MEJORA DE LAS TÉCNICAS DE MICROEXTRACCIÓN MEDIANTE EL DISEÑO DE NUEVAS MODALIDADES ASISTIDAS POR CO_2

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Trabajo presentado para aspirar al Grado de Doctor en Ciencias

EL DOCTORANDO

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TITULO: *Mejora de las técnicas de microextracción mediante el diseño de nuevas modalidades asistidas por CO2*

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CERTIFICAN: Que la Tesis Doctoral "**MEJORA DE LAS TÉCNICAS DE MICROEXTRACCIÓN MEDIANTE EL DISEÑO DE NUEVAS MODALIDADES ASISTIDAS POR CO_2**" ha sido desarrollada en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba, y que, a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajo.

Que Guillermo Lasarte Aragonés es el primer autor de todos los trabajos científicos presentados en esta Tesis Doctoral. De acuerdo con la normativa de esta Universidad y los acuerdos internos del Grupo de Investigación, el primer autor es responsable de la realización del trabajo experimental y de la producción del manuscrito. Además, ha participado activamente en las reuniones periódicas con los supervisores para evaluar y discutir los resultados obtenidos durante la Tesis Doctoral.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en la ciudad de Córdoba, a 30 de Septiembre de 2014.

Mª Soledad Cárdenas Aranzana **Rafael Lucena Rodríguez**

TÍTULO DE LA TESIS: Mejora de las técnicas de microextracción mediante el diseño de nuevas modalidades asistidas por CO_2

DOCTORANDO/A: Guillermo Lasarte Aragonés

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El licenciado Guillermo Lasarte Aragonés cursó con distinción la Licenciatura de Bioquímica y brillantemente los estudios del máster en Biotecnología Molecular, Celular y Genética. En el año 2010 accedió a una beca de formación de profesorado universitario para la realización de la Tesis Doctoral, cuya Memoria se presenta ahora para su defensa.

La temática de la misma ha sido el desarrollo de herramientas de microextracción basadas en el empleo de CO_2 como sustituto de aparatos o disolventes. Las metodologías propuestas comprenden tanto la microextracción en fase sólida como líquida, empleando tanto medios de extracción convencionales como nanoestructurados y nuevos disolventes.

El trabajo experimental realizado se ha materializado en cinco artículos científicos, publicados o enviados para su publicación a revistas especializadas del área. Se han realizado además dos artículos de revisión y un capítulo de libro. El doctorando ha asistido a 7 congresos nacionales e internacionales presentando un total de 8 comunicaciones en formato flash o cartel. A lo largo de estos años, ha adquirido formación en técnicas de microextracción, cromatografía de líquidos y de gases, tratamiento de muestras de agua para la determinación de distintas familias de compuestos. Además de las competencias técnicas inherentes al trabajo en el laboratorio, ha demostrado iniciativa, capacidad para la resolución de problemas, facilidad para el trabajo en equipo, capacidad para la planificación y dirección del trabajo a investigadores noveles así como para la innovación en la investigación.

Finalmente, ha colaborado en la impartición de clases prácticas y supervisión de dos trabajos fin de máster, apareciendo como firmante en los correspondientes artículos científicos, uno de ellos ya publicado. De esta forma ha adquirido las competencias docentes asociadas a la finalidad de la beca disfrutada.

Por todo ello la tesis doctoral reúne a nuestro juicio los requisitos de calidad y novedad exigibles a este tipo de trabajo, por lo que se autoriza la presentación de la misma.

Córdoba, 29 de septiembre de 2014

Firma del/de los director/es

Fdo.: Rafael Lucena Rodríguez

Fdo.: Mª Soledad Cárdenas Aranzana

Agradezco al Ministerio de Ciencia e Innovación la beca de formación de profesorado universitario (FPU) que ha hecho posible la realización de esta Tesis Doctoral.

Objeto

Las operaciones previas de tratamiento de muestra, dentro del conjunto del proceso de medida químico, representan una etapa crítica del mismo con una influencia significativa en la calidad de la información (bio)química suministrada. Su papel es esencial en la mejora de propiedades analíticas relevantes, como la selectividad y la sensibilidad, gracias al aislamiento y preconcentración de los analitos de interés.

Las técnicas de extracción han evolucionado en el contexto de la Química Analítica conforme a tres tendencias fundamentales: automatización, simplificación y miniaturización. En este sentido, el tratamiento de muestra ideal debe ser simple, rápido, con un coste reducido y seguro para el medio y el operador. A partir de estas líneas generales, surgen las técnicas de microextracción en fase sólida o líquida, cuyo objetivo principal es proporcionar información (bio)analítica de calidad cumpliendo con los requisitos citados.

Las técnicas de microextracción en fase sólida han estimulado el desarrollo de nuevos materiales con propiedades mejoradas. Así, se pueden encontrar materiales poliméricos de estructura variable con una gran superficie especifica de interacción, polímeros de reconocimiento molecular con una elevada selectividad por los analitos de interés o materiales nanoestructurados con excepcionales capacidades de interacción y superficie específica. En el campo de la microextracción en fase líquida, han surgido nuevos disolventes como los líquidos iónicos, con excelentes propiedades físico-químicas o los recientemente desarrollados disolventes conmutables, permitiendo reducir los volúmenes empleados y minimizar los riesgos para el medio y el operador.

De entre las modalidades de microextracción, tanto en fase líquida como sólida, una de las más respetuosas es la microextracción dispersiva. Esta modalidad permite establecer un íntimo contacto entre las fases donadora (muestra) y aceptora

(extractante) mediante la formación de dispersiones (en el caso de los líquidos) o suspensiones (en el caso de los sólidos) de finas gotas o partículas maximizando la superficie de contacto. Considerando esto, el objetivo principal de la Tesis Doctoral es el desarrollo de modalidades de microextracción dispersivas, tanto en fase sólida como en fase líquida, mediante el empleo de dióxido de carbono como agente dispersante o como mediador de la solubilidad de nuevos disolventes conmutables.

Específicamente, los objetivos de la Tesis Doctoral se pueden sintetizar en los puntos siguientes:

- Desarrollar nuevas modalidades de microextracción sólida dispersiva empleando la generación *in situ* de dióxido de carbono como agente dispersante mediante una reacción efervescente. En este contexto, se evaluará la versatilidad de la dispersión por efervescencia para sólidos poliméricos y materiales nanoestructurados.
- Evaluar el potencial de la efervescencia como agente dispersante de microvolúmenes de disolventes orgánicos en modalidades dispersivas en fase líquida y su co-dispersión con materiales magnéticos nanoestructurados para facilitar la separación y recuperación de la fase extractante.
- Abordar el potencial de disolventes conmutables en el campo de la microextracción en fase líquida empleando dióxido de carbono como mediador de la hidrofilicidad/hidrofobicidad de la fase extractante, en el rango de los microlitros.
- Minimizar el consumo de disolventes orgánicos en los procedimientos de extracción y la dependencia de aparatos para generar dispersiones o recuperar la fase extractante para el análisis de muestras ambientales.

Contextualización de las técnicas de microextracción

1. Generalidades

La química analítica es la rama de la ciencia encargada de proporcionar información relativa a la composición de los sistemas o muestras de interés mediante el denominado proceso de medida químico (PMQ). El PMQ se divide en tres etapas bien diferenciadas: (i) el conjunto de operaciones previas, que engloba la toma y el tratamiento de la muestra; (ii) la medida y transducción de la señal analítica; y (iii) la toma y análisis de los datos derivados. Si bien las etapas generales de este proceso están bien delimitadas y definidas, la naturaleza específica de cada una de ellas depende del problema analítico objeto de estudio y del tipo de información requerida. Atendiendo al problema analítico, se debe tener en consideración la naturaleza de la muestra (complejidad, estabilidad, estado de agregación y disponibilidad) y de los analitos de interés (características físico-químicas, abundancia y número total). En relación a la información, se tiene que definir el tipo de información deseada (estructural, cualitativa y/o cuantitativa) y la calidad de ésta (en términos de exactitud, coste y rapidez) [1]. Dada la cantidad y tipo de variables que definen el PMQ, no existe una estrategia única, se trata de una metodología flexible y adaptable al problema analítico en cuestión.

El tratamiento de la muestra tiene una incidencia decisiva en las propiedades analíticas. Influye en la exactitud (errores sistemáticos cometidos durante el proceso), precisión (errores aleatorios) y en las propiedades complementarias (rapidez, costes y factores humanos). También afecta a la selectividad (a través de la eliminación de interferentes) y sensibilidad (gracias a la preconcentración que se consigue de los analitos de interés) del PMQ [2].

A pesar de la constante innovación y sofisticación de la instrumentación disponible, la gran mayoría de los instrumentos son incompatibles con el análisis directo de las muestras. Esto se debe principalmente a dos factores: (i) la complejidad de la matriz de la muestra (habitualmente con un elevado número de interferentes

potenciales) y (ii) la baja concentración de la mayoría de analitos de interés. En este sentido, las denominadas técnicas de extracción tienen el objetivo de hacer compatibles las muestras con las técnicas instrumentales para determinar los analitos de interés.

La etapa de pretratamiento de muestra, en la práctica inevitable, suele considerarse como la etapa limitante del PMQ, esencialmente por los siguientes motivos:

- La complejidad y diversidad de las matrices de las muestras objeto de estudio hace imposible el establecimiento de una estrategia única, debiendo estas operaciones adaptarse a cada caso concreto.
- El rango de concentración y naturaleza química de los analitos de interés puede ser muy amplio, lo cual requiere una selección cuidadosa de las técnicas empleadas en cada caso.
- La mayoría de las operaciones de pretratamiento de muestra implican múltiples etapas bien definidas y en la mayoría de los casos con un alto grado de participación y manipulación por parte del operador. Este hecho tiene una doble influencia negativa, ya que puede generar errores accidentales y/o sistemáticos y consumir hasta el 80% del tiempo del PMQ.
- Conllevan un elevado consumo de disolventes orgánicos y reactivos en general, implicando un riesgo para el operador y el medio, a la vez que encarecen el PMQ.

De lo expuesto anteriormente, se pueden deducir los requisitos que debería cumplir el protocolo de pretratamiento de muestra *ideal:*

- *Simple y automatizable*; reduciendo la manipulación y el número de etapas para minimizar las posibles fuentes de error durante el proceso.
- *Rápido*; facilitando el flujo de trabajo del PMQ y permitiendo el análisis de un mayor número de muestras.
- *Barato*; reduciendo el coste total del PMQ.
- *Miniaturizable*; consiguiendo reducir al mínimo posible el consumo de disolventes y muestra.
- *Seguro*; limitando los riesgos para el operador y el medio.

2. Técnicas de extracción convencionales

Las técnicas de extracción en el pretratamiento de muestra se pueden clasificar en dos grandes familias en función de la naturaleza de la fase extractante: la ***extracción liquido-liquido*** (liquid-liquid extraction**, LLE**) y *la* ***extracción en fase sólida*** (solid phase extraction**, SPE**) [3].

La LLE se fundamenta en el reparto de uno o varios analitos entre dos fases líquidas inmiscibles en función de su diferente solubilidad en ambas. Se trata de una técnica de rutina clásica en los laboratorios de análisis, pero actualmente su papel se ha visto relegado debido a las connotaciones negativas de la técnica, fundamentalmente relacionadas con el elevado consumo de disolventes orgánicos y tiempo, con el consiguiente sobrecoste e impacto en el medio y el operador.

Tradicionalmente, la LLE se basa en el empleo de embudos de decantación en los cuales tiene lugar el intercambio de analito(s) entre las fases inmiscibles mediante etapas sucesivas de agitación, reposo y separación de fases. La transferencia de analitos se rige por la ley de distribución de Nernst a través de la constante de distribución (K_D), la cual establece que a presión y temperatura constantes, el cociente entre las concentraciones de un analito entre ambas fases inmiscibles es constante e independiente de la concentración inicial, siempre que dicho analito se encuentre en la

misma forma química en ambas fases. El valor de K_D depende de los disolventes seleccionados, siendo habitual que uno de ellos sea acuoso. A mayores valores de K_D, más favorecida está la transferencia de analito(s) a la fase orgánica.

La selección del disolvente que compondrá la fase extractante en los procesos de extracción líquido-líquido es crucial, puesto que determinará, *a priori*, la selectividad y eficiencia del proceso. Sin embargo, existen factores adicionales que pueden ajustarse para influir significativamente en el rendimiento global de la extracción y que dependen de la naturaleza química de los analitos de interés. De entre estos factores cabría destacar la influencia del pH, la presencia de agentes quelantes o la adición de sales (efecto *salting out*).

Si bien K_D se relaciona con los parámetros termodinámicos que rigen el proceso en equilibrio de la distribución de analitos entre las fases, la influencia de la componente cinética del proceso debe también considerarse. La cinética del proceso establece la velocidad a la que ocurre la transferencia de analitos entre las fases. Puede considerar o no la interconversión de especies químicas en formas solubles en una fase e insolubles en otra, que generalmente es una etapa rápida, y la velocidad de transferencia de las mismas entre las fases. De forma general, la transferencia de materia entre las fases se divide en tres etapas:

1. Difusión del analito desde la fase donadora a la interfase con la fase extractante.
2. Difusión en la interfase formada entre ambas fases.
3. Difusión del analito desde la interfase formada hasta la fase extractante.

De las tres etapas, el paso limitante es la difusión en la interfase, ya que depende del espesor de la misma. Habitualmente este proceso se favorece mediante la agitación continua, que suele provocar la aparición de una emulsión debida a la

inmiscibilidad de los disolventes, aumentando la superficie de contacto entre las fases y favoreciendo en última instancia la transferencia de materia.

La LLE suele ser un proceso secuencial en el que tienen lugar extracciones sucesivas con disolvente orgánico nuevo, que no ha estado en contacto con muestra ni analito, alcanzándose un nuevo equilibrio cada vez. Por esta razón, el consumo de disolvente orgánico y tiempo es muy elevado. Debido a esto, los procesos de LLE se caracterizan por bajos factores de preconcentración, lo cual limita su versatilidad. No obstante, es posible incluir una etapa adicional de evaporación-redisolución del extracto con el objetivo de mejorar la sensibilidad. Por estas razones, la LLE ha caído en desuso y ha sido sustituida por otras alternativas más simples y menos contaminantes, como la extracción en fase sólida.

La SPE comparte el mismo principio de partición del analito entre dos fases inmiscibles que la LLE, pero en este caso, una de las fases es sólida (sorbente) y la otra es un fluido. La base de la separación es similar al fundamento de la cromatografía de líquidos en columna. La fase sorbente podrá retener bien los interferentes presentes en la matriz o los analitos de interés, si bien es este segundo caso el más frecuente, ya que requiere una menor cantidad de sorbente y permite la preconcentración de los analitos. Si son los analitos los retenidos, posteriormente se separarán de esta fase mediante el lavado con un disolvente o eluyente. Recientemente, esta técnica ha ganado popularidad frente a la LLE debido a su versatilidad. Es frecuente encontrar en la bibliografía métodos que emplean SPE para muy diversos fines, desde purificación, enriquecimiento de trazas, desalado, hasta derivatización o fraccionamiento.

Los sorbentes se emplean en distintas configuraciones, siendo la más habitual el empaquetamiento en cartuchos. El abanico de sorbentes disponibles está en permanente crecimiento y centra gran parte de los esfuerzos de investigación. Tanto es así, que la selección del sorbente a utilizar en este tipo de procedimientos resulta fundamental para garantizar el éxito del proceso. A partir del conocimiento acerca de la

naturaleza química de los analitos de interés se puede seleccionar un sorbente polar, apolar, iónico o mixto.

La retención de analitos sobre el sorbente en las técnicas de SPE generalmente se basa en cuatro mecanismos generales, confiriendo a la técnica una gran versatilidad:

- Interacciones no polares por fuerzas de van der Waals.
- Puentes de hidrógeno.
- Interacciones polares de tipo dipolo-dipolo.
- Interacciones iónicas.

Los sorbentes más comunes que podemos encontrar en la bibliografía son básicamente de tres tipos: sílices enlazadas [3,4], basados en el carbono [5-7] y poliméricos [8-12]. Otros tipos de sorbentes para SPE desarrollados posteriormente son los inmunosorbentes y los polímeros de impresión molecular [13].

La SPE presenta una serie de ventajas claras frente a otras técnicas de pretratamiento de muestra. Entre ellas se puede destacar su elevada capacidad de recuperación de analitos con una alta selectividad. Además, los procedimientos empleados son rápidos y sencillos y en ocasiones completamente automatizables gracias al acoplamiento con sistemas de cromatografía de gases o líquidos. No obstante, es una técnica limitada para su empleo con analitos de naturaleza volátil. Presenta además ciertos problemas asociados al comportamiento físico del sólido empleado, como por ejemplo problemas de sobrepresión debido a la obturación total o parcial por acumulación de partículas presentes en la muestra, o la aparición de canales preferentes a elevados caudales de paso de muestra, que limitan la extracción.

3. Papel de la química verde en el tratamiento de muestra

La química analítica no ha estado al margen de los ideales de desarrollo eco-sostenible que impulsan el desarrollo científico-tecnológico actual. A partir de esta idea, nace en el año 1995 el concepto de "*Química Analítica Verde*" derivada del planteamiento del Prof. de la Guardia de métodos analíticos respetuosos con el medio [14]. Esta nueva corriente de la química analítica se enmarca dentro de la química verde propuesta por Anastas [15].

Al margen de los avances tecnológicos ligados al desarrollo de las técnicas instrumentales, cuyo fin último es el de satisfacer las necesidades en términos de calidad de los análisis químicos, la química analítica verde centra sus esfuerzos en reducir el impacto ambiental de los métodos de trabajo de los laboratorios analíticos.

Las líneas generales de actuación de la química verde se pueden resumir esencialmente en: (i) sustitución de reactivos tóxicos durante los procedimientos, (ii) reducción del consumo energético durante las determinaciones, (iii) mínimo consumo de reactivos y (iv) mínima generación de residuos. A partir de estas líneas fundamentales, Namieśnik y colaboradores proponen doce principios para la química analítica verde [16]:

1. Desarrollo de metodologías directas que permitan eliminar la etapa de pretratamiento de muestra.
2. Mínima cantidad de muestra necesaria y en un número de ellas mínimo, manteniendo la representatividad.
3. Medidas realizadas *in situ.*
4. Integración de las operaciones analíticas para reducir al mínimo el consumo de reactivos.
5. Empleo de métodos miniaturizados y/o automatizados.
6. Evitar la derivatización.

7. Reducción al mínimo la generación de residuos y gestión obligatoria.
8. Empleo preferente de metodologías que determinen varios parámetros y/o analitos simultáneamente frente a aquellas que lo hagan individualmente.
9. El consumo energético debe ser el mínimo posible para todo el procedimiento.
10. Empleo de reactivos que provengan de fuentes renovables.
11. Los reactivos tóxicos deben evitarse o sustituirse.
12. La seguridad del operador es primordial.

El principal inconveniente de la implantación de la química verde en los laboratorios de análisis es alcanzar un compromiso entre el rendimiento óptimo del PMQ y el cumplimiento de los requisitos de ésta. En términos generales, el objetivo de un PMQ debe ser resolver el problema analítico, y de manera trasversal, hacerlo de la forma más "*verde*" posible. La mayoría de las directrices de la Química Analítica Verde pueden provocar mermas en ciertos parámetros como la exactitud, precisión y sensibilidad tal como muestra la Tabla 1 (adaptada de [16]).

Tabla 1: Influencia de los principios de la química analítica verde sobre los parámetros analíticos

Etapa del proceso analítico	Parámetro del proceso analítico				
	Representatividad	Exactitud	Precisión	Selectividad	Sensibilidad
Muestreo					
Mínimo tamaño de muestra	↓	↓	↓	↓	↓
Número mínimo de muestras	↓	0	0	0	0
Tto. Muestra					
Sin derivatización	0	↓	↓	↓	↓
Mínimo consumo energético	0	↓	↓	↓	↓
Reactivos de fuentes renovables	0	↓	↓	↓	↓
Reactivos no tóxicos	0	↓	↓	↓	↓
Seguridad del operador	0	↓	↓	↓	↓
Medida					
Directa	↓	↓	↓	↓	↓
In-situ	↑*	↓	↓	↓	↓
Integración de operaciones y procesos	0	↑	↑	0	↑
Automatización	0	↑	↑	↑	↓
Instrumentos miniaturizados	↓	↓	↓	↓	↓
Multi-analitos/multi-medidas	0	0	0	0	0
Mínimo consumo energético	0	↓	↓	↓	↓

Nota: ↓; descenso, ↑; aumento, 0; neutral. * Según el procedimiento

4. Técnicas de extracción miniaturizadas

El elevado consumo de disolvente orgánico, reactivos y muestra inherente a los procesos de extracción líquido-líquido y en fase sólida impulsaron la miniaturización de estas técnicas de separación. En este apartado se van a comentar brevemente las modalidades de microextracción más relevantes atendiendo a la naturaleza de la fase extractante y la forma en la que esta se dispone en el proceso de extracción. Dada su mayor relación con la investigación desarrollada en esta Tesis Doctoral, las técnicas dispersivas se comentaran conjuntamente en una sección posterior.

4.1 Técnicas de extracción en fase sólida miniaturizadas

4.1.1 Microextracción en fase sólida

La prevalencia de la SPE sobre la LLE por las razones previamente comentadas, favoreció que las primeras técnicas de extracción miniaturizadas fueran en fase sólida. La ***microextracción en fase sólida*** (solid phase microextraction; ***SPME***) es una técnica propuesta a principios de los 90 por Arthur y Pawliszyn [17] y posteriormente comercializada por SUPELCO (Bellefonte, USA). Se basa en la utilización de una fibra cilíndrica de sílice fundida (1 cm de longitud y 0.1 mm de diámetro interno) recubierta con una fase de naturaleza polimérica (poli-dimetil siloxano, PDMS). En la SPME se establece un equilibrio entre la concentración de analito en la muestra (fase donadora) y el polímero extractante (fase aceptora). La manipulación de la muestra y el consumo de disolventes orgánicos es mínimo. Posteriormente, se induce la desorción térmica o elución mediante un disolvente orgánico de los analitos previa a la determinación analítica.

La extracción tiene lugar al introducir la aguja del dispositivo de SPME en un vial sellado con la muestra, presionar el émbolo de la jeringa de modo que la fibra queda sumergida directamente en la matriz de muestra acuosa (en la llamada modalidad de *inmersión directa o* ***DI-SPME***) o expuesta a su espacio de cabeza (en la modalidad de *espacio de cabeza o* ***HS-SPME***), ambas alternativas presentadas en la Figura 1. Transcurrido un tiempo definido, se retrae la fibra y se procede a la siguiente etapa de desorción/elución e inyección de los analitos.

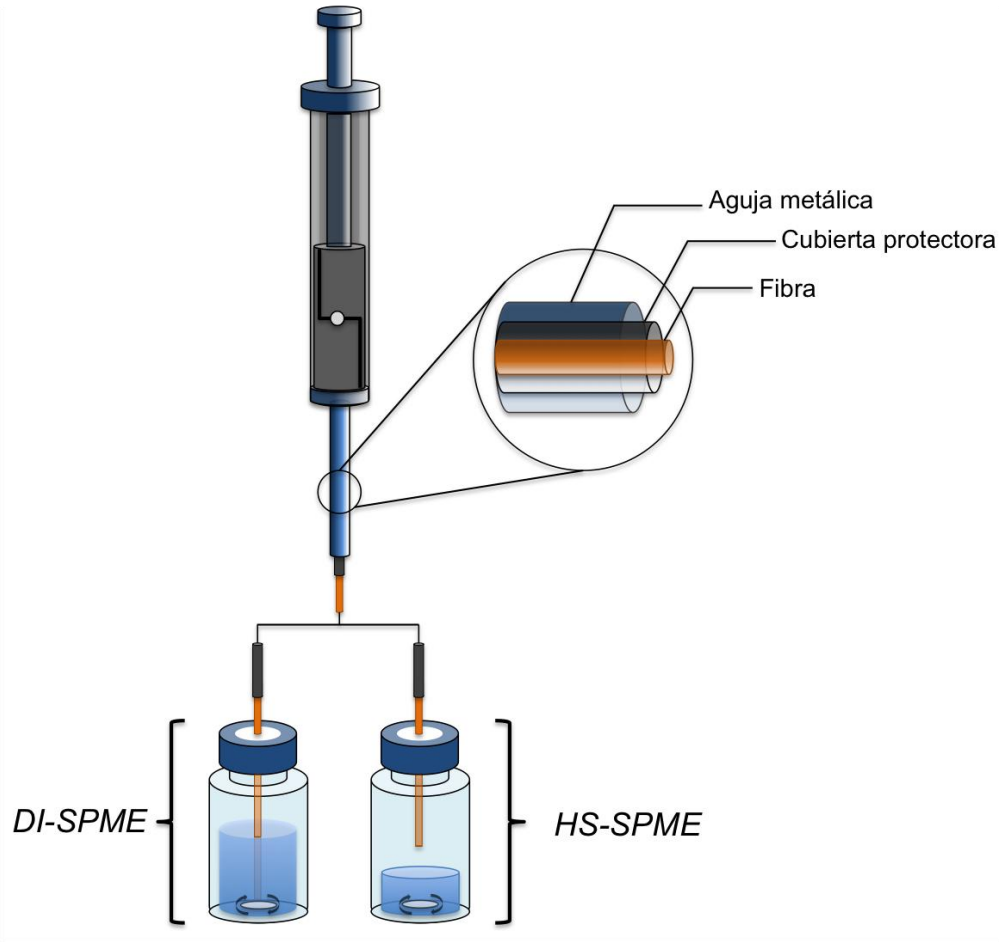

Figura 1:. Modalidades de microextracción en fase sólida con fibra

La modalidad de inmersión directa se recomienda cuando los analitos no son volátiles o la matriz no es excesivamente compleja. Generalmente los compuestos que se pueden extraer y preconcentrar con esta alternativa son no volátiles o poco volátiles. Si la muestra es más compleja pero el modo de inmersión directa es el que se debe emplear por la naturaleza de los analitos se puede proteger el sistema con una membrana polimérica, pero esto ralentiza la extracción. La modalidad de HS-SPME se recomienda para la determinación de analitos volátiles y semivolátiles en matrices

complejas con componentes no volátiles o de alto peso molecular como los ácidos húmicos o proteínas. Además permite añadir determinados reactivos para modificar ciertos parámetros de la muestra (pH, fuerza iónica) sin dañar la fibra.

En SPME, la cinética del proceso es crucial. Ésta controla el transporte de analitos desde la matriz de la muestra a la fibra (en la modalidad de inmersión directa) o hasta el espacio de cabeza y posteriormente a la fibra (en la modalidad de espacio de cabeza). Para aumentar la velocidad del proceso se puede agitar la muestra con vistas a potenciar la difusión de los analitos hacia la superficie de la fibra. Si se van a determinar analitos volátiles, se puede aumentar también la temperatura para favorecer aún más su transferencia al espacio de cabeza y de ahí a la fibra.

La transferencia de analitos desde la fibra al instrumento (generalmente un cromatógrafo) se puede realizar mediante el uso de un disolvente orgánico adecuado (cromatografía líquida o de gases) o mediante desorción térmica (directamente en el inyector de un cromatógrafo de gases). Esta última alternativa mejora la sensibilidad de la medida ya que evita la dilución inherente al uso de disolventes orgánicos. La SPME presenta una serie de ventajas frente a otras alternativas, como son:

- No requiere de disolventes orgánicos para el proceso de extracción.
- Es simple, ya que puede integrar el proceso de extracción e inyección en un mismo dispositivo.
- No requiere de grandes volúmenes de muestra.
- Su manejo es cómodo y fácilmente acoplable a diversos sistemas instrumentales como cromatografía de gases (GC), de líquidos (HPLC) o electroforesis capilar (CE).
- Es fácilmente automatizable e incluso se encuentra integrada en equipos instrumentales comerciales.

- Puede utilizarse con todos los tipos de muestras, ya sean gaseosas como por ejemplo aire [18-20] o aliento [21,22], líquidas como aguas o bebidas [23-27], o sólidas como sedimentos, alimentos, etc. [28-32].
- Se puede aplicar para la determinación de analitos volátiles y no volátiles.

Como inconveniente se puede destacar que debido a la limitada capacidad de las fibras por el reducido volumen del recubrimiento, en ocasiones no se alcanzan valores de límites de detección lo suficientemente bajos, sobre todo si la SPME se utiliza combinada con la cromatografía de líquidos [33].

4.1.2 Microextracción en fase sólida basada en unidades agitadas

Las técnicas de extracción tienen lugar bajo condiciones de difusión controlada, de acuerdo a la ley de Nernst a través de la constante K_D. En este sentido la agitación de la muestra mejora los procesos de difusión entre las distintas fases implicadas en el proceso de extracción. En la práctica, la agitación puede lograrse mediante un dispositivo externo o mediante la integración de estos elementos y los elementos de extracción en el mismo dispositivo.

La ***microextracción por sorción con barra magnética agitadas*** (stir bar sorptive extraction; ***SBSE***) es una técnica de extracción y preconcentración desarrollada en 1999 por Sandra y colaboradores [34]. Ésta se basa en los mismos principios de partición de los analitos entre la muestra y un sorbente polimérico que la SPME. El primer ejemplo (y hasta el momento único comercial) emplea un recubrimiento de 1 mm de espesor de PDMS (poli-dimetil siloxano) sobre una barra magnética agitadora o Twister™ [35].

El dispositivo de extracción se compone de tres capas diferentes: la primera y más interna, compuesta por el material magnético recubierto por una segunda y fina capa de vidrio que a su vez está recubierta por la tercera y última capa (24-100 µL) de PDMS que hace las veces de fase extractante. Actualmente se comercializan en dos

tamaños diferentes, según su diámetro externo: 10 mm x 3.2 mm y 40 mm x 3.2 mm. La selección de cada una de ellas dependerá del volumen de muestra a tratar, así la de 10 mm se emplea preferentemente para volúmenes entre 1 y 50 mL y la de 40 mm para volúmenes superiores.

El procedimiento de extracción es muy simple. El dispositivo se intruduce directamente en la muestra o bien se expone al espacio de cabeza de la misma. Una vez ha transcurrido el tiempo estimado para la extracción, el dispositivo se retira y se lava para eliminar cualquier exceso de muestra, se seca con un trozo de papel y los compuestos extraídos por la barra magnética se desorben térmicamente en la unidad de desorción y se introducen directamente en el inyector del cromatógrafo de gases o se eluyen químicamente con el disolvente orgánico apropiado para su análisis en un sistema de cromatografía líquida o de gases. Debido a la naturaleza del PDMS, los analitos que pueden extraerse y preconcentrarse con esta modalidad son generalmente apolares o poco polares [36].

La desorción térmica es la opción mayoritaria en las aplicaciones de SBSE desarrolladas actualmente. Para poder llevarla a cabo, se ha comercializado una unidad de desorción térmica que se acopla al sistema de inyección de un cromatógrafo de gases. Este sistema consiste básicamente en la combinación de dos inyectores de temperatura programable (PTV) conectados en serie para desorber y dirigir los analitos hacia el cromatógrafo de gases.

De igual modo, la SBSE puede combinarse con HPLC si se realiza la elución química mediante un disolvente orgánico. Todas las aplicaciones publicadas en este sentido se han realizado fuera de línea con HPLC debido a la ausencia hasta la fecha de interfases comerciales para el acoplamiento en línea. Así, el dispositivo se introduce después de la extracción en un determinado volumen de disolvente orgánico (100-1000 µl) y a continuación se procede a la inyección de una alícuota de este disolvente que contiene los analitos de interés. Este modo de desorción también es una alternativa

para el uso de la SBSE combinada con la GC si no se dispone de la unidad de desorción térmica. Es posible realizar una etapa previa a la inyección del extracto de evaporación/reconstitución en un volumen de disolvente menor para conseguir un aumento de la sensibilidad.

Al igual que en las otras modalidades de microextracción, las variables que afectan al proceso, tanto de preconcentración como de elución deben optimizarse para cada problema analítico para poder obtener los factores de enriquecimiento más elevados que permitan alcanzar los valores de sensibilidad requeridos.

A partir de la idea clásica de SBSE han surgido nuevas alternativas basadas en dispositivos innovadores. Tal es el caso de la ***microextracción mediante disco rotativo sorbente*** (rotating-disk-sorptive extraction; ***RDSE***), propuesta por Ritcher y colaboradores en 2009 [37]. Esta modalidad se desarrolló con el objetivo de minimizar el daño que se producía en el dispositivo preparado en el laboratorio cuando se empleaban altas velocidades de agitación durante la extracción. La unidad está formada por un disco de teflón que contiene el elemento magnético a lo largo de su diámetro y una película de PDMS unida mediante silicona al teflón. Este dispositivo puede girar hasta 1600 rpm, velocidad muy superior a la SBSE convencional.

Las membranas poliméricas en SBSE se emplearon por primera vez en 2009. En este caso se denominó ***extracción con membrana agitada*** (stir-membrane extraction; ***SME***) [38]. En esta modalidad, se integra la membrana y la agitación gracias a un montaje especial que la mantiene fija en el dispositivo de extracción representado en la Figura 2. La unidad gira en la muestra y los analitos quedan retenidos en la membrana mediante filtración. El vórtice creado durante la agitación impulsa los analitos hacia el centro de la unidad donde se encuentra la membrana. Los compuestos pueden determinarse directamente sobre la membrana mediante técnicas espectroscópicas o bien mediante técnicas cromatográficas, después de un proceso de elución de los mismos.

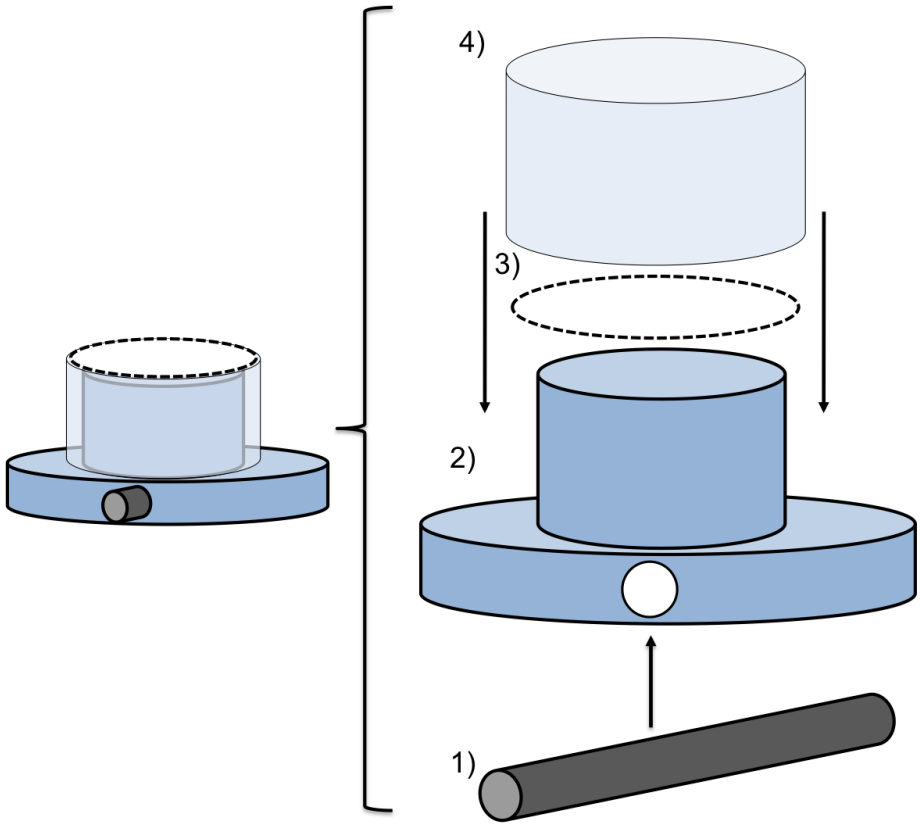

Figura 2: Unidad de extracción para SME. 1) barra metálica; 2) parte superior de un cartucho comercial de SPE; 3) membrana de PTFE; 4) sección precortada de una punta de pipeta de plástico de 5 mL.

Otra alternativa desarrollada en 2010 por Luo y colaboradores es la ***microextracción con varilla agitada*** (stir-rod-sorptive extraction; ***SRSE***) con el objetivo de minimizar el daño producido a los recubrimientos de polímeros de impresión molecular (molecularly imprinted polymers, MIPs) en las técnicas de SBSE [39]. El dispositivo, esquematizado en la Figura 3, consiste en una varilla metálica con un imán en el extremo sobre el cual se acopla un inserto de vidrio con un recubrimiento de MIPs. El dispositivo se introduce en el recipiente que contiene la muestra y se fija a este

mediante un tapón de goma. Esta modalidad permite reutilizar el dispositivo hasta 60 veces empleando una velocidad de agitación de 300 rpm.

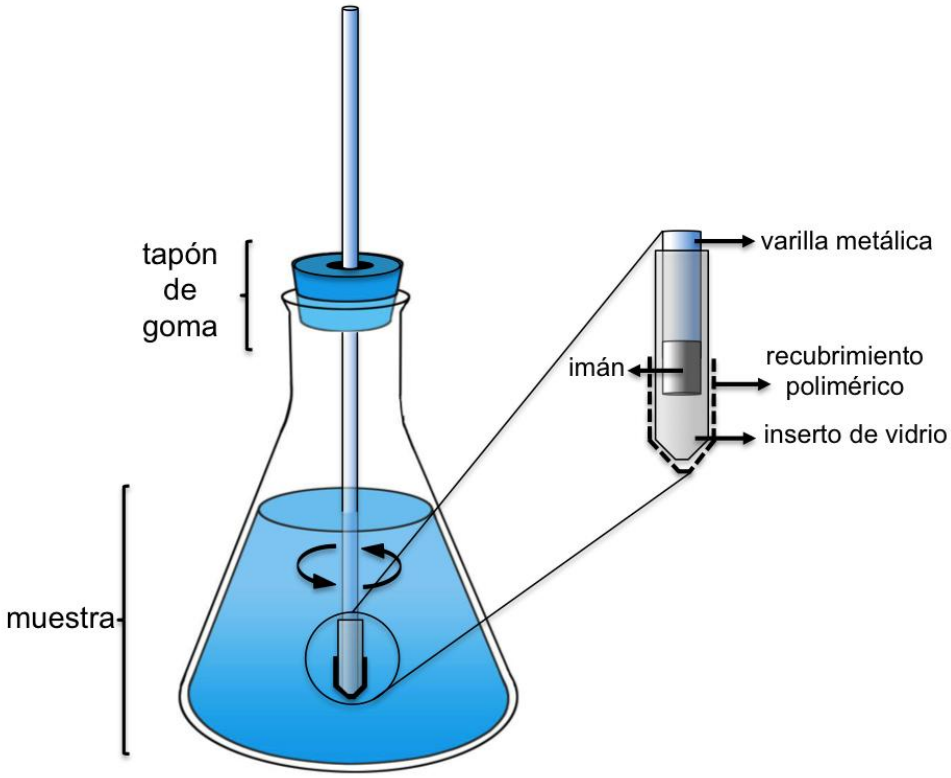

Figura 3: Dispositivo para la modalidad de SRSE

Por último, en 2011 apareció una adaptación de la SRSE y la RDSE. Huang y colaboradores proponen un dispositivo que emplea materiales monolíticos con una configuración de disco protegido por una unidad comparable a la presentada en la SME pero con una forma muy característica, la cual le da el nombre a la técnica de ***extracción agitada con sorbente en forma de pastel*** (stir-cake sorbent extraction, ***SCSE***) [40].

4.1.3 Otras técnicas de extracción en fase sólida miniaturizadas.

La ***microextracción en fase solida en tubo*** (***in tube-SPME***) es una adaptación automatizada de la SPE como muestra la Figura 4. En esta modalidad, la extracción tiene lugar en un capilar de sílice fundida cuyo volumen interno se ha recubierto parcial o totalmente con fase sorbente. Si el capilar se sitúa en el bucle de una válvula de inyección, se puede hacer pasar la muestra por su interior mediante una bomba HPLC y posteriormente eluir los analitos haciendo pasar el disolvente orgánico apropiado o fase móvil y analizarlos directamente con la técnica instrumental seleccionada. La longitud del capilar situado en la válvula oscila entre unos pocos centímetros hasta más de 50 cm, con lo que se puede hacer un ajuste muy especifico de la capacidad de extracción según el problema analítico planteado.

Esta variante presenta como ventajas que permite la automatización de todo el proceso de una forma fácil y robusta y que en general, se consiguen límites de detección inferiores a los obtenidos cuando se usan fibras en el acoplamiento de la SPME con HPLC tradicional. El principal inconveniente es que sólo puede utilizarse con muestras muy limpias ya que, de lo contrario, el capilar puede obstruirse fácilmente [41]. No obstante, es una alternativa muy versátil tanto en lo que se refiere a muestras como a analitos [42].

