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Estrategias metodológicas para la determinación de compuestos citostáticos en muestras ambientales

Methodological strategies for the determination of cytostatic compounds in environmental samples

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D. PABLO MARTEL ESCOBAR COORDINADOR DEL PROGRAMA DE DOCTORADO EN CALIDAD AMBIENTAL Y RECURSOS NATURALES

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha ..... tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "Estrategias metodológicas para la determinación de compuestos citostáticos en muestras ambientales" presentada por el doctorando D. Sergio Santana Viera y dirigida por las Doctoras Zoraida Sosa Ferrera y María Esther Torres Padrón

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a...de.....de dos mil.....

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

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Título de la Tesis

Estrategias metodológicas para la determinación de compuestos citostáticos en muestras ambientales

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La Directora, La Codirectora, El Doctorando,

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RESUMEN

El cáncer es una de las enfermedades más importantes del siglo XXI. Se trata de una enfermedad que produce un crecimiento descontrolado de células en una parte determinada del cuerpo. Una de las formas de combatir esta enfermedad consiste en la administración de ciertos medicamentos, conocidos como *compuestos antineoplásicos o agentes citostáticos*. El problema es que dichos medicamentos no son selectivos y dañan tanto a células sanas como tumorales. Estos compuestos, una vez administrados, son excretados por el paciente y pueden llegar al medioambiente a través de las plantas de tratamiento de aguas residuales (EDARs), donde pueden causar efectos adversos. Además, debido a sus características físico-químicas, pueden quedar adsorbidos en los lodos de las EDARs.

Por ello, existe una gran preocupación sobre la presencia de estos compuestos en muestras medioambientales, por lo que se han desarrollado e implementado metodologías técnicas analíticas para su determinación. Sin embargo, la optimización de procedimientos sensibles y selectivos capaces de extraer y determinar compuestos citostáticos en matrices complejas, como son las muestras ambientales, a bajas concentraciones, es difícil y constituye uno de los objetivos actuales de la química ambiental.

La presente Tesis Doctoral presenta los resultados de la optimización de metodologías de extracción y preconcentración para la determinación de compuestos citostáticos en muestras ambientales, usando tanto técnicas tradicionales como la extracción en fase sólida (SPE), como

nuevas técnicas miniaturizadas como la extracción por adsorción sobre tejidos químicamente modificados (FPSE) para la extracción en muestras líquidas y la extracción asistida por microondas (MAE) en muestras sólidas y biológicas. Todas ellas se han acoplado a cromatografía líquida de ultra resolución (UHPLC) con detección de espectrometría de masas en tándem (MS/MS) así como a la espectrometría de emisión atómica con plasma de acoplamiento inductivo y espectrometría de masas (ICP-MS).

A lo largo de esta Memoria se presentan los resultados y conclusiones obtenidos de la aplicación de los procedimientos optimizados en muestras de aguas residuales procedentes de estaciones depuradoras de aguas residuales (EDARs) de Gran Canaria, así como en muestras de aguas residuales tomadas en uno de los principales hospitales de la isla y en muestras de aguas de mar tomadas a la salida de los emisarios submarinos correspondientes a dichas EDARs.

También, los métodos optimizados se aplicaron a muestras de sedimentos marinos tomadas en las proximidades de los emisarios submarinos de las EDARs y lodos de la EDAR de la ciudad de Las Palmas de Gran Canaria.

Por último, además de conocer la presencia y distribución de los compuestos citostáticos en el medioambiente, es interesante conocer si representan una amenaza para los ecosistemas acuáticos. Para investigar dicho tema, se ha realizado un estudio sobre su presencia en muestras de peces capturados en las proximidades de emisarios submarinos, pertenecientes a diferentes niveles en la cadena trófica.

Varios compuestos citostáticos han sido detectados en las aguas residuales del hospital en niveles de $\mu g \cdot L^{-1}$. También se han detectado en influentes y efluentes de las EDARs en el rango de $ng \cdot L^{-1}$ hasta $\mu g \cdot L^{-1}$. Sin embargo, no fueron detectados en agua de mar ni en los peces que se alimentan en las proximidades de los emisarios submarinos. Por último, en los lodos y sedimentos marinos analizados no se detectaron compuestos citostáticos.

ABSTRACT

Cancer is one of the most important diseases of the 21st century. It is a disease that causes uncontrolled growth of cells in a certain part of the body. One of the ways to combat this disease is the administration of certain medications, known as *antineoplastic compounds* or *cytostatic agents*. The problem is that these medications are not selective and damage both healthy and tumour cells. These compounds, once administered, are excreted by the patient and can reach the environment through wastewater treatment plants (WWTPs), where they can cause adverse effects. In addition, due to their physical-chemical characteristics, they can be adsorbed in the sludge of the WWTPs.

Therefore, there is a big concern about the presence of these compounds in environmental samples. Consequently, different analytical methodologies have been developed and implemented for their determination. However, the optimization of sensitive and selective procedures capable of extracting and determining cytostatic compounds in complex matrices, such as environmental samples, at low concentrations still remains a challenge, and constitutes one of the current objectives of environmental chemistry.

This Doctoral Thesis presents the results regarding the optimization of different extraction and preconcentration methodologies for the determination of cytostatic compounds in environmental samples. Both, traditional extraction techniques such as solid phase extraction (SPE) and new miniaturized extraction techniques such as fabric phase sorptive extraction (FPSE) were optimized for extraction in liquid samples.

ABSTRACT

Microwave assisted extraction (MAE) was used as the extraction technique for solid and biological samples. All of them have been coupled to ultra-high resolution liquid chromatography (UHPLC) with tandem mass spectrometry detection (MS/MS) as well as atomic emission spectrometry with inductive coupling plasma and mass spectrometry (ICP-MS) for sample determination.

Throughout this Thesis, the results and conclusions obtained from the application of optimized procedures to samples of wastewater coming from wastewater treatment plants (WWTPs) in Gran Canaria are presented, as well as samples of wastewater taken in one of the main hospitals of the island. Samples of seawater taken at the exit of submarine emissaries of the WWTPs were also analysed.

In addition, the optimized methods were applied to marine sediment samples taken in the vicinity of the submarine outfalls of different WWTPs and sludge of the WWTP of the city of Las Palmas de Gran Canaria.

Finally, besides of knowing the presence and distribution of cytostatic compounds in the environment, it is interesting to know if they represent a threat to aquatic ecosystems. To investigate this issue, a study has been conducted to determine the presence of these compounds on fish samples caught in the vicinity of marine outfalls of different levels in the food chain.

Several cytostatic compounds have been detected in hospital wastewater at levels of $\mu g \cdot L^{-1}$. They have also been detected in influents and effluents of WWTPs in the range of $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$. However, they

were not detected in seawater or in fish that feed in the vicinity of marine outfalls. Finally, in the sludge and marine sediments analysed, the cytostatic compounds were not detected.

CAPÍTULO 1: INTRODUCCIÓN

Según la Organización Mundial de la Salud (OMS, World of Health Organisation, WHO) "*el cáncer se produce por la transformación de células normales en células tumorales en un proceso en varias etapas que suele consistir en la progresión de una lesión precancerosa a un tumor maligno.*" Esta enfermedad se caracteriza por el crecimiento descontrolado y desmesurado de células que pueden llegar a invadir tejidos cercanos. Las células cancerosas se diferencian de las células sanas en que no son tan especializadas por lo que siguen dividiéndose sin detenerse, ignorando las señales que les indican que deben morir [1].

El cáncer es una enfermedad provocada por cambios en los genes que controlan el crecimiento celular. Esta enfermedad puede ser heredada, puede aparecer de repente y/o puede deberse a factores ambientales, siendo estos últimos los más importantes. Durante el periodo 2011 –

2015, se detectaron 439 casos de cáncer por cada 100.000 personas, de los cuales, casi un tercio resultaron en muerte del paciente [2].

Uno de los tratamientos utilizados para combatir el cáncer es la quimioterapia mediante *compuestos citostáticos o antineoplásicos*. Después del tratamiento, gran parte del compuesto es excretado por el paciente, en su forma original o como producto de la metabolización del compuesto original, llegando a las aguas residuales y, posteriormente, siendo vertidos en los ríos, mares y océanos. Aunque las cantidades que llegan a las aguas residuales y al medio acuoso son muy pequeñas, su presencia debe ser evaluada, ya que por su forma de actuar (están diseñados para destruir o dañar las células), aun estando presentes en bajas concentraciones, pueden resultar perjudiciales para la biota.

Se debe, por tanto, desarrollar metodologías analíticas selectivas y sensibles para detectar y determinar, a bajas concentraciones, los analitos de interés de entre la multitud de interferencias que pueden existir en las muestras ambientales en la que se encuentran.

1.1. Cáncer y compuestos antineoplásicos

El cáncer es un grupo heterogéneo y numeroso de enfermedades caracterizadas por un crecimiento anormal de células [3]. Se puede considerar una enfermedad genética, ya que son los genes quienes dan órdenes a las células para que éstas sepan qué hacer. Cada célula contiene en torno a 30.000 genes. Sin embargo, solo se encuentran activos los genes específicos para la función que van a realizar. Por lo tanto, todas las células poseen los genes necesarios para dividirse y multiplicarse, aunque estos pueden estar desactivados [1].

La proliferación celular se produce de manera muy controlada durante el desarrollo del cigoto, según las necesidades del organismo. Este proceso por el que una célula se divide para dar lugar a dos células idénticas se conoce como *ciclo celular*.

El *ciclo celular* tiene cuatro fases, dos preparatorias y dos funcionales. La primera fase preparatoria (G1) es previa a la replicación del ADN. En ella, la célula dobla su masa y tamaño, comprueba que está preparada para la división y que las condiciones ambientales son las adecuadas. La siguiente fase (fase S) es en la que tiene lugar la replicación del ADN. Posteriormente, la fase G2 es el período entre la replicación del ADN y el inicio de la división. En este punto, la célula comprueba que se ha duplicado correctamente el ADN y ha duplicado su masa. Por último, la célula entra en la fase M (mitosis), donde se produce la propia división celular [4]. La Figura 1 muestra un esquema del ciclo celular.

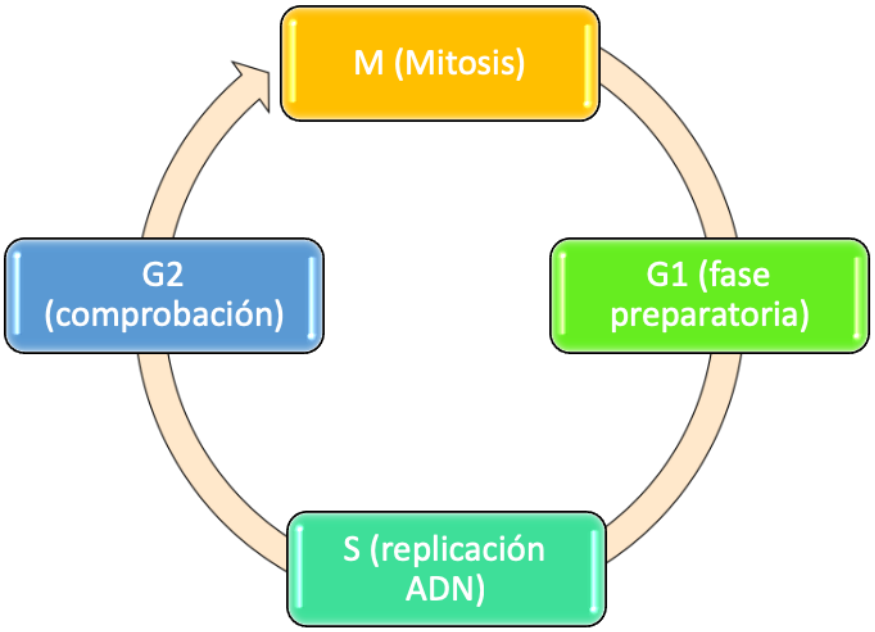

Figura 1. Etapas del ciclo celular

El cáncer se desarrolla a partir de un trastorno genético cromosómico en células individuales [5]. En algún momento del ciclo, se produce un error en algún gen de alguna célula que provoca una mutación en el ADN. Las mutaciones son comunes y suceden con frecuencia en nuestros tejidos y las células pueden detectar esta mutación y repararla pero, en algunas ocasiones, esta mutación tiene lugar en los genes que regulan la proliferación de la célula [1].

Como se comentó anteriormente, el **cáncer** provoca el *desarrollo anormal de células que han desarrollado mutaciones que impiden que mueran provocando un crecimiento descontrolado*. Las células sanas crecen, se dividen y mueren durante un tiempo determinado, pero la mutación que sufre una célula tumoral hace que se expanda sin control pudiendo llegar a sustituir tejidos sanos. Si llegan a invadir vasos sanguíneos y linfáticos pueden moverse a otros órganos y tejidos

distantes, provocando la **metástasis**. La capacidad de un cáncer de provocar metástasis dependerá de dónde se encuentre el cáncer y las características del mismo [6].

Hoy en día se considera que existen más de 400 cánceres diferentes, los cuales se agrupan en cuatro grupos: *carcinomas, sarcomas, cánceres de sangre y otros.* De entre ellos, los carcinomas son los más comunes, ya que el 80% de los cánceres son de este tipo. Este cáncer comienza en los epitelios, que son los tejidos que recubren las superficies externas e internas de nuestro cuerpo [1].

Los sarcomas comienzan en los huesos, cartílagos o tejidos blandos como músculos, nervios, vasos sanguíneos o tendones. En tercer lugar, se encuentran los cánceres de sangre, también llamados leucemias, que nacen en la médula ósea, dónde se forman las células de la sangre. El último grupo engloba, principalmente, los tumores del sistema nervioso central (cerebro, cerebelo y medula espinal) [1].

Se trata de una enfermedad de origen principalmente ambiental, ya que únicamente el 20% de los casos se considera hereditario. Algunas causas del cáncer pueden ser físicas (radiaciones), dieta (dentro de la cual se puede incluir el tabaco o el alcohol), virus, factores ambientales, etc. [3].

Los tratamientos contra el cáncer son, principalmente, tres: **cirugía, radioterapia y quimioterapia**. Así, la **cirugía** consiste en la extirpación del tejido canceroso. Es el tratamiento más antiguo, más arraigado y aún sigue siendo el más eficaz [7].

La **radioterapia** consiste en la aplicación de radiaciones y se aplica dependiendo de la sensibilidad del cáncer a dicha radiación. Algunos cánceres se pueden eliminar por completo con esta técnica, como el cáncer de laringe o el de próstata.

Por último, la **quimioterapia** engloba multitud de medicamentos que tienen por objetivo frenar la división celular, motivo por el cual resulta perjudicial también para las células sanas. Otras aplicaciones de la quimioterapia son la reducción del tamaño del tumor y que éste sea, finalmente, operado por cirugía o su uso después de la extirpación del cáncer, para impedir que el tumor vuelva a crecer [1]. A estos medicamentos se les conoce como *compuestos antineoplásicos o citostáticos.*

Según Greaves [5], el cáncer es una enfermedad global, esto es, en todo el mundo se producen al año unos ocho millones de diagnósticos de cáncer, independientemente de etnia o situación geográfica. Cuando para tratar el cáncer se usa quimioterapia hay que tener en cuenta que la propia naturaleza de estos medicamentos hace que sean muy peligrosos y, por ello, su uso tiene que estar controlado.

Dependiendo del tipo de cáncer, el tratamiento podrá consistir en uno o en una combinación de estos compuestos. Su combinación facilita la lucha contra el cáncer. Sin embargo, también puede tener como resultado la suma de las toxicidades de los compuestos ya que la combinación de ellos puede resultar más peligrosa que por separado. El tratamiento se suele suministrar por ciclos. De esta manera, se pretende que las células sanas puedan volver a su actividad y que las células tumorales se

recuperen en menor cantidad [8]. Hay que tener en cuenta que la dosis terapéutica es superior a la dosis tóxica [3].

Según la OMS [9], los compuestos antineoplásicos se clasifican en cinco grupos, según se muestra en la Tabla 1.

Tabla 1. Algunos compuestos antineoplásicos nombrados en esta tesis

		Código ATC	Nombre	Abreviatura
L01A AGENTES ALQUILANTES	**L01AA Análogos de la mostaza nitrogenada**	L01AA01	ciclofosfamida	CP
		L01AA02	clorambucil	CHLO
		L01AA03	melfalán	MELP
		L01AA06	ifosfamida	IF
L01B ANTIMETABOLITOS	**L01BA Análogos del ácido fólico**	L01BA01	metotrexato	MET
	L01BC Análogos de pirimidina	L01BC01	citarabina	CYT
		L01BC02	fluorouracilo	5-FU
		L01BC05	gemcitabina	GEM
		L01BC06	capecitabina	CAP
		L01BC07	azacitidina	AZA
L01C ALCALOIDES VEGETALES Y OTROS PRODUCTOS NATURALES	**L01CA Alcaloides vinca y análogos**	L01CA01	vinblastina	VINB
		L01CA02	vincristina	VINC
		L01CA04	vinorelbina	VINO
	L01CB Derivados de la podofilotoxina	L01CB01	etoposido	ETO
	L01CD Taxanos	L01CD01	paclitaxel	PAC
		L01CD02	docetaxel	DOC
	L01DB Antraciclinas y sustancias relacionadas	L01DB01	doxorubicina	DOX
		L01DB02	daunorubicina	DAU
		L01DB03	epirubicina	EPI
		L01DC03	mitomycin	MIT
L01X OTROS AGENTES ANTINEOPLASICOS	**L01XA compuestos de platino**	L01XA01	cisplatino	Cis-Pt
		L01XA02	carboplatino	Car-Pt
		L01XA03	oxaliplatino	Oxa-Pt
	L01XE Inhibidores de la proteína cinasa	L01XE01	tamoxifeno	TAM
		L01XE03	erlotinib	ERLO
		L01XE26	carbozantinib	CARBO
		L01XX19	irinotecan	IRI

Los agentes alquilantes (L01A) son compuestos químicos capaces de formar enlaces moleculares con ácidos nucleicos, proteínas y moléculas de bajo peso molecular. Dañan la capacidad de reproducción celular provocando un daño irreversible en el ADN [7]. Estos compuestos tienen un radical bis(cloroetil)amina, etilamina o nitrosourea y ceden sus grupos alquilo al componente celular para ejercer su actividad citotóxica. La ciclofosfamida (CP), uno de los compuestos seleccionados para esta tesis doctoral, posee un radical bis(cloroetil)amina (Figura 2).

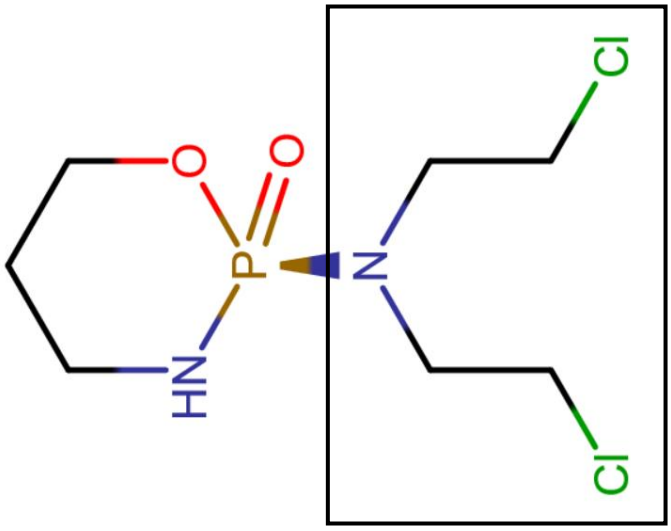

Figura 2. Ciclofosfamida marcado en un recuadro el radical bis(cloroetil)amina

Los **antimetabolitos (L01B)** son compuestos de bajo peso molecular que actúan frenando la división celular. Pueden parecerse químicamente a alguna sustancia que necesita la célula, engañando a esta y haciendo que lo incorpore a su metabolismo o inhiben alguna enzima necesaria para el metabolismo [8]. La Figura 3 muestra la similitud entre el metotrexato (MET) y el ácido fólico, dónde la única diferencia entre ellos son los radicales marcados en amarillo.

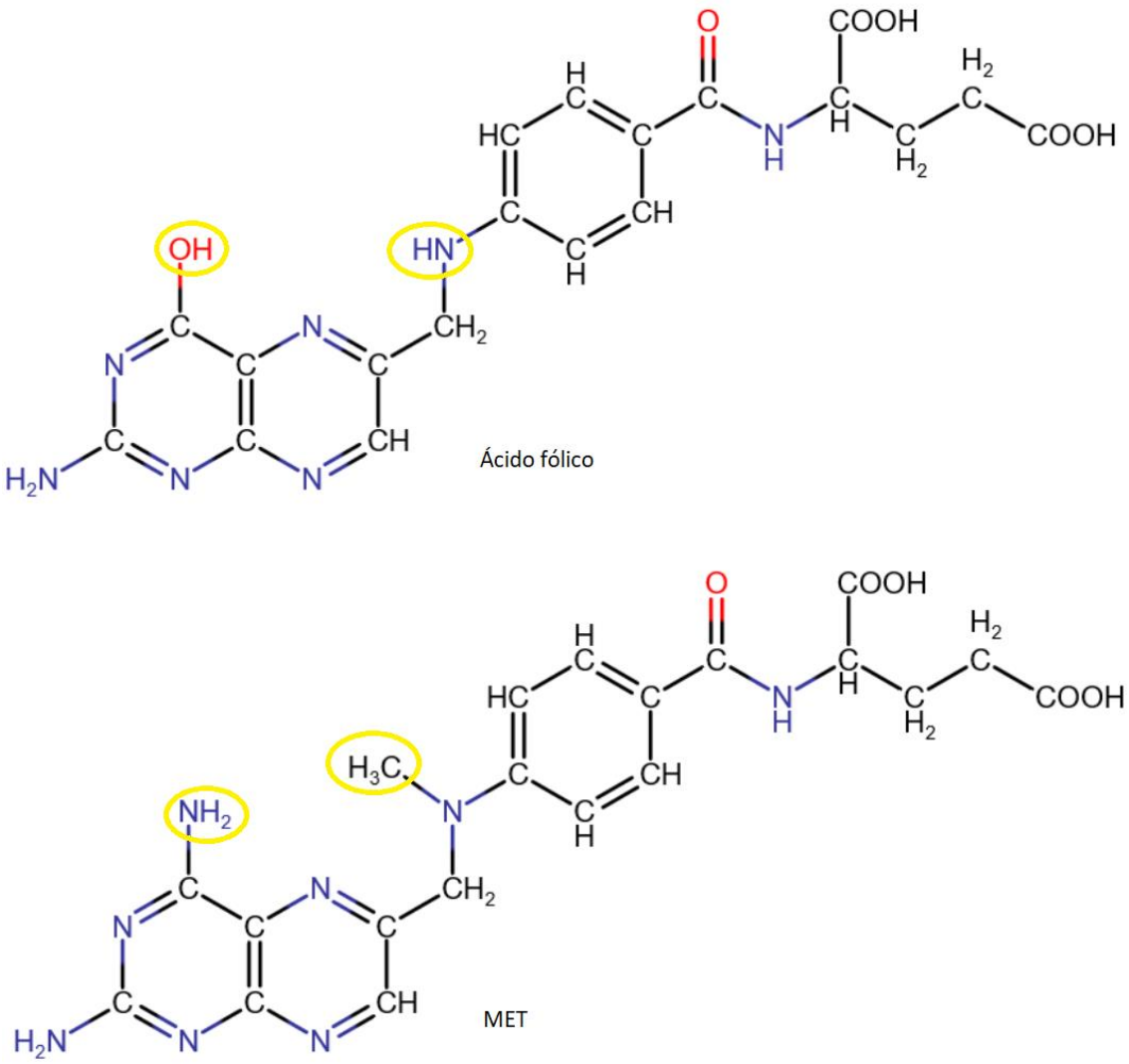

Figura 3. Diferencias entre MET y ácido fólico

Los **alcaloides vegetales (L01C)** son compuestos inhibidores de la mitosis celular que provienen de productos naturales [8]. Dentro de este grupo destacan los antibióticos citostáticos, los cuales son compuestos muy tóxicos que no se usarían nunca contra infecciones. Su objetivo es inactivar el ADN formando complejos estables con él [8].

En el último grupo encontramos **otros compuestos antineoplásicos (L01X)**, como los anticuerpos monoclonales que activan la muerte programada de las células [8]. Se encuentran incluidos los fármacos de

platino que, pese a su uso extendido, no se conoce con certeza su mecanismo de acción y se cree que es similar al de los fármacos alquilantes, es decir, destruyen las etapas del ciclo celular. También se ha demostrado que se unen a las proteínas citoplasmáticas y nucleares, potenciando sus efectos citotóxicos y antitumorales [10].

Debido a su forma de actuar hay que reducir al mínimo su exposición y, por lo tanto, tener un control exhaustivo de los mismos. No porque los fármacos vayan a ser vertidos de manera intencionada o no vayan a ser eliminados de la forma adecuada a través de un gestor autorizado, sino porque gran parte del medicamento es excretado por el paciente en la orina y las heces. Eso provoca que los compuestos originales, metabolitos o productos de transformación entren en el sistema de aguas residuales. Si bien, como veremos en capítulos posteriores de esta tesis, su concentración será muy baja, estos podrían causar efectos adversos a diferentes organismos.

Los compuestos citostáticos tienen características y propiedades físico-químicas muy diferentes. Los hay de bajo peso molecular como es el caso del 5-fluorouracilo (5-FU) con 130 u.m.a. [11] y de alto peso molecular como la vincristina (VINC) con 825 u.m.a. [12]. Algunos tienen constantes de disociación ácida negativas como el oxaliplatino (Oxa-Pt) (pK_a = -11.72) [13] y otros altas como la gemcitabina (GEM) (pK_a = 11.52) [14], coeficientes de partición octanol/agua altos como el tamoxifeno (TAM) (Log $K_{o/w}$ = 6.35) [15] hasta incluso negativos como el cisplatino (Cis-Pt) (Log $K_{o/w}$ = -8.24) [16]. Aunque se trate de una única familia de compuestos, sus características los hacen muy diferentes por lo que se dificulta su extracción y determinación simultáneas.

Las concentraciones a las que los compuestos antineoplásicos se encuentran en el medio son bajas, del orden de $\mu g \cdot L^{-1}$ en efluentes de hospitales, en $ng \cdot L^{-1}$ en las estaciones depuradoras de aguas residuales e inferiores a $ng \cdot L^{-1}$ en ríos [17]. Sin embargo, al tratarse de compuestos que pueden ser genotóxicos, mutagénicos, carcinogénicos, teratogénicos o fetotóxicos, es necesario el estudio de los efectos adversos que podrían causar estos medicamentos. Así, desde hace unos 10 años, se han publicado diferentes trabajos en dónde se estudian los efectos citotóxicos y genotóxicos de los principales medicamentos contra el cáncer. Si nos centramos en el medio marino, los estudios se han realizado, principalmente en algas, bacterias, crustáceos, mejillones y peces.

Así, algas y cianobacterias fueron expuestas a ifosfamida (IF), CP y sus metabolitos o productos de transformación por Česen et al [18]. Los compuestos padres resultaron no ser tóxicos a las concentraciones probadas para el alga *Pseudokirchneriella subcapitata* ni para la cianobacteria *Synecococcus leopoliensis*. Sin embargo, demostraron que el metabolito carboxi-ciclofosfamida era tóxico para la cianobacteria.

Zounková et al. [19] estudiaron la ecotoxicidad de cinco compuestos antineoplásicos (Cis-Pt, 5-FU, doxorubicina (DOX), etoposido (ETO) y CP) ampliamente usados en la bacteria P*seudomonas putida* y en el alga P*seudokirchneriella subcapitata*, realizando un test de inhibición del crecimiento, y en *Daphnia magna,* realizando un test de inmovilización aguda. Los resultados de este estudio indican que los compuestos Cis-Pt y 5-FU deben ser considerados como altamente tóxicos, DOX debe ser considerado como tóxico y ETO como dañino para el ecosistema marino. Los compuestos estudiados provocaron efectos significativos en la

mayoría de los ensayos pero a concentraciones relativamente altas ($\mu g \cdot L^{-1}$ – $mg \cdot L^{-1}$) comparadas con las que se detectan en el medioambiente. Sin embargo, señalan que la exposición crónica aún debe ser estudiada, así como los metabolitos y las descargas puntuales más altas debido a los tratamientos administrados.

Brezovŝek et al. [20] estudiaron la toxicidad de cuatro compuestos citostáticos (5-FU, Cis-Pt, imatinib (IMA) y ETO) y sus mezclas binarias sobre el alga *Pseudokirchneriella subcapitata* y la cianobacteria *Synechococcus leopoliensis*, encontrando un rango de toxicidad aguda similar al trabajo de Zounková et al [11]. Por otro lado, estudiaron las mezclas binarias 5-FU + Cis-Pt, 5-FU + IMA y Cis-Pt + ETO, y concluyeron que las mezclas de los compuestos citostáticos pueden tener efectos sinérgicos o antagónicos dependiendo de los compuestos y la especie.

Dado que, en muchas ocasiones, el tratamiento contra el cáncer consiste en aplicar una mezcla de compuestos y que los tratamientos que se aplican pueden ser diferentes, en las aguas residuales se espera encontrar una mezcla de compuestos anticancerígenos. A la vista de los resultados obtenidos, los autores sugieren que los datos de toxicidad de un solo compuesto no son suficientes para la predicción de las toxicidades acuáticas, ya que no se tienen en cuenta la sinergia de las posibles mezclas que puedan tener lugar.

Parrella et al. [21] estudiaron la exposición de los niveles más bajos de la cadena trófica a seis compuestos antineoplásicos (5-FU, capacitabina (CAP, Cis-Pt, ETO, IMA y DOX). Una vez más se demostró que la toxicidad aguda ocurre en concentraciones de $mg \cdot L^{-1}$, mucho mayor a las

determinadas en el medioambiente, pero la exposición crónica a concentraciones de $\mu g \cdot L^{-1}$ provocó un 50% de inhibición de la reproducción en crustáceos. Los autores de este trabajo también señalan la necesidad de estudiar los productos de transformación y metabolitos derivados de los compuestos citostáticos, así como las posibles sinergias que pueda haber entre ellos o con otros contaminantes del medio.

Borgatta et al. [22] estudiaron los efectos de dos metabolitos del TAM (endoxifen y 4-hidroxi-tamoxifen (4-OH-TAM)) en concentraciones de $\mu g \cdot L^{-1}$ en dos generaciones de *Daphnia pulex*. Los metabolitos provocaron efectos reproductivos y de supervivencia, aumentando la sensibilidad de *Daphnia pulex* a los metabolitos en la segunda generación. También disminuyó la tasa de crecimiento natural al aumentar las concentraciones de los metabolitos. En un estudio posterior, investigaron los efectos de TAM y 4-OH-TAM en cuatro generaciones de *Daphnia pulex* [23]. Encontraron que tanto el TAM como su metabolito 4-OH-TAM afectaron a la tasa intrínseca de aumento natural, la reproducción, el tamaño y la viabilidad, pero estos efectos no se magnificaron en las siguientes generaciones. La presencia de los dos contaminantes provocó efectos en la descendencia que no se observaron cuando se probaron los compuestos de manera individual. La segunda parte del experimento consistió en averiguar si los efectos de la exposición a estos contaminantes son reversibles. Para ello, pusieron en contacto *Daphnia pulex* con TAM y su metabolito y en generaciones posteriores los contaminantes fueron retirados. Observaron que en estos descendientes también hubo una reducción del tamaño y la reproducción.

Trombini et al. [24] estudiaron los efectos adversos del Cis-Pt en mejillones (*Mytilus galloprovincialis)* demostrando que, incluso concentraciones bajas, cercanas a las que se podrían detectar en efluentes de aguas residuales, provocan efectos adversos. Realizando ensayos similares en peces, Kovács et al. [25] expusieron dos generaciones de *Danio rerio* a concentraciones de 0.01, 1 y 100 $\mu g \cdot L^{-1}$ de 5-FU. Los resultados no mostraron efectos en el crecimiento, reproducción o supervivencia en el pez, pero sí se observaron cambios histopatológicos en el hígado y el riñón y efectos genotóxicos. Se determinaron daños en el ADN en las células sanguíneas y en el hígado, pero no en las branquias ni en las gónadas. Aunque la exposición crónica no afectó a la capacidad reproductiva, 5-FU puede provocar cambios degenerativos, incluyendo cáncer, que podrían afectar a la población de peces en varias generaciones.

Los efectos de los compuestos antineoplásicos CP, Cis-Pt, IF y 5-FU, solos y en combinaciones, fueron estudiados por Novak et al. [26] también en el hígado del pez cebra. Probaron la mezcla de los cuatro compuestos a las concentraciones máximas detectadas en efluentes de hospitales. La mezcla no resultó ser citotóxica ni genotóxica, pero indujo un aumento significativo en la formación de roturas de la cadena de ADN a concentraciones mucho más bajas que la producida por los compuestos individuales. Con estos resultados remarcaron la necesidad de establecer niveles tóxicos teniendo en cuenta la mezcla de compuestos y no cada uno de forma individual.

Es muy necesario tener en cuenta la exposición crónica, ya que varios trabajos sugieren que ciertos compuestos antineoplásicos pueden ser

persistentes en el medio acuático y presentar baja biodegradabilidad [27,28]. Por lo tanto, la continua entrada de estos compuestos a través de las aguas depuradas, principalmente, junto con su baja biodegradabilidad podría contribuir a un aumento de su concentración en las proximidades de los efluentes.

Las conclusiones generales de estos autores sugieren la necesidad de estudios multigeneracionales para comprender adecuadamente las consecuencias de la exposición prolongada a pequeñas dosis de compuestos antineoplásicos. Han demostrado efectos adversos a concentraciones inferiores a la concentración tóxica aguda, y cercana a las concentraciones que son detectadas en los efluentes de las estaciones depuradoras. Además, hacen un llamamiento al estudio de las sinergias por las mezclas de compuestos antineoplásicos que pueden llegar a ser más tóxicos que los compuestos individuales, y es como realmente se encontrarán en el medio. Por este mismo motivo también se hace necesario el estudio de los metabolitos y productos de degradación, ya que pueden llegar a ser más tóxicos que los compuestos padres.

Para llevar a cabo esta tarea, es necesario el desarrollo de procedimientos analíticos capaces de extraer y preconcentrar estos compuestos citostáticos de matrices complejas, como las aguas residuales, los lodos generados en la depuración y los organismos, para su posterior determinación y monitorización en el medioambiente. Únicamente analizando la variedad de compuestos citostáticos que llegan al medio y su intervalo de concentraciones se podrá conocer la magnitud del problema.

1.2. Técnicas de extracción de compuestos antineoplásicos en muestras medioambientales

1.2.1. Muestras líquidas

Desde hace años la comunidad científica se ha dado cuenta que una etapa fundamental de la determinación de micro-contaminantes es la extracción. Es una etapa laboriosa, que lleva bastante más tiempo que la determinación en sí misma pero que una equivocación en ella podría resultar en un importante error en la determinación [29]. Sin embargo, debido a las concentraciones tan bajas en las que se encuentran los analitos es prácticamente obligatorio realizar un proceso de extracción y preconcentración. La Extracción en Fase Sólida (SPE) es una técnica que ha ido sustituyendo y desplazando a la Extracción Líquido-Líquido (LLE) como técnica de extracción y preconcentración, ya que presenta ventajas como la reducción del uso de disolventes, de costes y del tiempo de extracción. Además, es selectivo, fácilmente automatizable, permite que los analitos queden retenidos en el sorbente, consiguiendo una alta preconcentración y eliminando interferencias. Los principales sorbentes usados son de fase reversa que actúan, principalmente, por fuerzas de Van der Waals [30]. Aun así, se puede encontrar algún trabajo en el que la extracción líquido-líquido ha sido empleada en la determinación de citostáticos, concretamente TAM aunque, posteriormente, fue purificado con cartuchos de SPE Oasis MCX [31].

En general, el proceso de extracción mediante SPE consta de cinco pasos generales, tal como se muestran en la Figura 4:

1º Acondicionamiento del cartucho

2º Paso de la muestra mediante vacío

3º Retención de los analitos de interés

4º Eliminación de interferencias

5º Elución de analitos mediante un disolvente orgánico apropiado

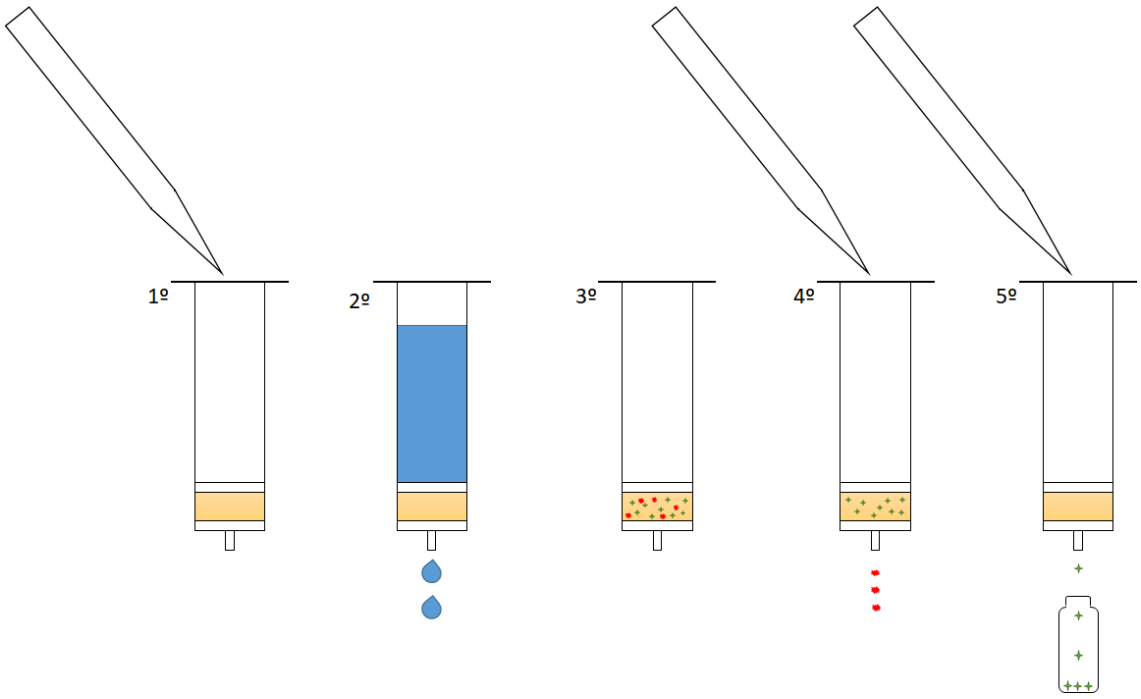

Figura 4. Esquema de procedimiento mediante Extracción en Fase Sólida

donde hay que optimizar las condiciones experimentales más adecuadas para cada una de estas etapas.

En el primer trabajo publicado donde se extraen compuestos antineoplásicos de muestras medioambientales se utilizan cartuchos CHROMABOND® C_{18} en la extracción de CP e IF de aguas residuales, obteniendo recuperaciones promedio en torno al 30% para CP y 39% para IF [32]. Steger-Hartmann et al. [32] demostraron la presencia de esos dos compuestos en aguas residuales en concentraciones de 24 $ng \cdot L^{-1}$ de IF y 146 $ng \cdot L^{-1}$ de CP. Además, demostraron que los tratamientos

convencionales de depuración de aguas residuales no son capaces de degradar estos compuestos, abriendo un amplio campo de estudio.

En posteriores trabajos, estos autores realizaron un estudio de IF [27] y de CP [33], en los cuales encontraron concentraciones de hasta 1,994 $\mu g \cdot L^{-1}$ de IF y hasta 4,486 $\mu g \cdot L^{-1}$ de CP en efluentes de hospitales. Con este resultado demuestran que una de las principales fuentes de entrada de compuestos antineoplásicos al medio es a través de las aguas residuales hospitalarias, ya que los pacientes reciben el tratamiento en los hospitales y gran parte de estos compuestos son excretados sin metabolizar. Por otro lado, encontraron concentraciones similares de ambos compuestos en la entrada y salida de la EDAR confirmando, de nuevo, que no son degradados por los tratamientos convencionales.

Kiffmeyer et al. [34] trató de optimizar un procedimiento de extracción para diez compuestos citostáticos de diferentes familias, con propiedades y características distintas. Por este motivo tuvo que probar diferentes versiones de cartuchos C_8 y C_{18} de fase reversa, cartuchos de modo mixto, de intercambio iónico, etc. En general, encontró que la mejor solución para la extracción de la mezcla de analitos fue usar cartuchos de fase reversa C_{18} o cartuchos de poliestireno divinilbenceno (PS-DVB), los cuales tratan de solucionar la extracción de compuestos polares de matrices acuosas medioambientales [35], llegando a obtener recuperaciones de hasta el 90%.

Otra opción a la hora de extraer compuestos farmacéuticos con diferentes propiedades es agrupando los compuestos de características similares y seleccionando el cartucho de SPE más adecuado para cada

grupo [36]. El problema es que, en este caso, sería necesario realizar 4 extracciones, lo cual conlleva un consumo de tiempo importante y, a su vez, una cantidad de muestra considerable (3.5 L). Sin embargo, otros autores han tomado esta decisión cuando se trata de extraer compuestos antineoplásicos de diferentes familias [37].

Los compuestos polares y muy solubles en agua siempre han resultado muy problemáticos para su extracción y preconcentración. Entre los compuestos anticancerígenos, destaca el 5-FU, un antimetabolito ampliamente utilizado, que fue extraído por primera vez por Mahnik et al. [38]. Para ello, usaron los cartuchos ENV$^+$, apropiados para este tipo de compuestos, obteniendo recuperaciones entre 80 – 96%. En un trabajo posterior [39], optimizaron un procedimiento para la extracción de tres antraciclinas (epirubin (EPI), DOX y daunorobicina (DAU)) mediante cartuchos de SPE C_8 logrando recuperaciones superiores 80%. Las aplicaciones de estos trabajos se realizaron, únicamente, en aguas hospitalarias.

Kovalova et al. [40] publicaron un trabajo sobre la extracción y determinación de compuestos citostáticos muy polares (5-FU, citarabina (CYT) y GEM) y dos metabolitos en muestras de aguas residuales de un hospital usando cartuchos Isolute ENV$^+$ para la extracción y Speedisk H_2O-philic SA-DVB como paso de limpieza. Hasta este momento, la mayoría de los trabajos sobre determinación de compuestos citostáticos se habían centrado en compuestos no polares que son retenidos y separados con facilidad en columnas C_{18}. Sin embargo, los compuestos muy polares, como los seleccionados por los autores de este trabajo, no son retenidos en este tipo de columnas, por lo que usaron columnas de interacción

hidrófila (HILIC). Para los compuestos 5-FU y el principal metabolito orinado de GEM, difluorodeoxyuridina (dFdU), se obtuvieron recuperaciones inferiores al 50% debido al uso de Speedisk H_2O-philic SA-DVB, que reduce el efecto matriz pero, a la vez, también reduce la recuperación. Sin un paso de limpieza, el efecto matriz hubiese hecho imposible la determinación de los compuestos. Pese a obtener buenos límites de detección, la aplicación se realizó únicamente en efluentes de hospitales. Usando estos mismos cartuchos (ENV^+), Ghafuria et al. [41] fueron capaces de extraer Cis-Pt, Car-Pt y Oxa-Pt, compuestos con coeficientes de partición octanol/agua negativos, obteniendo recuperaciones de entre el 0.70 – 0.78%.

Garcia-Ac et al. [42] fueron los primeros en aplicar la extracción en fase sólida on-line (on-line SPE) en la extracción y determinación de MET y CP junto a otros medicamentos. Este procedimiento se caracteriza porque los cartuchos de SPE están acoplados directamente al sistema cromatográfico. De esta manera, la extracción se realiza de forma automática e inmediatamente se dirige a la cabeza de la columna para proceder a la separación cromatográfica de los analitos. Debido a esta nueva configuración, la optimización del procedimiento está relacionada con el caudal de muestra, el volumen y el contenido de disolvente orgánico en la muestra. Uno de los inconvenientes de este procedimiento es que resulta complicado y casi imposible distinguir entre efecto matriz y recuperación. Por ello, estos autores presentaron dos fórmulas para tratar de evaluarlos, obteniendo recuperaciones de 148% para CP y 55% para MET.

A partir del año 2010, gracias al desarrollo del SPE online, se publicaron varios trabajos multi-residuo en los que se incluían compuestos antineoplásicos [43–45] o con compuestos antineoplásicos, exclusivamente [46,47].

A su vez, las publicaciones comenzaron a centrarse en la evaluación de mezclas de compuestos citostáticos, principalmente en aguas residuales, tanto de hospital como de estaciones depuradoras. En la mayoría de los trabajos, la extracción y preconcentración se realizó mediante SPE con cartuchos Oasis HLB [44,48,49].

Ferrando-Climent et al. [44] probaron cartuchos Oasis HLB y de intercambio iónico MAX y MCX para la extracción de MET, TAM, docetaxel (DOC) y placlitaxel (PAC), obteniendo los mejores resultados con el cartucho Oasis HLB, donde lograron recuperaciones entre el 46% y el 129%. En condiciones óptimas y para mejorar la recuperación de los compuestos, añadieron un agente quelante (Na_2EDTA) a una concentración 0.1M, para evitar las posibles interferencias de metales solubles con los compuestos. Finalmente, obtuvieron recuperaciones de 100 ± 20% para todos los compuestos. Con el mismo procedimiento de extracción estudiaron, en un trabajo posterior, efluentes de estaciones depuradoras y aguas de rio obteniendo recuperaciones entre el 58.4 – 129.7% y entre 70.9 – 127.0%, respectivamente [50].

Yin et al. [48] obtuvieron recuperaciones entre 51 – 105% para una mezcla de citostáticos que incluía MET, azatioprina, DOX, doxorrubicinol, VINC, IF, CP, ETO y procarbazina usando los mismos cartuchos. Gómez-Canela et al. [51] obtuvieron recuperaciones del 87% para CP usando

Oasis HLB, pero del 37% para EPI. Gómez-Canela et al. [52] probaron cuatro cartuchos (Oasis HLB, Oasis MCX, Isolute ENV$^+$ e Isolute C_{18}) para la extracción de 26 compuestos citostáticos en aguas residuales. Los mejores resultados los obtuvieron, nuevamente, para el cartucho Oasis HLB, con un amplio rango de recuperaciones (6 – 110%) ya que las propiedades físico-químicas de los compuestos son muy diferentes. Franquet-Griell et al. [53] también usaron Oasis HLB para la extracción de 19 compuestos citostáticos de agua de rio, logrando recuperaciones entre 29 – 111%, justificando la baja recuperación por la hidrólisis de ciertos compuestos que presentan baja estabilidad en agua.

Otros tipos de cartuchos también han sido utilizados para extraer seis compuestos citostáticos (bicalutamida, CAP, CP, doxifluridina, TAM y tegafur (TEG)), como es el caso de Azuma et al. [54] que emplea cartuchos Oasis MAX obteniendo recuperaciones de 63 – 124% y 52 – 115% para agua de rio y agua residual, respectivamente. Usando también cartuchos de intercambio iónico como Oasis WAX, en la extracción de 7 compuestos antineoplásicos de las mismas matrices, Santos et al. [55] obtuvieron recuperaciones entre el 31 – 105% en agua Milli-Q. Se observa, por tanto, que la principal desventaja de los cartuchos de intercambio iónico es la recuperación, aunque el efecto matriz disminuye.

Como ya se comentó, la dificultad de evaluar separadamente el efecto matriz y la recuperación en SPE on-line, hace que la recuperación se tenga que evaluar, por ejemplo, mediante cartuchos off-line. De esta forma, López-Serna et al. [43] estudió la recuperación obtenida, la cual resulta bastante baja para el antineoplásico analizado, TAM (<15%). La misma forma de evaluar la recuperación la utiliza Negreira et al. [46] obteniendo,

en esta ocasión, recuperaciones por encima del 70%. Rabii et al. [47] calcularon la recuperación comparando la concentración medida de una muestra contaminada con la concentración que, teóricamente, debería tener obteniendo buenas recuperaciones para casi todos los compuestos, excepto GEM, el compuesto más hidrofílico cuya recuperación fue inferior al 50%. El problema de calcular la recuperación de esta última forma en muestras complejas es que es imposible distinguir entre pérdida de señal por una baja recuperación en el cartucho o interferencias en la ionización.

Un nuevo enfoque en los últimos años en la química analítica ha sido el desarrollo de metodologías donde el uso de disolventes orgánicos se ve reducido. Este movimiento se conoce como "química analítica verde", cuyos principios pretenden elaborar y usar productos químicos menos peligrosos, prevenir la contaminación, reducir el uso de materias primas, productos, disolventes, reactivos, etc. [56].

Siguiendo estos principios comenzó una corriente de miniaturización, creando técnicas de microextracción que son capaces de muestrear, extraer y preconcentrar. En la optimización de estas nuevas técnicas miniaturizadas, y teniendo en cuenta el gran número de parámetros que afectan la extracción, se hace necesario alejarse de los procedimientos clásicos que no tienen en cuenta las interacciones de las variables [57].

Hasta 2016, según la revisión realizada [17], la aplicación de técnicas de microextracción para compuestos citostáticos no se había llevado a cabo. Los primeros trabajos de estas técnicas aplicadas a compuestos citostáticos se publicaron en 2017 sobre Extracción por Adsorción sobre

Tejidos Químicamente Modificados" (FPSE) [58] y en 2018 sobre microextracción por dispersión líquido-líquido (DLLME) [59].

La DLLME fue introducida en 2006 [60]. Se basa en la formación de finas partículas del disolvente de extracción dispersas en la fase acuosa. La centrifugación logra que las partículas de disolvente sedimenten. El principal inconveniente es el limitado número de extractantes que se pueden utilizar debido a las condiciones de extracción requeridas [61]. La DLLME dio como resultado la extracción de cuatro compuestos citostáticos (IRI, DOX, EPI y DAU) de muestras de aguas residuales de hospitales, obteniendo recuperaciones entre el 75 – 90% [59].

1.2.2. Muestras sólidas

La bibliografía consultada relacionada con la extracción de compuestos antineoplásicos en muestras sólidas es escasa. De hecho, existe menos de una decena de trabajos en los que se han estudiado estos compuestos en muestras como lodos, compost o sedimentos.

En el primer trabajo, que fue publicado por Ternes et al. [62] en 2005, se optimizó un método de Extracción Asistida por Ultrasonidos (USE) para una mezcla de compuestos farmacéuticos, en la que incluyeron dos compuestos antineoplásicos (IF y CP), en muestras de lodos. La técnica USE se basa en el uso de ondas ultrasónicas para aumentar la solubilidad de los analitos en el disolvente en el que se encuentran. En esta ocasión, fue usado, en primer lugar 4mL y 2mL de metanol, seguido de dos extracciones más con 2mL de acetona. Además, utilizaron un paso extra de preconcentración después de la extracción con cartuchos de SPE de

fase reversa C_{18}. De esta forma, lograron recuperaciones entre el 53 – 59% y 58 – 66% para IF y CP, respectivamente.

Esta técnica también fue usada para la extracción de ocho compuestos farmacéuticos, entre los que se encontraban CP e IF, en compost [63]. Lograron obtener una recuperación entre 87.3 – 97.3% para IF y entre 95 – 99.1% para CP, con límites de cuantificación (LOQ) de 2.05 $ng \cdot g^{-1}$ y 1.77 $ng \cdot g^{-1}$ respectivamente.

Azuma et al. [64] estudiaron la presencia de seis compuestos anticancerígenos en sedimentos de ríos de Japón. Para ello, aplicaron un protocolo basado en el uso de USE obteniendo recuperaciones entre 23 y 112% para sedimentos y entre 33 y 122% para material en suspensión. Los autores afirman haber detectado concentraciones de 0.319 $ng \cdot g^{-1}$ de bicalutamide, 0.392 $ng \cdot g^{-1}$ de doxifluridine y 0.250 $ng \cdot g^{-1}$ de TAM.

Okuda et al. [65] llevaron a cabo la extracción de 66 compuestos en lodos, entre los que incluyeron el CP, mediante la Extracción por Líquidos Presurizados (PLE) en lodos. Esta técnica se basa en las mejores propiedades en cuanto a transferencia de masa que poseen los líquidos cuando se encuentran a altas temperaturas y presiones, aumentando la eficiencia de extracción y reduciendo el tiempo de extracción. Para CP, la eficiencia de extracción estuvo en torno al 75%, y se detectó, por primera vez, en concentraciones de 12.5 $ng \cdot g^{-1}$ en el fango primario y 3 $ng \cdot g^{-1}$ en el fango de exceso.

Usando la misma técnica, Seira et al. [66] presentaron un trabajo con el objetivo de extraer tres compuestos citostáticos ampliamente utilizados

(CP, IF y TAM) en lodos. La optimización se realizó a través de un diseño experimental en donde encontraron dificultades para evaluar el TAM debido a importantes variaciones de su recuperación, por lo que éste fue finalmente descartado. Además, añadieron un paso de limpieza con cartuchos SPE Oasis MAX y Oasis MCX con el objetivo de eliminar interferencias de la matriz, el factor más limitante del proceso. Obtuvieron recuperaciones para el IF entre el 14 – 33% y para el CP entre el 24 – 47%, midiendo concentraciones de IF de entre 11.4 – 44.5 $ng \cdot g^{-1}$ y 12.6 $ng \cdot g^{-1}$ de CP.

Peysson y Vulliet [67] aplicaron QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) para la extracción de 136 compuestos en lodos, de entre los cuales se encontraban tres compuestos anticancerígenos: DAU, EPI y TAM. Obtuvieron, en general, bajas recuperaciones para los compuestos citostáticos (21 – 37% DAU, 4 – 5% EPI y 58 – 67% TAM) y no pudieron ser detectados en las muestras estudiadas.