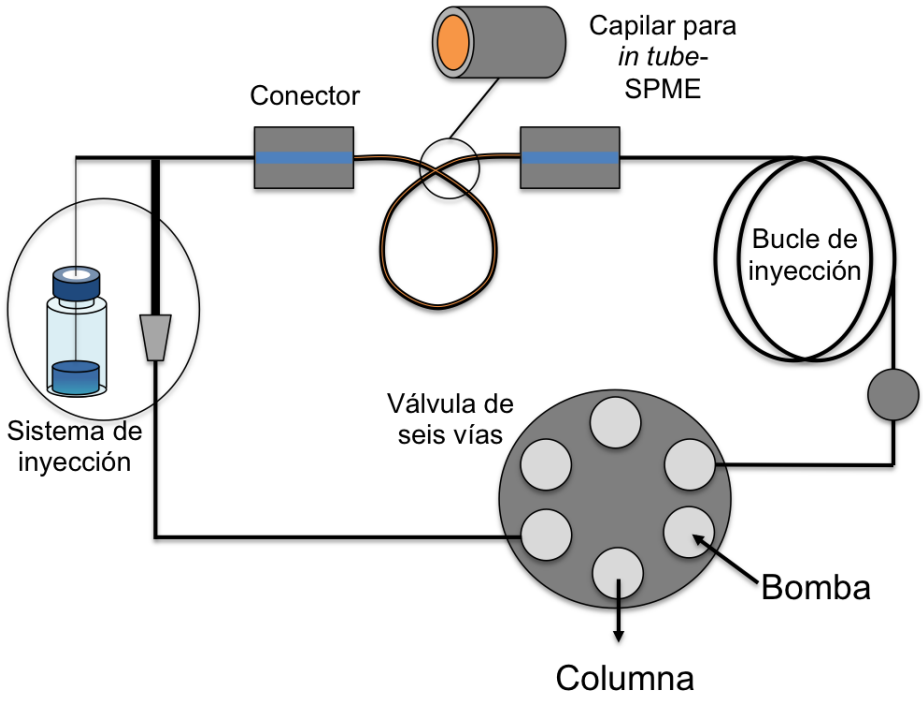

Figura 4: Sistema automatizado *in tube*-SPME

En la práctica, existen dos modalidades de trabajo, una en la que se utiliza un flujo de muestra continuo, y otra en la que se realizan ciclos de aspiración/expulsión de la muestra mediante una jeringa [43]. En todos los casos, además de los factores que afectan a la extracción en general, tales como pH, o valores de los coeficientes de distribución, es muy importante optimizar el caudal de muestra en función de los parámetros operacionales de la columna así como el número de ciclos de aspiración/expulsión de muestra si se quiere favorecer la situación de equilibrio y obtener el máximo rendimiento de extracción.

En el año 2003, Abdel-Rehim propone una nueva alternativa miniaturizada y automatizable empleando un automuestreador comercial. En esta modalidad,

denominada ***microextracción por sorbente empaquetado*** (microextraction by packed sorbent; ***MEPS***) [44], se emplean sorbentes convencionales de SPE pero a una escala considerablemente menor, pasando de los 1000 mg de media a apenas 2 mg. El sorbente se compacta en una jeringa o una punta de pipeta con un volumen de entre 100 y 250 µL, quedando confinado entre dos fritas. El sistema puede incluirse en un automuestreador, con lo que el acoplamiento con el sistema cromatográfico es total, permitiendo desarrollar una modalidad totalmente automatizable. Gracias a los dispositivos implementados, se pueden emplear pequeños volúmenes de muestra (10-250 µL) y eluyente (20-50 µL).

El procedimiento operativo consiste en hacer pasar la muestra a través del sorbente mediante aspiraciones con una jeringa, en tantos ciclos como sean necesarios para garantizar la extracción de los analitos de interés. Resulta además interesante destacar que, gracias a la automatización del proceso, el tiempo puede verse reducido drásticamente, no requiriendo más de 1-2 minutos. Además, los sorbentes empaquetados presentan una tasa de reusabilidad de más del doble de la habitual para los cartuchos de SPE [45].

En el mismo año, Pawliszyn y colaboradores desarrollaron una modalidad de SPME que denominaron ***microextracción en película delgada*** (thin film microextracion, ***TFME***). El objetivo de esta técnica es mejorar la sensibildad de la medida mediante el aumento de la superficie de contacto entre la fase extractante y la muestra [46].

En este caso, una fina lámina de PDMS se sujeta mediante una varilla de acero y el conjunto se pone en contacto con la muestra, bien por inmersión directa o por exposición al espacio de cabeza. Transcurrido el tiempo de extracción, la lámina de PDMS se enrolla sobre la varilla y se introduce en el inyector del cromatógrafo para proceder a la desorción térmica de los analitos.

Existen diferentes configuraciones según el tipo de muestra a tratar, ya sea líquida, sólida o gaseosa [47].

4.2 Técnicas de extracción miniaturizadas en fase líquida

El elevado consumo de disolventes orgánicos asociados a la extracción líquido-líquido convencional es uno de los principales inconvenientes de esta técnica. El desarrollo de la ***microextracción en fase líquida*** (liquid phase microextraction, ***LPME***) redujo el volumen de extractante al rango de los microlitros frente a los cientos de mililitros empleados en la modalidad clásicas. La primera propuesta de empleo de microvolúmenes de extractante se desarrolló a mediados de la década de los noventa por Liu y Dasgupta [48]. Los autores emplean una gota de disolvente orgánico suspendida en la punta de un capilar con el objetivo de absorber gases atmosféricos solubles como el NH_3 o el SO_2 de muestras gaseosas.

4.2.1 Técnicas de microextracción basadas en microgotas

En 1996, Liu y Dasgupta y Jeannot y Cantwell desarrollaron independientemente una de las modalidades de microextracción en fase líquida que más continuidad ha tenido en decadas posteriores; ***la microextracción en gota*** (single-drop microextraction, ***SDME***) [49,50]. Ambos grupos de investigación redujeron el volumen de fase extractante hasta los pocos microlitros, contenidos en forma de gota y puestos en contacto con la muestra. El grupo de Dasgupta propuso el empleo de una microgota (1.3 µL) de cloroformo suspendida sobre un capilar para extraer dodecil sulfato sódico (SDS) como par iónico de azul de metileno. Todo el sistema estaba controlado mediante una bomba peristáltica y acoplado en línea a un espectrofotómetro mediante fibra óptica. El grupo de Jeannot por su parte desarrolló la primera alternativa acoplada a una técnica cromatográfica (de gases) mediante el empleo de una varilla de teflón como soporte para una gota de octanol (8 µL). La SDME queda finalmente consolidada en 1997 con el segundo tabajo de Jeannot y Cantwell [51], en el que emplean una jeringa de GC para mantener suspendido el disolvente

durante la extracción e inyectarlo en el cromatógrafo. Entre las numerosas ventajas de la SDME se pueden citar las siguientes:

- Empleo de volúmenes de extractante reducidos, lo que repercute directamente en un menor coste del proceso, mayor seguridad y eficiencia de la extracción.
- Fácilmente automatizable
- Acoplable a técnicas cromatográficas

El éxito de una técnica de SDME depende de diferentes variables, entre las que destacan:

- *Elección del disolvente que compone la fase extractante*: la estabilidad de la microgota es un aspecto determinante de la precisión y eficiencia de la técnica. Las características del disolvente son clave en este sentido. Por ello, se utilizan aquellos disolventes con una elevada viscosidad capaces de mantener la gota en el dispositivo de extracción (sin comprometer la etapa de difusión de los analitos a través de esta), inmiscibles en agua, con temperaturas de ebullición elevadas y gran afinidad por los analitos.
- *Volumen de disolvente*: generalmente la cantidad de analito extraído aumenta con el volumen de extractante, pero volúmenes elevados generarán gotas de mayor tamaño y por tanto más inestables en la punta de la aguja o capilar. Por otra parte, cuanto menor es el volumen de extractante, mayor es el factor de preconcentración que se puede alcanzar. Por lo tanto, el volumen de fase extractante se debe seleccionar de forma que se maximicen tanto la cantidad de analito extraído como la estabilidad de la gota. En general, se recomienda no exceder los 3 μL de extractante.

Existen numerosas alternativas basadas en el empleo de SDME, siendo la forma más común de clasificación la que tiene en cuenta el numero de fases implicadas en el proceso, originándose sistemas de dos o tres fases tal y como se muestra en la Figura 5.

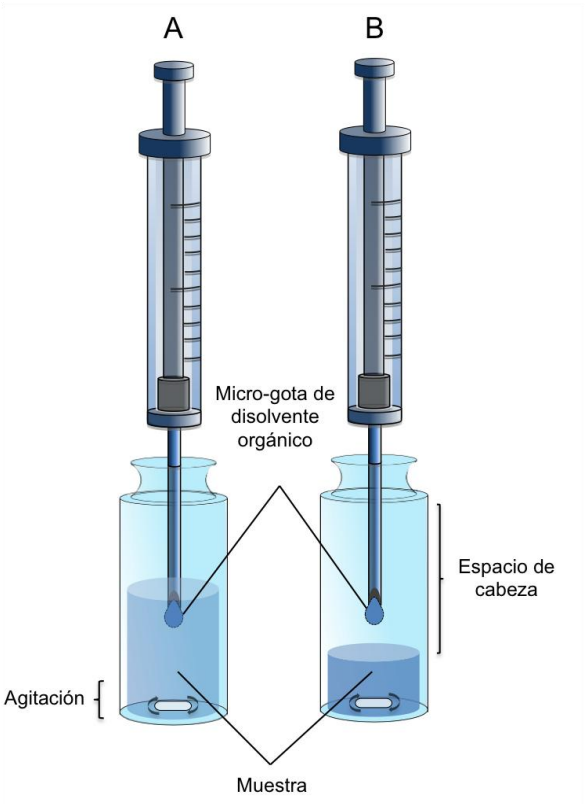

Figura 5:. SDME en su modalidad A) de dos fases (inmersión directa) y B) de tres fases (espacio de cabeza).

La modalidad de **dos fases** más común es la denominada ***microextracción en gota por inmersión directa*** (direct immersion single drop microextraction; ***DI-SDME***). Se emplea una jeringa que mantiene sumergida la punta de la aguja con el disolvente orgánico suspendido en el seno de una fase acuosa. Los disolventes más habituales

para esta modalidad son hexano y tolueno, debido a su compatibilidad con los sistemas cromatográficos. Por la naturaleza de estos disolventes, esta modalidad está especialmente indicada para la extracción y preconcentración de analitos apolares [52-54]. La máxima eficiencia de esta técnica se consigue cuando la muestra acuosa está en continua agitación. Esto renueva constantemente la interfase entre la gota y la fase donadora aumentando la transferencia de analito. A mayores velocidades de agitación mayor transferencia se producirá, pero es posible que tenga lugar la desestabilización de la propia gota debido a las turbulencias generadas.

Reduciendo aún más el volumen de las fases, en este caso de la fase donadora, se encuentra la modalidad de ***microextracción gota-a-gota*** (drop to drop single drop microextraction; ***DD-SDME***) propuesta en 2006 por Wu y colaboradores [55]. En este caso, ambas fases están en el rango de los microlitros, si bien la fase donadora presenta un volumen ligeramente superior para poder sumergir completamente la microgota de fase aceptora suspendida en la aguja de la jeringa. Al encontrarse la muestra en un volumen tan reducido, no se requiere agitación para alcanzar el equilibrio y la transferencia de materia es mucho más rápida. Sin embargo, la relación de volúmenes no permite alcanzar valores de preconcentracion elevados, lo que puede limitar su uso.

Liu y Lee presentaron en 2000 una nueva modalidad de ***microextracción en gota en flujo continuo*** (continuous flow single drop microextraction; ***CF-SDME***) [56]. En este caso, el extractante se inyecta en una cámara de vidrio mediante una micro-jeringa convencional y se mantiene a la salida de un tubo de teflón dentro de la misma. La muestra se bombea a la cámara a través del tubo de teflón y circula hacia un colector de desecho de forma que la gota de extractante está en contacto permanente con muestra nueva durante la extracción. La CF-SDME difiere de otras técnicas de microextracción precisamente en que la gota de extractante está completa y continuamente en contacto con muestra fresca. La difusión y las fuerzas que actúan

sobre la gota generadas por el flujo de muestra contribuyen significativamente a su eficacia. El empleo de válvulas de inyección de alta presión permite el control exhaustivo del tamaño y volumen de la gota, permitiendo alcanzar elevados factores de preconcentración. Una modificación interesante es la propuesta por Xia y colaboradores, en la cual la muestra no va al desecho sino que se recircula de nuevo a la cámara de contacto lo que permite emplear un volumen menor de muestra y una parcial automatización del proceso, puesto que no habría problema en dejar el sistema desatendido ya que no se quedaría sin muestra en ningún momento [57]. La modalidad de CF-SDME está especialmente indicada para la extracción de analitos apolares y ligeramente polares [58].

Los sistemas de **tres fases** suelen emplearse para muestras más complejas y el proceso es más tedioso que los anteriores. El sistema más empleado es la ***microextracción en gota en espacio de cabeza*** (head-space single drop microextraction; ***HS-SDME***). Para esta modalidad, la microgota de extractante se sitúa en el espacio de cabeza de una muestra acuosa o en una corriente gaseosa. En este caso, los disolventes seleccionados, al no estar en contacto con la fase acuosa, no pueden solubilizarse en ésta por lo que su elección es más flexible. Los más comunes son 1-octanol, hexadecano, dodecano y decano y se emplean para la extracción de analitos volátiles o semivolátiles [59]. Aunque la técnica sea acoplable a cromatografía de líquidos y gases, es esta segunda opción la más empleada debido a la naturaleza de los analitos que se pueden extraer [60].

En la modalidad de ***microextracción líquido-líquido-líquido en gota*** (liquid-liquid-liquid microextraction; ***LLL-ME***), los analitos se extraen mediante una fase orgánica y posteriormente se reextraen mediante una gota de otra fase acuosa. Fue propuesta en 1999 por Ma y Cantwell [61] y basa su funcionamiento en la ionización potencial de los analitos de interés. Para conseguir las dos extracciones sucesivas, se emplea un gradiente de pH entre las dos interfases que establece la fase orgánica y las

dos fases acuosas donadora (muestra) y aceptora final (microgota). Generalmente, se busca un pH para la muestra que mantenga los analitos en su forma neutra y un pH para la microgota que los mantenga en su forma iónica. El gradiente de pH se establece en función de la naturaleza ácido-base de los analitos de interés. Esta técnica es especialmente interesante para la determinación de compuestos ionizables de polaridad intermedia en muestras acuosas y es acoplable a HPLC .

En 2006, Lu y colaboradores propusieron una metodología para minimizar los inconvenientes inherentes al empleo de microgotas estáticas [62]. La alternativa se denominó ***microextracción con gota suspendida*** (directly-suspended droplet microextraction; ***DS-DME***). En esta estrategia, se sitúa una barra agitadora en fondo de un vial que contiene la muestra acuosa y se agita vigorosamente hasta generar un vórtice. En esas condiciones, se añade una microgota de un disolvente orgánico inmiscible con la fase acuosa en la superficie, que permanecerá en el centro del vórtice. El momento de agitación del sistema hará girar también a la microgota de disolvente incrementando la transferencia de materia.

Comparada con otras técnicas de microgota, la DS-DME permite mayor flexibilidad en la selección del disolvente o la velocidad de agitación. Esta metodología es muy simple y alcanza rápidamente el equilibrio. Disponiendo la microgota directamente sobre la muestra se evita el uso de sistemas de soporte como el teflón o la aguja anteriormente citadas. La principal desventaja de este método estriba en la recuperación de la microgota de la disolución una vez terminada la extracción. Emplear una microjeringa para recoger el disolvente orgánico queda prácticamente descartado, ya que arrastraría parte de la fase acuosa, haciéndolo incompatible con ciertas técnicas instrumentales como la cromatografía de gases. Para evitar este problema, Yamini y colaboradores propusieron una alternativa basada en la solidificación del disolvente orgánico [63]. Para ello, se emplea un disolvente con una temperatura de fusión próxima a la temperatura ambiente, entre 10 y 30°C. El sistema funciona como se ha

descrito y una vez transcurre el tiempo de extracción, la muestra se sumerge en un baño de hielo. El disolvente orgánico solidifica transcurridos aproximadamente 5 min, y se transfiere a un vial cónico donde se funde a temperatura ambiente, analizándose a continuación. Pocos disolventes presentan esta característica, por lo que esta alternativa es relativamente poco versátil. El 1-undecanol es el disolvente orgánico más comúnmente empleado en este tipo de metodología.

La SDME es por tanto una técnica rápida y sencilla que permite obtener factores de preconcentración elevados gracias al reducido volumen de extractante empleado, haciéndola además respetuosa con el medio y segura para el operador. Generalmente se trabaja alejado del equilibrio, ya que de lo contrario el tiempo de extracción sería muy elevado por los diferentes equilibrios que se deberían alcanzar en cada interfase presente, especialmente en las modalidades de tres fases.

4.2.2 Microextracción en fase líquida soportada

Una de las características de las técnicas de microextracción es que cuanto menor es el volumen de fase extractante, en principio se pueden alcanzar factores de enriquecimiento teóricos más elevados, con valores de recuperación cercanos al 100%. Sin embargo, si el volumen empleado es muy pequeño, su capacidad de extracción es baja, puesto que se satura rápidamente. En la modalidad de SDME descrita anteriormente, el volumen de extractante está limitado por la estabilidad de la gota. Por lo tanto, para emplear volúmenes mayores, es necesario desarrollar modalidades de microextracción que permitan acomodar mayores cantidades de fase extractante.

En este contexto aparece la técnica de ***microextracción en membrana líquida soportada*** (supported liquid membrane; ***SLME***). En este caso el disolvente orgánico se dispone formando una película delgada sobre una membrana que puede ser plana o una fibra hueca porosa. Esta segunda modalidad se denomina ***microextracción líquida protegida en fibra hueca*** (hollow fiber protected liquid phase microextraction; ***HF-***

LPME). La SLME fue propuesta en 1992 por Jönsson y colaboradores [64]. Esta alternativa utiliza una membrana porosa de teflón impregnada con un disolvente orgánico dispuesta entre dos piezas de teflón con canales prefabricados. Los canales cumplen la función de permitir el flujo de fase acuosa a ambos lados de la membrana. El dispositivo se conecta a una bomba y se hace circular muestra a un lado de la membrana y una fase aceptora al otro. El sistema completo se esquematiza en la Figura 6. Los analitos pasaran a través de la película de fase orgánica soportada en la membrana. En esta modalidad, el analito se encuentra en dos formas iónicas diferentes en las dos fases acuosas circulantes, por lo que una vez alcanza la fase aceptora no puede volver a la fase donadora. Habitualmente, la forma no iónica de los analitos está presente en la fase donadora, para poder difundir a través del disolvente orgánico. Para reducir el volumen de fase extractante esta suele ser estática, mientras que la fase donadora se bombea continuamente a través del dispositivo. Cuanto mayor sea la relación de volúmenes fase donadora:fase aceptora mayores serán los factores de preconcentración.

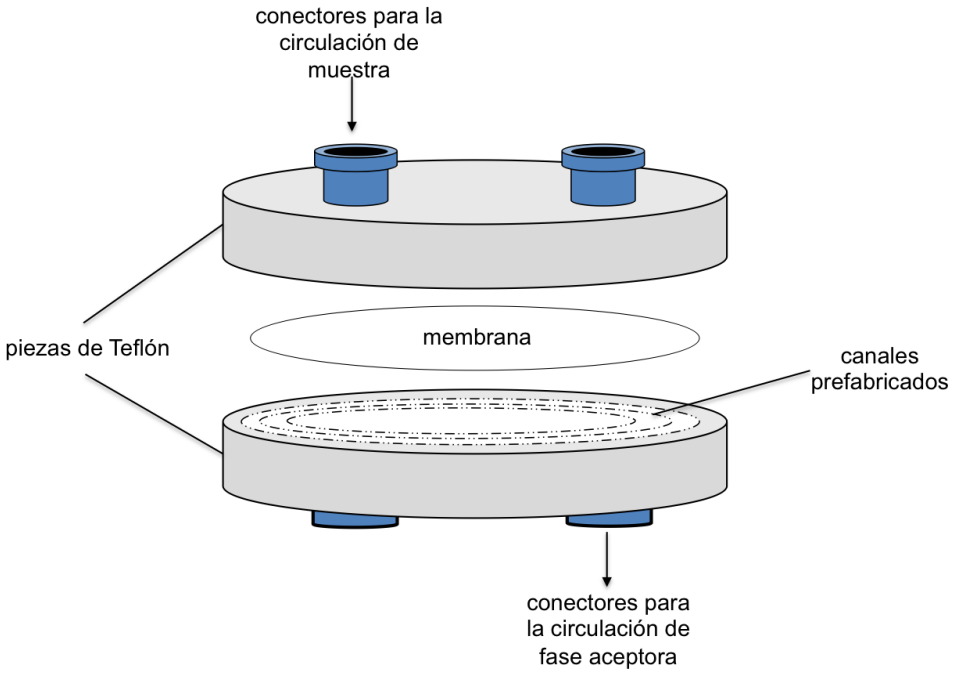

Figura 6: Representación esquemática de un dispositivo para SLME.

En 1999 el grupo de Pedersen-Bjergaard y Rasmussen [65] propuso la HF-LPME como una alternativa más robusta a la SLME. En esencia consiste en impregnar el interior y los poros de una fibra hueca con el disolvente orgánico (**modalidad de dos fases**) o bien disponer una fase acuosa en el lumen de la fibra hueca de manera que el medio orgánico ocupe únicamente los poros de la misma (**modalidad de tres fases**) como se detalla en la Figura 7. Una vez preparado el dispositivo, éste se sumerge en la muestra acuosa para llevar a cabo el proceso de extracción. Lógicamente, la modalidad de dos fases es apropiada para la extracción de compuestos apolares o de naturaleza hidrofóbica para su posterior determinación por cromatografía de gases, mientras que la modalidad de tres fases tiene mayor aplicabilidad para la determinación de

compuestos apolares ionizables, siendo su compatibilidad mayor con cromatografía de líquidos o técnicas electroforéticas.

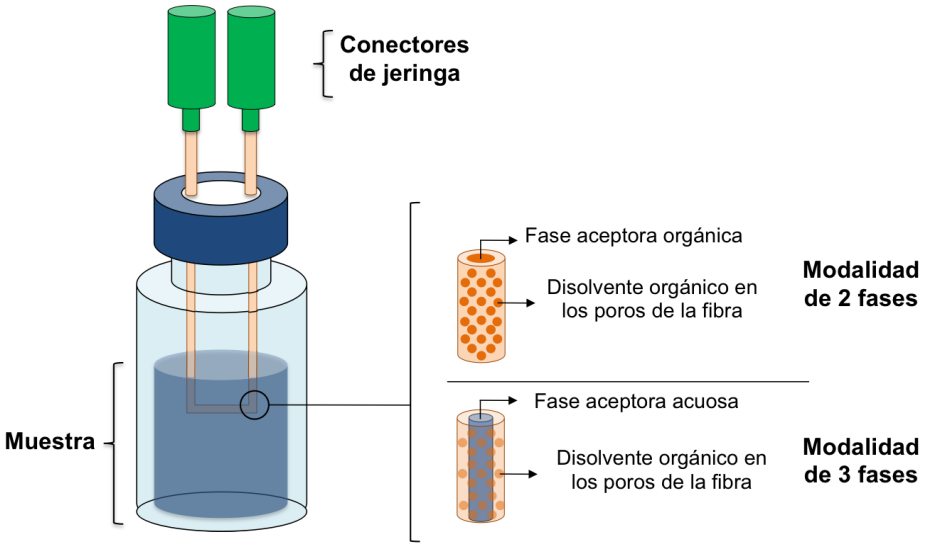

Figura 7:. Modalidades de microextracción líquida con fibra hueca

Los disolventes orgánicos empleados deben cumplir una serie de requisitos, de entre los que destacan: inmiscibilidad en agua, para evitar pérdidas; adecuada viscosidad, para quedar fuertemente retenidos en los poros de la fibra pero permitir la difusión de analitos a través de ellos; poco volátiles, para evitar pérdidas por evaporación; y presentar unos coeficientes de partición para los analitos que se van a extraer suficientemente elevados como para que la extracción sea eficiente.

Por último, es posible añadir *portadores* al sistema. Los portadores son reactivos añadidos de naturaleza moderadamente hidrofóbica que actúan formando pares iónicos con los analitos, favoreciendo su transferencia a la fase orgánica. Si el sistema es de tres fases, deberán además tener un contraión en la fase aceptora de

manera que libere al analito una vez alcance ésta. Los portadores más utilizados son las aminas como la trioctil-amina, para analitos ácidos y ácidos carboxílicos como el ácido octanóico para analitos con carácter básico.

4.3 Técnicas de microextracción dispersivas

Las llamadas técnicas de microextracción dispersivas son una novedosa alternativa que emplea bajos volúmenes de muestra y extractante. Presentan una elevada eficiencia, simplicidad operativa y elevados factores de preconcentración con un coste mínimo. El objeto de esta modalidad es el aumento de la superficie de contacto de una fase extractante (ya sea sólida o líquida) con la muestra mediante la división de esta en finas partículas o microgotas, generando una dispersión. Posteriormente, el extractante se recupera mediante centrifugación, filtración o campos mágneticos (si el sorbente presenta características magnéticas) y se analiza mediante una técnica instrumental apropiada.

La primera referencia a esta técnica aparece en el año 2003, en el que Anastassiades y colaboradores propusieron una nueva modalidad de SPE con el objetivo de limpiar muestras de impurezas tras un procedimiento de extracción sólido-líquido. Esta alternativa recibió el nombre de ***extracción en fase sólida dispersiva*** (dispersive solid phase extraction; ***DSPE***) [66]. Entre sus características fundamentales destacan su reducido coste, facilidad y rapidez operativa, robustez y seguridad para el medio y el operador. Estas se resumen en el acrónimo QuEChERS (por sus siglas en ingles: quick, easy, cheap, effective, rugged, y safe). En la práctica, el procedimiento cuenta con una primera etapa de extracción sólido-líquido de muestras complejas con una elevada concentración de potenciales interferentes. En una segunda etapa, estos interferentes se eliminan mediante el empleo de un sorbente apropiado de forma que se aumenta la selectividad del proceso. Este sorbente sólido se añade en finas partículas y se dispersa en el extracto orgánico mediante una vigorosa agitación para

favorecer el contacto del sorbente con las interferencias. Posteriormente se adiciona una mezcla de sales ($MgSO_4$ y NaCl) con el objetivo de reducir el contenido de agua en el extracto orgánico y favorecer la transferencia de analitos mediante el efecto de *salting out*. En su primera aplicación, se empleó para la determinación de plaguicidas en productos hortofrutícolas. En este caso, la técnica resultó tan eficiente como cualquier otro método propuesto hasta la fecha. El procedimiento se modificó posteriormente para extender su aplicabilidad a un mayor número de familias de plaguicidas [67, 68].

Recientemente, se ha empleado la DSPE para la preconcentración de los analitos en lugar de para la eliminación de las interferencias de la matriz. Con esta finalidad, se añade una pequeña cantidad de sorbente a la muestra líquida, para el aislamiento de los analitos. Una vez recuperado el sorbente por filtración o centrifugación, se añaden unos pocos microlitros de disolvente orgánico, tras la eliminación de la fase líquida y el secado. Tsai y colaboradores evaluaron las propiedades extractivas en modo dispersivo de varios sorbentes comerciales para la preconcentración de tetraciclinas en matrices de agua y leche empleando sorbentes poliméricos de sílice funcionalizada [69]. En 2009, Alcudia-León y colaboradores propusieron un método de DSPME empleando un sorbente comercial (Lichrolut EN) para la extracción y preconcentración de ácido benzóico y sórbico de zumos comerciales. En esta aproximación, se añaden 2 mg del sorbente a 5 mL de muestra y se agitan vigorosamente. Finalmente, el sorbente con los analitos retenidos se recupera por centrifugación y se analiza directamente mediante espectroscopía infrarroja con reflexión total atenuada [70].

La aparición de los materiales nanoestructurados ha permitido desarrollar nuevas modalidades dispersivas. El empleo de nanopartículas de carbono es un ejemplo de miniaturización en química analítica y gracias a sus propiedades como sorbentes se usan ampliamente en el campo de la microextracción. De entre las

nanoestructuras basadas en el carbono podemos destacar el empleo de nanotubos de carbono de pared múltiple (MWCNTs) en una técnica tipo QuEChERS para la determinación de varios plaguicidas en frutas y vegetales. El procedimiento se lleva a cabo empleando 10 mg de nanotubos en lugar de los sorbentes convencionales. Este tipo de sorbentes proporciona extractos más limpios y limites de detección de hasta 1 $\mu g\ g^{-1}$ [71].Otro ejemplo de nanoestructuras de carbono empleadas en DSPME son los nanocuernos de carbono de pared simple (SWCNHs). Para mejorar su solubilidad se emplean tras un proceso de oxidación (o-SWCNHs) por irradiación con energía microondas. A continuación, se prepara una dispersión que contiene 0.2 $g\ L^{-1}$ y se toma 1 mL para realizar la extracción. Esta modalidad se ha empleado para determinar PAHs en muestras de agua. Tras la extracción dispersiva, el material sorbente con los analitos extraídos se retiene en un filtro de nylon de 0.45 µm de tamaño de poro y se eluyen con hexano para su determinación mediante GC. En el mismo trabajo, los o-SWCNHs se comparan con otras nanoestructuras, nanotubos de carbono de pared múltiple oxidados (o-MWCNTs) y los nanoconos de carbono (CNCs). El éxito del empleo de nanopartículas de carbono en el ámbito de la microextracción en fase sólida dispersiva es vencer su tendencia a la agregación con vistas a mantener la dimensión nanométrica responsable de sus excelentes propiedades sorbentes.

Alternativamente al uso de nanomateriales de carbono, los MIPs modificados con nanopartículas de sílice permiten hacer uso de la elevada selectividad de los MIPs con las características de dispersabilidad y elevada área superficial de los nanomateriales. En este caso se han empleado para la extracción y preconcentración de herbicidas en muestras agrícolas [72] y hormonas en leche [73].

Por último, las nanopartículas metálicas se han incorporado al abanico de sorbentes disponibles para microextracción gracias a sus características como materiales nanoestructurados. El empleo de coloides de nanopartículas de oro (AuNPs) ha dado lugar a una modalidad denominada por los autores como ***nanoextracción en***

fase sólida (solid phase nanoextraction, ***SPNE***). En este caso, los autores emplean AuNPs de 20 nm de diámetro para extraer 16 PAHs de muestras acuosas, encontrando una relación entre el diámetro de las nanopartículas y la eficiencia de la extracción [74]. Los nanotubos de dioxido de titanio (TNTs) son nanoestructuras con propiedades muy interesantes, ya que se trata de nanomateriales inertes, muy estables y con una baja toxicidad y corrosividad. Presentan extremos abiertos en su estructura, ofreciendo una elevada superficie potencial de interacción con los analitos. García-Valverde y colaboradores han empleado los TNTs en el contexto de la DSPE recubiertos con carbono mediante un proceso hidrotérmico. Las denominadas c-TNTs se han empleado para la determinación de naproxeno y ketoprofeno de muestras de orina [75]. Gracias al recubrimiento de carbono, su dispersabilidad en muestras acuosas es muy superior a la que presentan las nanoestructuras de carbono de propiedades similares. Un grupo especialmente interesante dentro de los nanomateriales metálicos serían las nanopartículas magnéticas (NPMs). Su uso en microextracción está muy extendido gracias a la facilidad de recuperación del material sorbente mediante campos magnéticos. En el caso de las técnicas dispersivas, la primera aplicación de las NPMs desarrolló una estrategia para recuperar un disolvente orgánico (1-octanol) mediante la interacción hidrofóbica de los grupos hidroxilo superficiales de las nanopartículas [76]. La derivatización en superficie de NPMs es una alternativa que amplía la versatilidad del nanomaterial. Por una parte, se simplifica el procedimiento gracias a la facilidad de recuperación del sorbente; por otra, se dota a las NPMs de mayor capacidad de interacción con analitos de interés mediante la incorporación de recubrimientos funcionales que mejoren su selectividad. Además, el material hibrido conserva una gran dispersabilidad en agua, siendo necesaria únicamente una breve agitación durante el procedimiento de extracción. En este sentido, Reyes-Gallardo y colaboradores han usado las propiedades de las NPMs mediante la hibridación con un polímero comercial

(Lichrolut EN) para la extracción de compuestos nitroaromáticos [77] y nylon para la extracción de PAHs [78] en muestras de agua ambientales.

En cuanto a las alternativas dispersivas en fase líquida, Rezaee y colaboradores propusieron por primera vez la denominada ***microextracción líquido-líquido dispersiva*** (dispersive liquid-liquid microextraction; ***D-LLME***) en la que se emplean unos pocos microlitros de disolvente extractante junto con otro disolvente orgánico que actúa como agente dispersante [79]. La dispersión de los disolventes provoca la aparición de turbidez en la muestra acuosa. Los analitos hidrofóbicos se extraen con el disolvente orgánico y éste se separa de la fase acuosa mediante centrifugación para su análisis posterior.

En esta alternativa, el disolvente dispersante juega un papel fundamental en el desarrollo de la extracción. Su función es dispersar en finas gotas el disolvente extractante y puede representar hasta el 98% de la mezcla extractiva. Mediante la formación de microgotas, se aumenta la superficie de contacto entre las fases donadora y aceptora lo cual acelera considerablemente la transferencia de materia durante la extracción. Los disolventes empleados como agentes dispersantes suelen ser metanol, acetona, etanol y acetonitrilo, debido a su coste relativamente bajo. El extractante por su parte, debe ser poco soluble en la muestra pero soluble en el dispersante, de lo contrario se solubilizaría y no formaría microgotas. En términos de densidad, debe ser lo suficientemente distinta de la de la fase acuosa como para poder separarse cuantitativamente por centrifugación. Los extractantes habitualmente empleados son disolventes clorados, hidrocarburos o alcoholes de cadena larga, estos últimos menos tóxicos. Con la D-LLME se puede conseguir la extracción y preconcentración de analitos apolares y el acoplamiento directo con la cromatografía de gases. Si la técnica instrumental seleccionada es la cromatografía de líquidos, es recomendable la evaporación y reconstitución del extracto en un disolvente compatible con el sistema.

Entre los nuevos medios de extracción en este campo se puede citar el empleo de líquidos iónicos y disolventes supramoleculares. Los líquidos iónicos (ioinic liquids, ***ILs***) constituyen un grupo de disolventes no-moleculares formados por sales orgánicas que mantienen su estado líquido a temperatura ambiente. Esto les confiere unas propiedades físico-químicas únicas como son presión de vapor prácticamente nula, elevada viscosidad y estabilidad térmica. Puesto que son sales, sus propiedades dependen de los iones que los forman. Así, se pueden encontrar ILs miscibles o inmiscibles en agua y capaces de extraer analitos polares o apolares. Gracias a sus propiedades se pueden emplear como extractantes en técnicas dispersivas, dando lugar a la ***IL-DLLME*** [80]. Si el IL es insoluble en agua, puede emplearse sin necesidad de un agente dispersante, lo cual representa una clara ventaja frente a otras alternativas más contaminantes [81]. Por su parte los disolventes supramoleculares (SM) son líquidos micro o nano-estructurados que se generan a partir de una disolución de moléculas anfifílicas mediante un proceso secuencial de autoensamblaje. Jafarvand y colaboradores emplean un sistema ternario de ácido alquilcarboxílico/agua/ THF en el cual el ácido actúa como extractante y el tetrahidrofurano como dispersante [82] en la denominada ***SM-DLLME***. El proceso de extracción basado en estos disolventes se resume brevemente en tres etapas: (i) formación de micelas invertidas de ácido alquil-carboxílico en THF; (ii) ensamblado de los coacervados por dispersión de la mezcla anterior en la fase acuosa; (iii) separación de fases por centrifugación mediante unos tubos especiales [83]. La contribución más importante de esta modalidad es el amplio rango de polaridades de analitos al cual se puede aplicar, desde quelatos metálicos hasta analitos orgánicos.

La DLLME es una alternativa simple, rápida, barata y respetuosa con el medio. Su principal desventaja es, por una parte el empleo de elevados volúmenes de dispersante y la necesidad de emplear una centrifuga para lograr la efectiva separación de fases.

La investigación que se presenta en esta Tesis Doctoral se enmarca precisamente en las técnicas de microextracción dispersivas, tanto en fase sólida como líquida. Las propuestas que se han desarrollado se basan en el empleo de CO_2 generado en el medio como agente dispersante o como mediador para conseguir la solubilización de la fase extractante en la muestra.

En cada uno de los bloques experimentales de la memoria se profundizara en las particularidades de las técnicas de microextracción dispersivas presentadas.

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Bloque I: Técnicas de microextracción asistidas por efervescencia

La microextracción en fase sólida dispersiva (DSPE) se concibió originalmente como una estrategia para la limpieza de extractos orgánicos en contacto con muestras de naturaleza agroalimentaria. De forma general, emplea un sólido para retener las interferencias potenciales y una combinación de sales para facilitar la separación de fases y favorecer la transferencia de los analitos al medio orgánico.

Con el tiempo, ésta técnica ha ido ganando protagonismo como estrategia de pretratamiento de muestra para mejorar la sensibilidad de los PMQ en matrices muy diferentes, desde muestras de agua a muestras de suelo o compost. En este sentido, se emplean pequeñas cantidades de sorbente para extraer y preconcentrar analitos que se analizaran posteriormente tras una etapa de elución (D-µSPE). Existen diferentes sorbentes disponibles para este tipo de herramientas, siendo una elección crítica a la hora de optimizar el proceso. Generalmente este tipo de estrategias requieren un disolvente orgánico (acetonitrilo) que actúe como dispersante.

La DSPE/D-µSPE presenta numerosas ventajas, entre las que destacan la simplicidad operativa, el reducido consumo de disolventes y extractantes o la seguridad para el operador.

En este capítulo de la Memoria de Tesis Doctoral se presentan las alternativas de microextracción dispersiva evaluadas empleando la efervescencia como agente dispersante con el objetivo de eliminar el uso de disolventes orgánicos. El capítulo se inicia con una revisión de las técnicas de microextracción en fase sólida y a continuación se recogen los tres artículos científicos relacionados con esta temática. El primero de ellos emplea la efervescencia para la dispersión de un material polimérico, mientras que en el segundo son nanotubos de carbono los que se dispersan con ayuda de la efervescencia. El tercer artículo emplea nanopartículas magnéticas para la recuperación del extractante, 1-octanol, y en el que el CO_2 sustituye al disolvente dispersante.

Capítulo I: Dispersive micro-solid phase extraction

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Dispersive Micro Solid-Phase Extraction

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Abstract

The usefulness of dispersive techniques leans on their ability to maximize the interaction between the sample and the extractant phase, thus increasing the extraction efficiency. As far as dispersive solid phase extraction is concerned, it was initially developed to increase the selectivity of the analytical process because the solid was added to retain the potential interferents from the sample matrix. In spite of its efficient sample clean-up, the sensitivity is its Achille's heel as no preconcentration is usually carried out. Recently, the use of few milligrams of sorbent which is dispersed in a liquid sample for analytes isolation has raised a new miniaturized extraction technique, the so-called dispersive micro solid-phase extraction. This alternative is mainly focused on sensitivity enhancement. The chapter beings with a short contextualization of this extraction technique, followed by a brief description of the first approach in this context, viz, dispersive solid phase extraction. Next, the main contributions in the field of dispersive micro solid-phase extraction in context will be described on the basis of the nature of the solid used. Also, the combination with dispersive liquid phase microextraction and the expected evolution of this miniaturized extraction technique is included.

Keywords: Dispersive extraction; solid phase microextraction; miniaturization; dispersive sorbent, dispersive solid phase microextraction; conventional sorbents; carbonaceous solids; nanostructured sorbents; polymeric sorbents, molecularly imprinted polymers; silica-based sorbents, green chemistry; auxiliary energies; enrichment factors; dispersive liquid-liquid microextraction; dispersive microextraction techniques combination.

1. Introduction

The evolution of Analytical Chemistry has been leaded by three trends: automation, simplification and miniaturization. While the former is clearly consolidated in any analytical laboratory, the other two are still being developed. The preliminary operations are by far the target of these tendencies taking into consideration the influence they have in the basic (sensitivity, selectivity and precision) and productivity-related (cost, time, personal and environmental risks) analytical properties. Therefore, many research efforts are focused on the simplification and miniaturization of the sample pretreatment, as it is practically an unavoidable step of the analytical process.