Meeravali et al. [68] desarrollaron un procedimiento secuencial para la extracción de Cis-Pt, Car-Pt y hexacloroplatinato en muestras de suelo y determinación mediante absorción atómica. El procedimiento de extracción implica el uso de agua regia con un 20% de HCl a temperatura ambiente, seguido de una extracción por microondas con agua regia y HF. Después de la extracción, se aplicó una variación de extracción por punto de nube (CPE), usando Triton X-114 como surfactante, con la intención de preconcentrar los analitos. Con este procedimiento, se obtiene una recuperación muy alta para los compuestos de Pt (96 – 102%) y un límite de detección (LOD) de 0.5 $ng \cdot g^{-1}$.

Una vez que se ha demostrado la presencia de compuestos antineoplásicos en lodos de depuradoras y en los efluentes, es necesario estudiar qué puede ocurrir en las proximidades de los emisarios submarinos. La contaminación del ecosistema marino es un hecho demostrado, parte de esta contaminación se debe a la entrada de contaminantes emergentes y, en el caso de los compuestos citostáticos, esta entrada se produce, principalmente, a través de estaciones depuradoras. Estos compuestos son persistentes y pueden bioacumularse en diferentes tipos de organismos. Berlioz-Barbier et al. [69] estudiaron la posible bioacumulación de diferentes compuestos farmacéuticos entre los que se encontraba CP y TAM en invertebrados bentónicos mediante una técnica QuEChERS modificada y miniaturizada. Con este método, lograron recuperaciones del 70% para TAM y del 95% para CP, obteniendo un LOQ de 30 $ng \cdot g^{-1}$ para CP y 161.6 $ng \cdot g^{-1}$ para TAM. No se llegaron a detectar estos compuestos en las muestras analizadas.

1.3. Sistemas de detección para compuestos antineoplásicos

1.3.1. Espectrometría de masas

Después de la extracción y preconcentración de los analitos, tanto de matrices líquidas como sólidas, es necesario el uso de una técnica adecuada de separación y determinación.

La cromatografía líquida (CL) es la técnica preferida para la separación de compuestos no volátiles. El gran desarrollo de esta técnica se debe, fundamentalmente, a los avances conseguidos en la instrumentación, donde la cromatografía líquida de ultra alta resolución (UHPLC) es la forma más novedosa. Este nuevo formato es capaz de trabajar a presiones muy altas, logrando completar el cromatograma en menos tiempo y usando menor cantidad de disolventes orgánicos.

La espectrometría de masas (MS) es la técnica más aplicada en la detección y determinación de compuestos citostáticos [17]. La MS mide la masa de iones gaseosos que se producen a partir de las moléculas del analito siendo capaz de detectar, directamente, la masa del analito e, incluso, fragmentos de la molécula y seleccionando los iones en función de su relación masa/carga (m/z).

En los primeros trabajos publicados, los compuestos antineoplásicos eran separados y detectados mediante cromatografía de gases con espectrometría de masas (CG-MS) ya que la CL no cumplía las condiciones necesarias para muestras ambientales [32]. Sin embargo, para la aplicación de la CG, es necesario derivatizar los analitos, por lo que las mejoras en CL hace que esta primera técnica se haya visto desplazada en

pocos años y los posteriores trabajos usan, en su mayoría, CL. Aun así, ciertos compuestos como 5-FU han seguido siendo detectados y determinados por GC-MS [70].

La fuente de ionización seleccionada en CL, después de que los analitos se hayan separado en la columna, es fundamental para que entren como iones al detector de masas. Entre las diversas fuentes de ionización existentes, la ionización por electrospray (ESI) es la más utilizada en la determinación de compuestos antineoplásicos [17]. Esta técnica es capaz de producir iones de compuestos no volátiles o termolábiles y puede producir iones de carga múltiple por lo que el valor m/z de los iones de macromoléculas caen en el rango de los espectrómetros de masas más usados. La ESI es una de las interfaces más efectivas y exitosas para CL-MS ya que la ionización suave permite determinar la masa precisa de moléculas pequeñas [71].

La espectrometría de masas en tándem (MS/MS) ha supuesto una mejora de la tradicional al emplear un segundo espectrómetro de masas. La versión más avanzada de esta técnica es la espectrometría de masas de triple cuadrupolo (TQ-MS). En esta ocasión, el primer y tercer cuadrupolo son selectivos de masas (transmiten iones de un determinado valor m/z), mientras que el segundo cuadrupolo opera, únicamente, en modo radiofrecuencia (transmite iones de cualquier relación m/z), sirviendo de cámara de colisión. El primer cuadrupolo selecciona el ion precursor que se transmite a la cámara de colisión y el tercer cuadrupolo analiza los productos que salen de la celda de colisión [71].

Ternes et al. [72] comenzaron a aplicar CL-ESI-MS/MS para la determinación de IF y CP, no sólo en aguas residuales, sino también en agua de río y agua apta para el consumo humano. Pese a que en el trabajo no se habla de la posible pérdida de señal por las interferencias de la matriz, los resultados con CL-ESI-MS/MS muestran ser menos sensibles a las interferencias que con CG-MS ya que, por ejemplo, para el compuesto CP, el LOD en agua de río fue 50 $ng \cdot L^{-1}$ y en aguas residuales 250 $ng \cdot L^{-1}$ usando CG-MS, mientras que usando CL-ESI-MS/MS el LOD se mantuvo en 10 $ng \cdot L^{-1}$ en ambas matrices.

La evolución de las distintas técnicas de detección por espectrometría de masas ha promovido su aplicación en los distintos campos de la química analítica, solucionando algunos de los problemas que presentan la espectrometría de masas en tándem. La determinación de compuestos a nivel de trazas en muestras medioambientales mediante MS/MS muchas veces se ve limitada por la baja señal de la segunda transición, impidiendo la confirmación del analito.

Por ello, la Espectrometría de Masas con Tiempo de Vuelo (ToF-MS) trata de solucionar este inconveniente. Esta técnica mide el tiempo que tarda en llegar un ion desde la fuente de ionización hasta el detector. Todos los iones reciben la misma energía cinética pero, debido a que tienen distinta relación m/z, adquieren distintas velocidades [71].

Garcia-Ac et al [73] fueron los primeros en aplicar esta técnica a una mezcla de compuestos farmacéuticos entre los que se encontraban los antineoplásicos CP y MET. En dicho trabajo, los autores trataron de optimizar un procedimiento de extracción de volúmenes relativamente

grandes mediante SPE on-line para conseguir límites de detección que permitieran determinar dichos fármacos en aguas superficiales. Estos dos compuestos antineoplásicos, junto con otros fármacos, presentaban poca intensidad en su segunda transición y su confirmación era imposible mediante MS/MS. Sin embargo, de los dos compuestos, solo CP pudo ser detectado por CL-ToF-MS a niveles algo más alto que mediante MS/MS, lo que resultó ser un factor limitante de esta técnica. Sin embargo, usando ToF-MS se obtiene mayor resolución y peor sensibilidad.

El detector de trampa de iones cuadrupolo (QIT) trabaja a una presión relativamente alta para ser un espectrómetro de masas, la cual se mantiene por un flujo continuo de helio o argón en el interior del espectrómetro [71]. Esta instrumentación fue utilizada por Valcárcel et al. [74] para la detección de diversos compuestos farmacéuticos en aguas de rio, siendo el primer trabajo en detectar IF a 41 $ng \cdot L^{-1}$ en ríos.

Ferrando-Climent et al. [44] utilizaron la trampa de iones cuadrupolo lineal (Q-LIT) junto con su herramienta de adquisición de información (IDA)[44] en la determinación de diez compuestos antineoplásicos. La herramienta de adquisición de información se usó en el estudio de metabolitos y productos de transformación de los compuestos antineoplásicos detectados, concretamente hidroxi-tamoxifeno (el principal metabolito humano conocido de TAM), 4,4-dihidroxi desmetiltamoxifeno (segundo metabolito humano del TAM) y carboxifosfamida (un metabolito de la CP), mediante el peso molecular teórico de los iones en los cromatogramas. Aunque para una confirmación precisa haría falta el correspondiente patrón, los autores fueron capaces

de detectar estos metabolitos en aguas residuales de hospitales por primera vez.

Usando una combinación de dos detectores (TQ y Q-LIT), Negreira et al. [46] optimizaron un procedimiento para la extracción de 17 compuestos citostáticos entre los cuales se encontraban 4 metabolitos. Los resultados muestran la presencia de un metabolito, 6(α)-hydroxypaclitaxel (OH-PAC), pero no del compuesto padre PAC mostrando la necesidad de estudiar no solo los compuestos originales, sino también los metabolitos o productos de degradación que se pudieran formar.

El detector Orbitrap es uno de los detectores de masas más nuevos. Ofrece una alta sensibilidad a la fragmentación de la espectrometría de masas y bajos límites de detección en escaneo completo, además de una masa precisa tanto para calcular la composición elemental más favorable como de los iones producto. Gómez-Canela et al. [51] optimizaron un procedimiento de extracción de CP y EPI de aguas residuales, usando cartuchos de SPE como preconcentración de los contaminantes e inyección directa y Orbitrap como detector. En este trabajo llegan a obtener un LOQ de 3.1 $ng \cdot L^{-1}$ para CP y 85 $ng \cdot L^{-1}$ para EPI, mediante inyección directa. En un trabajo posterior, compararon MS/MS con Orbitrap para la detección de una mezcla de 26 compuestos citostáticos, encontrando que ambos equipos son suficientemente selectivos pero el Orbitrap es hasta 100 veces más sensible [75].

1.3.2. Otras técnicas instrumentales empleadas

Dependiendo del compuesto citostático que se quiera analizar es posible usar distintos sistemas de detección que puedan ser más

asequibles o sencillos de utilizar. En los primeros años del siglo XXI, la CG-MS siguió utilizándose como técnica de detección para otros compuestos citostáticos como TAM y 5-FU [31,76]. Para la determinación de 5-FU se ha usado la electroforesis capilar (CE) [37,38]. Esta técnica se basa en el movimiento de iones, sustancias neutras o migración pasiva que se repelen o son atraídas por un campo eléctrico dentro de un capilar [77].

Por otro lado, hay compuestos citostáticos que pueden ser determinados con un detector de fluorescencia (FD). Algunos compuestos determinados de esta forma son las antraciclinas EPI, DOX y DAU [37,39], [47]; con LOD que varían entre 50 $ng \cdot L^{-1}$ hasta 290 $ng \cdot L^{-1}$; el antineoplásico irinotecan (IRI) [47] y los compuestos ETO, melfalán (MELP) y vinblastina (VINB) [34], obteniendo LOD de 20 – 100 $\mu g \cdot L^{-1}$. Otro detector utilizado es el detector de matriz de diodo (DAD) para la determinación de los compuestos carmustina, clorambucilo (CHLO), Cis-Pt, CP, CYT, 5-FU y MET [34], logrando LOD que varían de 0.02 – 80$mg \cdot L^{-1}$.

Un grupo particular de medicamentos contra el cáncer, los compuestos citostáticos basados en platino, principalmente Cis-Pt, carboplatin (Car-Pt) y Oxa-Pt, suelen ser determinados por plasma acoplado inductivamente con espectrometría de masas (ICP-MS) [37,78–85]. En esta técnica la muestra se aspira en el conjunto de la antorcha, donde los solutos se desolvatan, se atomizan y se ionizan en un medio no oxidante y muy energético. Esta técnica también fue aplicada, pero con detector de espectrometría de emisión óptica (ICP-OES) por Ghafuria et al. [86] para la determinación de compuestos de platino en dos hospitales, con un LOD de 1000 $ng \cdot L^{-1}$.

Por otro lado, la separación de los compuestos de platino para su identificación como molécula y no como platino total ha sido bastante complicada. Como se comentó, el ICP-MS se puede acoplar a un sistema de cromatografía líquida, pero debido a la polaridad de los compuestos de platino estudiados, su separación con las columnas tradicionales C_{18} de fase reversa es muy complicada. Falter y Wilken [85] lograron separar e identificar Cis-Pt y Car-Pt usando columnas de fase reversa C_{18}, al igual que Hann et al. [80], quienes usaron una columna C_{18} basada en un pentafluorofenilpropilo como fase estacionaria para la separación de Cis-Pt, Car-Pt y Oxa-Pt. Los LOD en esta ocasión fueron 90 $ng \cdot L^{-1}$ para el Cis-Pt, 100 $ng \cdot L^{-1}$ para el Oxa-Pt y 150 $ng \cdot L^{-1}$ para el Car-Pt. Más recientemente, Vidmar et al. [84] separaron e identificaron gracias a una columna HILIC el compuesto Cis-Pt y un complejo hidrolizado del mismo $[PtCl(OH_2)(NH_3)_2]^+$. Los LOD para el Cis-Pt variaban entre 27.3 $ng \cdot L^{-1}$ y 172.6 $ng \cdot L^{-1}$.

1.3.3. Efecto matriz

El efecto matriz es un problema que afecta, sobre todo, a las muestras ambientales complejas. Se caracteriza por una supresión o aumento de la señal por las interferencias extraídas junto con los analitos, influyendo considerablemente en la calidad de los resultados. De hecho, algunos autores han optado por añadir un paso de limpieza para eliminar las interferencias, aunque ello significase reducir considerablemente la recuperación de sus compuestos [40]. Fue, a partir de 2012, cuando los investigadores que trabajan con mezclas de compuestos citostáticos o trabajos multi-residuo que incluyen compuestos citostáticos, empezaron a comentar los inconvenientes de este efecto, cómo evaluarlo y cómo tratar con él.

Gómez-Canela et al. [51] atribuyeron la baja recuperación de EPI a una posible supresión de la señal debido al efecto matriz o a la baja estabilidad del compuesto en una solución acuosa. Sin embargo, no se evaluó por lo que no se valoró cuál podría ser el verdadero efecto de la baja recuperación para ese compuesto.

Para evaluar el efecto matriz, Ferrando-Climent et al. [44] usaron la siguiente expresión:

$$ME\ (\%) = \left(\frac{W}{N}\right) * 100$$

dónde N es la pendiente de una curva de calibrado hecha con la fase móvil que se compara con W, la pendiente de una curva de calibrado hecha con los extractos obtenidos después de la extracción del analito (las llamadas Matrix Match Calibration (MMC)). De esta forma, un valor de efecto matriz superior al 100% indicaría aumento de la señal y un valor inferior al 100% indicaría supresión de la señal. En este trabajo, los autores obtuvieron valores de hasta un 13% de supresión de señal y hasta un 778% de aumento de la señal, lo cual remarca la importancia de este efecto, ya que los resultados pueden variar considerablemente.

En la mayoría de los trabajos de extracción de compuestos antineoplásicos de muestras ambientales se usaron cartuchos Oasis HLB. Estos cartuchos son capaces de extraer multitud de contaminantes simultáneamente. Por este mismo motivo arrastran muchas interferencias presentes en la matriz, sobre todo cuando se trabaja con matrices tan complejas como aguas residuales dando lugar, en la mayoría de los casos,

a supresión de la señal a la hora de su determinación [49]. Otros trabajos en los que se estudian aguas más “limpias” el efecto matriz resulta mucho menor, dando lugar a un aumento de la señal máximo del 49% y una supresión de señal máxima de 32% [53].

Usando cartuchos de intercambio iónico Oasis WAX, Santos et al. [55] lograron un efecto matriz bajo, ya que las interferencias extraídas por este cartucho son menores. En el caso del agua de rio analizada, prácticamente no se ve influenciada por el efecto matriz, ya que este varía entre el -20 – 13%, mientras que para el agua residual fue algo mayor, entre -25 – 46%, aun así, siendo menor que el obtenido por los cartuchos Oasis HLB.

En el caso del SPE online resulta mucho más complicado evaluar el efecto matriz. El proceso de extracción y determinación se realiza en continuo, la pérdida de señal del analito puede ser debida tanto a baja recuperación por el cartucho como a interferencias durante la ionización. Negreira et al. [46] evaluaron el efecto matriz comparando el área de los picos de muestras reales contaminadas, restándoles la posible cantidad de analito que pudiera contener la muestra real que no ha sido contaminada, con una muestra de agua de calidad HPLC contaminada, obteniendo pérdidas de analito de hasta el 90%. Finalmente tuvieron que usar compuesto marcados isotópicamente (ILC) para poder solucionar el problema, lo cual puede resultar muy caro cuando se trata de una mezcla de compuestos que tienen propiedades y comportamientos muy diferentes y haría falta prácticamente un ILC para cada uno de los analitos. Lopez-Serna et al. [43] evaluaron el efecto matriz de igual forma, ya que disponían de 51 estándares internos para sus 74 compuestos.

En los casos en los que no se dispone de estándar interno, la opción más adecuada es una MMC o adiciones estándar. Comparando los picos de muestras reales contaminadas con los picos del disolvente contaminado a la misma concentración obtuvieron que, para el TAM, en matrices relativamente limpias, no se producía prácticamente efecto matriz. Sin embargo, en influentes de estaciones depuradoras se perdía señal (-40% para el TAM).

La principal desventaja de esta forma de evaluar el efecto matriz es que es necesario suponer la misma recuperación en el cartucho tanto para una muestra limpia como para compleja. Esta retención puede no ser la misma, por lo que no se consigue aclarar si esa pérdida se producía durante la extracción o por interferencias durante la determinación.

Rabii et al. [47] usaron el método de adiciones estándar para compensar el efecto matriz. Para ello, dividió el área obtenida para un agua residual contaminada a una concentración dada, a la que se le restó el área de la cantidad de analito que pudiera haber en la muestra de agua residual, entre el área de un blanco de agua contaminado a la misma concentración. De esta forma, valores inferiores al 100% indican supresión de la señal, mientras que valores superiores al 100% indican aumento de la señal. El efecto matriz cuantificado en este trabajo varió entre 55 – 118%.

El efecto matriz es menor en el caso del uso de las técnicas de microextracción. Así, en el caso de la aplicación de la DLLME, la supresión de señal fue menor del 10% para los cuatro compuestos estudiados (DOX,

DAU, EPI e IRI) [59] y en el caso de la aplicación de la FPSE, se observó un aumento de la señal hasta un 40%, aproximadamente [58].

El efecto matriz está también presente en la extracción de analitos de muestras sólidas. En los primeros trabajos, este efecto no era mencionado ni evaluado, pero cuando se tiene en cuenta se observa que puede ser el factor más limitante y la técnica de extracción resulta ser el factor clave.

Cuando se optimizó un procedimiento de extracción mediante PLE de IF y CP en lodos, se encontró que el efecto matriz era el factor más limitante, por lo que tuvieron que usar estándares deuterados [66]. La aplicación de QuEChERS modificados para la extracción de TAM y CP, entre otros contaminantes, en invertebrados bentónicos mostró un importante efecto matriz, con una supresión de la señal de aproximadamente el 55% [69]. Lopez-Zavala et al. [63] trabajaron en la extracción de varios contaminantes en compost, entre los que se encontraban CP e IF, extrayéndolos con USE y los autores aseguraron que no se extrajeron prácticamente interferencias con el procedimiento optimizado y que el efecto matriz observado era muy bajo, inferior al ±5% para los compuestos citostáticos.

1.4. Fármacos citostáticos en muestras ambientales: Una actualización de los procedimientos de extracción y determinación

Como se ha comentado a lo largo de esta Introducción, los compuestos citostáticos albergan diferentes familias de compuestos con diferentes propiedades físico-químicas y de funcionamiento. El interés que despierta la problemática ambiental de estos compuestos no se puede estudiar en profundidad debido a la escasez de datos respecto a su presencia ambiental. Debido a ello, se hace necesario caracterizar su presencia y distribución en los diferentes compartimentos medioambientales, puesto que ello ayudará a crear futuras estrategias de remediación y prevención de la contaminación.

Como ya se ha comentado, los compuestos citostáticos se encuentran en las muestras ambientales a bajas concentraciones y su determinación es complicada debido a la complejidad de las mismas. Por ello, es necesario disponer de metodologías analíticas que permitan alcanzar la sensibilidad y selectividad adecuada para la determinación de estos compuestos en estas muestras, que incluya una reducción en el tiempo de análisis y en se pretratamiento.

Desde la publicación de Kosjek y Heath [87] en 2011, se han realizado diferentes mejoras en cuanto a técnicas de extracción y determinación, nuevas aplicaciones y nuevos enfoques para el análisis de los compuestos citostáticos. Por ello, se realizó un trabajo, en forma de artículo, en el que se llevó a cabo una actualización de las metodologías analíticas desarrolladas para el análisis de los compuestos citostáticos en diversas

matrices, ya que el número de trabajos relacionados con esta temática ha aumentado considerablemente en los últimos años.

De esta publicación cabe destacar que:

- La bibliografía respecto a la presencia de estos compuestos en muestras ambientales es escasa. La mayoría de los trabajos se han enfocado a la determinación en aguas residuales procedentes de las estaciones depuradoras de aguas residuales y aguas residuales hospitalarias y solo unos pocos han analizado su presencia en aguas de rio o aguas subterráneas.
- La Espectrometría de Masas y sus variantes son las técnicas de determinación de compuestos citostáticos más aplicadas. Por otro lado, frente a las técnicas de separación, se observa que la cromatografía de gases ha sido desplazada en favor de la cromatografía líquida, ya que evita la derivatización de las muestras, para conseguir la volatilización de los analitos.
- De las técnicas de extracción aplicadas hasta la fecha para compuestos citostáticos en muestras acuosas, la extracción en fase sólida (SPE) es la técnica más empleada. Se usan diferentes tipos de cartuchos, pero en general, el cartucho Oasis HLB es el más utilizado para la extracción de mezclas de compuestos de matrices medioambientales.
- Para la extracción en muestras sólidas, la bibliografía es escasa. En los trabajos publicados se ha empleado la Extracción por Ultrasonidos (UAE) y la Extracción con líquidos presurizados (PLE).

De acuerdo con nuestra revisión, las concentraciones encontradas en los efluentes de aguas residuales hospitalarias están en el orden de los $\mu g \cdot L^{-1}$, mientras que en aguas residuales de las estaciones depuradoras de aguas residuales, aguas superficiales y aguas de ríos, están en torno a los $ng \cdot L^{-1}$.

Cabe destacar que muchos de los trabajos se han orientado hacia la extracción de un mayor número de compuestos, con la mejor recuperación posible y en el menor tiempo. Por otro lado, se observa que, en muchos trabajos, los compuestos citostáticos no son detectados en el medio, probablemente porque sufren algún tipo de transformación o son excretados por los pacientes en forma de metabolitos. Es por eso que los trabajos más recientes se centran, también, en la búsqueda de metabolitos y productos de transformación.

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Cytostatic drugs in environmental samples: An update on the extraction and determination procedures

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ABSTRACT

Antineoplastic or cytostatic compounds are used to fight cancer. They are a broad group of organic compounds, possessing a diverse range of physico-chemical characteristics. Given the toxicity of these compounds, the development of reliable analytical methods for their analysis became necessary. Cytostatic compounds are found in very low concentrations, and their determination is even more complicated by the complex matrices in which they are bound. These are considered to be the emerging contaminants, and there is little knowledge about their products of degradation and their possible toxicity. Hence, it is essential to obtain data about the presence of cytostatic compounds and their metabolites in environmental samples. Obtaining such data will require advanced sampling techniques and analytical tools, including the latest separation and determination methods and instrumentation. In this overview, we discuss the current methods used for extraction and quantitative determination of these compounds in liquid and solid environmental samples.

Contents

Abbreviations: 5-FU, 5-Fluorouracil; ACT-D, actinomycin-D; A-GLU, Aminoglutethimide; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; araU, 1-β-d-arabinofuranoside; AZA, azathioprine; BLEO, bleomycin; BP1, 3′-O-desmethyl etoposide; BSA, bovine serum albumin; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CAP, capecitabine; Car-Pt, carboplatin; CE, Capillary Electrophoresis; CHLO, Chlorambucil; CIPRO, Ciprofloxacin; Cis-Pt, cisplatin; CP, cyclophosphamide; CPC, Cancerostatic Platinum Compounds; CYP, Cyproterone; CYT, cytarabine; DACAR, dacarbazine; DAD, Diode Array Detection; DAU, daunorubicin; dFdU, 2′,2′-difluorodeoxyuridine; Di-Pt, diaquacisplatin; DOC, docetaxel; DOX, doxorubicin; DOXOL, doxorubicinol; EI, electron impact; EPI, Epirubicin; ERLO, erlotinib; ESI, electrospray ionization; EtAc, Acetato de etilo; ETO, etoposide; F. A., Formic Acid; FD, Fluorescence Detector; FL, Florisil®; FLU, Fludarabine; GC, Gas Chromatography; GEM, gemcitabine; GOS, Goserelin; H-ESI, Heated Electrospray Ionization; HILIC, hydrophilic interaction liquid chromatography; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IDL, instrumental detection limits; IF, ifosfamide; IMA, imatinib; IRI, irinotecan; LC, Liquid Chromatography; LEU, Leuprolide; LLE, Liquid–Liquid Extraction; LOD:, Limit of Detection; LOQ:, Limit of Quantification; MAE, Microwave-assisted Extraction; MDL:, Method Detection Limit; MEG, Megestrol; MEL, Melphalan; MeOH, Metanol; MERCAP, mercaptopurine; MET, methotrexate; MI, Mitotic Indexes; MISPE, Molecularly imprinted solid-phase extraction; MIT, mitoxantrone; MIT-C, Mitomycin C; Mono-Pt, monoaquacisplatin; MS, Mass Spectrometry; MS/MS, tándem mass spectrometry; MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide; MTBSTFA, N-methyl-N-[tert-butyldimethylsilyl]-trifluoroacetamide; MTIC, 5-(3-N-methyltriazen-1-yl)-imidazole-4-car-boxamide; N. D., not detectable; OH-D-TAM, 4-hydroxy-N-desmethyl-tamoxifen; OH-MET, hydroxymethotrexate; OH-PAC, 6(-)-hydroxypaclitaxel; OH-TAM, (Z)-4-hydroxytamoxifen; Oxa-Pt, oxaliplatin; PAC, paclitaxel; PFBBr, pentafluorobenzyl bromide; PGC, porous graphitic carbon; PLE, Pressurized Liquid Extraction; PRED, Prednisone; PROCAR, procarbazine; QqLit, triple quadrupole–linear ion trap; QqQ, Triple quadrupole; QqTOF, coupled to quadrupole time-of-flight; RP-LC-FD, Reverse-phase Liquid Chromatography with Fluorescence Detector; SFE, supercritical fluid extraction; SPE, Solid-phase Extraction; TAM, tamoxifen; TCB, 1,2,3,5-tetrachloro-benzene; TEM, temozolomide; TF, trofosfamide; TFA, trifluoroacetic anhydride; TMS, trimethylsilyl; ToF, Time of Flight; TP, Transformation Product; UAE, Ultrasonic-assisted Extraction; UHPLC, Ultra-High Performance Liquid Chromatography; VINB, Vinblastine; VINC, vincristine; VINO, Vinorelbine; WWTP, wastewater treatment plant.

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1. Introduction

Antineoplastic or cytostatic agents are compounds used in chemotherapy to fight cancer. These agents have been designed to disrupt or prevent cell proliferation, usually by interfering with DNA synthesis [1]. Chemotherapy, along with surgical excision and irradiation, is one of the three main approaches established for treatment of this disease [2].

Cytostatic drugs can be classified according to their mechanism of action, with the most important groups being antimetabolites, DNA-interactive agents, antitubulin agents, molecular targeting agents, hormones, monoclonal antibodies and other biological agents [3]. This study provides an overview of the following most commonly used drugs:

- Antimetabolites are one of the oldest families of anticancer drugs; their mechanism of action is based on the interaction with the essential biosynthesis pathways. 5-Fluorouracil (5-FU) and mercaptopurine (MERCAP) are typical pyrimidine and purine analogues, respectively. Other antimetabolites, such as methotrexate (MET), interfere with the essential enzymatic processes of metabolism.
- DNA-interactive agents constitute one of the largest and most important anticancer drug families, acting through a variety of mechanisms:
  - Alkylating agents lead to the alkylation of DNA bases in either the minor or major grooves. Examples of these compounds include dacarbazine (DACAR), procarbazine (PROCAR) and temozolomide (TEM).
  - Cross-linking agents work via DNA binding, resulting in an intra-strand or inter-strand cross-linking of DNA. Platinum complexes (e.g. cisplatin (Cis-Pt), carboplatin (Car-Pt) and oxaliplatin (Oxa-Pt)) and nitrogen mustards (e.g. cyclophosphamide (CP) and ifosfamide (IF)) are the two main groups of this anticancer drug subfamily.
  - Intercalating agents act by binding of base pairs. This family of agents includes anthracyclines (e.g. doxorubicin (DOX) and epirubicin (EPI)), mitoxantrone (MIT) and actinomycin-D (ACT-D).
  - Topoisomerase inhibitors include irinotecan (IRI) and etoposide (ETO) compounds. These drugs inhibit the enzymes responsible for the cleavage, annealing and topological state of DNA.
  - DNA-cleaving agents, such as bleomycin (BLEO), interact with DNA and cause strand scission at the binding site.
- Antitubulin agents interfere with microtubule dynamics (i.e. spindle formation or disassembly), thus blocking the division of the nucleus and leading to cell death. The main members of this family include taxanes and vinca alkaloids [3].

Cytostatic compounds do not act selectively on the growth of cancer cells and rather act on all cells. Moreover, and paradoxically, these compounds are potentially carcinogenic. Due to their mode of action, virtually all eukaryotic organisms are vulnerable to damage, with teratogenicity being the greatest concern at low concentrations (ng L^{-1}) [4]. However, there are no threshold values to estimate the minimum harmful concentration, and the huge increase in cancer cases among the population has promoted the use of chemotherapy drugs. The number of cases was estimated at 3.2 million cases of cancer diagnosed for 2006 [5], while the forecast for 2012 was about 3.5 million [6]. According to the International Agency for Research on Cancer (IARC), 14.1 million new cancer cases and 8.2 million cancer-related deaths have been estimated per year worldwide, which makes cancer the second leading cause of death [7].

An important feature of cytostatic compounds is that they have low biodegradability in conventional wastewater treatments and thus are considered recalcitrant compounds [8–12]. The main access path of cytostatic agents in the medium is through effluents from hospitals. Although the current trend in chemotherapy is toward the non-hospitalization of patients, the hospitals, where chemotherapies are administered daily, remain a significant source of anticancer drug residues [13]. Once administered, these drugs are excreted in the urine or faeces; a portion of the drugs is metabolized and the rest remains as the original compound. In many cases, the effluents of hospitals are connected to the sewage system without any pretreatment. Therefore, effluents from wastewater treatment plants (WWTPs) could be considered to be the main source of introduction of these compounds and their metabolites into the aquatic environment.

The concentration of these drugs in the influents and effluents of WWTPs are generally in the ng L^{-1} range, but the consequences of this continued input are unknown [14]. Given the inherent nature of anticancer drugs, that is, to kill or inhibit the growth of cells, it is important to establish the risk from the presence of these compounds in the environment [15]. To determine the consequences, studies must be conducted regarding the presence of these drugs and the determination of their concentrations in the aquatic environment once they have entered the waste stream [16].

The risks of anticancer drugs to humans are not very clear mainly because of the lack of toxicity testing in terms of approach and tests to be used. Hence, residues of cytostatic compounds are the emerging pollutants in the environment. Because many of these drugs are genotoxic and they could cause adverse effects in aquatic ecosystems [17], and despite the necessity to obtain data that help us understand the risk of these contaminants in the environment [18], only a small number of studies exist regarding these compounds, with a limited amount of experimental data on the ecotoxicity [19]. As a consequence, one of the challenges in the analytical chemistry field is the development of fast and efficient procedures for the analysis of these emerging compounds.

Studies in fish demonstrate that exposure to 5-FU at relevant concentrations can damage their DNA integrity and induce massive whole-transcriptome changes, which might affect fish populations over long-term exposure of several generations [17]. The application of photo-oxidation processes such as ultraviolet (UV)/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 over 5-FU can eliminate parent compounds and their toxicity but transformation products formed can still be toxic. Therefore, toxicity screening after advanced treatment is recommendable [20].

CP and IF are persistent in the aquatic environment and they can reach drinking waters through the surface waters. Although it has been proven that they can react with the DNA and that the risk is higher for newborns and children than for adults, a safe threshold concentration regarding health effects could not be given [21].

The effects of CP, MET, 5-FU and Imatinib (IMA) over plant seedlings were evaluated and significant differences in the mitotic indexes (MIs) were observed in three of them (MET, 5-FU and CP), thus indicating their potential cytotoxic activity. All of them caused the formation of micronucleated cells indicating mutagenic potential. Moreover, assays performed for MET presented a high number of cell death, indicating that these compounds may affect the growth

and normal development of these plants [22]. Moreover, the study of the acute toxic and genotoxic properties of 5-FU, ETO, Cis-Pt, Car-Pt, vincristine (VINC) and CP concluded that all compounds caused genotoxic effects [23].

In this context, sensitive analytical methods capable of detecting these contaminants at low concentrations in the aquatic environment are essential. Furthermore, a high level of selectivity is required to avoid interference due to complex matrix components [24]. Although several traditional sample preparation methods are still in use, the trend in recent years is toward the development of more effective sample preparation methods.

To the best of our knowledge, both offline and online solid-phase extraction (SPE) are the preparation procedures that have been primarily used for the analysis of these drugs in liquid samples [25], including SPE with new materials. We only found one study wherein liquid–liquid extraction (LLE) was used for the extraction of cytostatic compounds [26] as a previous step for the purification using SPE. According to our literature review, microextraction techniques have not been used.

The bibliography regarding the presence of these compounds in environmental liquid samples is scarce. Most of the relevant works are focused on the analysis of sewage waters from WWTPs or hospital effluents, and only a few studies consider river water or groundwater samples for the analysis of cytostatic compounds.

Cytostatic drugs can also be accumulated in solid matrices, mainly in the sludges from the WWTPs; however, the information available about this accumulation is even scarcer than the information on the liquid samples. To the best of our knowledge, only three papers have been published that describe the use of ultrasonic-assisted extraction (UAE) [27,28] and pressurized liquid extraction (PLE) [27,29] techniques for the extraction of these compounds from sludges.

Different methodologies have been applied to determine some of these compounds such as inductively coupled plasma mass spectrometry (ICP–MS) [30] or capillary electrophoresis (CE) [31]. However, as seen in the last decade with most of the applications in environmental samples, gas chromatography (GC) and liquid chromatography (LC) combined with MS detection are the most important techniques used for the analysis of these compounds [14]. Thus, in this overview, we focused on the analytical methodologies for the determination of cytostatic compounds in environmental liquid and solid samples using GC and LC.

In this overview, we revise and update the trends in the extraction and determination of cytostatic drugs in environmental samples to update a previous review published in 2011 by Kosjek and Heath [2], where most of the studies on the determination in environmental samples were performed in 1990–2000. Table 1 shows some physico-chemical characteristics of the most detected cytostatic compounds in environmental samples.

2. Transformation products and metabolites

Besides the parent compound, active metabolites and transformation products (TPs) should be included in the determination methods because they can appear in the environment and might therefore contribute to their biotoxic and mutagenic potential effects [33]. Hence, some authors have begun to study the metabolites and TPs of cytostatic compounds. Different studies about the stability of cytostatic compounds have been carried out by Negreira et al. [34,35], demonstrating that half of the studied compounds were unstable in water. The experiments included different storage conditions, and it was found that –20°C is the most favourable temperature. However, some cytostatic compounds such as (5-(3-N-methyltriazen-1-yl)-imidazole-4-car-boxamide (MTIC), IMA, DOX, 6(α)-hydroxypaclitaxel (OH-PAC), paclitaxel (PAC), erlotinib (ERLO) and chlorambucil (CHLO)) were not stable for >1 month [34]. The authors concluded that the samples must be frozen as soon as possible in order to minimize degradation and erroneous measurements because unrealistically low values may be obtained for most compounds when they are stored at 4°C. In some cases, acidification may improve the stability but it may also affect negatively [35].

Chemical disinfectants can provoke degradation reaction in many compounds, generating by-products. Hence, ETO by-products in chlorinated water have been investigated and measured in wastewater samples, whereas parent compound (ETO) was not detected [36]. Similarly, Roig et al. [37] studied the behaviour of MET during chlorination in a batch trial, in order to detect the possible TPs. Using different spectroscopic and chromatographic techniques, they concluded that monochloro-MET is likely to be one of the main stable transformation products formed during chlorination process.

TPs of MET formed during biological breakdown process have also been identified, and 2,4-diamino-N_{10}-methyl-pteroic acid has been found to be most abundant and persistent. In the same study, other biotransformation reactions such as demethylation, oxidative cleavage of amine, cleavage of C–N bond, aldehyde to carboxylate transformation and hydroxylation were observed [38].

In the context of possible remediation, experiments concerning the degradation of 5-FU by three different advanced photo-oxidation processes (UV/H_2O_2, $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2) demonstrated that the original compound is quickly removed in all the irradiation experiments leading to the formation of six transformation products. Most of these products were formed and further eliminated during the reactions. In the same work, biodegradability studies demonstrated that 5-FU was not biodegraded, whereas the photolytic mixture formed in the UV/H_2O_2 treatment showed a noticeable improvement of the biodegradability [20].

Finally, it is worth noting that sometimes the metabolites generated from cytostatic compounds are of great interest from an environmental point of view compared to the parent molecules. For example, tamoxifen (TAM) is converted into the metabolites endoxifen or 4-hydroxy-N-desmethyltamoxifen (OH-D-TAM) and (Z)-4-hydroxytamoxifen (OH-TAM), which are 30- to 100-fold more potent than TAM. MET is metabolized and/or biodegraded to hydroxymethotrexate (OH-MET), while PAC is metabolized to OH-PAC [39]. 1-β-d-arabinofuranoside (AraU) and 2′,2′-difluorodeoxyuridine (dFdU) are also metabolites frequently studied in wastewater; they were found to present at higher concentrations than the parent compounds [40]. In hospital effluents, metabolites such as dFdU have been measured at high concentrations than 5-FU and gemcitabine (GEM) [41].

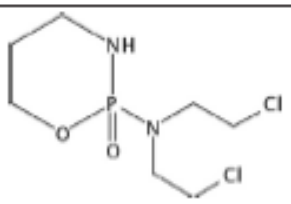
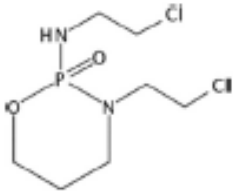
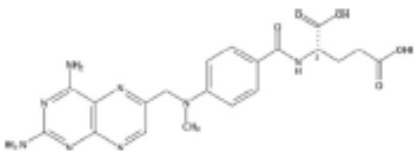
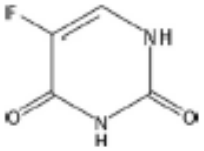
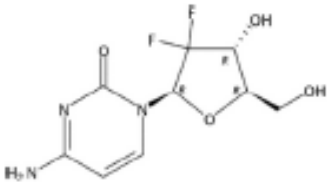
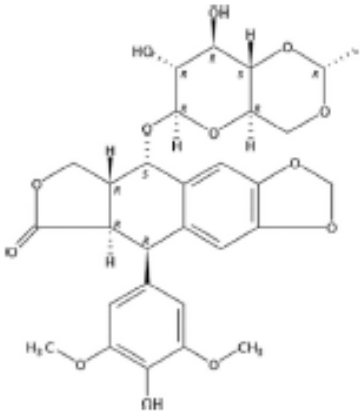
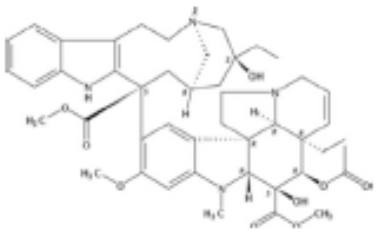
3. Determination methods

As previously stated, LC and CG followed by MS are nearly the sole determination techniques used for the determination of cytostatic drugs. Among these techniques, LC is the selected approach in >75% of cases.

The first determinations of cytostatic agents in wastewater were performed in the 1990s using derivatization with trifluoroacetic anhydride (TFA) in GC [10,42]. Despite the low concentrations of these compounds that can be measured using this technique (6–146 ng/L), almost all the subsequent analyses have been performed using LC to avoid the derivatization of little volatile compounds (generally with high molecular weight compounds) and the drawbacks related with the time of sample pretreatment and the incapability to determine very polar compounds or TPs [43].

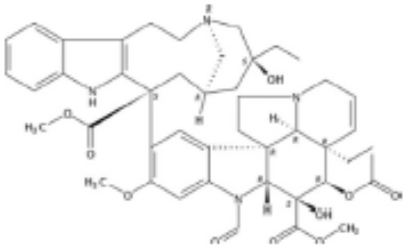
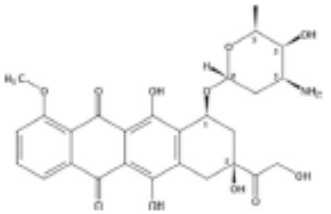
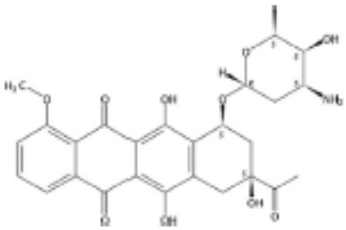
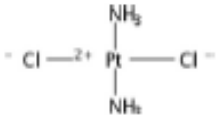
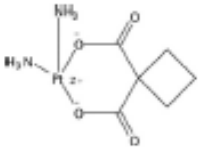
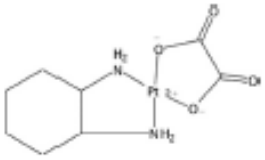
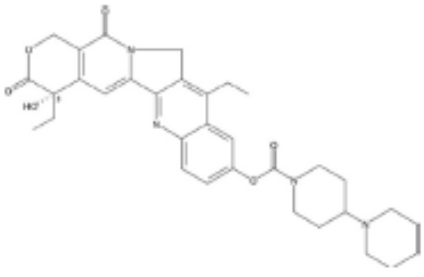
LC coupled to MS/MS with electrospray ionization (ESI) in positive mode is undoubtedly the most used technique in the determination of cytostatic compounds. Only Kovalova et al. [40] have used ESI in a negative mode and analysed 5-FU, cytarabine (CYT), GEM, araU and dFdU in wastewater. Weissbrodt et al. [41] used the same procedure with the addition of ESI in positive mode

Table 1
Characteristics of the most detected cytostatic compounds in environmental samples[a]

Name	CAS	Properties		Structure
cyclophosphamide	50-18-0	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	261.09 2.84 ± 0.20 49.5–53°C 336.1 ± 52°C 1.33 ± 0.1 g cm^{-3}	
ifosfamide	3778-73-2	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	261.09 1.44 ± 0.20 39–41°C 336.1 ± 52°C 1.33 ± 0.1 g cm^{-3}	
methotrexate	59-05-2	Molecular Weight: pKa (predicted): Melting Point: Density:	454.44 3.47 ± 0.10 195°C 1.536 ± 0.06 g·cm^{-3}	
fluorouracil	51-21-8	Molecular Weight: pKa (predicted): Melting Point: Density:	130.08 6.73 ± 0.10 282°C 1.53 ± 0.1 g cm^{-3}	
gemcitabine	95058-81-4	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	263.20 11.65 ± 0.70 72.6°C 482.7 ± 55.0°C 1.84 ± 0.1 g cm^{-3}	
etoposide	33419-42-0	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	588.56 9.94 ± 0.40 236–251°C 798.1 ± 60°C 1.55 ± 0.1 g cm^{-3}	
vinblastine	865-21-4	Molecular Weight: pKa (predicted): Melting Point: Density:	810.97 11.36 ± 0.60 211–216°C 1.37 ± 0.1 g cm^{-3}	

(continued on next page)

Table 1 (*continued*)

Name	CAS	Properties		Structure
vincristine	57-22-7	Molecular Weight: pKa (predicted): Melting Point: Density:	824.96 11.10 ± 0.60 218–220°C 1.40 ± 0.1 g cm^{-3}	
doxorubicin	23214-92-8	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	543.52 7.35 ± 0.60 204 °C 810.3 ± 65°C 1.61 ± 0.1 g cm^{-3}	
daunorubicin	20830-81-3	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	527.52 7.39 ± 0.60 208–209°C 770 ± 60°C 1.55 ± 0.1 g cm^{-3}	
cisplatin	15663-27-1	Melting Point: Density:	270°C 4E-6 g cm^{-3}	
carboplatin	41575-94-4	Melting Point:	217 °C	
oxaliplatin	61825-94-3			
irinotecan	97682-44-5	Molecular Weight: pKa (predicted): Melting Point: Boiling Point: Density:	586.68 11.20 ± 0.20 222–223°C 873.4 ± 65°C 1.40 ± 0.1 g cm^{-3}	

* Data obtained from [32].

for the analysis of GEM. In both papers, the same range of limits of detection (LODs: 0.9–9 ng L^{-1}) was obtained.

Orbitrap-MS coupled to heated-electrospray ionization (H-ESI) was used to analyse 26 cytostatic compounds commonly found in effluents from hospitals and wastewater [36,44]. In the study of metabolites of anticancer compounds, these compounds are detected in sewage treatment plants but not in effluents from hospitals. As a result, the technique of ultra-high-performance liquid chromatography (UHPLC) coupled to quadrupole–orbitrap–MS (Qq–Orbitrap–MS/MS) has been used to elucidate the degradation of ETO and the determination of its by-products in chlorinated waters [36]. This work showed that ETO degrades in few seconds into two products; the concentration in 3'-O-desmethyl etoposide (BP1), one of the reactions' by-products, is in the range of 14–33 ng L^{-1}.

Despite the use of the most innovative technologies, better results are not obtained in terms of the LODs. The lower LODs were obtained by Nebot et al. [45] and Llewellyn et al. [46], who observed levels of 0.03 ng L^{-1} in wastewater for TAM and CP and IF, respectively, both using SPE and LC with MS.

However, certain cytostatic compounds, specifically pyrimidine analogues, are often determined using GC–MS. Tauxe-Wuersch et al. [26] obtained LODs of 1 ng L^{-1} for TAM and between 15 and 30 ng L^{-1} for 5-FU in wastewater, which are higher than those obtained using LC (Table 2). A GC–MS method developed by Mullot et al. [47] provided an LOD of 12 ng L^{-1} using trimethylsilyl (TMS) to derivatize the 5-FU, which has been tested to prevent its decomposition to another nucleoside, fluoropyrimidine, during the derivatization step and/or chromatographic separation. 5-FU was also analysed by GC with LODs of 0.16 and 0.48 ng L^{-1} in surface and wastewater, respectively [48], which are closer to the lowest LOD value achieved in LC [46]. Moreover, three silylation reagents (N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N-methyl-N-[tert-butyldimethylsilyl]-trifluoroacetamide (MTBSTFA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)) and an alkylation reagent pentafluorobenzyl bromide (PFBBr) were tested for the derivatization of 5-FU in GC-MS with electron impact (EI). MTBSTFA was selected due to the best hydrolytic stability of the derivatives and the most favourable fragmentation and superior chromatographic response [48].

We also found an example of the analysis of cytostatic compounds using a fluorescence detector (FD) as the detection system [49], achieving limits of quantification (LOQs) ranging from 0.26 to 0.29 μg L^{-1}. The authors of this paper measured concentrations between 0.1 and 1.4 μg L^{-1} for EPI and between 0.1 μg L^{-1} and 0.5 μg L^{-1} for DOX in hospital effluents.

4. Extraction methods

4.1. Liquid samples

In recent decades, conventional procedures for the extraction from liquid samples, such as LLE, have been replaced by alternative procedures. Despite LLE providing high recoveries and good repeatability, the technique is operated manually, relatively time consuming, harmful (due to the use of large volumes of organic solvents that are frequently toxic) and is very expensive. To address these deficiencies, some alternative techniques have been developed, including SPE, which is the technique used for the extraction of cytotoxic compounds in liquid environmental samples [14].

The only paper using LLE for the determination of these compounds was published by Tauxe-Wuersch et al. [26] TAM and 5-FU were extracted with dichloromethane from hospital and urban wastewater samples. The solution was evaporated after the addition of 2 mL of methanol to avoid losses of TAM during rotary evaporation. After purification using SPE, the authors measured these compounds at concentration levels of 4 ng L^{-1} in wastewater.

SPE is a sample treatment procedure that enables the concentration and purification of analytes from solution via sorption on a solid sorbent [50], and it is widely used in the environmental analytical field because it involves extraction and preconcentration of the sample in a single step. Moreover, SPE can be automated (online SPE), which solves some of the limitations of the conventional procedure. Online SPE requires a smaller volume of sample and minimizes sample loss or contamination during handling, thus improving the reproducibility of the analysis. Another advantage is the reduction in the analysis time. Automated SPE also provides better sensitivity because the elution is performed using the chromatographic mobile phase, and all of the injected mass reaches the detector.

In the next section, we review different works using both offline and online SPE procedures, with all of the relevant experimental data presented in Table 2.

4.1.1. Offline SPE

Studies on the analysis of cytostatic compounds in wastewater were first performed in the 1990s. In the first of these reports, IF and CP were studied using a C_{18} cartridge and GC–MS [42]. Good LODs (7 ng L^{-1} for IF and 6 ng L^{-1} for CP) were obtained. They obtained recoveries between 30% and 39% and measured concentrations of 24 ng L^{-1} (IF) and 146 ng L^{-1} (CP) in the samples. 1,2,3,5-Tetrachlorobenzene (TCB) and trofosfamide (TF) were used as internal standards for the underivatized and derivatized samples, respectively. The authors indicated that the compounds might have undergone decomposition in the column, resulting in a loss of efficiency, because the response of the standards rapidly changed from one analysis to another for underivatized samples. Kümmerer et al. [10] used the same procedure and achieved 6 ng L^{-1} as the LOD for IF, detecting concentrations of 109 ng L^{-1} in the hospital effluents and concentrations between 6.2 and 9.3 ng L^{-1} in the wastewater. Subsequently, Ternes [51] and Ternes et al. [52] also used C_{18} cartridges for the extraction of IF and CP from wastewater, river water, and tap water. In the later work, the authors compared the detection levels between GC–MS and LC–MS. They achieved LODs of 100–250 ng L^{-1} using GC–MS, but the recoveries were not good (51–57%). Similar recoveries were obtained using LC/MS/MS, but they achieved LODs of 10 ng L^{-1} using dihydrocarbamazepine as the surrogate standard, which is better than those obtained using GC–MS.

Buerge et al. [53] extracted IF and CP from wastewater with reusable columns containing 10 mL of a macroporous polystyrene divinylbenzene adsorbent and LC–MS. The samples were fortified with an internal standard, $^{13}C_3$-caffeine (0.052 ng L^{-1}), achieving LODs in the range of 0.2–0.3 ng L^{-1} and recoveries of 74–102% for both compounds.

Tauxe-Wuersch et al. [26] tested different SPE cartridges to find the most efficient sorbent for the purification of TAM and 5-FU after LLE. Oasis MCX and ENV$^+$ cartridges were used for TAM and 5-FU, respectively, and PFBBr was chosen to derivatize the 5-FU in GC–MS. Concerning the determination in real samples, only TAM was detected in the wastewaters from hospitals, residential areas and from WWTPs. However, the concentrations of this drug were between the LOQ and the LOD (1 and 4 ng L^{-1}) of the technique.

ENV$^+$ cartridges have also been used by other authors in the extraction of pyrimidine analogues [40,41,47,48]. Kosjek et al. [48] conducted a study of 5-FU and its prodrug, capecitabine (CAP), showing that the latter is less degradable. In this work, using UHPLC coupled to quadrupole time-of-flight (QqTOF) –MS, the transformation of these two products was studied by proposing six product transformations for 5-FU and 10 for CAP. They measured concentrations in the range of 4.7–14 ng L^{-1} in wastewater and 35–92 ng L^{-1} in effluents from hospitals.

As mentioned earlier, cytostatic drugs in wastewater are found not only in their original form but also in the form of metabolites. Kovalova et al. [40] studied three cytostatic agents (5-FU, CYT and GEM) and two metabolites (araU and dFd)) in wastewater using Isolute ENV$^+$ cartridges and LC–MS/MS. Isotopically labelled internal standards [^{13}C] [$^{15}N_2$] uridine and [$^{15}N_2$] 5-fluorouracil were used, which is crucial not only for quantification but also for confirming the identity of the measured signal. Concentrations of 27 ng L^{-1} (5-FU), 38 ng L^{-1} (GEM) and 840 ng L^{-1} (dFdU) were found, demonstrating that the metabolite is present in higher concentrations.

Because the concentrations of cytostatic compounds in the effluents from hospitals are higher than those measured in WWTPs, their determination provides essential information about the inputs from this source into the medium. To determine 5-FU, GEM and dFdU, Weissbrodt et al. [41] performed an experiment using the same

Table 2
Methods for the determination of cytostatic compounds in liquid samples

Matrix	Compounds	Extraction condition	Determination	Recovery (%)	LOD (ng L^{-1})	Found concentration (ng L^{-1})	Ref.
Wastewater	IF, CP	Offline SPE Cartridge or column: C18 Conditioning step: 3 ml Hex, 3 ml MeOH, 3 ml water Elution solvent: 2 ml MeOH/acetone (95:5)	Technique: GC-MS Derivatization: 100 μL TFA	39 (CP) 30 (IF)	7 (IF) 6 (CP)	24 (IF) 146 (CP)	[42]
Wastewater Hospital effluents	IF	Same as reference [42]	Same as reference [42]		6	6.2–9.3 (WW) 109 (Hospital)	[10]
Wastewater	IF, CP	Offline SPE: Reusable cartridges	Technique: LC-TIS-MS Mobile phase: (A): water + 0.1% F. A.; (B): MeOH + 0.1% F. A.	74–102	0.2–0.3	1.4–11	[53]
Wastewater	TAM, 5-FU	Offline SPE as purification step after LLE Cartridge or column: Oasis MCX (TAM) Conditioning step: 6 ml MeOH, 1 ml Mili-Q water Elution solvent: MeOH/NH_4OH (95:5) Cartridge: ENV+ (5-FU) Conditioning step: 12 ml MeOH, solution 0.01 mol/L KH_2PO_4 (pH 5 with KOH) Elution solvent: 4 × 3 ml MeOH	Technique: GC-MS Derivatization: 1 ml ACN, 100 μL Milli-Q water with 25% K_2CO_3	81 (TAM) 73 (5-FU)	1 (TAM) 15–30 (5-FU)	4 (TAM) N.D. (5-FU)	[26]
Wastewater	CP, IF	Offline SPE Cartridge or column: Strata-X: Conditioning step: 12 ml EtAc, 12 ml water Elution solvent: 2 × 5 ml MeOH or EtAc	Technique: LC–ESI–MS Mobile phase: (A): water + 0.1% F. A.; (B): MeOH + 0.1% F. A.	57–70	0.03–0.12 (CP) 0.05–0.09 (IF)	4–11 (CP) 2 (IF)	[46]
Wastewater River water	5-FU, GEM, CP, IF, CYT, MET, PAC, ETO, IRI, DOC, EPI, DOX, VINO, MIT-C	Offline SPE Cartridge: Oasis HLB Conditioning step: 3 ml MeOH, 3 ml deionized water Elution solvent: 1 ml MeOH	Technique: LC–ESI–QqQ–MS Mobile phase: (A): ACN + 0.1% F. A.; (B) solution 15 mM ammonium formate + 0.1% F. A.		0.1–38	1.2–15	[55]
Wastewater Hospital effluents	CP, EPI	Offline SPE Cartridge or column: Oasis HLB Conditioning step: 10 ml MeOH, 15 ml HPLC water Elution solvent: 15 ml MeOH.	Technique: LC–ESI–Orbitrap–MS Mobile phase: (A): water + 0.1% F. A.; (B): MeOH + 0.1% F. A.	37–107	0.35–85	5.73–24.8[a]	[54]
Wastewater Hospital effluents	5-FU, CAP	Offline SPE Cartridge or column: Isolute ENV+ Conditioning step: 6 ml MeOH, 6 ml deionized water Elution solvent: 3 × 2 ml MeOH	Technique: GC–MS Derivatization: MTBSTFA		0.16 0.48 (WW)	4.7–14 (WW) 35–92 (hospital)	[48]
Wastewater	5-FU, CYT, GEM, araU, dFdU	Offline SPE Cartridge or column: Isolute ENV+: Conditioning step: 15 ml MeOH, 15 ml solution 10 mM ammonium acetate (pH 6) Elution solvent: MeOH	Technique: LC–ESI–MS/MS (-) Mobile phase: (A): solution 30 mM ammonium acetate and ACN (2/3); (B): ACN	72–127	0.9–9[b]	27 (5-FU) 38 (GEM) 840 (dFdU)	[40]
Wastewater Hospital effluents	5-FU, GEM, dFdU	Same as reference [40]	Technique: LC–ESI–MS/MS Mobile phase: (A): 1% ACN + 0.5% A.F. + 0.1% solution ammonium formiate; (B): ACN	54–118	0.9–9[b]	0.9–839 (hospital) N.D. (WW)	[41]
Hospital effluents	EPI, DOX, DAU	Offline SPE Cartridge or column: C8 Conditioning step: 5 ml MeOH, 5 ml water, 5 ml PBS 2% BSA Elution solvent: 1.5 ml MeOH/$CHCl_3$ (1:2)	Technique: RP–LC–FD Mobile phase: (A): 10 mM buffer K-di-hydrogen phosphate pH 2; (B): ACN	80–90	0.26–0.29[c]	0.1–1.4 (EPI)[a] 0.1–0.5 (DOX)[a]	[49]
Hospital effluents	EPI, DOX, DAU	Same as reference [49]	Same as reference [49]		0.26–0.29[c]	0.3–0.6 (DOX)[a]	[12]
Hospital effluents	Cis-Pt, Car-Pt, Oxa-Pt, Mono-Pt, Di-Pt	Same as reference [61]	Technique: LC-ICP-MS Mobile phase: (A): solution ammonium formate 20 mM + 4% MeOH; (B): water; (C): MeOH	90–116	0.09–0.15[a]	0.3–1.7 [a]	[62]
Hospital effluents	Cis-Pt, Car-Pt, Oxa-Pt, DOX, DAU, EPI	EPI, DOX, DAU → same as reference [49] CPC → same as reference [61]	EPI, DOX, DAU → same as reference [49] CPC → same as reference [62]		0.09–0.15 (CPC)[a] 0.05 (DOX)[a] 0.06 (DAU)[a]	0.26–1.35 (DOX)[a] 30.6–41.3 (CPC)[a]	[11]
Hospital effluents	Cis-Pt, Car-Pt (4)	Same as reference [61]	Same as reference [62]			38.1[a]	[63]

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Table 2 (*continued*)

Matrix	Compounds	Extraction condition	Determination	Recovery (%)	LOD (ng L^{-1})	Found concentration (ng L^{-1})	Ref.
Hospital effluents	5-FU	Offline SPE Cartridge or column: ENV+ Conditioning step: 6 ml MeOH, 6 ml deionized water Elution solvent: 2 × 5 ml MeOH	Technique: GC–MS Derivatization: TMS	93–101	12	0.09–4[a]	[47]
Hospital effluents	MET, AZA, DOX, DOXOL, VINC, IF, CP, ETO, PROCAR	Offline SPE Cartridge or column: Oasis HLB Conditioning step: 6 ml MeOH, 6 ml ultra-pure water Elution solvent: 6 ml MeOH/water (80:20) Cartridge or column: Oasis WAX Conditioning step: 6 ml MeOH, 6 ml water Elution solvent: 7 ml MeOH/water (60:40), 5 ml MeOH/water (40:60) + 0.1% F. A.	Technique: UHLPC–ESI–MS/MS Mobile phase: (A): ultra-pure water + 0.1% F. A.; (B): ACN	51–105	2–20	17 (MET) 15 (AZA) 151 (IF) 100 (CP) 42 (ETO)	[56]
Wastewater	Same as reference [56]	Same as reference [56]	Same as reference [56]	51–104 (inf.) 45–109 (eff.)	0.6–7 (inf.) 0.5–3.5 (eff.)	8.5–14.5 (CP) 9.0–16.4 (IF) 1.6–18.1 (MET)	[57]
Hospital effluents Wastewater	CP, IF, DOC, PAC, ETO, VINC, TAM, MET, AZA, CIPRO	Offline SPE Cartridge or column: Oasis HLB Conditioning step: 5 ml MeOH, 5 ml water + 0.1F. A. Elution solvent: 5 + 5 ml MeOH	Technique: UHPLC–ISS–QqLit Mobile phase: (A): water + 0.1% F. A.; (B): ACN	46–124	0.8–24	14.725–0.133[a]	[16]
Hospital effluents Wastewater	CP, CHLO, MEL, IF, FLU, CYT, GEM, CAP, VINB, VINC, ETO, PAC, DOC, DOX, DAU, EPI, IMA, ERLO, IRI, LEU, GOS, TAM, A-GLU, MEG, CYP, PRED	Offline Cartridge or column: Oasis HLB Conditioning step: 6 ml MeOH, 6 ml solution 100 mmol/L NH_4OAc Elution solvent: 6 ml MeOH, 6 ml F. A.:MeOH (5:95)	Technique: LC–HESI–Orbitrap–MS Mobile phase: (A): water + 0.1% F. A.; (B): ACN + 0.1% A.F.	6–110	0.7–356[d]	Hospital effluents 86.2 (IF)[a] 4.71 (CP)[a] 0.73 (IRI)[a] WW: 0.22 (CP, MEG)[a]	[44]
River water	CP (18)	Offline SPE: Cartridge or column: LiChrolut 100 RP-18 Conditioning step: 6 ml hexane, 3 ml acetone, 6 ml MeOH, 2 ml water pH 2 Elution solvent: 3 × 3 ml MeOH	Technique: LC–ESI–MS/MS Mobile phase: (A): ACN; (B): solution 20 mM ammonium acetate + 0.1% F. A.		1–10 (Surface) 5–20 (WW)	4–8	[64]
Wastewater	CP, MET (30)	Offline SPE Cartridge or column: Oasis MCX Conditioning step: 6 ml MeOH, 3 ml Milli-Q water, 3 ml water pH 2 Elution solvent: 2 ml MeOH, 2 ml MeOH + 2% NH_3, MeOH + 0.2% NaOH Cartridge or column: Lichrolut EN Conditioning step: 6 ml MeOH, 6 ml Milli-Q water Elution solvent: 3 ml MeOH, 3 ml EtAc	Technique: LC–TIS–MS/MS Mobile phase: (A): Milli-Q water + 0.1% F. A. pH 2; (B): ACN	106 (CP) 76 (MET)	1.9 (CP) 0.83 (MET)	2.1–9 (CP) 12.6 (MET)	[66]
Wastewater	IF, CP (32)	Same as reference [52]	Same as reference [52]		10	N.D.	[51]
Wastewater River water Tap water	CP, IF (22)	Offline SPE Cartridge or column: C18 Conditioning step: 3 × 2 ml Hex, 3 × 2 ml MeOH, 1 ml water pH 7.5 Elution solvent: 4 × 1 ml MeOH	Technique: LC–ESI–MS/MS Mobile phase: isocratic flow (0.4 mL/min); water/ACN (68.5/31.5 v/v) + 10 mmol/L ammonium acetate pH 5.7 at room temperature	51–57	10		[52]
Groundwater	IF, CP (60)	Offline SPE Cartridge or column: PPL Blond-Elut Elution solvent: 5 ml MeOH	Technique: LC–ESI–MS/MS Mobile phase: (A): solution 20 mM ammonium acetate in Milli-Q water; (B): 20 mM ammonium acetate in ACN/MeOH (2:1)	71–102 (CP) 73–87 (IF)	32 (CP) 14 (IF)	N.D.	[68]
Wastewater	Same as reference [66]	Same as reference [66]	Same as reference [66]		Same as reference [66]	N.D.	[67]

(*continued on next page*)

Table 2 (*continued*)

Matrix	Compounds	Extraction condition	Determination	Recovery (%)	LOD (ng L^{-1})	Found concentration (ng L^{-1})	Ref.
Wastewater	5-FU (18)	Offline SPE Cartridge or column: Phenomenex Strata-X Conditioning step: 5 ml MeOH, 5 ml Milli-Q water pH 2 Elution solvent: 7 ml ACN	Technique: GC-MS Derivatization: 180 μL K_2CO_3 10% pH 10.5, 70 μL PFBBr			N.D.	[58]
Wastewater	TAM (12)	Offline SPE Cartridge or column: Strata X Conditioning step: 5 ml MeOH, 5 ml deionized water Elution solvent: 5 ml acetone, 2 × 5 ml MeOH	Technique: LC–ESI–MS/MS Mobile phase: (A): water; (B): MeOH; (C): ammonium acetate 10 mM; (D): acetic acid	29	0.03	N.D.	[45]
Wastewater Tertiary of R.O.	IF, CP (18)	Offline SPE Cartridge or column: Strata X Conditioning step: 6 ml EtAc, 6 ml MeOH, 12 ml water pH 7 Elution solvent: 4 ml ACN, 4 ml MeOH, 3 ml EtAc, 1.5 ml ACN	Technique: LC-ESI-MS/MS Mobile phase: (A): MeOH + 1% F. A.; (B): water + 0.4% F. A.	167–96 (IF) 106–98 (CP)	136 (IF)[e] 93 (CP)[e]	<L.D.	[59]
Wastewater Surface water	CP, MET (5)	Online SPE Cartridge or column: Strata X Conditioning step: 1000 μL/min X% acetic acid in 2.5% MeOH and 97.5 % water Elution solvent: mobile phase (200 μL/min)	Technique: SPE–ESI–LC-MS/MS Mobile phase: (A): water +0.2% acetic acid; (B): ACN	148–120 (CP) 55–125 (MET)	9–20	9 (CP) 59 (MET)	[71]
Tap water Surface water	CP, MET (14)	Online SPE Cartridge or column: Strata X	Technique: SPE–LC–ESI–ToF–MS/MS Mobile phase:(A): water + 0.1% F. A.; (B): MeOH + 0.1% F. A.	60–109	0.6–6	1–2	[24]
Wastewater	CP, MET (5)	Online SPE Cartridge or column: Strata X Conditioning step: 0.1% acetic acid in MeOH/Milli-Q water (1:40) 1 ml/min. Elution solvent: mobile phase	Technique: SPE–LC–ESI–MS/MS Mobile phase: (A): Milli-Q water + 0.1% acetic acid; (B): ACN		5–38		[72]
Wastewater	TAM (74)	Online SPE Cartridge or column: HySphere Resin GP Conditioning step: 2 ml MeOH, 2 ml water (5 ml/min) Elution solvent: mobile phase (0.3 ml/min)	Technique: SPE-LC-ESI-MS/MS Mobile phase: (A): ACN + 0.1% F. A.	ABS: 10.36–14.34 REL: 74.16–131.3	0.63–5.41[d]	N.D.	[73]
Groundwater	TAM (95)	Same as reference [73]	Same as reference [73]	ABS: 74.16–131.33 REL: 10.36–10.7		26.9–72. 7	[74]
Hospital effluents	CIPRO, CP, IF (68)	On-Line SPE Cartridge or column: Isolute ENV+ and Oasis HLB Conditioning step: nano-pure water + 0.1% F. A. Elution solvent: mobile phase	Technique: SPE–LC–ESI–MS Mobile phase: (A): HPLC water + 0.1% F. A.; (B): MeOH	90–149 (CIPRO) 103–134 (CP) 101–152 (IF)	16–3500 (CIPRO) 9–110 (CP) 2–26 (IF)	0.151–0.958[a]	[75]
Superficial water Groundwater Wastewater	GEM, TEM, MET, IRI, IMA, IF, CP, ERLO, ETO, DOX, CAP, TAM, PAC, OH-MET, OH-D-TAM, OH-TAM, OH-PAC	On-Line SPE Cartridge or column: HySphere Resin GP10 Conditioning step: 1 ml MeOH, 1 ml water. Elution solvent: mobile phase (0.2 ml/min)	Technique: SPE–LC–ESI–MS/MS Mobile phase: (A): ultra-pure water + 0.1% F. A.; (B): MeOH + 0.1% F. A.	72 – 119	5–180	2.1–29.7	[33]
Wastewater Hospital effluents	Same as reference [33]	Same as reference [33]	Same as reference [33]	Same as reference [33]		2–180	[39]
Wastewater	CP, GEM, IF, MET, IRI, EPI	Online SPE Cartridge or column: Hypersil Gold PFP Conditioning step: water + 0.1% F. A. Elution solvent: mobile phase (350 μL/min)	Technique: SPE–ESI–LC–MS Mobile phase: (A): water + 0.1% F. A.; (B): ACN/MeOH (50:50) + 0.1% F. A.	>70 (except GEM)	4–20	13 (CP) 60 (MET)	[76]
Chlorinated water	ETO	Same as reference [33]	Technique: UHPLC–HESI–Qq–Orbitrap–MS/MS Mobile phase: (A): ultra-pure water; (B): MeOH	97–119 (ETO) 69–116 (BP1)	0.3–1.11 (ETO) 0.26–0.80 (BP1)	14–33	[36]

[a] μgβL^{-1}.
[b] Limit of Quantification (LOQ).
[c] μgβL^{-1}, LOQ.
[d] Method Detection Limit (MDL).
[e] Instrumental Detection Limits (IDL).
N.D. = Not Detectable; LOD = Limit of Detection; F.A. = Formic Acid; MeOH = Methanol; EtAc = ethyl acetate.

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procedure used by Kovalova et al. [40] in sewage and hospital effluents, measuring concentrations from 0.9 to 839 ng L^{-1} in the effluents from hospitals, with the highest concentrations for the dFdU metabolite. Mullot et al. [47] also used cartridges of Isolute ENV+ to determine 5-FU in hospital effluents; concentrations in the range of 0.09–4 μg L^{-1} were determined. They used GC–MS/MS for quantification, but LC–diode array detection (DAD) was used for validation and recovery determination. Quantification was performed using an internal standard (5-fluorouracil-6C [13]), with recoveries in the range of 93–101%, which are better than those found in wastewater (54–118% [41]).

Oasis HLB cartridges have also been widely used for the extraction of these compounds. Ferrando-Climent et al. [16] developed a method for the determination of cytostatic agents and their metabolites in hospital effluents and WWTP influents using UHPLC coupled to a triple quadrupole–linear ion trap (QqLit)–MS with isotopically labelled compounds as the internal standards. They obtained concentrations of up to 14 μg L^{-1} in the hospital effluents. Gomez-Canela et al. [54] identified CP and EPI using LC–Orbitrap–MS, revealing their presence in WWTP influents and hospital and urban effluents at concentration levels ranging from 5.73 to 24.8 μg L^{-1}; these concentration levels are higher than those determined by other authors in wastewaters, with values rarely reaching the level of hundreds of ng L^{-1} (Table 1). The same authors, using the same instrumentation [44], analysed 26 commonly cytostatic compounds in effluents from hospitals and wastewater, obtaining concentrations up to 86 μg L^{-1}. Martin et al. [55] investigated 14 cytostatic drugs (5-FU, GEM, CP, IF, CYT, MET, PAC, ETO, IRI, docetaxel (DOC), EPI, DOX, vinorelbine (VINO) and mitomycin C (MIT-C)) in wastewater and river water using the same cartridge and LC coupled to a triple quadrupole (QqQ)–MS and measuring concentrations in the range of 1.2–15 ng L^{-1}. Yin et al. [56] developed a method for the determination of MET, azathioprine (AZA), DOX, doxorubicinol (DOXOL), VINC, IF, CP, ETO and PROCAR in the effluents from hospitals using the OASIS HLB cartridge and UPLC–ESI–MS/MS. They achieved LODs of between 2 and 20 ng L^{-1} and recoveries between 51% and 105%. This same procedure was used by the same authors for the determination of these compounds in wastewater with recoveries of 51–104% in influents and 45–109% in effluents [57]. The LODs in this case were between 0.6 and 7 ng L^{-1} in the influents and 0.5 and 3.5 ng L^{-1} in the effluents. For the majority of drugs, significant differences were observed in the matrix effect values between different water samples; therefore, in this case, they used the standard addition method.

Multi-residue methods for different types of compounds have been reported using Strata-X cartridges, although in many cases, the results were not good for the extraction of cytostatic compounds. Yu et al. [58] used GC–MS to identify 18 compounds, including 5-FU, but 5-FU was not found in their sewage sample study. Two internal standards, 4,4-di-tert-butylbiphenyl-d_5 and 1-phenylnonane-d_3 were used. Nebot et al. [45] investigated 12 of these drugs in wastewater, including TAM, achieving a maximum recovery of 29% and a LOD of 0.03 ng L^{-1} for this compound; however, cytostatic compounds were not detected in real samples. This cartridge was also used to determine IF and CP from wastewater, including reverse osmosis as tertiary treatment, but they were found in concentrations below the detection limit [59]. Deuterated standards were employed to take into account and correct the matrix effects.

Mahnik et al. [49], in a later study, attempted to determine EPI, DOX and daunorubicin (DAU) via LC with FD using C_8 columns in wastewater samples. Because anthracyclines are easily adsorbed on matrices, such as plastic and glass, which leads to irreproducible analytical results, they applied the hypothesis that the addition of bovine serum albumin (BSA) would result in linear calibration curves, as observed with matrices such as plasma [60]. The recoveries were in the range of 80–90% with LODs of 50–60 ng L^{-1}. In their following study [12], they determined anthracyclines (EPI, DOX and DAU) in hospital wastewaters with the same procedure, measuring concentrations of 0.3–0.6 μg L^{-1} for DOX.

Different cytostatic agents (Cis-Pt, Car-Pt, Oxa-Pt, DOX, DAU and EPI) in effluents from hospitals were investigated by Lenz et al. [11]. The C_8 columns were selected for the extraction of the anthracyclines (DOX, DAU and EPI); detection was conducted using LC–FD, employing the procedure developed by Mahnik et al. [49], and concentrations of 0.26–1.35 μg L^{-1} were obtained for DOX. Cis-Pt, Car-Pt and Oxa-Pt, belonging to the so-called cancerostatic platinum compounds (CPCs), were extracted by the procedure developed in a previous work [61], wherein ^{115}In was used as an internal standard for the quantification of platinum. The detection method for CPCs was previously developed by Hann et al. [62] in a work wherein CPCs were determined in hospital effluents by LC–ICP–MS; concentrations of total Pt were measured to be between 0.3 and 1.7 μg L^{-1}. Parallel to this work, the same authors presented another study wherein a membrane treatment plant was installed for hospital effluents [63], focusing on the removal of CPCs. To determine the efficiency of this system, the above-mentioned methods [61,62] were applied to determine the CPCs; the concentrations of total Pt were found to be 38.1 μg L^{-1} in the influent and 18.7 μg L^{-1} in the effluent of the pilot plant.

Metcalfe et al. [64,65] analysed 18 pharmaceutical compounds, including CP, in river water. They sampled a large geographical distribution, making it impossible to spike effluent samples with surrogate standards prior to SPE extraction. In this case, an internal standard (dihydrocarbamazepine) for neutral drugs was added before the evaporation of samples to check the precision of the procedure. The extraction was performed using LiChrolut 100 RP-18 cartridges, resulting in CP concentrations of 4–8 ng L^{-1} [64]. Castiglioni et al. [66,67] investigated MET and CP among 30 compounds in urban wastewater using salbutamol-d_3 as the internal standard. In their first study [52], they tested different cartridges, Oasis MCX for MET and LiChrolut EN for CP, detecting concentrations between 2.1 and 12.6 ng L^{-1}. In a subsequent paper, the authors applied this optimized procedure for the determination of the same compounds in other WWTPs, but cytostatic compounds were not found [67].

Sacher et al. [68] analysed IF and CP among 60 other pharmaceuticals compounds in groundwater using 2,3-dichlorophenoxyacetic acid as the internal standard for the overall procedure. PPL Blond-Elut cartridges followed by LC–MS/MS were chosen for IF and CP, but these cytostatic compounds were not detected in real samples.

The lowest LODs were achieved by Llewellyn et al. [46] (varying between 0.03 and 0.12 ng L^{-1} for CP and between 0.05 and 0.09 ng L^{-1} for IF in different WWTPs) and Nebot et al. [45] (0.03 ng L^{-1} for TAM in wastewater). Strata-X cartridges were used in both cases, followed by LC–MS/MS. An extra cleaning step with cartridges by Florisil® (FL) was used by Llewellyn et al. [46], with recoveries between 57 and 70%, followed by LC–MS/MS. Furthermore, CP concentrations of 3.7 ng L^{-1} and 3.5 ng L^{-1} before and after UV treatment, respectively, were measured.

4.1.2. Online SPE

Sample preparation is the drawback of the analytical methods regarding the uncertainly and accuracy of analytical data. Therefore, it is necessary to minimize the number of steps to reduce both time of analysis and the error sources. The development of online SPE methods coupled to LC–MS/MS has been shown to improve method sensitivity, reduce sample pretreatment and analysis time and increase the number of samples that can be analysed simultaneously [69]. Although the online SPE process is one of the most promising techniques, reverse-phase sorbents show some limitations, such as poor extraction of quite polar compounds [67],

Alternative phases may be used in online SPE to obtain the desired selectivity (for example, hydrophilic interaction liquid chromatography (HILIC), porous graphitic carbon (PGC) or molecularly imprinted solid-phase extraction (MISPE)) [70], but combining different analytes with a wide range of hydrophobicities may lead to problems. As a result, low recovery and poor resolution occur.

Most environmental monitoring programs have the goal to analyse the maximum number of compounds while spending minimal resources. This goal was the objective of Garcia-Ac et al. [71], who determined CP and MET, among five other pharmaceuticals, in residual and surface water using online SPE–LC–ESI–MS/MS with Strata × cartridges. LODs in the range of 9–20 ng L^{-1} were obtained using the standard addition method. Recoveries were in the range of 55–125% for MET and 148–120% for CP, and concentrations of 9 ng L^{-1} (CP) and 59 (MET) ng L^{-1} were measured.

In a subsequent paper [24], the same authors attempted to determine these cytostatic compounds (CP and MET) among 14 pharmaceutical compounds in drinking and surface water using SPE–LC–ToF–MS with the same Strata × cartridges. They achieved recoveries between 60% and 109% and LODs of 0.6–6 ng L^{-1}, which were better than those of the previous work. For drinking water, internal calibration was used, whereas the standard addition method and an internal standard ($^{13}C_3$-atrazine) were used for surface water samples. The authors claim that the standard additions were necessary to compensate for the imprecision caused by the matrix effect over the ESI process; thus, they measured concentrations of 1–2 ng L^{-1} for CP and MET. In another study, using the same cartridge, they compared the three forms of atmospheric pressure ionization most used in LC–MS/MS (atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and ESI) [72]; ESI was found to be most effective for the five compounds studied, including CP and MET. The LODs were higher than those obtained in the previous work (from 5 to 38 ng L^{-1}).

In another multi-residue method, López-Serna et al. [73] studied 74 pharmaceutical compounds, including TAM, using SPE–LC–MS/MS with ESI in a positive ion mode. They applied an internal standard calibration, adding 37 surrogates to correct the losses during the SPE, as well as the matrix effects. The chosen cartridges were HySphere Resin GP, but cytostatic compounds were not detected in the wastewater samples. In 2013, the authors published another work using the same procedure, in which TAM, among 95 other pharmaceutical compounds and transformation products, were determined in groundwater [74]. They measured concentrations of 26.9–72.7 ng L^{-1} with absolute recoveries of approximately 10% and relative recoveries from 74 to 131%.

Kovalova et al. [75] used an online SPE process with two cartridges (Isolute ENV+ and Oasis HLB) for the analysis of a total of 68 substances, including three cytostatic drugs (ciprofloxacin (CIPRO), CP and IF) in the hospital effluents, in which a pilot water treatment plant using bio-membranes was installed. Concentrations of these cytostatic drugs were detected from 0.151 μg L^{-1} to 0.958 μg L^{-1}.

Negreira et al. [33] published several works regarding the determination of cytostatic agents and their metabolites using an online SPE–LC–MS/MS. In the first paper (the first multi-cytostatic analysis), they analysed 13 compounds (GEM, TEM, MET, IRI, IMA, IF, CP, ERLO, ETO, DOX, CAP, TAM and PAC) and four metabolites (hydroxymethotrexate, desmethylhydroxytamoxifen, hydroxytamoxifen and hydroxypaclitaxel) in superficial water, groundwater and wastewater using a Resin HySphere GP10 SPE cartridge. Quantification was performed via the isotope dilution method using 15 different isotope-labelled compounds. They obtained LODs between 5 and 180 ng L^{-1} and recoveries in the range of 72–119%. In the samples, concentrations between 2.1 and 29.7 ng L^{-1} were measured. In the same year, they presented a study of the stability of cytostatic agents in water [34]. Many of these compounds were found to be unstable in water, which could explain the lack of methods and studies for the quantitative analysis of many of them. The following year, with the same methodology, they attempted to determine 13 cytostatic agents and four metabolites in sewage and hospital effluents [39]. Quantification was performed using the stable isotope dilution method. For DOX and TAM, isotope-labelled analogues were not available, hence their quantification was performed using erlotinib-d_6 hydrochloride and 4-hydroxy-ethyl-tamoxifen-d_5, respectively, as the internal standards. Finally, the authors measured concentrations ranging between 2 and 180 ng L^{-1}.

CP, GEM, IF, MET, IRI and EPI in wastewater were determined by Rabii et al. [76] using an online SPE–LC–MS with Hypersil Gold PFP cartridges and using the standard addition method and an internal standard (atrazine-^{13}C). LODs ranging from 4 to 20 ng L^{-1} were achieved. Concentrations of 13 ng L^{-1} (CP) and 60 ng L^{-1} (MET) were measured in the real samples, with recoveries > 70%, except for GEM.

4.2. Solid samples

Volatilization, biodegradation and sorption onto sludge are processes that can affect the concentration of the analytes during treatment with activated sludge in the WWTPs, which depends on both compounds and sludge physico-chemical properties. It is said that volatilization is usually negligible for pharmaceuticals because of their low Henry's constant [77]. While biodegradation implies complete elimination of the contaminants, sorption onto sludge can be considered as their displacement from the aqueous to the solid phase.

In solid environmental samples, the extraction and purification of these complex matrices are frequently the most tedious and time-consuming parts of the analytical process, due to the large number of interferences and the strong interactions between the analytes and the sample. Moreover, their analysis represents a difficult task because of the usually low concentration at which the target compounds are present in such samples. Removing interferences and enriching the target compounds to detectable concentrations are necessary to obtain high recoveries.

Soxhlet and ultrasound-assisted extraction (UAE) are common methods for the extraction of the emerging contaminants from solid samples [29]. However, the use of microwave-assisted extraction (MAE) or more advanced techniques, such as pressure liquid extraction (PLE) and supercritical fluid extraction (SFE), are becoming important extraction techniques for environmental samples. In most of the cases, the extraction step is not selective enough, and a clean-up step is mandatory, with SPE being the most commonly used.

To our knowledge, only three works related to the analysis of cytostatic compounds in solid environmental samples have been published (Table 3). In all of these works, sewage sludges have been analysed using LC–MS/MS or UHPLC–MS/MS. No study was found regarding the presence of these compounds in other environmental solid samples, such as marine sediments affected by sewage outfalls. In the literature, UAE and PLE were the only techniques used for the extraction of these compounds from solid samples.

Ternes et al. [28] extracted two cytostatic compounds (IF and CP) using UAE with MeOH/acetone, followed by a clean-up with a C_{18} cartridge and LC–MS/MS detection, obtaining an LOD of 20 ng g^{-1} for both compounds. The authors claimed that the variations related to the matrix effects were efficiently compensated using the surrogate standard dihydrocarbamazepine and that the losses could be caused mainly by the extraction because the SPE used for clean-up was quantitative, with recoveries >90%. Applying the method to sewage sludges, relative recoveries of 59–95% for IF and 66–106% for CP in activated sludges and absolute recoveries of 53–105% for IF and 58–116% for CP in digested sludges were obtained.

Moreover, Okuda et al. [27] investigated the presence of 66 pharmaceutical compounds in sewage sludge, including two cytostatic compounds (CP and CIPRO). Two methods of extraction were tested,

Table 3
Methods for the determination of cytostatic compounds in solid samples

Matrix	Compounds	Extraction condition	Determination	Recovery (%)	LOD (ng g^{-1})	Found concentration (ng g^{-1})	Ref.
Sludge	CP, IF	Technique: UAE: 4× 5 min Solvent extraction 1 × 4 ml MeOH, 1 × 2 ml MeOH, 2 × 2 ml acetone, addition of surrogate standard. Clean-up: SPE C18	Technique: LC-ESI-MS Mobile phase: (A): 5 mmol/L aqueous ammonium acetate (pH 5.7) and ACN (90:10, v/v); (B): 400 mL eluent A + 600 mL ACN	52–78	20		[28]
Sludge	CP, CIPRO (66)	Technique: PLE (used for CP) Solvent extraction: Water/MeOH, 9:1 Technique: UAE (used for CIPRO) Solvent extraction: Water (pH 2)	Technique: LC–MS/MS; UHPLC–MS/MS	85 (CIPRO) 75 (CP)		20–500 (CIPRO) 30–5 (CP)	[27]
Sludge	IF, CP, TAM	Technique: PLE Solvent extraction: MeOH/water (65/35) Clean-up: Oasis MAX and MCX	Technique: UHPLC–ESI–MS/MS Mobile phase: (A): ultra-pure water/ACN (90:10) with 1 mM NH_4CH_3COO + 0.3% F.A.; (B): ACN	95–108 (IF) 94–100 (CP)	3.5–74 ng L^{-1} (IF) 2.5 – 51 ng L^{-1} (CP)	11.4–42.5 (IF) 12.6 (CP)	[29]

LOD = Limit of Detection; F.A. = Formic Acid; MeOH = Methanol.

PLE and UAE followed by SPE as a clean-up step, using Oasis HLB cartridges and LC–MS/MS or UHPLC–MS/MS. PLE with water at pH = 2 was chosen for CP, whereas UAE with water:MeOH (9:1, v/v) was used for CIPRO. The recoveries obtained were 75% for CP and 85% for CIPRO. In both cases, the extracts were diluted with ultra-pure water, and EDTA–Na_2 was added prior to the SPE step. Concentrations of 20 ng g^{-1} in the primary sludge and 500 ng g^{-1} in the excess sludge were measured for CIPRO, and concentrations of 30 ng g^{-1} in the primary sludge and 5 ng g^{-1} in excess sludge were measured for CP.

In a subsequent study, IF, CP and TAM were extracted from sludges using PLE and determined by UHPLC–MS/MS [29]. The sample was placed into the cell with MeOH:water (65:35, v/v) at 85 bars and 100°C for 9 min and four cycles. The extract was evaporated to 5 mL and dissolved in 200 mL of ultra-pure water to pass it through Oasis MAX and later Oasis MCX cartridges. Elution in the Oasis MCX cartridge was performed with MeOH for neutral cytostatic drugs (IF and CP) and with 6 mL of 2% NH_4OH in acetone for the basic compound (TAM) in two different fractions. The extract was evaporated to dryness and then redissolved in the mobile phase. The deuterated standard was spiked at the beginning to compensate for the possible losses. Recoveries in the range 95–108 % (IF) and 94–100% (CP) were obtained, reaching LODs of 3.5–74 ng L^{-1} (IF) and 2.5–51 ng L^{-1} (CP). Concentrations of 12.6 μg kg^{-1} for CP and 14.4–42.5 μg kg^{-1} for IF were found in the sludges.

5. Conclusions and future trends

Because of the mutagenic and genotoxic potential of cytostatic compounds, it is essential to determine the concentration at which these compounds are found in the environment to place them under surveillance. A large number of cytostatic compounds has been studied, with CP being the most investigated compound, followed by IF and MET. Despite the large number of compounds that have been studied, various cytostatic compounds have not been detected in sewage, with CP, IF, TAM, 5-FU, EPI, MET and DOX being the most commonly detected, possibly because they are the most frequently used anticancer drugs.

The main sources of contamination of these compounds are considered to be hospital effluents. However, some authors do not agree with the majority opinion, and they claim that the levels of cytostatic agents in effluents from hospitals and in the influent of WWTPs are similar [16,39]. According to our review, the difference in concentrations between hospital effluents and wastewater is remarkable: in hospitals, the effluent concentrations are measured at the μg L^{-1} level, whereas in wastewater, surface water and river water, the concentrations are at the ng L^{-1} level.

Due to the low concentrations at which cytostatic compounds are found, the use of techniques for purification and preconcentration becomes mandatory. In addition, more selective and sensible detection methods are required. The combination most frequently used for the analyses of these compounds in liquid samples is SPE followed by LC–MS/MS.

Online strategies have been implemented in recent years because of their absolute advantages in terms of repeatability, efficiency and speed. However, one of the limitations of the online SPE technique is that a sufficient variety of sorbents is not available. Future trends should be focused on developing new extraction materials capable of extracting polar analytes and suitable for multi-residue analysis of compounds with a wide range of hydrophobicity levels.

Regarding the analysis of cytostatic compounds in solid samples, the literature is too scarce, thus making it mandatory to develop new methods for the analysis of these compounds, not only in sludges but also in marine sediments, and even in organisms close to the outfalls of sewage waters. Wider and deeper environmental monitoring of cytostatic drugs is essential to determine the presence and distribution of these pharmaceutical compounds, which will inform the establishment of policies of control of these drugs in the near future.

Moreover, often, methods for the determination of cytostatic compounds are applied, but these compounds are not found in the samples; therefore, further studies should investigate the possible transformation of these compounds into other products. In fact, as indicated earlier, some studies have indicated the metabolite concentrations in sewage samples that are considerably higher than the concentration of the parent compounds.

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CAPÍTULO 2: OBJETIVOS

Tal y como se ha descrito, la previsión del aumento de la incidencia del cáncer conllevará un mayor consumo de medicamentos que combatan esta enfermedad. La introducción de estos compuestos en el medioambiente tiene lugar, principalmente, a través de las estaciones depuradoras de aguas residuales, debido a su incompleta eliminación.

Los compuestos citostáticos están presentes en el medio como una mezcla de sustancias en matrices ambientales complejas con muchas interferencias, por lo que se hace necesario el desarrollo de metodologías que permitan su determinación, a niveles traza.

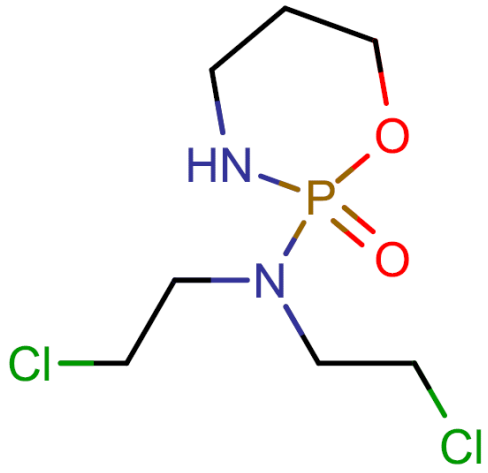
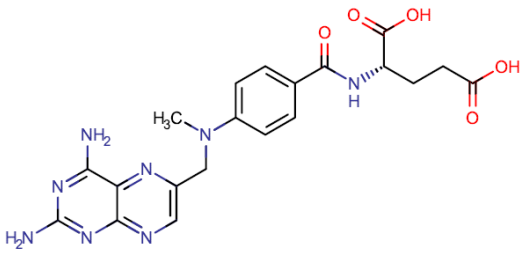
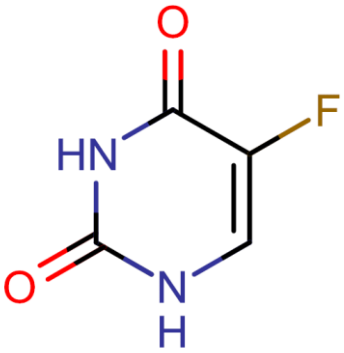
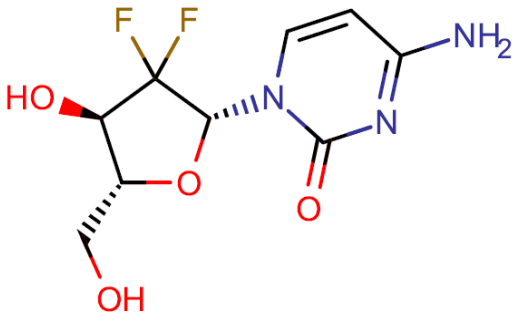
Si bien, el estudio de alguno de estos compuestos se ha realizado en muestras de aguas residuales, la mezcla de ellos no está tan bien documentada. Por otro lado, los estudios en muestras sólidas son

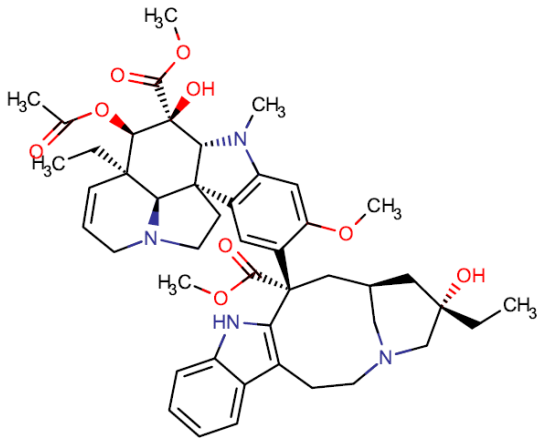
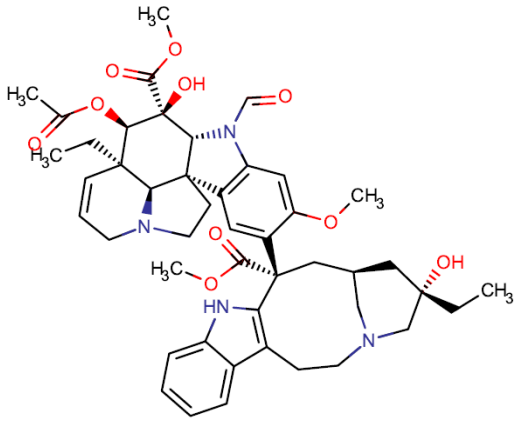
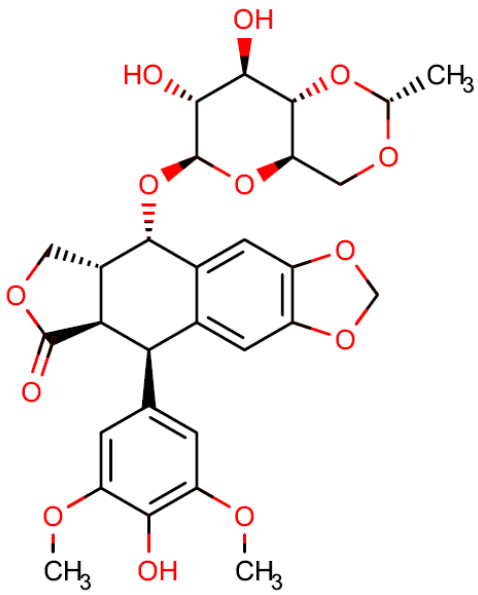
bastante escasos y se pretende aumentar el conocimiento en dicho campo.

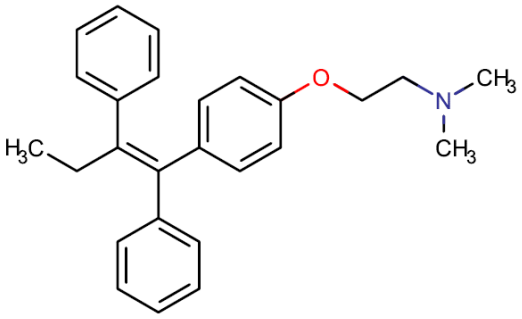
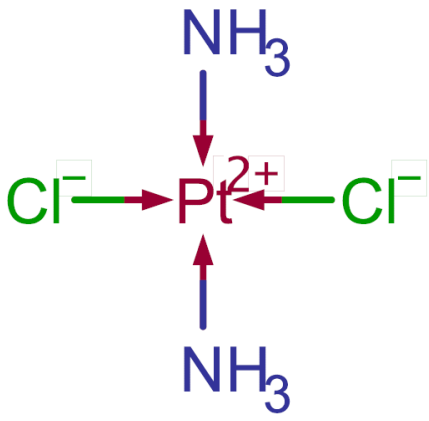
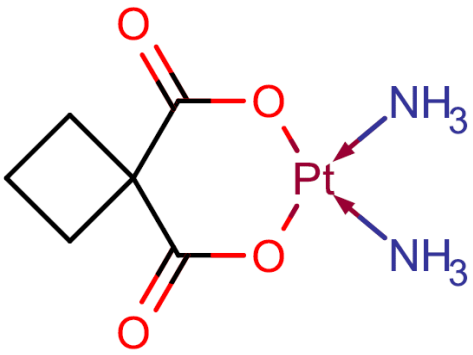
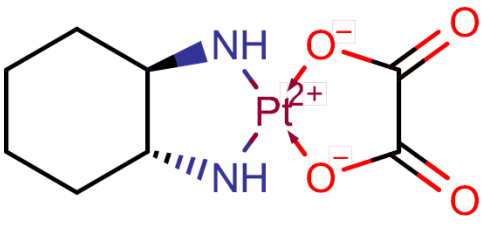
Las metodologías que se utilicen para su extracción, deben ser respetuosas con el medioambiente, deben ser rápidas y extraer la mayor cantidad de compuestos antineoplásicos a la vez y suficientemente sensibles para detectarlos a niveles de traza o ultratraza. El establecimiento de estos procedimientos beneficiará a la sociedad, ya que permitirá el monitoreo de los distintos compartimentos del medioambiente donde pueden estar presentes estos medicamentos.

Por ello, el objetivo general de esta Tesis es aportar una visión amplia y consolidada del conocimiento sobre métodos de extracción y determinación que permitan estudiar la presencia y distribución de los compuestos citostáticos en el medioambiente. Para llevar a cabo este objetivo, es necesario el desarrollo y optimización de metodologías de análisis altamente sensibles y selectivas para la evaluación y monitorización de compuestos citostáticos en muestras medioambientales. En concreto, estudiaremos su presencia en efluentes de hospitales donde los pacientes son tratados con compuestos antineoplásicos, en aguas residuales y lodos procedentes de estaciones depuradoras de aguas residuales, así como agua de mar, sedimentos marinos y peces tomados o capturados en las proximidades de los emisarios submarinos. Se han seleccionado compuestos antineoplásicos de diferentes familias, ampliamente utilizados en el tratamiento contra los tipos de cáncer más comunes:

Tabla 2. Compuestos citostáticos seleccionados para la Tesis (información obtenida de chemicalize.org).

Familia	Compuesto	Abreviatura	Estructura
Agentes alquilantes	Ciclofosfamida	CP	
Antimetabolitos	Metotrexato	MET	
	5-fluorouracilo	5-FU	
	Gemcitabina	GEM	

Alcaloides vegetales	Vinblastina	VINB	
	Vincristina	VINC	
	Etoposido	ETO	

Otros agentes antineoplásicos	Tamoxifeno	TAM	
	Cisplatino	Cis-Pt	
	Carboplatino	Car-Pt	
	Oxaliplatino	Oxa-Pt	

De acuerdo con lo expuesto, los objetivos específicos de esta Tesis Doctoral son:

CAPÍTULO 2: OBJETIVOS

I. Establecer las condiciones óptimas de separación cromatográfica mediante cromatografía líquida de ultra resolución (UHPLC) y acoplada para la detección de los compuestos antineoplásicos seleccionados, principalmente por espectrometría de masas en tándem (MS/MS).

II. Optimizar e implementar una metodología basada en la extracción en fase sólida (SPE) para monitorizar compuestos antineoplásicos en muestras líquidas ambientales (aguas residuales y agua de mar).

III. Desarrollar y optimizar un procedimiento basado en la técnica de microextracción por adsorción sobre tejidos químicamente modificados (FPSE) en muestras de aguas residuales.

IV. Desarrollar y optimizar una metodología basada en la extracción en fase sólida para compuestos citostáticos de platino en muestras de aguas residuales y determinación por plasma acoplado inductivamente y espectrometría de masas (ICP-MS).

V. Desarrollar y optimizar procedimientos de extracción de compuestos en matrices sólidas mediante la aplicación de la extracción asistida por microondas (MAE) en lodos procedentes de estaciones depuradoras de aguas residuales (EDAR) y sedimentos (arena).

VI. Aplicar la extracción asistida por microondas (MAE) para la extracción de compuestos antineoplásicos de matrices biológicas. Se estudiarán los músculos y las vísceras de peces que se alimentan en las proximidades de emisarios submarinos.

CAPÍTULO 3: PARTE EXPERIMENTAL Y RESULTADOS

3.1. Análisis simultáneo y sistemático de fármacos citostáticos en muestras de aguas residuales mediante cromatografía líquida de ultra resolución y espectrometría de masas en tándem

Como ya hemos comentado, se prevé que el consumo de los compuestos citostáticos aumente en los próximos años. Son compuestos mutagénicos [88] frente a los cuales no se ha establecido una concentración mínima segura y pueden causar efectos adversos a los organismos que se encuentren en el agua por su exposición prolongada, aun estando en bajas concentraciones, por lo que se hace necesaria su monitorización en el medio ambiente.

La principal fuente de entrada al medio es a través de las aguas residuales, tanto de hospitales, donde los pacientes son tratados y, a veces, ingresados, como la de las casas de pacientes que han salido del hospital después del tratamiento, o, incluso, muchos de ellos son tratados en casa. Las estaciones depuradoras de aguas residuales actuales están diseñadas para reducir la carga de materia orgánica, pero no para la eliminación de este tipo de compuestos químicos, los cuales pueden ser detectados en los efluentes y, en algunos trabajos, hasta en aguas de rio [17].

El objetivo de este trabajo fue estudiar la presencia y distribución de compuestos citostáticos en las aguas residuales y el agua de mar próxima a los emisarios submarinos. Para ello, la extracción de los compuestos se llevó a cabo mediante la extracción en fase sólida (SPE) seguida por su determinación por cromatografía líquida de ultra resolución acoplada a la espectrometría de masas en tándem (UHPLC-MS/MS).

La SPE es una de las técnicas más empleadas para la extracción y preconcentración de contaminantes emergentes en muestras ambientales. Existen, principalmente, cuatro compañías que comercializan estos cartuchos con sorbentes poliméricos similares (C_{18} de fase reversa) pero con ciertas variaciones que hace que sea necesario la selección adecuada del cartucho para la mezcla de compuestos que se pretende analizar. En el caso de los medicamentos contra el cáncer, existen diferentes familias de citostáticos con diferentes estructuras y propiedades que se usan dependiendo del tipo de cáncer a tratar y, en ocasiones, combinaciones de ellos para un mismo cáncer. Por tanto, es necesario seleccionar el cartucho adecuado para la mezcla de compuestos y optimizar adecuadamente las condiciones de extracción.

Se realizó una optimización de SPE comparando los cuatro cartuchos comerciales de diferentes características que son los más usados para la extracción de contaminantes emergentes en muestras ambientales (Bond Elut, Isolute ENV+, Oasis HLB y Strata-X). Se seleccionaron ocho compuestos citostáticos de diferentes familias ampliamente utilizados en quimioterapia (ETO, VINB, GEM, 5-FU, MET, TAM, CP y VINC). Se realizó un diseño experimental con cada uno de los cartuchos estudiando las variaciones producidas en el rendimiento de la extracción al modificar el pH, la fuerza iónica y el volumen de muestra con el objetivo de encontrar el cartucho más idóneo y las mejores condiciones de extracción para la mezcla de compuestos seleccionada. Como resultado, se obtuvieron dos procedimientos de SPE, uno con cartuchos Oasis HLB para aguas residuales y otro con cartuchos Bond Elut para agua de mar.

Una vez optimizadas las condiciones de extracción se obtuvo un método reproducible, con desviaciones de reproducibilidad entre el 4 – 15% y variaciones de repetibilidad entre 6 – 16% para todos los compuestos estudiados a distintas concentraciones. Se obtuvieron recuperaciones en las distintas aguas analizadas (influente y efluente de depuradoras y agua de mar) entre 50 – 150% dependiendo de la matriz, el compuesto y la concentración. Finalmente, se obtuvieron límites de detección entre 2 – 104 $ng \cdot L^{-1}$ en influentes, 3 – 78 $ng \cdot L^{-1}$ en efluentes y 1 – 5 $ng \cdot L^{-1}$ en agua de mar.

Para comprobar la aplicabilidad del método desarrollado, se tomaron muestras en unos de los principales hospitales de la isla, en cuatro estaciones depuradoras de diferentes zonas de la isla, con distintos tratamientos de depuración, y en tres emisarios submarinos de las tres depuradoras de mayor volumen analizadas, en un monitoreo trimestral durante dos años. Con este estudio se pretende contribuir, no solo al conocimiento de la presencia y distribución de estos compuestos en aguas residuales, sino también al conocimiento existente sobre su eliminación bajo diferentes tratamientos.

Se detectaron cuatro compuestos en aguas residuales de hospital: ETO, en concentraciones entre 375.8 y 619.9 $ng \cdot L^{-1}$, CP a 1218 $ng \cdot L^{-1}$, VINC a 1851 $ng \cdot L^{-1}$ y VINB a 1835 $ng \cdot L^{-1}$ detectándose, por primera vez, el compuesto vinblastina en aguas residuales hospitalarias.

Respecto a las aguas residuales de las EDARs, se detectaron los compuestos ETO y CP. Las concentraciones de ETO a la entrada de la depuradora fueron relativamente altas (de hasta 5141 $ng \cdot L^{-1}$). Sin

embargo, no se detectó a la salida, lo que podría significar que el compuesto podría sufrir degradación. La CP, por el contrario, se detecta únicamente en la salida (hasta 91.25 $ng \cdot L^{-1}$) debido, probablemente, a que el efecto matriz en la entrada es mayor y la señal se pierde entre las interferencias. Estos resultados indican que los tratamientos convencionales no son adecuados para la eliminación total de estos compuestos en las aguas residuales. Por último, en los emisarios submarinos, no se detectaron ninguno de los compuestos, probablemente por el factor de dilución.

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Simultaneous and systematic analysis of cytostatic drugs in wastewater samples by ultra-high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Cytostatic drugs are compounds used to fight cancer, which may be excreted after administration to patients, and eventually reach wastewater. Given the high incidence of cancer and the properties of drugs, the drug concentrations in water systems should be evaluated.

We present the optimization, development and application of a solid phase extraction (SPE) method for the determination of eight cytostatic compounds of different classes in wastewater and seawater samples. We compared four SPE cartridges prior to their determination by Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry. For wastewater samples, the Oasis HLB cartridge gave the best recoveries, which were higher than 65% in most cases, achieving limit of detections (LODs) of 1.68–103.95 ng·L^{-1}. In seawater samples, the Bond Elut cartridge afforded the best recoveries > 70%, with LODs of 0.95–5.14 ng·L^{-1}.

The optimal procedure was applied in four hospital wastewater effluent samples taken during one year, and in different influents and effluents from wastewater treatment plants and seawater from marine outfalls taken in eight campaigns during two years, in Gran Canaria island (Spain). Results showed that etoposide was present in influents of wastewater treatment plants in several months and different wastewater treatment plants and hospital effluents in the range 375.8–5141 ng·L^{-1}, while cyclophosphamide was present in some months in effluents from only one wastewater treatment plant and hospital effluents in the range 55.94–1212 ng·L^{-1}. Vinblastine and vincristine were detected in one sample of hospital at concentrations of 1836 ng·L^{-1} and 1851 ng·L^{-1}, respectively.