The determination of a family of compounds in a given sample usually involves its isolation from the matrix (to increase the selectivity) and preconcentration (for sensitivity enhancement) prior to the instrumental measurement. According to the above commented tendency, the introduction of novel microextraction techniques is highly recommended in order to reduce the amount of sample and organic solvents as well as the time, cost and manipulation needed. This tendency is in good agreement with the principles of the green chemistry often described in the literature as the three Rs: replace, reduce and recycle [1] Solid phase microextraction and liquid phase microextraction can be considered as consolidated miniaturized sample treatment techniques, being their fully acceptance in routine laboratories dependant on their automation or even integration with the analytical instrumentation.

The main requirement of any microextraction technique is that the extractant media used should be highly efficient in analyte extraction taking into account the reduced amount used. In this context, dispersive-based procedures have gained relevance in the last years as they enhance the contact surface area between the sample and the extractant and thus the kinetics of the overall extraction procedure while reducing the amount/volume of the solid/liquid extractant phase needed [2].

2. Dispersive Solid Phase Extraction

Dispersive solid-phase extraction (DSPE) was first proposed by Anastassiades et al. in 2003 as a powerful tool to enhance the selectivity of the measurement [3]. Briefly, an initial leaching of the sample with an organic solvent (typically acetonitrile) is carried out. Then, a mixture of salts ($MgSO_4$, NaCl) is added to facilitate both, the analyte migration from the sample to the organic media and the phases' separation. Then, few grams of an active solid (viz. dispersive solid) are added to the mixture to retain potential interferents from the sample matrix. Primary secondary amine (PSA) is the most commonly used while graphitized carbon black (GCB), RP-C_{18} and alumina can be used but to a lesser extent. The nature of the solid is crucial in the process as the capability of interaction with the analytes must be reduced to the minimum in order to maintain the maximum sensitivity (e.g. no losses of the analytes during this clean up must occur). After a centrifugation step, the organic phase can be directly used or conditioned, usually by an evaporation/redissolution step, prior to the instrumental measurement step, commonly by a chromatographic technique.

This general procedure was mainly designed to analyze horticultural samples on account of the complexity of the matrices and the low concentration of the target compounds (mainly pesticides). The presence of co-extracted compounds in the chromatogram makes difficult the unequivocal identification of the pesticides, even when detectors with highly discrimination capabilities, such as mass spectrometry, are employed. In this context, the DSPE is known as QuEChERS, acronym of quick, easy, cheap, effective, rugged and safe, its main analytical features.

The former procedure was modified by Lehotay et al. in 2005 to improve the recovery of certain pesticides [4]. Recently, Anastassiades et al. have reported a second evolution of their initial proposal to facilitate the application of the DSPE to samples with different water and acid contents [5]. DSPE has also been used to enhance the

selectivity of the analysis of environmental samples (soil and compost) to determine pesticides.

The excellent performance of DSPE has also been exploited to improve sensitivity. The miniaturized approach of this dispersive extraction technique is the main focus of this chapter.

3. Dispersive Micro Solid Phase Extraction

The main goal of dispersive micro solid-phase extraction focused on the isolation and preconcentration of the target analytes is to achieve the complete disaggregation of the sorbent inside the sample matrix. This fact compensates the low amount of sorbent used, usually in the low milligram level. This step is commonly assisted by an external energy (vortex, ultrasounds or microwave) or by using the appropriate solvent or chemical reagent. Depending on the nature of the solid selected, oxidation or functionalization can also be used to increase its solubility and/or interaction with the target compounds. Additionally, reversible interaction between the sorbent and the analytes is mandatory in order to elute them properly (either thermally or chemically) prior to their instrumental determination, often by a chromatographic technique.

The advances in this microextraction modality are leaded by the incorporation of micro and nanomaterials as extraction phases. This fact leans on their excellent sorbent properties, which derived from their reduced size and high surface area, which maximized the interaction with the target analytes. Also, the wide sorption mechanisms available can be cited as a clear advantage. In this section, the most relevant applications of dispersive micro-solid phase extraction based on the use of micro/nanoparticles are presented, classified by the nature of the solid material (see Figure 1). Magnetic nanoparticles, which are also used in this context, are not included as chapter 10 is fully devoted to their role in analytical microextraction techniques.

3.1. Poymeric solids

Alcudia et al. were the first in demonstrating the usefulness of dispersive microsolid phase extraction for in sorbent surface attenuated total reflection infrared (ATR-IR) detection of sorbic and benzoic acids in fruit juices [6]. The dispersive extraction allows the selective isolation of the analytes from the sample, allowing their preconcentration prior to the IR detection. The extraction efficiency surpasses that provided by its counterparts as (i) the interaction sorbent-analyte is favoured with the stirring step and (ii) the detection is directly carried out on the sorbent, so the dilution associated to the chemical elution is avoided.

In this application, the sorbent selection was critical, as it must fulfill two requirements. The first one is the favourable interaction with the analytes in order to isolate them from the sample. The second one is its compatibility with the IR detection of the analyte i.e. it should not absorb all the photons coming from the IR source. Two typical reversed-phase sorbents, C_{18} (silica-based) and LiChrolut-EN (polymeric) were evaluated, being the latter the most compatible with the IR detection. The sorbent amount used in the dispersive μ-SPE was also critical as the surface monitored depends on the limited penetration depth of the evanescent wave. In this case, an amount of 2 mg of LiChrolut-EN resulted in the best results. Finally, a comparison of the performance of SPE and dispersive μ-SPE demonstrated the better sensitivity of the dispersive approach by a factor of 10.1 for sorbic acid and 4.1 for benzoic acid. In 2011, Garg et al. applied the proposed dispersive μ-SPE-ATR-FTIR method to determine methylphosphonate in waters. In this case, the sorbent used was OASIS-HLB, a polystyrene-co-divinylbenzene-co-polyvynilpirrolidine-based material [7].

The homogeneous dispersion of the sorbent inside the sample matrix is usually accomplished by vortering the mixture for a given time. Sometimes, the addition of few microliters of a slurry of the sorbent in an organic solvent is selected to ensure the

maximum dispersibility of the particles in the sample. Both approaches present some limitations regarding the need for an external apparatus (vortex) or the potential interference of the organic media selected for the initial dispersion of the solid, mainly increasing the solubility of the analytes in the sample, thus reducing the extraction efficiency. Our research group has proposed a green alternative for the efficient dispersion of the sorbent in the sample matrix [8]. The so-called effervescence-assisted μ-SPE requires the preparation of a tablet containing the effervescence precursors (sodium carbonate as carbon dioxide source and a phosphate salt as proton donor) as well as the sorbent, in this case, OASIS HLB. The tablet is placed inside of a glass syringe and, when the 10 mL of sample is aspirated, the effervescence process begins. The release of CO_2 yields a homogeneous dispersion of the sorbent. The composition of the tablet in both, nature and amount of precursors is critical as it determines the extraction time (viz. the time required for complete tablet dissolution). The novel microextraction approach was evaluated using the determination of nitroaromatic compounds in waters as model analytical problem. The effervescence assisted dispersive μ-SPE was ca. 3 times more sensitive as regards conventional SPE. Also, it was more efficient than vortex-assisted dispersion of the OASIS HLB sorbent.

3.2 Silica-based Solids

The conventional reversed-phase solid RP-C_{18}, has also been proposed as sorbent in dispersive μ-SPE [9]. In this specific application, the solid is previously dispersed in acetone to a final concentration of 10 g/L. This fact allows the appropriate spreading of the particles inside the sample, a cloud of fine particles being immediately observed. Both, the solvent and the final concentration are key factors. The solvent should fulfill the following requirements: (i) miscibility with the sample; (ii) to yield a stable suspension of the sorbent; and (iii) no interference during the extraction process. On the other hand, the concentration used must be high enough to reduce the

amount of organic phase added to the sample while remaining stable and homogeneous with the time. The operational procedure is rather simple: 500 µL of the RP-C_{18} suspension in acetone were sprayed in 12.5 mL of sample, previously added to a dedicated stainless steel interface fitted with a cotton bead. The liquid phase is eliminated under vacuum, being the solid retained in the interface. Then, the interface is coupled to the injection port of a gas chromatograph and the analytes are thermally eluted by means of a focused heated air stream (300ºC, 2 min). The continuous flow of an helium stream carried the analytes to the chromatographic column and further to the mass spectrometer for unequivocal identification and quantitation. The thermal desorption of the analytes increased the method sensitivity as the dilution inherent to the chemical elution is avoided. In fact, the limits of detection (LODs) for the selected polycyclic aromatic hydrocarbons (PAHs) were in the range 0.1-0.2 µg/L with relative standard deviations lower than 8.4 %.

Tsai et al. compared the performance of silica and polymeric-based sorbents in the dispersive µ-SPE format using tetracyclines (TCs) as target analytes [10]. The comparison includes the dispersion of the sorbents in organic and aqueous media. Up to 16 sorbents with different functional groups: primary amine, secondary amine, carbonyl or ion exchange groups were evaluated. In both cases, the extraction recoveries of TCs were higher in acetonitrile than in deionized water, being the results of silica-based sorbents better in both media. Among the sorbents studied, primary-secondary amine, NH_2, DSC-WCX and Su-WCX provided quantitative extraction of TCs from acetonitrile extracts, being PSA selected as optimum for the dispersive microextraction configuration. The method was analytically characterized, being the limits of detection between 0.7 ng/mL and 3.5 ng/mL with recovery values in the range 96-106.6 % and the precision (expressed as relative standard deviation) lower than 4.4 %. In addition, no interferences from other matrix components were detected in the

chromatographic analysis, which demonstrates the usefulness of the proposed dispersive μ-SPE for the given analytical problem.

3.3 Metallic nanoparticles

As it has been mentioned before, the efficiency of the miniaturized extraction techniques relies on the surface area of the sorbent material available for analytes interaction. This requirement is not fulfilled by conventional sorbents but it is an outstanding property of nanostructured materials.

In the dispersive context, the first nanoparticles used were gold nanoparticles (AuNPs) although the extraction was named by the authors as solid phase nanoextraction (SPNE). In this case, commercial AuNPs colloids were used for the extraction of PAHs [11]. A very interesting study correlates the extraction efficiency with the AuNPs diameter. They observed that, although the number of molecules extracted per nanoparticle increased with the particle diameter and the total surface area of Au, the inverse tendency was observed if the extracted molecules per gram of Au and nanometer squared of surface area of Au are considered. Therefore, it can be concluded that the smaller particles were more efficient for the extraction and the 20 nm AuNPs were selected as optimum. This fact demonstrated the relevance of the nanometric size in miniaturized extraction procedures. The analytical performance was excellent using only 500 μL of water sample and HPLC with fluorescent detection for the determination of 16 PAHs [11] or benzo[a]pyrene by laser excited time resolved Shpol'skii spectrometry [12].

Titanium nanotubes (TNTs) have been used in different areas thanks to their inertness, stability, non-toxicity, low cost and corrosion resistance [13,14]. The structure of TNTs consists of TiO_2 sheets rolled and separated by H^+ ions [15]. They are open-ended structures, which confers to the TNTs with large internal, external and interlayer

interactions surfaces/spaces. They have been used as-grown bare-TNTs, functionalized for selectivity increase or combined with metallic nanoparticles [16].

In 2014, García-Valverde et al. synthesized TNTs which were further coated with a carbon layer following an hydrothermal process. The so-called carbon coated TNTs (c-TNTs) were evaluated using the determination of naproxen and ketoprofen in biological samples [17]. The c-TNTs were characterized by TEM (morphology) FTIR (monitoring of the superficial carbonization process) thermogravimetry and elemental analysis (carbon content) and X-ray powder diffraction (to evaluate the cristalinity of the material). In addition to their easy, low cost synthetic process, another favourable feature of c-TNTs is their better dispersibility in aqueous media in comparison with that of carbonaceous nanoparticles. The dispersive μ-SPE was carried out in a dedicated unit that: (i) is adapted to low sample volumes; (ii) integrates extraction and elution of analytes; and (iii) allows dispersion of the sorbent in the sample. As it can be seen in Figure 2, 10 mg of the c-TNTs are placed in a pipette tip section and confined by a cotton bead. Then, a 1 mL plastic syringe is connected to the tip for sorbent conditioning, sample loading, washing and elution steps. The dispersion of the solid takes place in the syringe body. By using this unit, limited-volume biological samples such as saliva can be processed. Moreover, the nanomaterial is easily recovered after extraction. The comparison of the performance of c-TNTs and MWNTs under the optimized conditions revealed that the c-TNTs exhibited a 2-fold extraction recovery thanks to its better dispersibility in aqueous and organic media.

Khodadoust et al. have proposed the use of NiZnS nanoparticles loaded on activated carbon (NiZnS-AC) as sorbent for the dispersive μ-SPE of carbamates pesticides from waters [18]. In this case, the complete dispersion of the nanoparticles (15.5 mg) requires the use of a vortex assisted platform (5.5 min) followed by ultrasonication (5.5 min). The extraction is carried out on a 3 ml end cap glass pipette fitted with a filter to isolate the solid from the liquid phase under vacuum. Elution is

carried out with acetone, a solvent changeover step to methanol being required prior to liquid chromatographic analysis. The hybrid nanoparticles were characterized in terms of surface area, pore volume and size and nanoparticle size. As far as the analytical features are concerned, the miniaturized extraction configuration allows the quantification of bendiocarb and promecarb in tap, river and mineral waters at the trace level.

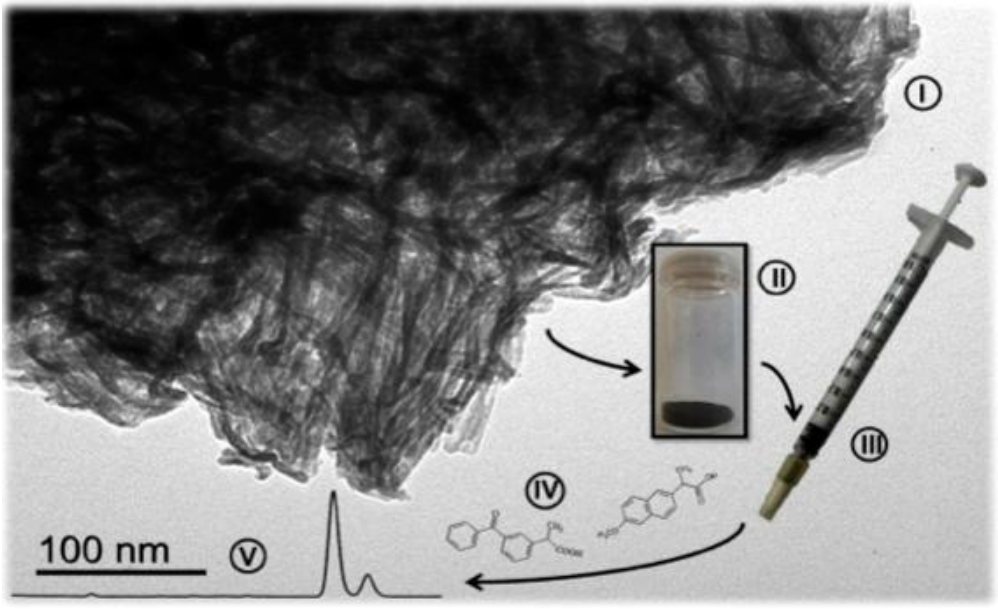

Figure.2. Schematic representation of the dispersive-µSPE procedure using c-TNTs. (I) TEM micrograph of the c-TNTs; (II) solid obtained after treatment; (III), proposed extraction unit; (IV) target analytes; and (V) HPLC-UV chromatogram.

3.4 Carbon-based material.

The discovery of the different allotropic nanostructured forms of carbon has been a cornerstone in many scientific areas, including analytical nanoscience and nanotechnology and analytical chemistry. The outstanding properties of carbon nanomaterials have been extensively pointed out in the literature [19]. In the specific

field of analytical chemistry, fullerenes, carbon nanotubes and nowadays graphene, have contributed to the development of more competitive (bio)chemical measurement processes. Additionally, the miniaturization of the whole procedures can be highlighted.

The incorporation of carbon nanostructures to miniaturized extraction devices has resulted in the development of more efficient microextraction units, thanks to the excellent sorbent properties of these nanomaterials. However, it is well-known that this property is inherent to the nanometric dimensions and, therefore, the aggregation of the nanoparticles must be avoided. This aggregation results from the insolubility of carbon nanomaterials in aqueous and organic media, being especially relevant for their reference allotropic from, carbon nanotubes. This fact limits their use in conventional extraction devices such as SPE cartridges or in miniaturized units which require the flow of sample through them as the presence of nanoparticle bundles (i) reduces the efficiency of the process; and (ii) results in system overpressure. No doubt, the use of miniaturized extraction devices which immobilized the carbon nanoparticles on an inert or active surface is the simplest alternative to exploit their sorbent properties in the microextraction context. Excellent reviews have been published on the topic [20,21].

Taking into account that the bundle formation is usually exhibited by as-grown carbon nanotubes, surface modification is a core procedure to prevent carbon nanoparticles from aggregation as well as to provide additional selectivity when they are used as extracting media. In the case of carbon nanoparticles, oxidation (to generate hydroxyl, carboxyl or carbonyl groups) and more scarcely functionalization with different active groups have been proposed to facilitate the use of carbon nanoparticles in dispersive μ-SPE by increasing their solubility in aqueous media.

The first application described in the literature in this context used MWNTs as reversed-dispersive sorbent in a conventional QuEChERS multiresidue analysis [22]. The proposed method follows the steps defined in DSPE but substituting the conventional

sorbent (PSA, GBC or RP-C_{18}) by 10 mg of MWNTs. The use of nanostructured material resulted in cleaner extracts compared to PSA even at the lower concentrations, with comparable recoveries. However, the better clean-up reduces the limits of detection up to 1 μg/g level. On the negative side, the non-reusability of the material and its relatively high cost should be noted.

Single walled carbon nanohorns (SWNHs) have been studied as sorbent in dispersive μ-SPE. They are smaller in size but tend to form stable aggregates of dahlia shape with an average diameter of 60-80 nm. These aggregates provide additional interaction sites which increase the adsorption capacity of the SWNHs. To increase the aggregates solubility in water and polar organic solvents, they can be oxidized by microwave irradiation [23]. For the dispersive μ-SPE, a dispersion of o-SWNHs at a concentration of 0.2 mg/mL in Milli-Q water was firstly prepared. It was sonicated for 60 min and centrifugated to remove potential non-functionalized material. The dispersion was tested using PAHs as model analytes and environmental waters as sample [24]. For extraction, 1 mL of the dispersion was added to 10 mL of water sample, being the vial stirred for 2 min at 1600 rpm, an homogeneous dispersion being immediately formed. The o-SWNHs enriched with the PAHs were retained on a 0.45 μm disposable nylon filter and the analytes were further eluted with 100 μL of hexane. The identification and quantitation of the analytes was carried out by gas chromatography-mass spectrometry. The performance of o-SWNHs was compared with that obtained with carbon nanocones and oxidized carbon nanotubes under the optimized experimental conditions. As a general conclusion, it can be said that the conical nanoparticles provided the highest recoveries thanks to their better dispersibility in water. Moreover, it was also observed the increased efficiency of the dispersive μ-SPE using carbon nanocones with increasing number of aromatic rings in the PAH molecule thanks to the larger dimensions of this nanostructure as regards SWNHs, which permits

the analyte interaction in the inner part of the nanoparticle. This fact difficulties the elution, giving a poor reproducibility as a result.

A green alternative to disperse carbon nanotubes in water is the previously described effervescence assisted μ-SPE. The potential of the CO_2 to avoid MWNTs aggregation was evaluated by Lasarte-Aragonés et al. using triazines as target analytes [25]. Neither oxidation/functionalization nor external energy source, such as ultrasounds, microwave or vortex is needed for sorbent dispersion. The effervescent tablet is prepared by mixing the carbon nanotubes with the effervescence precursors in a glass mortar and 250 mg of the homogeneous powder is compressed in a manual hydraulic press. Each tablet contains ca. 7.5 mg of carbon nanotubes. The dispersion of carbon nanotubes was evaluated under four experimental conditions, in which the sorbent and effervescence precursors were added in different formats (simultaneously, sequentially, in powder and in a tablet). As it can be seen in Figure 3, the best results were obtained when the ingredients were added in the form of effervescence tablet. Under these conditions, absolute recoveries in the range from 48% o 75% were achieved, which are too high for a microextraction technique.

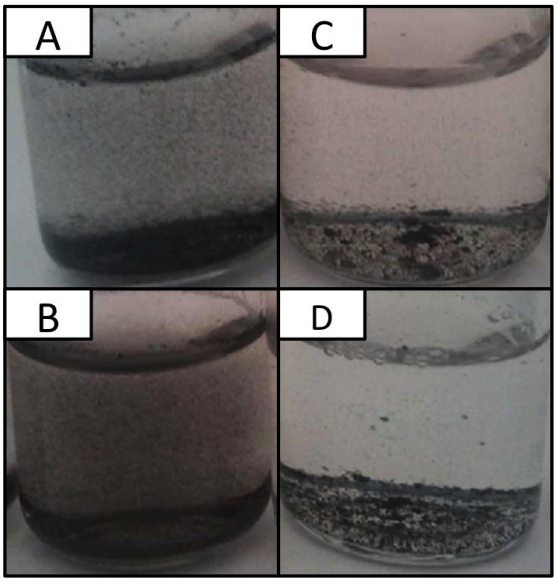

Figure 3 Dispersions obtained after the addition of the effervescence precursors and the carbon nanotubes in powder (A), both in a tablet (B), carbon nanotubes without the effervescence precursors (C), and the tablet containing the effervescence precursors and the carbon nanotubes added after tablet dissolution.

Carbon nanotubes have been extensively used for the determination of organic, aromatic compounds, as they can be easily retained by means of π-π interactions. However, inorganic species (e.g. metal ions) can also be preconcentrated previous formation of a chelate with an organic complexing agent. The final hydrophobic complex is adsorbed on the nanoparticle surface by means of Van der Waals forces and hydrophobic interactions. Both, multiwalled carbon nanotubes [26] and graphene [27] have been used in the dispersive format to determine metal ions at the trace and ultratrace levels by energy dispersive X-ray fluorescence spectrometry (EDXRF). In both applications, ammonium pyrrolidine dithiocarbamate (APDC) was selected as chelating reagent. Once retained, the nanoparticles enriched with the analytes were collected onto a filter and directly measured by EDXRF. Nickel, cobalt, copper and lead were extracted using this methodology. In the view of the results, graphene is better sorbent as lower limits of detection were obtained using even less

amount of sorbent (0.4 mg of graphene versus 1 mg of MWNTs) although it requires the presence of Triton X-100 for the extraction.

Carbon molecular sieves such as Carboxen have also been proposed as sorbent in dispersive μ-SPE. N-nitrosamines were first extracted from meat by microwave assisted extraction using 30 mL of 0.0025 M sodium hydroxide and further diluted up to 50 mL with deionised water prior to the dispersive procedure. In the second step, 100 mg of Carboxen were added and agitated in a vortex for 30 min at the maximum speed. After extraction, the solid with the analytes was separated in a filter and then eluted with 200 μL of dichloromethane and subjected to gas chromatographic-mass spectrometric analysis under chemical ionization mode. Two carbon molecular sieves, Carboxen 1000 and 1003 with different surface area and pore diameter were evaluated, being the former selected for the extraction. The authors conclude that the analytes were determined at the trace level with good precision, wide liner range and detection limits at the low ng/g, being a good alternative for the determination of nitrosamines in complex food matrices [28].

Finally, mesoporous carbon COU-2 has also been used for the determination of cloxacillin in waters [29]. The mesoporous materials are characterized by a high surface area, large pore volume, tunable mesoporous channels, well defined pore-size distribution and changeable surface properties [30]. COU-2 material presents a wormlike mesostructure with highly ordered mesopores [31]. For this application, 100 mg of COU-2 were added to 50 mL of sample and only agitation for 1 min was required for analyte extraction. Aliquots of 300 μL of methanol were used as eluent. The eluate was evaporated to dryness and reconstituted in water for HPLC analysis. The authors compared the performance of the mesoporous material with that of activated carbon. No peaks were obtained in this case owing to the strong interaction analyte-sorbent.

The high surface area and the mesopore volume of COU-2 facilitates the diffusion of the analyte from the solid to the eluent during desorption. The analytical

characteristics of the dispersive μ-SPE were comparable to those of conventional SPE by using less amount of sorbent.

3.5 Molecularly imprinted polymers

The use of selective sorbents is increasing in Analytical Chemistry, thans to the simplification of the whole (bio)chemical measurement process. Molecularly imprinted polymers (MIPs) are built-in functionalized material, specifically designed for the isolation of a given molecule (template), around which the sorbent is fabricated (printed). The synthesized solid is highly selective as retention depends on shape, size and functionality criteria. Nanoscience and nanotechnology have played a key role in this particular area, as the extraction efficiency of MIPs is increased when they are prepared at the nanoscale level, viz. nanoMIPs, thanks to the morphology and accessibility of the recognition sites. Several synthetic processes have been proposed for the fabrication of nanoMIPs. Among them, the use of composite beads is highly popular as it permits to control the particle size as the polymeric material (silica or latex) is used as core shell, being the recognition element coated on its surface as an homogeneous layer. Following this method, molecularly imprinted polymer layer-coated silica nanoparticles have been designed towards sulfonylurea herbicides [32] in soil and crop samples, 17β-estradiol [33] and oestrogens in milk samples [34]. The imprinted nanoparticles exhibited a high selectivity, large adsorption capacity and fast kinetics. The sorption capacity of the SiO_2-MIP is higher than that of the non-imprinted polymers.

Molecularly imprinted microbeads have also been used for the determination of sulfamethazine by capillary electrophoresis. The microparticles were prepared via precipitation polymerization using the analyte as template, methacrylic acid as functional monomer and ethylene glycol dimethylacrylate as cross-linking monomer.

The microparticles were highly dispersible in aqueous solution, so, they are

very convenient for dispersive μ-SPE. The extraction was carried out at room temperature, stirring the sample plus the MIPs-microbeads for 5 min. The sorbent was separated by centrifugation and the analyte was eluted by 300 μL of methanol:acetic acid [35]. The dispersive method is highly selective with relative recoveries between 89-110% in different milk samples. The sensitivity was increased ca. 300 times compared to direct CE-UV analysis.

4. Combined Dispersive Microextraction Techniques

The analysis of complex samples usually requires extensive sample treatments. This is unavoidable if the analytes are presented at the trace levels when, in addition to sample clean-up, high preconcentration factors are mandatory. To fulfill these requirements, sequential extractions are usually implemented.

Taking into account the high efficiency of dispersive techniques, they can be used in the same analytical process combining both modalities, liquid and solid phase microextraction. In this way, the limitations of both techniques are overcome. In general, the dispersive solid phase extraction is used as clean up while the dispersive liquid phase microextraction is used for analyte preconcentration. The combined used of both dispersive methodologies at the microscale level is very scarce, however.

Zhou et al. have proposed a one step sample preparation technique which combines solid and liquid dispersive microextraction for the determination of organochlorine pesticides (OCPs) in tobacco [36]. The so-called hybrid field assisted solid-liquid-solid dispersive extraction (HF-SLSDE) consists of three phases: two solids (sample matrix and dispersive sorbent) and one liquid (extraction solvent). The clean-up is carried out on the sorbent while the analytes are isolated and preconcentrated on the solvent. The term hybrid field refers to the auxiliary energies used during the extraction: ultrasounds (US), microwave (MW) and stirring. Their application is aimed

to accelerate the extraction process. The temperature generated in the extraction vessel is controlled by an infrared sensor.

Several dispersive sorbents, namely Florisil, neutral alumina, underivatized silica, diatomaceous earth and activated carbon were considered and the clean-up ability together with the analytes adsorption were jointly evaluated. As expected, silica and diatomaceous earth showed a low efficiency in interference removal while the adsorption on activated carbon was non-selective and low extraction yields of the analytes were obtained. Therefore, Florisil was the best dispersive sorbent in terms of clean-up ability and OCPs extraction yields. As far as the solvent is concerned, hexane, ethyl acetate, acetone and hexane:ethyl acetate (9:1, v:v) mixture were tested. The most polar solvents were able to elute polar impurities from Florisil, which results in dirty chromatograms. Therefore, hexane was selected as optimum.

The authors also compared the effect o the hybrid field (MW-US) with that of using the auxiliary energies separately. The lowest recoveries and the poorest precision were obtained when ultrasound irradiation was employed. The microwave assisted SLSDE provided intermediate results. That means that the influence of the temperature owing to microwave energy is more important than the increased pressure resulted from cavitation. However, the hybrib field (MW-US) is more powerful than the single field.

Another interesting issue studied by the authors is the microwave absorptive property of Florisil. The heating rate of the hexane with Florisil was ca. 1.8 times higher than that in absence of the sorbent. That means that it is capable of absorbing the radiation and diffunding the heat to the solvent. This result in a fast temperature rising, thus improving the extraction speed and efficiency. The analytical figures of merit obtained are good enough for the given analytical problem.

In the green chemistry context, the effervescence can also be used to disperse a few microliters of an organic solvent which is recovered by the aid of magnetic

nanoparticles after the extraction [37]. In this case, the combination of dispersive techniques results in a reproducible collection on the liquid phase avoiding the need for centrifugation. Moreover, the efficient dispersion of the extractant by means of the CO_2 generated in the sample matrix does not require additional organic phase which usually interferes in the extraction equilibrium by increasing the analyte solubility in the sample. The bare Fe_3O_4 magnetic nanoparticles can be easily synthesized in the laboratory. The extraction mixture is prepared as follows: 2.5 mL of glacial acetic acid (H^+ donor), 200 µL of 1-octanol (extractant) and 100 mg of bare Fe_3O_4 magnetic nanoparticles were sonicated for 5 min to achieve efficient homogenization. Aliquots of 250 µL of this mixture were used in each extraction. The procedure is schematically shown in Figure 4. For analytes isolation and preconcentration, the aqueous standard or the water sample was enriched with sodium carbonate (CO_2 source) and then, the extractant mixture was slowly added by means of a syringe, the needle being completely immersed in the sample. In this way, the extractant and the nanoparticles were homogeneously distributed inside the sample while the effervescence takes place (ca. 5 s). By using an external magnet, the 1-octanol coated magnetic nanoparticles with the extracted analytes were separated from the sample, the target compounds being eluted using 100 µL of methanol and the magnet for nanoparticles isolation. This procedure was more efficient than the sequential addition of the effervescence precursors and the liquid-solid extractant phase. Also, the substitution of the effervescence by a conventional solvent-aided dispersion using acetonitrile provided lower extraction recoveries. The enrichment factors achieved for the selected triazines were in the range 21-185 with the limits of detection low enough to determine these herbicides in environmental water samples.

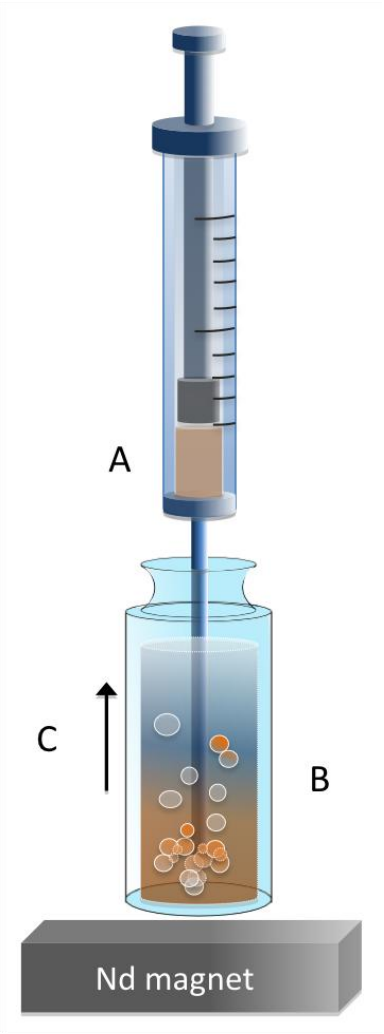

Figure 4. Schematic representation of the procedure followed for the effervescence assisted liquid phase microextraction. (A) 250 µL MNPs + 1-octanol in acetic acid (H^+ donnor); (B) Carbonated sample (CO_3^{2-} donnor); (C) Extractant mixture is inyected at the vial bottom and effervescence disperses the solvent up-through the sample

5. Future Trends

The applicability of dispersive micro solid phase extraction should be increased in the next years following two different research lines. First, the relevance of nanostructured solids in this context is unquestionable. Their outstanding sorbent properties yield high extraction recoveries using only a few milligrams of solid. The expected advances in Nanoscience and Nanotechnology will help to increase the applicability of dispersive micro solid phase extraction as the new nanostructured solids synthesized will be applied to novel analyte-sample binomials.

Next, the combination of dispersive micro solid phase extraction with other miniaturized extraction techniques is also a relevant research line. As it has been pointed out before, this strategy minimized or even overcomes the shortcomings of the individual dispersive technique. Up to now, the most successful combination uses a conventional DSPE with dispersive LPME. However, in order to miniaturize the whole analytical process, the sequential implementation of dispersive μSPE and LPME must be studied. Although the advantages of magnetic nanoparticles is unquestionable in this context, thanks to the simplification inherent, carbon-based, molecularly imprinted or polymeric nanostructured materials must be studied, as their potential is clearly demonstrated in the miniaturization and simplification of the analytical process [38].

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Capítulo II:

Effervescence-assisted dispersive micro-solid phase extraction

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Effervescence-assisted dispersive micro-solid phase extraction

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Abstract

Extraction techniques are surface dependent processes since their kinetic directly depends on the contact area between the sample and the extractant phase. The dispersion of the extractant (liquid or solid) increases this area improving the extraction efficiency. In this article, the dispersion of the sorbent at the very low milligram level is achieved by effervescence thanks to the in-situ generation of carbon dioxide. For this purpose a special tablet containing the effervescence precursors (sodium carbonate as carbon dioxide source and sodium dihydrogen phosphate as proton donor) and the sorbent (OASIS-HLB) is fabricated. All the microextraction process takes place in a 10 mL-glass syringe and the solid, enriched with the extracted analytes, is recovered by filtration. Acetonitrile was selected to elute the retained analytes. The extraction mode is characterized and optimized using the determination of five nitroaromatic compounds in water. The absolute recoveries of the analytes were in the range 61-85% while relative recoveries close to 100% in all cases, which demonstrates the absence of matrix effect on the extraction. These values permit the determination of these analytes at the microgram per liter range with good precision (relative standard deviations lower than 6.1 %) using ultra performance liquid chromatography (UPLC) combined with ultraviolet (UV) detection as instrumental technique.

Keywords: Dispersive micro-solid phase extraction, effervescence, nitroaromatic compounds

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1. Introduction

Extraction techniques play an important role in many analytical methodologies since they comprise the isolation and/or preconcentration of the target analytes from the sample matrix with the intrinsic selectivity and sensitivity improvements. The efficiency of those techniques is defined by thermodynamic and kinetic factors. In this sense, the distribution constant defines the potential transference of the target analyte from the donor (the sample) to the acceptor (the extractant) phase while the kinetic influences the rate at which this distribution takes place [1]. Solid phase extraction (SPE) techniques are surface-dependent processes as their efficiency directly depends on the particle size and the surface area of the sorbent [2]. In fact, in most of the cases, the potential extractability is not fully exploited due to the trend to aggregation of solid particles, which dramatically reduces their active area [3]. This behavior is more pronounced when the particle size is reduced to the nanoscale as it has been observed with carbon nanotubes [4] or magnetic nanoparticles [5]. Moreover, aggregation is critical when the amount of sorbent is reduced to a few milligrams, even micrograms, as the sorbent capacity is very limited.

In the SPE and μ-SPE context the disaggregation of the sorbent particles by dispersion increases the surface area of the extractant, improving the overall extraction efficiency. Based on this, Anastassiades *et al* proposed in 2003 the dispersive solid phase extraction (DSPE) technique [6], which was focused on selectivity improvement at first. DSPE has found its main application field in the multiresidue determination in food samples. DSPE is also known as QuEChERS, an acronym of its main characteristics: quick, easy, cheap, effective, rugged and safe. The extraction mode has been recognized by the Association of Official Analytical Chemists (AOAC) as an international method for multiclass pesticide determination in fruits and vegetables [7,8]. Against this traditional view, DSPE can be also employed for sensitivity enhancement. In this

approach, the analytes are extracted in the sorbent, being finally eluted or even determined in the sorbent by spectroscopic techniques [9,10].

DSPE procedures present two critical steps: the dispersion and the final phases´ separation. The dispersion of the sorbent into the sample, or its extract, is usually assisted by an external energy source and therefore special apparatus, like a vortex, are required. Moreover, the use of organic solvents has also been proposed for dispersion but these substances may interfere in the extraction process enhancing the solubility of the target analytes in the sample and therefore reducing the extraction efficiency [11]. On the other hand, phases´ separation is usually performed by centrifugation, which is very effective, but it makes the overall procedures time consuming. In this sense, the development of DSPE methodologies using magnetic nanoparticles [12,13] has simplified in a large extent this negative aspect. The development of new dispersion processes that avoid the use of organic solvents and even external apparatus could be an important research field that allows the development of on-site dispersive solid phase approaches, especially for environmental analysis. In fact, on-site solid phase extraction has been proposed as a simple way to ensure the sample integrity during transport and storage while simplifies both processes [14]. On-site SPE reduces the sample volume as only the sorbent with the extracted analytes, has to be stocked up [15].

Nitroaromatic compounds, such as 2,4-dinitrotoluene (as propellant) or nitrotoluenes (as explosives) are widely used in military related facilities, but also they are used in mining or building industry. They can be introduced in the environmental compartments by dumping practice, or leaching of unexploded ordnance [16]. Nitroaromatic compounds are very toxic and genotoxic even at low concentration to many biological groups, especially to mammals, in which some carcinogenic activity has been determined [17,18]. Their determination and quantification is especially important in zones where those activities take place. The matrix of the sample is

usually complex, and the compounds are heterogeneously distributed, so a time-consuming sampling process is required, followed by an extraction step. Due to their environmental relevance, the US-EPA (United States Environmental Protection Agency) develops a standard method for the determination of selected group of nitroaromatic compounds in waters and soils using LC-UV as instrumental technique [19].

This article deals with the use of effervescent tablets in dispersive micro-solid phase extraction. In this sense, the dispersion of the sorbent is assisted by the carbon dioxide produced when the tablet components are dissolved in water. In fact, the final product only produces an increase in the ionic strength that does not interfere with the extraction of the target analytes. The determination of selected nitroaromatic compounds (4-nitrotoluene; 1,4-dinitrobenzene; nitrobenzene; 2,4-dinitrotoluene and 4-nitrobenzaldehyde) in water samples has been selected as model analytical problem. First of all, the proposed configuration is optimized taking into account the tablet composition and the chemical variables. Finally the methodology is characterized in terms of linearity, sensitivity, precision and accuracy.

2. Experimental section

2.1 Reagents and samples

All reagents were of analytical grade or better. Nitroaromatic hydrocarbons (4-nitrotoluene; 1,4-dinitrobenzene; nitrobenzene; 2,4-dinitrotoluene and 4-nitrobenzaldehyde) were purchased from Sigma-Aldrich (Madrid, Spain). Stock standard solutions were prepared in acetonitrile (Panreac, Barcelona, Spain) at 200 mg L^{-1} and stored at 4°C. Working solutions of the target analytes were prepared by the appropriate dilution of the stocks in Milli-Q water (Millipore Corp., Madrid, Spain) or acetonitrile as required.

Water samples from the Genil River were collected in amber glass bottles without headspace and stored at 4°C until analysis.

Sodium dihydrogenphosphate and sodium carbonate purchased from Sigma-Aldrich, were used as components of the effervescent tablets.

OASIS HLB, purchased from Waters (Madrid, Spain) was employed as sorbent for analytes extraction. OASIS HLB is a polyvinylpyrrolidone-divinylbenzene copolymer with a surface of 830 m^2 g^{-1} and a particle size in the interval 30-60 μm.

2.2 OASIS HLB-effervescent tablets preparation

The effervescent tablets were prepared in different and well-defined steps. Initially, 837.5 mg of sodium dihydrogenphosphate and 412.5 mg of sodium carbonate, previously desiccated at 90°C for 2 h in an oven (Binder, Madrid, Spain), were mixed. Then, 25 mg (amount needed for 5 tablets) of OASIS HLB were added and the solids were manually blended in a glass mortar until a homogeneous and fine powder is obtained. An accurately weighted amount of 250 mg was compressed in a manual hydraulic press (Perkin-Elmer, Madrid, Spain) at 12 Ton for 60 min. The resulting tablets (102 mm ID) were stored in an inert atmosphere.