1. Introduction

The most studied emerging pollutants that fall under the category of pharmaceutical chemicals include antibiotics, analgesics and hormonal compounds [1]. However, a significant group of pharmaceutical compounds, such as anti-cancer drugs, has not yet been fully examined. Anti-cancer compounds are designed to kill cancerous cells or reduce their growth [2]. The International Agency for Research on Cancer [3] has estimated that in 2012, there were > 14 million cases of cancer globally, distributed as indicated in Fig. 1, and has predicted that cancer incidents will increase in the future, thereby increasing the use of these drugs. However, cytostatic agents can cause unwanted side effects because of their genotoxic, mutagenic and carcinogenic activities. They can cause cell death by acting directly on DNA, blocking cell growth, or by recruiting cytotoxic cells [4].

According to the World Health Organization's Collaborating Centre for Drug Statistics Methodology [5], there are five groups of cytostatic agents. Depending on the type of cancer and its stage, the patient may be treated with chemotherapy. For example, gemcitabine (GEM) and etoposide (ETO) are used for the treatment of lung cancer; cyclophosphamide (CP), 5-fluorouracil (5-FU), methotrexate (MET), tamoxifen (TAM) and GEM are used for the treatment of breast cancer; 5-FU is also used for the treatment of colon cancer; and vinblastine (VINB) is used in the treatment of bladder cancer. [6]. The first group of cytostatic agents comprises alkylating agents, which include the widely used CP or ifosfamide (IF). The antimetabolite group includes MET (a folic acid analogue) and pyrimidine analogues, such as cytarabine (CYT), 5-FU, capacitabine (CAP) and GEM. Plant alkaloids and other natural products make up the third group, which includes vinca alkaloids and their analogues, such as VINB, vincristine (VINC) and podophyllotoxin derivatives like ETO. Cytotoxic antibiotics and related substances represent the fourth group, which includes doxorubicin (DOX),

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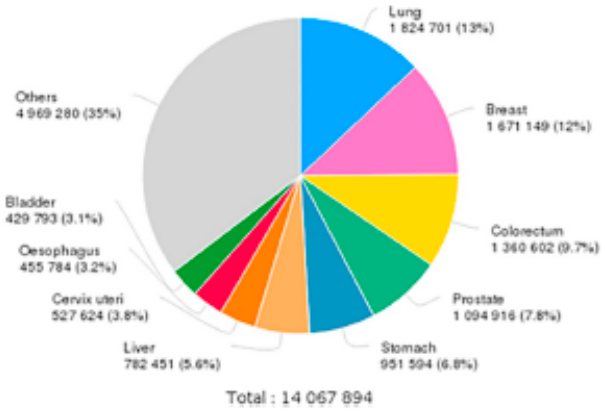


Fig. 1. Estimated number of incidence cases, 2012 [3].

daunorubicin (DAU) and epirubicin (EPI). The last group is other cytostatic agents, where TAM, platinum compounds and protein kinase inhibitors can be found.

Once administered to a cancer patient, the cytostatic agent can be excreted without chemical modification and can enter the water system. The possible health and environmental effects of these compounds, even at low concentrations, are presently unknown. In addition, the metabolites and transformation products of these drugs could be even more dangerous, so they should also be studied [7].

Most wastewater treatment plants discharge their effluents into lakes or seas, but there are very few optimized procedures for the detection of cytostatic compounds in sea or river waters. In this way, Martin et al. [8] detected CYT and GEM, among other compounds, at concentrations 13 and 2.4 $ng{\cdot}L^{-1}$, respectively, and Buerge et al. [9] detected CP in concentrations between 0.05 and 0.17 $ng{\cdot}L^{-1}$ and IF in concentrations between 0.08 and 0.14 $ng{\cdot}L^{-1}$ in surface waters. In the same kind of waters, Ferrando-Climent et al. [10] detected four cytostatic compounds (TAM, CP, MET and ciprofloxacin) in concentrations that ranged between 5 and 102 $ng{\cdot}L^{-1}$. In other studies, cytostatic compounds were measured among other pharmaceutical compounds. For instance, CP was detected by Zuccato et al. [11] at concentrations between 2.2 and 10.1 $ng{\cdot}L^{-1}$ and Metcalfe et al. [12] at concentrations of 5 $ng{\cdot}L^{-1}$, both in river waters, while Valcárcel et al. [13] detected IF in concentrations between 12.8 and 41 $ng{\cdot}L^{-1}$, also in surface waters.

To the best of our knowledge, no study has been conducted on seawater, being this ecosystem where wastewater treatment plants discharge their treated wastewaters on islands such as the Canary Islands (Spain).

Due to the low concentrations of these pharmaceutical agents, extraction and preconcentration techniques, such as solid phase extraction (SPE), are required. It is necessary to determine which cartridge offers the best retention for the target compounds while separating them from the interferences of the matrix, especially in complex matrices such as residual water. For example, Strata-X cartridges were used to extract the alkylating agents CP and IF [14]; however, for the extraction of pyrimidine analogues (5-FU, CYT and GEM [15] or 5-FU and CAP [16]), the cartridge Isolute ENV$^+$ seems to be the best option. For the extraction of anthracyclines, such as DOX, DAU and EPI, C8 cartridges were used by Mahnik et al. [17].

In each of these cases, only one family of cytostatic agents was selected, and a process for that group was optimized. However, to analyse a larger group of the most widely used cytostatic agents, a method that can extract a variety of compounds with different structures and physicochemical properties must be developed.

The aim of this study is, on one hand, to optimize an extraction procedure for the preconcentration and determination of cytostatic agents used to treatment the most common types of cancer in samples of wastewater and seawater. We present the development and application of a SPE method for the determination of eight cytostatic compounds of different classes (ETO, VINB, GEM, 5-FU, MET, TAM, CP and VINC). On the other hand, we studied the occurrence of these drugs in samples of wastewater effluents from hospital taken between July 2017, and April 2018, and different influents and effluents from wastewater treatment plants and seawater samples from marine outfalls taken between July 2016, and April 2018. Some of these compounds are very consumed annually in Spain, as is the case of TAM) (200 kg/year), MET (75 kg/year) and CP (25 kg/year) [18]. According to the scientific community, the research of cytostatic compounds in environmental samples should focus on the mixture of cytostatic compounds, since depending on the cancer type, several cytostatic compounds can be used at the same time and not only one cytostatic families with similar properties., that's why we select compounds from different families of cytostatics. Furthermore, we compared four cartridges with different characteristic (Isolute ENV$^+$, Strata-X, Oasis HLB and Bond Elut) for the extraction and preconcentration of these cytostatic drugs prior their detection and determination by Ultra High Performance Liquid Chromatography tandem Mass Spectrometry (UHPLC-MS/MS). All cartridges were evaluated trough a 3^2 experimental design, examining three different pHs and ionic strengths. Then the cartridges that offered the best results for each matrix were selected. The results were compared with similar works, and the difficulties and drawbacks of the simultaneous extraction of structurally diverse compounds in complex matrices was discussed.

2. Experimental

2.1. Material and reagents

Bond Elut Styrene divinyl-benzene cartridges (6 cc, 500 mg) were purchased from Agilent (Madrid, Spain), Isolute ENV+ cartridges (6 cc, 500 mg) hydroxylated polystyrene–divinylbenzene copolymer were purchased from NET INTERLAB (Madrid, Spain), Oasis HLB cartridges (6 cc, 500 mg) polymeric reversed-phase sorbent were purchased from Waters (Barcelona, Spain) and Strata-X *N*-vinylpyrrolidone cartridges (6 cc, 500 mg) were purchased from Phenomenex España (Madrid, Spain).

HPLC-grade methanol (MeOH), HPLC-grade acetonitrile, LC–MS grade MeOH, LC–MS grade water and formic acid used to adjust the pH of the mobile phase were all obtained from Panreac Quimica (Barcelona, Spain). Ultrapure water was obtained using a Milli-Q system (Milli-pore, Bedford, MA, USA). The cytostatics MET, GEM, 5-FU, VINB and VINC were purchased from Cymit-Quimica (Barcelona, Spain), whereas CP, TAM and ETO were purchased from Sigma-Aldrich (Madrid, Spain). All compounds had a purity > 97%. Stock solutions containing 1000 $mg{\cdot}L^{-1}$ of each analyte were prepared by dissolving the compound in MeOH, and the solutions were stored in glass-stoppered bottles at −20 °C in the dark. Working standard solutions were prepared daily. Some chemical characteristics and structures of the selected compounds are shown in Table 1.

2.2. Sample collection

Wastewater samples were collected, on one hand, from the effluent and influent of four wastewater treatment plants in Gran Canaria Island, Spain, which each use different treatment processes. Table 2 shows the type of treatment of each wastewater treatment plant, the design flow and the equivalent population.

On the another hand, wastewater samples were also taken at the effluents of one of the main hospitals of the Gran Canaria island.

Seawater samples were collected from the marine outfalls (MO) of the three conventional wastewater treatment plants (WWTP2, WWTP3 and WWTP4), located in the coast of Gran Canaria island. These water samples were taken in the exit of the marine outfalls at the depth of the

Table 1
Structure and properties of the selected cytostatic compounds [19–26].

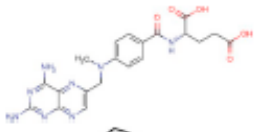
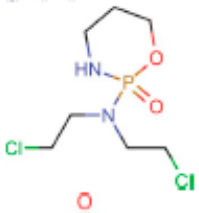
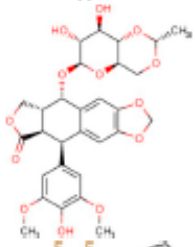
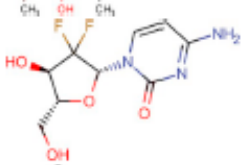
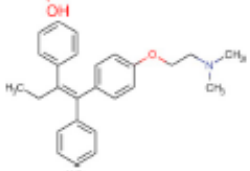
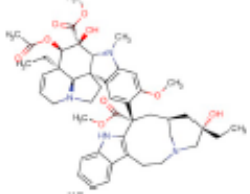
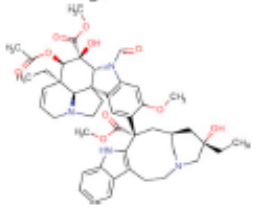
Compound	Molar mass ($g \cdot mol^{-1}$)	pK_a	Log $K_{o/w}$	Structure
MET	454.447	3.25	−0.24	
CP	261.08	13.43	0.1	
5-FU	130.078	7.18	−0.66	
ETO	588.562	9.33	1.16	
GEM	263.201	11.52	−1.47	
TAM	**371.524**	**8.76**	**6.35**	
VINB	810.989	10.87	4.18	
VINC	824.972	10.85	3.13	

Table 2
Characteristics of the wastewater treatment plants studied.

WWTP	Treatment	Design flow ($m^3 \cdot d^{-1}$)	Equivalent population
WWTP1	Membrane bioreactor	700	7000
WWTP2	Activated sludge	34,800	290,000
WWTP3	Activated sludge	7000	70,000
WWTP4	Activated sludge + reverse osmosis as tertiary treatment	6000	60,000

ocean and on the surface by a professional scuba diver.

All samples were taken in amber glass bottles that were rinsed beforehand with ultrapure water. All samples were immediately acidified with hydrochloric acid to a pH between 2.5 and 3.5 to avoid microbial degradation and then stored in a refrigerator at 4 °C until analysis. The samples were collected every three months during a two-years span. Sampling was carried out between July 2016, and April 2018, in four wastewater treatment plants both in the effluent and the influent and 3 samples of seawater on the surface and 3 in depth of each submarine outfalls of the main wastewater treatment plants (WWTP2, WWTP3 and WWTP4). Hospital wastewater effluent samples were taken only during one year, from July 2017 until April 2018.

A matrix effect significantly impacted the analytical parameters of these samples, and it will be treated in depth in the next section. Due to the loss of analytical signal caused by the matrix interference, the detection limits achieved were not sufficiently low to detect cytostatic compounds in wastewater, the concentrations in which are usually in the $ng \cdot L^{-1}$ range [27]. For this reason, an extra step was introduced in which extracts of 5 mL of MeOH, from the samples of wastewater and seawater, were brought to dryness and then reconstituted in 1 mL of MeOH.

2.3. Equipment and chromatographic conditions

All cartridges were conditioned with 5 mL of MeOH and 5 mL of Milli-Q water, except for the Oasis HLB cartridges, which needed no conditioning step. The extraction was performed under vacuum at a rate of one drop per second. After the extraction, the cartridges were washed with 5 mL of Milli-Q water and 5 mL of MeOH. All analyses were performed in triplicate ($n = 3$).

An ACQUITY UPLC system equipped with a triple quadrupole detector with an ESI interface controlled by MassLinx Mass Spectrometry software was used. The system consisted in a 2777 autosampler, a Binary Solvent Manager and a column manager, all from Waters Chromatography (Barcelona, Spain). The electrospray ionization was performed in positive mode (except for 5-FU, which was performed in negative mode) and detection parameters were optimized using standard solutions of each compound. The optimum detection parameters values were as follows: capillary voltage of 3.5 kV, cone voltage of 40 V and source and desolvation temperature of 120 and 400 °C, respectively. Nitrogen was used as the desolvation gas at a flow of $1000\ L \cdot h^{-1}$ and argon was employed as the collision gas.

The sample volume that was injected into the chromatographic system was 10 µL, and the analyte separation was performed using a mobile phase of water (A) and MeOH (B), both adjusted to 0.1% (v/v) of formic acid. The analytical column was a Phenomenex Luna Omega Polar 50 × 2.1 mm, with a particle size of 1.6 µm (Phenomenex, Madrid, Spain) operated at room temperature.

The flow was set at $0.4\ mL \cdot min^{-1}$ and remained constant throughout the chromatographic run. The gradient started at 40% A, 60% B (v/v) and increased to 90% A in 0.5 min. Then it decreased to 0% A in 0.5 min and remained at 100% B for 1 min. Over 1 min, the gradient returned to its initial conditions (40%A, 60%B (v/v)), and it remained constant for 2 min more to stabilize the flow. The full chromatographic separation was completed in 4 min.

Table 3
Mass spectrometer parameters for the determination of selected cytostatic compounds.

Compound	T_R (min)	Precursor ion (m/z)	Cone voltage (ion mode)	Quantifier MRM, m/z (collision potential, V)	Qualifier MRM, m/z (collision potential, V)
ETO	1.30	589.5	40 V (ESI+)	229.21 (15)	185.26 (20)
VINB	0.43	812.8	40 V (ESI+)	224.07 (45)	124.39 (43)
GEM	0.52	264.2	35 V (ESI+)	112.11 (20)	87.01 (20)
5-FU	0.41	129.1	30 V (ESI-)	58.19 (19)	85.92 (19)
MET	0.48	455.1	30 V (ESI+)	308.14 (15)	175 (17)
TAM	1.39	372.3	40 V (ESI+)	72.12 (20)	129.17 (20)
CP	1.29	261.2	30 V (ESI+)	140.04 (20)	106.06 (18)
VINC	0.41	825.8	40 V (ESI+)	140.17 (55)	122.18 (60)

For the quantitative analysis, multiple reaction monitoring parameters were optimized for each compound. The optimizations of the quantification and confirmation of fragment ions and of the detection parameters for each compound was performed by directly infusing a standard of 1 mg·L^{-1} in MeOH at a flow rate of 10 µL·min^{-1} into the mass spectrometry detector.

The mass spectrometer parameters for the determination of target analytes are shown in Table 3. Precursor ions were $[M-H]^+$ in the positive ion mode (ESI +) for all compounds, with the except of CP, which was $[M]^+$, and 5-FU, which was $[M-H]^-$ obtained using the negative ion mode (ESI-).

3. Results and discussion

In an SPE procedure, several experimental variables, including type of sorbent, sample volume, sample pH, the wash step and elution conditions, must be evaluated to achieve the maximum extraction efficiency of the target analytes. Milli-Q water samples were spiked with 10 µg·L^{-1} of each analyte. The elution was performed with 5 mL of MeOH at a rate of one drop per 2 s.

3.1. Optimization of SPE procedure

The cartridge experiments were carried out using a Minitab® 17.1.0. First, a 2^3 experimental design (3 variables, 2 levels) was performed with an Oasis HLB cartridge, because it is a hydrophilic-hydrophobic balanced cartridge that has more sensitivity to the polarity of analytes. The three variables were sample volume (100 and 250 mL), pH (3 and 11, adjusted with HCl and NaOH) and ionic strength (adding NaCl, 0 and 10% (*w/v*)). The results showed a *p*-value positive in most of cases related with the sample volume. This means that an increase in the sample volume will benefit the extraction, but a volume higher than 250 mL can be reached the breakthrough volume and in addition, in real wastewater samples clog the cartridge, so a limit of 250 mL of sample volume was chosen for the rest of the assays. The results also showed that an acidic pH benefited the extraction with Oasis HLB. Hence, the 3^2 experimental design (2 variables at 3 levels) carried out for Oasis HLB used the pHs 2, 4 and 6, while, for those rest of the cartridges used the pHs 2, 6 and 10. In relation to the ionic strength, some compounds were positively affected and others were negatively affected, so the same conditions were tested in all the cartridges (0%, 5% and 10%). Table 4 summarizes the conditions of each trial, and results are shown in Fig. 2.

The efficiency of extraction is shown in the Fig. 2 by the ratio between the eluted extract of the SPE cartridges and a MeOH standard at the same concentration. 5-FU does not appear to be retained by any of the cartridges under any of these conditions, GEM was poorly extracted, probably due to low octanol / water partition coefficients. The rest of the compounds with higher octanol / water partition coefficients were better retained. For these reason, 5-FU and GEM were excluded for the later studies.

The extraction efficiencies of VINB and VINC were above 100%, probably due to some interaction between the compounds and the polymer of the cartridge under certain conditions that increased in the signal in the detector. The rest of the compounds appeared to behave normally and were extracted at higher or lower efficiencies depending on the extraction conditions. The selected cytostatic compounds have pKas that range from 3.25 to 13.43 [19,20], which means that their behaviour will vary greatly under different extraction conditions. Fig. 2 demonstrates that the pH and ionic strength conditions that produced very high extraction efficiencies for some compounds produced very low extraction efficiencies for others. For this reason, when working with compounds with so much variety in their structures and properties, it is prudent to look for conditions that are acceptable for most of them. In this case, we selected the Oasis HLB cartridge for the wastewater samples and the Bond Elut cartridge for the seawater samples, since they presented the best extraction efficiencies for most of the compounds at ionic strengths of 0 and 5% (*w/v*) (adjusting with NaCl), respectively. In accordance with these results, the optimum conditions for the Oasis HLB procedure were sample volume = 250 mL, pH = 4 ± 0.1 and 0% ionic strength, and for the Bond Elut procedure were sample volume = 250 mL, pH = 2 ± 0.1 and the ionic strength of seawater, which is close to 5%.

Table 4
Experimental design conditions for the different cartridges.

N° Assay	Oasis HLB		Isolute ENV, Strata-X, Bond Elut	
	pH	Ionic strength (%, w/v)	pH	Ionic strength (%, w/v)
1	2 ± 0.1	0	2 ± 0.1	0
2	2 ± 0.1	5	2 ± 0.1	5
3	2 ± 0.1	10	2 ± 0.1	10
4	4 ± 0.1	0	6 ± 0.1	0
5	4 ± 0.1	5	6 ± 0.1	5
6	4 ± 0.1	10	6 ± 0.1	10
7	6 ± 0.1	0	10 ± 0.1	0
8	6 ± 0.1	5	10 ± 0.1	5
9	6 ± 0.1	10	10 ± 0.1	10

Three different eluents (MeOH, ACN and 50:50 *v/v* ACN:MeOH) were tested at three different volumes (5, 2 and 1 mL) in both cartridges under selected conditions. Fig. 3 shows how the extraction efficiency of each compound varied with the eluents and elution volumes used in Oasis HLB cartridge. Because 5 mL of MeOH achieved acceptable extractions for most of the compounds, it was selected for the remainder of the assays.

Due to the loss of analytical signal caused by the matrix interference, an extra step was introduced in which extracts of 5 mL of MeOH, from the samples of wastewater and seawater, were brought to dryness and then reconstituted in 1 mL of MeOH, reaching a preconcentration factor of 250.

3.2. Quality control levels

A Milli-Q water standard calibration curve was prepared with the mixture of compounds using nine points with concentrations between 1

Fig. 2. Results of the experimental design for the different compounds and cartridges.

and 750 $\mu g \cdot L^{-1}$. Linearity was calculated as the relationship between peak area and concentration, obtaining correlation coefficients higher than 0.9902. The analytical parameters were first performed in Milli-Q water at two concentrations (0.5 and 2 $\mu g \cdot L^{-1}$), obtaining the results shown in Table 5 for Oasis HLB (a) and for Bond Elut (b) SPE cartridges. These two levels were chosen since these concentrations are among which cytostatic compounds are mostly detected both in wastewater and hospital effluents [27].

The recovery was calculated as the ratio between the peak areas of the extract that was spiked before the extraction and the peak areas of the extract that was spiked after the extraction [28]. The Limit of detection (LOD) and Limit of Quantification (LOQ) were calculated using the signal-to-noise ratio of each individual peak. The LOD is defined as the lowest concentration that gives a signal-to-noise ratio > 3, whereas LOQ is defined as the minimum concentration giving a signal-to-noise > 10. Due to the differences of the compounds, LOD and LOQ ranged widely.

A good recovery (> 80%) was obtained for Oasis HLB in most cases, except for the compounds 5-FU and GEM, which could not be extracted. The intra-day and inter-day standard deviation were < 10% and 12% respectively for all compounds. In Bond Elut cartridges recoveries higher than 70% were obtained for almost all the compounds, except for GEM and 5-FU that cannot be extracted with this cartridge under the selected conditions. The intraday and interday standard deviation is < 15% and 16% respectively. It can therefore be said that the method is reproducible. LODs obtained were between 0.6 and 7.46 $ng \cdot L^{-1}$ for Oasis HLB, depending on the compound, and between 0.5 and 8.78 $ng \cdot L^{-1}$ for Bond Elut, depending on the compound. LODs lower than 10 $ng \cdot L^{-1}$ were obtained in both cartridges for the selected compounds.

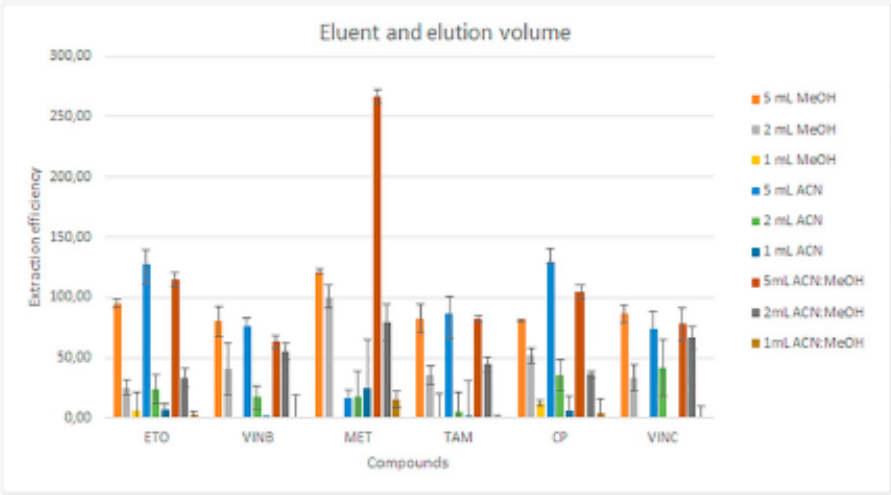


Fig. 3. Selection of the eluent and elution volume for Oasis HLB.

Table 5
Analytical parameters of selected cytostatic compounds in Milli-Q water for (a) Oasis HLB and (b) Bond Elut cartridges.

Compound	Recovery (%) (n = 3)		RSD (%) Intraday (n = 6)		RSD (%) Interday (n = 3)		LOD ($ng \cdot L^{-1}$)	LOQ ($ng \cdot L^{-1}$)
	0.5 $\mu g \cdot L^{-1}$	2 $\mu g \cdot L^{-1}$	0.5 $\mu g \cdot L^{-1}$	2 $\mu g \cdot L^{-1}$	0.5 $\mu g \cdot L^{-1}$	2 $\mu g \cdot L^{-1}$		
a)								
ETO	77	72	6	4	6	7	0.86	5.72
VINB	65	83	6	4	12	10	3.10	20.6
MET	92	91	5	8	7	11	0.60	4.03
TAM	88	86	6	4	9	10	1.75	11.6
CP	90	64	6	8	7	9	2.20	14.6
VINC	116	96	10	5	11	11	7.46	49.7
b)								
ETO	91	142	10	9	13	13	2.15	7.17
VINB	68	78	12	5	16	13	2.25	7.50
MET	131	99	11	9	14	9	8.78	29.3
TAM	70	73	9	13	15	16	0.50	1.67
CP	104	122	11	7	2	8	0.90	2.99
VINC	65	94	15	12	11	13	2.11	7.03

Table 6
Recovery (%) of cytostatic compounds in samples from effluent and influent of a wastewater treatment plant and seawater from marine outfalls.

Compound	Effluent			Influent			Seawater		
	0.5 $\mu g \cdot L^{-1}$	1 $\mu g \cdot L^{-1}$	2 $\mu g \cdot L^{-1}$	0.5 $\mu g \cdot L^{-1}$	1 $\mu g \cdot L^{-1}$	2 $\mu g \cdot L^{-1}$	0.5 $\mu g \cdot L^{-1}$	1 $\mu g \cdot L^{-1}$	2 $\mu g \cdot L^{-1}$
ETO	nd	76.23	77.16	nd	136.5	51.71	120.0	113.6	129.4
VINB	97.49	80.11	100.2	nd	nd	91.69	90.65	142.1	79.28
MET	47.02	69.99	67.98	53.51	67.01	85.55	134.9	130.7	102.2
TAM	49.55	91.35	66.56	nd	76.72	67.05	68.58	113.9	78.16
CP	82.44	107.8	91.69	45.00	83.81	77.72	132.5	133.0	122.6
VINC	nd	115.8	149.1	nd	nd	81.53	102.9	133.8	94.85

*nd: not detected.
*Average of three determinations.

Analytical parameters in wastewater and seawater were performed at three concentrations (0.5 $\mu g \cdot L^{-1}$, 1 $\mu g \cdot L^{-1}$ and 2 $\mu g \cdot L^{-1}$). Recovery obtained to three appropriate concentration levels for the effluents and influents of wastewater treatment plants and for seawater are shown in Table 6.

Recovery values in the wastewater were acceptable for most compounds (ranging from 65%–100%) under different concentrations; however, some of them were slightly lower than those obtained with Milli-Q water due to the great amount of interferences. The recovery of VINC increased in both types of samples, likely due to the "salting-out" process. In Fig. 2, it can be observed that this compound's recoveries also increased when salt was added to the samples to increase their ionic strength. This effect is probably also behind the other recoveries in seawater samples that ranges above 100%. For this study, the range of recoveries varied between 80%–140% for almost all compounds at different concentrations.

The LODs and LOQs of the different compounds, calculated for effluent and influent of wastewater from wastewater treatment plant and seawater from marine outfalls are shown in Table 7. These results were in a range acceptable for the concentrations in which the compounds

Table 7
LODs and LOQs of cytostatic compounds in wastewater and seawater.

Compound	Effluent		Influent		Seawater	
	LOD ($ng{\cdot}L^{-1}$)	LOQ ($ng{\cdot}L^{-1}$)	LOD ($ng{\cdot}L^{-1}$)	LOQ ($ng{\cdot}L^{-1}$)	LOD ($ng{\cdot}L^{-1}$)	LOQ ($ng{\cdot}L^{-1}$)
ETO	16.36	54.53	67.51	225.0	1.06	3.55
VINB	32.61	108.7	104.0	346.5	3.66	12.20
MET	11.04	36.78	3.51	11.69	5.14	17.13
TAM	2.66	8.88	5.16	17.22	0.95	3.15
CP	3.40	11.34	1.68	5.60	3.10	10.33
VINC	77.88	259.6	45.92	153.1	1.96	6.54

were detected and were in agreement with the results obtained by other authors [29,30].

3.3. Matrix effects in wastewater and seawater

The matrix effect greatly hampers the detection of cytostatic compounds in wastewater samples. It is a common drawback in complex matrices such as wastewater, especially when attempting to extract and detect compounds with greatly varying structures or many compounds simultaneously. In these cases, cleaning the sample to avoid interference is not a viable option because it leads to loss of the target compounds. The negative matrix effects (suppression of the signal) observed by Martin et al. [8] ranged around 30% for most compounds, but reached up to 80% for some compounds. Yin et al. [31] obtained an average of suppression of the signal due to the matrix effect around 80%, although in some cases it rose to 95%.

For some compounds, such as VINC, the matrix effects had the opposite result, increasing of the signal more than double. Ferrando-Climent et al. [32] observed both amplification (up to seven times the supposed value) and suppression of the signal and propose two possible ways to deal with the matrix effect. The first is by using internal standards. However, Ferrando-Climent et al. were no able to correct the matrix effect of all analytes by using internal standards. Internal standards can be expensive since they must be available and similar to the molecules that are been investigated. The second way was to perform a Matrix Match Calibration (MMC) that takes into account the effects of interferences on detecting the analytes.

In our work, a MMC was performed to take into account the interferences extracted during the SPE that suppressed of the signal in wastewater. In this case, wastewater (both influent and effluent) and seawater extracts, were dried with nitrogen and then reconstituted with MeOH and the appropriate amount of analyte to perform the calibration curve. In some cases, these signals were suppressed up to 99%, but most cases the suppression was around 90%, as seen in Table 8. Matrix effect has been calculated following the formula of F. Gosetti et al. [33].

$$ME = 100 - \left(\frac{B}{A} * 100\right),$$

where ME is defined as the Matrix Effect, B is the signal of the compound already extracted and A is the signal of compound directly injected in the mobile phase.

On the other hand, as observed in the initial tests, the presence of salt significantly influences the extraction efficiency. The matrix effect in seawater does not always result in the suppression of the signal, but instead amplifies it in some cases, which is shown as a negative matrix effect. In this study, the matrix effect was more variable, and the low presence of interferences facilitated the detection of the compounds. A MMC was performed to deal with this problem as well. The results obtained cannot be compared with any other work, since no optimization of these cytostatic compounds in seawater has been found in literature. For these reasons, we prepared MMC for every matrix (effluent, influent and seawater) to correct the matrix effect during the quantification of every compound in environmental samples.

Table 9
Concentration of cytostatic compounds in wastewater samples.

Compound	Point	Sampling campaign	Concentration ($ng{\cdot}L^{-1}$)*
ETO	Hospital effluent	January 2018	375.8 ± 35
		April 2018	619.9 ± 48
	WWTP1 (influent)	July 2017	3816 ± 260
	WWTP2 (influent)	October 2017	5141 ± 256
		January 2018	4337 ± 93
	WWTP3 (influent)	January 2018	3376 ± 592
		April 2018	874.9 ± 161
	WWTP4 (influent)	October 2017	987.6 ± 91
		January 2018	4888 ± 151
CP	Hospital effluent	April 2018	1218 ± 45
	WWTP2 (effluent)	October 2016	91.25 ± 14
		October 2017	65.97 ± 1.4
		January 2018	55.94 ± 6.9
VINB	Hospital effluent	April 2018	1835 ± 181
VINC	Hospital effluent	April 2018	1851 ± 136

(*) Average of three determinations.

3.4. Determination of cytostatic compounds in wastewater and sea water samples

The optimized method was applied to samples from four wastewater treatment plants in Gran Canaria island (Spain) taken during eight sampling campaigns like was indicated in the experimental section. The results are shown in Table 9.

As it can be observed, etoposide is the compound that has been detected in higher concentrations and in more occasions. It was detected in hospital effluents and in influents samples from all WWTPs, but not in the effluent, which could indicate degradation of the compound during the treatment. ETO reacts quickly with free chlorine [34] and this may be the reason why it is not detected in the effluents, since it is common the use of chlorine or sodium hypochlorite in the WWTPs as a disinfectant.

Cyclophosphamide was detected in hospital effluent in one occasion (during the month of April 2018) and in WWTP2 effluent, likely because the matrix effect is much higher in the influent. For that reason, it

Table 8
Matrix effect (%) of cytostatic compounds in wastewater and seawater samples.

Compound	Effluent			Influent			Seawater		
	0.5 $\mu g{\cdot}L^{-1}$	1 $\mu g{\cdot}L^{-1}$	2 $\mu g{\cdot}L^{-1}$	0.5 $\mu g{\cdot}L^{-1}$	1 $\mu g{\cdot}L^{-1}$	2 $\mu g{\cdot}L^{-1}$	0.5 $\mu g{\cdot}L^{-1}$	1 $\mu g{\cdot}L^{-1}$	2 $\mu g{\cdot}L^{-1}$
ETO	nd	99.18	97.54	99.85	99.77	99.83	−6.42	19.80	−100.8
VINB	97.85	99.32	92.38	nd	95.78	98.02	49.83	75.50	87.93
MET	87.25	93.82	84.12	92.66	98.18	96.73	66.78	80.38	85.84
TAM	85.29	90.05	80.20	nd	98.50	98.51	−75.68	3.79	−34.21
CP	91.80	89.22	84.20	98.25	97.61	98.81	−85.17	−45.54	−17.11
VINC	nd	93.35	89.59	nd	92.87	94.10	73.96	74.38	84.86

*nd: not detected.

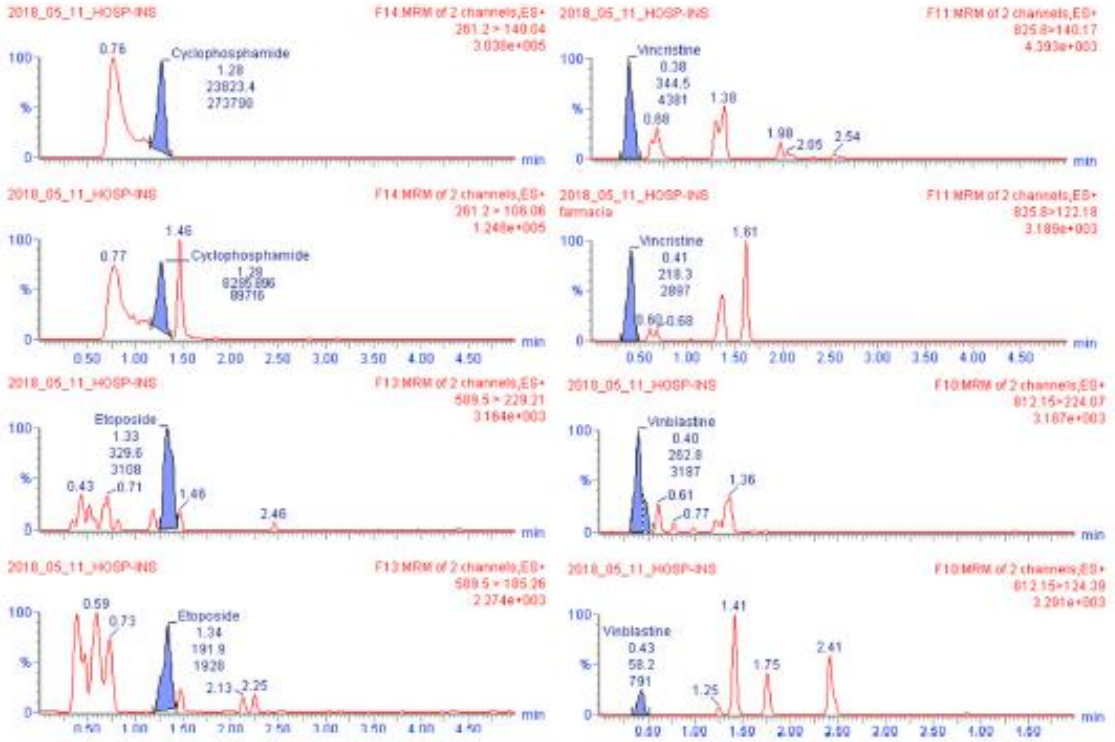


Fig. 4. Chromatogram of the detected cytostatic compounds in hospital effluents in April 2018.

could be concluded that the conventional treatment of activated sludge is not sufficient for CP's degradation. CP is a notable cytostatic, not only because of its high consumption, but also because of its resistance to oxidation by ozone or OH radicals [35]. Vinblastine and Vincristine were also detected in the hospital effluents during the month of April 2018. However, the fact that the compounds were not detected in half of the samples could indicate that the use of these medicines is not constant throughout the year. In Fig. 4, the chromatogram relevant to a sample from hospital effluents for the month of April 2018, is shown, as an example.

The optimized methodology was also applied to the study of the presence of these cytostatic compounds in three marine outfalls of the main treatment plants of the island (WWTP2, WWTP3 and WWTP4) and located in different points of Gran Canaria coast. However, no positive results were obtained. According to the bibliography, river samples present concentration levels of these compounds up to 100 times lower than those found in effluents of wastewater treatment plants and so probably in seawater the factor of dilution will be higher.

4. Conclusions

Due to the increase in cases of cancer and the anticipation that it will continue to increase, the consumption of cytostatic compounds is expected to increase accordingly. These compounds could cause important side effects upon excretion into water systems, so it is necessary to develop procedures capable of detecting them. For this work, a group of commonly used drugs against the most familiar diagnosed cancers was selected, and the aim of the study was to measure their concentrations in the influents and effluents of wastewater treatment plants and in their marine outfalls. The detection of compounds by electrospray ionization is very sensitive to interference, so working with complex matrices is very challenging. In addition, when analysing a heterogeneous group of compounds in environmental samples, the matrix effect is observed. In most cases, this produces a reduction in the signal, which means a higher LOD due to the presence of impurities. This can affect the compound's recovery and the reproducibility of results, which necessitates developing a method for accounting for it. We decided to use a MMC to correct for it in the quantification of the real samples.

A sensitive method, based on SPE extraction and UHPLC-MS/MS quantification, was optimized and validated for the simultaneous determination of different cytostatic compounds in wastewater and seawater samples from marine outfalls. The compounds in wastewater had absolute recoveries higher than 65% in most of cases, and their LODs ranged between 1.68 and 103.95 $ng \cdot L^{-1}$ depending on the compound. The compounds in seawater had absolute recoveries higher than 68% for all compounds, and their LODs ranged between 0.95 and 5.14 $ng \cdot L^{-1}$ depending on the compound. The repeatability and reproducibility of the method for both matrices was satisfactory.

Finally, the optimal method was applied to eight sampling campaigns for detecting etoposide in the influents of the wastewater treatment plants at concentrations between 874.9 and 5141 $ng \cdot L^{-1}$, cyclophosphamide in the effluents at concentrations between 55.94 and 91.25 $ng \cdot L^{-1}$. In hospital wastewater effluents we detected etoposide at concentrations of 375.8 and 619.9 $ng \cdot L^{-1}$, cyclophosphamide at concentrations of 1218 $ng \cdot L^{-1}$, vinblastine at concentrations of 1836 $ng \cdot L^{-1}$ and vincristine at concentrations of 1851 $ng \cdot L^{-1}$. However, the studied cytostatic drugs were not detected in seawater samples, likely due to the high dilution factor in this medium.

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3.2. Optimización de un método de Extracción por Adsorción sobre Tejidos Químicamente Modificados acoplado a cromatografía líquida de ultra resolución en tándem con espectrometría de masas para la determinación de residuos de medicamentos citostáticos en aguas ambientales

Como ya se ha comentado en la introducción de esta Tesis Doctoral, debido a las bajas concentraciones a la que se detectan los compuestos citostáticos en las muestras ambientales, es necesario el uso de técnicas de extracción y preconcentración previas a su determinación. Tradicionalmente, esta extracción y preconcentración se ha realizado mediante SPE pero como ya se ha discutido, se trata de una técnica cuyo principal inconveniente es el tiempo de extracción que conlleva, además de la cantidad de muestra necesaria para la extracción y el consumo de disolventes orgánicos.

Tratando de buscar alternativas viables, los investigadores se centran en el desarrollo de nuevas técnicas de extracción, más beneficiosas para el medioambiente (principalmente, por el menor consumo de disolventes), que simplifiquen el proceso de extracción. Siguiendo estos principios, los investigadores Abuzar Kabir y Kenneth G. Furton desarrollaron un procedimiento de microextracción con el nombre de “Extracción por Adsorción sobre Tejidos Químicamente Modificados” (FPSE) [89].

Esta técnica de microextracción consiste en un tejido de 2.5 x 2 cm recubierto con disolventes orgánicos-inorgánicos de alta eficiencia, que se pone en contacto directamente con la muestra durante un tiempo determinado, siendo 60 minutos en esta aplicación. Pasado este tiempo, el tejido se extrae de la muestra y se pone en contacto con el disolvente

orgánico adecuado para su elución. El tiempo de elución, en esta ocasión, fue de 5 minutos usando tan solo 1 mL de metanol, por lo que el extracto pudo ser inyectado directamente en el sistema cromatográfico. La Figura 5 muestra un esquema simplificado de la técnica:

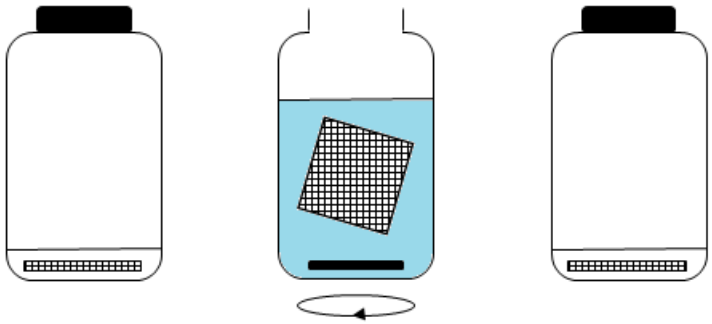

Figura 5. Extracción por Tejidos Químicamente Modificados

Hasta la fecha de publicación de este artículo, no se había aplicado ninguna técnica de microextracción para la extracción y preconcentración de compuestos citostáticos. En este trabajo se aplica, por primera vez, un procedimiento de microextracción en la extracción de compuestos citostáticos en aguas residuales.

Para ello, en primer lugar, se probó la capacidad de adsorción de seis tejidos distintos para la mezcla de compuestos citostáticos seleccionados. Se eligió el tejido que adsorbía la mayor cantidad de compuestos en mayor proporción y, a partir de ahí, se comenzó la optimización de todos los parámetros que afectan al proceso de absorción/desorción mediante un diseño experimental. Se seleccionaron las cuatro variables que podrían afectar en mayor medida a la extracción: tiempo de extracción, pH, volumen de muestra y tiempo de desorción. Se analizaron a dos niveles (2^4). Seleccionadas las dos variables cuya combinación afectaba en mayor

medida el proceso de extracción, pH de la muestra y tiempo de extracción, se realizó un segundo diseño experimental a tres niveles (3^2). Finalmente, se establecieron las condiciones óptimas de extracción: 10mL de muestra con un 0% de NaCl para modificar la fuerza iónica, puestos en contacto con el tejido durante 60 minutos a 1000 rpm y eluídos con 1mL de metanol durante 5 minutos. Debido a las propiedades físico-químicas de los compuestos, se tuvieron que seleccionar dos pHs diferentes para el volumen de muestra: pH = 8 para ETO, CP y TAM y pH = 10 para VINB y VINC.

En estas condiciones, mediante esta técnica podemos extraer cinco compuestos citostáticos ampliamente utilizados en terapias contra el cáncer (ETO, CP, VINC, VINB y TAM) de manera reproducible (con desviaciones estándar menores al 12%) y alcanzando límites de detección de entre 0.2 hasta 80 $ng \cdot L^{-1}$, equiparables a aquellos logrados con técnicas de extracción exhaustivas como la SPE y recuperaciones relativas superiores al 40%. Además, el efecto matriz observado varía entre 30% de supresión de señal y 40% de aumento de señal, lo cual es menor al obtenido cuando se trabaja con cartuchos de SPE y compuestos con propiedades y características muy diferentes como son los compuestos citostáticos.

Los medicamentos citostáticos se administran, en muchas ocasiones, en los hospitales. Aunque cada vez se tiende más a que los pacientes reciban el tratamiento en casa, o se vayan a casa poco después de recibir el tratamiento, los hospitales siguen siendo una importante fuente de entrada de este tipo de compuestos en el medio ambiente. Por ese motivo, este método de microextracción se aplicó, una vez optimizado,

tanto a muestras de aguas residuales procedentes de la EDAR de Las Palmas de Gran Canaria, como a aguas residuales de uno de los principales hospitales de la isla.

Por último, tras la aplicación del método a muestras de aguas residuales de distinto origen (EDAR y hospital), se detectó concentraciones de 2.6 $\mu g \cdot L^{-1}$ de ETO en el efluente del hospital, pero ningún compuesto fue detectado en la EDAR estudiada.

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Full length article

Optimization and application of fabric phase sorptive extraction coupled to ultra-high performance liquid chromatography tandem mass spectrometry for the determination of cytostatic drug residues in environmental waters

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ABSTRACT

Every year, hundreds of tons of organic pollutants reach the environment through effluents released from wastewater treatment plants worldwide, and many of these compounds have harmful effects on the aquatic ecosystem. A new class of emerging pollutants of high concern is cytostatic drugs, which are designed to treat different types of cancers by attacking cells. Environmental concentrations of cytostatic drugs are known to be in the range of ng L^{-1}, and for this reason, it is imperative to develop analytical methods of extraction and preconcentration to allow for subsequent instrumental analysis of these drugs.

In this work, a rapid, simple and green method for the analysis of seven cytostatic drug compounds that are commonly used in anti-cancer therapies was developed using a novel extraction process based on a powerful miniaturized technique, fabric phase sorptive extraction (FPSE) coupled to ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS).

The major parameters that affect the extraction process were optimized. The new method shows good linearity, with a relative standard deviation (RSD) of less than 12%. Relative recoveries higher than 40% were obtained for the studied compounds, and the detection limit of the method was within the values at which these compounds are usually found in environmental water (0.20 ng L^{-1} to 80 ng L^{-1}). The Limit of Quantification ranged from 0.68 to 267 ng L^{-1}. Significant suppression of the signal due to the matrix effect, a common shortcoming attributed to interference from the extraction process as well as the use of ionization mode, was not observed. Subsequently, the method was applied to real wastewater samples from an effluent obtained from a hospital area and three wastewater treatment plants located in Gran Canaria Island, Spain.

1. Introduction

Cytostatic agents are a group of emerging pollutants that could inhibit cell growth. For that reason, these compounds have high environmental relevance due to their potentially negative effects on eukaryotic organisms cells. These compounds constitute a large group of organic compounds with many chemical and structural differences and hence it is very challenging to obtain a reliable and sensitive analytical method for cytostatic residues in different sample matrices. Little is known about the mechanism of how low concentrations of these compounds could affect fauna and flora, but they can be extremely harmful to living organisms, even more so if these compounds are present in organisms as a mixture. [1,2]. According to the National Cancer Institute (NCI), some of the most prolific cancers in recent years, including lung, chest, bladder, non-Hodgkin's lymphoma, pancreas, etc., are treated with medicines such as methotrexate, gemcitabine, tamoxifen, cyclophosphamide, vinblastine and vincristine [3]. These drugs can be detected at very low concentrations (ng L^{-1}) in the aquatic environment and could be very persistent due to their continuous entry into the environmental water [4]. As a result, over the past decade, there has been

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0021-9673/

an increasing concern on the presence of cytostatic drugs, their behaviour and their environmental distribution [5].

As with other emerging pollutants, cytostatic agents can enter into the aquatic environment mainly through effluents from wastewater treatment plants and hospitals [6], and urinary excretion is the main contributory pathway of various cytostatic drugs that appear in the water cycle [7]. Their consumption mostly occurs in hospitals, but there are increasing levels in domestic wastewaters that are attributed to out-patients' consumption [1] and, as with other many pharmaceuticals, they are not completely metabolized and subsequently excreted as unchanged entities [8]. Due to their chemical properties, they are less likely to be degraded or removed by conventional wastewater treatment plants and more likely to reach the aquatic environment [9].

Some works have reported the genotoxicity of cytostatic compounds (5-fluorouracil, cytarabine and gemcitabine) observing that the concentration values in which these substances could produce substantial damage (44 $\mu g L^{-1}$–200 $mg L^{-1}$) [8] is close to the concentrations observed in hospital sewage water in China, where concentrations of up to 10.65 $\mu g L^{-1}$ of ifosfamide were measured [10]. A study of the toxic effects of cyclophosphamide, methotrexate, 5-fluorouracil and imatinib in plants found that all might affect the normal development and growth of plants, but the concentrations in which they are found in the environment (in the range of $ng L^{-1}$) are not likely to cause death [2]. A study on the toxic and genotoxic properties of the most widely used cytostatic agents (5-fluorouracil, etoposide, cisplatin, carboplatin, vincristine sulfate and cyclophosphamide) had similar results. Environmental concentrations are significantly lower ($ng L^{-1}$) than are needed to cause adverse effects in plants, but the effluent of hospital areas, which are discharged into the sewer network without prior treatment, could produce an increase of the concentration of these compounds in the sewage. Therefore, genetic material of aquatic organisms exposed to wastewater treatment plants outfalls could be adversely affected [11].

In vitro tests have been carried out with bacteria and algae showing damage at ambient concentrations similar to those found in hospital effluents and showing that a cytostatic mixture is even more harmful [12]. Moreover, the toxicity of cytostatic compounds is not only limited to the parent compounds but also the metabolites, due to the fact these metabolites may be more hazardous to certain species [13].

Cytostatic compounds are known to be found at very low concentrations in the aquatic environment and therefore warrant the development of an efficient extraction and preconcentration process prior to their determination. Solid phase extraction (SPE) is the most used technique to extract them from liquid samples [10,14–16]. However, SPE has some drawbacks that include the use of a high volume of organic solvents, the requirement of special laboratory equipment to carry out the operation, inability to perform the process in the field, the need of an extra step of solvent evaporation and sample reconstitution in most cases and the long extraction times. Currently, to make analytical chemistry greener, researchers are increasingly favouring more environmentally friendly analytical procedures, simplifying the extraction process [17], and developing microextraction methods. As a response to the global call for greenifying sample preparation technologies, Kabir and Furton [18] recently developed fabric phase sorptive extraction (FPSE), which is considered as a microextraction technique [19]. This technique consists of a fabric media (2.5 cm × 2.0 cm) coated with high-efficiency sol-gel hybrid inorganic-organic sorbents that is directly submerged into the sample containing the target analytes. This technique shows substantial advantages in comparison with other microextraction techniques by increasing the analyte retention capacity through higher sorbent loading and the reduction of extraction times by increasing the primary contact surface of the extraction device for rapid analyte-extraction sorbent interaction. Moreover, it shows important advantages by minimizing the use of organic solvents in the sample preparation. In addition, it can be introduced directly into the sample without requiring prior filtration or matrix clean-up exercises, and it can be used with other techniques such as sonication and magnetic stirring [19,20].

This novel technique have been applied successfully for the determination of different emerging pollutants as triazine herbicides [21], non-steroidal anti-inflammatory drugs [22], alkyl phenols [23], oestrogens [20], benzotriazole UV stabilizers [24] and androgens and progestogens [25] in environmental samples.

The most widely used separation and determination technique for cytostatic compounds is high performance liquid chromatography tandem mass spectrometer [9,10,15,16,26–28], usually with an electrospray ionization source. However, considering the need to analyse metabolites or degradation products that can be formed from these compounds, some studies have developed liquid chromatography with high-resolution mass spectrometry methods [14,29,30].

In this work, a rapid, simple and green method for the analysis of seven cytostatic compounds (Table 1) that are commonly used in anti-cancer therapies has been developed using fabric phase sorptive extraction (FPSE) coupled to UHPLC–MS/MS in environmental samples. Moreover, it is the first attempt to apply a microextraction technique to cytostatic compounds in wastewater [31]. The optimization process was performed through a 2^4 (four variables at two levels) experimental design, where the variables of pH, sample volume, extraction time and desorption time were optimized, followed by a 3^2 (two variables at three levels) experimental design where the two most dependent variables of the process were studied. Ionic strength, different eluents and volumes of elution were also studied.

Subsequently, the applicability of the new method to the analysis of cytostatic compounds in environmental samples was verified on wastewater from a hospital effluent and wastewater treated with different techniques from wastewater treatment plants located in Gran Canaria, Spain.

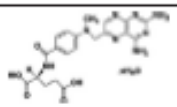
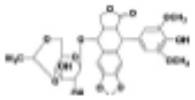
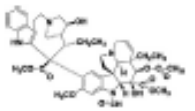
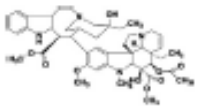
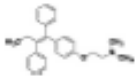
2. Experimental

2.1. Materials and reagents

The substrate used in creating sol-gel sorbent coated FPSE media was unbleached muslin cotton (100% cellulose) purchased from Jo-Ann Fabric (Miami, FL, USA). Organic polymers (polyethylene glycol 300 (PEG 300), Carbowax 20M (CW20M), polycaprolactone triol (Pcaptriol), and UCON™ HTF 14), sol-gel precursors (methyltrimethoxysilane (MTMS) and 3-cyanopropyltriethoxysilane), organic solvents (acetone and methylene chloride), sodium hydroxide, and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caprolactone-dimethylsiloxane-caprolactone block polymer (Cap-DMS-Cap) was obtained from Gelest, Inc. (Morrisville, PA, USA).