2.3 Extraction procedure

The extraction procedure, which is schematically presented in Figure 1, consists of various well-defined steps. First of all, the tablet is introduced in a 10 mL glass-syringe, which will act as extraction unit. Then, 10 mL of sample are aspirated into the syringe where the effervescence of the tablet takes place. During this process, the OASIS HLB sorbent is effectively dispersed into the sample enhancing the contact between the sample and extractant. After this, the sorbent is recovered by filtration, being subsequently dried. The analytes are eluted by means of 500 μL of acetonitrile, which are collected in a 2 mL glass vial for the further UPLC® analysis.

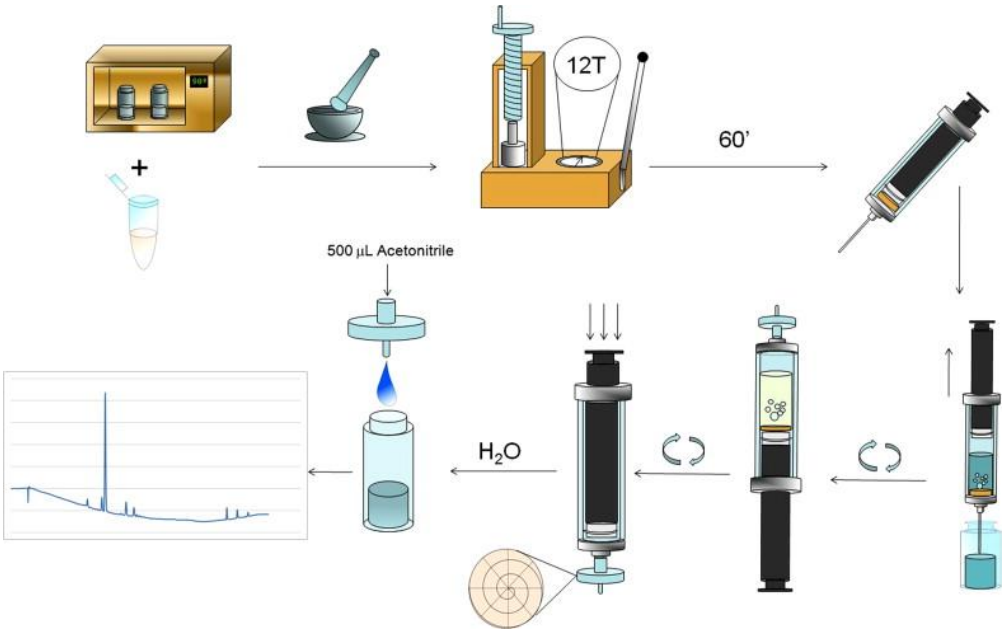

Figure 1. Description of the general analytical procedure of the effervescence-assisted dispersive micro-solid phase extraction.

2.4 Chromatographic separation

Chromatographic analyses were carried out on a Waters-Acquity™ Ultra Performance LC system (Waters Corp., Madrid, Spain) using an Acquity UPLC® BEH C18 column (1.7µm, 2.1 mm x100 mm) maintained at 54°C. The mobile phase consisted of (A) water and (B) acetonitrile at a flow rate of 0.5 mL min^{-1} using a gradient elution program. The initial composition was fixed at 90 % A, the percentage being decreased to 25 % in 7.5 min. The injection volume was 1 µL with partial loop mode. The separated analytes were determined using a PDA eλ (extended wavelenght) Detector (Waters). System control was achieved with Empower software.

3. Results and discussion

Effervescent tablets have three main components, namely: (a) a carbon dioxide source; (b) a proton donor compound and (c) an active compound, which is pretended to be delivered/dispersed. The mixture of the two first components in water releases carbon dioxide that favors the solubility/dispersion of the active compound. This effect, which is evaluated in the present article, may be exploited in dispersion based solid phase microextractions to accelerate the kinetic of the process.

3.1 Composition of the sorbent effervescent tablets

The composition of the effervescent tablet is a key-aspect in the overall procedure. For their fabrication, sodium carbonate (C) and sodium bicarbonate (Bi) were selected as carbon dioxide sources while citric acid (Ci), ascorbic acid (A) and sodium dihydrogenphosphate (P) were evaluated as acid reagents. Six tablets, considering all the possible combinations were prepared by triplicate (n=3), maintaining the appropriate ratio on account of stoichiometry of the corresponding chemical reaction. The total weight of the tablet was 250 mg, as it was the maximum capacity for the press. For the evaluation of the extraction capability of each tablet, 1 mg of OASIS HLB sorbent was added and mixed with the effervescence precursors.

Tablets should be moisture free in order to maintain the effervescent capacity and therefore an initial thermal treatment, desiccation of the ingredients, is required. It was observed that ascorbic and citric acids were less stable at temperatures higher than 90°C. For this reason, the use of both organic acids may require more complex drying processes (e.g. inert atmospheres, low temperatures and longer times). Moreover, as it is well known, carbonate is more thermally stable than bicarbonate.

In the same way, the stability of the tablets, including their hygroscopicity, is also a critical aspect. This parameter was evaluated at three different times (24, 120 and 190 h), studying the evolution of the tablet weight in the laboratory atmosphere.

The results, which are shown in Table 1, indicate that the combination between sodium dihydrogenphosphate and sodium carbonate present the lower weight variation. This aspect is quite important since the effervescent activity is affected by the hygroscopicity of the tablet. In this sense, moisture reduces and even inhibits the effervescence as it was observed for example for Ci-C and P-Bi mixtures after 192 h. This time can be extended by their storage under an inert atmosphere.

Table 1. Main characteristics of the tablets fabricated using different effervescent components and 1mg of OASIS-HLB.

Composition		Characteristics				
Carbon dioxide source	Proton donor	Eff. Time[a]	ΔpH[b]	Stability (%ΔW)[c] 24 h	120 h	192 h
Sodium carbonate	Citric acid	1.3	1.5	0.43	1.01	1.25
Sodium bicarbonate	Citric acid	1.8	1.1	0.52	0.81	0.81
Sodium carbonate	Ascorbic acid	2.0	1.2	-0.23	-0.14	-0.52
Sodium bicarbonate	Ascorbic acid	2.3	0.6	-0.29	1.71	0.90
Sodium carbonate	Sodium dihydrogenphosphate	4.5	0.2	0.55	0.52	0.55
Sodium bicarbonate	Sodium dihydrogenphosphate	19.0	-0.7	0.87	2.18	4.80

[a] Eff. Time: effervescent time in minutes, [b] ΔpH: pH increment after the tablet effervescence,[c] ΔW: weight variance of the tablet at different times. Expressed as percentage.

Effervescent time was also studied since it defines the extraction time. The values (see Table 1) were in the interval between 1.26 to 19 min depending on the composition of the tablet. In principle, the influence of the effervescent time on the extraction efficiency may be contradictory since a faster effervescence involves a vigorous dispersion but also a lower contact time between the sorbent and the analytes. In order to evaluate the effect of tablet composition on the extraction efficiency, six aqueous standards containing nitrobenzene at a concentration level of 500 μg L^{-1} were extracted using all the possible tablets with 1 mg of OASIS HLB. The

results are presented in Figure 2. According to the chromatographic analysis, the P-C combination provides the best extraction efficiency in a reasonable time. A deep study of the data shows a linear relation between the peak area and the effervescent time for Ci-C, Ci-Bi, P-C and A-C combinations. In this range, larger extraction times involve higher recoveries, which indicate a strong enough effervescence. By contrast, very low extraction efficiency is observed for P-Bi due to the poor dispersion of the sorbent. Only the A-Bi combination has an unexpected behavior.

Finally, the pH variation after the effervescent process was also studied. The results, which are also shown in Table 1, indicate negligible variations.

According to all the results the P-C combination was selected as the optimum tablet composition.

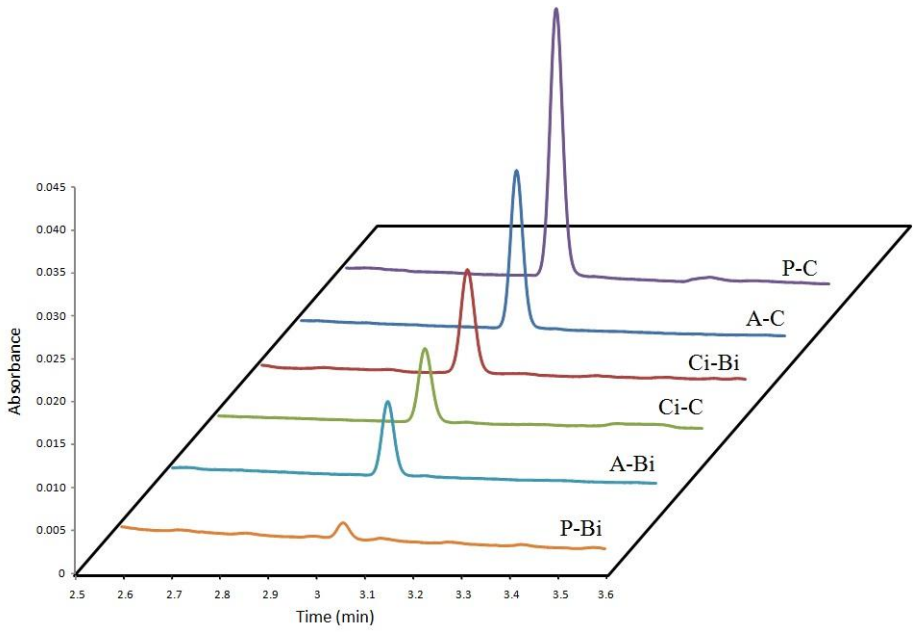

Figure 2.Effect of the effervescent mixture on the extraction of nitrobenzene as model analyte. C, sodium carbonate; Bi, sodium bicarbonate; Ci, citric acid; A, ascorbic acid; and P, sodium dihydrogenphosphate.

3.2 Amount of OASIS HLB sorbent and elution process

Once selected the components of the effervescent mixture, the amount of sorbent in each tablet was studied in the interval from 1 to 10 mg. As expected, the analytical signal increase when higher amount of sorbent were used. However, the integrity of the tablets is directly affected by the quantity of OASIS HLB solid as the particles reduce the cohesion between the tablet components. For this reason, the amount of OASIS HLB was fixed to 5 mg as a compromise between sorbent capacity and tablet stability.

Acetonitrile and methanol were evaluated as eluents of the extracted analytes from the OASIS HLB particles, the first one providing the best recoveries. The elution volume was studied in the interval from 50-1000 µL, being the maximum peak areas obtained for 500 µL. The main aim of this article is to propose an efficient method for analytes isolation based on a dispersive procedure. For this reason, no further improvements (for example evaporation and reconstitution in a lower volume) were considered in the elution process.

3.3 Analytical figures of merit

Once optimized, the proposed procedure was evaluated, facing up a specific model problem, such as the determination of five nitroaromatic explosives (namely, 4-nitrotoluene; 1,4-dinitrobenzene; nitrobenzene; 2,4-dinitrotoluene and 4-nitrobenzaldehyde) in waters. The main figures of merit are summarized in Table 2. A calibration graph was constructed for each analyte by extracting in triplicate six working aqueous standards containing all the analytes at different concentrations in the range from 10 µg L^{-1} to 1 mg L^{-1}. The limits of detection, which were calculated using a signal-to-noise ratio of 3, varied between 1.8 µg L^{-1} (4-nitrobenzaldehyde) and 7 µg L^{-1} (nitrobenzene). The repeatability of the method was evaluated at 50 µg L^{-1} in triplicate with results (expressed as relative standard deviation) in the range from 0.5 %

(2,4-dinitrotoluene) to 6.1% (4-nitrotoluene). The inter-day precision was evaluated at the same concentration level using 5 independent extractions, the result being better than 8.6% for almost all the analytes. The absolute recovery of the method was also calculated for each analyte. It refers to the percentage of total analyte that can efficiently extracted by the sorbent and finally eluted with acetonitrile. The values obtained are in the interval of 61-85%. These values are between the exhaustive extraction of SPE and the low values of microextraction techniques (even below 5% in solid phase microextraction).

Table 2. Figures of merit for the determination of the selected nitroaromatic compounds in water.

Analyte	Linear range (µg/L)	R[a]	LOD[b] (µg/L)	RSD[c] (%)		Absolute extraction Recovery (%) ± SD[d]
				Intra-day	Inter-day	
4-nitrotoluene	15-1000	0.999	4.5	6.1	8.6	61 ± 4
1,4-dinitrobenzene	15-1000	0.998	3.7	0.9	1.8	78 ± 1
nitrobenzene	20-1000	0.999	7.0	1.2	2.2	74 ± 1
2,4-dinitrotoluene	20-1000	0.999	6.1	0.5	3.1	83 ± 2
4-nitrobenzaldehyde	10-1000	0.999	1.8	1.3	1.7	85 ± 3

[a] R: regression coefficient, [b] LOD: limit of detection, [c] RSD: relative standard deviation, [d]SD: standard deviation

In order to validate the proposed method, a river water sample was analyzed. As a result, no signals for the target analytes were obtained. Then, a recovery test was carried out by spiking the water sample with the five analyte at two concentrations levels (50 and 200 µg L^{-1}) each one being analyzed by quintuplicate. The results are listed in Table 3. As it can be seen, for all the analytes the recoveries were next to 100% regardless the concentration studied. This demonstrates the absence of interferences from the sample matrix.

Table 3. Analysis of a river water sample spiked with the target nitroaromatic compounds

Analyte	Concentration added (µg/mL)	Recovery (%±SD[a], n=5)	Concentration added (µg/mL)	Recovery (%±SD[a], n=5)
4-nitrotoluene	50	101±4	200	103±4
1,4-dinitrobenzene	50	100±6	200	100±2
nitrobenzene	50	96±4	200	100±2
2,4-ninitrotoluene	50	97±4	200	97±2
4-nitrobenzaldehyde	50	96±5	200	94±5

[a] SD, Standard deviation

3.4 Final evaluation of the effervescence-assisted dispersive µ-SPE approach

First of all, the proposed methodology was compared with conventional solid phase extraction. Taking into account the small amount of sorbent, the SPE procedure was developed immobilizing 5 mg of HLB in a syringe filter similar to the employed in the effervescence assisted extraction. For comparative purposes, the potential retention of the target analytes in the filter was also considered. Moreover, the ionic strength and pH of the sample were fixed at the same value for all the experiments. The comparative chromatograms as well as the experimental conditions are described in Figure 3 for 1,4-dinitrobenzene and 4-nitrobenzaldehyde. These assays were performed at a final concentration of 50 µg L^{-1}, and each one was made by triplicate. According to the results, the effervescence-based procedure enhances (ca. 3 times) the signal of the target analytes due to an efficient dispersion of the sorbent. On the other hand, the filter does not contribute to the extraction of the nitroaromatic compounds.

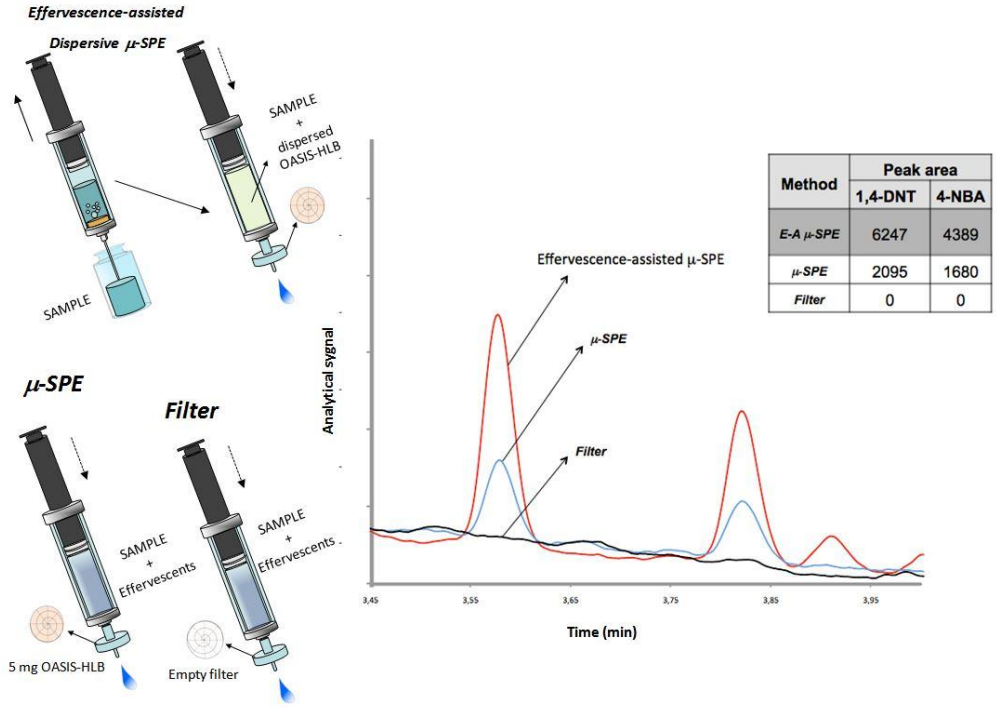

Figure 3. Comparison of the effervescence-assisted dispersive micro-solid phase extraction with conventional solid phase extraction. For comparative purposes, the contribution of the filter is also shown.

Finally, the effervescence-assisted procedure was compared to the conventional dispersive procedure, in which a vortex stirrer is employed for the sorbent dispersion. As shown in Figure 4, the effervescence-assisted extraction provides a better analytical signal not requiring any additional apparatus to be performed. The facility of dispersion of a given sorbent directly depends on its particle size among other factors. In this sense, nanometric particles have a high trend to aggregation while micrometric particles are easily dispersed. Although Oasis HLB sorbent (particle size in the range 30-60 μm) corresponds to the latter class, the effervescence even improves the dispersion. The use of nanoparticles will be the focus

of our further investigations since this type of material will be a challenge for the effervescence performance.

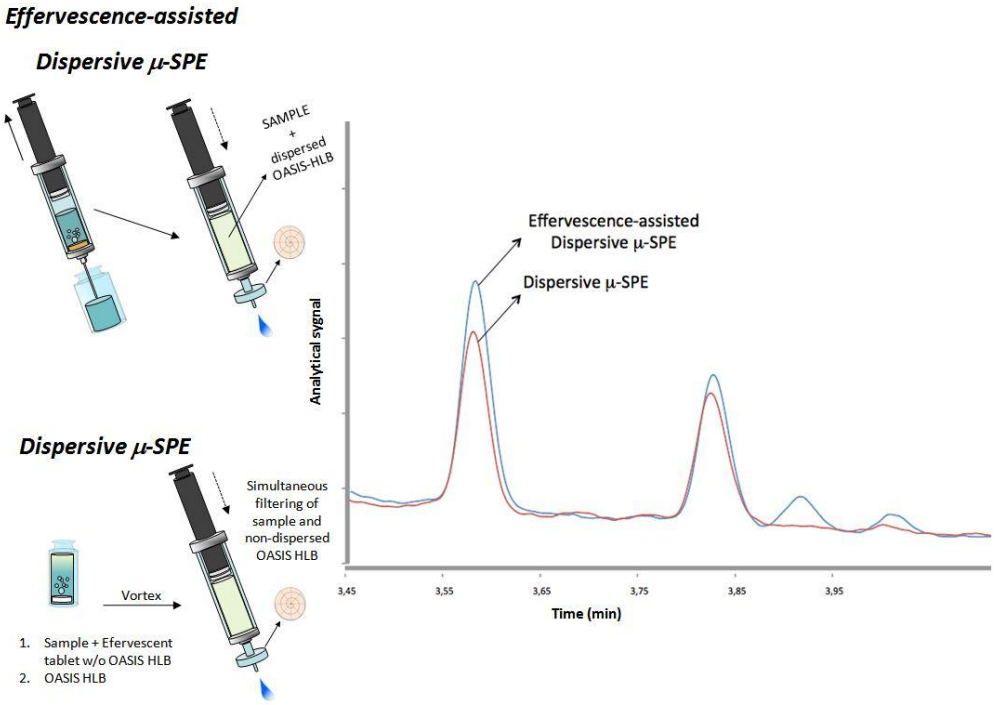

Figure 4. Comparison the effervescence-assisted dispersive micro-solid phase extraction with the conventional dispersive procedure.

4.Conclusions

In this article a new extraction procedure, called effervescence-assisted dispersive micro-solid phase extraction, is presented and characterized. The new approach is very simple since the extraction is performed using a simple syringe, a filter and a lab-prepared effervescent tablet. The tablet preparation requires ca. 1 hour, which can be considered as a limitation of the method. However, this time does not

affect to the overall analysis time as the tablets can be prepared in advance and maintained under an inert atmosphere until their use.

The simplicity of the manifold may facilitate the on-site extraction of environmental samples making easier their storage, transport and even favoring the stability of the target analytes. The new approach is also versatile since the composition of the tablets, including the effervescent compounds and the sorbent, can be changed according to the given analytical problem.

The proposed method can be compared with other analytical methodologies described for the determination of nitroaromatic compounds in waters (Table 4) in terms of sensivity, precision and recoveries (absolute and/or relative). In this sense, the effervescence-assisted μSPE provides a limit of detection comparable with those which use in fiber-SPME. The best sensivity is achieved with conventional SPE. In this particular case, up to 500 mL of sample can be loaded in the extraction cartridge packed with 500 mg of sorbent and the analytes were eluted using 5 mL of acetonitrile.

Concerning the microextraction techniques, the use of PDMS/DVB fiber [21] allows the detection of the analytes at the very low microgram per liter level using 20 mL of sample. The extraction procedure requires ca. 1 h instead of the 5 min needed in the EA-μSPE approach. Regarding the recoveries, the present method is the best option among the available alternatives.

Table 4. Comparision of the proposed effervescence microextraction method with reported approaches for the determination of nitroaromatic compounds in water

Method	Sample volume (mL)	Extraction time (min)	Recovery (%)	LOD[a] (μg/L)	RSD[b] (%)	Ref
SPME-LC-UV	25	65	67.2 - 121.9	1 - 10	10.2 - 27.2	[16]
SPME-LC-UV	6	30	n/a	0.6 - 120	1.2 - 4.8	[20]
SPME-LC-UV	20	62	85.5 - 97.2	0.17 - 0.92	1.5 - 3.5	[21]
SPE-LC-UV	500	n/a	75.9 - 100.3	0.03 - 0.29	3.1 - 13.12	[22]
EA-μSPE-LC-UV	10	5	94 - 103	1.8 - 7.0	1.8 - 8.6	Present method

[a]LOD: limit of detection, [b]RSD: relative standard deviation, n/a: not available

The presented method has been analytically characterized in terms of sensitivity, precision and recovery. We are aware that the limits of detection are not outstanding for the target analytical problem. Different alternatives will be evaluated to face-up this initial limitation such as:

(a) The use of larger sample volumes, which will require the design of new extraction vessels.

(b) The use of lower elution volumes, which directly depends on the final analytical instrumentation. The elution process can be enhanced by the design of new interfaces that eventually allow the on-line elution of the analytes and their direct injection in the instrument.

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Capítulo III:

Effervescence assisted carbon nanotubes dispersion for the micro-solid phase extraction of triazine herbicides from environmental waters

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ORIGINAL PAPER

Effervescence assisted carbon nanotubes dispersion for the micro-solid phase extraction of triazine herbicides from environmental waters

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Abstract

Extraction techniques are surface dependent processes since their kinetic directly depend on the contact area between the sample and the extractant phase. The dispersion of the extractant (liquid or solid) increases this area improving the extraction efficiency. In this article, the dispersion of a nanostructured sorbent at the very low milligram level is achieved by effervescence thanks to the in-situ generation of carbon dioxide. For this purpose, a special tablet containing the effervescence precursors (sodium carbonate as carbon dioxide source and sodium dihydrogenphosphate as proton donor) and the sorbent (multiwalled carbon nanotubes, MWCNTs) is prepared. All the microextraction steps take place in a glass beaker containing 100 mL of the sample. After the extraction, the MWCNTs, enriched with the extracted analytes, are recovered by vacuum filtration. Methanol was selected to elute the retained analytes. The extraction mode is optimized and characterized using the determination of nine herbicides in water samples as model analytical problem. The absolute recoveries of the analytes were in the range 48-76% while relative recoveries were close to 100% in all cases. These values permit the determination of these analytes at the low microgram per liter range with good precision (relative standard deviations lower than 9.3 %) using ultra performance liquid chromatography (UPLC) combined with ultraviolet (UV) detection.

Keywords: Effervescence assisted dispersion, multiwalled carbon nanotubes, triazines, water samples.
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Effervescence assisted carbon nanotubes dispersion for the micro-solid phase extraction of triazine herbicides from environmental waters

1. Introduction

Carbon-based nanoparticles have been extensively used as sorbent materials in both solid-phase extraction (SPE) and solid phase microextraction (SPME) approaches due to their exceptional properties such as their large surface area and their ability to interact with target analytes by different mechanisms [1, 2]. Moreover, the wide variety of carbon allotropes, each one characterized by their own properties, makes these nanostructures a very versatile sorptive material. Carbon nanotubes (CNTs) are by far the most exploited carbon-based nanoparticles in the extraction context [3] although other allotropes have also been used including fullerenes [4, 5], nanodiamonds [6], graphene [7], carbon nanocones [8, 9] and carbon nanohorns [10, 11]. Carbon nanotubes, which were firstly described by Ijima as a sub-product in fullerene synthesis [12], consist of a graphite sheet rolled up to create a tube with a high length-diameter ratio. Owing to their structure and composition, CNTs may interact with target analytes by an array of interactions comprising, among others, hydrogen bonding, π-π interactions and van der Waals forces [1]. In addition, their easy carboxylation allows the introduction of carboxylic groups on the surface, which can be further derivatized to incorporate special functional groups, extending their application field [13].

Although CNTs have been successfully used in conventional SPE, based on the sorbent packing into a cartridge [14, 15], their aggregation tendency limits their potential since it involves a dramatic reduction of the real superficial area and also produces back-pressure on the extraction devices [16]. On one hand, the immobilization of the CNTs in the surface of an inert support, like silica microparticles [17], or their location in a filter minimize the negative effect of the aggregation in the extraction yield [18]. On the other hand, the efficient dispersion of the CNTs in the

sample matrix is also a valuable alternative to take advantage of the potential of this material in sorptive extraction.

Dispersive solid phase extraction (DSPE), which was first proposed by Anastassiades *et al.*, is a consolidated sample treatment technique especially for the multiresidue determination in food analysis [19]. This technique, which is also known by the acronym QuEChERS (for quick, easy, cheap, effective, rugged and safe), is mainly focused on the interferents removal and therefore on selectivity enhancement. However, the dispersion of low amounts of sorbent in the sample can be also employed to isolate the target analytes with a view to improving the sensitivity [20]. The use of CNTs in DSPE necessarily involves their efficient disaggregation, which is extremely tricky when aqueous samples are processed. CNTs disaggregation can be achieved by the chemical modification of their surface either by the covalent immobilization of polar functional groups [21] or by wrapping this surface with surfactants [22, 23] or especial polymers [24]. On the other side, the dispersion can be also assisted by an external energy source, like ultrasounds, which preserve the original surface of CNTs [25] although long irradiation times may produce imperfections on the structure [26]. Carbon nanostructures have been extensively used for extraction and preconcentration of many different analytes [27] from waters samples, including triazines [28].

Triazines are used as herbicides to control of broad leaf and grassy weeds in agricultural crops around the world. These herbicides are soil-applied with half-lives in the range of weeks to several months and they can arrive to aquatic media. Due to their high toxicity triazines have been included on the list of human carcinogens, while simazine and its metabolites present serious adverse effects such as hormone disrupting, birth defects, reproductive cancers and weight loss of mother and embryos during gestation [29-31]. Triazines are persistent pollutants in the environment even when they are used at very low concentrations due to their high solubility and to their

special physicochemical properties. These properties involves their strong interaction with the soil by forming hydrogen bonds between the triazine amine groups and the electronegative centers presents in the organic matter of the soil such as quinone, ketonic, and aldehyde groups [32]. Their control and monitoring in environmental waters is desirable and it has been usually performed by gas chromatography-mass spectrometry (GC-MS) [33], high performance liquid chromatography combined with UV [34] or MS [35], and capillary electrophoresis [36]. Due to their very low concentration level in natural or drinking water samples and the low limits imposed by the European Union (EU) and the United States Environmental Protection Agency (US-EPA), a sample preconcentration is required. Solid phase extraction (SPE) has demonstrated effectiveness thanks to its high enrichment factors and low organic solvent usage. Followed by LC-UV it has been extensively used in the determination and quantification of triazine herbicides with reasonable sensitivity in the past decade [37]. The SPME alternative avoids the major disadvantages of the SPE, reducing even more the use of expensive organic solvents and the amount of sorbents and permits to obtain higher enrichment factors to achieve the low detection limits imposed by regulatory agencies [34].

In this article, effervescence assisted dispersive micro-solid phase extraction (Dμ-SPE) [38] is evaluated to achieve the disaggregation of CNTs in an aqueous media. In this case, carbon dioxide release is used as in-situ dispersive force able to produce the dispersion of unmodified CNTs avoiding the usage of any organic solvent or surfactant. The different variables involved in the extraction process, especially the sorbent amount and sample volume, are considered in depth in the optimization process. The approach is finally characterized and compared with other published methodologies for the same analytical problem.

2. Experimental

2.1 Reagents and samples

All reagents were of analytical grade or better. Sigma–Aldrich (Madrid, Spain) provided the triazine herbicides: simazine, (SMZ); simetryn, (SMT); atrazine, (ATZ); secbumeton, (SBM); prometon, (PMT), terbumeton, (TBM); propazine, (PPZ); prometryn, (PMT) and terbutryn (TBT). Stock standard solution for each analyte was prepared in acetonitrile (Panreac, Barcelona, Spain) at a concentration 1000 mg L^{-1} and stored in the dark at 4ºC. Working solutions of the target analytes were prepared by the appropriate dilution of the stocks in Milli-*Q* water (Millipore Corp., Madrid, Spain) or acetonitrile as required.

Sodium dihydrogenphosphate, sodium carbonate and multi-walled carbon nanotubes (purity >95%, diameter in the range of 6-9 nm and average length of 5 µm) were purchased also from Sigma–Aldrich and they were used for the preparation of the effervescent tablets.

Three water samples were used to evaluate the method performance including river water (Genil River; Córdoba, Spain), well water (Montalban; Córdoba, Spain) and tap water (Córdoba, Spain). All samples were collected in amber glass bottles and stored at 4º until analysis.

2.2 Apparatus

Effervescence precursors were desiccated for 2 h at constant temperature of 90 ºC in an oven (Binder, Madrid, Spain) prior to the tablet fabrication which was performed using a manual hydraulic press (Perkin- Elmer, Madrid, Spain).

Vacuum filtration was performed in a glass filter holder (Millipore Corp, Madrid, Spain) with PTFE tape membrane (100 µm in thickness and 0.5 µm of normal pore size) in order to recover MWCNTs after extraction.

The elution process was carried out inside an ultrasonic bath (model 3510, Branson, Connecticut, USA) with a glass syringe (SGE Analytical Science, Melbourne, Australia) caped with a 0.45 µm syringe nylon filter to avoid the loss of MWCNTs which could potentially damage the UPLC® system.

Chromatographic analyses were carried out on a Waters-AcquityTM Ultra Performance LC system (Waters Corp., Madrid, Spain) using an Acquity UPLC® BEH C18 column (1.7 µm particle size, 2.1 mm × 100 mm) maintained at 45°C. The mobile phase consisted of (A) water and (B) acetonitrile at a flow rate of 0.5 mL min^{-1} using a gradient elution program. The initial composition was fixed at 75% A, the percentage being decreased to 40% in 10 min. The injection volume was 1 µL with partial loop with needle overfill mode. The separated analytes were determined using a PDA eλ (extended wavelength) detector (Waters) at 223 nm. System control was achieved with Empower software.

2.3 Carbon nanotubes-effervescent tablets preparation

The effervescent tablets were prepared in different and well-defined steps according to our previous work [38]. Initially, 1625 mg of sodium dihydrogenphosphate and 825 mg of sodium carbonate, previously desiccated at 90 ºC for 2 h in an oven were mixed. Then, 75 mg (amount needed for ten tablets) of MWCNTs were added and the solids were manually blended in a glass mortar until a homogeneous and fine powder was obtained. An accurately weighted amount of 250 mg, per tablet, was compressed in a manual hydraulic press at 12.5 ton for 15 min. The resulting tablets (102 mm ID) were stored in an inert atmosphere or immediately used for extraction.

2.4 Extraction procedure

The extraction procedure, which is presented in Figure 1, is as follows. First of all, the tablet is introduced in a 250 mL glass beaker containing 100 mL of the aqueous standard or the sample. As a consequence of the dissolution and reaction of the effervescent compounds, carbon dioxide is released dispersing the MWCNTs into the sample. After sonication (1 min), the MWCNTs containing the extracted analytes are recovered by filtration in a polytetrafluoroethylene (PTFE) tape (which was previously conditioned and washed with 10 mL of methanol). Vacuum was applied during 5 min to dry the tape before the elution of the analytes.

In order to elute the extracted analytes, the PTFE tape containing the MWCNTs is recuperated and introduced in an empty glass syringe (10 mL). Later on, 2.5 mL of methanol are aspirated and got into close contact with the filter. The elution is assisted by ultrasounds (1 min). The eluate is finally passed through a 0.45 μm disposable syringe filter to remove potential particles. The extraction and elution steps take ca. 13 min to be performed.

To improve the sensitivity of the determination, the eluate is dried under a nitrogen stream and the residue is redisolved in 100 μL of methanol and placed on a "Total Recovery ®" vial (Waters Corp.) for UPLC® analysis.

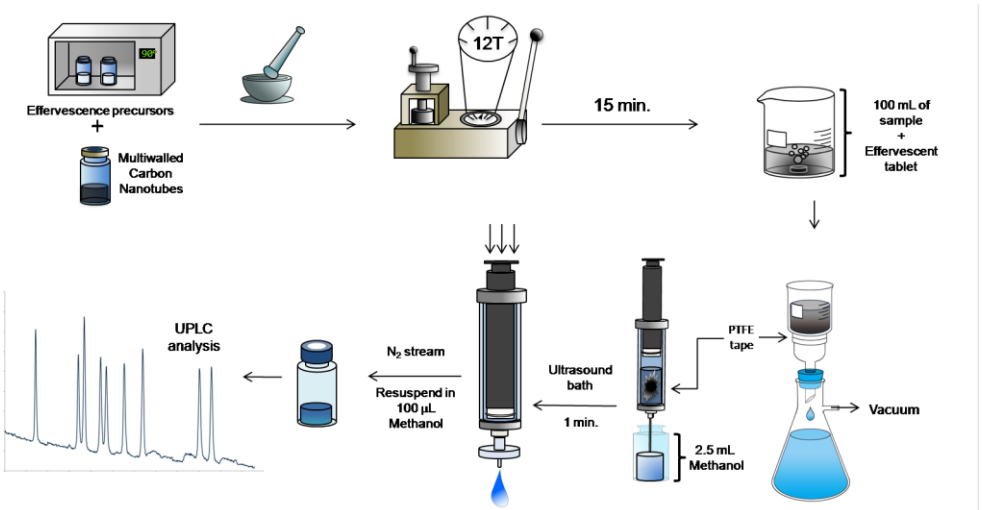

Figure 1: Description of the extraction procedure. For details, see text.

3. Results and discussion

The effervescent tablets employed in this Dμ-SPE have three main components, namely: (a) a carbon dioxide source; (b) a proton donor compound and (c) an active sorbent, which is intended to be dispersed. The appropriate selection of the three elements is crucial in order to achieve a good isolation and enrichment of the target analytes. In a previous study, the combination of sodium carbonate (as carbon dioxide source) and sodium dihydrogenphosphate (as proton donor compound) in stoichiometric ratio, provides a product with a minimum higroscopicity, a high mechanical stability and minimum pH variation during dissolution [38]. Moreover, this composition offers an adequate effervescence time, which finally determines the contact time between the sorbent and the analytes. A brief discussion regarding the tablet composition is presented in supplementary material (Table S1).

In this article, multiwalled carbon nanotubes (MWCNTs) are used as sorbent. Taking into account their aggregation tendency, the dispersion of the material is

mandatory. Figure 2 presents the pictures obtained for the dispersion of 5 mg of MWCNTs in 5 mL of water under different experimental conditions, namely:

(A) The dispersion was performed adding a powder that contained sodium carbonate (162.5 mg), sodium dihydrogenphosphate (82.5 mg) and MWCNTs (5 mg).
(B) The dispersion was performed adding a tablet (compressed powder) that contained sodium carbonate (162.5 mg), sodium dihydrogenphosphate (82.5 mg) and of MWCNTs (5 mg).
(C) The dispersion was performed adding directly 5 mg of MWCNTs.
(D) The dispersion was performed in two steps. First, a tablet containing sodium carbonate (162.5 mg) and sodium dihydrogenphosphate (82.5 mg) was added to the vial. After the effervescence, 5 mg of MWCNTs were added to the vial.

Before taking the photographs, the vials were mechanically agitated in a vortex during 1 min, and they left to stand during 2 min. As it can be seen, the best dispersions were achieved when the effervescence precursors and MWCNTs were added together. Moreover, the tablet (Figure 2B) provided a better dispersion compared to the powder (Figure 2A) due to the close contact between effervescence precursors and MWCNTs. As it was expected, MWCNTs were poorly dispersed in water (Figure 2C) and aqueous solution containing the effervesced precursors (Figure 2D). This fact corroborates that it is the effervescent process, and not the final ionic strength, the responsible for the efficient dispersion of the MWCNTs.

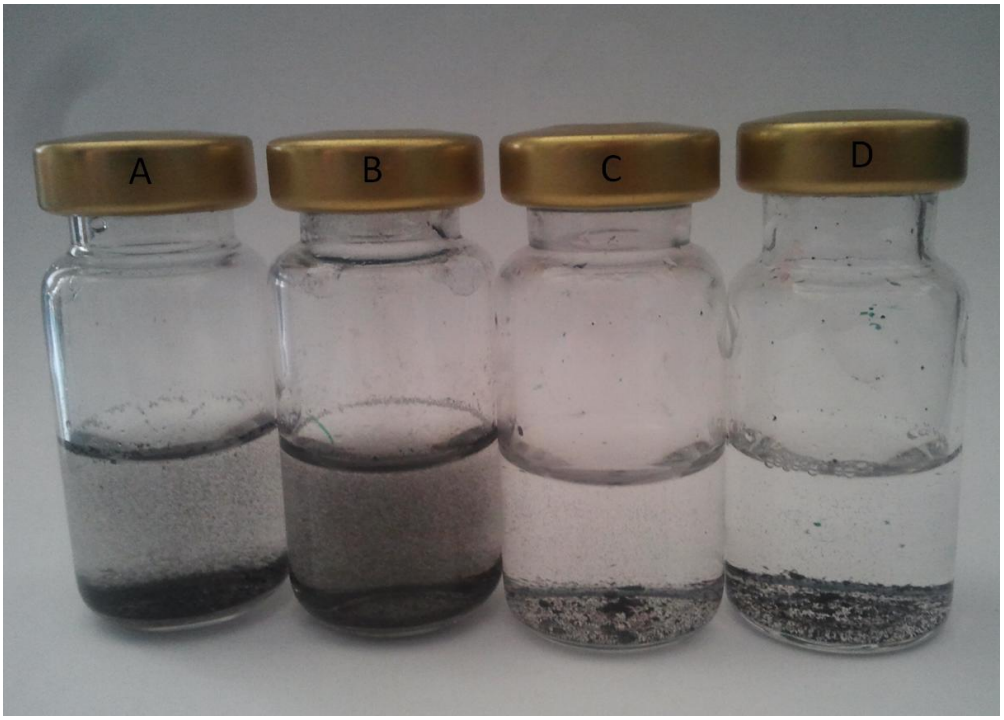

Figure 2: Dispersion of the MWCNTs under different experimental conditions: A, B, C and D. For further details, see text

3.1 Amount of carbon nanotubes and sample volume

The sample volume and the sorbent amount are crucial in any extraction SPE procedure. Moreover, both variables are usually connected, as when higher sample volumes are processed, higher amounts of sorbent are required in order to effectively isolate the analytes. For this reason, both variables were optimized together considering five different sample volumes (10, 25, 50, 100 and 250 mL) and five different amounts of MWCNTs (0.5, 2.5, 5.0, 7.5 and 10 mg). The maximum sample volume studied was limited to 250 mL in order to have an acceptable sample throughput. The optimization process was performed taking into consideration all the target analytes but for simplicity Figure 3 only shows the results obtained for simazine.