A Barnstead NANOPure Diamond (Model D11911) deionized water system (Thermofisher Scientific, Waltham, MA, USA) provided ultra-pure deionized water (18.2 MΩ) for sol-gel synthesis. Centrifugation of different solutions to obtain particle free sol solution for the sol-gel coating was performed in an Eppendorf Centrifuge Model 5415 R (Eppendorf North America, Hauppauge, NY, USA). Thorough mixing of all solutions was achieved using a Fisher Scientific Digital Vortex Mixture (Fisher Scientific, Waltham, MA, USA). A 2510 Branson Ultrasonic Cleaner (Branson Ultrasonics, Inc., Danbury, CT, USA) was employed to obtain bubble free sol solution.

Table 1
Chemical structures and pertinent physico-chemical properties of selected cytostatic compounds.

Compound	Molecular Weight, g/mole	Chemical Structure	$LogK_{ow}$	pKa
Methotrexate (MET)	454.477		−2.20	4.7
Gemcitabine (GEM)	263.201		−2.01	5.27; 11.24
Etoposide (ETO)	588.562		0.60	9.8
Cyclophosphamide (CP)	261.083		0.63	N/A
Vincristine (VINC)	824.972		2.82	5.0; 7.4
Vinblastine (VINB)	810.989		3.70	5.4; 7.4
Tamoxifen (TAM)	371.524		6.30	8.87

Ultrapure water used was provided by a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol, LC–MS methanol, LC–MS water and formic acid used to adjust the pH of the mobile phase were all obtained from Panreac Quimica (Barcelona, Spain). Cytostatics methotrexate (MET), gemcitabine (GEM), vinblastine (VINB), and vincristine (VINC) were purchased from Cymit-Química (Barcelona, Spain), whereas cyclophosphamide (CP), tamoxifen (TAM), and etoposide (ETO) were purchased from Sigma-Aldrich (Madrid, Spain). All compounds present a purity greater than 97%. Stock solutions containing 1000 mgL^{-1} of each analyte were prepared by dissolving the compound in methanol, and the solutions were stored in glass-stoppered bottles at −20 °C in the dark. Working standard solutions were prepared daily.

2.2. Sample collection

Samples were collected from the effluent of three wastewater treatment plants in Gran Canaria Island, Spain, which each use different treatment processes. Two of the treatment plants analysed, which use conventional activated sludge treatments, treat the water of 290,000 (WWTP1) and 180,000 equivalent population (WWTP2), and they are located in high-density population areas of Gran Canaria (Spain). The other treatment plant studied (WWTP3) is designed to treat the wastewater of a rural area of the north part of the island (7000 equivalent population) using a membrane bioreactor technology. Moreover, untreated wastewater from an effluent of the hospital area of Las Palmas de Gran Canaria was also taken. The samples were taken in amber glass bottles rinsed beforehand with ultrapure water. Then, the samples were immediately acidified with hydrochloric acid at a pH between 2.5–3.5 to avoid microbial degradation and stored in a refrigerator at 4 °C until analysis.

2.3. Instrumentation and chromatographic conditions

To determine the target compounds, an ACQUITY UPLC system equipped with a triple quadrupole detector with an ESI interface controlled by MassLinx Mass Spectrometry software was used. The system consisted in a Binary Solvent Manager (BSM), a column manager and a 2777 autosampler, all from Waters Chromatography (Barcelona, Spain). The electrospray ionization was performed in positive mode, and detection parameters were optimized using standard solutions of each compound. The optimal MS/MS parameter values were as follows: capillary voltage of 3.5 kV, cone voltage of 40 V, source and desolvation temperature of 120 and 400 °C, respectively and desolvation gas at a flow of 1000 $L\,h^{-1}$. Nitrogen was used as desolvation gas, and argon was employed as collision gas.

The analytical column was a Phenomenex Luna Omega Polar 50 × 2.1 mm, with a particle size of 1.6 μm (Phenomenex, Madrid, Spain) operating at room temperature. The sample volume injected in the chromatographic system was 10 μL, and the analyte separation was performed using water and methanol, both adjusted with 0.1% (v/v) of formic acid, at a flow rate of 0.3 mL/min in gradient mode.

The mobile phase was water (A) and methanol (B) both with 0.1% formic acid, in gradient mode at 0.3 mL/min. The gradient started at 90:10 (v/v) and was increased to 100% methanol in 3 min. Then, it remained 100% of methanol for 0.5 min and returned to 90:10 in 1.5 min. Finally, it stayed at that ratio for 1 min to stabilize the pressure of the chromatographic system, completing the chromatographic separation in 6 min.

For the quantitative analysis, multiple reaction monitoring (MRM) parameters were optimized for each compound. The mass spectrometer parameters for the determination of target analytes are shown in (Table 2). Precursor ions were $[M-H]^+$ in positive ion mode (ESI+) for all compounds, with the except of CP, which was

Table 2
Mass spectrometer parameters for the determination of selected cytostatic compounds.

Compound	Precursor ion (m/z)	Cone voltage (ion mode)	Quantification ion, m/z (collision potential, V)	Confirmation ion, m/z (collision potential, V)	Ratios of response
MET	455.1	30 V (ESI+)	308.1 (15)	175 (17)	0.02
GEM	264.2	35 V (ESI+)	112.1 (20)	87.0 (20)	0.10
ETO	589.5	40 V (ESI+)	229.2 (15)	185 (20)	0.19
CP	261.2	30 V (ESI+)	140.0 (20)	106 (18)	0.34
VINC	825.8	75 V (ESI+)	122.4 (55)	156 (55)	0.87
VINB	812.8	75 V (ESI+)	224.2 (42)	355 (30)	0.03
TAM	372.3	40 V (ESI+)	72.12 (20)	129 (20)	0.01

$[M]^+$. The optimization of the quantification and confirmation of fragment ions and the detection parameters of each compound were performed by directly infusing a standard of 1 mg L^{-1} in methanol at a flow rate of 10 μL/min into the mass spectrometry detector.

2.4. Preparation of sol-gel sorbent coated fabric phase sorptive extraction media

The commercial 100% cotton cellulose muslin fabric that was used as the substrate for creating designer sol-gel sorbent coatings contained residual finishing chemicals, dust and other debris accumulated on its surface over the period of time and needed thorough cleaning. In addition, the surface hydroxyl functional groups of the cellulose fabric require activation to maximize the loading of sol-gel sorbents during the chemical sorbent coating process. This was accomplished following a rigorous cleaning process adopted in our laboratory and described elsewhere [20]. Briefly, a 50 cm^2 (10 cm × 5 cm) of the fabric was soaked and cleaned with water, followed by treatment with 1.0 M NaOH for 1 h and 0.1 M HCl for 1 h, respectively. The chemically treated and cleaned fabric was then dried in an inert atmosphere for 12 h and stored in an air-tight container until it was coated with sol-gel sorbent.

The design of the sol solution used to create the sol-gel sorbent coating on the substrate surface primarily depends on the polarity/functionality of the target analytes. Taking the polarity/functional makeup and other physicochemical characteristics of the target analytes (as presented in Table 1) into consideration, a number of sol-gel sorbents were synthesized including: sol-gel CN CW20M (CN-CW20M), sol-gel methyl CW20M (M-CW20M), sol-gel methyl polycaprolactone triol (M-Pcaptriol), sol-gel methyl UCON (M-UCON), sol-gel methyl polyethylene glycol 300 (M-PEG300) and sol-gel methyl caprolactone-dimethylsiloxane-caprolactone (M-Cap-DMS-Cap). Sol solutions constitute of an organic polymer, a sol-gel precursor, a solvent system, a catalyst and water. All sol solutions in the current study were prepared using methyltrimethoxysilane (MTMS) as the sol-gel precursor, trifluoroacetic acid (TFA) as the acid catalyst, a mixture of acetone and methylene chloride (50/50, v/v) as the solvent system and water for hydrolysis. However, in the sol-gel CN CW20M coating, 3-cyanopropyl triethoxysilane was used as the sol-gel precursor. The molar ratio between sol-gel precursor, organic polymer, acetone, methylene chloride, TFA and water was optimized and maintained at (1:0.012:3.26:3.74:1.25:3) for CN-CW20M, (1:7.1 × 10^{-3}: 3.26:3.74:1.25:3) for M-CW20M, (1:0.48: 3.26:3.74:1.25:3) for M-Pcaptriol, (1:0.10:3.26:3.74:1.25:3) for M-UCON and (1:0.02: 3.26:3.74:1.25:3) for M-Cap-DMS-Cap), respectively.

The preparation of sol solution for the sol-gel coating is described in detail elsewhere [20,32]. The fabric substrates were kept inside the sol solution for 4 h. At the end of the residence time in the sol solution, the coated fabric was removed from the solution and was kept in a desiccator overnight for solvent evaporation and aging of the coating. The sol-gel coated FPSE media was then rinsed with methylene chloride: acetone (50/50, v/v) mixture under sonication to remove unreacted and unbonded residual coating ingredients from the fabric surface. The cleaned FPSE media coated with sol-gel sorbents were then stored in an air-tight container so that they did not accumulate unwanted analytes from the environment.

2.5. Fabric phase sorptive extraction optimization

The six different fabric media that were tested are shown in Table 3. Firstly, to clean and activate the coating of the fabric media, the coated media were immersed in 2 mL of a mixture of methanol and acetonitrile (50/50, v/v) for 5 min and then washed in 2 mL of water for 5 min. To make a prior selection of which fabrics could be efficient for the target compounds, adsorption tests were performed with the six fabrics. This test consisted of measuring the concentration of the analytes in the spiked Milli-Q water samples before and after extraction with the fabrics for one hour. In a second step, to evaluate the desorption efficiency, the analytes were eluted from the fabrics in 1 mL of methanol for 5 min, and the methanol was measured to check the presence of the analytes. In light of the results obtained in the elution tests, two fabric media were selected for later experimental designs.

Finally, the extracts were injected in the chromatographic system to evaluate the extraction recoveries. After the extraction and elution step, the fabric media were cleaned in 2 mL of a mixture of methanol and acetonitrile (50/50, v/v) for 5 min and placed in a flat surface to dry for 10 min.

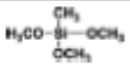
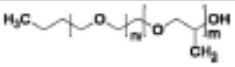
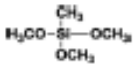
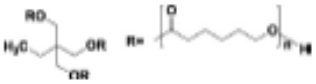
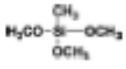
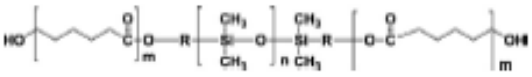
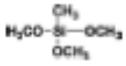
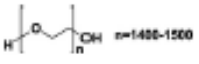
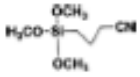
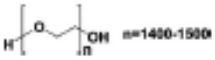
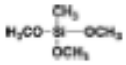
3. Results and discussion

3.1. Selection of fabric phase sorptive extraction substrate and sorbent chemistries

Unlike conventional extraction and microextraction techniques, where the substrates (silica particle for SPE and fused silica glass rod for SPME) merely hold the extraction sorbent often via physical immobilization, fabric phase sorptive extraction heavily relies upon the hydrophilic/hydrophobic properties of the substrates that consequently complement the overall polarity/selectivity of the FPSE device. Among many potential candidates as the viable substrates for FPSE, 100% cotton cellulose muslin fabric was selected as the substrate due to its hydrophilic surface property that may exert higher affinity towards the majority of target analytes, which are known as polar.

Sol-gel synthesis allows facile chemical incorporation of organically modified inorganic precursor and organic polymer into a three-dimensional network of composite sorbent material system. The selectivity/polarity of the resulting hybrid inorganic-organic composite depends on both the inorganic precursor and the organic polymer. It appears that the selectivity of FPSE medium is not only governed by the polarity of the organic polymer, but is also dependent upon the organic ligand connected to the inorganic precursor (methyl, ethyl, phenyl, etc.) as well as the hydrophilic/hydrophobic

Table 3
Selected fabric phase sorptive extraction sorbents used in the current study.

Sorbent	Sol-gel Precursor	Organic Polymer	Polarity
Sol-gel UCON			Highly polar
Sol-gel polycaprolactone triol			Highly polar
Sol-gel caprolactone-dimethylsiloxane-caprolactone block polymer			Medium polar
Sol-gel CW20M			Highly polar
Sol-gel cyanopropyl CW20M			Highly polar
Sol-gel polyethylene glycol 300			Highly polar

interaction exerted by the fabric substrate, which collectively determine the extraction efficiency of the FPSE media.

Design of the sol solution for creating sol-gel sorbent chemically bonded to the substrate primarily depends on the characteristics of the target analytes. The selectivity or extraction efficiency of fabric phase sorptive extraction media can be designed by simultaneously exploiting the polarity of the organic polymer, functional ligand of the organically modified sol-gel precursor and the hydrophilicity/hydrophobicity of the fabric substrate. Due to the wide polarity range of the target analytes (log K_{ow} values ranging from −2.20 to 6.30), a number of sol-gel sorbent coatings were created and tested, including highly polarity CN-CW20M, M-CW20M, M-Pcaptriol, M-PEG300 and medium polarity M-UCON and M-Cap-DMS-Cap. The presence of the cyanopropyl functional groups in the CN-CW20M sorbent makes the sorbent more polar. On the other hand, the presence in methyl functional groups in the 5 other sorbents allows exploitation of London dispersion during analyte extraction in addition to the typical dipole-dipole interaction and hydrogen bonding interactions.

3.2. Selection of optimum fabric coatings

To evaluate the extraction efficiency, a sample of Milli-Q water with the mixture of the target analytes were prepared and their concentration was measured before and after the extraction, assuming that the difference in the concentration is due to the compounds being adsorbed in the fabric coated with different sol-gel sorbents. The extraction efficiency, expressed as the ratio of the initial concentration to the final concentration in the Milli-Q water sample, is shown in Fig. 1. As seen from the extraction efficiency bars presented in Fig. 1 of different sol-gel sorbents, extraction efficiencies vary widely for different analytes. TAM, VINB and VINC appear to be easily extracted by any of the FPSE, unlike GEM, MET and CP, which only have extraction efficiencies greater than 30% with CN-CW 20M.

Desorption efficiency was evaluated by desorbing the analytes retained in the FPSE media using 1 mL of methanol for 5 min. From the obtained results, it was found (Fig. S1) that some fabrics (Caprolactone Triol, PEG 300, and CN-CW 20M), in spite of having good absorption of the compounds, showed only good desorption efficiencies for three compounds (higher than 50%). Finally, only two FPSE media (M-CW20M and M-UCON) showed desorption efficiencies greater than 60% for most of the compounds and also good adsorption efficiencies, so they were selected for the optimization of the extraction process.

3.3. Optimization of the experimental conditions of the selected FPSE

There are several parameters that could affect to the extraction on FPSE, such as extraction time, sample volume, sample pH and ionic strength of the samples or desorption time and volume. To evaluate the influence of the different variables in the extraction process a 2^4 experimental design was carried out with four variables at two levels to determine the correlation between them. These four variables were extraction time (10 and 40 min), sample volume (10 and 20 mL), pH (3 and 9) and desorption time (5 and 10 min) with methanol, for a final concentration of 500 μg L^{-1} of the target compounds. To evaluate the importance of these variables to the extraction efficiency of the method, Pareto charts of the standardized effects of the variables were built. As seen in Fig. 2, the variables that affect the extraction procedure more were sample pH and extraction time. Moreover, bivariate correlations were calculated to establish better levels for the variables. All the compounds had a positive correlation in relation to the time of extraction, which indicates that greater times of extraction produce better extraction rates. In general, in relation to pH, the correlations were positive, so it was also decided to choose basic pH, and in contrast, sample volume had a negative correlation, so the lowest volume was selected. Finally, regarding desorption times, the correlation was in most cases near to zero or slightly negative, which indicated that short desorption times were appropriate.

To evaluate the improved conditions of the variables that showed a high correlation between them and most affected the extraction method, a 3^2 experimental design was carried out by studying three pH values (6, 8 and 10) and three extraction times (20, 40 and 60 min). Because of the differences in the physicochemical properties, the best conditions for some cytostatic were not as optimal for others, so an intermediate point was chosen, which resulted in a compromise of acceptable values for the greatest number of compounds (Fig. 3).

All FPSE sorbent chemistries investigated in the current project have been developed to extract only neutral species of organic analytes. However, as seen in Table 1, some cytostatic drugs are either weakly acidic or weekly basic or possess both acidic and basic functional groups. These compounds are found in a partially dissociated

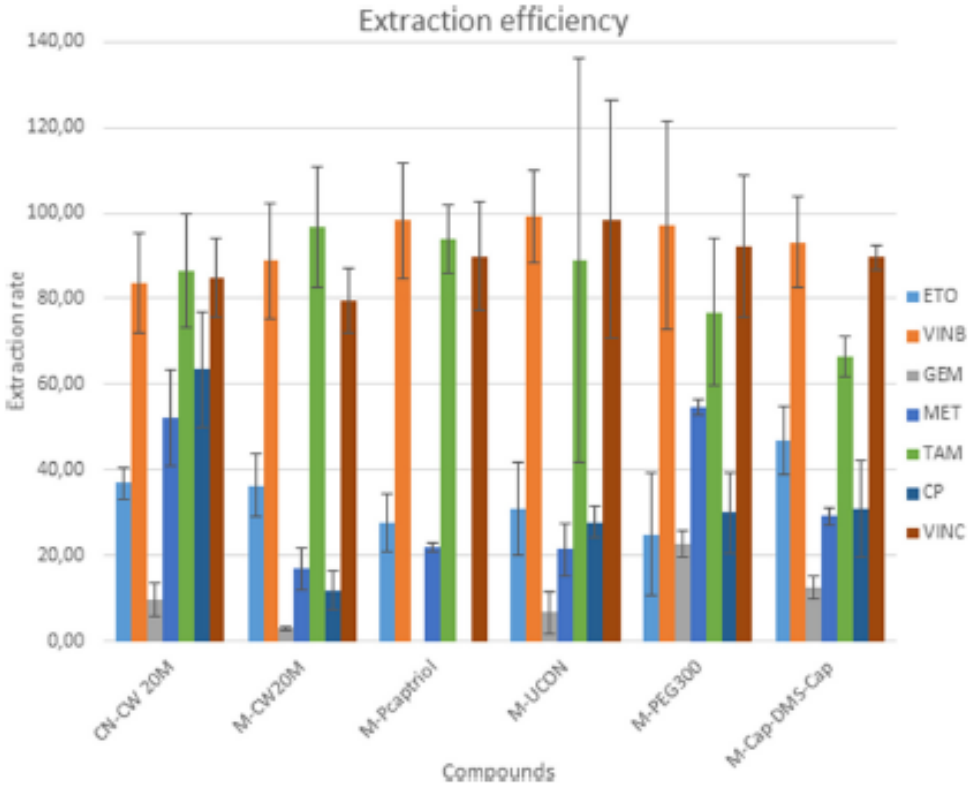


Fig. 1. Extraction efficiency of six selected fabric phase sorptive extraction sorbents (n = 3).

state in aqueous samples and can be forced to form their neutral species by adjusting the matrix pH value. Due to the wider range of the *pKa* values of cytostatic drugs, it is almost impossible to find a matrix pH value that would maximize the neutral species content of all the target analytes. For this reason, two pH values (8 and 10) were selected for M-CW 20M; however, the rest of the conditions were the same: sample volume of 10 mL, extraction time of 60 min at 1000 rpm and elution time of 5 min

Modification of ionic strength of the sample matrix by adding salts such as sodium chloride, sodium sulfate, potassium carbonate into aqueous sample matrix is a common practice to enhance extraction efficiency of many organic analytes except highly polar analytes [33]. If the ionic strength of the water is modified by adding salt, the extraction efficiency is modified as well, either increasing or decreasing this efficiency (processes known as "salting-out" and "salting-in", respectively) [34,35]. Addition of salt in the aqueous sample matrix reduces the solubility of many organic compounds and leads to higher extraction onto the FPSE medium. However, the inverse salting-in effect can reduce the analyte mass transfer rate from the bulk to the FPSE medium because of the salt-analyte interaction, resulting in longer extraction equilibrium time. Therefore, the net effect of ionic strength modification should be assessed by empirical experiments. As such, the impact of ionic strength was evaluated by adding sodium chloride at three levels (0, 5 and 10% *w/v*). The results showed that the addition of salt produces a negative effect on the recoveries so a 0% ionic strength was chosen as the optimum condition.

Furthermore, three different elution solvents (methanol (100%), acetonitrile: methanol (50% – 50%, *v/v*) and acetonitrile (100%)) were tested to evaluate all the key variables of the extraction process. The best recoveries were obtained with pure methanol, and the presence of acetonitrile produced wide chromatographic peaks that were more difficult to quantify (Fig. S2). For this reason, methanol was selected as desorption solvent.

M-CW20M was the fabric media that achieved better results, with a range of absolute recoveries between 25 and 90%. These recoveries were better than those obtained with M-UCON, which were in the range of 20–70%, so M-CW20M was selected to evaluate the analytical parameters of the method. As previously stated, because of the differences between the physicochemical structures and properties of cytostatic compounds, two pHs were selected to perform extractions using this fabric media. ETO, TAM and CP were extracted at pH 8, whereas VINB and VINC were extracted at pH 10. GEM and MET showed very low extraction yields so they were dismissed from the analysis. The optimal final conditions were a sample volume of 10 mL with an ionic strength of 0%, an extraction time of 60 min, and elution with 1 mL of methanol for 5 min.

3.4. Analytical parameters

For the calculation of linearity, a standard curve was set up in Milli-Q water with 8 points between 1 $\mu g\,L^{-1}$ and 500 $\mu g\,L^{-1}$ of the mixture of analytes. Linearity was calculated as the ratio between the peak areas and concentration of each compound, and correlation coefficients greater than 0.998 were obtained in all cases.

Study of repeatability and reproducibility of the method was performed in Milli-Q water and effluents from three WWTPs with spiked samples with the target analytes at two concentration levels (2.5 and 10 $\mu g\,L^{-1}$). Intraday repeatability of the method was evaluated using six measurements of each sample whereas inter-day reproducibility was evaluated using a triplicate of each sample during three days. The results were very satisfactory and, in all cases, relative standard deviations were less than 12%, in both types of water.

The limits of detection (LOD) and limits of quantification (LOQ) of the method were calculated using the signal-to-noise ratio of each individual peak. The LOD is defined as the lowest concentration that gives a signal-to-noise ratio greater than 3, whereas LOQ is defined as the minimum concentration giving a signal-to-noise

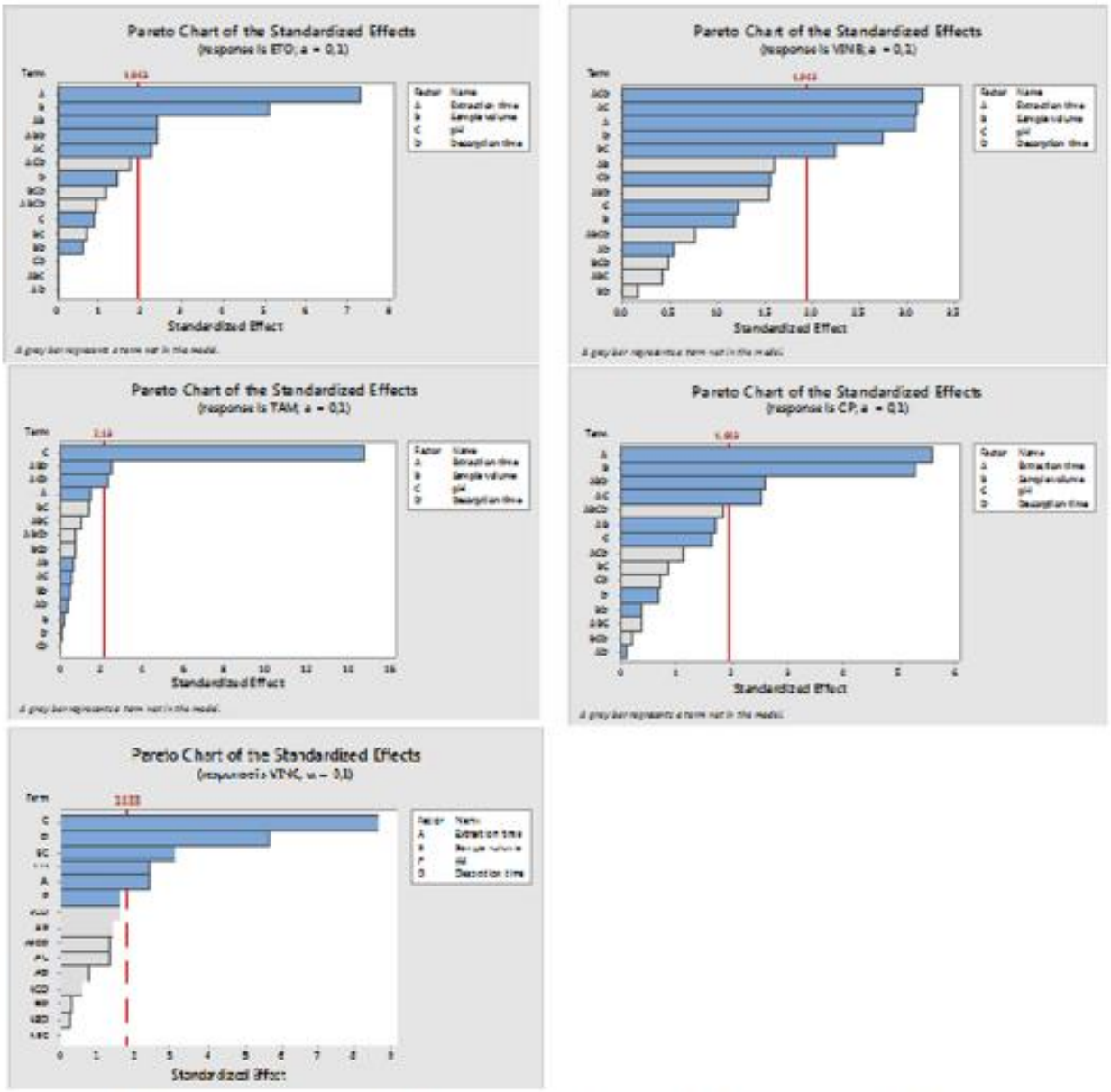


Fig. 2. Pareto charts of standardized effects of the variables studied in the 2^4 experimental design for the selected fabric media (M-CW20M) (n = 3).* A gray bar represents a term not in the model.

greater than 10. Table 4 shows the results obtained for both types of water, where the LOD varied in the range of 0.2 ng L^{-1} to 100 ng L^{-1}. There are no large differences between the limits obtained in the different wastewater and Milli-Q water samples, demonstrating that the method shows good selectivity.

Recoveries were studied in Milli-Q water and wastewater at two different concentrations (2.5 and 10 μg L^{-1}). Recovery was calculated as the ratio between the peak areas of the extract spiked before extraction and the peak areas of the extract spiked after extraction [36].

Absolute recoveries in Milli-Q water ranged between 40%–90% except for TAM, which had recoveries between 20%–40%. Relative recoveries in wastewater were calculated from absolute Milli-Q recoveries. As seen in Table 4, the recoveries obtained varied from 40% to 100% in most cases. It is also observed that the recoveries of WWTP3, which used a bioreactor membrane technology as its purification system, had better results (relative recoveries between 40 and 102%, except for TAM) than the other wastewater treatment plants that are based on traditional activated sludge treatments and have a mixed input of both urban and industrial wastewaters.

The relative recovery of TAM is higher than the other analytes because of the presence of salt, which favoured the extraction.

3.5. Matrix effect

Mass spectrometry with electrospray ionization source detection is often influenced by the composition of the matrix in which the compounds are found, producing a decrease or increase of the analytical signal [37]. For this reason, it is very important to evaluate the matrix effect of the different samples studied. To carry out this evaluation, the analytical signals of the spiked extracts after extraction and the signals of the analytes dissolved in methanol were compared. For the calculation of the matrix effect, the following formula was applied:

$$\text{Matrix effect} = \frac{A - (B - C)}{A} * 100$$

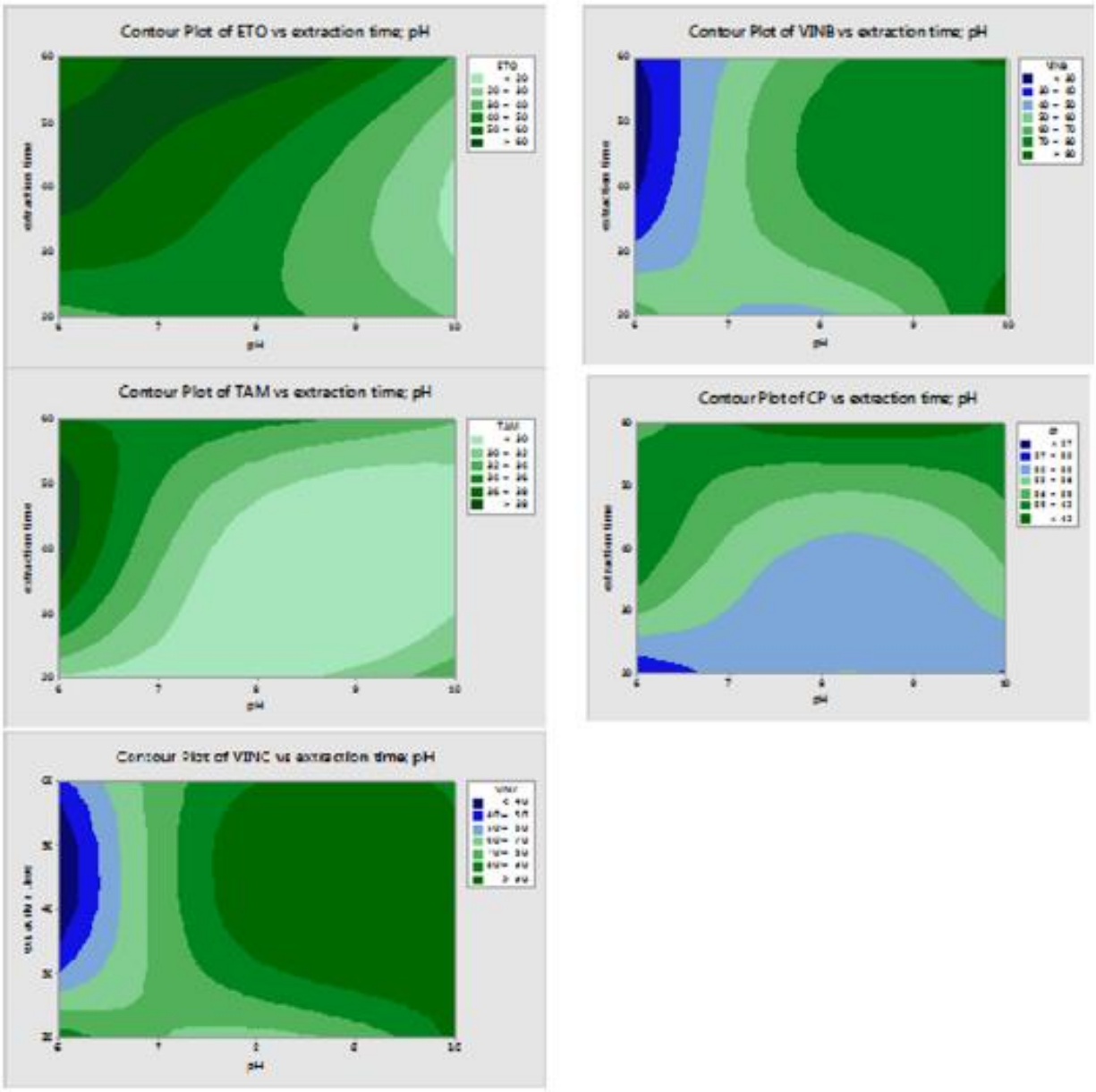


Fig. 3. Surface responses of the variables studied in the 3^2 experimental design of the selected fabric media (M-CW20M) (n = 3).

Where:

- A = peak area of the standard solution
- B = peak area of the extract spiked after extraction
- C = peak area of the real sample

It could be said that ETO and CP do not have a matrix effect. There was a signal enlargement in some samples and a signal suppression in others, but the values were lower than or close to 20%, which is considered to have no matrix effect (Table 5). A suppression of the signal in TAM is observed, whereas in VINB and VINC, the opposite, a signal enlargement is observed.

Regarding to the salinity of the sample, the extraction of certain cytostatic compounds (TAM, VINB and VINC) may be affected. In the study of ionic strength, tests showed a similar behaviour, TAM signal increased due to the addition of salt, but VINB and VINC signals decreased. Nevertheless, the matrix effect can be due to the variation either to the ionic strength or to any impurity in the wastewater.

3.6. Suitability comparison between fabric phase sorptive extraction and solid phase extraction

Solid phase extraction (SPE) is considered the gold standard among all contemporary sorbent-based sample preparation techniques. Solid phase extraction requires an absolutely particle free sample matrix to prevent clogging of the sorbent bed. As such, environmental water, which may contain particles, debris, and biomasses, has to be filtered first before passing through the SPE sorbent bed. This extra step may lead to loss of analytes. In addition, SPE requires a relatively high volume of organic solvent to quantitatively elute the extracted analytes. Subsequently, the eluate undergoes solvent evaporation and sample reconstitution into a small volume of organic solvent, which is not only time consuming but also implicated in the loss of analytes and poor data quality.

Fabric phase sorptive extraction has addressed the shortcomings often encountered in SPE. It does not require a clean, particle free sample matrix. Due to the flexibility of the FPSE media, it can be introduced directly into the sample vial. It also minimizes the

Table 4
Analytical parameters of the different water samples (n=3).

Compound	Milli-Q							
	LOD[a] ng L^{-1}	LOQ[b] ng L^{-1}	Absolute Recovery (%)		Intraday RSD (n = 6)		Interday RSD	
			2.5 μg L^{-1}	10 μg L^{-1}	2.5 μg L^{-1}	10 μg L^{-1}	2.5 μg L^{-1}	10 μg L^{-1}
ETO	7.403	24.68	74.87	58.21	5.04	6.65	10.0	10.8
CP	3.825	12.75	41.87	37.30	4.02	6.12	6.25	10.2
VINC	98.04	326.8	92.26	66.77	5.34	8.14	11.0	5.85
VINB	39.93	132.8	64.41	47.39	8.39	10.9	8.78	11.9
TAM	0.093	0.309	37.10	22.68	3.96	12.5	9.12	9.05

Compound	WWTP1				WWTP2				WWTP3			
	LOD ng L^{-1}	LOQ ng L^{-1}	Relative Recovery (%)		LOD ng L^{-1}	LOQ ng L^{-1}	Relative Recovery (%)		LOD ng L^{-1}	LOQ ng L^{-1}	Relative Recovery (%)	
			2.5 μg L^{-1}	10 μg L^{-1}			2.5 μg L^{-1}	10 μg L^{-1}			2.5 μg L^{-1}	10 μg L^{-1}
ETO	5.025	16.75	66.76 (±1.40)	70.22 (±4.70)	28.62	95.42	44.49 (±8.51)	55.07 (±16.4)	5.439	18.13	78.57 (±8.74)	77.56 (±4.31)
CP	6.230	20.77	70.20 (±16.1)	64.95 (±6.41)	27.14	90.46	40.50 (±15.2)	44.86 (±9.88)	6.635	22.12	62.38 (±7.61)	68.66 (±6.34)
VINC	48.10	160.3	42.18 (±0.97)	57.53 (±10.9)	79.98	266.6	42.18 (±9.22)	55.25 (±4.74)	31.53	105.1	42.24 (±5.87)	82.87 (±13.3)
VINB	21.54	71.79	76.86 (±12.7)	59.06 (±4.46)	53.41	178.0	30.18 (±21.2)	47.11 (±2.46)	66.33	221.1	51.11 (±25.4)	101.8 (±8.94)
TAM	0.539	1.797	124.3 (±4.15)	200.5 (±11.1)	0.203	0.677	81.90 (±4.06)	126.0 (±6.26)	0.579	1.931	116.7 (±7.99)	186.6 (±8.94)

[a] Limit of Detection.
[b] Limit of Quantification.

Table 5
Matrix effects demonstrated by environmental water collected from different wastewater treatment plants (%RSD) (n=3).

Compound	Concentration	WWTP1	WWTP2	WWTP3
ETO	2.5 μg L^{-1} pH = 8	2.9 (4.2)	−23.1 (3.3)	10.4 (10.6)
	10 μg L^{-1} pH = 8	12.1 (9.3)	1.2 (12.3)	21.8 (2.5)
CP	2.5 μg L^{-1} pH = 8	−10.8 (9.3)	−14.8 (13.0)	−3.9 (4.9)
	10 μg L^{-1} pH = 8	7.9 (4.0)	−3.1 (6.1)	22.3 (2.0)
VINC	2.5 μg L^{-1} pH = 10	1.9 (0.6)	17.1 (10.8)	−42.7 (10.1)
	10 μg L^{-1} pH = 10	−18.2 (3.6)	−42.73 (4.2)	−10.6 (3.9)
VINB	2.5 μg L^{-1} pH = 10	−15.6 (7.3)	−2.8 (6.4)	−11.2 (4.2)
	10 μg L^{-1} pH = 10	−42.3 (5.8)	−38.2 (8.6)	−31.3 (8.6)
TAM	2.5 μg L^{-1} pH = 8	9.0 (4.6)	−23.1 (4.0)	8.3 (4.0)
	10 μg L^{-1} pH = 8	27.6 (7.9)	7.5 (1.8)	29.3 (3.9)

number of glassware/sample containers used in sample preparation and thus eliminates any risk of potential analyte loss. At the end of the extraction, the FPSE media is introduced into a small volume of organic solvent (500–1000 μL) for eluting the extracted analytes, achieving an enrichment factors up to 10 times. As such, FPSE maintains the analyte preconcentration factor achieved during the extraction and it eliminates the time consuming solvent evaporation and sample reconstitution step from the sample preparation work-flow. The whole analytical process, including sample preparation, extraction, preconcentration and determination, is performed in about 90 min.

In Table 6, a comparison is shown of the results obtained in this work with other studies that have been performed with offline SPE (Oasis HLB cartridges) [10,14,15,38] that obtained recoveries and LODs in many cases closer to those obtained by FPSE.

3.7. Applications in wastewater treatment plants

To evaluate the precision, selectivity and sensitivity of the developed FPSE method, three wastewater samples from the secondary treatment of three wastewater treatment plants and water samples from a hospital effluent were analysed. After the analysis, it could be determined that none of the cytostatic compounds were detected in the wastewater from the effluent of the wastewater treatment plants, although ETO was detected in one of the two points sampled in the hospital at 2.6 μg L^{-1}.

Because of the lack of positive results in real samples for the rest of the target analytes and to check the efficiency of the method, the water samples were spiked with target compounds at a concentration of 2.5 μg L^{-1}. As seen in Fig. 4, all the compounds under study could be detected without significant matrix effect. Relative recoveries ranged between 38 and 102%, (except for TAM) depending on the sample and the compound. In light of the results, the developed FPSE method showed a very good selectivity, with high signal-to-noise ratios between the analytical signal and the background noise of the chromatogram, as well as very good repeatability and extraction recoveries.

4. Conclusions

To our knowledge, this is the first attempt to apply the FPSE microextraction technique for extraction and preconcentration of cytostatic compounds present in water samples. Six different FPSE media were tested and the one that extracted the highest number of compounds at higher extraction rates was chosen as optimum. All parameters involved in the extraction (sample ionic strength, pH and volume, extraction time, type and volume of the eluent, and elution time) were optimized. In addition, all the chromatographic and detection parameters of the target compounds were optimized.

The fabric phase sorptive extraction is therefore presented as a fast, sensitive and simple method for the analysis of cytostatic compounds in wastewater. The detection limits of the method are within the values at which cytostatic compounds are usually found in the aquatic environment and with values similar to those obtained with classical SPE techniques. Moreover, the method has been shown to be reproducible with RSD of less than 12% and

Table 6
Comparison of the results with FPSE and SPE.

Extraction technique	Extraction time (min)	Extraction volume (ml)	Absolut recoveries	LODs ng L^{-1}	Reference
FPSE	60	10	30% (CP) 50% (ETO) 42% (VINC) 50% (VINB) 51% (TAM)	6.2 (CP) 5 (ETO) 48.1 (VINC) 21.5 (VINB) 0.5 (TAM)	
SPE	83	250	[a]85% (CP) [a]105% (ETO)		[15]
SPE	20	100	51–105% (CP, ETO, VINC)	2 (CP) 5 (ETO) 20 (VINC)	[10]
SPE	50	50	58–46% (CP) 47–64% (ETO) 90–93% (VINC) 49–48% (TAM)	2.4 (CP) 24 (ETO) 7.4 (VINC) 0.8 (TAM)	[38]
SPE	100	100	79% (CP) 69% (VINC) 110% (VINB) 43% (TAM)	4.4 (CP) 5.2 (VINC) 4.9 (VINB) 0.7 (TAM)	[14]

[a] Process efficiency.

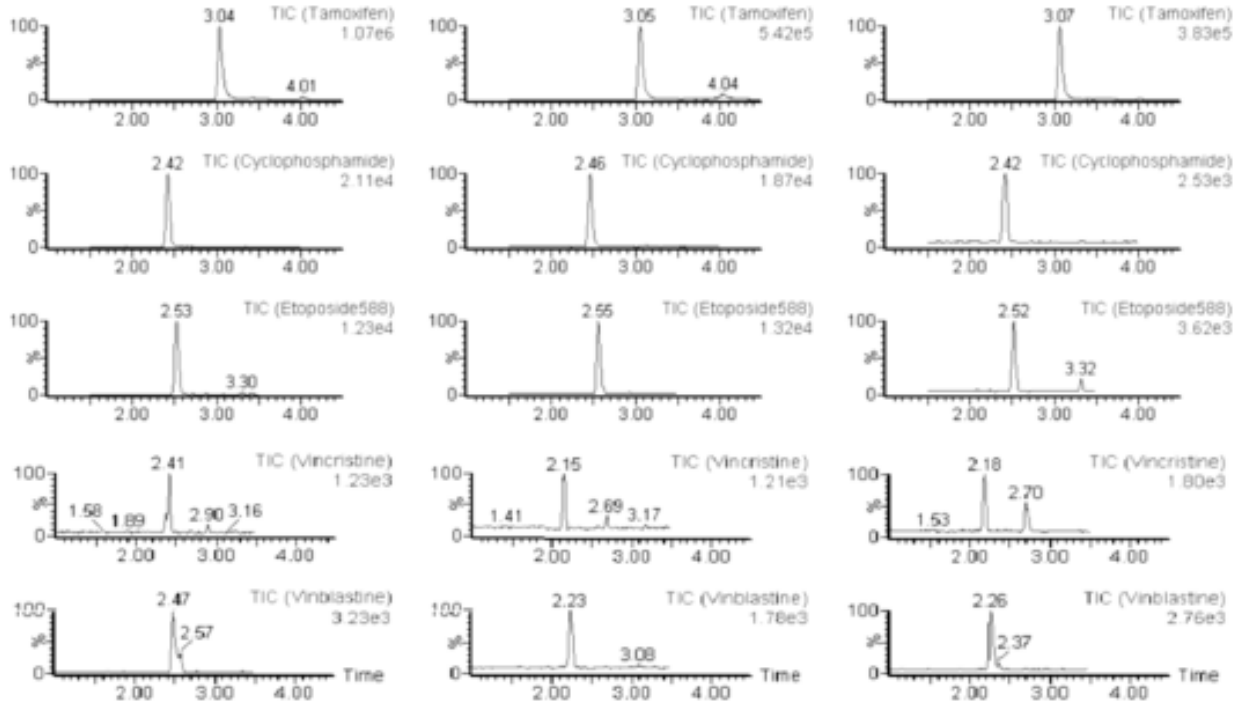


Fig. 4. Chromatogram of the spiked samples in the three different effluent from the three wastewater treatments plants (n = 3).

acceptable recoveries that were between 40 and 50% in most cases for real samples. Significant matrix effects were not observed. The method was applied to real wastewater samples, detecting concentrations of 2.6 μg L^{-1} of ETO in hospital effluents but no cytostatic compounds were detected in WWTP effluents. However, spiked samples were analysed, proving that the method had good selectivity, repeatability and extraction recoveries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2017.10.070.

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3.3. Determinación de compuestos citostáticos de platino en aguas residuales por ICP-MS después de su extracción por intercambio iónico

Los compuestos citostáticos de platino son de los medicamentos contra el cáncer más ampliamente utilizados [90]. Son compuestos que pueden causar efectos adversos en organismos acuáticos a muy bajas concentraciones [24,26] pero, a su vez, no están bien estudiados en matrices medioambientales. La mayoría de los trabajos se han centrado en su determinación en efluentes de hospitales, pero no en EDARs.

Creemos que esta falta de estudios se debe a que no se han optimizado métodos de extracción y preconcentración adecuados, ya que se trata de compuestos altamente polares que no pueden ser extraídos con los cartuchos convencionales de SPE. Por este motivo, se decidió aplicar un enfoque diferente utilizando cartuchos de SPE de intercambio iónico para su extracción y su posterior determinación por plasma acoplado inductivamente con espectrometría de masas (ICP-MS).

Se preparó una mezcla de los tres compuestos citostáticos de platino más utilizados (Cis-Pt, Car-Pt y Oxa-Pt). En primer lugar, se realizó un diseño experimental donde se estudiaron las variables que podrían afectar a la extracción: pH, fuerza iónica y volumen de muestra, a dos niveles, en los cuatro tipos de cartuchos de intercambio iónico comerciales, esto es, intercambio aniónico fuerte y débil e intercambio catiónico fuerte y débil. Se encontró que el cartucho de intercambio aniónico fuerte era el único capaz de retener los compuestos citostáticos de platino. Se fijaron, por tanto, como condiciones más idóneas pH=3, fuerza iónica = 0% y 250mL de muestra.

Una vez hemos logrado retener los compuestos en el cartucho, se realizaron diferentes ensayos para su elución con diferentes disolventes (agua Milli-Q, acetonitrilo (ACN) y MeOH con diferentes porcentajes de NH_3), encontrando como disolvente más adecuado 5mL de metanol con un 10% de NH_3

Se obtuvieron unos valores de efecto matriz muy bajos (inferiores al 24%) que, como hemos visto, es uno de los principales inconvenientes en la extracción de micro-contaminantes de muestras de aguas residuales. Hemos obtenido una buena reproducibilidad y repetividad con desviaciones inferiores al 15% y un LOQ de 0.74 $ng \cdot L^{-1}$.

Debido a la presencia de materia orgánica en las muestras de aguas residuales se tuvo que realizar una elución en dos etapas: 1) 5mL de agua Milli-Q (con un 5% NH_3) y 5mL de metanol (con un 5% HCOOH) y 2) 5mL de metanol (con un 10% NH_3). De esta forma se obtuvo una recuperación relativa en agua residual entre el 47% y el 90%

El procedimiento aquí optimizado es el primer procedimiento validado para la extracción de compuestos citostáticos de platino de aguas residuales. Este procedimiento se aplicó en muestras de aguas residuales tomadas en uno de los principales hospitales de la isla y en muestras de aguas residual tomadas a la entrada y a la salida de la EDAR de la ciudad de Las Palmas de Gran Canaria. Los muestreos se realizaron durante 1 año cada 3 meses. Se detectaron concentraciones de CPCs en todas las muestras analizadas, encontrándose las concentraciones más altas a la salida del hospital, entre 81.94 – 13913 $ng \cdot L^{-1}$ e inferiores en la estación

depuradora, donde las concentraciones se encontraban entre 3.97 – 75.79 $ng \cdot L^{-1}$.

Los resultados de este estudio han sido enviados para su consideración como publicación a la revista Journal of Analytical Atomic Spectrometry, la cual se situaba en el primer cuartil y con un IF=3.646 en el año 2018.

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DETERMINATION OF CYTOSTATIC PLATINUM COMPOUNDS IN WASTEWATER BY ICP-MS AFTER ION EXCHANGE EXTRACTION (JA-ART-01-2020-000004)

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DETERMINATION OF CYTOSTATIC PLATINUM COMPOUNDS IN WASTEWATER BY ICP-MS AFTER ION EXCHANGE EXTRACTION

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DETERMINATION OF CYTOSTATIC PLATINUM COMPOUNDS IN WASTEWATER BY ICP-MS AFTER ION EXCHANGE EXTRACTION

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ABSTRACT

Cytostatic platinum compounds (CPCs) are pharmaceutical compounds widely used in chemotherapy. However, these compounds have important side effects and can be toxic to the biota once they are excreted by patients and reach the aquatic medium, even at low concentrations.

Most of the works have focused on the determination of CPCs in hospital wastewaters using inductively coupled plasma mass spectrometry (ICP-MS). However, the determination of CPCs in samples from wastewater treatment plants (WWTPs) is very limited, probably due to the difficulty of extracting such hydrophilic compounds from these complex aqueous matrices.

This paper presents a new optimised and developed method for the extraction and preconcentration of CPCs in wastewater samples based on ion exchange solid phase extraction and their determination by ICP-MS.

Under the optimal conditions, the procedure has good reproducibility and repeatability (with deviations lower than 15%), with a relative recovery between 47–90% and a low matrix effect (lower than 24%). We have obtained the lowest limit of quantification achieved up until now (0.74 $ng \cdot L^{-1}$), thus allowing the determination of CPCs in new matrices. The described method was used for the determination of CPCs in

wastewater from a WWTP and hospital wastewater of Gran Canaria Island (Spain). We have detected concentrations between 81.94–13913 ng·L^{-1} in hospital effluents and between 3.97–75.79 ng·L^{-1} in wastewater treatment plants.

1. INTRODUCTION

Cytostatic platinum compounds (CPCs) are widely used anticancer drugs. More than 50% of cancer patients are treated with them or a mixture of them with other medications[1]. The oldest of them is cisplatin (Cis-Pt), a cytostatic compound widely used in the treatment of different types of cancer, such as testicular, ovarian and lung cancer[2]. Attempting to mitigate its side effects, other platinum-based compounds have been developed, including oxaliplatin (Oxa-Pt) and carboplatin (Car-Pt), which are also widely used against different types of cancer[3]. The problem with cytostatic compounds and CPCs in particular is that they can be carcinogenic, teratogenic and/or mutagenic, and thus, the evaluation of their presence in the aquatic environment is necessary.

High percentages of these compounds or their active metabolites are excreted through the urine[4]. Vyas et al.[5] suggests that the greatest proportion (around 75%) of platinum excreted by patients takes place outside the hospital, and they even foresee concentrations of 0.1 ng·L^{-1} in environmental waters due to the effluents from the wastewater treatment plants (WWTPs). Predicted environmental concentrations of Johnson et al.[6] indicate the possible presence of Oxa-Pt in the range of ng·L^{-1} in sewage, although the limits of detection (LODs) reached for actual methods do not achieve these levels, and thus, it has not been possible to corroborate their hypothesis. For this reason, it is necessary to develop a method in order to achieve lower LODs than those obtained to date in order to quantify and monitor CPCs at low concentrations predicted in samples, such as WWTP effluents or river water.

Given that Cis-Pt is a compound widely used in anticancer therapies and that it has important adverse effects, different papers have studied its effects on the environment. *Zebrafish* liver cells were exposed to four cytostatic compounds including Cis-Pt, with an increase in DNA strand breakage formation found at low concentrations, concluding that side effects in aquatic organisms may be considered[7]. *Mytilus galloprovincialis* mussels were exposed to 0.1 $\mu g \cdot L^{-1}$ Cis-Pt, which resulted in changes in the antioxidant capacity, causing oxidative stress in the digestive gland and the gills as well as neurotoxicity and DNA damage[8].

The determination of CPCs have been carried out mainly by inductively coupled plasma mass spectrometry (ICP-MS) in samples from effluents from hospitals[4,9–12]. Measured concentrations of up to 762 $\mu g \cdot L^{-1}$ platinum were obtained[12–16]. In all of these works, no procedures have been developed for the extraction and preconcentration of platinum compounds before their analysis, which have been carried out for the study of other cytostatic compounds. For that, it is necessary to optimise a preconcentration procedure, mostly using solid phase extraction (SPE)[17], to reach the limits of detection and quantification in which cytostatic compounds are usually present in the influents and/or effluents of the WWTPs. To our knowledge, only Ghafuri. et al[18] have tried to apply ENV+ SPE cartridges for the preconcentration of CPCs to determine these compounds in WWTPs, groundwater and drinking water using an non-specified EPA method. They achieved recoveries of between 0.70–0.78%. They were able to measured concentrations of CPCs in the ranges of 0.27–0.94 $\mu g \cdot L^{-1}$ and 0.11–0.28 $\mu g \cdot L^{-1}$ in influents and effluents of WWTPs, respectively, confirming the prediction of Johnson et al.[6]. However, CPC concentrations in groundwater and drinking water were lower than their limit of quantification, which ranged from 0.009 to 0.017 $\mu g \cdot L^{-1}$. If the hypothesis

of Vyas et al.[5] is correct, lower LODs are necessary for the evaluation of CPCs in environmental waters.

CPCs are highly polar compounds, which are difficult to extract using conventional SPE cartridges, and probably, for this reason, there are not developed extraction methods for them. Ion exchange sorbents could be a very useful alternative to solve the retention problem that very polar compounds present with the most typically used reversed phase cartridges[19]. The main disadvantage in the use of ion exchange sorbents for the extraction of CPCs is the variable range of recoveries: between 64–124% for river water; 52–115% for wastewater[20] and 31–105% for Milli-Q water[21]. However, ion exchange sorbents have been successfully used before in the extraction of different pharmaceuticals from wastewater samples, achieving better limits of detection by reducing the matrix effect[22–26].

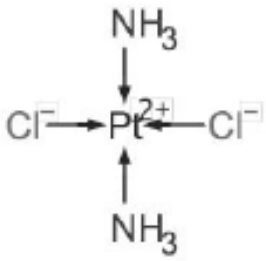
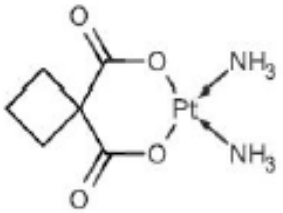
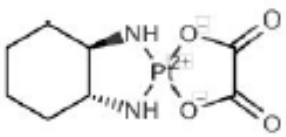
The aim of this work is to optimise an extraction and preconcentration procedure for CPCs based on ion exchange sorbents prior to their determination by ICP-MS to reach the lowest limits of detection and to be able to perform their monitoring in WWTPs and hospital effluents. For that, different ion exchange cartridges were tested and optimised through an experimental design for choosing the one with the best results and finding the optimal extraction conditions. Then, the optimised method was applied to determine these compounds in various environmental wastewater samples, and in this way, it is intended to alleviate the lack of knowledge about the presence of CPCs at low concentrations in this kind of matrix.

2. EXPERIMENTAL

2.1. MATERIALS AND REAGENTS

Ultrapure water used was provided by a Milli-Q system (Milli-pore, Bedford, MA, USA). Methanol of HPLC grade was purchased from VWR (France). HCl and NaOH were used to modify the pH. The ionic strength was modified by the addition of NaCl (%, w/v). Carboplatin (Car-Pt) was purchased from Cymit-Química (Barcelona, Spain), and cisplatin (Cis-Pt) and oxaliplatin (Oxa-Pt) were purchased from Sigma-Aldrich (Madrid, Spain). Stock solutions were prepared by dissolving every compound in Milli-Q water at a concentration of 240 $mg \cdot L^{-1}$. A working solution was prepared daily. The structures and properties of the compounds are shown in Table 1.

Table 1. Properties and structures of the CPCs studied[27–29].

Name	Properties		Structure
Cisplatin	Molar mass:	300.05 $g \cdot mol^{-1}$	
	Log $K_{O/W}$:	-8.24	
Carboplatin	Molar mass:	371.256 $g \cdot mol^{-1}$	
	Log $K_{O/W}$:	-8.47	
Oxaliplatin	Molar mass:	395.278 $g \cdot mol^{-1}$	
	Log $K_{O/W}$:	-2.81	
	Acid pK_a:	-11.72	

	Basic pK_a: 6.53	

The SPE cartridges Oasis MCX (strong cation exchange), Oasis WCX (weak cation exchange), Oasis MAX (strong anion exchange) and Oasis WAX (weak anion exchange) were kindly provided by Waters (Barcelona, Spain). After extraction, the determination was carried out with an ICP-MS instrument (iCAP RQICP-MS) from Thermo Fisher. The conditions of determination are summarised in Table 2.

Table 2. ICP-MS conditions

RF Power (W)	1550
Plasma gas flow ($L \cdot min^{-1}$)	14
Nebulizer gas flow ($L \cdot min^{-1}$)	1.065
Auxiliary gas flow ($L \cdot min^{-1}$)	0.8
Nebulizer	Quartz
Spray chamber	Cyclonic
Number of replicates	3
Reaction cell	Collision cell
Reaction gas	He
Reaction gas flow ($L \cdot min^{-1}$)	4.64

2.2. SAMPLE COLLECTION

Wastewater samples were sampled from the influent and effluent of a WWTP and from the effluent one of the most important hospitals of Gran Canaria Island (Spain). The samples were collected from a WWTP located in the city of Las Palmas de Gran Canaria (Gran Canaria Island, Spain), which has a population of almost 400,000 inhabitants.

The wastewater samples from the hospital were taken at two different points: point 1 collected wastewater from the palliative unit, among others, and point 2 collected wastewater from the pharmacy and oncology departments, among others. In both cases the samples were taken every three months from October 2018 to July 2019. All samples

were taken in 2.5 L amber bottles and acidified at a pH in the range of 2.5–3.5 in less than 1 hour after intake. Subsequently, the samples were stored refrigerated at -4°C until analysis. Before extraction, samples were filtered up to a size of 0.65 µm.

3. RESULTS AND DISCUSSION

3.1. OPTIMISATION OF THE ION EXCHANGE SOLID PHASE EXTRACTION

For this work, we carried out a systematic optimisation through different experimental designs. In all cases, we used four exchange cartridges: strong cation exchange cartridges (Oasis MCX), weak cation exchange cartridges (Oasis WCX), strong anion exchange cartridges (Oasis MAX) and weak anion exchange cartridges (Oasis WAX)

To choose the most suitable conditions for the extraction of the compounds being studied, a 2^3 (three variables at two levels) experimental design was used using Minitab® 17.1.0. In order to study the significance of each variable and the correlation/interaction between them, the pH (3–9), sample volume (100–250 mL) and ionic strength (0–10% w/v of NaCl) were tested for all of the selected SPE cartridges (n=3). Milli-Q water was spiked with a mixture of the three CPCs (Cis-Pt, Car-Pt and Oxa-Pt) at a concentration of 2.5 $\mu g \cdot L^{-1}$ for all them. After extraction, all samples were dried under nitrogen and reconstituted in 5 mL of Milli-Q water + 2% HNO_3 before being injected into the ICP-MS. Results of the adsorption efficiency of every cartridge, measured as the relationship between the concentration of CPCs in Milli-Q water after extraction and a standard of the same concentration in Milli-Q water (2% HNO_3), are shown in Table 3.

Table 3. Adsorption efficiency (%) of the first experimental design

N° Assay	Sample volume (mL)	pH	Ionic strength	Adsorption efficiency (%)			
				MCX	WCX	MAX	WAX
1	100	3	0	47.9	16.3	N.R.	3,44
2	250	3	0	48,6	10.3	3.77	1.77
3	100	9	0	42.4	19.6	10.1	5.43
4	250	9	0	44.8	27.0	8.69	N.R.
5	100	3	10	9.57	15.4	1.86	4.47
6	250	3	10	N.R.	3.14	N.R.	5.65
7	100	9	10	N.R.	N.R.	N.R.	0.20
8	250	9	10	N.R.	7.29	9.51	N.R.

*N.R.: not retained

The results show that the adsorption efficiencies for the MAX and WAX cartridges are very low in all conditions. The WCX cartridge obtains poor adsorption efficiencies (less than 30%) that are affected negatively by the presence of salt. For the MCX cartridges, it was found that the addition of NaCl to increase the ionic strength impairs the retention of analytes on the sorbent. In addition, it seems that the pH and volume variation does not affect the retention since in all of them, the extraction efficiency is approximately 45%. Although MCX cartridges at a pH=3 seem to obtain the best adsorption, the results obtained at a pH=9 were slightly lower. Therefore, to confirm that a pH=3 is the best option, different pHs were tested with 0% ionic strength and a sample volume of 250 mL. Figure 1 shows that the best adsorption rates were achieved with a pH = 3. Therefore, Oasis MCX cartridges at a pH = 3, 0% ionic strength and a sample volume of 250 mL were selected for subsequent studies.

Under the optimal conditions, an adsorption efficiency of 45% of the CPCs onto the Oasis MCX cartridges was obtained. Relative recoveries were calculated taking into account the adsorption achieved.

Next, different elution solvents (MeOH, ACN and water) and mixtures of them were tried to extract the CPCs retained on the cartridge. None of them were able to extract the retained compounds. It could happen that the compounds were permanently retained on

the cartridges, which is one of the main disadvantages of using strong ionic exchange sorbents, and thus, solvents with different additives were tested. Then, different percentages of ammonia (0%, 5%, 10%, 15%, 20% and 25%) (v/v) in methanol were tested for the elution of the retained compounds. Good elution efficiencies were obtained with a percentage of 10% (v/v) ammonia. Different volumes of elution (between 5–12.5 mL) were also tested, and it was observed that the recovery decreased slightly when the elution solvent volume was higher. Thus, 5 mL was fixed as the best elution volume, which also reduces the evaporation time and the use of a large volume of organic solvents. In these conditions, the theoretical preconcentration factor was 22.5 times, taking into account the retention in the cartridge.

3.2. ANALYTICAL PARAMETERS

To test the applicability of the method, the analytical parameters were evaluated in the optimal extraction conditions. External calibration curves were prepared in the CPC concentration range of 12.5 $ng \cdot L^{-1}$ to 10000 $ng \cdot L^{-1}$. The linearity was calculated with excellent correlation coefficients (r^2) higher than 0.999.

The relative recoveries were studied using four different concentrations of CPCs in Milli-Q water corresponding to the different concentration levels at which CPCs are expected to be found in WWTPs: 100 $ng \cdot L^{-1}$, 500 $ng \cdot L^{-1}$ and 1000 $ng \cdot L^{-1}$. The relative recoveries were calculated by comparing the signal of the extract of a spiked sample and the extract of a blank sample spiked after extraction. All experiments were performed in triplicate (n=3). As can be seen in Table 4, the recoveries ranged between 64–89% in Milli-Q water.

Table 4. Analytical parameters in Milli-Q water

Concentration ($ng \cdot L^{-1}$)	Relative recovery (%)	RSD intraday (%)	RSD interday (%)

100	75	14	15
500	64	6	10
1000	66	3	14

To evaluate the precision of the method, the intra-day (n=6) and inter-day (n=3) relative standard deviations (RSD) were determined. The results are shown in Table 4. The results were satisfactory, obtaining intraday RSD in the range of 3–14% and interday RSD in the range of 10–15%.

The limit of quantification (LOQ) was established as the lowest point in the calibration curve, which is 0.74 $ng \cdot L^{-1}$, taking into account the preconcentration factor achieved. This value is suitable, taking into account the expected concentrations in wastewater.

After testing the analytical parameters in Milli-Q water, we determined the analytical parameters in wastewater from the influent and effluent of a WWTP. The matrix effect was studied by comparing the signal of Milli-Q water spiked with the extract of wastewater spiked after SPE. We found a very low matrix effect (Table 5). Recoveries were slightly lower (between 27 and 45%), and this can be explained by the presence of matrix interferences in the wastewater, which could reduce the effectiveness of the adsorption of the target compounds or because CPCs can remain adsorbed on the organic matter when the extract is dried.

Table 5. Matrix effect (%) in wastewater

Concentration ($ng \cdot L^{-1}$)	Influent	Effluent
100	3,66	1,91
500	24,4	10,9
1000	6,37	9,16

To improve the extraction efficiency in wastewater, an elution in two steps was optimised. In these assays, all samples were spiked to a final concentration of CPCs of 1000 ng·L^{-1}. The results are shown in Table 6.

Table 6. Results of the elution in two steps

Elution 1	Relative recovery (%)	Elution 2	Relative recovery (%)	TOTAL
5 mL MeOH	10	5 mL MeOH (10% NH_3)	38	48
10 mL MeOH	12	5 mL MeOH (10% NH_3)	40	52
5 mL MeOH (5% HCOOH)	9	5 mL MeOH (10% NH_3)	48	57
10 mL MeOH (5% HCOOH)	13	5 mL MeOH (10% NH_3)	42	55
5 mL Milli-Q water + 5 mL MeOH (5% HCOOH)	13	5 mL MeOH (10% NH_3)	36	49
5 mL MeOH (5% HCOOH) + 5 mL Milli-Q water	14	5 mL MeOH (10% NH_3)	31	45
5 mL Milli-Q water (5% HCOOH) + 5 mL MeOH (5% HCOOH)	10	5 mL MeOH (10% NH_3)	34	44
5 mL MeOH (5% HCOOH) + 5 mL Milli-Q water (5% HCOOH)	11	5 mL MeOH (10% NH_3)	46	57
5 mL MeOH (5% HCOOH) + 5 mL Milli-Q water (5% NH_3)	50	5 mL MeOH (10% NH_3)	10	60
5 mL Milli-Q water (5% NH_3) + 5 mL MeOH (5% HCOOH)	40	5 mL MeOH (10% NH_3)	28	68

Taking into account these results, we decided to perform the elution in the following two steps: a first elution with 5 mL of MeOH (5% HCOOH) + 5 mL Milli-Q water (5% NH_3) and a second elution with 5 mL of MeOH (10% NH_3). The results of the recoveries in the wastewater influent and effluent are shown in Table 7.

Table 7. Relative recovery (%) in influent and effluent water from WWTP.

Concentration (ng·L^{-1})	Influent	Effluent
100	82	90
500	47	56
1000	82	66

The procedure shows very good limits of detection, which are lower than those obtained to date, as well as good reproducibility and repetitiveness, almost without the presence of a matrix effect. Sometimes, it is preferable to work with a lower recovery if it means less interference to achieve a lower detection limit for the method[19]. Relative recoveries of CPCs between 47–90% were obtained for the influents and effluents of wastewater. In this way, we were able to study CPCs from hospital wastewater but also from influents and effluents of WWTPs.

3.3. ANALYSIS OF WASTEWATER SAMPLES

Samples of wastewater were taken during one year from October 2018 to July 2019 every three months in a wastewater effluent from a hospital of the island and in a WWTP (influent and effluent). The hospital wastewaters were analysed directly by ICP-MS without preconcentration. Samples of the WWTP were preconcentrated by SPE before their determination by ICP-MS. Each extraction was performed triplicate (n=3). The results are shown in Table 8.

Table 8. Concentrations obtained in the analysis of samples from the WWTP and hospital samples

Sample	Date	Point	Concentrations ($ng \cdot L^{-1}$)
Hospital effluent	OCT'18	Point 1	2282 ± 6,85
		Point 2	13913 ± 28
	JAN'19	Point 1	86,59 ± 0,87
		Point 2	92,40 ± 1,02
	APR'19	Point 1	Not taken
		Point 2	81,94 ± 0,90
	JUL'19	Point 1	104,1 ± 0,41
		Point 2	448,8 ± 3,14
WWTP	OCT'18	Influent	27,01 ± 3,22
		Effluent	75,79 ± 5,61
	JAN'19	Influent	22,49 ± 3,69
		Effluent	74,02 ± 4,17
	ABR'19	Influent	3,97 ± 0,25
		Effluent	56,08 ± 2,10

	JUL'19	Influent	38,68 ± 5,68
		Effluent	71,01 ± 4,55

As has been proven before, it is possible to analyse the CPC concentrations in the wastewater effluent from a hospital without the use of a preconcentration step since the concentrations from the hospital are large enough. We have analysed two different points of the hospital, and we have detected a higher concentration of CPCs in the part from the oncology and pharmacy units than in the part from the palliative unit. On the other hand, due to the low concentration of CPCs in the WWTP, it makes it necessary the use of a steps of the extraction and preconcentration of the contaminants. With the procedure developed here, we have been able to study the presence of CPCs in the influent and effluent of a WWTP. We have found slightly higher concentrations in the effluent than in the influent. This may be due to the higher content of organic matter in the influent that strongly retains CPCs, and these cannot be eluted. In the case of the effluent, the organic matter content is lower, and thus, the extraction efficiency is better, detecting a greater amount of CPCs in the corresponding samples. However, the concentrations are similar, and more studies should be carried out to confirm these results.

4. CONCLUSIONS

Cytostatic platinum compounds are compounds that have been shown to be cytotoxic and genotoxic; however, their concentrations in wastewater are not well documented, especially not as well as for cyclophosphamide, etoposide or tamoxifen, for example. This is probably because the methodologies optimised to date do not reach an adequate detection limit.

In order to analyse CPCs in wastewater samples from a WWTP, we have optimised and developed a new approach of extraction and preconcentration of very polar

compounds using ion exchange sorbents, a strong cation exchange cartridge, prior to their determination by ICP-MS. We have optimised all of the variables that affect the process, and with this procedure we have managed to extract and preconcentrate CPCs, obtaining a method limit of quantification of 0.74 $ng \cdot L^{-1}$, with a low matrix effect and low intraday and interday deviations. This procedure allows us to measure and monitor concentrations of CPCs in samples from WWTPs as well as in samples from hospital effluents.

The method has been satisfactorily applied to real samples, and CPCs were detected in the range of 81.94–13913 $ng \cdot L^{-1}$ in effluent samples from a hospital and between 3.97 $ng \cdot L^{-1}$and 75.79 $ng \cdot L^{-1}$ in wastewater treatment plant samples.

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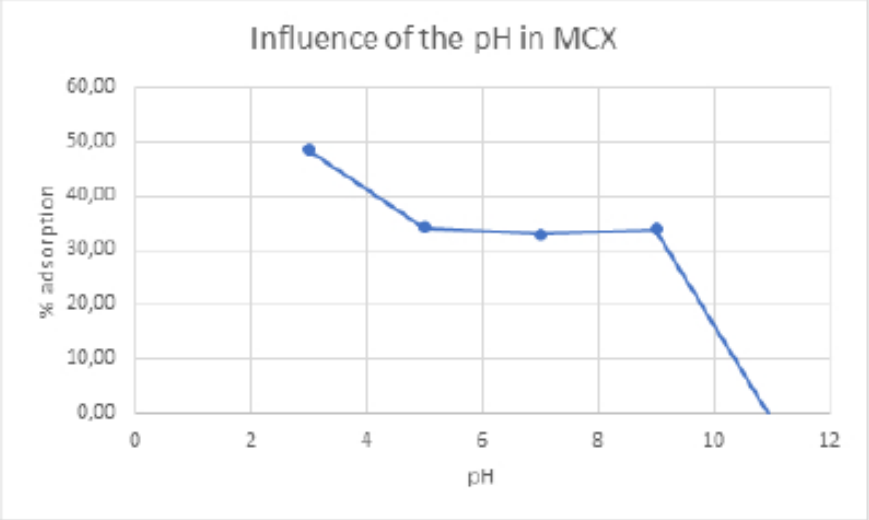

Figure 1. Influence of the pH on the adsorption efficiency of MCX cartridges.

3.4. Compuestos citostáticos en lodos y sedimentos extraídos mediante extracción asistida por microondas y determinación por UHPLC-MS/MS

La mayoría de los trabajos relacionados con la determinación de compuestos antineoplásicos en muestras ambientales se han realizado sobre muestras de aguas residuales, por lo que la información sobre la presencia de estos compuestos en muestras sólidas es muy escasa. En los trabajos publicados, se han detectado concentraciones de CP en lodos [65], CP e IF en lodos [66] y bicalutamida, doxifluridina y TAM en sedimentos [64].

El objetivo de este trabajo es contribuir al conocimiento sobre la presencia de compuestos antineoplásicos en lodos y sedimentos. Para ello, hemos optimizado un procedimiento de extracción basado en la extracción asistida por microondas (MAE), ya que ha demostrado ser efectiva en la extracción de micro-contaminantes de muestras sólidas, seguido de su posterior determinación mediante UHPLC-MS/MS [91]. En esta ocasión, la mezcla de compuestos seleccionada contiene VINB, VINC, GEM, MET, 5-FU, ETO, CP y TAM.

Se desarrolló un diseño experimental con todas las variables que podrían afectar el proceso de extracción: tiempo y temperatura de extracción, tipo y volumen de disolvente, y cantidad de muestras. Los resultados obtenidos mostraron que la temperatura y el tiempo de extracción no eran variables que afectasen en gran medida el proceso de extracción. El disolvente más adecuado debía ser metanol y que un volumen mayor favorecía la extracción al igual que una cantidad de muestra menor. Pese a que los lodos y los sedimentos son matrices

diferentes, el resultado del diseño experimental fue el mismo para ambas matrices. Por tanto, se eligieron como condiciones más adecuadas 5 minutos de tiempo de extracción a 60°C, utilizando 50mg de muestra y 14mL de metanol.

La extracción de los compuestos 5-FU y MET no se pudo lograr en ninguna de las matrices, quizás debido a su polaridad y a que se ven muy perjudicados por el efecto matriz. Los compuestos VINB y VINC al reducir la concentración para evaluar los parámetros analíticos, no se pudieron detectar.

La recuperación en lodos fue mejor que en sedimentos, llegando a extraer cuatro compuestos citostáticos (ETO, GEM, CP y TAM) con recuperaciones de entre el 65 – 122% a las concentraciones seleccionadas (0.5 $ng \cdot g^{-1}$, 1 $ng \cdot g^{-1}$ y 2 $ng \cdot g^{-1}$). En sedimentos, el compuesto ETO se extrajo con baja recuperación (10 – 23%), mientras que para los otros compuestos, las recuperaciones obtenidas variaron entre el 49 – 109%. En relación al efecto matriz, en general, fue menor en sedimentos que en lodos, excepto para el compuesto GEM, que tiene una importante supresión de la señal en ambas matrices. En relación a la desviación de la reproducibilidad y repetibilidad, estas fueron inferiores al 15% y 18%, respectivamente.

Finalmente, el procedimiento se aplicó a muestras de sedimentos tomadas en las proximidades de tres emisarios submarinos de tres EDARs de Gran Canaria diferentes y a lodos procedentes de la EDAR de Las Palmas de Gran Canaria. Los muestreos se realizaron cada 3 meses durante 2 años. No se llegaron a detectar ninguno de los compuestos

analizados, probablemente debido a que su concentración era muy baja y estaba por debajo del límite de detección.

Los resultados de este estudio han sido enviados para su consideración como publicación a la revista Analytical and Bioanalytical Chemistry, la cual se encontraba en el primer cuartil con un IF=3.286 en el año 2018.

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CYTOSTATIC COMPOUNDS IN SLUDGE AND SEDIMENT: EXTRACTION AND DETERMINATION BY A COMBINATION OF MICROWAVE ASSISTED EXTRACTION AND UHPLC-MS/MS

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ABSTRACT

Cytostatic compounds are an important group of micro-pollutants since they are used to kill cells or stop cell division. For this reason, they are also considered mutagenic. Several cytostatic compounds have been detected in hospital effluents, in the influents and effluents of wastewater treatment plants, and even in river water. However, their detection in solid matrices is very scarce.

In this work, we have developed a new procedure based on microwave assisted extraction (MAE) for the extraction of cytostatic compounds from sludge and sediment before determination by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). To develop this procedure, we have chosen a group of eight widely used cytostatic compounds and carried out a systematic experimental design to optimise the extraction conditions.

Under these optimal conditions, the studied cytostatic compounds are extracted with good sensitivity, with recoveries ranging from 65 – 122% in sludge and recoveries varying between 49 – 109% in sediment, with the exception of etoposide, which has a

lower recovery from these types of samples. The limits of detection were from 0.42 – 79.8 ng·g^{-1} in sludge and from 0.10 – 87.5 ng·g^{-1} in sediment. Intraday and interday relative standard deviations (RSDs) were below 15% and 18%, respectively, in both matrices at the tested concentrations.

The total procedure was applied to samples of sludge taken from the main wastewater treatment plant (WWTP) of the island of Gran Canaria (Spain) and for sediment samples obtained close to the marine outfalls of different wastewater treatment plants for the same island.

1. INTRODUCTION

Concerns have been raised in recent years about the possible adverse effects caused by pharmaceutical compounds that are not completely degraded by wastewater treatment plants. A group of pharmaceutical compounds of special interest are cytostatic compounds. Consumption of these compounds has been increasing [1] because they are designed to stop cell division or kill cells; however, they affect all cells, not only cancerous cells. In addition, it has been demonstrated that cytostatic compounds can cause side effects at a very low concentration due to prolonged exposure [2–6].

Several cytostatic compounds have been detected in aqueous samples [7–11], and they may not necessarily be biodegradable [12, 13]; therefore, these compounds could also be adsorbed within sludge and sediment [14]. The bulk of the papers reporting environmental concentrations of cytostatic compounds have been carried out in liquid samples [15]. Nevertheless, some studies suggest that they may also be absorbed in sludge, and for that reason, certain cytostatic compounds do not appear in effluents [14]. Moreover, only a few papers report optimised procedures for the extraction of these compounds from solid samples.

Ternes et al. carried out the first optimised extraction from solid samples in 2005. In this work, the studied pharmaceutical compounds were divided into three groups using different procedures for each one. The group in which cytostatic compounds were found used ultrasonic solvent extraction (USE) for the extraction and HPLC-MS/MS for the determination. Two cytostatic compound were studied: ifosfamide (IF) and cyclophosphamide (CP), reaching a recovery of 59% and 66%, respectively, and a limit of quantification (LOQ) of 20 $ng \cdot g^{-1}$ for both; these compounds, however, were not detected in the real samples [16].

Okuda et al. detected between 1 – 10 $ng \cdot g^{-1}$ of CP using pressurised liquid extraction (PLE) and LC-MS/MS in sludge samples, where they studied several pharmaceutical compounds. They obtained a recovery of 70% for CP and a LOQ of 0.66 $\mu g \cdot L^{-1}$, detecting concentrations of approximately 30 $ng \cdot g^{-1}$ in the real samples [17]. Seira et al. optimised a method for the extraction of CP and IF from sludge with PLE and UHPLC-MS/MS. They included a sample purification step by solid phase extraction using a tandem of Oasis MCX and MAX cartridges. At the beginning, they also included the compound tamoxifen (TAM). However, this compound showed strong variability, and therefore, it was excluded. They achieved a relative recovery of 97 – 100% for CP and 95 – 108% for IF. They found the matrix effect to be the most limiting step, which reduces the efficiency of the method to 22 – 38% (CP) and 15 – 28% (IF). In spite of the matrix effect, they reached a limit of detection of 2.5 – 4.7 $ng \cdot g^{-1}$ for CP and 3.9 – 7.9 $ng \cdot g^{-1}$ for IF, measuring concentrations of 12.6 $ng \cdot g^{-1}$ CP and 11.4 – 42.5 $ng \cdot g^{-1}$ IF in the real samples [18].

Peysson and Vulliet determined 139 compounds from sludge using a QuEChERS method followed by HPLC-ToF-MS. Included in the mixture of compounds were daunorubicin (DAU), epirubicin (EPI) and TAM. During the first attempts, these compounds were extracted with low recoveries: 21 – 37% DAU, 4 – 5% EPI, and 58 –

67% TAM, which was probably due to the large quantity of compounds they wanted to extract simultaneously. The compounds were also not detected in real samples [19].

López-Zavala and Reynoso-Cuevas developed a USE and UHPLC/MS-MS procedure for the determination of pharmaceutical compounds in compost, including IF and CP. IF was extracted with a recovery of 87.3 – 97.3%, while CP had a recovery of 95.0 – 99.1%, achieving a LOD of 0.66 $ng \cdot g^{-1}$ for both compounds. The intraday and interday reproducibility was also studied, achieving a deviation lower than 12.7% for these compounds. No matrix effect was detected [20].

Azuma et al. investigated six cytostatic compounds (bicalutamide, capecitabine, cyclophosphamide, doxifluridine, tamoxifen and tegafur) among other compounds contained in river sediment. They applied USE and UPLC/MS-MS for the analysis and were able to achieve LODs between 2.7 – 3.5 $ng \cdot kg^{-1}$ with recoveries that ranged from 42 – 114%, depending on the compound. They detected concentrations of 391 $ng \cdot kg^{-1}$ bicalutamide, 391 $ng \cdot kg^{-1}$ doxifluridine and 250 $ng \cdot kg^{-1}$ TAM in real samples [21].

We consider that it is important to monitor the presence of cytostatic compounds in wastewater treatment plant sludge and sediment from marine outfalls. For this, the aim of the work is to develop an analytical methodology based on microwave assisted extraction coupled to ultra-high-performance liquid chromatography with tandem mass spectrometry detection (MAE-UHPLC-MS/MS) for the analysis of eight cytostatic compounds, which belong to different families of compounds and are widely use in cancer therapies. Some of these cytostatic compounds have been previously detected in the effluent of wastewater treatment plants in the studied geographical area [22].

Microwave assisted extraction (MAE) has been demonstrated to be a fast method capable of extracting micro-pollutants from a solid matrix using small amounts of sample

and solvent volume [23]. We have optimised the whole procedure through a systematic experimental design. Once optimised, the method was applied to the analysis of sludge samples from the main wastewater treatment plant of Gran Canaria (Spain), which receives wastewater from the main hospital that uses chemotherapy treatments. Samples from marine sediment in the vicinity of three marine outfalls from different wastewater treatment plants on the same island were also analysed.

2. EXPERIMENTAL

2.1. MATERIALS AND REAGENTS

HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), LC–MS grade MeOH, LC–MS grade water and formic acid used to adjust the pH of the mobile phases were obtained from Panreac Química (Barcelona, Spain). The etoposide (ETO), cyclophosphamide (CP) and tamoxifen (TAM) antineoplastic compounds were purchased from Sigma-Aldrich (Madrid, Spain). The cytostatic compounds 5-fluorouracil (5-FU), gemcitabine (GEM), methotrexate (MET), vincristine (VINC) and vinblastine (VINB) were purchased from Cymit-Química (Barcelona, Spain). Properties of the selected compounds are listed in Table 1. Stock solutions containing 1000 $mg \cdot L^{-1}$ of each analyte were prepared by dissolving the compound in methanol. All solutions were stored in glass-stoppered bottles at –20 °C in the dark. Working standard solutions were prepared weekly at 10 $\mu g \cdot g^{-1}$.

2.2. EQUIPMENT AND CHROMATOGRAPHIC CONDITIONS

A Titan MPS microwave equipment with 16 vessels was purchased from PerkinElmer (Madrid, Spain). An ACQUITY UPLC system equipped with a triple quadrupole detector with an ESI interface, controlled by MassLinx Mass Spectrometry software, was used for instrumental determination. The chromatographic system consisted of a Binary Solvent

Manager, a 2777 autosampler and a column manager, all from Waters Chromatography (Barcelona, Spain). The electrospray ionisation was performed in positive mode (except for 5-FU, which was performed in negative mode) and detection parameters were optimised using standard solutions of each compound. The optimum detection parameter values were as follows: capillary voltage of 3.5 kV, cone voltage of 40 V and source and desolvation temperature of 120 and 400 °C, respectively. Nitrogen was used as the desolvation gas at a flow rate of 1000 $L \cdot h^{-1}$, and argon was employed as the collision gas. Chromatographic conditions are shown in Table 2 and Table 3.

Multiple reaction monitoring parameters were optimised for each compound to carry out the quantitative analysis. The optimisation of the quantification and confirmation ions, as well as the detection parameters, was performed for each compound by direct infusion of 1 $mg \cdot L^{-1}$ standard solutions in MeOH at a flow rate of 10 $\mu L \cdot min^{-1}$. Precursor ions were $[M+H]^+$ in the positive ion mode (ESI +) for all compounds, with the exceptions of CP, which was $[M]^+$, VINB, which was $[M+2H]^{2+}$ and 5-FU, which was $[M-H]^-$. The conditions of each compound in the mass spectrometer are summarised in Table 4.

2.3. SAMPLE COLLECTION AND TREATMENT

Sludge samples were taken from the main wastewater treatment plants (WWTPs) of the island (WWTP1), located in Las Palmas de Gran Canaria, every three months from July 2016 to April 2018. Samples were taken in glass bottles and frozen at -20 °C. Prior to analysis, samples were lyophilised, ground and sieved to 0.3 mm.

Sediment samples were taken in the vicinity of three marine outfalls from three WWTPs from Gran Canaria (Spain). WWTP1 and WWTP 2 use conventional treatment of activated sludge, while WWTP3 also performs reverse osmosis as a tertiary treatment following activated sludge treatment. The samples were collected in glass bottles,

lyophilised, sieved to 0.3 mm and stored in a fridge at 4°C until analysis. The samples were taken every three months over a duration of two years, spanning from July 2016 to April 2018.

The sludge used for the optimisation of the method was collected from WWTP1 before starting to take samples quarterly. Sediment used for the optimisation was taken from a secluded beach on the island of Gran Canaria.

3. RESULTS AND DISCUSSION

3.1. OPTIMISATION OF MICROWAVE ASSISTED EXTRACTION THROUGH EXPERIMENTAL DESIGN

MAE is strongly influenced by several parameters, such as the weight of the sample, the composition and volume of the extractant, the temperature of the microwave and the time of the extraction. The optimisation was performed through experimental design using Minitab® software, version 17.1.0. The experimental design was performed with 5 variables at 2 levels: weight (100 and 250 mg), solvent volume (7 and 12 mL), time (5 and 10 minutes), temperature (60 and 80 °C) and solvent (methanol or acetonitrile). This methodology was employed in order to study the contributions of each variable individually, as well as in relation to the other variables, to determine the extraction efficiency. Both the sludge and the sediment were spiked to a concentration of 10 $\mu g \cdot g^{-1}$ with a mixture of cytostatic compounds. All extractions were performed in triplicate (n=3). The efficiency of the extraction was calculated as the relationship between the signal obtained after extraction and a standard in methanol at the same concentration. The results of all extractions are presented in the Supplementary material (Tables S1 and S2). Regardless of the matrix, the obtained results appear to be quite similar. 5-FU was not extracted under any of the conditions tested, probably due to its polarity. For this reason,

5-FU was excluded. The compounds MET, VINC and VINB are poorly extracted under the conditions tested, and GEM as well as ETO are extracted in a few assays. CP and TAM are readily extracted under the conditions tested.

A study of the variables that affect the extraction was performed by using Pareto charts (Figures 1 and 2). We focus on a combination of two variables that would affect the procedure. For many compounds, we can observe that weight of the sample and the type of solvent are critical variables. The results show that the MeOH extraction is better than extraction with ACN, as shown as in Supplementary material. Thus, we chose to employ MeOH in subsequent assays. Time and temperature factors do not greatly affect the extraction, and according to Pearson's correlation (Table 5), it is not clear whether a higher or lower value benefits the extraction. For this reason, we set the extraction time at 5 minutes, which makes the extraction faster, and an extraction temperature of 60 °C was used since a lower temperature is reached faster.

Because the combination of sample weight and solvent volume variables were found to affect the extraction, these two variables were chosen for the next experimental design (3^2, two variables at three different levels). We chose 8, 11 and 14 mL for the solvent volume and used 50, 75 and 100 mg of sample.

The results of the 3^2 experimental design are shown in Tables S3 and S4 and in Figures 3 and 4. In sludge, the compound VINC was not extracted under the conditions tested. VINB was extracted under some conditions, as was MET. ETO, GEM, TAM and CP were more effectively extracted using a low sample weight and 14 mL of solvent volume. In sediment, MET was not extracted and VINB and VINC practically neither. This observation may be due to the very complex nature of their structures, which have the ability to create strong and stable bonds with a range of molecules. The compounds

ETO, GEM, TAM and CP have a similar behaviour to compounds found in sludge; these compounds were more effectively extracted at low sample weights and higher MeOH solvent volumes. It was necessary to reach a compromise and to select the two values that give rise to the best analytical signals for all target compounds in both type of samples. Finally, we chose the following as the optimal conditions for extraction from both sludge and sediment: 50 mg of sample, extracted with 14 mL of MeOH, heated at 60 ºC for 5 minutes. Taking into account all these results, we have chosen four cytostatic compounds (ETO, GEM, CP and TAM), which enhance the signal necessary for detecting lower concentrations.

3.2. ANALYTICAL PARAMETERS

The linearity, recovery, repeatability, limits of detection (LODs) and limits of quantification (LOQs) of the MAE-UHPLC-MS/MS method were evaluated to ensure the precision, accuracy and selectivity of the developed method (Tables 6 and 7). Due to the presence of the matrix effect, matrix match calibration (MMC) was performed in every of matrix from 0.2 $\mu g \cdot g^{-1}$ to 12 $\mu g \cdot g^{-1}$ (10 points).

The linearity was calculated using the relationship between the areas and concentrations of compounds, with correlation coefficients (r^2) higher than 0.990 obtained in both matrices.

Recoveries were performed at three different concentrations—0.25 $\mu g \cdot g^{-1}$, 1 $\mu g \cdot g^{-1}$ and 2 $\mu g \cdot g^{-1}$—under the optimal conditions. Recovery was calculated as the ratio between the spiked sample before extraction and the blank sample spiked after extraction (eq. 1). All extractions were performed in triplicate.

$$Recovery = \frac{sample\ spiked\ before\ extraction}{sample\ spiked\ after\ extraction} \times 100 \text{ (eq. 1)}$$

It was observed that the recoveries were better in sludge than in sediment. The recoveries from sludge ranged from 65 – 122% for the analysed compounds at the concentrations tested. For sediment, ETO was poorly extracted, with recoveries between 10 – 23%, while the rest of the compounds (GEM, CP and TAM) were extracted with recoveries ranging from 49 – 109%, as is shown in Table 7.

Regarding the matrix effect, in the analysis of complex matrices, such as sludge or sediment extracts, it is common for the signal to suffer some type of modification, which may result in suppression or enlargement of the signal when electrospray ionisation is used in the mass spectrometer detector because of the interferences extracted along with the target compounds.

Matrix effects were calculated as the ratio of blank sample spiked after extraction against a standard sample in methanol at the same concentration (eq. 2).

$$Matrix\ effect = \frac{Standard\ in\ MeOH - sample\ spiked\ after\ extraction}{Standard\ in\ MeOH} \times 100 \text{ (eq. 2)}$$

Following this equation, a signal suppression would have a positive value; if this value is close to zero, it means there is a small matrix effect, and a negative value means that there will be an enlargement of the signal. As we expected, the matrix effect is more pronounced in sludge than sediment except for GEM, which displays high signal suppression due to the matrix in both. For CP and TAM, the signal suppression in sludge is similar. However, for ETO, the signal varies from enlargement to suppression. In the case of sediment, ETO exhibits a constant suppression of the signal for the concentrations tested, TAM has almost no influence on the matrix, and the signal for CP ranges from enlargement to suppression.

Intraday precision was evaluated by performing six extractions in the same day, and interday precision was studied by performing three extractions on three different days. The relative standard deviation for intraday measurements were below 15% in both matrixes for all compounds at the tested concentrations. For interday measurements, the relative standard deviation was slightly higher but below 18% for all compounds, as is shown in Tables 6 and 7.

The LOD was calculated as the minimum concentration that gives a signal/noise ratio higher than 3, while the LOQ was defined as a signal/noise ratio higher than 10. As we expected, the LOD was lower for TAM and CP in sediment than in sludge. However, because GEM and ETO have lower recovery in sediment, they have a better LOD in sludge. The LODs of GEM, CP and TAM in sludge ranged between 0.42 – 1.20 $ng \cdot g^{-1}$, and the LOD of ETO was 79.8 $ng \cdot g^{-1}$. In sediment, the LODs of TAM and CP were 0.10 and 0.77 $ng \cdot g^{-1}$, respectively. The LOD for GEM was 7.97 $ng \cdot g^{-1}$, and for ETO, the LOD was 87.5 $ng \cdot g^{-1}$.

3.3. ANALYSIS OF SLUDGE AND SEDIMENT SAMPLES

The optimised and developed MAE-UHPLC-MS/MS method was applied to study the presence of the target cytostatic compounds in sludge from a wastewater treatment plant and in marine coastal sediment close to marine outfalls.

Sludge and sediment samples were collected every three months over two years (from July 2016 to April 2018) in the main WWTP of the island of Gran Canaria (Spain) and in the nearest of three marine outfalls from three different WWTPs, respectively. WWTP1 is located in the northeast of the island (28° 6′ 36.263″ N, 15° 24′ 25.47″ W), WWTP2 is located in the east (27° 49′ 19.43″ N, 15° 25′ 5.963″ W) and WWTP3 is located in the south (27° 45′ 45.742″ N, 15° 31′ 29.017″ W). All WWTPs use

conventional treatment methods for activated sludge. WWTP1 is the main wastewater treatment plant of the island, and it is located in the capital city of the island. This WWTP receives wastewater from two hospitals that treat patient with cytostatic compounds, which is the reason we collected sludge samples from WWTP1.

Overall, the proposed method was applied to 8 sludge samples and 24 sediment samples. The target cytostatic compounds were not detected in any of the analysed samples. For the sludge, these results are in agreement with previous papers, which have not detected this kind of compound in sludge [16, 19], probably due to the complexity and lack of homogeneity of sludge samples. In the case of sediment, this result may be due to the dilution effect of this type of compound in the seawater near marine outfalls. Only one author reported detecting cytostatic compounds in sediment after achieving a LOD one thousand times lower than reported in the other papers [21].

4. CONCLUSIONS

Cytostatic compounds have been studied mainly in environmental liquid samples, but there is a lack of knowledge about their presence in environmental solid samples. In this work, we have developed, for the first time, a procedure for the extraction of cytostatic compounds from sludge and sediment based on microwave assisted extraction (MAE) combined with ultra-high-performance liquid chromatography with tandem mass spectrometry detection (MAE-UHPLC-MS/MS) for their determination.

Under optimal conditions, we are able to extract different cytostatic compounds from sludge and sediment with good recoveries and sensitivity compared with the few previous published works in this field. To deal with the matrix effect, we have performed two matrix match calibrations, one for each matrix. Intraday and interday relative standard deviations were below 15% and 18%, respectively, in both solid samples. With this

method, we have achieved a sub-ng·g^{-1} level limit of detection for these compounds, a range in which some of the compounds have been detected in real matrices.

In addition, we have applied the developed procedure for monitoring sludge from the main wastewater treatment plant of the capital city of the island, along with sediment collected from three different marine outfalls of three wastewater treatment plants on the island of Gran Canaria (Spain). None of the cytostatic compounds studied were detected in the samples analysed. These compounds are used only to treat certain, so their concentration is probably below the LOQ. However, the method proposed here is valid for both matrices (sludge and sediment) under the same extraction conditions. This simple procedure also provides good LODs without a clean-up step, and requires only a low amount of sample and only five minutes of extraction time.

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Figure 1: Pareto charts in sludge

Figure 2: Pareto charts in sediments

Figure 3: Contour plots of sludge for selected analytes

Figure 4: Contour plots of sediment for selected analytes

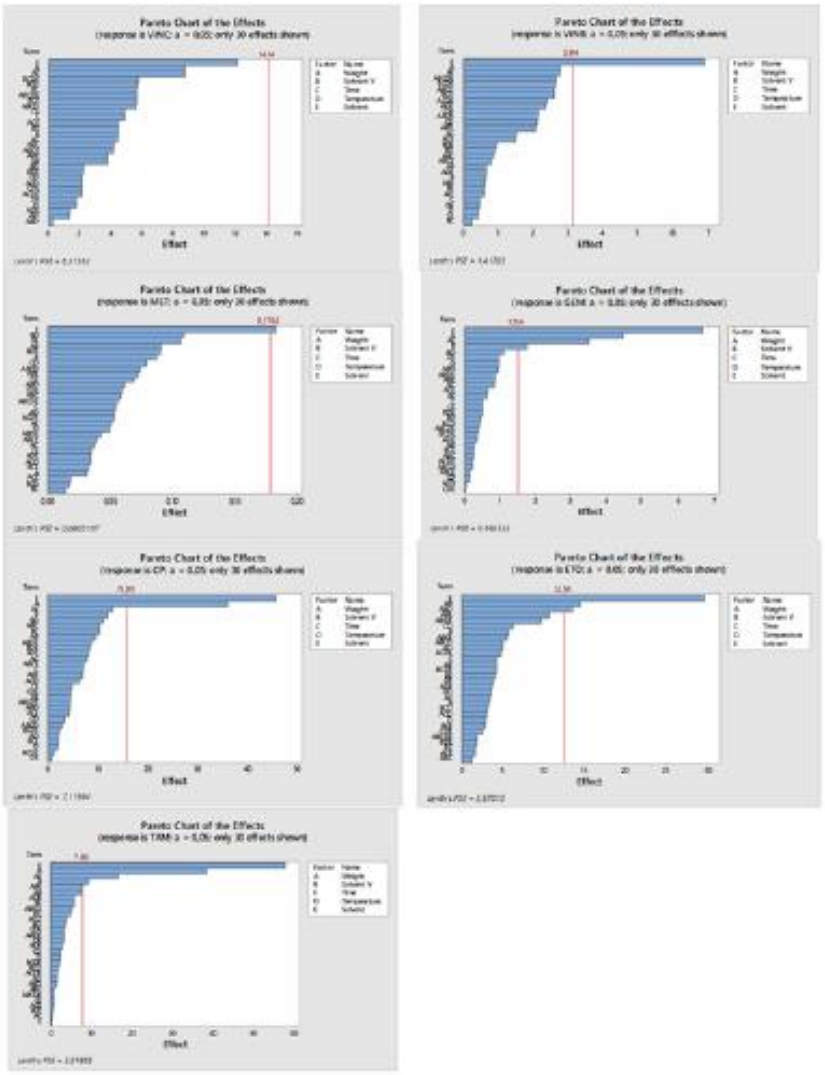


Figure 1: Pareto charts in sludge

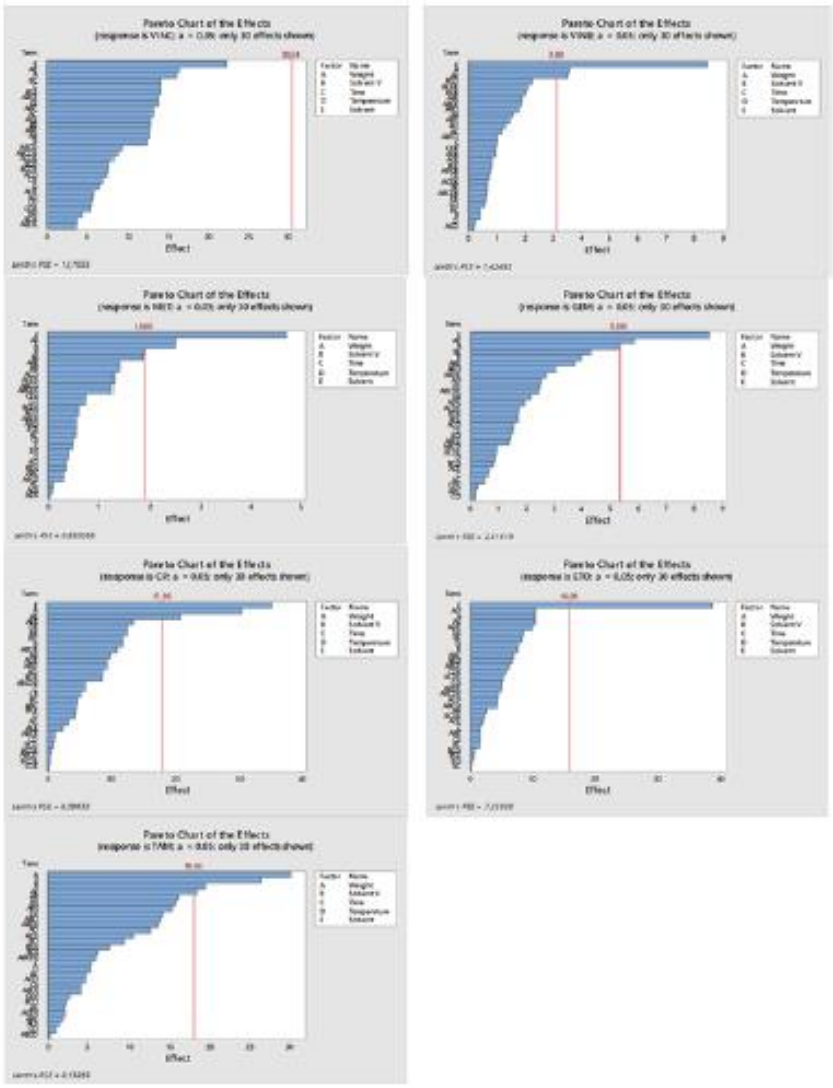


Figure 2: Pareto charts in sediments

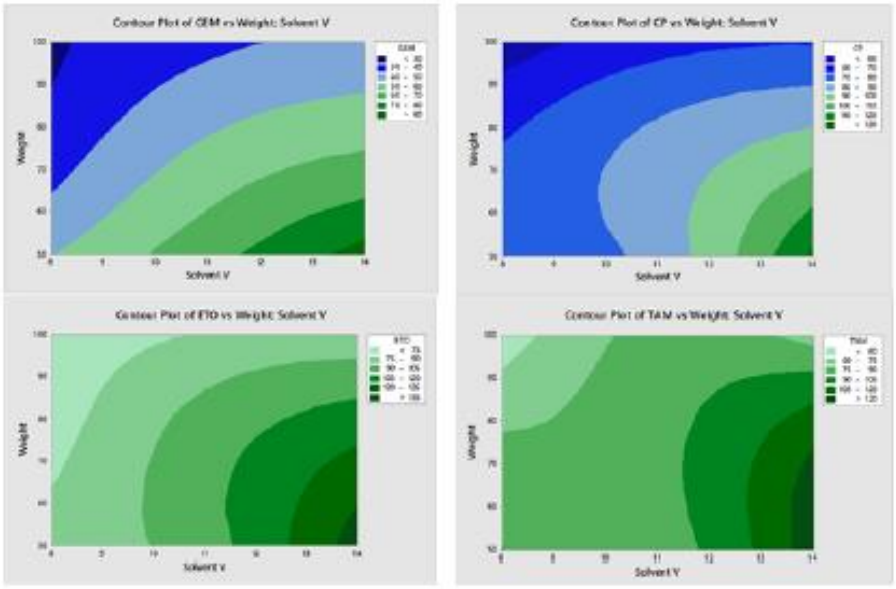


Figure 3: Contour plots of sludge for selected analytes

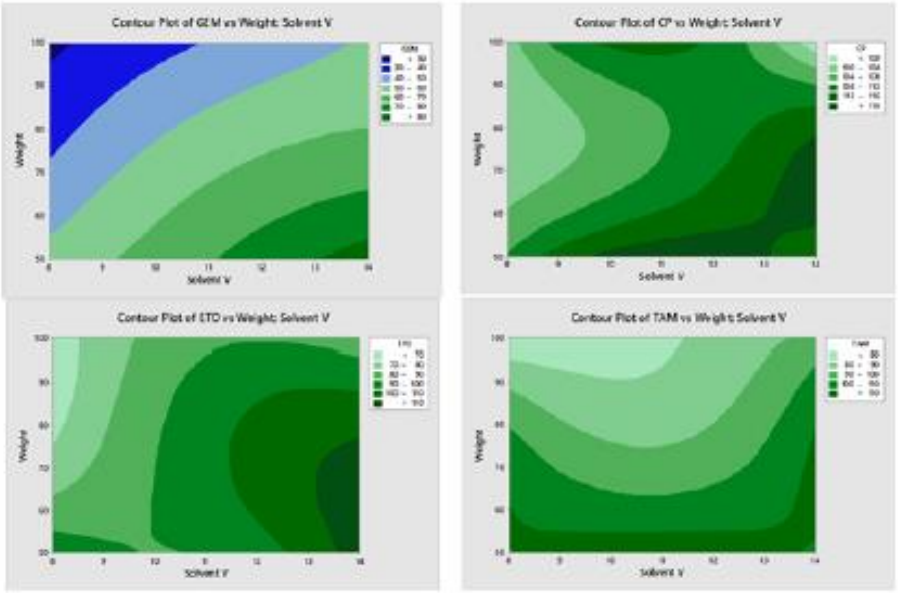
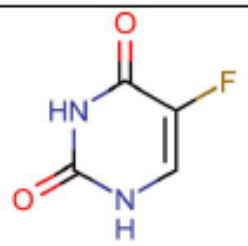
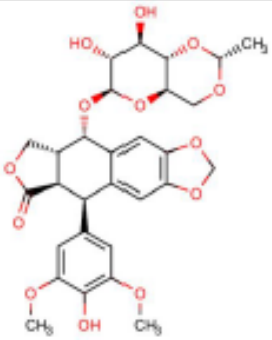
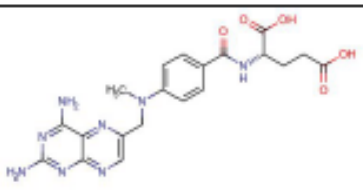


Figure 4: Contour plots of sediment for selected analytes

Table 1. Structure and properties of the selected cytostatic compounds [24–31]:

Compound	Properties		Structure
5-FU	Molar mass ($g \cdot mol^{-1}$)	130.078	
	pK_a	7.18	
	Log $K_{o/w}$	-0.66	
CP	Molar mass ($g \cdot mol^{-1}$)	261.08	
	pK_a	13.43	
	Log $K_{o/w}$	0.1	
ETO	Molar mass ($g \cdot mol^{-1}$)	588.562	
	pK_a	9.33	
	Log $K_{o/w}$	1.16	
GEM	Molar mass ($g \cdot mol^{-1}$)	263.201	
	pK_a	11.52	
	Log $K_{o/w}$	-1.47	
MET	Molar mass ($g \cdot mol^{-1}$)	454.447	
	pK_a	3.25	
	Log $K_{o/w}$	-0.24	

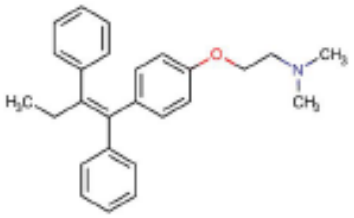
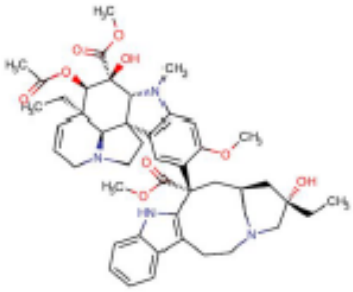
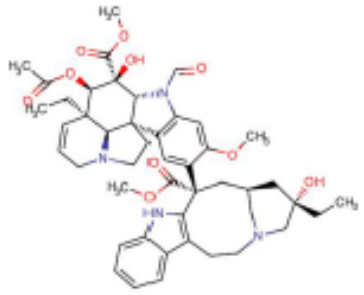
TAM	Molar mass ($g \cdot mol^{-1}$)	371.524	
	pK_a	8.76	
	Log $K_{o/w}$	6.35	
VINB	Molar mass ($g \cdot mol^{-1}$)	810.989	
	pK_a	10.87	
	Log $K_{o/w}$	4.18	
VINC	Molar mass ($g \cdot mol^{-1}$)	824.972	
	pK_a	10.85	
	Log $K_{o/w}$	3.13	

Table 2. Liquid chromatographic condition

Parameters	Applied Condition
Sample volume	10 µL
Column	Phenomenex Luna Omega Polar (50 mm x 2.1 mm x 1.6 µm) (Phenomenex, Madrid, Spain)
Mobile phase	A: water with 0.1% of formic acid B: MeOH with 0.1% of formic acid
Flow rate	0,4 mL·min^{-1}
Column temperature	Room temperature
Chromatography mode	Gradient elution

Table 3. Steps of gradient mode

Time (Min)	% Eluent A	% Eluent B
0	40	60
0,5	90	10
1	0	100
2	0	100
3	40	60
4.5	40	60

Table 4. Mass spectrometer parameters for the chosen cytostatic compounds.