The results obtained for the rest of the analytes are presented in the supplementary material section (Figures S-1, S-2, S-3, S-4, S-5, S-6, S-7 and S-8). Although each analyte provided a different analytical signal, depending on its extraction efficiency and instrumental sensitivity, the results presented a similar tendency when sample volume and sorbent amount were studied. The optimum conditions are slightly different for all the analytes and they are finally selected as a compromise.

On the one hand, Figure 3A shows the univariant effect of the MWCNT amount on the extraction of the target analyte. In this case, the sample volume is 100 mL while the signals for different sorbent amounts are presented. As it was expected, the analytical signal increases with the sorbent amount since the extraction capacity directly depends on the quantity of MWCNTs. In fact, the analytical signal increases linearly up to 7.5 mg, although this linear relationship is not observed for higher sorbent amounts. This fact, which can be ascribed to a less efficient dispersion of the sorbent, makes the absolute extraction recovery lower when 10 mg of MWCNTs are used. According to these results, 7.5 mg of MWCNTs was selected as the optimum value, thus reducing the sorbent requirement.

On the other hand, Figure 3B shows the univariant effect of the sample volume on the extraction of the target analytes. In this graph, the amount of MWCNTs is 10 mg while different volumes of an aqueous standard containing the analyte at 500 μg L^{-1} are presented. As it can be observed, the signal increases linearly up to 100 mL although this linear relationship is not observed for higher sample volumes. This fact, which can also be ascribed to a less efficient dispersion of the MWCNTs, makes the absolute extraction recovery lower when 250 mL of sample are processed. According to these results, 100 mL was selected as the optimum value. This selection also simplifies the storage of the samples until their analysis.

Finally, Figure 3C shows the bivariant effect of both the sample volume and the MWCNTs on the analytical signal by means of a contour surface graph. This graph corroborates the previously explained behaviors.

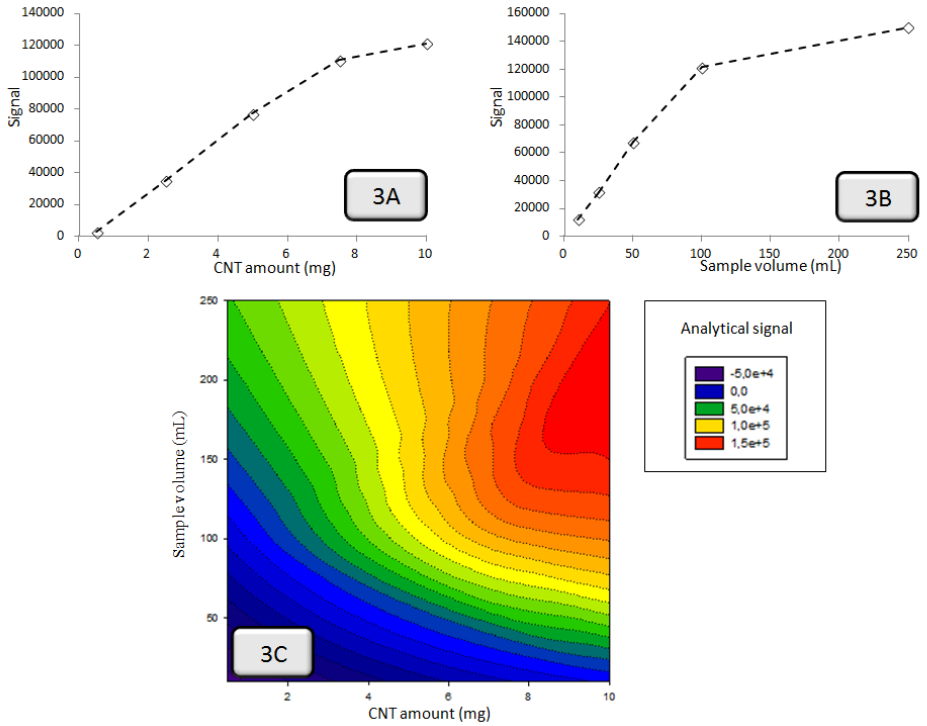

Figure 3: Effect of the sorbent amount (A) and sample volume (B) on the analytical signal obtained for a standard solution containing simazine at 500 µg L^{-1}. Data presented in (A) are obtained maintaining the sample volume fixed at 100 mL while data presented in (B) are obtained maintaining the sorbent amount fixed at 10 mg. The bivariant effect of both variables is also shown (C).

3.2 Extraction time

In this procedure, the extraction time is defined as the time lap between the addition of the tablet to the sample and the final recovery of the MWCNTs. This time comprises the effervescent process, the sonication of the sample and the final filtration

of the MWCNTs. On the one hand, the effervescent time depends mainly on the dissolution of the tablet components and it lasts ca. 3 min. On the other hand, the filtration depends on the vacuum pressure and it takes ca. 5 min for 100 mL of sample. Finally, the sonication time was studied in a wide interval (from 1 to 5 min), and a negligible effect was observed. This fact indicates a quick extraction of the analytes by the MWCNTs showing the high efficiency of the dispersion process.

3.3 Elution process

Once the analytes have been conveniently extracted, they should be eluted for the subsequent chromatographic analysis. Poor elution values were observed when the eluent was directly passed though the filter containing the retained MWCNTs. This aspect was ascribed to the non-homogeneous distribution of the sorbent in the PTFE tape due to the low amount of MWCNTs. In fact, the sorbent makes difficult the flow of the solvent, and therefore the solvent tends to pass through those zones with a less amount of MWCNTs. In such conditions, the contact of the eluent and the sorbent is limited.

For this reason, the elution was performed using an ultrasonic bath. After the extraction, the PTFE tape with the MWCNTs is transferred to a syringe and an appropriate volume of eluent is aspirated. The syringe is sealed and submerged in an ultrasonic bath for one minute, which is enough to achieve the elution. Three different solvents (methanol, acetonitrile and acetone) were evaluated as eluents. The results showed that methanol provided the best elution results not only in the absolute recovery values but also in their precision. In fact the average precision for the analytes elution with methanol was ca. 5.1 %, expressed as relative standard deviation (RSD), while the precision values obtained for acetonitrile and acetone were 8.3 % and 9.8 % respectively.

Finally, the elution volume was studied bearing in mind that the final extract will be evaporated for sensitivity enhancement. Therefore, the volume should be higher enough to provide an efficient elution but lower enough to allow a rapid evaporation. The elution volume was studied in the interval from 1 to 10 mL, 2.5 mL being finally selected as a compromise between both effects.

3.4 Analytical figures of merit

Once optimized, the proposed procedure was evaluated, facing up a specific model problem, such as the determination of nine triazine herbicides (namely, simazine, simetryn, atrazine, secbumeton, prometon, terbumeton, propazine, prometryn, and terbutryn) in waters. The main figures of merit are summarized in Table 1. A calibration graph was constructed for each analyte by extracting in triplicate six working aqueous standards containing all the analytes at different concentrations levels. The limits of detection, which were calculated using a signal-to-noise ratio of 3, varied between 0.15 μg L^{-1} (simazine) and 0.40 μg L^{-1} (terbumeton). The repeatability of the method was evaluated at two different concentration levels: closer to the limit of quantification of each analyte and at 10 μg L^{-1}. The results, which are shown in Table 1 and expressed as RSD, shows values in the range of 8.2-13.9 % and 3.9-9.3% in both conditions, respectively.

Moreover, Table 1 summarizes the absolute extraction recovery (AER) values obtained with the proposed extraction procedure. If these data are represented against the logarithm of the octanol/water partition coefficient ($logK_{ow}$), a linear relationship can be obtained (R=0.97) for SMZ, SMT, ATZ; PPZ, PMT and TBT. This relationship suggests an interaction based on dispersion forces. However, the AER are not clearly related to the logKow for SBM, PMT and TBM. Interestingly, the latter compounds present a similar chemical structure containing a methoxy functional group.

Table 1: Analytical figures of merit for the determination of the selected triazine herbicides in water using effervescence assisted microsolid phase extraction.

Analyte	LOD[a] (µg L^{-1})	LOQ[b] (µg L^{-1})	Linear range (µg L^{-1})	R[c]	Precision expressed as RSD[d] (%) at LOQ[b] (n=3)	Precision expressed as RSD[d] (%) at 10 µg L^{-1} (n=5)	AER[e] (%)
Simazine	0.15	0.50	0.5-100	0.999	10.8	7.9	48
Simetryn	0.35	1.20	1.2-100	0.991	8.2	6.8	56
Atrazine	0.30	0.90	0.9-100	0.999	11.8	5.8	61
Secbumeton	0.25	0.90	0.9-100	0.998	10.1	6.8	61
Prometon	0.15	0.50	0.5-100	0.998	13.8	4.9	49
Terbumeton	0.40	1.25	1.3-100	0.999	13.9	4.1	70
Propazine	0.30	1.00	1-100	0.997	9.1	3.9	60
Prometryn	0.20	0.65	0.7-100	0.999	9.5	9.3	68
Terbutryn	0.35	1.15	1.2-100	0.998	9.5	7.0	75

[a] LOD, limit of detection, [b] LOQ: limit of quantification, [c] R: regression coefficient, [d] RSD: relative standard deviation, [e] AER: absolute extraction recovery

Figure 4 shows a typical chromatogram obtained for the extraction of a river sample spiked with the nine analytes at 10 µg L^{-1}. A small overlapping of the SMT-ATZ and SBM-PMT peaks appear for this sample. This overlapping, which can be due to the sample matrix, does not appear in the rest of the standards and samples.

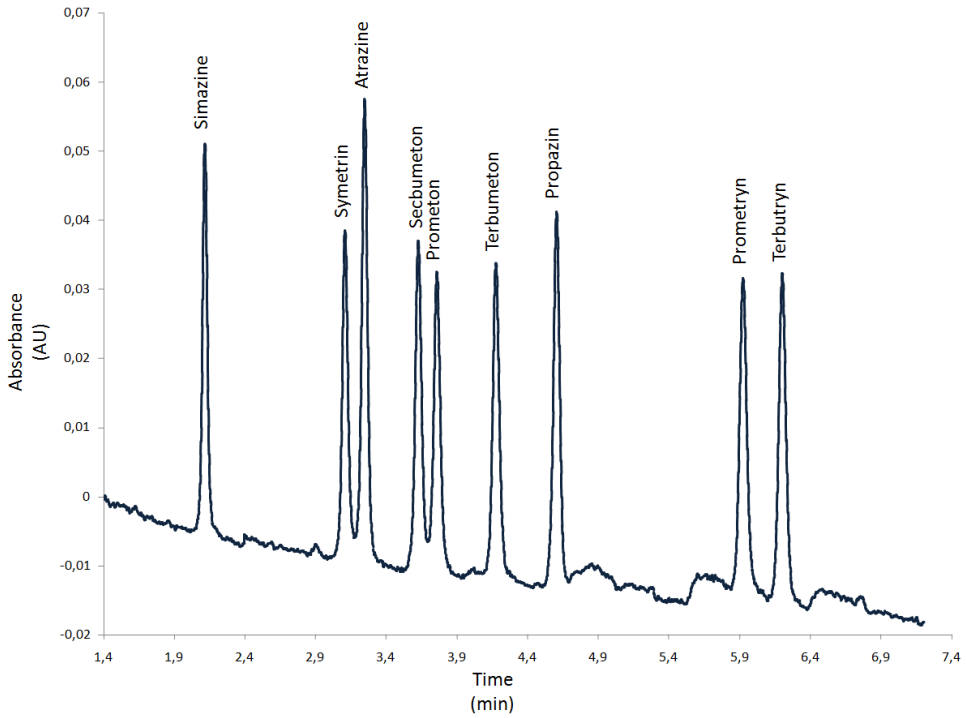

Figure 4: Typical chromatogram obtained for the extraction of a river water sample spiked with the target analytes at a concentration of 10 μg L^{-1}

3.5 Analysis of water samples

In order to evaluate the applicability of the proposed method for the determination of triazine herbicides from waters, three different water samples (river, tap and well) were analysed. As a result, no herbicides were found on the samples. Then, the samples were spiked with the selected herbicides at 10 μg L^{-1} and each sample was analyzed in triplicate. The results obtained are listed in Table 2. As it can be seen, the relative recoveries fulfil the 70-130% recovery criterion [39], except for propazine in tap water samples. The reduction of the recovery in propazine, as well as

for simazine and atrazine, in chlorinated water has been observed for other authors [40]. The results shows potential of the proposed effervescence assisted Dμ-SPE method for the extraction of the triazines from natural environmental waters.

Table 2: Relative recovery study performed on real samples spiked with the analytes at a concentration of 10 μg L^{-1}.

	Water sample (R%[a]±SD[b])		
Analyte	River	Tap	Well
Simazine	92± 7	77± 3	93 ± 9
Simetryn	101±9	91± 7	106 ± 11
Atrazine	101±13	82 ± 6	97 ± 7
Secbumeton	109 ± 12	97 ± 12	98 ± 13
Prometon	108 ±7	86 ± 8	98 ± 17
Terbumeton	89± 10	89 ± 4	94 ± 10
Propazine	85± 7	63 ± 5	94 ± 12
Prometryn	92 ± 9	72 ± 6	101 ± 9
Terbutryn	87 ± 15	72 ± 7	93 ± 11

[a]R%: Extraction Recovery, [b]SD: Standard Deviation (n=3)

4. Conclusions

In this paper, an effective approach for the dispersion of MWCNTs in μ-SPE is proposed. In this case, effervescent assisted Dμ-SPE extraction is employed for the extraction of nine herbicides from water samples. The dispersion, which is achieved by the in situ generation of a carbon dioxide stream, does not require a previous derivatization of the nanostructured sorbent or the use of especial reagents (organic solvents or surfactants). The dispersion process enhances the interaction between the sorbent and the analytes, which results in a good extraction efficiency with absolute recoveries in the range from 48 to 75 %. The whole procedure is very simple since it

uses common labware. In this sense, the recovery of the MWCNTs with the extracted analytes is achieved using commercial PTFE tape as filter, which reduces the cost per analysis.

On the negative side, the use of ultrasounds is required to achieve the elution of the analytes from the MWCNTs retained in the filter. This fact limits the development of a completely on-site methodology and it will be considered in further investigations. In addition, when high sample volumes are processed the use of ultrasound improves the dispersion of the sorbent. The extraction process takes ca. 13 min to be developed which is a little bit longer than conventional SPE approaches. However, the present approach requires less material (7.5 mg of sorbent) than its SPE counterparts and allows the efficient dispersion of MWCNTs.

The proposed method fulfills the sensitivity requirements established by US-EPA which demands a limit in the range from 1 to 10 μg L^{-1} for selected herbicides in municipal drinking waters samples). However, the methodology should be improved to respond to the more restrictive EU requirements which establish a limit of 0.1 μg L^{-1} for individual herbicides and 0.5 μg L^{-1} for total herbicides content for drinking waters and 1-3 μg L^{-1} for surface waters. This improvement should be focused on the instrumental technique (e.g MS) or related variables (e.g larger injection volumes) since the AER (and therefore the preconcentration factors) obtained with the proposal are near to the maximum values.

Finally, Table 3 compares the proposed method with other counterparts proposed for the resolution of the same analytical problem [28, 33-35, 41-42]. These approaches cover a wide range of extraction and instrumental techniques as well as target analytes. For simplicity, the discussion has been focused on the determination of simazine and atrazine, which are considered in all these approaches. According to the results, the new proposal is one of the most rapid extraction procedures, which could

be interesting when a high number of samples should be processed. Moreover, the accuracy and precision levels are comparable with the other approaches.

The sensitivity can be considered the weak factor in this comparison, as it was previously indicated, since some alternatives provide lower detection limits. This fact can be explained if the instrumental technique is considered in the discussion. On the one hand, the use of MS as instrumental technique, combined both with GC [28, 33, 42] or LC [35], provides the best sensitivity levels. On the other hand, the combination of liquid chromatography with UV detection [34, 41] results to be the less sensitive alternative. In this sense, the new proposal provides similar or better detection limits than those given by the latter approaches.

Table 3: Comparison of the proposed extraction procedure with other published methods for the extraction of simazine and atrazine from water samples.

Extraction technique	Sample volume (mL)	Sorbent amount (mg)	Sorbent type	Elution step	Instrumental technique	Extraction time (min)	LOD[a] (μg L^{-1})	Relative Recovery (%)	Precision	Ref
Dispersive μ-SPE	10	0.2	oxidized single wall carbon nanohorns	Methanol (200 μL)	GC-MS	2	0.06-0.1	72-105	<9.3[b]	[28]
SPE	300-1000	360	C18 cartridge	Ethyl Acetate (2x2mL)	GC-MS	>30	0.005-0.00167	90.5	3.5[b]	[33]
SPME	10	n/a	Graphene coated steel wire	Acetone (50 μL)	HPLC-DAD	>30	0.2	88-94.4	3.3[b]	[34]
SPE	1000	250	OASIS HLB	10 % Methanol in methyltertbutyl ether	HPLC-MS	>200	0.0001-0.0005	92.7-95	---	[35]
SPME	3	n/a	Fiber PDMS/DVB or CW/TPR	Acetonitrile/acetate buffer (35:65)	HPLC-UV	>20	1.5-3.3	84.4-112.9	<8.8[b]	[41]
SPE	200	30	MWCNT on disk filter	Acetone (5mL)	GC-MS	n/a	0.0025-0.005	87-110	<6.9[b]	[42]
Effervescence assisted MWCNT dispersion	100	7.5	MWCNT	Methanol (2.5mL)	UPLC-DAD	3	0.15-0.3	77-101	<7.9[b]	Present method

[a] LOD, Limit of Detection, [b] RSD values, [c] SD values

Acknowledgments

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Effervescence assisted carbon nanotubes dispersion for the micro-solid phase extraction of triazine herbicides from environmental waters

Supplementary Material

1. Tablet composition.

The effervescent tablet is composed by three different substances, namely: the carbon dioxide source, the proton donor compound and the sorbent which is intended to be dispersed. In this composition, the two first elements play a key role since they affect to the effervescence performance, that is, to the sorbent dispersion efficiency. Moreover, the tablet composition has a great influence in other collateral, but

important, aspects such as its hygroscopicity, the pH variation observed in the sample as a consequence of the tablet dissolution, and the chromatographic baseline which is directly affected by the final products formed during the effervescence. In our previous work [38], we studied in depth the tablet composition using different carbon dioxide precursors and proton donor compounds. The combination of sodium carbonate and sodium dihydrogenphosphate in stoichiometric ratio provided the best results which are summarized in Table S-1

Table S-1: Characteristics of the effervescent tablet

Components	Sodium carbonate, sodium dihydrogenphosphate
Proportion	Stoichiometric
Effervescence time	4.5 min
ΔpH	0.2
Hygroscopicity (%ΔW at 72 h)	0.55

In this case, the tablet has a slight influence on the sample pH which can be interesting for the extraction of ionisable compounds. The hygroscopicity is also negligible and it is constant at least in the period of time evaluated (from 24 to 72 hours). Special attention should be given to the effervescence time since it influences not only the extraction time but also the dispersion efficiency. The selected tablet provides a very good compromise between both aspects.

2. Effect of the sample volume and sorbent amount on the extraction of the analytes

For brevity, the manuscript only presents the data obtained for simazine which is selected as representative analyte. Although all the analytes presented similar behaviors, the optimum conditions are slightly different for all of them. In the following figures, the data obtained for all the analytes assayed are presented.

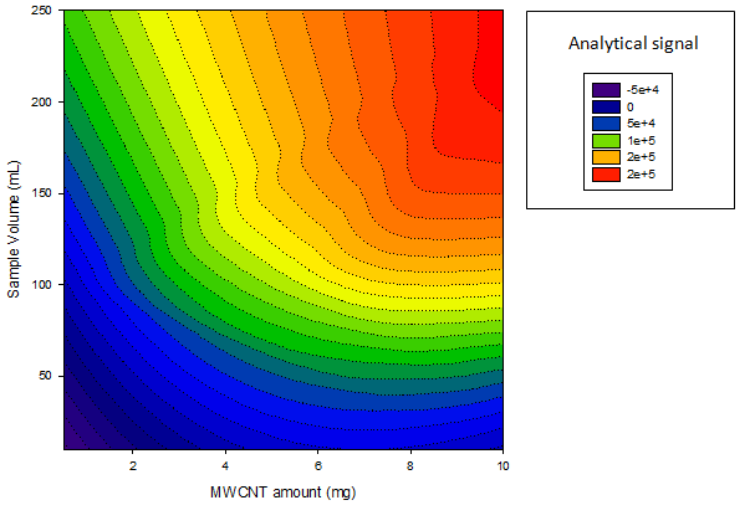

Figure S-1: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing simetryn at 500 µg L^{-1}.

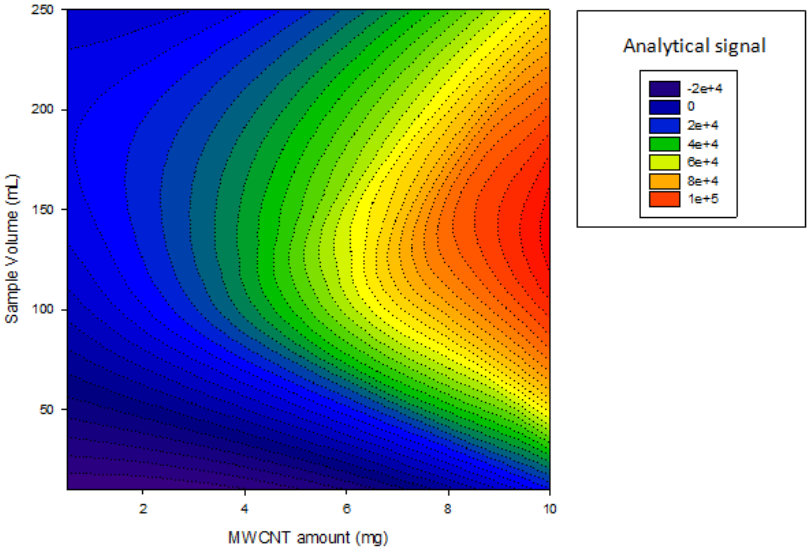

Figure S-2: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing atrazine at 500 µg L^{-1}

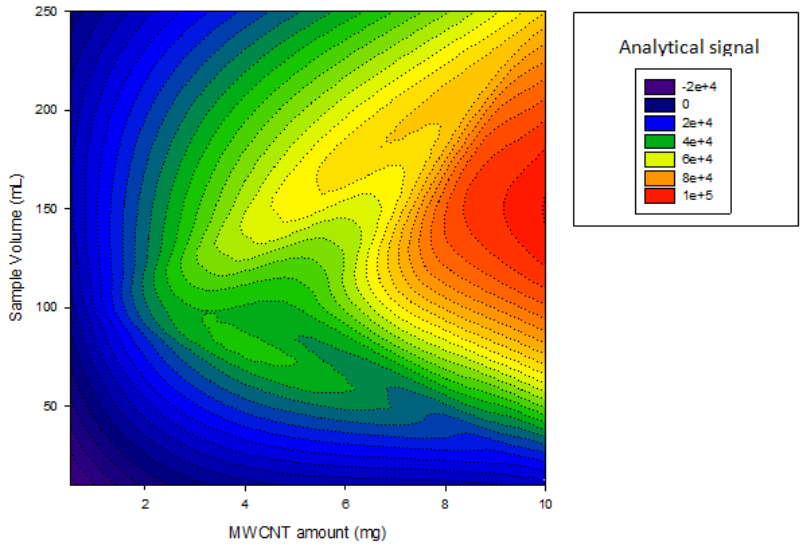

Figure S-3: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing secbumeton at 500 µg L^{-1}

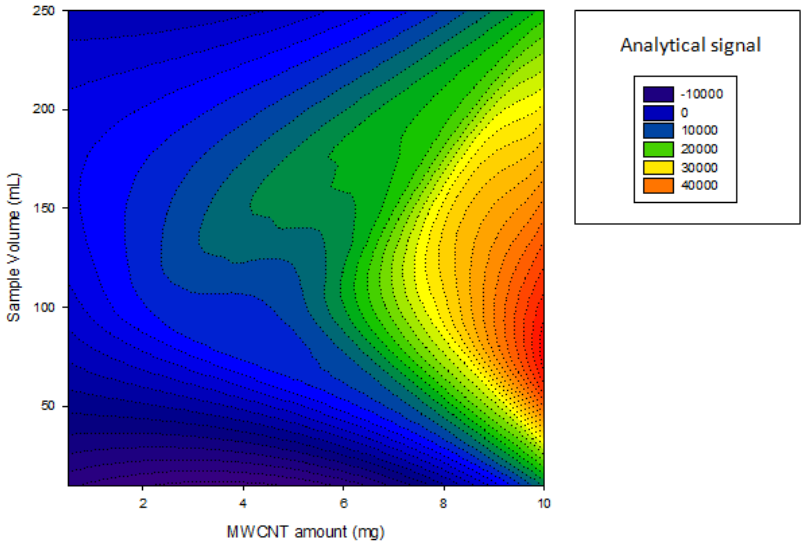

Figure S-4: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing prometon at 500 µg L^{-1}.

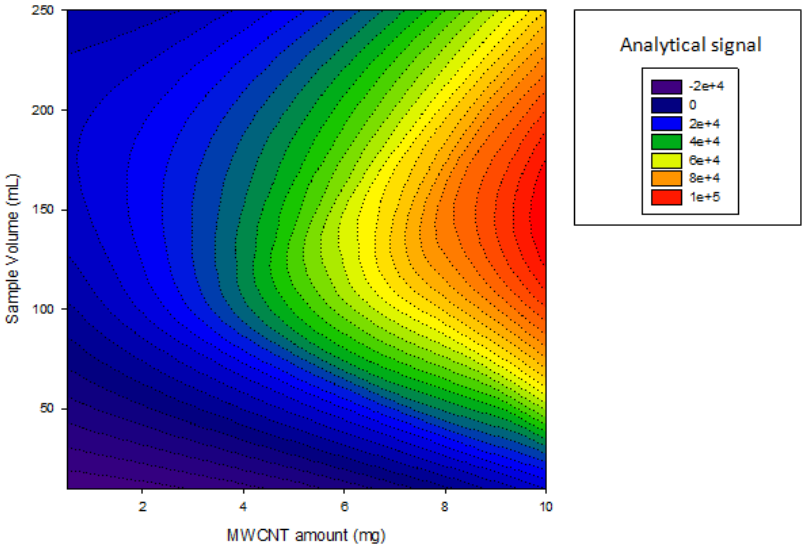

Figure S-5: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing terbumeton at 500 µg L^{-1}.

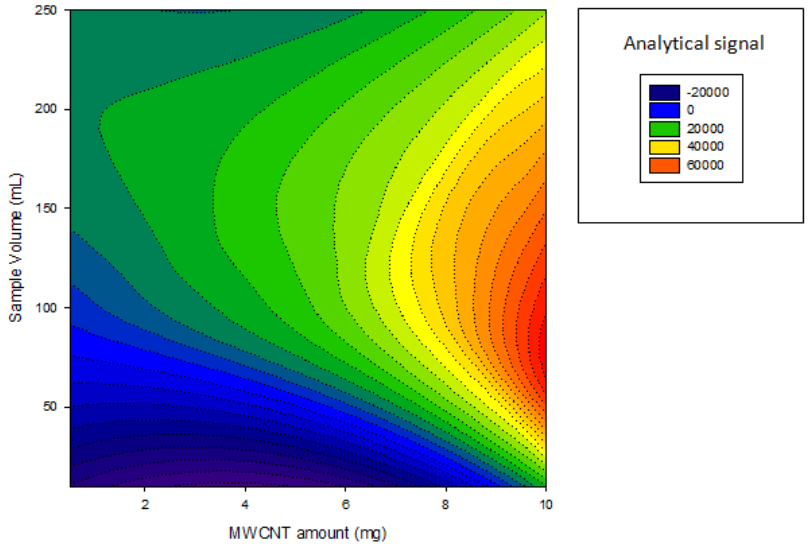

Figure S-6: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing propazine at 500 µg L^{-1}.

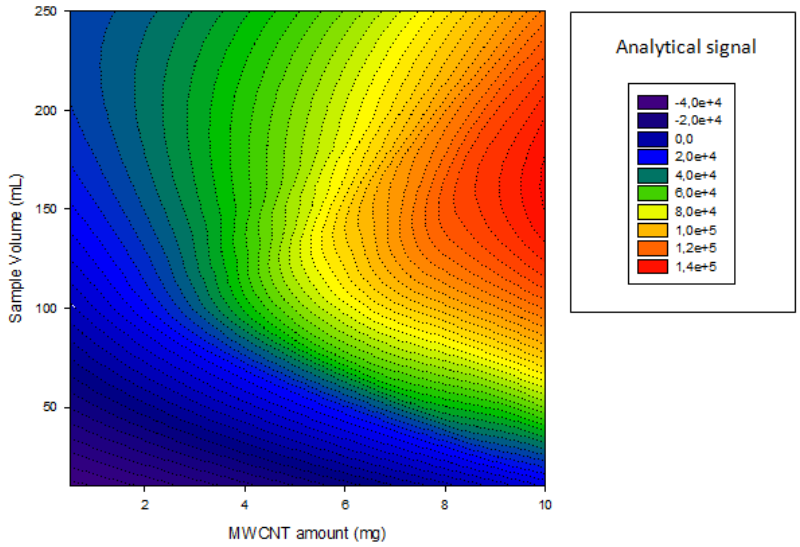

Figure S-7: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing prometryn at 500 µg L^{-1}.

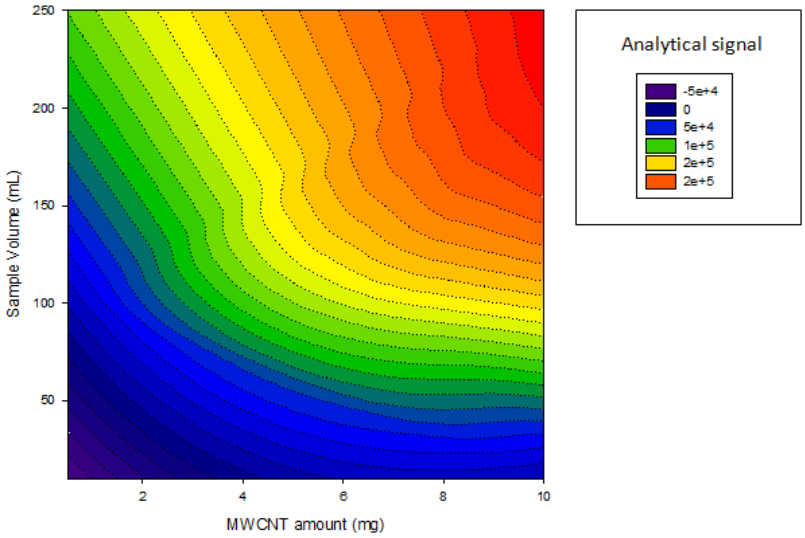

Figure S-8: Bivariant effect of the sorbent amount and sample volume on the analytical signal obtained for a standard solution containing terbutryn at 500 µg L^{-1}.

Capítulo IV:

Effervescence-assisted dispersive liquid-liquid microextraction with extractant removal by magnetic nanoparticles

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Effervescence assisted dispersive liquid-liquid microextraction with extractant removal by magnetic nanoparticles

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Abstract

In this article, effervescence assisted dispersive liquid-liquid microextraction with extractant removal by magnetic nanoparticles is presented for the first time. The extraction technique makes use of a mixture of 1-octanol and bare Fe_3O_4 magnetic nanoparticles (MNPs) in acetic acid. This mixture is injected into the sample, which is previously fortified with carbonate, and as a consequence of the effervescence reaction, CO_2 bubbles are generated making possible the easy dispersion of the extraction solvent. In addition, the MNPs facilitates the recovery of the 1-octanol after the extraction thanks to the interaction between hydroxyl groups present at the surface of the MNPs and the alcohol functional group of the solvent. The extraction mode has been optimized and characterized using the determination of six herbicides in water samples as model analytical problem. The enrichment factors obtained for the analytes were in the range 21-185. These values permit the determination of the target analytes at the low microgram per liter range with good precision (relative standard deviations lower than 11.7 %) using gas chromatography (GC) coupled to mass spectrometry (MS) as analytical technique.

Keywords: Effervescence assisted dispersive liquid-liquid microextraction, magnetic nanoparticles, triazines, water samples.
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1. Introduction

Microextraction techniques, which resulted from the evolution of classic sample treatment towards simplicity and miniaturization are, nowadays, usual tools in the development of competitive analytical methodologies [1, 2]. The large variety of techniques, each one possessing special properties, covers a wide range of potential applications, which indicates the great versatility of these approaches. Among all the available techniques, those based on dispersive forces, which can be developed equally in the solid and liquid phase microextraction formats, are characterized by their efficiency and rapidity [3]. Both characteristics are derived from the extraction fundament, which is based on the efficient dispersion of the extractant, a sorbent or a solvent, into the sample. The dispersion process maximizes the contact sample/extractant surface enhancing the mass transference and improving the extraction kinetics.

Dispersive liquid-liquid microextraction (DLLME), which was proposed by Rezaee *et al.* in 2006, [4] is based on the efficient dispersion of an extractant solvent into the sample in the form of fine droplets with enhanced sample/solvent contact surface. DLLME has been extensively used due to their efficiency, with extremely high absolute recoveries, and rapidity. Classic DLLME involves two subsequent steps, solvent dispersion and recovery.

The dispersion of the solvent is usually assisted by an external chemical compound or by an additional energy source such as mechanical stirring [5], ultrasound [6], microwaves [7] or heating [8]. The chemical dispersion requires a disperser solvent usually in the mL range to create a cloudy solution of the extractant solvent. The disperser solvent may participate in the partition distribution of the analytes since it increases their solubility in the aqueous phase and therefore it can reduce the potential efficacy of the technique.

The recovery of the solvent after the extraction usually requires a centrifugation step, although other alternatives have been proposed [9], which may slow down the overall extraction procedure. Shi et al. proposed the use of magnetic nanoparticles to improve the classic DLLME performance [10]. After the conventional DLLME step, a magnetic sorbent is dispersed into the sample interacting by hydrophobic forces with the organic solvent. In a final step, the solvent-sorbent combination is recovered by using an external magnet. A similar approach has been recently proposed for the extraction of nonylphenol in waters [11].

In this paper, effervescence, which has been successfully applied in dispersive solid phase extraction [12, 13], is proposed to improve classic DLLME reducing its main limitations. The extraction solvent dispersion is chemically assisted by means of an effervescent reaction while its final recovery is performed by means of bare iron oxide nanoparticles. For this purpose, an extraction mixture (containing 1-octanol as extractant, acetic acid as effervescence precursor and iron oxide nanoparticles) is introduced into the sample, to which sodium carbonate is previously added to promote the effervescent reaction. The new proposal has been evaluated using the extraction of some herbicides from waters as model analytical problem.

2. Experimental section

2.1. Reagents and samples

All reagents were of analytical grade or better. Sigma–Aldrich (Madrid, Spain) provided the triazine herbicides: prometon, (PMT); terbumeton, (TBM); secbumeton, (SBM); simetryn, (SMT); prometryn, (PMT) and terbutryn (TBT) and sodium carbonate required for the effervescent reaction. Stock standard solutions for each analyte were prepared in methanol (Panreac, Barcelona, Spain) at a concentration of 1000 mg L^{-1} and stored in the dark at 4ºC. Working solutions of the target analytes were prepared by

the appropriate dilution of the stocks in Milli-Q water (Millipore Corp., Madrid, Spain) or methanol as required.

Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) and ammonia were used for the synthesis of the magnetic nanoparticles (Fe_3O_4). Tetraethyl orthosilicate (TEOS) and ethanol were employed for covering the magnetic core with a protective silica-based coating. Finally, octadecyltriethoxysilane and toluene were used to introduce hydrophobic (C_{18}) groups on the nanoparticles surface. Glacial acetic acid and 1-octanol were used for the preparation of the effervescent mixture. All these reagents were supplied by Sigma–Aldrich.

Three water samples were used to evaluate the method performance including river water (Genil River; Córdoba, Spain), well water (Montalban; Córdoba, Spain) and tap water (Córdoba, Spain). All samples were collected in amber glass bottles and stored at 4ºC until analysis. The samples were not filtered before their extraction.

2.2 Apparatus

Gas chromatographic/mass spectrometric analyses were carried out on an Agilent (Palo Alto, CA) HP6890 gas chromatograph equipped with an HP5973 mass spectrometric detector based on a quadrupole analyzer and an electron multiplier detector. System control and data acquisition was achieved with HP1701CA MS ChemStation software. Chromatographic separations were performed on a fused silica capillary column (30m×0.25mm i.d.) coated with 5% phenyl-95% methyl polysiloxane (film thickness 0.25µm) (Supelco, Madrid, Spain) using helium (6.0 grade, Air liquid, Seville, Spain) at a flow rate of 1 mL min^{-1} as carrier gas. The column temperature program was as follows: 2 min at 40 °C, raised up to 170°C at 40°C min^{-1}, then immediately ramped at 2 °C min^{-1} up to 200 °C. Finally, the temperature increased up to 260°C at 10°C min^{-1} (kept for 2 min). A splitless injection mode was selected for the

introduction of 2 µL of extracts or standards by means of an autosampler (Gerstel, Mülhein an der Ruhr, Germany). The injector was kept at 280 °C.

Electron impact ionization (70eV) was used for analytes fragmentation. The quadrupole mass spectrometer detector was operated in selected ion monitoring mode, recording the following fragment-ions characteristic of each analyte: 210 for PMT and TBM; 196 for SBM; 213 for SMT; 241 for PMT and 226 for TBT. The MS source and quadrupole temperatures were kept at 230°C and 150°C, respectively. The peak areas were used for quantification of individual analytes.

2.3 Synthesis of magnetic nanoparticles

Fe_3O_4 magnetic nanoparticles were obtained following a previously described co-precipitation method [14]. Briefly, $FeCl_3 \cdot 6H_2O$ (24 g) and $FeCl_2 \cdot 4H_2O$ (9.8 g) were dissolved in 100 mL of Milli-*Q* water under nitrogen atmosphere. The solution was vigorously stirred at 80ºC in a water bath. Next, 50 mL of ammonia (25% w/w) were dropwise added producing a black precipitate of iron oxide magnetic nanoparticles (MNPs). The MNPs were separated by the application of a magnetic field with an external neodimium (Nd) magnet (Supermagnete, Gottmadingen, Germany), washed with Milli-*Q* water to remove the unreacted chemicals and finally dried.

Bare MNPs were covered with a silica coating using TEOS. For this purpose, 1 g of magnetic nanoparticles were placed in a beaker containing 50 mL of ethanol and 4 mL of water, the pH of the suspension being adjusted to 9.0 with ammonia. Then, 2 mL of TEOS were added and the suspension was stirred overnight under a nitrogen atmosphere. Finally, the silica-protected magnetic nanoparticles ($Fe_3O_4@SiO_2$) were recovered with an external Nd magnet, thoroughly washed with water and dried. A weight increase of ca. 3% was observed in this step.

To prepare $Fe_3O_4@SiO_2@C18$ nanoparticles, silica protected MNPs were dispersed in 50 mL of anhydrous toluene in the presence of 0.5 mL of

octadecyltriethoxysilane. The mixture was sonicated for 5 min and refluxed for 12 h. Fe_3O_4@SiO_2@C18 nanoparticles were washed several times with ethanol to remove toluene and unreacted products and dried, yielding a fine brown powder.

2.4 Extraction mixture preparation

The mixture required to perform nine separate extractions is prepared by mixing 2.5 mL of glacial acetic acid, 200 µL of 1-octanol and 100 mg of bare Fe_3O_4 magnetic nanoparticles. The mixture is then placed in an ultrasound bath (Mod. 3510 Branson, Connecticut, USA) during 5 min to achieve the efficient suspension of the MNPs. This suspension remains stable for at least 24 h. An aliquot of 250 µL is used for the extraction of the triazine herbicides.

2.5 Extraction procedure

The extraction procedure, represented in Figure 1, is as follows. First of all, 20 mL of the aqueous standard/sample is homogeneously mixed with 2.5 mL of 3 mol L^{-1} sodium carbonate aqueous solution in a 25 mL glass vial. Subsequently, 250 µL of the extraction mixture are slowly added to the vial by means of a syringe, placing the needle at the bottom of the vial without generating excessive pressure with the plunger. In this way, the effervescence occurs from down to the top of the vial and the extractant (1-octanol) and the magnetic nanoparticles are homogeneously distributed into the standard/sample. Once the effervescent reaction has taken place (less than 5 s per extraction) a Nd magnet is placed along the vial wall to achieve the separation of the 1-octanol-coated magnetic nanoparticles enriched with the analytes. This recovery process takes ca.1 min. The aqueous phase is then discarded and the particles are gently washed with Milli-*Q* water by means of a pipette. Finally, 100 µL of methanol are added to the vial and the MNPs are redispersed for analytes elution. The

particles are again separated from the organic phase by means of the external magnet and the methanolic phase containing the eluted analytes is transferred to a vial for GC-MS analysis.