Compound	T_R (min)	Precursor ion (m/z)	Cone voltage (ion mode)	Quantifier MRM, m/z (collision potential, V)	Qualifier MRM, m/z (collision potential, V)
5-FU	0.41	129.1	30V (ESI-)	58.19 (19)	85.92 (19)
VINC	0.41	825.8	40V (ESI+)	140.17 (55)	122.18 (60)
VINB	0.43	812.8	40V (ESI+)	224.07 (45)	124.39 (43)
MET	0.48	455.1	30V (ESI+)	308.14 (15)	175 (17)
GEM	0.52	264.2	35V (ESI+)	112.11 (20)	87.01 (20)
CP	1.29	261.2	30V (ESI+)	140.04 (20)	106.06 (18)
ETO	1.30	589.5	40V (ESI+)	229.21 (15)	185.26 (20)
TAM	1.39	372.3	40V (ESI+)	72.12 (20)	129.17 (20)

Table 5. Pearson's correlation of the variables studied.

Compound	Sediment				Sludge			
	Weight	Volume	Time	Temperature	Weight	Volume	Time	Temperature
VINC	-0.325	0.167	0.180	-0.067	-0.260	0.198	0.227	-0.107
VINB	-0.245	0.057	-0.042	0.038	-0.307	-0.038	0.086	0.020
MET	-0.082	-0.232	0.145	0.132	-0.333	0.248	-0.066	0.055
GEM	-0.474	0.187	0.055	0.103	-0.337	0.252	-0.040	0.055
CP	-0.523	0.173	-0.103	-0.029	-0.476	0.181	-0.093	-0.069
ETO	-0.075	-0.134	-0.100	-0.068	-0.208	-0.149	-0.033	0.155
TAM	-0.516	0.125	0.002	0.103	-0.461	0.297	-0.083	-0.082

Table 6. Analytical parameters of proposed method in sludge

		Recovery (%)	Matrix effect (%)	RSD Intraday (%)	RSD Interday (%)	LOD ($ng \cdot g^{-1}$)	LOQ ($ng \cdot g^{-1}$)
GEM	**0.25 $\mu g \cdot g^{-1}$**	122,2	87,86	6,43	12,5	0,58	1,92
	1 $\mu g \cdot g^{-1}$	101,1	91,32	11,3	12,2		
	2 $\mu g \cdot g^{-1}$	80,57	94,79	9,16	7,67		
CP	**0.25 $\mu g \cdot g^{-1}$**	75,16	69,10	10,1	13,2	1,20	4,00
	1 $\mu g \cdot g^{-1}$	101,4	66,95	2,95	7,87		
	2 $\mu g \cdot g^{-1}$	74,55	72,36	4,94	4,24		
ETO	**0.25 $\mu g \cdot g^{-1}$**	99,03	-15,72	8,83	12,6	79,8	266
	1 $\mu g \cdot g^{-1}$	97,81	26,06	13,2	9,5		
	2 $\mu g \cdot g^{-1}$	84,29	69,61	14,4	16,4		
TAM	**0.25 $\mu g \cdot g^{-1}$**	82,92	53,06	14,6	12,9	0,42	1,39
	1 $\mu g \cdot g^{-1}$	79,53	54,23	9,68	9,51		
	2 $\mu g \cdot g^{-1}$	64,91	53,40	8,66	7,05		

Table 7. Analytical parameters of proposed method in sediment

		Recovery (%)	Matrix effect (%)	RSD Intraday (%)	RSD Interday (%)	LOD ($ng \cdot g^{-1}$)	LOQ ($ng \cdot g^{-1}$)
GEM	**0.25 $\mu g \cdot g^{-1}$**	49,13	93,31	15,2	7,83	7,97	26,56
	1 $\mu g \cdot g^{-1}$	101,1	91,87	10,3	13,9		
	2 $\mu g \cdot g^{-1}$	59,75	93,77	8,20	11,7		
CP	**0.25 $\mu g \cdot g^{-1}$**	84,42	48,83	8,33	16,8	0,77	2,56
	1 $\mu g \cdot g^{-1}$	109,0	-0,89	14,6	13,0		
	2 $\mu g \cdot g^{-1}$	65,69	29,92	7,33	11,5		
ETO	**0.25 $\mu g \cdot g^{-1}$**	9,94	48,59	N.D.	N.D.	87,46	291,55
	1 $\mu g \cdot g^{-1}$	15,19	20,57	12,8	17,8		
	2 $\mu g \cdot g^{-1}$	22,96	43,79	12,0	16,1		
TAM	**0.25 $\mu g \cdot g^{-1}$**	88,29	11,46	5,04	9,09	0,10	0,32
	1 $\mu g \cdot g^{-1}$	104,84	1,64	10,9	10,9		
	2 $\mu g \cdot g^{-1}$	55,17	-2,95	6,83	9,79		

Table S1: Results of the 2^5 experimental design in sediment expressed as extraction efficiency (%)

Order	Weight (mg)	Solvent V (ml)	Time (min)	Temperature (°C)	Solvent	CP	ETO	TAM	GEM	MET	VINB	VINC
1	100	7	5	60	MeOH	71.44	74.89	89.74	8.68	0.00	12.08	20.94
2	250	7	5	60	MeOH	54.11	31.41	49.37	3.83	0.00	2.95	2.41
3	100	12	5	60	MeOH	169.79	0.00	127.35	12.15	0.00	0.00	0.00
4	250	12	5	60	MeOH	55.25	51.49	57.35	4.93	0.08	4.03	4.65
5	100	7	10	60	MeOH	77.86	53.10	95.23	9.67	0.52	8.75	5.57
6	250	7	10	60	MeOH	35.83	1.81	56.45	2.22	0.00	3.33	0.00
7	100	12	10	60	MeOH	110.19	0.00	140.65	15.18	0.00	13.68	70.16
8	250	12	10	60	MeOH	47.39	48.11	51.67	5.73	0.47	6.86	8.51
9	100	7	5	80	MeOH	91.24	43.85	96.67	11.14	0.64	6.65	5.41
10	250	7	5	80	MeOH	54.71	20.09	56.80	5.16	0.24	4.47	5.19
11	100	12	5	80	MeOH	118.32	48.11	132.57	14.03	0.22	27.98	19.97
12	250	12	5	80	MeOH	43.42	13.23	57.30	6.14	0.00	0.82	0.00
13	100	7	10	80	MeOH	97.04	45.72	100.20	14.41	0.48	6.28	18.72
14	250	7	10	80	MeOH	62.77	33.09	59.41	5.38	0.43	5.82	3.20
15	100	12	10	80	MeOH	70.90	28.96	102.52	16.90	0.00	2.30	27.15
16	250	12	10	80	MeOH	61.80	35.10	55.10	4.79	0.00	4.70	3.38
17	100	7	5	60	ACN	33.59	22.81	26.24	2.85	0.00	0.00	0.00
18	250	7	5	60	ACN	14.64	0.00	9.40	0.23	0.00	0.00	0.00
19	100	12	5	60	ACN	43.00	0.00	29.58	0.60	0.00	0.39	0.00
20	250	12	5	60	ACN	35.97	20.15	23.84	4.96	0.00	0.00	0.03
21	100	7	10	60	ACN	37.49	0.00	30.50	2.15	0.00	0.00	0.00
22	250	7	10	60	ACN	19.25	11.57	14.57	1.03	0.00	0.00	0.03
23	100	12	10	60	ACN	45.02	0.00	31.49	1.74	0.00	0.00	0.00
24	250	12	10	60	ACN	21.59	0.00	13.13	3.23	0.15	0.00	0.04
25	100	7	5	80	ACN	35.37	0.00	35.35	1.80	0.00	0.00	0.18

Order	Weight (mg)	Solvent V (ml)	Time (min)	Temperature (°C)	Solvent	CP	ETO	TAM	GEM	MET	VINB	VINC
26	250	7	5	80	ACN	12.66	0.00	11.22	0.80	0.00	0.02	0.00
27	100	12	5	80	ACN	68.45	0.59	49.07	4.70	0.00	0.00	0.00
28	250	12	5	80	ACN	10.97	0.00	11.43	0.83	0.00	0.08	0.00
29	100	7	10	80	ACN	42.20	0.00	45.13	2.43	0.00	0.00	0.00
30	250	7	10	80	ACN	20.86	0.00	13.90	1.06	0.00	0.01	0.02
31	100	12	10	80	ACN	32.24	0.00	39.64	4.33	0.00	0.00	0.00
32	250	12	10	80	ACN	17.48	0.00	16.17	0.81	0.00	0.00	0.00

Table S2: Results of the 2^5 experimental design in sludge expressed as extraction efficiency (%)

Order	Weight (mg)	Solvent V (mL)	Time (min)	Temperature (°C)	Solvent	CP	ETO	TAM	GEM	MET	VINB	VINC
1	100	7	5	60	MeOH	53.10	37.38	42.43	8.27	2.58	21.17	22.53
2	250	7	5	60	MeOH	34.02	54.92	23.14	4.44	1.75	4.76	7.68
3	100	12	5	60	MeOH	164.17	28.16	170.62	35.50	16.43	0.00	0.00
4	250	12	5	60	MeOH	33.08	33.75	25.51	3.81	1.50	5.81	9.82
5	100	7	10	60	MeOH	58.66	58.12	41.10	8.11	2.74	16.69	14.62
6	250	7	10	60	MeOH	25.49	7.78	34.59	3.99	1.21	4.66	6.37
7	100	12	10	60	MeOH	123.05	2.92	135.09	27.24	5.66	16.85	168.96
8	250	12	10	60	MeOH	28.14	28.83	19.13	4.20	2.54	6.79	5.71
9	100	7	5	80	MeOH	66.18	72.55	47.21	11.15	4.83	10.19	6.03
10	250	7	5	80	MeOH	29.17	35.88	20.35	4.54	2.05	2.71	4.71
11	100	12	5	80	MeOH	80.24	64.08	70.04	13.91	7.45	12.94	19.23
12	250	12	5	80	MeOH	38.11	3.56	52.28	16.08	5.20	10.53	0.00
13	100	7	10	80	MeOH	56.48	72.14	39.71	10.89	5.77	12.99	11.54
14	250	7	10	80	MeOH	33.71	41.55	23.31	4.39	1.92	6.49	7.71
15	100	12	10	80	MeOH	55.74	65.11	61.56	18.73	12.38	12.87	67.74
16	250	12	10	80	MeOH	25.76	28.13	20.02	2.88	1.54	4.87	9.72
17	100	7	5	60	ACN	25.58	4.42	28.29	1.38	0.00	0.00	0.00
18	250	7	5	60	ACN	8.25	0.00	11.98	0.29	0.00	0.00	0.00
19	100	12	5	60	ACN	22.13	0.00	35.75	0.00	0.00	0.46	0.00
20	250	12	5	60	ACN	17.82	0.00	17.20	1.13	0.00	1.79	0.00
21	100	7	10	60	ACN	20.51	0.00	21.03	2.28	0.00	0.00	0.00
22	250	7	10	60	ACN	10.84	0.00	11.34	0.79	0.00	0.17	0.40
23	100	12	10	60	ACN	19.43	0.00	26.21	0.00	0.00	0.00	0.00
24	250	12	10	60	ACN	14.70	6.17	14.77	1.28	0.06	1.64	1.18
25	100	7	5	80	ACN	28.59	0.00	27.09	4.36	0.00	0.00	0.00

Order	Weight (mg)	Solvent V (mL)	Time (min)	Temperature (°C)	Solvent	CP	ETO	TAM	GEM	MET	VINB	VINC
26	250	7	5	80	ACN	24.94	0.00	31.60	6.15	0.00	2.06	2.47
27	100	12	5	80	ACN	31.68	3.29	37.52	2.10	0.00	2.19	0.00
28	250	12	5	80	ACN	14.31	0.00	17.34	1.68	0.00	0.00	0.00
29	100	7	10	80	ACN	38.22	0.00	39.34	5.14	0.00	2.83	2.20
30	250	7	10	80	ACN	16.84	0.00	16.70	1.57	0.00	1.55	0.44
31	100	12	10	80	ACN	23.79	0.00	33.99	3.27	0.00	2.36	0.00
32	250	12	10	80	ACN	24.25	0.87	33.73	9.81	0.00	0.00	0.00

Table S3: Results of the 3^2 experimental design in sludge

Order	Weight (mg)	Solvent V (mL)	Time (min)	Temperature (°C)	Solvent	ETO	GEM	TAM	CP
1	50	11	5	60	MeOH	99.04	66.52	83.04	84.22
2	50	8	5	60	MeOH	82.93	49.76	90.48	74.45
3	100	14	5	60	MeOH	80.49	42.95	68.56	69.19
4	75	14	5	60	MeOH	118.33	59.51	118.27	95.49
5	50	14	5	60	MeOH	142.67	84.13	128.57	122.41
6	100	8	5	60	MeOH	62.75	28.56	50.04	54.05
7	100	11	5	60	MeOH	76.04	37.62	79.08	65.86
8	75	8	5	60	MeOH	70.04	34.47	77.02	70.64
9	75	11	5	60	MeOH	97.72	51.62	85.88	83.33

Table S4: Results of the 3^2 experimental design in sediment

Order	Weight (mg)	Solvent V (mL)	Time (min)	Temperature (°C)	Solvent	ETO	GEM	TAM	CP
1	50	11	5	60	MeOH	90.96	69.33	116.07	118.96
2	50	8	5	60	MeOH	97.36	53.28	109.60	108.32
3	100	14	5	60	MeOH	83.43	51.02	93.46	96.82
4	75	14	5	60	MeOH	111.30	63.46	111.48	117.22
5	50	14	5	60	MeOH	115.13	84.78	108.68	112.04
6	100	8	5	60	MeOH	63.65	28.16	72.48	102.29
7	100	11	5	60	MeOH	87.55	40.66	78.46	113.63
8	75	8	5	60	MeOH	70.14	38.97	103.83	101.06
9	75	11	5	60	MeOH	97.01	55.93	90.00	107.79

3.5. Extracción asistida por microondas para la determinación de compuestos antineoplásicos en peces de mar

La descarga de aguas depuradas tratadas se produce, en la mayoría de los casos, en ríos y/o mares. Estas aguas depuradas, pese a su tratamiento, son una fuente de nutrientes que atraen a los peces a alimentarse en las cercanías de los emisarios submarinos, las cuales, pese a cumplir con la normativa actual, contienen muchos contaminantes emergentes. En la bibliografía existente, se señala, que la eliminación de compuestos citostáticos resulta insuficiente. En trabajos previos, se han observado concentraciones de hasta 13.1 $\mu g \cdot L^{-1}$ de CP [51] y 58 $ng \cdot L^{-1}$ de TAM [50] en estaciones depuradoras de aguas residuales, los cuales podrían ser absorbidos por los peces que se alimentan en aguas cercanas al emisario y entrar en la cadena trófica.

La presencia de estos compuestos en el medioambiente es, de por sí preocupante, pero ésta se ve acrecentada si consideramos sus posibles efectos sobre los organismos acuáticos.

Con esta hipótesis de trabajo, se plantea la optimización de un método de extracción y determinación de estos compuestos citostáticos en peces que vivan y se alimentan en las proximidades de los emisarios submarinos.

Se comentó anteriormente que la bibliografía relacionada con la extracción de compuestos antineoplásicos en muestras sólidas es bastante escasa. Únicamente se ha realizado sobre lodos, compost y sedimentos y, principalmente, con ultrasonidos o líquidos presurizados. Las metodologías referentes al análisis de muestras biológicas presentan una problemática propia derivada del propio origen de la muestra. Los tejidos

biológicos poseen grandes cantidades de lípidos y proteínas, lo cual puede complicar enormemente el análisis de estas muestras.

En este trabajo, hemos optimizado una metodología de extracción asistida por microondas (MAE), que resulta ser rápida, consume poca cantidad de disolvente y requiere menos cantidad de muestra que otros métodos.

La optimización se realizó mediante un diseño experimental en el que se estudiaron las variables que afectan al proceso de extracción (tiempo, temperatura de extracción, volumen de disolvente y cantidad de muestra) obteniendo, en las condiciones óptimas, un procedimiento capaz de extraer, simultáneamente, 12 muestras, usando 50mg de muestra en cada vaso, 7mL de metanol y 5 minutos de extracción a 55°C. Posteriormente, los extractos pasaron por un cartucho de eliminación de fosfolípidos y proteínas, se secaron con nitrógeno y se reconstituyeron en 1mL de metanol. El análisis de los extractos se realizó mediante el sistema de UHPLC-MS/MS.

La optimización se realizó en muestras de boga (*Boops boops).* En condiciones óptimas, dos compuestos ampliamente utilizados en quimioterapia, CP y TAM, fueron extraídos con recuperaciones entre el 74 – 122%, alcanzado límites de detección de 1.3 y 0.8 $ng \cdot g^{-1}$, respectivamente. Los compuestos VINB y VINC fueron extraídos con recuperaciones entre el 53 – 112%, logrando un límite de detección de 46 y 536 $ng \cdot g^{-1}$, respectivamente.

Para la aplicación y validación del método de extracción, se capturaron peces situados en distintos niveles de la cadena trófica (*Sphoeroides marmoratus*, *Boops boops* y *Sphyraena viridensis*), de los cuales, los dos últimos son considerados como alimento en Canarias [92], en las proximidades de tres emisarios submarinos alrededor de la isla de Gran Canaria. Estos muestreos también se realizaron durante dos años cada tres meses.

El método fue aplicado en diferentes tejidos de peces, en músculo y vísceras. Este estudio de tejidos por separado buscaba evaluar la distribución de los compuestos estudiados en un mismo organismo y ver su posible acumulación. En los diferentes tejidos analizados no se detectó la presencia de compuestos antineoplásicos, por lo que para comprobar la aplicabilidad del método se contaminó músculo y vísceras de los otros dos peces capturados (*Sphoeroides marmoratus* y *Sphyraena viridensis*) con CP y TAM a una concentración de 0.5 $\mu g \cdot g^{-1}$. En todos los casos, la recuperación se mantuvo entre el 75 y el 124%, comprobando que el método optimizado es válido para distintas especies de peces y tejidos.

Hasta la fecha, solo se ha realizado este estudio referente a la acumulación de compuestos citostáticos en peces. Como se ha mencionado, *Boops boops* y *Sphyraena viridensis,* son considerados como alimento en Canarias, por lo que la población humana puede quedar expuesta a través de la alimentación a los compuestos citostáticos.

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Original Research Article

Microwave assisted extraction for the determination of antineoplastic compounds in marine fish

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ABSTRACT

Presence of antineoplastic compounds in the marine environment could cause adverse effects even at very low concentrations. The bulk of the produced literature at present has focused on the detection of these compounds in wastewater samples, while only a few papers tackled the more complex solid samples and until now, to the best of our knowledge, none has investigated marine organisms. This work presents the development, optimisation and application of an analytical method for the extraction and determination of the four antineoplastic compounds; cyclophosphamide, vincristine, vinblastine and tamoxifen, from fish tissues by microwave assisted extraction and ultra-high-performance liquid chromatography coupled to tandem mass spectrometry. Under optimal conditions, recoveries ranged between 60% and 120%, while intra- and inter-day precision were in the 5.0–19.4% and 12.0–20.4% ranges, respectively. Method detection and quantification limits were in the ranges 0.8–1.3 and 2.7-4.3 ng·g^{-1} dry weight for tamoxifen and cyclophosphamide, and 46.3–536 and 154-1787 ng·g^{-1} dry weight for vinblastine and vincristine, respectively. The developed analytical method was successfully applied for the extraction and determination of target compounds from muscle and liver tissues belonging to three different fish species. The results did not highlight any contamination by target antineoplastic molecules in the investigated fish tissues.

1. Introduction

Pollution of the marine ecosystem is a topic of foremost environmental concern, since a multitude of emerging pollutants continuously pours into the seas, potentially causing adverse effects on the marine ecosystem (Montesdeoca-Esponda et al., 2018). Marine biota run the risk of coming into contact with xenobiotic compounds, especially those organisms that live and/or feed in the vicinity of wastewater treatment plants (WWTPs) marine outfalls (Álvarez-Muñoz et al., 2015; Cunha et al., 2015; Emnet et al., 2015; Langford et al., 2015; Peng et al., 2015).

Although there are references to antineoplastic compounds in the literature, we are unaware of any studies that analyse their role on marine organisms. The main concerns are related to their mode of action in general and their non-specificity in particular, which allows them to damage both healthy and cancerous cells indiscriminately (Parrella et al., 2014). Their effects emphasize the importance of these substances owing to their continuous release and possible persistence into the environment (Isidori et al., 2016). These drugs, once metabolized and excreted by patients after treatment, are able to reach the inlet of WWTPs (Franquet-Griell et al., 2017b). Biological processes commonly implemented in WWTPs are not suitable for the removal of this kind of micropollutants. Accordingly, human metabolites and parent molecules can be found in WWTP effluents (Santana-Viera et al., 2016) and reach the marine environment, eventually causing damages to the marine biota, especially near WWTP marine outfalls (Luo et al., 2014).

A number of papers have reported on the presence of antineoplastic compounds in WWTP effluents (Gómez-Canela et al., 2014; Yin et al., 2010). Conversely, only a few studies have focused on WWTP sludge and river waters (Buerge et al., 2006; Ferrando-Climent et al., 2014; Martín et al., 2011; Metcalfe et al., 2003; Valcárcel et al., 2011; Zuccato et al., 2000), whereas no data are currently available on sea water.

The most recent studies concerning the environmental occurrence of antineoplastic compounds have highlighted the high level of detection of cyclophosphamide and tamoxifen, which represent two of the most widely employed anticancer molecules within the classes of alkylating agents and hormonal regulators, respectively. Isidori et al. conducting a widespread campaign across Spain and Slovenia, found both analytes in the effluents from activated sludge WWTPs of the two countries

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(7–17 ng L^{-1}) (Isidori et al., 2016). Moreover, for both compounds, they determined poor removal efficiencies, suggesting their scarce biological degradation.

Franquet-Griell et al. and Ferrando-Climent et al. also referred to the presence of the aforementioned analytes in two Spanish rivers: Besòs (Franquet-Griell et al., 2017a) and Ter (Ferrando-Climent et al., 2014). Their initial research suggested that the presence of both analytes was related to the role of WWTP effluents as sources of contamination, which spanned the 0.5–25 ng·L^{-1} and 5–13 ng·L^{-1} ranges, for tamoxifen and cyclophosphamide, respectively. The role of WWTP effluents as the sole source of cyclophosphamide release in surface water was also highlighted by Ferrando-Climent et al., who determined that the concentrations to be included ranged from 9 and 20 ng·L^{-1}. However, the same study also emphasized that the occurrence of tamoxifen (12–42 ng L^{-1}) was not only related to the punctual WWTP discharge, but also attributed to the consequence of diffuse contamination due to its in veterinary practices.

Among the other antineoplastic compounds covering a large portion (about 45% of the total) of the anticancer pharmaceutical market (Civjan, 2012) vinca alkaloids such as vinblastine and vincristine stand out as some of the oldest and most established compounds of proven cytotoxic activity. Their unique action mode has enabled them to be used, both individually and in combination regimes, for the treatment of a variety of cancers (leukaemia, testicular teratoma, lung, bladder and breast cancer being a few examples) (Mann, 2002; Moudi et al., 2013). Given the poor literature coverage of these analytes, there are only a few studies addressing their quantitation into the environment (Ferrando-Climent et al., 2013; Yin et al., 2010). The concentrations determined in water samples fall in the 20–50 ng L^{-1} range. A recent study (Negreira et al., 2016) highlighted their poor degradability during chlorination treatments. Potential foetal and embryonal toxicities have also been suggested for vinblastine and vincristine (Al-Ahmad and Kümmerer, 2001; Negreira et al., 2016). Sorption onto solids has already been reported in the literature (Kosjek and Heath, 2011; Kümmerer, 2008) for these compounds. Molecules of similar sorption tendencies have been shown to magnify their environmental concentrations up to three orders (from ng·L^{-1} to the ng·g^{-1}) when shifting from the seawater to the marine biota compartment (Emnet et al., 2015; Langford et al., 2015; Sang and Leung, 2016). Thus, potential for bioaccumulation and biomagnification through the trophic chain can be reasonably inferred (Montesdeoca-Esponda et al., 2018) since log K_{OW} values of these compounds stand approximately between 5 and 6, depending on the water pH (typically ranging from 7 to 8).

Studies of acute toxicity on different marine organisms show, in general, that the minimum concentrations needed for antineoplastic compounds to be toxic are in the mg·L^{-1} range (Białk-Bielińska et al., 2017; Brezovšek et al., 2014; Parrella et al., 2014). Although the actual concentrations to which marine organisms may be exposed through the effluents of the WWTPs are lower than those mentioned above, chronic exposure to low concentrations of a mixture of these analytes may cause adverse effects (Kosjek and Heath, 2011). For instance, Trombini et al. exposed mussels (*Mytilus galloprovincialis*) at concentrations of 100 ng·L^{-1} of cisplatin (Cis-Pt) through a 14 day period, observing DNA damage, neurotoxicity, oxidative stress in the digestive glands and changes in their antioxidant capacity.(Trombini et al., 2016) and the exposure to 120 ng·L^{-1} of tamoxifen and its 4-hydroxy derivative produced variation in size, reproduction and viability in *Daphnia pulex* (Borgatta et al., 2016).

The two major issues encountered in the determination of antineoplastic compounds are: I) matrix interferences and II) low concentration levels. Therefore it is necessary to apply methodologies that encompass both extraction and preconcentration processes to enhance the methods' sensitivity towards the targeted compounds, in order to obtain better chromatographic responses.

The bibliography related to the extraction of antineoplastic drugs from solid samples is, at present, very scarce. A handful of methods have been developed for their extraction in sediments, compost or sludge by using either ultrasound-assisted extraction (UAE) (Azuma et al., 2017; López Zavala and Reynoso-Cuevas, 2015; Ternes et al., 2005) or pressurized liquid extraction (PLE) (Okuda et al., 2009; Seira et al., 2013). Among them, only Azuma et al. were able to detect bicalutamide, doxifluridine, and tamoxifen at concentrations of 0.391 ng·g^{-1}, 0.392 ng·g^{-1} and 0.25 ng·g^{-1}, respectively, in river sediments using UAE (Azuma et al., 2017). Lopez-Zavala and Reynoso-Cuevas and Ternes et al. developed a method for the extraction of antineoplastic drugs among other compounds in compost (López Zavala and Reynoso-Cuevas, 2015) and sludge (Ternes et al., 2005). In both papers, the antineoplastic compounds were not detected in the samples but other compounds were determined. In sludge, Seira et al. detected ifosfamide and cyclophosphamide at concentrations of 11.4 – 42.5 ng·g^{-1} and 12.6 ng·g^{-1}, respectively, using PLE (Seira et al., 2013), while Okuda et al. were able to detect cyclophophamide, together with other non-antineoplastic pharmaceuticals (Okuda et al., 2009). However, no microwave-assisted extraction (MAE) method has been developed for the analysis of cytostatic compounds in solid matrixes. Moreover, no methods have at present been proposed for the determination of antineoplastic in marine biota. MAE is a technique that uses relatively low volumes of organic solvents as well as short extraction times, and has already been successfully applied to the extraction of hormones from fish tissue (Guedes-Alonso et al., 2017).

The objective of this work was to develop a MAE-based procedure for the analysis of cyclophosphamide (CP), vincristine (VINC), vinblastine (VINB) and tamoxifen (TAM) in various fish tissues, coupled to their determination by Ultra-High-Performance Liquid Chromatography tandem Mass Spectrometry (UHPLC-MS/MS).

The various parameters that affect the extraction method (i.e. extraction time, microwave power, extractant volume, sample weight), were evaluated using a statistical experimental design. The validated method was used to determine the above-mentioned pollutants in hepatic and muscular tissues belonging to three different fish species (*Boops Boops*, *Sphoeroides marmoratus* and *Sphyraena viridensis*) from growing trophic chain levels.

2. Experimental

2.1. Materials and reagents

Ultrapure water was obtained using a Milli-Q system (Milli-pore, Bedford, MA, USA). LC–MS grade methanol (MeOH), LC–MS grade water and formic acid used to adjust the pH of the mobile phases, as well as HPLC-grade MeOH were all obtained from Panreac Química (Barcelona, Spain). The CP and TAM antineoplastic compounds were purchased from Sigma-Aldrich (Madrid, Spain). VINB and VINC were purchased from Cymit-Química (Barcelona, Spain). All compounds had a purity grade above 97%. Stock solutions containing 1000 mg L^{-1} of each analyte were prepared by dissolving the compound in methanol, and the solutions were stored in glass-stoppered bottles at − 20 °C in the dark. Working standard solutions were prepared daily. The SPE cartridge Phree Phospholipid Removal for the cleaning step was purchased from Phenomenex España (Madrid, Spain).

2.2. Sample collection, preparation and extraction

The three selected fish species, *Sphoeroides marmoratus*, *Sphyraena viridensis* and *Boops boops*, were caught in the vicinity of the marine outfalls from WWTPs. All fish are lean fish with low fat content (González Pérez et al., 2004). The marine outfalls studied were at the following coordinates: 28° 6′ 36.263″ N, 15° 24′ 25.47″ W (in the northeast of Gran Canaria), 27° 49′ 19.43″ N, 15° 25′ 5.963″ W (in the southeast of Gran Canaria) and 27° 45′ 45.742″ N, 15° 31′ 29.017″ W (in the south of Gran Canaria).The samples remained frozen (-20 °C) until they were lyophilised. The tissue samples of each fish were

manually separated into muscle and liver prior to the lyophilisation procedure. Each part of each fish was then crushed and sieved to a particle diameter smaller than 0.3 mm and then stored into a refrigerator (4 °C) until analysis. The *boops boops* muscle used for the optimisation were spiked weekly with different concentrations of the analytes dissolved in methanol. The extraction was performed by MAE. At the optimised conditions, twelve vessels with 50 mg of sample and 7 mL of MeOH were placed inside the microwave. The temperature was set to increase from room temperature (25 °C) to 55 °C at a 5 °C per minute gradient, remaining at 55 °C for 5 min. In order to reduce the matrix effect, the supernatant was recovered (about 6 mL) and then passed through a phospholipid removal cartridge (Phree Phospholipid Removal cartridge from Phenomenex) previously washed with 1 mL of MeOH, using an SPE vacuum manifold. The cleaned-up extract underwent an evaporation step to dryness by means of a gentle nitrogen flow (about one hour) with the aim of achieving better detection limits. The extracts were finally reconstituted in 1 mL of MeOH and filtered at 0.2 µm before LC–MS/MS analysis.

2.3. Experimental design

A 2^4 experimental design was initially carried out, where extraction time, extraction temperature, solvent volume and sample weight were the variables, leading to 16 extractions. The variables with the strongest impact on the process were identified, and then we performed a 3^2 experimental design with 9 extractions. All extractions were performed in triplicate.

2.4. Equipment and chromatographic conditions

A Titan MPS microwave equipment with 16 vessels was purchased from PerkinElmer (Madrid, Spain). An ACQUITY UPLC system equipped with a triple quadrupole detector with an ESI interface, controlled by MassLinx Mass Spectrometry software, was used for instrumental determination. The chromatographic system consisted of a Binary Solvent Manager, a 2777 autosampler and a column manager, all from Waters Chromatography (Barcelona, Spain). The electrospray ionization was performed in positive mode for all compounds. The optimised detection parameter values were as follows: a cone voltage of 40 V, a capillary voltage of 3.5 kV, a source temperature of 120 °C and a desolvation temperature of 400 °C. Nitrogen was used as the desolvation gas at a flow of 1000 L·h^{-1} and argon was employed as the collision gas.

The analytical column adopted for the chromatographic separation was a Phenomenex Luna Omega Polar 50 × 2.1 mm, with a particle size of 1.6 µm (Phenomenex, Madrid, Spain) operated at room temperature. The mobile phase consisted in water with 0.1% of formic acid (A) and MeOH with 0.1% of formic acid (B) at a flow of 0.3 mL·min^{-1}. The total time of the chromatogram was set at 5 min in gradient mode, starting at 40% of A and increasing its percentage up to 90% during the first 0.5 min. Afterwards, A was set to decrease to 0% in 0.5 min, remaining stationary at 0% for another minute; then returning to initial conditions (40% A) in the next minute, remaining in these conditions for 2 min to equilibrate the flow. The injection volume was set at 10 µL. Table 1 shows the characteristics of the selected compounds and their retention time.

Multiple reaction monitoring parameters were optimised for each compound to carry out the quantitative analysis. The optimisation of the quantification and confirmation ions, as well as detection parameters was performed for each compound by direct infusion of 1 mg L^{-1} standard solutions in MeOH at a flow rate of 10 µL·min^{-1}. The mass spectrometer parameters for the determination of target analytes are shown in Table 2. Precursor ions were $[M+H]^+$ in the positive ion mode (ESI +) for all compounds, with the exceptions of CP, which was $[M]^+$ and VINB, which was $[M+2H]^{2+}$.

Table 1
Physicochemical properties[a] and retention time of the targeted antineoplastic compounds.

Compound	Molar Mass (g·mol^{-1})	pK_a	Log K_{OW}	t_R (min)
CP	261.09	2.84	0.73	1.29
VINC	824.96	11.10[b] - 7.90[c]	5.54	1.30
VINB	810.97	11.10[b] - 7.90[c]	5.74	1.31
TAM	371.51	8.69	4.36	1.40

[a] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02(© 1994–2018 ACD/Labs).
[b] Most acidic pKa.
[c] Most basic pKa.

Table 2
Mass spectrometer parameters for the determination of selected antineoplastic compounds.

Compound	Precursor ion (m/z)	Cone voltage (V)	Quantification ion, m/z (collision potential, V)	Confirmation ion, m/z (collision potential, V)	Response ratio
CP	261.2	30	140.04 (20)	106.06 (18)	0.40
VINC	825.8	75	122.40 (55)	156.00 (55)	0.60
VINB	812.8	75	224.07 (43)	124.39 (45)	0.20
TAM	372.3	40	72.12 (20)	129.17 (20)	0.02

3. Results and discussion

3.1. Optimisation of the microwave assisted extraction (MAE)

The experimental design was planned and executed using Minitab® software, version 17.1.0. First, a 2^4 factorial design was performed evaluating the effects of four independent variables at two levels: extraction time (5 and 10 min), extraction temperature (50 and 60 °C), solvent volume (7 and 11 mL of MeOH) and sample weight (100 and 500 mg dry weight). Only methanol was used as an extractant since previous studies (Santana-Viera et al., 2017) confirmed it to be the most suitable solvent.

Fish tissue was initially spiked with the proper amount of analyte to achieve a theoretical final concentration in the extracts of 250 µg·L^{-1}. All the analyses were done in triplicate (n = 3). Evaluation of the results was performed drawing Pareto charts for each of the compounds with the studied variables, enabling us to single out the variables that were most affected the extraction process (Fig. 1). The units of X-axis are the area of the peaks divided by the volume of solvent used in each test to normalize the results. The red line marks the statistical significance; the values on the right have statistical significance. We also analysed Pearson's correlation to determine which of the two levels studied favoured the extraction. Pearson's correlation values are shown in Table 3. In this figure, the four variables are represented in addition to their combination. The X-axis represents the effect of the variable on the extraction process.

The Pareto charts reveal that solvent volume and sample quantity was the combinationthat most affected the processes. On the other hand, the Pearson's correlation showed negative values for both of these variables, suggesting that lower solvent volumes and lower weights would positively affect the extraction's efficiency. The technical specifications of the equipment indicate that the minimum solvent volume allowed for each vessel could not exceed the 7 mL mark, so a value of 7 mL of MeOH was fixed for the subsequent extractions. Therefore, this restriction on the volume led us to consider a subsequent experimental design was would hone in on the amount of sample to be extracted. The sample weight influence was tested with 50, 100 and 150 mg aliquots. Time and temperature were the least affected by the extraction process, except for VINB. Low values derived from the

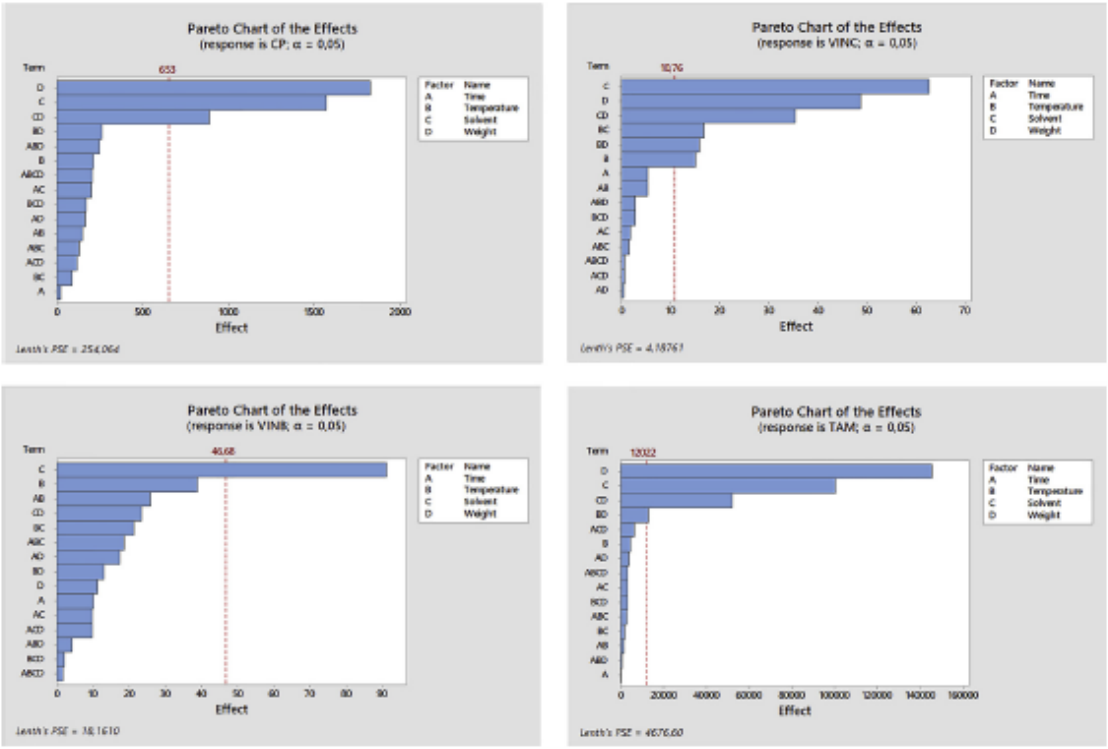

Fig. 1. Pareto charts of the different compounds with the different variables.

Table 3
Pearson's correlation of the studied compounds.

Variables	CP	VINC	VINB	TAM
Time	0.008	−0.059	0.090	−0.002
Temperature	−0.081	0.164	−0.344	−0.025
Solvent volume	−0.594	−0.684	−0.807	−0.542
Sample Weight	−0.693	−0.532	−0.098	−0.786

Pearson's correlations led to uncertainty on whether higher or lower values would improve extraction efficiency. A fixed extraction time of 5 min was therefore chosen with temperature taking on values of 50, 55 and 60 °C.

$A3^2$ experimental design was then carried out with sample quantity and temperature at three levels.

Contour plots for each compound are shown in Fig. 2. The region with dark green colour shows the area where the extraction is best for each compound. The figure shows that the optimal conditions for the extraction of each compound are not the same, so we tried to look for a satisfactory compromise. In general, low amounts of fish samples and medium temperatures seemed to be the best options for almost all of the compounds. As such we selected a lower domain of 50 mg aliquot of dried fish tissue and an extraction temperature of 55 °C.

Taking into account the obtained results, the final extraction conditions were selected as follows: 50 mg of sample, 7 mL of MeOH and 5 min of extraction at 55 °C. Since strong matrix effects were observed, a clean-up step aimed at the removal of phospholipids and proteins was carried out by SPE, eluting the extracts through a Phree Phospholipid Removal cartridge. We also sought to achieve better detection limits by an additional nitrogen flow desiccation and reconstitution step (in 1 mL of MeOH) was added, resulting in a LOQ and a LOD 7 times lower.

3.2. Analytical parameters

Different parameters were calculated to evaluate the precision and accuracy of the proposed method. The strength of the observed matrix effects necessitated a Matrix Matched Calibration (MMC) for quantification. MMC curves were drawn by spiking the extracts of blank fish tissue after extraction, evaporating it to dryness and reconstituting it. MMC was performed between 0.2 μg·g^{-1} and 5 μg·g^{-1} with determination coefficients ranging from 0.974 to 0.99. The analytical parameters were derived considering four concentration levels: 0.2 μg g^{-1}, 0.5 μg g^{-1}, 2 μg·g^{-1} and 5 μg·g^{-1}. The selected levels are similar to those used in works with antineoplastic compounds and solid biological samples, such as rat tissue (Bandu et al., 2015), mouse spleen (Sadagopan et al., 2001) and rat liver (Ju et al., 2015; Zhao et al., 2011).Recoveries were calculated as the ratio between the concentration that we measured of the spiked sample after extraction and the initial concentration of the compound in the spiked blank fish tissue. The matrix effect was calculated as the difference between the spiked solution of blank extract and a standard of methanol (Gosetti et al., 2010).

$$ME = 100 - \left(\frac{B}{A} * 100\right),$$

where B is the signal of the compound already extracted and A is the signal of compound directly injected in the mobile phase. According to this equation, we will have a positive matrix effect when a suppression of the signal is effectively produced, and a negative matrix effect when the interference of the matrix causes an increase of the signal. Intraday precision of the method was studied by performing 6 extractions at the same concentration on the same day (n = 6), while inter-day precision of the method was evaluated by performing extractions on 3 different days (n = 3). Limits of detection (LODs) and limits of quantification (LOQs) were calculated using the signal-to-noise ratio of each

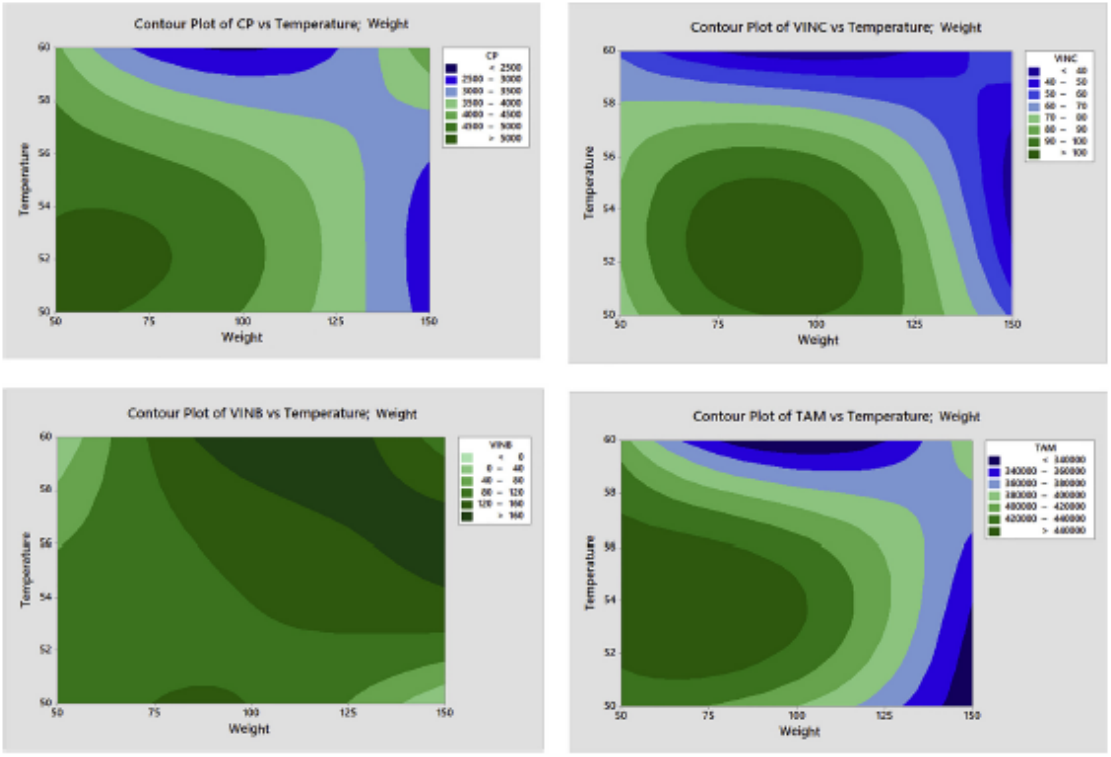

Fig. 2. Contour plots of the different antineoplastic compounds.

Table 4
Recovery, matrix effect, intraday and interday RSD, LOQs and LODs in muscle and liver of *Boops boops* (n = 3).

Muscle	Concentration ($\mu g\,g^{-1}$)	CP	VINC	VINB	TAM
Relative recovery (%)	**0.2**	106	nd	nd	74
	0.5	122	nd	nd	95
	2	109	53	65	86
	5	117	106	62	87
Matrix effect (%)	**0.2**	46	nd	nd	48
	0.5	35	nd	nd	35
	2	−28	−60	92	−34
	5	−34	−40	−116	−86
RSD Intraday (%)	**0.2**	14.4	nd	nd	5.0
	0.5	19.4	nd	nd	11.6
	2	10.7	4.0	14.0	12.9
	5	15.2	11.6	15.6	14.4
RSD Interday (%)	**0.2**	20.4	nd	nd	13.6
	0.5	19.8	nd	nd	16.8
	2	14.4	14.0	18.1	14.4
	5	15.7	12.0	16.5	14.5
LOD ($ng\,g^{-1}$)		1.3	536	46	0.8
LOQ ($ng\,g^{-1}$)		4.3	1787	154	2.7
Liver	**Concentration ($\mu g\,g^{-1}$)**	**CP**	**VINC**	**VINB**	**TAM**
Relative recovery (%)	**0.2**	94	nd	nd	116
	0.5	91	nd	80	111
	2	114	59	96	118
	5	82	113	60	101
Matrix effect (%)	**0.2**	59	nd	nd	83
	0.5	25	nd	−64	76
	2	31	−99	−11	65
	5	74	8	−48	11
LOD ($ng\,g^{-1}$)		1,3	189	99	0,5
LOQ ($ng\,g^{-1}$)		4,4	631	329	1,8

nd = not detected.

individual peak. LOD was defined as the minimum concentration giving a signal-to-noise ratio greater than 3, whereas LOQ was defined as the lowest concentration that gives a signal-to-noise ratio greater than 10. Results are shown in Table 4.

Analytical parameters were also studied in the liver tissue from the same fish to compare the differences between those two matrices. Results are shown in Table 4.

CP has a recovery between 106–122% with a matrix effect that decreases the signal at low concentrations, while producing a signal enhancement at higher concentrations. A recovery value could not be established for the VINC and VINB compounds at low concentrations since the signal was too low. Recovery range of VINC was between 53–106%, registering a matrix-induced signal enhancement at higher concentrations, while recovery ranges of VINB were between 62–65%, in spite of having a large signal suppression. TAM showed good recovery, over 70%. Residual standard deviation was less than 20% for all compounds. LODs and LOQs were between 0.8 – 536 $ng\,g^{-1}$ and 2.7 – 1787 $ng\,g^{-1}$ respectively.

The relative recovery in liver was similar to that obtained in the previous case. There were some variations on the matrix effects entity, which could be reasonably expected. It caused a greater signal suppression on the CP and TAM compounds; on the contrary, compounds such as VINC and VINB were positively influenced (signal enhancement) making it possible for VINB to be detectable at lower concentrations. LODs and LOQs obtained in liver are similar to those obtained in muscle.

3.3. Comparison with previously published methods addressing target antineoplastics in solid matrices

Table 5 illustrates the main characteristics of the analytical method herein proposed in comparison with those provided by previously published procedures using various sample preparation techniques for

Table 5
Main characteristics of the analytical method proposed in this study, in comparison with those provided by elsewhere published methods focusing on solid matrixes and including the same cytostatics herein addressed.

Matrix	Sample/Extractant (g/mL)	Extraction	Clean-up	Total Analysis Time (min)	Analytes	LOQs ($ng \cdot g^{-1}$)	Ref.
Fish tissue	1/20	MAE [a]	SPE-PLR [d]	80	CP TAM	4.3 2.7	This study
Sludge	1/1	USAE [b]	SPE-C18	≥150 [f]	CP	20	(Ternes et al., 2005)
Sludge	7/2	PLE [c]	SPE-SAX/SCX [e]	≥200 [f]	CP	6.1 – 12	(Seira et al., 2013)
Compost	1/10	USAE [b]	Ultra-freezing	≥40 [f]	CP	1.8	(López Zavala and Reynoso-Cuevas, 2015, p.)
Sediments	5/1	USAE [b]	no clean-up	≥100 [f]	CP TAM	0.011 0.010	(Azuma et al., 2017)
Breast tumour tissue	1/8	Centrifugation	SPE Bond-Elut C_2	≥ 35 [f]	TAM	60	(MacCallum et al., 1997)
Rat spleen	1/10	Homogenised	No clean-up	≥ 23 [f]	CP	1250	(Sadagopan et al., 2001)
Benthic invertebrates	1/160	Miniaturised QuEChERS	dSPE	≥ 15 [f]	CP TAM	30 161.6	(Berlioz-Barbier et al., 2014)

[a] Microwave assisted extraction; [b] Ultrasound assisted extraction; [c] Pressurized liquid extraction; [d] Phospholipid removal solid-phase extraction; [e] tandem strong cation and strong anion exchange solid-phase extraction; [f] total analysis time extrapolated by information reported in the original papers; [g] dispersive solid phase extraction.

the analysis of target antineoplastic compounds in different solid matrices. In this regard, it should be recalled that this study is the first one focusing on the development of an analytical procedure for antineoplastics in fish, whereas other papers in the literature deal with sludge, compost and sediments. The comparison is nevertheless relevant since compost, sediments and above all sludge are complex matrices containing a high percentage of organic matter, thus representing to some extent a similar matrix to the marine organisms investigated in this study. It should also to be noted that, to the best of our knowledge, for VINB and VINC no method development was attempted on solid matrices and therefore no comparison is possible.

Ternes et al proposed a method based on ultrasound assisted extraction, followed by SPE clean-up with C18 cartridges and LC-ESI-MS/MS instrumental analysis for the determination in biological sludge of a number of pharmaceuticals, including CP (Ternes et al., 2005). The method was quite time-consuming (about 150 min.) and the limit of quantification of CP was only $20\ ng \cdot g^{-1}$, notwithstanding the high weight-to-volume ratio adopted (1/1), probably due to the low sensitivity of the mass analyser. No information was provided regarding the matrix effect.

Seira et al. adopted PLE followed by a complex tandem SPE clean-up using strong anion and strong cation exchangers for the analysis of CP in various kinds of biological sludge (Seira et al., 2013). The tandem SPE procedure was employed to limit the extent of the matrix interferences, by way of selective analyte retention, even though it raised the analysis time over the 200-minute mark. Notwithstanding this, high matrix-induced ionisation suppressions were observed, probably also as a consequence of the remarkable weight-to-volume ratio (7/2) and the limit of quantification was at ppb level, ranging from 6.1 to $12\ ng \cdot g^{-1}$, depending on the sludge analysed. In this regard, it should also be noted that much higher suppressive effects and consequently much lower sensitivity was observed for strongly dewatered sludge (LOQ $= 128\ ng \cdot g^{-1}$), evidencing how a heightened matrix complexity represents a challenge in analytical chemistry.

The PLE approach for the analysis of various pharmaceuticals (including CP) in WWTP sludge was also proposed and adopted by Okuda et al. Unfortunately, no detailed information is available on both the analytical procedure and its figures of merit, including matrix effect and sludge-related limits of quantification (Okuda et al., 2009).

López Zavala et al. employed a rapid Ultrasonic Solvent Extraction (USE) procedure followed by ultra-freezing (−70 °C) and decantation steps for the recovery of CP, among other pharmaceuticals, from compost (López Zavala and Reynoso-Cuevas, 2015). The very low matrix interferences reported (i.e. limited to a ± 5% for all the encompassed analytes) could be ascribed to either the peculiar clean-up protocol or the less complex characteristics of compost, as a matrix subjected to an extensive biological digestion process. The limit of quantification of CP was $1.8\ ng \cdot g^{-1}$, being therefore fully comparable with the one herein obtained.

Low quantification limits were reported by Azuma et al. for a number of pharmaceuticals, including CP and TAM, in river sediments, using USAE as extraction technique, without any clean-up step (Azuma et al., 2017). The major factors influencing the high sensitivity is probably due to the high weight-to-volume ratio employed during the extraction procedure (5/1) and the supposed low complexity of the investigated matrix, since no mention to matrix effect evaluation was reported in the manuscript.

The TAM compound was extracted from breast tumour by crushing 50 mg of the tumour tissue and centrifuging it for 10 min at 3000 rpm to subsequently separate the supernatants by SPE. However, the matrix effect was not mentioned (MacCallum et al., 1997). Related to CP, Sadagopan et al. developed an extraction procedure from spleen of rats (Sadagopan et al., 2001). The tissue was homogenised and incubated during 20 min at 50 °C and then frozen at -70 °C. They claim to have obtained minimal interference despite the fact that no cleaning step had been taken.