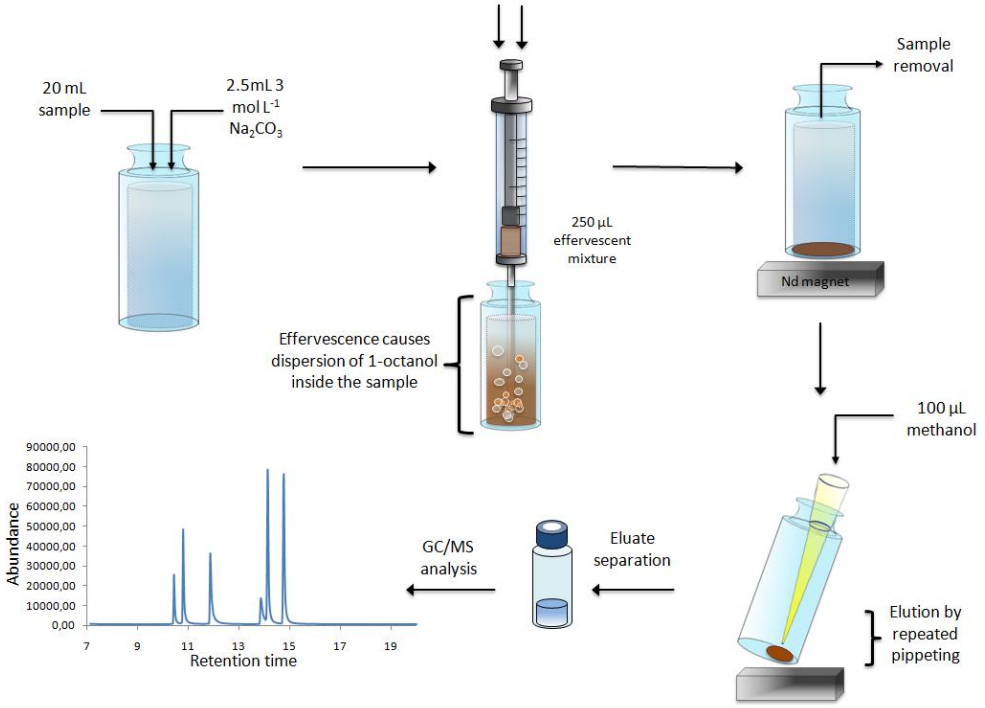

Figure 1: Schematic diagram of Effervescence assisted dispersive liquid-liquid microextraction with extractant removal by magnetic nanoparticles.

3. Results and discussion

The main issue regarding dispersion techniques is the homogeneous distribution of the extractant in the sample. The use of an organic solvent as disperser must comply with certain requirements in terms of miscibility with the sample and the extractant. Moreover, its use could reduce the extraction efficiency increasing the analyte solubility in the aqueous phase and making difficult the transfer to the

extractant. In this way, our group has developed a green, solvent-free and efficient alternative based on the effervescence-assisted dispersion of sorbents of different nature [13,14].

In this work, effervescence is selected as the dispersive force for the extractant organic solvent (1-octanol) and the magnetic nanoparticles required for its recovery, avoiding the employment of external apparatus or another organic solvent to achieve the dispersion during the extraction process. The intensity of the effervescent reaction is responsible for dispersion and the amount of the effervescent precursors, sodium carbonate and acetic acid, should be controlled. In this case, sodium carbonate is added previously to the sample while acetic acid is included in the extraction mixture. It should be indicated that acetic acid does not remain in the solution since it is transformed in sodium acetate.

3.1 Evaluation of the dispersion procedure

The efficient dispersion of the extraction solvent into the sample is a key aspect in a DLLME process. In the proposed procedure, the extraction mixture consists of three main elements, namely: (a) the proton donor solvent; (b) the extractant, 1-octanol, and (c) the magnetic nanoparticles employed for the extractant solvent recovery. These elements can be simultaneously or sequentially added to the samples. In the first approach, called one-step procedure, a mixture of the three elements is directly dispersed into the sample. In the latter approach, called two-step procedure, a mixture of the proton donor and extractant solvents is injected into the sample and the MNPs are added in a second step to recover the extractant solvent. The comparison of both procedures is shown in Figure 2A. For comparative purposes, the performance of the method was also compared with that obtained using a conventional disperser solvent, instead of the proton donor solvent, such as acetonitrile. In this assay C_{18}-coated MNPs were used for the recovery of the extractant. On the one hand, as it can

be observed in Figure 2A, the use of acetic acid leads to the best results thanks to the effervescence reaction, which improves the solvent dispersion. On the other hand, the one-step procedure provides superior results since the MNPs are also efficiently dispersed.

Taking into account the results obtained, the one-step procedure using acetic acid as disperser and bare MNPs was selected for further assays. It should be noted that 1-octanol is the responsible for the analytes extraction since negligible analytical signals are obtained when this solvent is not present in the extraction mixture.

3.2 Selection of magnetic nanoparticles

Once the dispersion mode was selected, the use of bare or C_{18}-coated magnetic nanoparticles was evaluated. The selection of the MNPs must be done taking into account of two aspects; (i) analytical signal values obtained by chromatographic analysis; and (ii) mechanical behavior, which influences the reproducibility of the method. The two nanoparticles were selected considering the different interaction that they can establish with 1-octanol. Therefore, for C_{18}-coated MNPs, the interaction is hydrophobic, while bare Fe_3O_4 nanoparticles retains the 1-octanol by means of the residual hydroxyl groups on their surface. As it can be seen in Figure 2B (upper panel), the effervescent mixture is completely different depending on the nature of the nanoparticles. Thus, bare nanoparticles results in homogeneous extractant mixture, while C_{18}-coated aggregates after ultrasound irradiation, which difficult their use. Fig. 2B (middle panel) shows the result of a completed effervescent dispersion. As it can be seen, bare MNPs form a homogeneous aqueous dispersion and the C_{18}-coated remain at the top of the sample surface, limiting the interaction with 1-octanol during extraction. In addition, C_{18}-coated MNPs, tend to aggregate and adhere to the PTFE pipette tip during washing and elution steps (Figure 2B, lower panel) and also to the glass vial used during extraction. Figure 2C compares the extraction performance when

the two types of MNPs are used. Bare MNPs give a slight improvement in sensitivity and precision compared with those results provided by C_{18}-coated MNPs. Finally, an extraction using the different MNPs without 1-octanol was carried out to ensure that the extractive potential is due to the organic solvent and not to the MNPs nature. The analytical signals obtained for these extractions, which were negligible compared with the combined MNPs/organic solvent (data not shown), corroborates this fact. Bare MNPs were selected as optimum material for further studies.

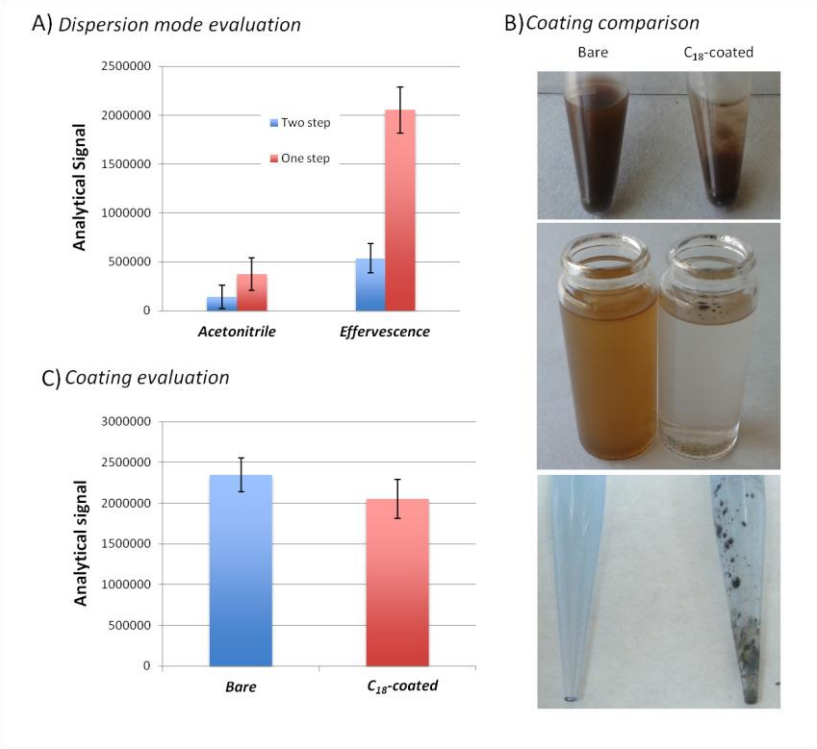

Figure 2:. Evaluation of the dispersion procedure and the nature of the MNPs. A) Dispersion mode evaluation; B) Visual comparison of the MNPs and C) Comparison of the MNPs in terms of analytical signal. For details, see text.

3.3 Volume of 1-octanol and MNPs amount

Several references corroborate the presence of hydroxyl groups over the surface of bare Fe_3O_4 nanoparticles [15, 16]. According to this, the most probable interaction between those functional moieties and 1-octanol occurs through the hydrogen bonds. Therefore, in order to maximize the amount of 1-octanol that can be efficiently recovered by the MNPs, both variables were optimized together considering three different 1-octanol volumes (10, 15 and 20 µL) and three different MNPs amounts (5, 10 and 20 mg). The maximum MNPs quantity is limited to 20 mg since higher amounts can clog the needle. The optimization process was performed taking into consideration all the target analytes at a concentration level of 50 µg L^{-1}. By way of example Figure 3 shows the bivariant effect of both variables on the extraction of terbumenton, which is presented as an example since all the analytes present a similar behaviour. The optimum conditions were finally selected as a compromise between the analytical signal and the mechanical behaviour of the system during extraction. As it was expected, the analytical signal was higher when larger extractant volumes were used, since the extraction capacity directly depends on the quantity of 1-octanol. In fact, the analytical signal increases up to 20 µL, although this relationship is not observed for higher solvent volumes. This fact can be ascribed not only to a less efficient dispersion of the 1-octanol, but also to the difficult of recovering a limited amount of MNPs. On the other hand, the analytical signal increases with the MNPs amount up to 10 mg, remaining constant up to 20 mg. The syringe is clogged when higher amounts of MNPs are used, thus reducing the efficiency of the procedure.

According to these results, 20 µL of 1-octanol and 10 mg of MNPs were selected as the optimum values.

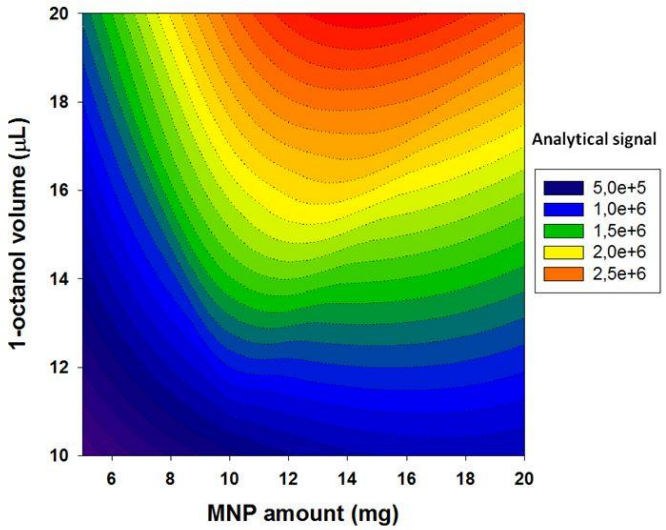

Figure 3: Bivariant effect of the extractant volume and MNPs amount on the analytical signal of terbumeton.

3.4 Elution process

Once the 1-octanol-coated MNPs and extracted analytes are separated from the aqueous phase, an elution step with an organic solvent is required to remove the analytes from the 1-octanol phase. According to our own experience and the extractant nature, methanol was selected as eluent. Initially, an ultrasound-assisted elution was evaluated, but poor recovery values were obtained and the chromatographic resolution was inappropriate. This result can be ascribed to the breakage of the 1-octanol/magnetic nanoparticles interaction due to the ultrasound, which led to introduction of 1-octanol in the chromatographic system. For this reason, 100 µL of methanol (the volume was selected as a compromise between complete coverage of the MNPs and minimum analyte dilution) are introduced in the glass vial and slowly mixed with the MNPs by means of a pipette. Finally, an external magnet is applied to separate the methanol from the MNPs and 2 µL of the extract are injected in the chromatographic system.

3.5 Analytical figures of merit

The optimized procedure was applied to the determination of six triazine herbicides (namely, prometon, terbumeton, secbumeton, simetryn, prometryn, and terbutryn) in environmental waters. The main figures of merit are summarized in Table 1. A calibration graph was constructed for each analyte by extracting in triplicate six working aqueous standards containing all the analytes within the concentration interval from 0.08 to 50 μg L^{-1}. The enrichment factors, which were calculated by comparing the calibration graphs before and after the extraction process, were in the range from 21 (for simetryn) to 185 (for prometon). The limits of detection, which were calculated using a signal-to-noise ratio of 3, varied between 0.02 μg L^{-1} (prometon, simetryn and terbutryn) and 0.06 μg L^{-1} (terbumeton and secbumeton). The repeatability of the method was evaluated at two concentration levels (10 μg L^{-1} and LOQ). The results, which are shown in Table 1 and expressed as RSD, were in the range of 7.8-11.7 % at 10 μg L^{-1} and in the range of 6-14% at LOQ.

Table 1: Analytical figures of merit for the determination of the selected analytes in water using effervescence assisted dispersive liquid-liquid microextraction with extractant removal by magnetic nanoparticles.

Analyte	LOD[a]	LOQ[b]	R[c]	Precision expressed as RSD[d] (%)		EF[e]
				at 10 µg L^{-1}	at LOQ	
Prometon	0.02	0.08	0.996	9.8	6.0	185
Terbumeton	0.06	0.2	0.995	11.1	11.7	45
Secbumeton	0.06	0.2	0.997	11.7	11.0	38
Simetryn	0.02	0.08	0.998	8.2	11.5	21
Prometryn	0.04	0.13	0.997	7.8	14.0	110
Terbutryn	0.02	0.08	0.999	8.9	9.7	127

[a] LOD: limit of detection expressed in µg L^{-1}, [b]LOQ: limit of quantification expressed in µg L^{-1}, [c]R: correlation coefficient, [d]RSD: relative standard deviation, [e]EF: enrichment factor

3.6 Analysis of water samples

In order to evaluate the applicability of the proposed method for the determination of the target analytes from waters, three different water samples (river, tap and well) were analysed. As no analytes (concentrations below the LOD) were found in the samples, a recovery study was carried out at the concentration level of 10 µg L^{-1} and each sample was analyzed in triplicate. The obtained results are listed in Table 2. As it can be seen, the relative recoveries fulfils the 70-130% recovery criterion [17], which demonstrates that effervescence is able to disperse the extractant and the particles even in presence of suspended material from the sample matrix, specially in river water samples. The results show the potential of the proposed effervescence assisted method for the *in situ* extraction of the triazines from natural environmental water samples.

Table 2: Relative recovery study performed on real samples spiked with the analytes at a concentration of 10 µg L^{-1}.

	Water sample (R%[a]±SD[b])		
Analyte	River	Tap	Well
Prometon	91± 16	100± 8	88± 3
Terbumeton	91± 11	107 ± 4	99± 3
Secbumeton	101 ±13	98± 8	94 ± 12
Simetryn	113± 9	97 ± 15	99± 2
Prometryn	110± 6	84± 3	92± 5
Terbutryn	125± 12	94± 1	107± 6

[a]R%:Extraction Recovery (calculated at 10 µg L^{-1}), [b]SD:Standard Deviation (n=3)

By way of example, Figure 4 shows a typical chromatogram obtained for the extraction of a river water sample spiked with the six analytes at 10 µg L^{-1}. As it can be observed, before each peak a shoulder is observed. This shoulder is due to the presence of small amounts of 1-octanol in the methanolic extract, which probably generates a slight partition of the injection plug within the chromatographic system. In fact, the presence of this shoulder is not observed when pure methanolic standards are injected in the chromatograph.

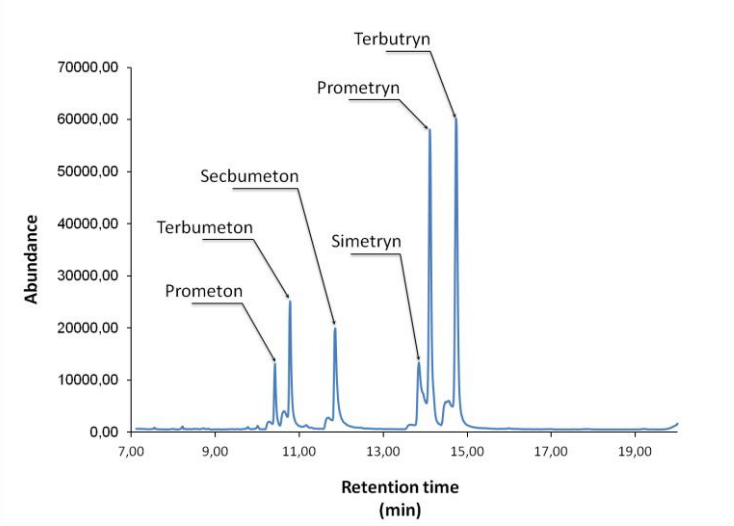

Figure 4: Typical chromatogram obtained for the extraction of a river water sample spiked with the six analytes at 10 µg L^{-1}

4. Conclusions

The presented method is a valuable, effective, dispersive liquid-phase microextraction technique, which makes use of the *in situ* generation of CO_2 as dispersive force whose efficiency was previously demonstrated as alternative for the dispersion of polymeric [13] or nanostructured sorbents [14]. The proposed method uses an organic solvent (1-octanol) as extractant at very low volume (20 µL) solubilized in an extraction mixture which contains a proton donor, such as acetic acid, and bare iron oxide nanoparticles to favor the extractant recovery. The sample is previously homogeneously mixed with a CO_2 source, such as sodium carbonate. When the effervescent extractant mixture is added to the sample an immediate and homogeneous dispersion of the 1-octanol within the sample is achieved. It results in good extraction efficiency with enrichment factors in the range from 21 to 185. Complete recovery of the extractant is critical in order to maximize the method sensitivity and precision. In this case, the introduction of Fe_3O_4 magnetic nanoparticles in the extractant mixture results in a fast, efficient method to separate 1-octanol from aqueous phase thanks to the interaction between hydroxide residues present in the surface of the nanoparticles and the hydroxyl group of the octanol chain. The analyte extraction is very fast and simple since it uses common labware and a commercial external magnet. The simplicity of the procedure makes it a viable alternative for on-site extraction of aqueous environmental samples. The proposed method uses a low sample volume, which facilitates handling and storage when on site extraction is required.

Acknowledgments

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Bloque II: Técnicas de microextracción basadas en disolventes conmutables mediadas por CO_2

La extracción líquido-líquido convencional (LLE) es una de las técnicas analíticas de pretratamiento de muestra más extendidas. Formalmente, consiste en una serie de etapas de extracción, para muestras acuosas de matriz más o menos compleja, con fracciones de disolvente orgánico frescas o de creciente polaridad. El resultado es un conjunto de extractos enriquecidos en cantidades crecientes de analito o de familias de estos [1]. Este tipo de procedimientos, con tantas etapas implicadas, son tediosos y consumen gran cantidad de recursos y tiempo. Además, emplean grandes volúmenes de disolventes orgánicos, tóxicos y costosos y generan gran cantidad de residuos.

Existen distintas alternativas para solventar los problemas asociados a la LLE. En su mayoria, han tratado de reducir el número de etapas y el consumo de disolventes. Entre las alternativas más destacadas podemos restaltar la microextracción basada en gotas (SDME) [2-4] o en fibras huecas [5]. Sin embargo, desde 2006, existe una alternativa aun más versátil que las anteriores. Rezaee y colaboradores [6] desarrollaron la microextracción líquido-líquido dispersiva en la que una mezcla de un disolvente miscible con agua (dispersante) y un disolvente inmiscible (extractante) se inyectan en una muestra acuosa y de manera que se dividen en finas gotas en forma de emulsión. Mediante este proceso, la superficie de contacto entre la fase extractante y la muestra es extraordinariamente elevada, lo cual simplifica el proceso y reduce el tiempo de extracción. Desde entonces, se han desarrollado numerosas variantes. La mayoría de avances han estado centrados en el tipo de disolvente extractante empleado. En este sentido, los líquidos iónicos han ganado protagonismo gracias a sus extraordinarias propiedades físico-químicas [7]. Sin embargo, también podemos encontrar alternativas basadas en la solidificación del disolvente orgánico extractante que se recupera posteriormente por centrifugación [8].

La mayoría de estas técnicas presentan dos limitaciones; por una parte requieren de un disolvente dispersante adicional y por otra, la separación de los microvolúmenes de fase extractante requieren un procedimiento adicional de

decantación o centrifugación. Para evitar estos problemas asociados, se desarrollo la *microextracción líquido-líquido homogénea* (homogeneous liquid-liquid microextraction, *HLLME*). Esta herramienta sigue un principio similar a la DLLME, con la salvedad de que evita el empleo de disolvente dispersante. En su lugar, hace uso de disolventes que son miscibles con la fase acuosa de la muestra y se vuelven inmiscibles mediante un proceso químico como *salting-out* [9, 10], cambios de pH [11, 12] o formación de pares iónicos [13].

Sin embargo, aunque la separación de fases en la HLLME puede conseguirse químicamente, los extractantes empleados deben cumplir una serie de requisitos. Entre los más importantes podemos destacar que deben ser menos densos que el agua, para poder formar una fase separada fácilmente recuperable. Además, aunque hay alternativas, la mayoría de procedimientos de este tipo implican la separación por cambios de pH, siendo pocos candidatos los que cumplen estos requisitos [14].

Recientemente se han aplicado a procesos industriales nuevos disolventes (denominados conmutables) que manifiestan una revolucionaria propiedad que puede ser muy útil para procedimientos de microextracción [15]. Los disolventes conmutables alternan una forma hidrofílica y otra hidrofóbica, en función de la presencia de CO_2 en la fase acuosa. Esto implica que, en presencia del gas, son totalmente miscibles con agua en un ratio 1:1, formando una fase homogénea. Una vez el gas es retirado de la disolución, se transforma en una forma hidrófoba inmiscible con agua.

En este capítulo de la memoria de Tesis Doctoral se aborda por primera vez el empleo de estos disolventes conmutables en técnicas de microextracción. En la primera contribución, se evaluó su viabilidad en sistemas de extracción miniaturizados, mientras que en la segunda, se abordó la resolución de un problema analítico concreto como es la determinación de triazinas en aguas de distinta procedencia. Las propiedades y características de este tipo de disolventes se comentaran en detalle en la

sección de resultados y discusión correspondiente a la aplicación desarrollada durante la realización de esta Tesis Doctoral.

El desarrollo de la microextracción líquido-líquido busca, por tanto, procedimientos simples, con un número reducido de etapas y con un bajo consumo de disolventes. Adicionalmente, una de las líneas más interesantes de estas metodologías es la eliminación de la dependencia de sistemas adicionales (tales como centrifugas) para conseguir la separación del extractante. Gracias al desarrollo de nuevas modalidades, esta etapa puede lograrse químicamente, agilizando significativamente el proceso [16R].

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Capítulo V: Use of switchable solvents in the microextraction context

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Use of switchable solvents in the microextraction context

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Abstract

In this article a new homogeneous liquid-liquid microextraction alternative, based on the use of switchable hydrophilicity solvents (SHS), is presented for the first time. The extraction technique makes use of a water-immiscible solvent (N,N-Dimethylcyclohexylamine) that can be solubilized in 1:1 ratio using CO_2 as reagent. After the extraction, phases' separation is induced by the addition of sodium hydroxide, which produces a change on the ionization state of the amine, centrifugation not being necessary. The extraction technique has been optimized and characterized using the determination of benz[a]anthracene by fluorimetric measurements in water samples as model analytical problem. Although the native fluorescence of the compound is quenched in the organic phase, this attenuation is reduced by diluting the extractant (1:1) in acetic acid. The fluorescence intensity is 35 % higher in the SHS-acetic acid mixture than that obtained in pure methanol. The proposed method allows the determination of the target analyte with limit of detection of 0.08 µg/L and good precision (relative standard deviation of 6.7% at the limit of quantification level). The recoveries were in the range of 72-100% fulfilling the Environmental Protection Agency criterion. Finally, the potential use of this microextraction technique in combination with gas chromatography is shown for several polycyclic aromatic hydrocarbons.

Keywords: Liquid phase microextraction, Green chemistry, switchable hydrophilicity solvents, polycyclic aromatic hydrocarbons.

1. Introduction

Dispersive liquid-liquid microextraction (DLLME), introduced by Rezaee et al. in 2006 [1], consists of the introduction of an extractant/disperser mixture in the aqueous sample to form a cloudy solution where the extractant is divided into small droplets increasing its surface to volume ratio. This fact positively affect to the extraction kinetics, which directly depends on the contact surface between the extractant phase and the sample. After the extraction, the cloudy state must be broken, usually by centrifuga- tion, in order to isolate the extractant phase prior to its analysis. DLLME presents a great variety of advantages (easy operation, expeditiousness, low consumption of solvent and samples, etc.) that makes them a suitable sample treatment technique in many applications fields.

Homogeneous liquid-liquid extraction (HLLE) involves the complete solubilisation of the extractant in the aqueous sample making the contact surface between phases infinitely large [2–4]. The solubilisation of the organic solvent is performed exploiting its chemical properties or using an external energy source like heat or ultrasounds . The phaseś separation , when the real extraction takes place, is chemically or physically induced. Among the chemical alternatives, the salting out effect [5–7], pH changes [8,9] and ion pairing [10] are the usual ones while the reduction of the temperature in the extraction vessel can be highlighted among the latter. Although HLLE presents excellent characteristics, the extraction becomes tedious when heating/cooling cycles are employed. Moreover, special reagents are required when ion pairing is performed.

Microextraction techniques, which can be considered under the Green Chemistry [11,12] realm since they reduce the consumption of extractant materials, have evolved in the last years. The development of new sorbent/solvent has been a key research line in this context. Jessop et al. firstly investigated the behaviour of the so-called switchable hydrophilicity solvents (SHS) [13,14] for industrial applications [15].

They found solvents that exhibit a complete solubilisation in water under the presence of carbon dioxide, while the solvents become immiscible when the gas is purged from the solution [16]. The non-toxic nature of carbon dioxide as well as its low cost makes it an ideal phase transition trigger in extraction techniques . In addition, the use of SHS allows the extraction of the analytes in a homogeneous phase and it permits the easy phases´ separation without additiona l apparatus. This process can be developed in industrial procedures due to their macroscale nature but its transference to the analytical context, especially to the microextraction field, has not been yet considered. On the one hand, the production of stable mixtures of the SHS and aqueous phases in the microscale would require special devices like extraction vessels with an exhaustive control of the CO_2 pressure. On the other hand, the purge of the CO_2 may produce extractant losses by projection (solvent dragged by the purge gas) or evaporation. The latter effect is especially important when volatile SHS are employed, although non-volatile SHS are available [17]. Extractant losses are not acceptable when low solvent volumes are employed in the microextraction context.

In the present work, the adaptation of the SHS to the micro- extraction context is proposed. The solubilisation of the solvent into the aqueous phase is performed prior to the extraction using dry ice as reagent, which provides a stable homogeneous SHS/ water mixture. The final separation is achieved using a common reagent like sodium hydroxide. A polycyclic aromatic hydrocarbon (PAH), benz[a]anthracene, is employed as model analyte, its native fluorescence being exploited for the determination. The use of the SHS in combination with acetic acid enhances the fluorescence of the target analyte compared with a conventional medium such as methanol.

2. Experimental section

2.1 Chemicals

All reagents were of analytical grade or better. Benz[a]anthracene, acetic acid (HOAc) and N,N-dimethylcyclohexylamine (DMCA) were purchased from Sigma-Aldrich (Madrid, Spain). Stock standard solution of the analyte was prepared in methanol (Panreac, Barcelona, Spain) at 500 mg L^{-1} and stored at 41C. Working olutions were prepared by the appropriate dilution of the stock in Milli-Q water (Millipore Corp., Madrid, Spain), methanol, DMCA or DMCA/HOAc mixtures, as required.

Dry ice, in the form of pellets of 3 mm, purchased from Cordogas (Cordoba, Spain) was employed for the solubilisation of the DMCA in water . NaOH from Sigma - Aldrich was employed to induce phase´s separation . A 20 mol L^{-1} NaOH solution was daily prepared and employed in the extraction procedure.

Tap and river water (Genil river, Córdoba, Spain) samples were collected in amber glass bottles without headspace and stored at 41C until analysis. The samples were not filtered prior their analysis. Bottled water samples were purchased from a local market.

2.2 Apparatus

Fluorescence analyses were carried out on a fluorescence spectrophotometer model F-2500 (Hitachi Ltd., Tokyo, Japan) equipped with a Xenon lamp. The excitation and emission wave- lengths were set at 289 nm and 388 nm, respectively, using 10 nm selector slits in both cases. The photomultplier tube voltage was set at 400 V. Data acquisition was performed using FL solutionss software from Hitachi Ltd.

2.3 Amine-aqueous phase preparation

The procedure is presented in Figure 1. First, 100 mL of Milli-Q water and 100 mL of DMCA are placed in a 1 L glass bottle with screw cap, forming a two phases

system (Figure. 1A). Then, 10 g of dry ice are added and the glass bottle is tightly closed. The high volume of the bottle employed in this synthesis allows the use of this amount of dry ice without a risky increase of the internal pressure. Undissolved CO_2 remains at the top of the bottle and the amine phase became cloudy (Figure. 1B). The mixture is stirred for 5 min in order to favour the solubilisation of the carbon dioxide. After this time the biphasic system remains but the volume phases ratio varies due to the partial solubilisation of DMCA in water (Figure. 1C). This stage is repeated ten times until a single phase is observed, which corresponds to a 1:1 (v:v) water/ DMCA solution (Figure. 1D). The solution loses its turbidity 1 h later after the last dry ice addition.

Two hundred millilitre of the 1:1 (v:v) water/ DMCA solution can be easily prepared and this volume allows the development of more than 200 extractions since each one requires only 750 µL of the mixture.

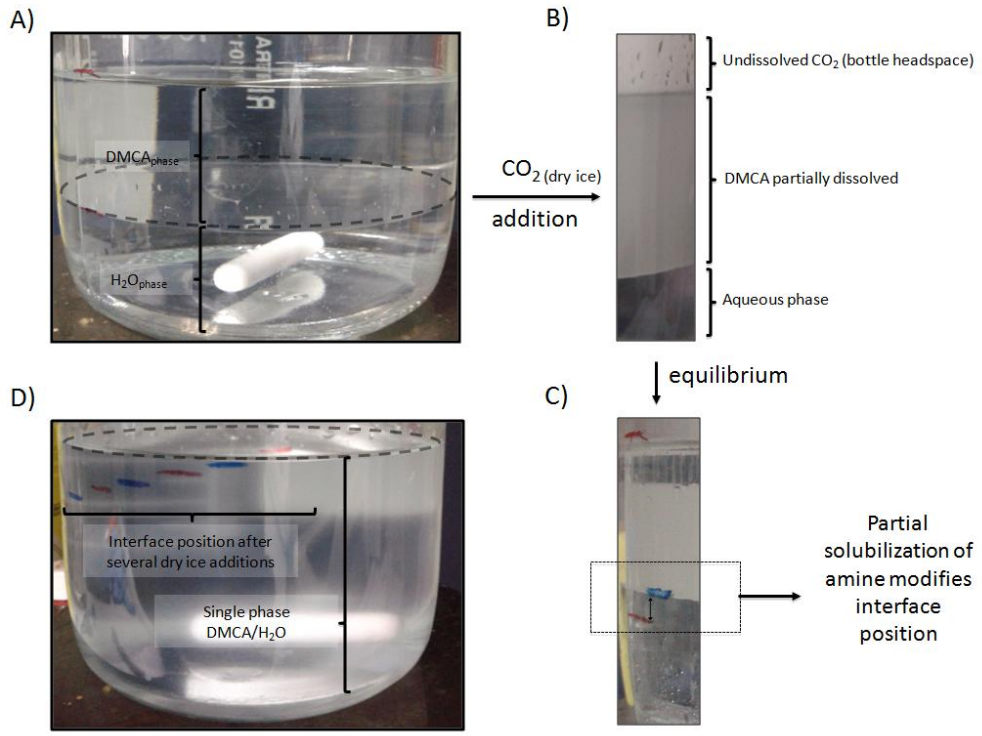

Figure 1: Steps of the DMCA dissolution in water. (A) Initial state where the two immiscible phases are observed; (B) Detail of the bottle after the addition of 10 g of dry ice where three phases (two liquid and one gaseous) are observed; (C) Detail of the bottle after the solubilisation of the CO_2. The change in the interface position is shown; (D) Final state after the several dry ice additions showing a unique liquid phase.

2.4 Extraction procedure

The extraction, schematised in Figure. 2, consists of various well-defined steps. First of all, 750 µL of aqueous amine are added to 10 mL of aqueous standard or water sample previously located in a glass test tube. The mixture is agitated in a vortex for 15 s, until a homogeneous phase is observed. After this, 1 mL of a 20 mol L^{-1} NaOH solution is added and cloudy solution appears. The solution is shaken again in the vortex for 15 s, and the test tube is left to stand for 5 min to achieve phase separation. Three hundred microlitre of DMCA are recovered and transferred to a glass vial for analysis.

For fluorescence analysis, a previous 1:1 (v/v) dilution in acetic acid is required to enhance sensitivity.

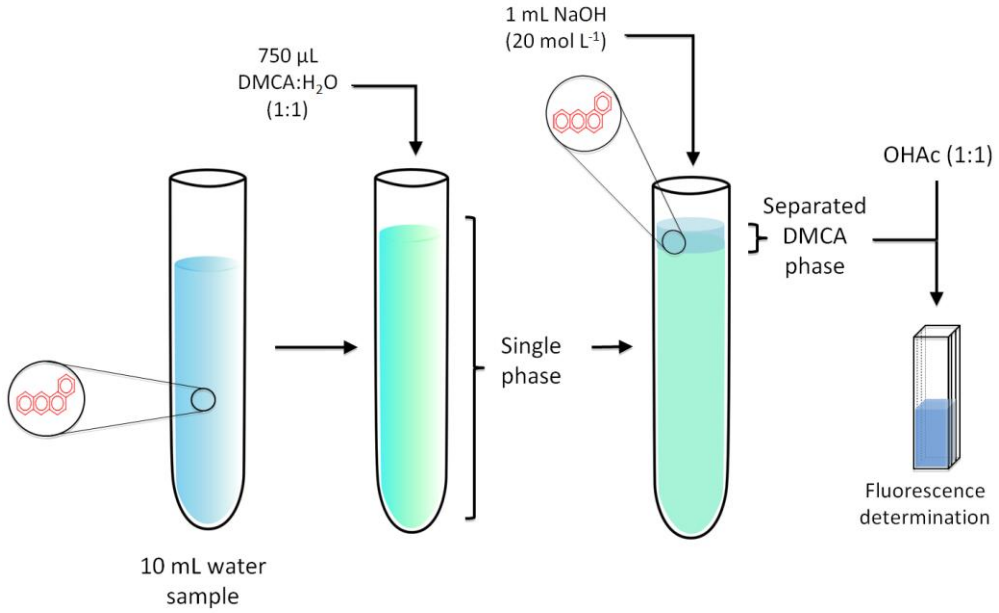

Figure 2: Schematised extraction procedure.

3. Results and discussion

In its neutral form, DMCA can be considered as a moderate non-polar solvent, taking into consideration that the logarithm of the octanol/water partition coefficient (log K_{ow}) is equal to 1.95 [18]. Thanks to its nature, DMCA can be employed for the extraction of hydrophobic analytes from aqueous samples and therefore benz[a] anthracene has been selected as model analyte. As a polycyclic aromatic hydrocarbon, benz[a]anthracene presents a native fluorescence that allows its sensitive determination.

Some preliminary assays were performed in order to evaluate the potential of fluorescence measurements for the determination of the target analyte. Qualitative information (pictures) was obtained under UV radiation of the solutions using a commercial lamp and quantitative measurements performed in a fluorescence spectrophotometer. For interpretation purposes, it is necessary to highlight that the pictures were obtained with an excitation wavelength of 365 nm while the quantitative measurements were obtained at the optimum excitation and emission wavelengths of benz[a]anthracene (289 nm and 388 nm, respectively).

The fluorescence of the analyte in pure DMCA was firstly evaluated at a concentration of 1 mg L^{-1} and it was compared with that obtained in methanol. As it can be observed in Figure. 3, the fluorescence of the analyte is quenched in the presence of DMCA. This quenching can be avoided if acetic acid is added to the DMCA phase. Moreover, the fluorescence in the DMCA /HOAc mixture is a 35% higher than that obtained in methanol.

To improve the sensitivity of the determination, the influence of the DMCA/HOAc ratio on the fluorescence of benz[a]anthracene was evaluated. The results, which are shown in Figure 4 point out a fluorescence enhancement in DMCA /HOAc mixtures compared to the pure solvents. The spectra obtained are similar in shape in the different media as it can be observed in Figure. S1 (supplementary content), although the S/N ratio is higher for intermediate DMCA/HOAc ratio. According to the results, a 1:1 ratio was selected as optimum value which involves a 1:1 (v:v) dilution of the DMCA in acetic acid before the fluorimetric analysis of the sample extracts.

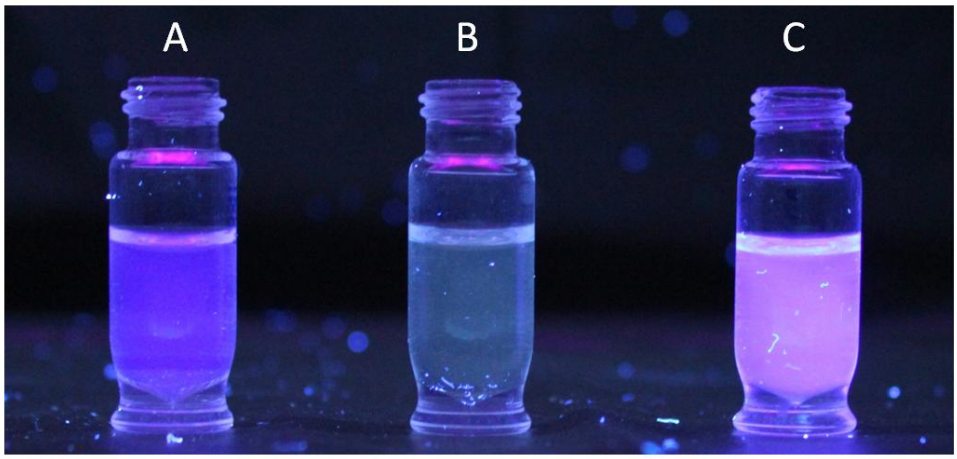

Figure 3: Vials containing standard solutions of benzo[a]anthracene at the concentration of 1 mg L^{-1} prepared in different solvents, namely, (A) methanol, (B) DMCA and (C) DMCA/HOAc 1:1 (v:v). The picture has been obtained under UV irradiation at 365 nm.

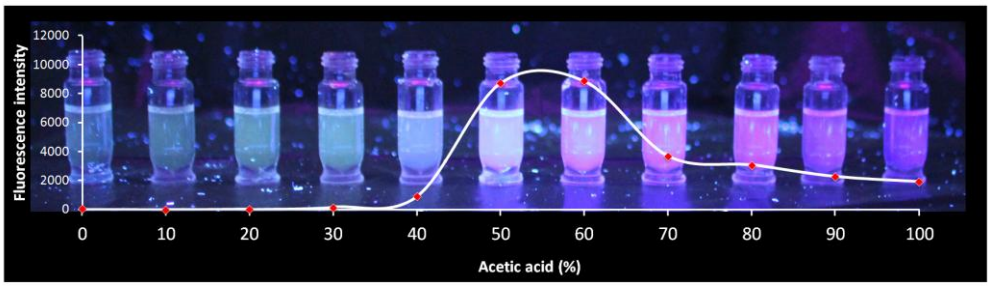

Figure 4: Study of the DMCA/HOAc ratio on the fluorescence of benzo[a]anthracene. The fluorescence intensity values for different standards containing the analyte at the same concentration level but in different media are presented. The picture shows the vials emission under UV radiation at 365 nm.