In marine biota samples, Berlioz-Barbier et al. developed a procedure based on miniaturised QuEChERS for the extraction of several pharmaceutical compounds, including TAM and CP (Berlioz-Barbier et al., 2014). In this work, the authors obtained recoveries about 74–80% for the TAM and CP antineoplastic compounds and also studied the matrix effect as a key piece to validate a method and that must be quantified, obtaining a suppression of the signal between 25–50% for CP. The matrix effect was not estimated for the TAM compound. The authors use Dispersive Solid Phase Extraction to reduce the matrix effect; however, to deal with the matrix effect, they also opt for a MMC.

It can be seen above that an increase in the complexity and organic content of the sample (i.e. from sediment to biological samples) results in higher quantification limits.

3.4. Determination of antineoplastic compounds in fish tissues

To demonstrate the applicability of the method, liver and muscles of two other fish species (*Sphoeroides marmoratus*, *Sphyraena viridensis*) were spiked with the target analytes, after having verified the absence of antineoplastic compounds in the samples. To do this, 250 mg of each fish tissue was spiked at $500\ ng \cdot g^{-1}$ of the antineoplastic mixture. In Fig. 3, a chromatogram of spiked *Sphoeroides marmoratus* and *Sphyraena*

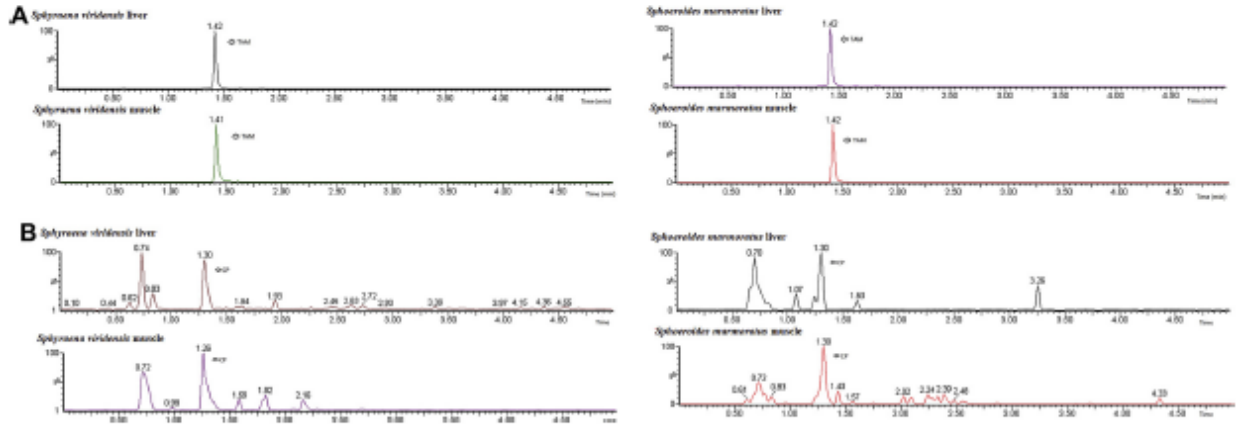

Fig. 3. a): Spiked *Sphoeroides marmoratus* and *Sphyraena viridensis* with 500 ng·g^{-1} of TAM. b): Spiked *Sphoeroides marmoratus* and *Sphyraena viridensis* with 500 ng·g^{-1} of CP.

Table 6
Extraction efficiency of TAM and CP at 0.5 µg·g^{-1} in *Sphoeroides marmoratus* and *Sphyraena viridensis* muscle and liver (n = 3).

Fish tissue		TAM	CP
***Sphoeroides marmoratus* muscle**	Recovery (%)	105	124
	Matrix effect (%)	−22	78
***Sphoeroides marmoratus* liver**	Recovery (%)	111	93
	Matrix effect (%)	−44	53
***Sphyraena viridensis* muscle**	Recovery (%)	75	109
	Matrix effect (%)	−83	87
***Sphyraena viridensis* liver**	Recovery (%)	79	93
	Matrix effect (%)	−104	38

viridensis liver and muscle is shown for TAM and CP at 500 ng·g^{-1}.

Relative recovery and matrix effect were studied in each tissue of each fish of the other two species to demonstrate the method's applicability. The results are shown in Table 6

The optimised procedure was applied to samples from three fish species caught in the vicinity of three WWTPs marine outfalls on the island of Gran Canaria during the month of October 2017. Muscular and hepatic tissues were studied. A total of eighteen samples were analysed, however no antineoplastic compounds were detected.

4. Conclusions

Antineoplastic compounds have been detected elsewhere in river and surface waters at concentrations close to the values that could cause adverse effects in marine environments (Ferrando-Climent et al., 2014; Franquet-Griell et al., 2017a). Accordingly, saltwater fishes are exposed to these organic micropollutants, thus representing an interesting, and yet unexplored, subject of investigation for our target analytes.

In this work, a method for the determination of the antineoplastics cyclophosphamide, vincristine, vinblastine and tamoxifen in various fish tissues, based on MAE followed by UHPLC-MS/MS has been developed, validated and applied. To the best of our knowledge, this is the first time that MAE has been used for the extraction of antineoplastic compounds from solid matrix and, specifically, the first attempt at their extraction and quantification in fishes. The main advantages brought forward by MAE are the low solvent volumes necessary (7 mL in this case) and the short extraction times (only 5 min). Extraction recoveries fall in the106–122% and 74–95% rangesfor cyclophosphamide and tamoxifen, respectively. The recoveries found for vincristine and vinblastine were lower: 53–112% and 62–65%, respectively. Intraday and inter-day precisions were studied, obtaining residual standard deviation below 20%. Finally, theoretical detection limits between 0.8 and 1.3 ng·g^{-1} were obtained for tamoxifen and cyclophosphamide, respectively; while 46.3 and 536 ng·g^{-1} were obtained for vinblastine and vincristine, respectively in muscle and 0.5 and 1.3 ng·g^{-1} were obtained for tamoxifen and cyclophosphamide, respectively; while 99 and 189 536 ng·g^{-1} were obtained for vinblastine and vincristine, respectively in liver.

The method herein proposed represents a noteworthy procedure in the field of analytical chemistry. In fact, the proposed procedure investigates for the first time the recovery of a wide-polarity group of antineoplastic compounds, from an extremely complex matrix, not yet investigated for target analytes, adopting a cheap, green and high throughput extraction procedure (12 samples at a time).

This paper offers the possibility of detecting at ng levels some of the most widely used antineoplastic drugs in marine biota, and thus is the beginning to further research inantineoplastic drugs' extraction and quantification from complex biological marine media.

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CAPÍTULO 4: CONCLUSIONES

De los estudios realizados en la presente Tesis Doctoral, e incluidos en esta Memoria, se pueden extraer las siguientes conclusiones:

A. Ha sido necesario el desarrollo de diversas metodologías de análisis de compuestos citostáticos basadas en diferentes técnicas extracción y determinación mediante cromatografía líquida de ultra resolución acoplada a espectrometría de masas (UHPLC-MS/MS) en muestras de diferente naturaleza (sólidas y líquidas).

B. Para la optimización y desarrollo de las metodologías de extracción y preconcentración para el análisis de muestras de agua se optó por el uso de técnicas de microextracción y por la extracción en fase sólida, concluyendo que:

I. La extracción en fase sólida ofrece una mejor preconcentración debido a que permite el uso de volúmenes de muestra superiores. Se obtuvo, en esta ocasión, límites de detección de entre 1 – 104 $ng \cdot L^{-1}$ para los compuestos estudiados en las diferentes matrices líquidas. Se obtuvo una buena recuperación y bajas desviaciones. Sin embargo, el efecto matriz afectaba considerablemente la determinación de los compuestos estudiados.

II. El uso de nuevas metodologías de extracción miniaturizadas permitió la extracción y preconcentración de cinco compuestos citostáticos de muestras de aguas residuales. La extracción es más rápida que con técnicas más convencionales como la SPE, ofreció una buena repetividad y reproducibilidad, recuperaciones relativas superiores al 40% y un efecto matriz menor al observado con SPE. Se obtuvieron límites de detección adecuados para el estudio de dichos compuestos en muestras de aguas residuales, sin embargo, el factor de preconcentración obtenido fue de 10, menor que el obtenido en SPE (250).

III. La extracción en fase sólida con cartuchos de intercambio iónico resultó ser la forma más adecuada para la extracción y preconcentración de compuestos citostáticos de platino (CPCs) que no se había podido realizar hasta la fecha. Se obtuvo recuperación relativa entre 47 – 90% en aguas residuales y una absorción de los CPCs en los cartuchos del 45%, encontrando como mayor inconveniente la retención de los compuestos en el cartucho.

C. Para la optimización y desarrollo de las metodologías de extracción y preconcentración para el análisis de muestras sólidas se optó por el uso de la extracción asistida por microondas, ya que:

I. Con esta técnica, hemos sido capaces de extraer cuatro compuestos antineoplásicos, simultáneamente, en lodos y sedimentos. Para la mayoría de los compuestos, en ambas matrices, el LOD se mantuvo por debajo de $ng \cdot g^{-1}$.

II. Se ha demostrado que es una técnica adecuada para la extracción de compuestos antineoplásicos en tejidos de peces. Los LODs variaron según los compuestos, pero se mantuvieron en el rango de $ng \cdot g^{-1}$, siendo válidos para el estudio de peces que pudieran estar contaminados. Además, el método demostró ser aplicable para distintos tipos de peces.

III. Se caracteriza por ser una técnica rápida (extracciones de 5 minutos), que ofrece buena reproducibilidad y repetitividad, así como buenas recuperaciones con una cantidad pequeña de muestra.

D. De la optimización y desarrollo de metodologías de separación y determinación:

I. La cromatografía líquida de ultra resolución (UHPLC) proporciona una adecuada separación de los diferentes compuestos citostáticos para su determinación. Sin embargo, debido a la polaridad de los mismos, se hace necesario el uso de columnas C_{18} modificadas que puedan retener compuestos muy

polares. Aun así, no se pudo retener los compuestos citostáticos basados en platino; para ellos sería necesario emplear otro tipo de columna como HILIC.

II. La espectrometría de masas de triple cuadrupolo (TQ-MS) ofreció una alta sensibilidad a los compuestos estudiados, que pudieron ser detectados a niveles de $ng \cdot L^{-1}$.

III. El uso de la técnica de plasma acoplado inductivamente con espectrometría de masas (ICP-MS) resultó ser la forma más adecuada para la determinación de compuestos citostáticos de platino. El platino tiene cinco isótopos y la abundancia relativa del mayor de ellos es del 33%, por lo que su determinación por MS/MS resulta complicada, ya que al seleccionar un valor m/z determinado perdemos bastante información.

E. De la aplicación de las metodologías:

I. Se han detectado por primera vez compuestos citostáticos en las aguas residuales de Canarias.

II. Las concentraciones fueron más altas en efluentes de hospitales donde se detectaron concentraciones de ETO de 375.8 hasta 2600 $ng \cdot L^{-1}$, de CP de 1218 $ng \cdot L^{-1}$, de VINC de 1851 $ng \cdot L^{-1}$ y por primera vez se detectó VINB en aguas residuales a una concentración de 1835 $ng \cdot L^{-1}$. Igualmente se detectaron concentraciones de entre 81.94 – 13913 $ng \cdot L^{-1}$ de CPCs en las aguas residuales hospitalarias.

III. Se estudiaron hasta cuatro EDARs diferentes dónde se detectó el compuesto ETO, siempre en la entrada, con concentraciones de entre 874.9 $ng \cdot L^{-1}$ hasta 5141 $ng \cdot L^{-1}$. La CP, por el contrario, únicamente se detecta en las salidas de la EDAR de la ciudad, y no en la entrada, a concentraciones entre 55.94 y 91.25 $ng \cdot L^{-1}$. De igual forma, la concentración de CPCs fue superior a la salida de la depuradora, con concentraciones entre 56,08 – 75,79 $ng \cdot L^{-1}$, que en la entrada, con concentraciones entre 38,68 – 3,97 $ng \cdot L^{-1}$. Pese a detectarse diferentes concentraciones de compuestos citostáticos en los efluentes de las EDARs, no se detectaron en aguas de mar tomadas en los emisarios submarinos probablemente debido al factor de dilución.

IV. En relación a muestras sólidas, la aplicación del método de extracción asistida por microondas a lodos procedentes de la EDAR de Las Palmas de Gran Canaria no dio resultados positivos. Tampoco se detectaron concentraciones de compuestos citostáticos en los sedimentos tomados en los emisarios submarinos ni en los peces capturados en las proximidades de los emisarios submarinos. Se trata de unos compuestos con una aplicación muy concreta por lo que su uso es menor al de otros contaminantes emergentes y probablemente, si estuviesen en las matrices sólidas estudiadas, estarían por debajo del límite de cuantificación.

CHAPTER 4: CONCLUSIONS

From the studies carried out in this Doctoral Thesis, and included in this Report, the following conclusions can be drawn:

A. It has been necessary to develop various methodologies for the analysis of cytostatic compounds based on different extraction and determination techniques using ultra-high performance liquid chromatography tandem mass spectrometry UHPLC-MS/MS in samples of different nature (solid and liquid).

B. For the optimization and development of extraction and preconcentration methodologies for the analysis of liquid samples we used solid phase extraction and one microextraction technique, concluding that:

   I. Solid phase extraction offered better preconcentration because it allowed the use of higher sample volumes. In this

work, limits of detection ranging from 1 – 104 $ng \cdot L^{-1}$ were obtained for the compounds studied in the different liquid matrix. A good recovery and low deviations were also obtained. However, the matrix effect affected significantly the determination of the compounds studied.

II. The use of new miniaturized extraction methodologies allowed the extraction and preconcentration of five cytostatic compounds from wastewater samples. The extraction was faster than with more conventional techniques such as SPE, it offered good repeatability and reproducibility, relative recoveries greater than 40% and a matrix effect lower than SPE. However, the preconcentration factor obtained was 10, lower than that obtained in SPE (250). Appropriate detection limits were obtained for the study of the already mentioned compounds in wastewater samples.

III. Solid phase extraction with ion exchange cartridges proved to be the most suitable way for the extraction and preconcentration of cytostatic platinum compounds (CPCs) that had not been possible to date. Relative recovery between 47-90% in wastewater and an absorption of the CPCs of 45% in cartridges was obtained, finding the retention of the compounds in the cartridge as a major drawback.

C. For the optimization and development of extraction and preconcentration methodologies for solid sample analysis, the use of microwave-assisted extraction was chosen, since:

I. With this technique, we have been able to extract four antineoplastic compounds, simultaneously, in sludge and sediment. For most of the compounds, in both matrices, the LOD remained below $ng \cdot g^{-1}$.

II. It has proven to be a suitable technique for the extraction of antineoplastic compounds in fish tissues. The LODs varied according to the compounds, but remained in the range of $ng \cdot g^{-1}$, being valid for the study of fish that could be contaminated. In addition, the method demonstrated to be applicable for different types of fishes.

III. It is characterized by being a rapid technique (5 minute extractions), which offers good reproducibility and repeatability, as well as good recoveries with a small amount of sample.

D. On the optimization and development of separation and determination methodologies:

I. Ultra-High Resolution Liquid Chromatography (UHPLC) provided adequate separation of the different cytostatic compounds for its determination. However, due to their polarity, it was necessary to use modified C_{18} columns that could retain very polar compounds. Even so, the platinum-based cytostatic compounds could not be retained; for them it would be necessary to use another type of column such as HILIC.

II. Triple quadrupole mass spectrometry (TQ-MS) offered high sensitivity to the compounds studied, which could be detected at levels of $ng \cdot L^{-1}$.

III. The use of inductively coupled plasma with mass spectrometry (ICP-MS) proved to be the most suitable form for the determination of cytostatic platinum compounds. Platinum has five isotopes and the relative abundance of the greatest of them is about 33%, so its determination by MS/MS was complicated, because selecting a given m/z value we may have lost a lot of information.

E. On the application of the methodologies:

I. Cytostatic compounds have been detected for the first time in the wastewater of the Canary Islands.

II. The concentrations were higher in hospital effluents where ETO concentrations of 375.8 to 2600 $ng \cdot L^{-1}$, CP of 1218 $ng \cdot L^{-1}$, VINC of 1851 $ng \cdot L^{-1}$ were detected and, for the first time, VINB was detected in wastewater at a concentration of 1835 $ng \cdot L^{-1}$. Likewise, concentrations between 81.94 - 13913 $ng \cdot L^{-1}$ of CPCs were determined in hospital wastewater.

III. Up to four different WWTPs were studied where the ETO compound was detected, always at the entrance, with concentrations ranging between 874.9 $ng \cdot L^{-1}$ to 5141 $ng \cdot L^{-1}$. The CP, on the other hand, was detected only at the effluent of the WWTP, but not at the entrance, at concentrations

between 55.94 and 91.25 $ng \cdot L^{-1}$. Similarly, the concentration of CPCs was higher in the effluent of the WWTP, with concentrations between 56.08 – 75.79 $ng \cdot L^{-1}$, than at the influent, with concentrations between 38.68 – 3.97 $ng \cdot L^{-1}$. Although different concentrations of cytostatic compounds were detected in the effluents of WWTPs, they were not detected in seawater taken in submarine outfalls, probably due to the dilution factor.

IV. In relation to solid samples, the application of the microwave assisted extraction (MAE) method to sludge from the WWTP of Las Palmas de Gran Canaria did not give positive results. Nor were concentrations of cytostatic compounds detected in the sediments and fishes taken near submarine outfalls. These are compounds with a very specific application so their use is lower than other emerging pollutants and probably, if they were present in the solid matrix studied, they would be below the limit of quantification.

ANEXOS

ANEXO I. Acrónimos

Abreviatura	Nombre
4-OH-TAM	4-hidroxi-tamoxifen
5-FU	5-Fluoracilo
AZA	Azacitidina
CAP	Capecitabina
CARBO	Carbozantinib
Car-Pt	Carboplatino
CE	Electroforesis capilar
CG	Cromatografía de gases
CHLO	Clorambucilo
Cis-Pt	Cisplatino
CL	Cromatografía líquida
CP	Ciclofosfamida
CPE	Extracción por punto de nube
CYT	Citarabina
DAD	Detector de matriz de diodo
DAU	Daunorobicina
dFdU	Difluorodeoxyuridina
DLLME	Microextracción por dispersión líquido-líquido
DOC	Docetaxel
DOX	Doxorubicina
EDAR	Estación depuradora de aguas residuales
EPI	Epirubicina
ERLO	Erlotinib
ESI	Ionización por Electrospray
ETO	Etoposido
FD	Detector de fluorescencia
FPSE	Extracción por Adsorción sobre Tejidos Químicamente Modificados
GEM	Gemcitabina
HILIC	Columnas de interacción hidrófila
ICP	Plasma acoplado inductivamente
IDA	Herramienta de adquisición de información
IF	Ifosfamida
ILC	Compuesto marcados isotópicamente
IMA	Imatinib
IRI	Irinotecan
LLE	Extracción Líquido-Líquido

LOD	Límite de detección
LOQ	Límite de cuantificación
MAE	Extracción Asistida por Microondas
MELP	Melfalán
MET	Metotrexato
MIT	Mitomycin
MMC	Matrix Match Calibration
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tándem
OES	Espectrometría de emisión óptica
OH-PAC	6(α)-hydroxypaclitaxel
OMS	Organización Mundial para la Salud
Oxa-Pt	Oxaliplatino
PAC	Paclitaxel
PLE	Extracción con líquidos Presurizados
PS-DVB	Poliestireno divinilbenceno
QIT	Trampa de iones cuadrupolo
Q-LIT	Trampa de iones cuadrupolo lineal
QuEChERS	Rápido, Fácil, Barato, Efectivo, Robusto y Seguro
SPE	Extracción en Fase Sólida
TAM	Tamoxifeno
TEG	Tegafur
ToF-MS	Espectrometría de Masas con Tiempo de Vuelo
TQ	Triple cuadrupolo
UHPLC	Cromatografía Líquida de Ultra Alta Resolución
USE	Extracción Asistida por Ultrasonidos
VINB	Vinblastina
VINC	Vincristina
VINO	Vinorelbina

ANEXO II. Índice de tablas y figuras

Índice de figuras:

Índice de tablas:

ANEXO III. Publicaciones de la tesis doctoral

1. **AUTORES:** S. Santana-Viera, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J. J. Santana-Rodríguez.

   TÍTULO: Cytostatic drugs in environmental samples. An update on the extraction and determination procedures.

   REVISTA: TRENDS IN ANALITYCAL CHEMISTRY

   VOLUMEN: 80

   PÁGINAS: 373 – 386

   FECHA: 2016

2. **AUTORES:** S. Santana-Viera, R. Guedes-Alonso, Z. Sosa Ferrera, J. J. Santana-Rodríguez, A. Kabir, K. G. Furton.

   TÍTULO: Optimization and application of Fabric Phase Sorptive Extraction coupled to Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry for the determination of cytostatic drug residues in environmental waters.

   REVISTA: JOURNAL OF CHROMATOGRAPHY A

   VOLUMEN: 1529

   PÁGINAS: 39 – 49

   FECHA: 2017

3. **AUTORES:** S. Santana-Viera, P. Hernández-Arencibia, Z. Sosa Ferrera, J. J. Santana-Rodríguez.

   TÍTULO: Simultaneous and systematic analysis of cytostatic drugs in wastewater samples by ultra-high performance liquid chromatography tandem mass spectrometry.

   REVISTA: JOURNAL OF CHROMATOGRAPHY B

   VOLUMEN: 1110 – 1111

   PÁGINAS: 124 – 132

   FECHA: 2019

4. **AUTORES:** S. Santana-Viera, L. Marzullo, M. E. Torres-Padrón, M. Del Bubba, Z. Sosa-Ferrera, J. J. Santana-Rodríguez.

   TÍTULO: Microwave assisted extraction for the determination of antineoplastic compounds in marine fish.

   REVISTA: JOURNAL OF FOOD COMPOSITION AND ANALYSIS

   VOLUMEN: 82

   PÁGINAS: 103241 – 103249

   FECHA: 2019

5. **AUTORES:** S. Santana-Viera, M. E. Torres-Padrón, Z. Sosa-Ferrera, J. J. Santana-Rodríguez.

   TÍTULO: Determination of cytostatic platinum compounds in wastewater by ICP-MS after ion exchange extraction

   REVISTA: JOURNAL OF ANALYTICAL ATOMIC SPECTROMETRY

   VOLUMEN: *Enviado*

   PÁGINAS:

   FECHA: 2020

6. **AUTORES:** S. Santana-Viera, J. Tuček, M. E. Torres-Padrón, Z. Sosa-Ferrera, J. J. Santana-Rodríguez, R. Halko.

   TÍTULO: Cytostatic compounds in sludge and sediment: extraction and determination by a combination of microwave assisted extraction and UHPLC-MS/MS

   REVISTA: ANALYTICAL AND BIOANALYTICAL CHEMISTRY

   VOLUMEN: *Enviado*

   PÁGINAS:

   FECHA: 2020

ANEXO IV. Contribuciones a congresos de la tesis doctoral

1. **AUTORES:** S. Santana-Viera, R. Guedes-Alonso, Z. Sosa-Ferrera, A. Kabir, K. G. Furton, J. J. Santana-Rodríguez

   TÍTULO: Comparison of different Fabric Sorptive Extraction Media applied to the extraction and pre-concentration of antineoplastic compounds in wastewater

   TIPO DE PARTICIPACIÓN: Póster

   CONGRESO: INTERNATIONAL CONFERENCE ON WATER: FROM POLLUTION TO PURIFICATION

   LUGAR DE CELEBRACIÓN: KOTTAYAM, KERALA (INDIA)

   FECHA: Diciembre de 2016

2. **AUTORES:** S. Santana-Viera, P. Hernández-Arencibia, R. Guedes-Alonso, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Application of a SPE multiresidue method for the determination of antineoplastic drugs in wastewater samples

   TIPO DE PARTICIPACIÓN: Póster

   CONGRESO: 23rd INTERNATIONAL SYMPOSIUM OF SEPARATION SCIENCE

   LUGAR DE CELEBRACIÓN: VIENNA (AUSTRIA)

   FECHA: Septiembre de 2017

3. **AUTORES:** S. Santana-Viera, R. Guedes-Alonso, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Optimization of an on-line SPE with UPLC-MS/MS method for the determination of antineoplastic compounds in wastewater

   TIPO DE PARTICIPACIÓN: Póster

   CONGRESO: 23rd INTERNATIONAL SYMPOSIUM OF SEPARATION SCIENCE

   LUGAR DE CELEBRACIÓN: VIENNA (AUSTRIA)

   FECHA: Septiembre de 2017

4. **AUTORES:** S. Santana-Viera, R. Guedes-Alonso, Z. Sosa-Ferrera, J. J. Santana-Rodríguez, A. Kabir, K. G. Furton.

TÍTULO: Application of a microextraction technique based on Fabric Phase Sorptive Extraction to the analysis of cytostatics drugs in wastewaters

TIPO DE PARTICIPACIÓN: Oral de 5 minutos.

CONGRESO: 2nd INTERNATIONAL CAPARICA CONFERENCE ON POLLUTANT TOXIC IONS AND MOLECULES

LUGAR DE CELEBRACIÓN: CAPARICA (PORTUGAL)

FECHA: Noviembre de 2017

5. **AUTORES:** S. Santana-Viera, P. Hernández-Arencibia, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Extraction and determination of antineoplastic compounds in waters from marine outfalls by Solid Phase Extraction and Ultra-High Performance Liquid Chromatography

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: INTERNATIONAL CONFERENCE ON WATER: FROM POLLUTION TO PURIFICATION (ICW2018)

   LUGAR DE CELEBRACIÓN: KOTTAYAM, KERALA (INDIA)

   FECHA: Diciembre de 2018

6. **AUTORES:** S. Santana-Viera, L. Marzullo, M. E. Torres-Padrón, M. Del Bubba, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Optimisation of a Microwave Assisted Extraction procedure combined with Liquid Chromatography tandem Mass Spectrometry for the determination of tamoxifen and cyclophosphamide in fish tissues

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: SETAC EUROPE 29th ANNUAL MEETING

   LUGAR DE CELEBRACIÓN: HELSINKI (FINLAND)

   FECHA: Mayo de 2019

COMPARISON OF DIFFERENT FABRIC PHASE SORPTIVE EXTRACTION MEDIA APPLIED TO THE EXTRACTION AND PRECONCENTRATION OF ANTINEOPLASTIC COMPOUNDS IN WASTEWATER

S. Santana-Viera, R. Guedes-Alonso, Z. Sosa-Ferrera, K. G. Furton, A. Kabir, J.J. Santana-Rodríguez.
Instituto Universitario de Estudios Ambientales y Recursos Naturales (i-UNAT), Universidad de Las Palmas de Gran Canaria, 35017, Las Palmas de Gran Canaria, Spain, e-mail: josejuan.santana@ulpgc.es

1 INTRODUCTION

Antineoplastic or cytostatic drugs are compounds used in chemotherapy and they are designed to interfering in the growth of cells making them potentially carcinogenic[1]. As many other pharmaceuticals compounds, they reach the environment through wastewaters because of an inefficient removal treatment[2]. The low concentration in which they are present in wastewaters becomes mandatory the use of extraction and preconcentration techniques which allows the measurement and determination of them[3]. In this work, we developed a microextraction process based on Fabric Phase Sorptive Extraction (FPSE)[4].

2 EXPERIMENTAL PROCEDURE

1°
- Eighteen FPSE media with different characteristics were tested
- The different FPSE were immersed in 10ml of water that contained the cytostatic drugs during 1h.
- The extraction efficiency: $\frac{\text{concentration in water after the extraction}}{\text{concentration in water before the extraction}} * 100$
- Determination technique: ultra-high performance liquid chromatography system (with a Phenomenex Luna Omega Polar 50 x 2.1mm, 1.6µm. column) coupled to a triple quadrupole mass spectrometer (UHPLC-MS/MS).

CYTOSTATIC COMPOUNDS	
COMPOUND	ABBREVIATION
Etoposide	ETO
Vinblastine	VINB
Gemitabine	GEM
Fluorouracil	FLU
Methotrexate	MET
Tamoxifen	TAM
Cyclophosphamide	CP
Vincristine	VINC

FPSE media tested	
CN-CW 20M	Chitosan
UCON TMOS	MTMOS-Super Polar
TMOS_CAP TRIOL	Super Polar
CN PEG 300	UCON
TMOS-CW 20M	PEG 300
Acetoxy-PEG 300	CAP-DMS-CAP
CW 20M	PTHF
Caprolactone Triol	PEG CW 20M
Silica	PDMDPS

3 RESULTS

2°
The best extraction efficiency were achieved by this 6 FPSE

CN-CW20M	PEG 300	Caprolactone Triol
UCON	CW 20M	CAP-DMS-CAP

Adsorption efficiency greater than 60% for almost half of the compounds and more than 20% for almost everyone

OPTIMIZATION OF THE EXPERIMENTAL CONDITIONS

3°
- Experimental Design for UCON and CW 20M
  - 2^4
    - Extraction time: 10min, 40min
    - Sample volume: 10ml, 20ml
    - pH: 3, 9
    - Desorption time: 5min, 10min

4°
- Experimental Design for UCON and CW 20M
  - 3^2
    - Extraction time: 20, 40 and 60 minutes
    - pH: 2, 6 and 10 (UCON)
    - pH: 6, 8 and 10 (CW 20M)

4 CONCLUSION

- To our knowledge this is the first attempt to apply the FPSE microextraction technique for extraction and preconcentration of antineoplastic compounds present in water samples.
- Due to the differences between their physicochemical structures and properties, two pHs were selected. Etoposide (ETO), Tamoxifen (TAM) and Cyclophosphamide (CP) were extracted at pH 8 whereas Vinblastine (VINB) and Vincristine (VINC) were extracted at pH 10.
- The best FPSE was "CW 20M"
- The optimal conditions for the extraction were: sample volume: 10 ml, extraction time: 60min, ionic strength: 0%; and for the desorption were: eluent volume: 1ml of MeOH, elution time: 5min.
- In the optimal conditions the PFSE CW 20M achieve recoveries between 75 – 37% and RSDs between 6 – 12%.

ACKNOWLEDGEMENT

This work was supported by funds provided by the Spanish Ministry of Economy and Competitiveness, Research Project CTM2015-66095-C2-1-R.
Sergio Santana-Viera would thank to Fundación Acuorum for his research grant

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3. S. Santana-Viera, S. Montesdeoca-Esponda, Z. Sosa-Ferrera and J. J. Santana-Rodríguez, *TrAC Trends Anal. Chem.* 80, 373–386 (2016).
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INTERNATIONAL CONFERENCE ON WATER: FROM POLLUTION TO PURIFICATION

Kottayam, Kerala (India), diciembre de 2016

APPLICATION OF A SPE MULTIRESIDUE METHOD FOR THE DETERMINATION OF ANTINEOPLASTIC DRUGS IN WASTEWATER SAMPLES

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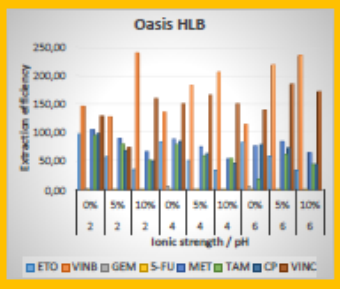
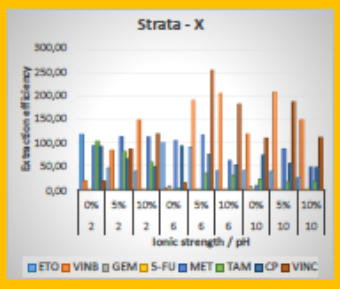
INTRODUCTION

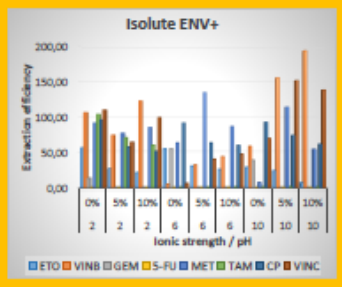
The physicochemical properties of the antineoplastic compounds, makes it very difficult to extract several of them at a time. For example, for the determination of cyclophosphamide (CP) and ifosfamide (IF), two nitrogen mustard analogues, the Strata-X cartridges were used successfully[1]. The Isolute ENV+ cartridges were used in the extraction and preconcentration of pyrimidine analogues such as fluorouracil (5-FU) and capecitabine (CAP)[2]. However, when determining a larger group of antineoplastics of different categories all cartridges should be tested to find the best of them. In this work, eight antineoplastic compounds: Etoposide (ETO), Vinblastine (VINB), Gemcitabine (GEM), 5-FU, Methotrexate (MET), Tamoxifen (TAM), CP and Vincristine (VINC) were studied under different extraction conditions with 4 different cartridges.

EXPERIMENTAL

Due to the fact that a large volume of sample would clog the cartridges when working with waste water, 250mL was decided for the experimental design. Then, a 3^2 experimental design, with two variables (pH and ionic strength) at three levels were carried out with all cartridges.

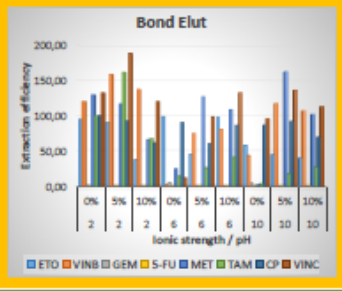




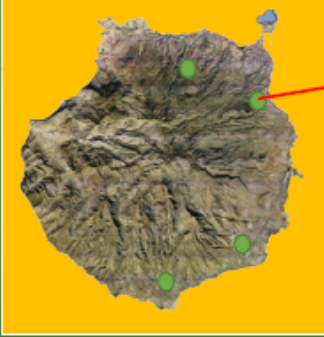


APPLICATION IN WASTEWATER WITH OASIS HLB

Optimum conditions			
Sample volume	250 mL	Elution solvent	Methanol
Sample pH	4	Elution volume	5 mL dried under a stream of nitrogen and reconstituted in 1 mL
Sample ionic strength	0%		



Wastewater samples from four WWTPs of the island of Gran Canaria was taken in the months of October, January and April.



CP was detected in the months of October (91,25ng/L and January (86,30ng/L).

CONCLUSIONS

- A method for the determination of antineoplastic compounds has been optimized and applied to real wastewater samples.
- In spite of the suppression of the signal due to the matrix, good LODs, between 1,34 ng/L and 77,88 ng/L (effluent) and between 0,84 ng/L and 103,95 (influent), were achieved.
- CP was detected in the effluents of the main wastewater treatment plant of the island in the months of October and January, at concentrations of 91.25 and 86.30 ng/L, respectively.

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[2] J. Martín, D. Camacho-Muñoz, J.L. Santos, I. Aparicio, E. Alonso, J. Sep. Sci. 34 (2011) 3166–3177

Acknowledgements
This work was supported by funds provided by the Spanish Ministry of Economy and Competitiveness [Research Project CTM2015-66095-C2-1-R]. Sergio Santana-Viera and Rayco Guedes-Alonso thanks the University of Las Palmas de Gran Canaria for his research grant.

23rd INTERNATIONAL SYMPOSIUM OF SEPARATION SCIENCE

Viena (Austria), septiembre de 2017

OPTIMIZATION OF AN ON-LINE SPE WITH UPLC-MS/MS METHOD FOR THE DETERMINATION OF ANTINEOPLASTIC COMPOUNDS IN WASTEWATER

S. Santana-Viera, R. Guedes-Alonso, Z. Sosa-Ferrera, J.J. Santana-Rodríguez.
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INTRODUCTION

In the last decade the number of works that have detected antineoplastic compounds in samples of environmental waters has increased[1]. Concern over these compounds has grown because the effects they can cause are still unknown, but could be very damaging and even more the mixture of them[2]. Concentrations in wastewater samples are in the range of ng L^{-1}, so it is necessary to use highly sensitive techniques such as UPLC-MS / MS and extraction and preconcentration techniques such as on-line SPE. In this work, all the parameters than affect the process have been optimised (sample volume, load wash solvents, sample loading solvent and pH), for the extraction and determination of Etoposide (ETO), Gemcitabine (GEM), Methotrexate (MET), Tamoxifen (TAM), Cyclophosphamide (CP), Vincristine (VINC) and vinblastine (VINB).

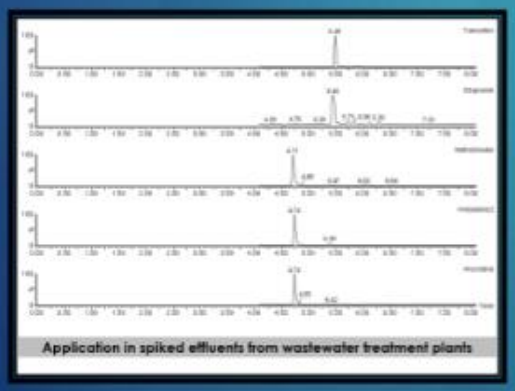
EXPERIMENTAL

Sample loading solvents: Three different loading solvents were tested at 5mL, 4mL, 3mL, 2mL and 1mL of volume of injection:
- Milli-Q water + 0,01% formic acid (v/v) (pH=3,11) (3mL of volume injection)
- Milli-Q water without additives (pH=5,78) (3mL of volume injection)
- Milli-Q water + 0,1% NH_3 (v/v) (pH=10,67) (5mL of volume injection)

After obtaining the best volume of injection for each phase of load they were compared to know what would be optimal

pH of the sample: Three different pH of the sample were tested (4, 7 and 10)

Sample wash step: Different proportions of organic solvent were tested to select the best composition.


Application in spiked effluents from wastewater treatment plants

OPTIMUM CONDITIONS AND SCHEME OF THE PROCESS

Injection of 3mL of the sample at pH=4

While the chromatographic separation, cartridge 1 is cleaned and cartridge 2 is conditioned with the load phase

Load phase (Milli-Q water + 0,01% F.A.) is loading into the SPE cartridge at 2mL/min

Analytes are eluted with the mobile phase (40% Water + 0,1% F.A./ 60% MeOH + 0,1% F.A.)

Sample loaded in the SPE is washed to eliminate interferences with 95% Milli-Q water / 5% MeOH

CONCLUSIONS

- All parameters affecting the process, such as loading phase, sample injection volume, sample pH and interference wash are optimized.
- The complete method, which involves extraction, preconcentration, separation and detection, takes place in 8.1 minutes.

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[2] A. Garcia-Ac, P.A. Segura, C. Gagnon, S. Sauvé, J. Environ. Monit., 2009, 11, 830–838.

ACKNOWLEDGEMENTS

This work was supported by funds provided by the Spanish Ministry of Economy and Competitiveness [Research Project CTM2015-66095-C2-1-R]. Sergio Santana-Viera and Rayco Guedes-Alonso thanks the University of Las Palmas de Gran Canaria for their research grant.

23rd INTERNATIONAL SYMPOSIUM OF SEPARATION SCIENCE

Viena (Austria), septiembre de 2017

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Química

Grupo de Análisis Químico MedioAmbiental

i-UNAT
INSTITUTO UNIVERSITARIO DE ESTUDIOS AMBIENTALES Y RECURSOS NATURALES

APPLICATION OF A MICROEXTRACTION TECHNIQUE BASED ON FABRIC PHASE SORPTIVE EXTRACTION TO THE ANALYSIS OF CYTOSTATICS DRUGS IN WASTEWATERS

S. Santana-Viera[1*], R. Guedes-Alonso[2], Z. Sosa-Ferrera[2], J.J. Santana-Rodríguez[2], A. Kabir[3], Kenneth G. Furton[3].

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2nd INTERNATIONAL CAPARICA CONFERENCE ON POLLUTANT TOXIC IONS AND MOLECULES

Caparica (Portugal), noviembre de 2017

EXTRACTION AND DETERMINATION OF ANTINEOPLASTIC COMPOUNDS IN WATERS FROM MARINE OUTFALLS BY SOLID PHASE EXTRACTION AND ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Sergio Santana-Viera, Patricia Hernández-Arencibia, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez.
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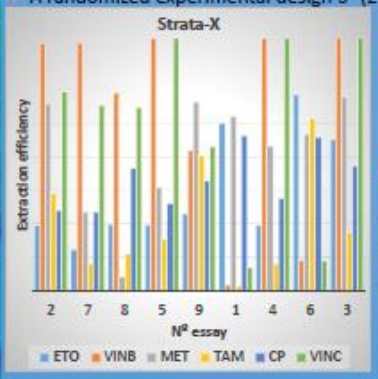
INTRODUCTION

- Concentrations in the $\mu g \cdot L^{-1}$ range of several of antineoplastic compounds have been detected in effluents from hospitals in the range of $\mu g \cdot L^{-1}$ and in influents and effluents from wastewater treatment plants in the range of $ng \cdot L^{-1}$ [1]. The fact that the antineoplastic compounds are detected in effluents means that the treatment plants are not efficient in the elimination of these compounds causing them to be detected in river and surface water [2,3].
- On the other hand, it has been shown that exposure to low concentrations (120 $ng \cdot L^{-1}$) of Daphnia pulex to tamoxifen causes adverse effects [4], which is a concentration no much higher than those found in some effluents, where the mixture of several antineoplastic compounds can be even worse than separately.
- For this reason it is necessary to optimise sufficiently sensitive techniques for the detection of said compounds in different water bodies.

EXPERIMENTAL

- Bond Elut, Isolute ENV+, Oasis HLB and Strata-X cartridges were tested.
- Six antineoplastic compounds were selected: Etoposide (ETO), Vinblastine (VINB), Methotrexate (MET), Tamoxifen (TAM), Cyclophosphamide (CP) and Vincristine (VINC)
- A sample volume of 250mL was selected, since a higher volume could clog the cartridges.
- A randomized experimental design 3^2 (2 variables at 3 levels) was carried out with the pH and ionic strength.



- Essay nº 2 was selected (pH = 2, ionic strength = 5%), since it offered good results for most compounds.
- Because the ionic strength of seawater is close to 5%, we continued working with the ionic strength of seawater.

ANALYTICAL PARAMETERS AT 0.5 $\mu g \cdot L^{-1}$

Compound	Recovery	LOD ($ng \cdot L^{-1}$)	LOQ ($ng \cdot L^{-1}$)	Matrix effect	RSD Intraday	RSD Interday
ETO	120,03	1,06	3,55	-6,42	10,34	13,33
VINB	90,65	3,66	12,20	49,83	12,34	15,52
MET	134,88	5,14	17,13	66,78	11,03	13,93
TAM	68,58	0,95	3,15	-75,68	9,27	15,44
CP	132,52	3,10	10,33	-85,17	10,63	1,92
VINC	102,93	1,96	6,54	73,96	14,68	10,93

CONCLUSIONS

- A sensitive method, based on SPE extraction and UHPLC-MS/MS quantification, was optimized and validated for the simultaneous determination of different antineoplastic compounds in seawater samples from marine outfalls.
- Absolute recoveries higher than 68% for all compounds were obtained and LODs ranged between 0.95 – 5.14 $ng \cdot L^{-1}$.
- Due to the matrix effect it is necessary to perform a matrix match calibration for quantification.
- Seawater samples were analysed from three marine outfalls taken at the bottom of the sea and the surface in several months; however, no antineoplastic compounds were detected.

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This work was supported by funds provided by the Spanish Ministry of Economy and Competitiveness [Research Project CTM2015-66095-C2-1-R]. Sergio Santana-Viera thanks the University of Las Palmas de Gran Canaria (Spain) for his Ph.D. student grant.

INTERNATIONAL CONFERENCE ON WATER: FROM POLLUTION TO PURIFICATION

Kottayam, Kerala (India), diciembre de 2018

OPTIMISATION OF A MICROWAVE ASSISTED EXTRACTION PROCEDURE COMBINED WITH LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF TAMOXIFEN AND CYCLOPHOSPHAMIDE IN FISH TISSUES

SETAC EUROPE 29th ANNUAL MEETING
26-30May 2019 | Helsinki, Finland
One Environment. One Health. Sustainable Societies.

Sergio Santana-Viera[1], Luca Marzullo[2], M.E. Torres-Padrón[1], Massimo del Bubba[2], Zoraida Sosa-Ferrera[1], José Juan Santana-Rodríguez[1]

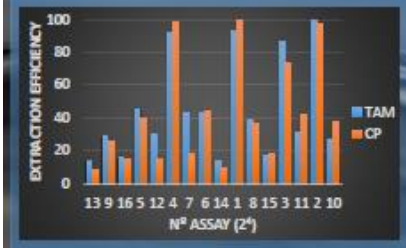
INTRODUCTION

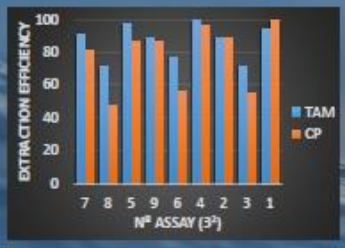
Human activities produce pollutants that come from wastewater treatment plants due to an ineffective treatment in the elimination of certain compounds. Antineoplastic compounds are some of them, which are drugs used as treatments against cancer and can cause important adverse effects if they reach the environmental waters [1]. They have been detected in wastewater and also river water, however, to date, no procedure has been optimized for the extraction of antineoplastic compounds from fish tissue [2].

EXPERIMENTAL

A microwave assisted extraction (MAE) followed my Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry (UHPLC-MS/MS) procedure was optimised with muscle from *Boops boops* .

First, an experimental design 2^4 was performed with 4 variables (temperature, time, solvent volume and sample weight) at 2 levels. Then a 3^2 experimental design was carried out with the variables temperature and sample weight at three levels. Finally, the optimal conditions were: 50 mg of fish; T=55ºC; time=5min; solvent volume=7mL of MeOH, clean-up step, dried and reconstituted in 1mL. Analytical parameters were performed at 4 concentrations levels: 0.2, 0.5, 2 and 5 $\mu g \cdot g^{-1}$.





	TAM	CP
Recovery (%)	74 – 95	106 – 122
Matrix effect (%)	-86 – 48	-34 – 46
RSD Intraday (%)	<14.4	<19.4
RSD Interday (%)	<16.8	<20.4
LOD ($ng \cdot g^{-1}$)	0.8	1.3
LOQ ($ng \cdot g^{-1}$)	2.7	4.8

APPLICATION

Different fish were analysed corresponding to different levels of the trophic chain caught in the vicinity of three submarine outfalls of the island of Gran Canaria. No antineoplastic compounds were detected. However, to prove the applicability of the method, muscle and liver of each of them were spiked at a concentration of 0.5 $\mu g \cdot g^{-1}$ obtaining the recoveries showed in the table:

Fish recovery (%)	Tissue	TAM	CP
Boops boops	Muscle	95	122
	Liver	111	91
Sphoeroides marmoratus	Muscle	105	124
	Liver	111	93
Sphyraena viridensis	Muscle	75	109
	Liver	79	93

CONCLUSIONS

- **A method for the determination of antineoplastic compounds from fish tissue has been developed, validated and applied for the first time.**
- **To our knowledge, this is the first time that MAE is applied for the extraction of antineoplastic compounds from solid matrix.**
- **In optimal conditions, we are able to extract 12 samples in 5 minutes, with good recoveries for different fishes and achieving ng levels of some of the most widely used antineoplastic drugs.**

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SETAC EUROPE 29th ANNUAL MEETING

Helsinki (Finlandia), mayo de 2019

ANEXO V. Otras publicaciones

1. **AUTORES:** R. Guedes-Alonso, S. Santana-Viera, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Molecularly imprinted solid-phase extraction coupled with ultra-high-performance liquid chromatography and fluorescence detection for determination of oestrogens and their metabolites in wastewater

   REVISTA: JOURNAL OF SEPARATION SCIENCE

   VOLUMEN: 38(22)

   PÁGINAS: 3961 – 3968

   FECHA: 2015

2. **AUTORES:** R. Guedes-Alonso, S. Santana-Viera, S. Montesdeoca-Esponda, C. Afonso-Olivares, Z. Sosa-Ferrera, J. J. Santana-Rodríguez.

   TÍTULO: Application of microwave-assisted extraction and ultra-high performance liquid chromatography–tandem mass spectrometry for the analysis of sex hormones and corticosteroids in sewage sludge samples

   REVISTA: ANALYTICAL AND BIONANALYTICAL CHEMISTRY (ABC)

   VOLUMEN: 408

   PÁGINAS: 6833 – 6844

   FECHA: 2016

3. **AUTORES:** S. Montesdeoca-Esponda, R. Guedes-Alonso, S. Santana-Viera, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Applications of fabric phase sorptive extraction to the determination of micropollutants in liquid samples

   REVISTA: SEPARATIONS

   VOLUMEN: 5 (3)

   PÁGINAS: 35 – 47

   FECHA: 2018

4. **AUTORES:** S. Santana-Viera S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Analytical methodologies for the determination of cytostatic compounds in environmental matrices

   LIBRO: FATE AND EFFECTS OF ANTICANCER DRUGS IN THE ENVIRONMENT

   EDITORIAL: SPRINGER

   PÁGINAS: 169 – 195

   FECHA: 2020

ANEXO VI. Otras comunicaciones presentadas en congresos

1. **AUTORES:** S. Santana-Viera, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: An approach to microextraction techniques applied to the determination of emerging contaminants in environmental liquid samples

   TIPO DE PARTICIPACIÓN: Oral

   CONGRESO: INTERNATIONAL MEETING OF ENVIRONMENTAL AND PHARMACEUTICAL ANALYSIS (IMEPA 2014)

   LUGAR DE CELEBRACIÓN: LAS PALMAS (ESPAÑA)

   FECHA: Diciembre de 2014

2. **AUTORES:** R. Guedes-Alonso, S. Santana-Viera, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J. J. Santana- Rodríguez

   TÍTULO: Optimization of molecularly imprinted solid phase extraction (MISPE) coupled with UHPLC-FD for the determination of estrogens in wastewaters

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: SETAC EUROPE 24TH ANNUAL MEETING

   LUGAR DE CELEBRACIÓN: LAS PALMAS (ESPAÑA)

   FECHA: Mayo de 2015

3. **AUTORES:** S. Santana-Viera, R. Guedes-Alonso, C. Afonso-Olivares, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Optimization of microwave assisted extraction combined with ultra-high performance liquid chromatography with fluorescence detection for the determination of estrogens in wastewater sludge samples

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: 42ND INTERNATIONAL SYMPOSIUM ON HIGH PERFORMANCE LIQUID PHASE SEPARATIONS AND RELATED TECHNIQUES (HPLC 2015)

   LUGAR DE CELEBRACIÓN: GINEBRA (SUIZA)

   FECHA: Junio de 2015

4. **AUTORES:** S. Santana-Viera, R. Guedes-Alonso, C. Afonso-Olivares, S. Montesdeoca-Esponda, M. E. Torres-Padrón, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Analysis of organic pollutants in micro-plastics

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: MICRO 2016: FATE AND IMPACT OF MICROPLASTICS IN MARINE ECOSYSTEMS: FROM THE COASTLINE TO THE OPEN SEA

   LUGAR DE CELEBRACIÓN: LANZAROTE (ESPAÑA)

   FECHA: Mayo de 2016

5. **AUTORES:** R. Guedes-Alonso, S. Santana-Viera, C. Afonso-Olivares, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Analysis of hormones in sludge samples using microwave-assisted extraction and ultra-high performance liquid chromatography tandem mass spectrometry

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: 31ST INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY

   LUGAR DE CELEBRACIÓN: UNIVERSITY COLLEGE CORK (IRELAND)

   FECHA: Agosto de 2016

6. **AUTORES:** R. Guedes-Alonso, S. Santana-Viera, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Optimization of a microwave-assisted extraction method coupled to ultra-high-performance liquid chromatography tandem mass spectrometry for the determination of steroid hormones in fish

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: 2nd INTERNATIONAL CAPARICA CONFERENCE ON POLLUTANT TOXIC IONS AND MOLECULES

   LUGAR DE CELEBRACIÓN: CAPARICA (PORTUGAL)

   FECHA: Noviembre 2017

7. **AUTORES:** P. Škvára, S. Santana-Viera, S. Montesdeoca-Esponda, J. J. Santana-Rodríguez, A. Vojs-Staňová

   TÍTULO: SPE UHPLC-ESI-MS/MS method development for 5-fluorocytosine, 5-fluorouracil and 5-fluorouridine analysis in complex environmental samples

   TIPO DE PARTICIPACIÓN: Oral

   CONGRESO: 24th INTERNATIONAL SYMPOSIUM ON SEPARATION SCIENCES

   LUGAR DE CELEBRACIÓN: JASNÁ (ESLOVAQUIA)

   FECHA: Junio 2018

8. **AUTORES:** S. Montesdeoca-Esponda, S. Santana-Viera, M. Guerra-Santana, J. Rodríguez-Pulido, P. Garcia-Jiménez

   TÍTULO: Desarrollo de la metodología del aula invertida y del aprendizaje basado en proyectos en las prácticas de laboratorio del grado en ciencias del mar

   TIPO DE PARTICIPACIÓN: Oral

   CONGRESO: InnoEducaTIC

   LUGAR DE CELEBRACIÓN: LAS PALMAS DE GRAN CANARIA (ESPAÑA)

   FECHA: Noviembre 2018

9. **AUTORES:** S. Montesdeoca-Esponda, R. Guedes-Alonso, S. Santana-Viera, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

   TÍTULO: Extraction and determination of personal care products adsorbed on microplastics

   TIPO DE PARTICIPACIÓN: Poster

   CONGRESO: SETAC EUROPE 29th ANNUAL MEETING

   LUGAR DE CELEBRACIÓN: HELSINKI (FINLANDIA)

   FECHA: Mayo 2019

10. AUTORES: S. Montesdeoca-Esponda, S. Santana-Viera, M. Guerra-Santana, J. Rodríguez-Pulido, P. Garcia-Jiménez

TÍTULO: Análisis de una propuesta sustentada por el aula invertida y el aprendizaje basado en proyectos

TIPO DE PARTICIPACIÓN: Oral

CONGRESO: InnoEducaTIC

LUGAR DE CELEBRACIÓN: LAS PALMAS DE GRAN CANARIA (ESPAÑA)

FECHA: Noviembre 2019

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Las Palmas de Gran Canaria
Abril 2020