3.1 Solvent solubility switching

The aqueous solubility of DMCA can be switched controlling the CO_2 concentration in the extraction unit. In industrial applications, this concentration can be controlled playing with the CO_2 pressure, special vessels being necessary. In the microextraction context, this alternative is not feasible at all due to the high sample to extractant ratio required if a good preconcentration factor is intended to be obtained.

The solubilisation of the DMCA can be achieved using dry ice as CO_2 source (see experimental section) and conventional glass- ware. The water/DMCA mixture resulted to be stable at least for 10 months.

Once the extractant phase is added to an aqueous sample, the phases separation should be induced to recover the extractant with the isolated analyte. Several alternatives, including physical and chemical methods, were evaluated for this purpose. These methods are schematically presented in Figure 5 and they are described in the supplementary content. Among all the procedures, the addition of a 20 mol L^{-1} sodium hydroxide solution provided the best results due to its efficiency (viz. quantitative recovery of DMCA), rapidity (5 min were enough to observe phase separation) and simplicity (no centrifugation was required).

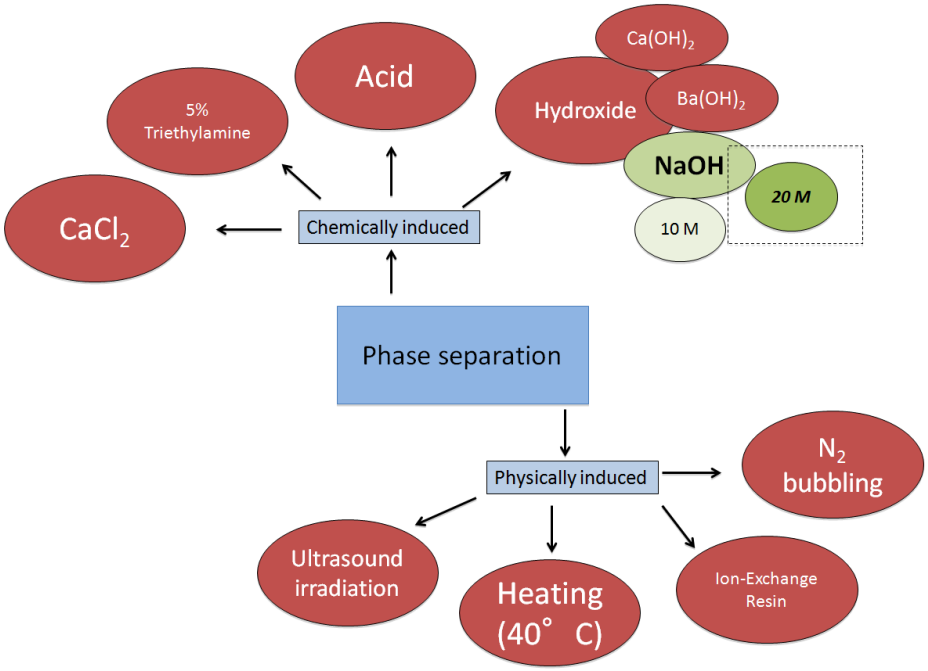

Figure 5: Different alternatives evaluated to induce phases separation. For further details, read the supplementary content.

3.2 Extractant volume

For simplicity, the aqueous sample volume was fixed to 10 mL, which allowed the use of conventional test tubes. The effect of the volume of DMCA aqueous phase on the extraction of the analyte was studied in depth. Three different volumes, namely, 1000 µL, 750 µL and 500 µL, were evaluated in triplicate. Lower volumes were non-practical since the recovery of the solvent was unfeasible while larger volumes induced an excessive dilution of the analyte. Each volume was evaluated in triplicate using fluorescent measurements. As it can be observed in Figure S2 (supplementary content), the higher signals are obtained when 500 µL of the extractant are employed. However, 750 µL is selected as the optimum value as a compromise between sensitivity and precision.

3.3 Analytical figures of merit

The optimised procedure was applied to the determination of benz [a]anthracene in water samples. A calibration graph was constructed for the analyte by extracting in triplicate five working aqueous standards within the concentration interval from 0.1 to 5 µg L^{-1}. The limit of detection, which was calculated as the method detection limit (MDL) [19], is 0.08 µg L^{-1}. The MDL value was obtained in a iterative process (starting with a spiked concentration of 0.5 µg L^{-1}) and using seven independent replicates (n=7). The repeatability of the method, which was evaluated at a concentration level of 0.5 µg L^{-1}, resulted to be 6.7% (expressed as relative standard deviation). In order to evaluate the applicability of the proposed method for the determination of benz[a]anthracene in waters, three different water samples (river, tap, and bottled) were analysed. As no positive results were obtained, a recovery study was performed at a concentration level of 1 µg L^{-1}. The results, which are listed in Table 1, fulfilled the 70–130% recovery criterion recommended by US-EPA, except in the case

of tap water. This sample has a relatively high chlorine content, which is a well-known quencher agent and can reduce the fluorescence intensity [20].

We realise that its direct combination with fluorescence spectroscopy presents a limited selectivity since it would not be capable to discriminate among different PAHs. The potential of the technique can be clearly demonstrated if it is combined with a chromatographic technique allowing the multi-determination of compounds. Although it was not the main objective of this article, Figure 6 shows the chromatogram (chromatographic conditions are described in the supplementary content) obtained for a water sample spiked with four PAHs (namely: benz[a]anthracene, benzo[a]pyrene, benzo[b] fluoranthene and chrysene) at 5 μg L^{-1} after their extraction by the proposed method. As it can be observed, all the analytes are effectively extracted under the optimised conditions for benz[a] anthracene. For GC/MS analysis, it is not necessary the dilution of the DMCA with acetic acid.

Table 1: Relative recovery study performed on real samples spiked with benz[a]anthracene at a concentration of 1 mg L^{-1}.

Analyte	Water sample (R%[a] ±SD[b])		
	River	Tap	Bottled
benz[a]anthracene	100 ± 9	72 ±10	87 ± 10

[a]R%. Extraction Recovery, [b]SD,Standard Deviation (n=3)

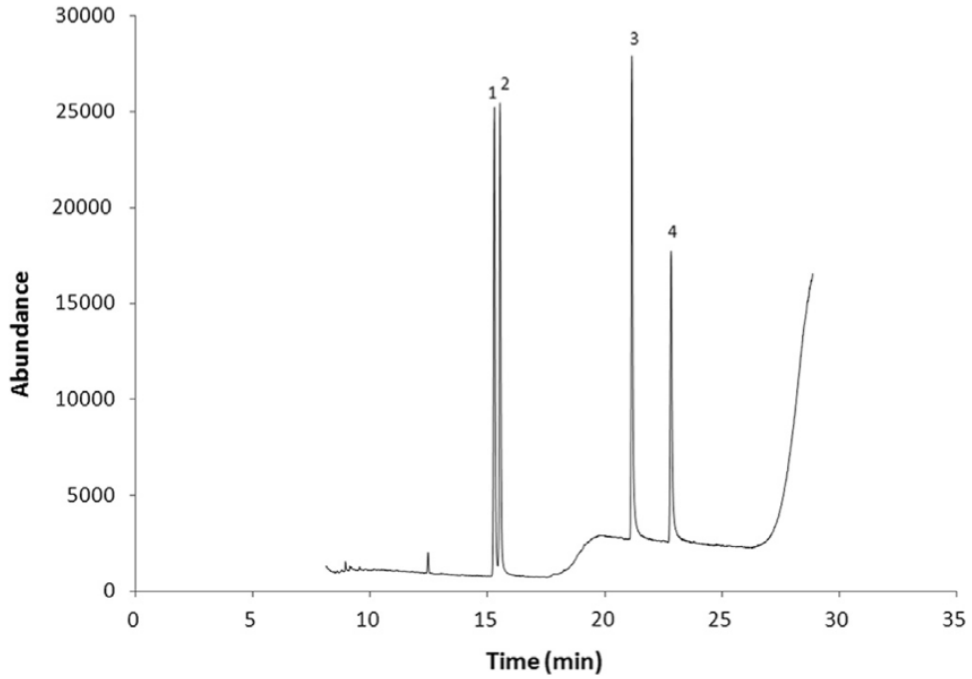

Figure 6: Chromatogram obtained for the extraction of a water sample spiked with four PAHs (benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene and chrysene) at 5 µg L^{-1} and direct injection of the separated DMCA phase (2 µL injection volume).

4.Conclusions

In this article, the use of the so-called switchable hydrophilicity solvents in homogeneous liquid-liquid microextraction is presented for the first time. The proposal uses a low volume (375 µL) of pure DMCA as extractant. The native amine, which is immiscible in water, is solubilised using dry ice as reagent yielding a stable (at least for 10 months) solution. This solubilisation can be reverted, inducing the phases´ separation, by means of sodium hydroxide, which is a common and relatively cheap reagent. Additional centrifugation is not required.

Benz[a]anthracene has been selected as model analyte due to its polarity and native fluorescence. The target compound can be easily extracted to the DMCA phase,

although its direct fluorimetric determination in this medium is not feasible due to a quenching process. This quenching has been successfully avoided using acetic acid; the fluorescence being actually 35% higher than that obtained in a conventional solvent such as methanol. The developed methodology permits the determination of the analytes with a low limit of detection (0.08 μg L^{-1}), good precision (RSD better than 6.7%) and accuracy.

According to the results , the proposed technique presents high extraction efficiency, it is simple and fast and it does not require complex or special labware for phases´ separation.

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Appendix A. Supporting information

1. Evaluation of the phase separation process

The solubilization of DMCA amine in an aqueous medium is achieved using carbon dioxide as trigger. In order to develop an extraction protocol, the solvent should be separated from the aqueous sample after the extraction, allowing in this way the determination of the extracted analytes. Different alternatives have been evaluated in order to induce phase's separation in a rapid and simple way. The assays were performed in glass test tubes (15 mL in volume) containing 13 mL of an aqueous solution of DMCA, previously solubilized using carbon dioxide. The total volume of DMCA in the tubes was fixed to 1.5 mL to make easier the observation of the phase´s separation.

1.1 Chemically induced separations

Several chemicals were evaluated in order to induce the insolubility of the amine in the aqueous phase. Each chemical was supposed to act by a different mechanism:

I. Calcium salts ($CaCl_2$) produce the insolubilization of carbonate in water and may break down the interaction amine-carbonate.
II. Amines (triethylamine) may displace DMCA inducing its separation.
III. Acids (HCl) remove carbonate and bicarbonate from the solution in the form of CO_2 and therefore may induce phases separation.
IV. Alkaline compounds induce a change on the chemical state of the DMCA to its non-charged form, inducing in this way the phase´s separation

Among all these alternatives, only the use of alkaline reagents induces a quantitative phase separation. Sodium hydroxide gave the best results, compared to

that obtained with $Ca(OH)_2$ and $Ba(OH)_2$, especially when it is used at high concentration (20 mol L^{-1}).

1.2 Physically induced separations

Several physical treatments were evaluated to induce phases separation.

I. *Ultrasound irradiation*. Ultrasounds may release the dissolved CO_2 and then turn the DMCA insoluble in aqueous phase. After 1h of ultrasound irradiation, an emulsion was observed which made the phase´s separation unfeasible.

II. *Heating at 40°C*. The increase of the vial temperature may induce a removal of the CO_2 dissolved in the solution. After 1 h of heating a slight phase separation was observed. However, the phase´s separation was not complete due to losses of the amine by evaporation.

III. *Ion-exchange resin*. The use of an anionic exchanger may decrease the concentration of carbonate and bicarbonate in the sample making the amine insoluble. For this purpose, 1 g of Amberlite was immersed into the aqueous phase for 12 h. No phase´s separation was observed in such conditions.

IV. *N_2 bubbling*. This method may induce removal of the CO_2 dissolved in the solution in mild conditions. However, it did not provide good results, since the process was too without quantitative separation.

2. Emission spectra of benz[a]anthracene in different media

As it is indicated in the manuscript, the influence of the DMCA /HOAc ratio on the fluorescence of benz[a]anthracene was evaluated to improve the sensitivity of the determination. The pictures and quantitative results are presented in Figure 4. In the obtaining of these quantitative results, the emission spectra of benz[a]anthracene in different media containing a different DMCA /HOAc ratio were obtained in a fluorescence spectrophotometer using 289 nm as excitation wavelength. The spectra showed a similar shape with almost the same emission bands although the signal to

noise ratio was higher for intermediate DMCA /HOAc ratios. By way of example, Figure S1 shows the emission spectra obtained in four different media.

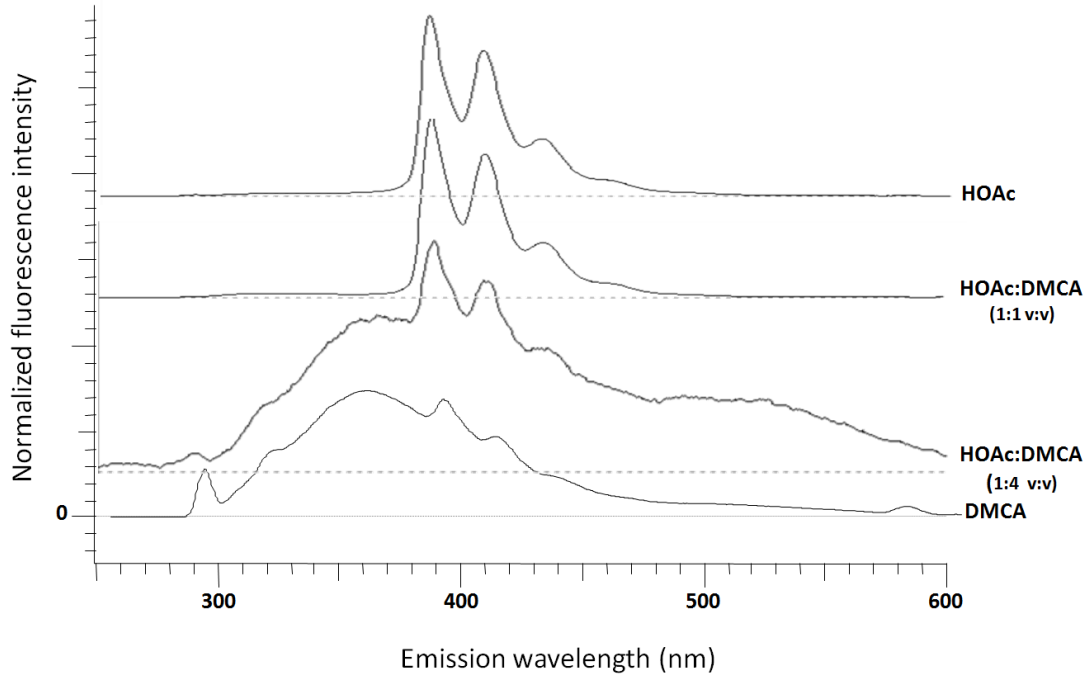

Figure S1: Effect of the DMCA /HOAc ratio on the emission spectra of benz[a]anthracene.

3. Gas chromatography-mass spectrometry analysis

Gas chromatographic/mass spectrometric analyses were carried out on an Agilent (Palo Alto, CA) HP6890 gas chromatograph equipped with an HP5973 mass spectrometric detector based on a quadrupole analyzer and an electron multiplier detector. System control and data acquisition was achieved with HP1701CA MS ChemStation software. Chromatographic separations were performed on a fused silica capillary column (30m×0.25mm i.d.) coated with 5% phenyl–95% methyl polysiloxane (film thickness 0.25 µm) (Supelco, Madrid, Spain) using helium (6.0 grade, Air liquid,

Seville, Spain) at a flow rate of 1 mL min^{-1} as carrier gas. The column temperature program was as follows: 0.8 min at 70°C, raised up to 180°C at 70°C min^{-1}, then immediately ramped at 7°C min^{-1} up to 230°C (kept for 8 min). After this, temperature was raised at 40°C min^{-1} up to 260°C (kept for 8 min). Finally, the temperature increased up to 300°C at 25°C min^{-1} (kept for 1 min). A split injection mode (1:10 ratio) was selected for the introduction of 2 µL of extracts or standards by manual injection. The injector was kept at 225°C. Electron impact ionization (70 eV) was used for analyte fragmentation. The quadrupole mass spectrometer detector was operated in selected ion monitoring mode, recording the following m/z ions: 228 and 252 for benz(a)anthracene, 277 and 252 for benz(a)pyrene, 276 and 252 for benz(b)fluoranthene and 253 and 229 for chrysene. The MS source and quadrupole temperatures were kept at 230°C and 150°C, respectively.

4. Effect of the volume of DMCA aqueous phase on the extraction of the analyte

Three different volumes, namely: 1000 µL, 750 µL and 500 µL, were evaluated in triplicate. Lower volumes were non-practical since the recovery of the solvent was unfeasible while larger volumes induced an excessive dilution of the analyte. Each volume was evaluated in triplicate using fluorescent measurements. As it can be observed in Figure S2 (supporting information), the higher signals are obtained when 500 µL of the extractant are employed. However, 750 µL is selected as the optimum value as a compromise between sensitivity and precision.

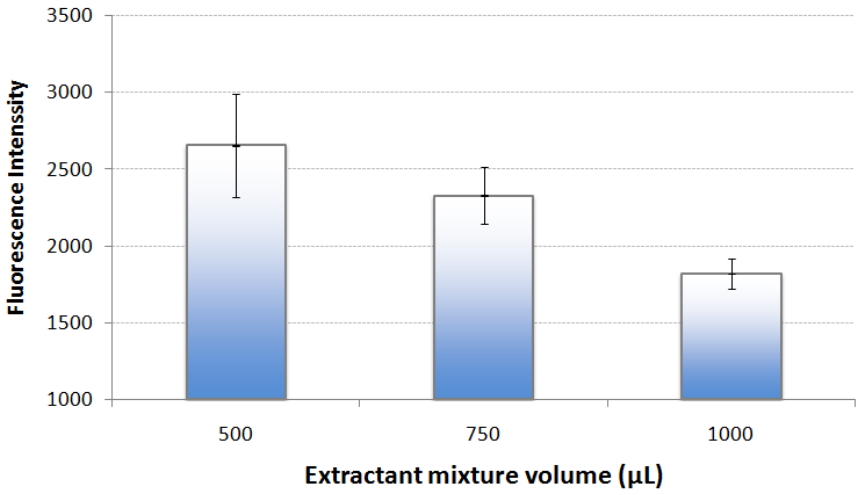

Figure S2: Effect of the extractant mixture volume on the analyte extraction

Capítulo VI: Usefulness of switchable hydrophilicity solvents for the homogeneous liquid-liquid microextraction of triazine herbicides from environmental water samples

JOURNAL OF SEPARATION SCIENCE

Usefulness of switchable hydrophilicity solvents for the homogeneous liquid-liquid microextraction of triazine herbicides from environmental water samples*

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Abstract

In this article an homogeneous liquid-liquid microextraction alternative, based on the use of switchable hydrophilicity solvents (SHS), is presented. The extraction technique makes use of 125 µL of a water-immiscible solvent (N,N-Dimethylcyclohexylamine) that can be solubilized in the aqueous phase in 1:1 ratio using CO_2 as reagent. After the extraction, phases' separation is induced by the addition of sodium hydroxide, which produces a change on the ionization state of the amine, centrifugation not being necessary. The extraction technique has been optimized and characterized using the determination of triazine herbicides by gas chromatography/mass spectrometry in water samples as analytical problem. The presence of metallic ions in environmental waters as interferents is easily avoided by addition of EDTA before the microextraction procedure. The proposed method allows the determination of the target analytes at the low microgram per liter range with good precision (relative standard deviation lower than 12.5 %).

Keywords: Liquid phase microextraction, Green chemistry, switchable hydrophilicity solvents, triazine herbicides.
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1. Introduction

Microextraction techniques, which resulted from the evolution of classic sample treatment towards simplicity and miniaturization, are nowadays usual tools in the development of competitive analytical methodologies [1,2]. The use of microextraction techniques for sample preparation reduces the number of errors that commonly result from classical multi-stage procedures, limits the negative impact on the environment and the health of analytical chemists performing laboratory work [3].

Since 2006, dispersive liquid-liquid microextraction (DLLME) represents an alternative to classical liquid phase extraction procedures thanks to the drastic reduction of organic solvent consumption. This technique, takes advantage of a small volume of extractant phase dispersed by means of an organic solvent (disperser) injected directly into de sample. The disperser solvent is miscible with both extractant and sample, and generates small droplets thus maximizing the contact between phases [4,5].

The main limitations of DLLME are the requirement for an additional organic solvent and the difficult for the recovery of the extractant phase. Several alternatives have been proposed to overpass these limitations. New approaches based on solidification of floating organic solvent drop, facilitates the extractant recovery [6]. Other solution includes the use of ionic liquids as acceptor phase without the need for a disperser solvent [7].

Another powerful technique, based on the same principle of DPLME is the so-called homogeneus liquid liquid microextraction. The main difference of this alternative compared with previous proposals is that the solvent forms a single phase with the donnor one. This methodology extracts the target analytes from an homogeneous solution into a very small volume resulting after the phase separation phenomenon [8]. The phases' separation step could be induced chemically by pH shift [9], salting out process [10] or ion pair formation [11].

Recently, a new group of compounds, namely switchable hydrophilicity solvents (SHS) has been introduced into the extraction field [12-15]. This family of compounds with switchable behaviour englobes amines and amidines with the capacity of alternate its hydrophilicity state as a consequence of the presence of CO_2 in solution [16]. The overall process comprises an acid-base reaction with hydrated CO_2/carbonic acid and the native hydrophobic form of the SHS. As a result, the SHS forms a carbonated salt, which is water-soluble. The SHS can be mixed with water samples and easily separated from the aqueous phase by removing CO_2 from solution by bubbling another gas [17]. Alternatively, the hydrophilicity switch can be also triggered by altering the charge of the SHS by pH shift [18], which is specially useful in the microextraction context, reducing the potential losses by evaporation or sparklings during CO_2 removal process.

In this paper, a SHS-HLLE, is used to improve classic DLLME reducing its main limitations. The solubilisation of the solvent into the aqueous phase is carried out prior to the extraction using dry ice as reagent, which provides a stable homogeneous SHS/water mixture usable as extractant phase. The presented method takes advantage of a water soluble extractant phase and pH shift as phases' separation trigger. The selected SHS is N,N-dimethylcyclohexylamine (DMCA), which has been previously characterized by Jessop et al [19], has been evaluated by our research group as extractant in HLLE using benz[a]anthracene as model analyte [18]. Its potential for the extraction of partially hydrophobic compounds, such as triazine herbicides from environmental waters is evaluated.

2. Experimental

2.1 Chemicals

All reagents were of analytical grade or better. Sigma-Aldrich (Madrid, Spain) provided the triazine herbicides: prometon, (PMT); terbumeton, (TBM); secbumeton, (SBM); simetryn, (SMT); prometryn, (PMT); and terbutryn (TBT). Stock standard solutions of the analytes were prepared in methanol (Panreac, Barcelona, Spain) at 500 mg L^{-1} and stored at 4**°**C. Sodium ethylenediaminetetraacetate dihydrate (EDTA) and N,N-dimethylcyclohexylamine (DMCA) were also purchased from Sigma–Aldrich. Working standard solutions were prepared by the appropriate dilution of the stocks in Milli-Q water (Millipore Corp., Madrid, Spain) or methanol as required.

Dry ice, in the form of pellets of 3 mm, purchased from Cordogas (Cordoba, Spain) was employed for the solubilisation of the DMCA in water. Sodium hydroxide from Sigma-Aldrich was employed to induce phase´s separation. A 20 mol L^{-1} NaOH solution was daily prepared and used in the extraction procedure.

Bottled, tap and river water (Guadalquivir river, Córdoba, Spain) samples were collected in amber glass bottles without headspace and stored at 4**°**C until analysis. The samples were not filtered prior their analysis. Bottled water samples were purchased from a local market.

2.2 Apparatus

Gas chromatographic/mass spectrometric analyses were carried out on an Agilent (Palo Alto, CA) HP6890 gas chromatograph equipped with an HP5973 mass spectrometric detector based on a quadrupole analyzer and an electron multiplier detector. System control and data acquisition was achieved with HP1701CA MS ChemStation software. Chromatographic separations were performed on a fused silica capillary column (30m×0.25mm i.d.) coated with 5% phenyl–95% methyl polysiloxane (film thickness 0.25 µm) (Supelco, Madrid, Spain) using helium (6.0 grade, Air Liquid, Seville, Spain) at a flow rate of 1 mL min^{-1} as carrier gas. The column temperature program was as follows: 2 min at 40°C, raised up to 170°C at 40°C min^{-1}, then

immediately ramped at 2°C min^{-1} up to 200°C. Finally, the temperature increased up to 260°C at 10°C min^{-1} (kept for 2 min). A splitless injection mode was selected for the introduction of 2 µL of extracts or standards by manual injection. The injector was kept at 280°C.

Electron impact ionization (70 eV) was used for analytes fragmentation. The quadrupole mass spectrometer detector was operated in selected ion monitoring mode, recording the following m/z fragment ions characteristic of each analyte: 210 for PMT and TBM; 196 for SBM; 213 for SMT; 241 for PMT and 226 for TBT. The MS source and quadrupole temperatures were kept at 230 and 150°C, respectively. The peak areas were used for quantification of individual analytes.

2.3 Amine aqueous-phase preparation

The procedure to obtain the water-soluble amine phase is described in a previous work [R]. In brief, 100 mL of Milli-Q water and 100 mL of DMCA are placed in a 1 L glass bottle with screw cap, forming a two phases system. Additions of 10 g each of dry ice are performed until a unique phase of aqueous amine is observed. After this process a volume of 200 mL of aqueous miscible amine is obtained. The monophasic system remains stable for 12 months.

2.4 Extraction procedure

The extraction consists of various well-defined steps. First of all, 250 µL of aqueous amine are added to 10 mL of aqueous standard of the trazine herbicides. The flask is manually shaken until an homogeneous phase is observed. Then, 1 mL of a 20 mol L^{-1} NaOH solution is added and a cloudy solution appears. The solution is manually shaken again, and the flask is left to stand for 5 min to achieve phases' separation. 100 µL of DMCA are recovered and transferred to a glass vial. In the case of water samples, the addition of EDTA at a final concentration of 0.1 mol L^{-1} is required to avoid the

precipitation of cations at the very alkalyne pH required for phases' separation. In both cases, a 1:2 dilution in methanol of the final DMCA extract is performed to enhance chromatographic separation.

3. Results and discussion

As moderate non-polar solvent, DMCA can be employed for the extraction of hydrophobic [18] and partially hydrophobic analytes from aqueous samples. For this reason, the extraction of triazines herbicides from environmental waters using SHS-HLLE has been evaluated. Solvent solubility switch must be chemically induced to prevent losses due to sprinkles out of the extraction vessel.

3.1 Extractant volume

The initial conditions selected for the SHS-HLLE procedure were those described in a previous work [R]. For the presented alternative, the extraction vessel is a 10 mL volumetric flask. The geometry of the extraction vessel increases the height of the separated DMCA phase, which facilitates the recovery of low volumes. As a result, the volume of extractant phase can be lower than that previously studied. For this reason, three different volumes of aqueous amine, namely: 1000 µL, 500 µL and 250 µL (which correspond to 500, 250 and 125 µL of DMCA respectively), were evaluated in triplicate. As it can be observed in Figure 1, the higher analytical signals were obtained when 250 µL of the aqueous amine were employed. According to the error bars, the lower volume could be considered less precise, but the sensitive is much better than that of the other volumes evaluated. Therefore, 250 µL is selected as extractant phase volume.

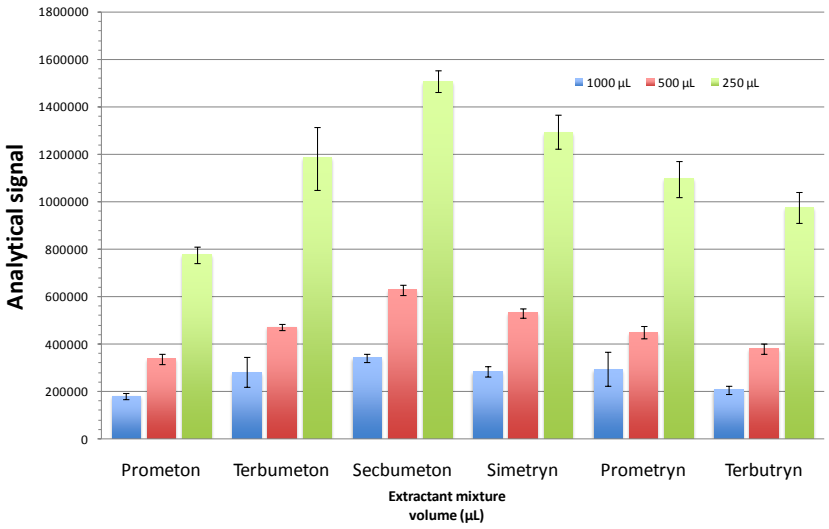

Figure 1: Effect of the extractant mixture volume on the analytical signal

3.2 Analytical figures of merit.

The optimized SHS-HLLE procedure was applied to the determination of triazine herbicides in water samples. A calibration graph was constructed for each analyte by extracting in triplicate five working aqueous standards within the concentration interval from 0.1 to 10 µg L^{-1}. The main analytical figures of merit are sumarized in Table 1. The sensitivity of the method was evaluated according to the limit of detection (LOD) and the method detection limit (MDL) defined by US-EPA [20]. The MDLs were in all cases in the microgram per litre range and varied between 0.22 µg L^{-1} (prometon) and 0.53 µg L^{-1} (secbumeton). The LODs, based on signal-to-noise ratio (*S*/*N*) of 3, ranged from 0.1 µg L^{-1} (prometon) and 0.37 µg L^{-1} (terbumeton). A good correlation between MDLs and LODs values were observed for all the analytes. The repeatability of the method was evaluated at a concentration level of 0.5 µg L^{-1} and the RSDs were in the range of 3.1-12.5%.

Table 1: Analytical figures of merit for the determination of the selected triazine herbicides in water using switchable solvents as extractant phase.

Analyte	MDL ($\mu g\ L^{-1}$)	LOD[b] ($\mu g\ L^{-1}$)	R[c]	Precision expressed as RSD[d] (%) (n=5)
Prometon	0.22	0.10	0.999	10.7
Terbumeton	0.29	0.37	0.998	9.5
Secbumeton	0.53	0.25	0.994	3.1
Simetryn	0.23	0.33	0.993	5.6
Prometryn	0.34	0.34	0.993	12.5
Terbutryn	0.25	0.23	0.997	8.9

[a] MDL: method detection limit [b] LOD: limit of detection[c] R: correlation coeficient[d] RSD: relative standar deviation

3.4 Analysis of water samples

In order to evaluate the applicability of the proposed method for the determination of triazines in water samples, three different matrices (river, tap, and bottled) were analysed. In order to evaluate the accuracy of the method, a recovery study was performed at a concentration level of 10 $\mu g\ L^{-1}$. During the pretreatment of the samples the appearance of a white precipitate on the interphase is observed. The precipitate difficults the recovery of the amine, which results in a poor extraction recovery when the extracts are analysed. Initially, the nature of the precipitate can be adscribed to the formation of insoluble metallic hydroxides due to the highly alkaline pH required for the phases' separation. To verify this, a standard containing triazine herbicides and a calcium salt giving a final calcium concentration of 80 $mg\ L^{-1}$ was processed following the optimized procedure. Under these conditions, the standard showed the same behavior than that observed for the water samples. Chromatographic analysis of the standards containing calcium showed a lower extraction efficiencies than those obtained without the metallic cation. To eliminate the interference of the metallic hydroxides in the environmental water samples a chelating agent was added. Different concentrations of EDTA viz, 0.01, 0.05 and 0.1 $mol\ L^{-1}$ were added to a standard containing the triazine herbicides at a concentration of 10 $\mu g\ L^{-1}$. As a result, the recovery increases linearly with the EDTA concentration. In order to assess the

complete chelating of the metallic ions present in environmental samples, the highest concentration of EDTA (0.1 mol L^{-1}) was selected as optimum. Under these conditions, no precipitate was observed in the samples. Table 2 compares the extraction recoveries obtained in presence and absence of EDTA. As a visual comparison, Figure 2 shows the differences for the same river sample in presence and absence of EDTA.

Table 2: Relative recovery study performed on real water samples spiked with the analytes at a concentration of 10 µg L^{-1}.

Analyte	Water sample (R%[a]± SD[b])					
	Tap		River		Bottled	
	w/o EDTA	w/ EDTA	w/o EDTA	w/ EDTA	w/o EDTA	w/ EDTA
Prometon	35±4	65±3	39±7	80±1	57±3	77±6
Terbumeton	35±7	66±3	42±3	77±1	54±8	77±7
Secbumeton	37±2	75±3	45±6	73±6	56±4	73±7
Simetryn	43±7	66±12	36±7	72±2	49±8	67±5
Prometryn	37±6	61±1	45±6	69±1	56±15	93±1
Terbutryn	35±15	74±3	41±14	71±1	54±12	75±5

[a]R%: extraction recovery (at 10 µg L^{-1} level), [b]SD: standard deviation (n = 3).

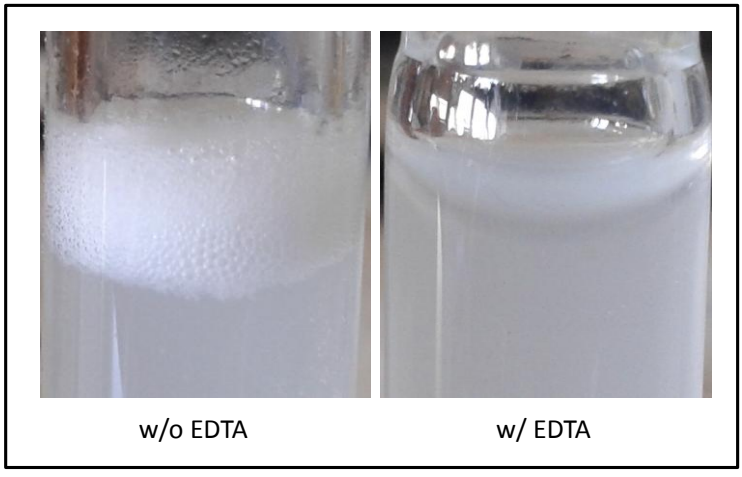

Figure 2: Comparison between river water samples in absence (left) and presence (right) of 0.1 mol L^{-1} EDTA

4. Conclusions

In this article, a switchable hidrophilicity solvent (SHS) is employed as extractant in a homogeneous liquid-liquid microextraction (HLLE) procedure. The proposal uses a very low volume (125 µL) of pure DMCA as extractant for 10 mL of water samples. The native amine is hydrophobic but it can be easily switched to its hydrophilic form by adding CO_2 into the biphasic system formed with water. Introduction of dry ice pellet has been the form selected to solubilise CO_2 because of its simplicity (it does not require gas supply or additional tubing) and reduced cost. The aqueous mixture of amine obtained following this procedure is completely stable in time, not reverting to its hydrophobic form. However, the phases´ separation can be induced in order to extract and preconcentrate the target analytes. As previously stated [18], sodium hydroxide is the selected chemical alternative for phases' separation, additional centrifugation not being required.

According to the results, the proposed technique presents high extraction efficiency, it is simple and fast and it does not require complex or special labware for phases´ separation.

The proposed method fulfills the sensitivity requirements established by US-EPA, which demands a limit in the range from 1 to 10 µg L^{-1} for the selected herbicides in municipal and drinking waters samples.

The matrix effect ascribed to insoluble metallic hydroxide can be easily avoided by adding of 0.1 mol L^{-1} of EDTA to the environmental samples prior to the extraction. As a consequence of the chelation of the cations, the recovery and precision for all the studied samples were considerably higher.

Acknowledgments

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Bloque III: Discusión de los resultados

En este apartado de la Memoria de Tesis Doctoral se detallan los resultados más significativos derivados del desarrollo experimental de la misma. De manera general, ha consistido en el desarrollo y evaluación de nuevas alternativas de microextracción dispersiva, tanto en fase sólida como líquida. Para ello, los trabajos experimentales desarrollados se han agrupado en función del papel que juega el dióxido de carbono en el proceso de extracción. En este sentido, se ha empleado el dióxido de carbono como agente dispersante, mediante la reacción efervescente en fase acuosa o como responsable de la solubilización en fase acuosa de los llamados disolventes conmutables. Todas las alternativas desarrolladas se han evaluado en función de su rendimiento analítico en el contexto del análisis de muestras ambientales. Finalmente, todas han sido comparadas entre sí en función de los principios de la Química Analítica Verde.

1.Energías auxiliares en procesos de microextracción

En general, el éxito de un procedimiento de microextracción en fase liquida o sólida depende del grado de contacto entre las fases donadoras y aceptoras. Las técnicas dispersivas permiten obtener un elevado contacto entre las fases mediante el empleo de un agente dispersante (físico o químico) que permite alcanzar el equilibrio entre las fases de forma rápida y eficaz.

La energía de ultrasonidos se puede emplear en distintas etapas del PMQ [1]. El mecanismo de actuación de la radiación ultrasónica en mezclas de fases inmiscibles se basa en el efecto de cavitación. La implosión de las burbujas generadas produce ondas de choque en el líquido circundante y genera desplazamientos de materia a alta velocidad, que pueden causar la disrupción de las gotas vecinas de las burbujas que colapsan. Mediante este fenómeno, el tamaño de las gotas de extractante se reduce drásticamente aumentando la superficie de contacto. Ejemplos destacados basados en el empleo de ultrasonidos son la ***extracción por emulsificación asistida por***

ultrasonidos (ultrasound–assisted emulsification extraction, ***USAEE***) [2] y la ***microextracción–emulsificación asistida por ultrasonidos*** (ultrasound–assisted emulsification-microextraction, ***USAEME***) [3]. Ambas técnicas emplean dos fases líquidas inmiscibles (muestra y extractante) para formar una emulsión de microgotas, incrementando la superficie de contacto entre ambas. El empleo de ultrasonidos permite desarrollar extracciones eficientes en un corto periodo de tiempo.

El equipo necesario para la extracción por ultrasonidos consiste en un baño ultrasónico comercial o una sonda, aunque es más eficiente el empleo de sondas [4]. Este hecho limita, en cierta medida, la aplicabilidad de estas técnicas para la extracción *in situ* de analitos de interés de muestras ambientales. Además, la adquisición y mantenimiento de este equipamiento genera un sobre coste adicional.

Otra forma ampliamente extendida de dispersar una fase extractante en una muestra acuosa es la agitación mediante el empleo de agitadores tipo vortex. La agitación con vortex de dos fases inmiscibles da lugar a la ***microextracción líquido-líquido asistida por vortex*** (vortex assisted liquid-liquid microextraction, ***VALLME***). Por norma general, el empleo de un vortex para producir la dispersión tiene un coste económico menor que en el caso de los ultrasonidos, con la ventaja adicional de que se generan dispersiones termodinámicamente más inestables, facilitando la separación de fases [5-8]. Estas dos alternativas permiten la eliminación del disolvente dispersante, además de favorecer la separación de fases mediante la coalescencia inducida durante la agitación [9]. Los disolventes tóxicos que se emplean en ambas alternativas pueden sustituirse por tensioactivos, dando lugar a las variantes conocidas como ***microextracción asistida por ultrasonidos mejorada con tensioactivos*** (ultrasound-assisted surfactant-enhanced emulsification microextraction; ***UASEME***) [10,11] y ***microextraccion líquido-líquido asistida por vortex mejorada con tensioactivos*** (vortex-assisted surfactant-enhanced emulsification liquid–liquid microextraction; ***VSLLME***) [12-14].

Otras estrategias menos extendidas para dispersar el extractante son el empleo de microondas y la diferencia de presión. En el caso de la aplicación de microondas, es frecuente encontrarla en técnicas de microextracción dispersiva que emplean líquidos iónicos como extractante. Xu y colaboradores propusieron el empleo de microondas en combinación con líquidos iónicos para extraer formaldehido de bebidas mediante IL-DLLME [15]. En este caso, la irradiación con microondas produce un aumento de temperatura y la aparición de una turbidez en la muestra debido a la presencia del liquido iónico. La cinética de extracción se ve favorecida por el doble efecto térmico y de elevada superficie de contacto. Aunque el empleo de microondas elimine el empleo de disolventes para generar la dispersión [16], su uso esta menos extendido que el vortex o la radiación de ultrasonidos. Por otra parte, en 2012 el empleo de aire dio lugar a la técnica denominada ***microextracción líquido-líquido asistida por aire*** (air assisted liquid-liquid microextraction, ***ALLME***) [17]. La técnica emplea una jeringa que inyecta y aspira repetidamente 15 µL de 1,1,2,2-tetracloroetano (1,1,2,2-TCE) como extractante en 10 mL de muestra en un tubo cónico de centrifuga. El extractante con los analitos extraídos se recupera por centrifugación y se analiza mediante GC. El empleo de volúmenes tan reducidos de extractante es muy recomendable, ya que, por una parte, permite alcanzar factores de preconcentración elevados y por otra, minimiza los riesgos ambientales y el coste del proceso.

A pesar de las ventajas de estas alternativas, la recuperación del extractante con los analitos suele requerir una etapa de centrifugación. Si bien la centrifugación es una herramienta extendida en los laboratorios de análisis, su uso puede considerarse una limitación en términos de tiempo y coste, además de resultar inviable para su empleo en análisis *in situ*.

Si bien en el ámbito de la DLLME, el empleo de energías auxiliares está extendido para conseguir la homogénea dispersión de la fase extractante, en el caso de la DSPME no se contempla tan exhaustivamente. Esto se debe a que se emplea

generalmente en procesos de limpieza de muestra para mejorar la selectividad del proceso, siendo necesaria la simple agitación física para conseguirlo. La dispersabilidad de sólidos en DSPME implica una dificultad cuando se emplean nanoestructuras, debido principalmente a la elevada tendencia a la agregación que éstas presentan. Por ello, es frecuente emplear suspensiones del material nanoestructurado en un disolvente orgánico que actuará como dispersante, pero esto puede repercutir negativamente en la eficacia del proceso de extracción, como ya se mencionó en la introducción de esta Memoria.

2. Empleo de la efervescencia para el aumento de la superficie de contacto

Desde que fue propuesta por Annastasiades [18], la extracción en fase sólida dispersiva ha evolucionado considerablemente. Si bien fue propuesta como una estrategia de limpieza para matrices complejas, es posible emplear el mismo planteamiento experimental para extraer y preconcentrar analitos de interés consiguiendo mejorar la selectividad y sensibilidad del proceso [19]. Los procesos dispersivos aumentan la superficie de contacto entre el extractante y la muestra, mejorando sensiblemente la cinética del proceso.

La evolución de la técnicas dispersivas corre en paralelo con la química verde, especialmente si consideramos que estos procesos son:

- Más eficientes, con la consiguiente reducción de costes.
- Más respetuosos con el medio, gracias al empleo de disolventes no contaminantes o incluso la eliminación de los mismos ya sean como fases extractantes o agentes dispersantes.
- Miniaturizados, en línea con los dos puntos anteriores, reduciendo el coste por análisis, los residuos generados y aumentando la sensibilidad del proceso.

2.1 Empleo de la efervescencia como fuerza dispersante de materiales sólidos

Durante la realización de esta Tesis Doctoral, se planteó el empleo de la efervescencia generada *in situ* en una muestra acuosa con el objeto de dispersar la fase extractante.

La efervescencia es una reacción de intercambio de protones entre un donador de iones carbonato (CO_3^{2-}) y un ácido en fase acuosa. La consecuencia directa de esta reacción es la liberación de dióxido de carbono gaseoso en el medio.

La liberación de CO_2 en el seno de una disolución tiene como consecuencia un aumento de la presión en el interior de la misma hasta que éste abandona la fase acuosa. Este proceso puede emplearse para dispersar principios activos, muy común en la industria farmacéutica, o en nuestro caso, para dispersar un sólido.

El intenso burbujeo que se genera en la disolución es el responsable de dispersar la sustancia de interés. La presencia de los precursores efervescentes en el proceso puede ser conjunta (mediante una única adición de los dos componentes simultáneamente) o por separado (si se encuentran en fases separadas que son añadidas secuencialmente).

Para dispersar un sólido con fines extractivos en una fase acuosa, se añade simultáneamente con los precursores efervescentes de manera que la disolución de estos libera CO_2 como agente dispersante dando lugar a la modalidad denominada ***microextraccion en fase sólida dispersiva asistida por efervescencia*** (effervescence-assisted dispersive micro solid phase extraction; ***EA-DμSPE***).

2.2 Precursores efervescentes

La eficiencia del proceso de dispersión depende directamente de las propiedades físico-químicas de los precursores y de su proporción. Por esta razón, la composición de la pastilla efervescente se optimiza, teniendo en cuenta los siguientes factores:

- *Velocidad de reacción*: la velocidad con la que ocurre la reacción y la liberación de CO_2 determina la intensidad de la dispersión. Sin embargo, una reacción demasiado violenta puede provocar proyecciones y pérdidas de muestra y extractante. Del mismo modo, determinará el tiempo de extracción, puesto que influye en el tiempo de contacto entre las fases.
- *Variación de pH*: en función de las proporciones de los precursores efervescentes, el pH de la muestra puede variar debido al carácter ácido/base de los componentes. Una variación acusada de pH puede ser desfavorable para la extracción de determinados analitos. Cuanto menor sea el rango de variación de pH, menor influencia tendrá este parámetro en la extracción.
- *Estabilidad*: la mayoría de los componentes tienen cierto carácter higroscópico. La hidratación de estos durante el almacenaje o preparación de la pastilla puede limitar su reactividad, y por tanto disminuir la eficiencia dispersiva.

Se evaluaron distintos candidatos a precursores efervescentes tal como recoge la Tabla 1. En todos los casos, es necesario eliminar la humedad de los reactivos antes de la preparación de la pastilla. Esto se consigue mediante un tratamiento térmico almacenando los componentes a temperaturas de hasta 90°C durante al menos 8 horas. Eliminar la humedad de estos reactivos puede plantear un problema, debido a que a elevada temperatura algunos de ellos, como los ácidos cítrico y ascórbico pueden descomponerse. En el caso del ácido ascórbico, existe el problema de la sensibilidad al oxígeno. El ácido ascórbico es un agente antioxidante, y por tanto puede reaccionar con el oxígeno del aire y descomponerse. Los dos donadores de CO_3^{2-} estudiados, carbonato y bicarbonato sódico, presentaban distintas estabilidades térmicas, siendo el carbonato el más estable.

La estabilidad de las combinaciones se evaluó mediante el estudio de la higroscopicidad de la mezcla en forma de pastilla. Para ello se estudió la ganancia de

peso (en porcentaje) en el tiempo (24, 120 y 192 h). La tabla muestra como la pastilla más estable fue la compuesta por carbonato de sodio y el dihidrógeno fosfato de sodio, presentando la menor variación en peso a tiempo final. Además, las mezclas que incluían ascórbico presentaron una pérdida de peso, fenómeno que puede atribuirse a la descomposición de éste, efecto más importante que la ganancia de peso por humedad. En el caso del carbonato, menos higroscópico que el bicarbonato, se observa que la pérdida de peso es prácticamente inexistente en el tiempo, por lo que esta combinación de precursores es la más estable. Para el bicarbonato el efecto es justamente el opuesto, al presentar una higroscopicidad mayor, la ganancia de peso por humedad es mayor. Este efecto es persistente en todas las mezclas que contienen bicarbonato.

Tabla 1: Principales características de las pastillas efervescentes en función de los precursores

Composición		Característica				
Fuente de CO_2	Donador de H^+	Tiempo Ef.[a]	ΔpH[b]	Estabilidad (%ΔP)[c]		
				24 h	120 h	192 h
carbonato de sodio	ácido cítrico	1.3	1.5	0.43	1.01	1.25
bicarbonato de sodio	ácido cítrico	1.8	1.1	0.52	0.81	0.81
carbonato de sodio	ácido ascórbico	2.0	1.2	-0.23	-0.14	-0.52
bicarbonato de sodio	ácido ascórbico	2.3	0.6	-0.29	1.71	0.90
carbonato de sodio	dihidrogenofosfato de sodio	4.5	0.2	0.55	0.52	0.55
bicarbonato de sodio	dihidrogenofosfato de sodio	19.0	-0.7	0.87	2.18	4.80

[a] Tiempo Ef.: tiempo de efervescencia (min).[b] ΔpH. variación de pH al final de la reacción ΔP, variación del peso con el tiempo, expresado como porcentaje.

El empleo de la pastilla efervescente en una modalidad de microextracción en fase sólida dispersiva se ha desarrollado con el objetivo de emplearse para la extracción *in situ* de analitos de interés en muestras acuosas. Para ello, la extracción se lleva a cabo empleando dos dispositivos: una jeringa de vidrio con una capacidad de 10 mL y un filtro estándar de jeringa de nylon de tamaño de poro de 0.45 µm para retener

el sorbente con los analitos extraídos después de la extracción. El uso de la jeringa limita ciertamente el volumen de muestra que puede analizarse mediante el método propuesto, sin embargo, simplifica significativamente el proceso. Para la extracción, la pastilla con el sorbente se deposita en el interior de la jeringa y con ésta se aspira el volumen de muestra como muestra la Figura 1. Cuando la pastilla entra en contacto con la fase acuosa, comienza la reacción efervescente y el sorbente se dispersa eficazmente a lo largo de la columna de muestra dentro de la jeringa. El tiempo de extracción queda definido para esta aplicación como el tiempo en el que están en contacto sorbente y muestra por medio de la efervescencia, en este caso, inferior a 5 minutos. En el interior de la muestra queda el sólido en suspensión en íntimo contacto con ella. Para recuperarlo, se acopla el filtro de jeringa y se presiona el émbolo hasta que toda la muestra ha sido retirada. El sorbente con los analitos extraídos queda retenido en el filtro y puede almacenarse y posteriormente realizar la elución de los analitos de interés con el disolvente apropiado. Almacenar los filtros tras el proceso de extracción es más simple que transportar la muestra para realizar la extracción en el laboratorio. Además, ciertos analitos pueden descomponerse durante el proceso de almacenaje debido a reacciones químicas con los componentes de la matriz de la muestra, reacciones fotoquímicas o biológicas. Además, el transporte de muestras acuosas suele requerir el empleo de refrigeración, que si bien puede minimizar los efectos anteriores, implica un coste adicional.

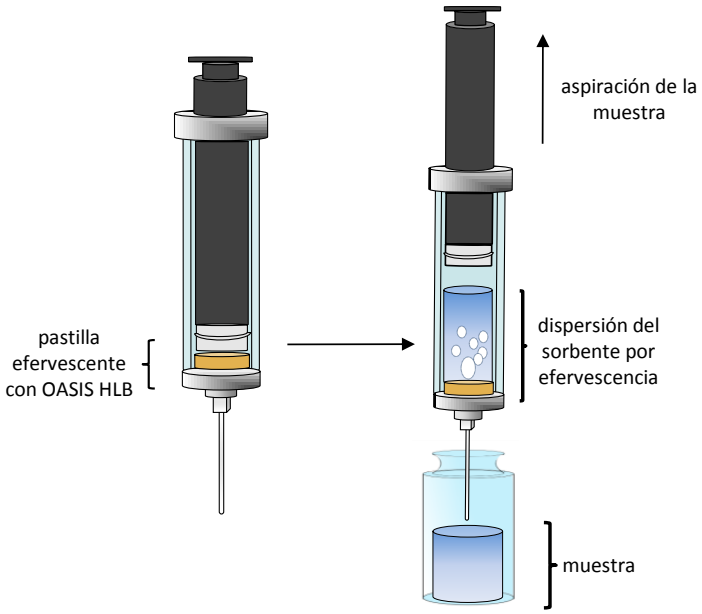

Figura 1: Esquema del proceso de extracción con pastilla efervescente y sorbente polimérico.

2.3 Evaluación de la sinergia entre efervescencia y sólidos poliméricos y nanoestructurados

El componente extractivo de la pastilla, puede ajustarse en función de los requerimientos de la aplicación analítica para la que se vaya a emplear. En esta Tesis Doctoral, el primer problema analítico seleccionado fue la determinación de compuestos nitroaromáticos en agua. El sorbente comercial OASIS HLB (Waters Corp.) está especialmente indicado para este tipo de compuestos. Se trata de un copolímero macroporoso [poli (divinilbenceno-co-N-vinilpirolidona)] en forma de microesferas de un tamaño de 30-60 µm.

Con el extractante seleccionado, se estudió la sinergia con las distintas combinaciones de precursores efervescentes y se optimizó la cantidad incluida en la pastilla utilizando un estandar acuoso de nitrobenzeno (NB) a una concentración de

500 µg L^{-1} como referencia para la comparación. Para ello, se mezcló 1 mg de sorbente con los precursores efervescentes para producir una pastilla de 250 mg totales y se compactaron con la prensa manual hasta 12.5 T (máxima capacidad de la prensa). La Figura 2 muestra los resultados del análisis cromatográfico de cada una de las combinaciones, destacando el tiempo de extracción/efervescencia en cada uno de ellos. Como era de esperar, a mayor tiempo de contacto, más eficiente es la extracción, con la excepción de la combinación de bicarbonato de sodio con dihidrogenofosfato de sodio y de ácido ascórbico y bicarbonato de sodio. En el primer caso, aunque el tiempo de desintegración de la pastilla de estos dos componentes sea tan elevado, la liberación de CO_2 ocurre de forma muy lenta, por lo que la dispersión del sorbente no es eficaz. En el segundo, sin embargo, el tiempo de efervescencia similar al observado para otras combinaciones pero con señales analíticas más bajas, es un reflejo de la inactivación del ácido ascórbico causada por el oxigeno ambiental anteriormente mencionada. Finalmente, la combinación de carbonato de sodio como fuente de CO_2 y de dihidrogenofosfato de sodio como donador de protones con el sorbente fue la combinación seleccionada. De acuerdo con los resultados mostrados en la tabla, es la combinación más estable que da lugar a tiempos de desintegración suficientes como para proporcionar las señales analíticas más elevadas.

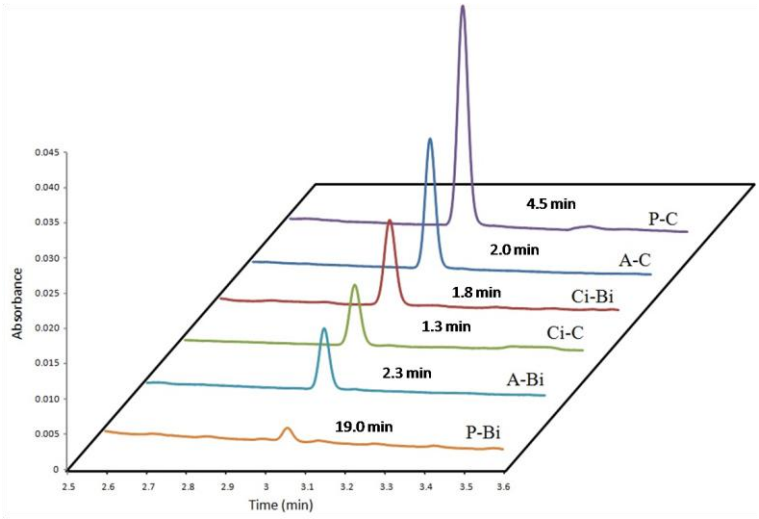

Figura 2: Comparación de las señales analíticas obtenidas para el análisis de un estándar acuoso de nitrobenceno (500 µg L^{-1}) y su correspondiente tiempo de extracción (P; dihidrogenofosfato de sodio, A; ácido ascórbico, Ci; ácido cítrico, Bi; bicarbonato de sodio, C; carbonato de sodio).

La cantidad de sorbente se evaluó en el intervalo de 1 a 10 mg por pastilla. La señal analítica obtenida a partir de las extracciones con cantidades crecientes de sorbente fue directamente proporcional a las mismas. Sin embargo, debido al tamaño de partícula del sorbente, a medida que se incrementó la cantidad en la pastilla, la integridad de ésta disminuye. Pese a la presión empleada para conseguir la compactación de los componentes, la pastilla se vuelve quebradiza por la distorsión que generan las partículas de sorbente, dificultando su manejo. Por esta razón, se optó por un compromiso entre la integridad de la pastilla y la capacidad sorbente, seleccionándose 5 mg como cantidad óptima.

La misma estrategia puede seguirse para la fabricación de pastillas efervescentes con sorbentes nanoestructurados como componente extractivo. En este caso se estudió la incorporación de nanotubos de carbono de pared múltiple (MWCNTs). Los MWCNTs presentan características físico-químicas que los convierten en sorbentes muy versátiles. Sin embargo, su empleo en estrategias dispersivas está

limitado debido a su persistente tendencia a la agregación, lo cual limita su capacidad sorbente. Habitualmente, se recurre a estrategias de funcionalización que modifican sus propiedades y mejoran su dispersabilidad. En esta Tesis Doctoral se ha empleado material sin funcionalizar para evaluar su dispersión mediante efervescencia.

Una de las principales diferencias entre los sorbentes poliméricos y nanoestructurados es su tamaño. Debido a esto, es posible incorporar una cantidad mayor de sorbente sin provocar alteraciones estructurales en la pastilla. Para el caso de los MWCNTs, la cantidad añadida a la pastilla determina el volumen de muestra que puede ser procesado. Si bien en el primer ejemplo de EA-DμSPE, el volumen de muestra venía determinado por el sistema de extracción, en este caso dicha variable puede optimizarse. El estudio de ambos parámetros se llevó cabo considerando la extracción de triazinas en muestras acuosas. Se estudió un intervalo de volúmenes de 10-250 mL de muestra y unas cantidades de sorbentes de 0.5-10 mg por pastilla. Es importante destacar que la recuperación del sorbente en este caso requiere un sistema de filtración a vacío, debido principalmente a dos factores:

1) el manejo de elevados volúmenes de muestra mediante jeringas resulta complejo y generalmente irreproducible.
2) las cantidades mayores de sorbente saturan fácilmente los filtros y generan problemas de presión.

Por estas razones se empleó un sistema de filtración a vacío con cinta de teflón comercial (PTFE) como elemento de retención para el sorbente.

La Figura 3 muestra el efecto de cada variable por separado (Figura 3A; cantidad de sorbente, Figura 3B; volumen de muestra) y en conjunto (Figura 3C) para el caso de un estándar acuosa de simazina (500 μg L^{-1}) como analito modelo. El comportamiento es similar en todos los casos estudiados. En función del gráfico de

contorno, se seleccionó la combinación de 7.5 mg de sorbente por cada 100 mL de muestra. El análisis conjunto de estas variables permite optimizar la cantidad de sorbente requerido para un volumen de muestra en concreto, siempre que se mantengan los precursores efervescentes en las mismas proporciones.

La microextracción en fase sólida dispersiva asistida por efervescencia para sorbentes micro y nanoestructurados se comparó con variantes de extracción en fase sólida convencional y dispersiva no asistida por efervescencia, corroborando que la dispersión mediante efervescencia en la muestra es más eficiente que las otras alternativas analizadas. En el caso de sorbentes nanoestructurados la efervescencia no sólo es preferible en términos de dispersabilidad, sino que permite obtener dispersiones más estables del material.

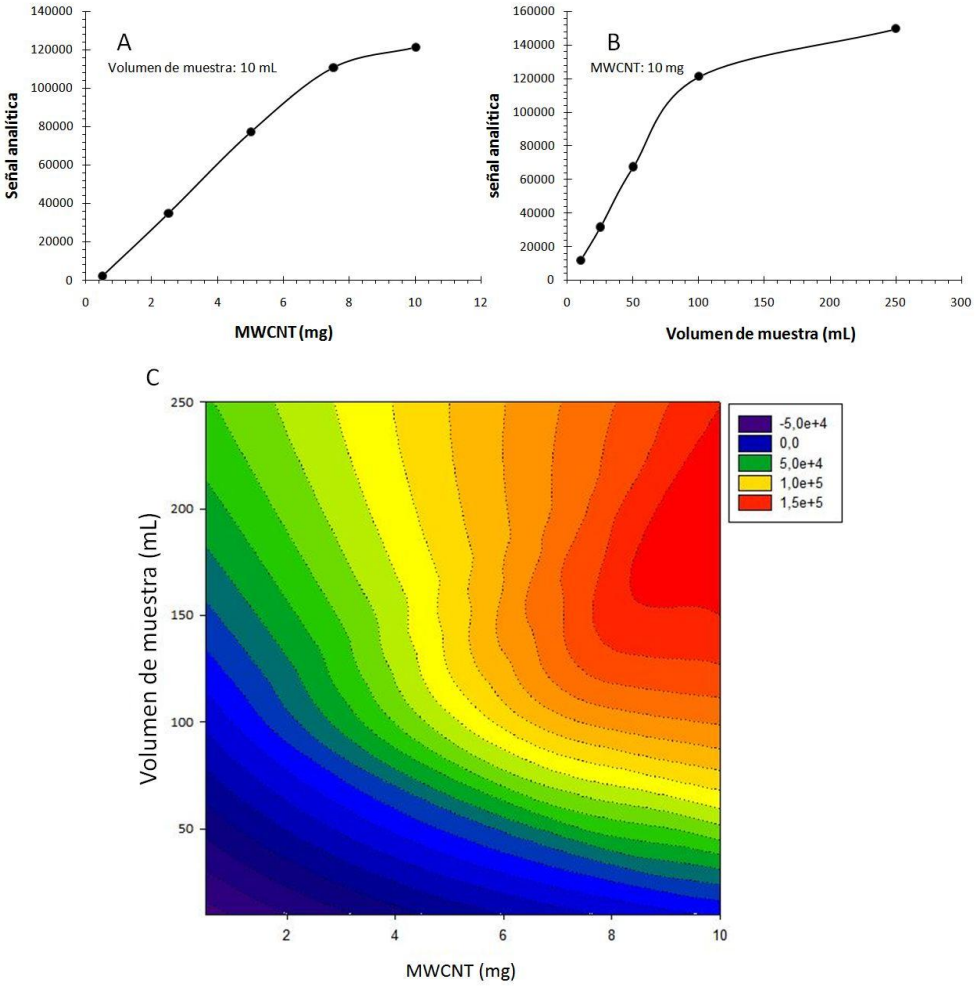

Figura 3: efecto de la cantidad de sorbente (A) y del volumen de muestra (B) individualmente y en conjunto (C)

La Figura 4 muestra la comparación entre distintas alternativas de dispersión,) la dispersión se genera mediante la disolución de la mezcla que constituye la pastilla sin prensar (Figura 4A; mediante la pastilla efervescente (Figura 4B); empleando los nanotubos directamente sobre el agua (Figura 4C) y en dos etapas, primero disolviendo los componentes efervescentes y posteriormente los nanotubos (Figura 4D). Todos los viales se agitaron mecánicamente durante un minuto y posteriormente se dejaron en

reposo durante otros dos minutos, previa a la toma de la imagen. Como puede observarse, el empleo conjunto de los componentes en forma de pastilla genera mejores dispersiones y más estables en el tiempo, siendo la efervescencia la responsable de este hecho (Figura 4B) y no la fuerza iónica generada (Figura 4D). Esto es especialmente relevante, puesto que el material empleado presenta muy baja dispersabilidad en agua y una elevada tendencia a la agregación.

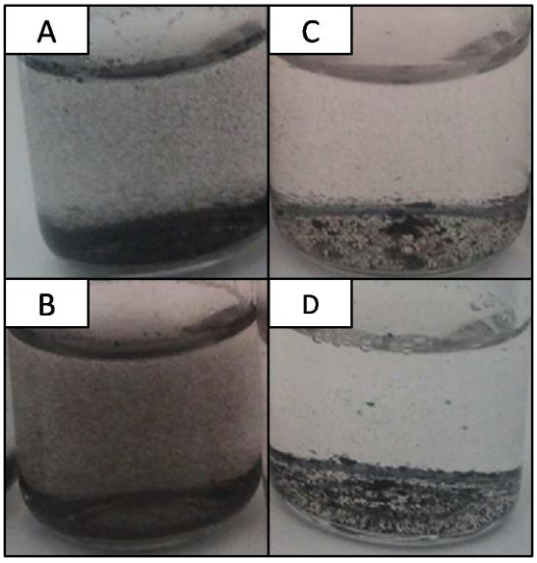

Figura 4: Dispersión de MWCNTs en las distintas condiciones experimentales. Ver detalles en el texto.

La pastilla completa puede prepararse previamente al tratamiento de muestra, permitiendo reducir el tiempo empleado en cada extracción. Para almacenar las pastillas es necesario minimizar su exposición a la humedad ambiente, aun siendo los componentes los menos higroscópicos. Para ello, se almacenan en viales herméticos en atmosfera de N_2.

En la microextracción en fase sólida dispersiva asistida por efervescencia, el sorbente sólido se retiene en un soporte tras la extracción. Una que se aísla con los

analitos extraídos, se realiza la elución de los mismos. En el caso de los sorbentes poliméricos, la retención se realiza mediante un filtro de jeringa (tamaño de poro: 0.2 µm) directamente acoplado al sistema de extracción. Por otra parte, el sorbente nanoestructurado requiere un filtro a partir de cinta de teflón convencional para poder procesar un volumen de muestra superior mediante un sistema de filtración por vacío. La configuración del filtro de jeringa permite realizar, en el mismo sistema, la elución mediante un disolvente orgánico. En el caso del sorbente polimérico se optó por 500 µL de acetonitrilo como eluyente. El mayor tamaño del filtro empleado para retener el sorbente requiere confinarlo en una jeringa donde pueda quedar cubierto de disolvente orgánico para la elución de los analitos. Para ello, se aspiran con la jeringa que contiene el filtro, 2.5 mL de eluyente (en este caso metanol), y se sonica la jeringa para favorecer la transferencia de los analitos al eluyente. Puesto que el volumen de elución es relativamente grande, es necesario introducir un paso de evaporación-redisolución para conseguir una mejora de la sensibilidad en el posterior análisis por cromatografía de líquidos.

Las principales características analíticas de los métodos de microextracción en fase sólida asistida por efervescencia se muestran en la Tabla 2. No obstante, en la sección 7.1 se abordara el problema analítico seleccionado en el caso de muestras ambientales.

Tabla 2: Principales características analíticas de los métodos presentados.

Sorbente	Analitos	Volumen de muestra (mL)	LdD[a] (µg L^{-1})	DER[b] (%)	FE[c]
OASIS HLB	Compuestos nitroaromáticos	10	1.8-7	1.7-8.6	13-17
MWCNTs	Triazinas	100	0.15-0.40	3.9-9.3	480-755

[a] LdD: límite de detección, [b] DER: desviación estándar relativa, [c] FE: factor de enriquecimiento

3. Empleo de la efervescencia para dispersar fases líquidas y recuperación mediante interacción con nanoestructuras magnéticas

La reacción de intercambio de protones responsable de la liberación de CO_2 por efervescencia puede tener lugar en fase heterogénea mediante una pastilla que contenga los componentes, como ya se ha mencionado, o en fase homogénea si los precursores efervescentes ya se encuentran disueltos en las fases implicadas en el proceso. Este segundo ejemplo es especialmente interesante para la dispersión de fases extractantes líquidas, permitiendo que la efervescencia sea la fuerza generadora de la dispersión. Durante el desarrollo de esta Tesis Doctoral se estudió la posibilidad de utilizar la efervescencia para el desarrollo de una modalidad de microextracción líquido-líquido dispersiva asistida por efervescencia.

En el caso de la microextracción líquido-líquido, existen numerosas alternativas para generar dispersiones de forma eficiente, como ha quedado resaltado en el apartado 1 de este capítulo y a lo largo de la introducción de esta Memoria de Tesis Doctoral. En el contexto de nuestra investigación, el empleo de la efervescencia como agente dispersante plantea una alternativa respetuosa con el medio y con un potencial analítico interesante.

A diferencia de lo que ocurre en el caso de las dispersiones de sólidos mediante el empleo de una pastilla efervescente, los precursores necesarios en fase líquida no pueden estar en contacto hasta el momento de la extracción, ya que reaccionarían y quedaría anulada la capacidad dispersante. Por consiguiente, para hacer uso de la capacidad del CO_2 generado mediante efervescencia, cada una de las fases implicadas (muestra y extractante) deberán contener uno de los precursores.

3.1 Precursores efervescentes

La modalidad de ***microextracción liquido-liquido asistida por efervescencia*** (effervescence assisted dispersive liquid-liquid microextraction, ***EA-DLLME***) emplea un

ácido orgánico débil como donador de protones (ácido acético, HAc) y carbonato de sodio como donador de CO_2. Para lograr la reacción completa y evitar interferencias de los precursores no consumidos, ambos se deben encontrar en proporciones estequiométricas. Concretamente, en el procedimiento optimizado para un volumen de muestra/estándar acuoso de 20 mL se deben añadir 2.5 mL de una disolución de carbonato de sodio 3 mol L^{-1} y sobre esta se añadiran 250 µL de una mezcla de ácido acético y extractante.

3.2 Evaluación del extractante y de la sinergia con nanopartículas magnéticas

Se seleccionó el disolvente orgánico 1-octanol como fase extractante debido a su inmiscibilidad en fases acuosas y su capacidad para extraer compuestos apolares y moderadamente polares [20-22]. En las técnicas de microextracción, cuanto menor sea la cantidad de fase extractante empleada, mayores serán los factores de preconcentración obtenidos, con la consiguiente mejora de la sensibilidad del método. Sin embargo, en el caso de las modalidades de extracción líquido-líquido en general, esto puede representar un inconveniente. El empleo de pequeños volúmenes de extractante supone una dificultad añadida, ya que suele requerir la intervención de etapas de centrifugación para lograr la separación cuantitativa de las fases.

Con el objetivo de evitar el empleo de dispositivos adicionales durante el proceso de extracción y reducir el número de etapas implicadas en el mismo, se planteó el empleo de nanopartículas magnéticas (NPMs) como elemento recuperador del disolvente orgánico.

Las NPMs han suscitado un gran interés en el campo de la Química Analítica a lo largo de los últimos años. Su composición incluye átomos metálicos (hierro, níquel, magnesio, manganeso o cobalto) y sus óxidos (simples o mixtos). Son nanomateriales simples de sintetizar que permiten obtener distribuciones de tamaño uniformes y altos momentos magnéticos. Además, se trata de materiales superparamagneticos, es decir,

sus propiedades magnéticas se manifiestan únicamente en presencia de un campo magnético externo.

Los nano y micromateriales magneticos son herramientas muy útiles en el campo de la microextracción para retener, preconcentrar y separar diferentes analitos de interés en matrices complejas [23-26]. En el caso de los nanomateriales, presentan una gran relación superficie/volumen, son fácilmente dispersables y presentan ciertas propiedades físicas que pueden ser útiles adicionalmente en la etapa de detección.

A pesar de sus numerosas ventajas, los nanomateriales magnéticos presentan un inconveniente: su superficie debe modificarse químicamente para obtener herramientas selectivas y estables. Las NPMs manifiestan cierta inestabilidad intrínseca en el tiempo, debido a su tendencia a la oxidación. Este proceso afecta negativamente a sus propiedades magnéticas y a su dispersabilidad. Por todo ello, es frecuente encontrar NPMs funcionalizadas en superficie con tensioactivos, polímeros, sílice o carbón cuya estabilidad se ve sensiblemente mejorada. Además de las anteriores, pueden aplicarse distintas modificaciones en superficie para mejorar la selectividad, especialmente para el tratamiento de muestras de interés biológico [27-29].

En primer lugar se evaluó el modo de dispersión en función del número de pasos implicados en el proceso tomando como referencia un estándar acuoso de terbumeton a un nivel de concentración de 50 µg L^{-1} como analito modelo. Se estudió el proceso de microextraccion en dos modalidades; modalidad de una etapa, en la que el disolvente orgánico y las NPMs se añaden simultáneamente y la modalidad en dos etapas [30], en la que se añaden secuencialmente. Así mismo, se evaluó la influencia de la efervescencia en la dispersabilidad del disolvente, comparándolo con la dispersión mediante un disolvente orgánico (acetonitrilo). Como muestra la Figura 5, la efervescencia es más eficiente para generar dispersiones, especialmente si el proceso implica una única etapa. Mediante la efervescencia se evita el uso de disolventes

orgánicos tóxicos y costosos y gracias a la eficiencia del proceso en una etapa se simplifica el procedimiento.

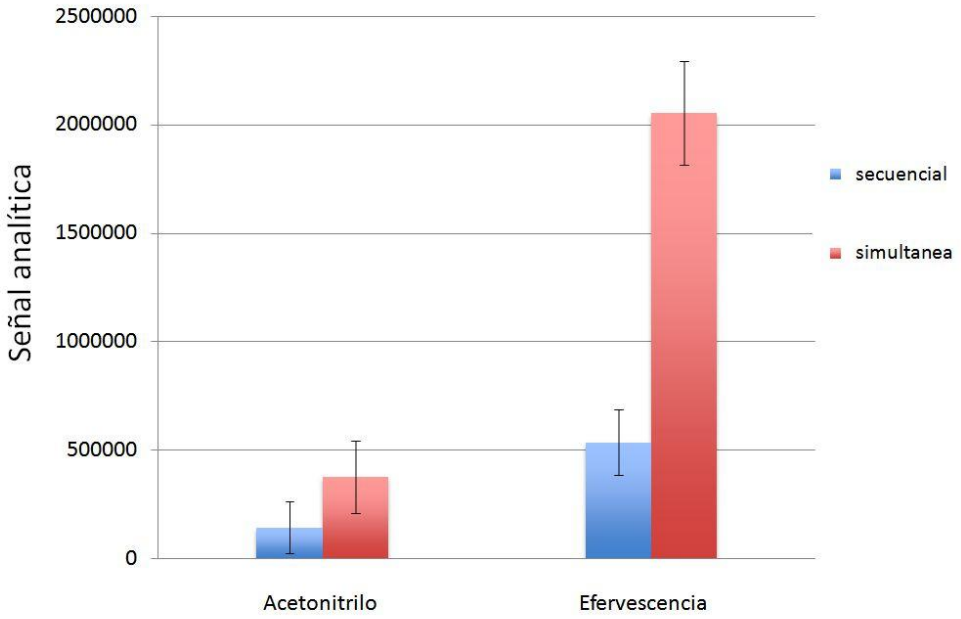

Figura 5. Comparación del proceso de dispersión de la fase extractante y las NPMs

Una vez seleccionado el modo en el que se genera la dispersión, se evaluaron dos clases de NPMs de distinto recubrimiento que podrían interaccionar mediante dos mecanismos con el 1-octanol, NPMs de Fe_3O_4 sin recubrimiento y con recubrimiento de C_{18}. Los distintos tipos de recubrimiento permiten dos tipos de interacción:

- El recubrimiento de C_{18} permite una interacción hidrofóbica con la cadena hidrocarbonada del 1-octanol. En la bibliografía podemos encontrar ejemplos de interacción entre el 1-octanol y NPMs hidrofóbicas [30, 31].

- Las NPMs sin recubrimiento poseen grupos hidroxilo en superficie que pueden interaccionar mediante puentes de hidrógeno con el grupo alcohol del 1-octanol [32,33].

Además del tipo de interacción con el extractante, el recubrimiento confiere a las NPMs un comportamiento radicalmente diferente en términos de dispersabilidad. La Figura 6 muestra una comparación entre ambos materiales. El panel superior muestra el material dispersado en HAc y con 20 µL de 1-octanol; el panel intermedio revela el comportamiento de la dispersión efervescente en 20 mL de fase acuosa y el panel inferior el aspecto de la punta de micropipeta empleada para durante el proceso. Ésta comparación visual muestra como el recubrimiento hidrofóbico de C_{18} incrementa la tendencia a la agregación de las NPMs, dando lugar a un material de baja dispersabilidad. Por su parte las NPMs sin recubrimiento dan lugar a dispersiones homogéneas. Además, la tendencia a adherirse a la punta de pipeta de PTFE durante la elución o el lavado puede reducir la reproducibilidad del proceso. Incluir en el procedimiento una etapa de lavado permite eliminar analitos fisiadsorbidos sobre la superficie de las partículas y el 1-octanol que no esté interaccionando directamente con el material.

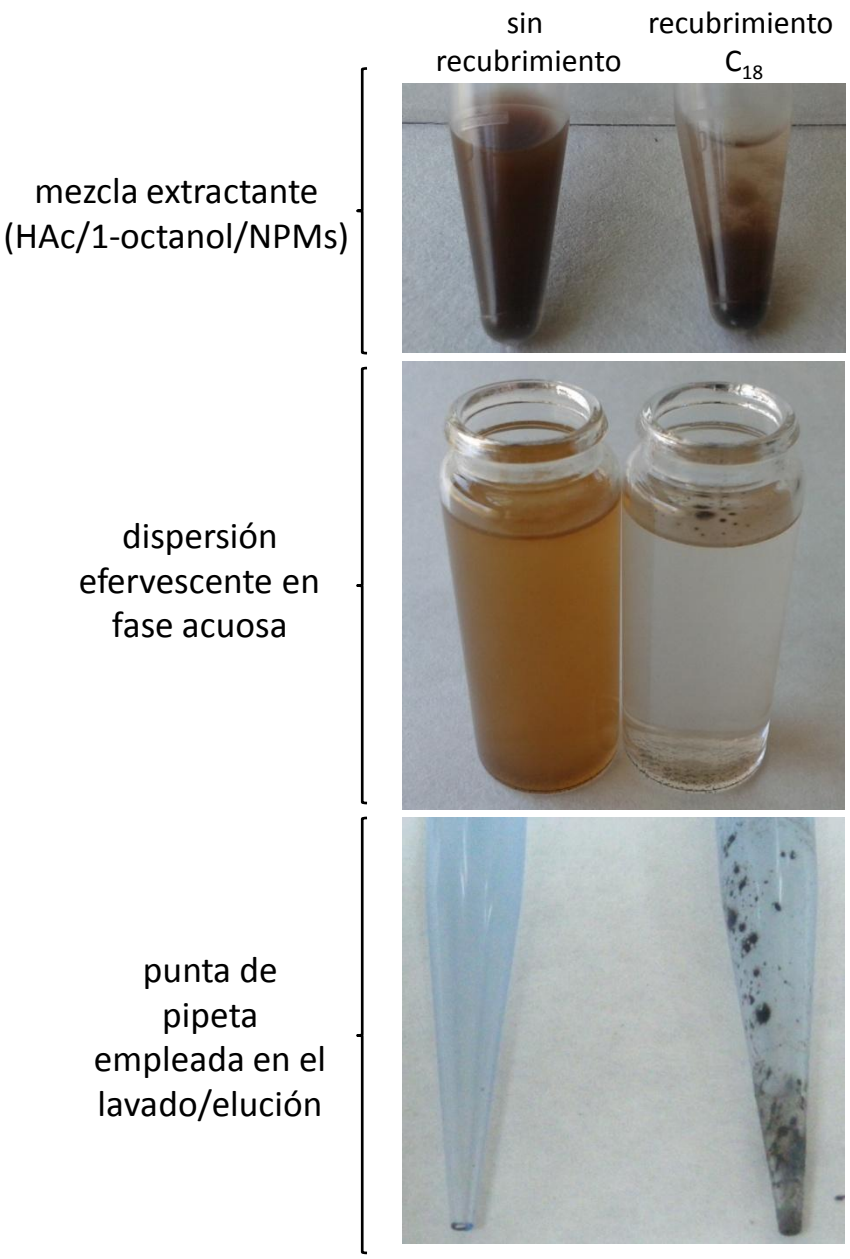

Figura 6: Comparación del comportamiento de las NPMs sin recubrimiento y con recubrimiento de C_{18}.

En términos de señal analítica (mediante el análisis por GC/MS) las nanopartículas sin recubrimiento proporcionaron una ligera mejora en cuanto a sensibilidad y precisión.

3.3 Evaluación de la capacidad de las NPMs para retener 1-octanol

La optimización completa del proceso de microextracción requiere ajustar el volumen de 1-octanol y NPMs. Considerando como el tipo de interacción más probable del 1-octanol con las NPMs aquella que tiene lugar a través de los grupos hidroxilo superficiales mediante puentes de hidrógeno, cierta cantidad de material magnético será capaz de atrapar un volumen limitado de disolvente. Por esta razón, estas dos variables del proceso deben evaluarse conjuntamente. El volumen de extractante y de material magnético se estudió en los intervalos 10-20 µL y 5-20 mg respectivamente. La cantidad máxima de NPMs se fijó en función del sistema de introducción de la mezcla extractante en la muestra, en este caso, una jeringa. Cantidades superiores podrían, ciertamente, retener mayor cantidad de disolvente orgánico, pero también obturar la aguja del dispositivo. El estudio de las variables se realizó mediante el análisis de estándares acuosos de triazinas a una concentración de 50 µg L^{-1}. La Figura 7 muestra el efecto bivariante de ambas variables para el terbumetón como analito modelo. Efectivamente, la señal analítica aumenta con la cantidad de disolvente orgánico empleado ya que aumenta la capacidad de extracción. En lo referente a la cantidad de NPMs la señal se incrementa hasta 10 mg, permaneciendo prácticamente constante hasta 20 mg. Considerando estos resultados, se seleccionó 20 µL y 10 mg como combinación más eficiente.

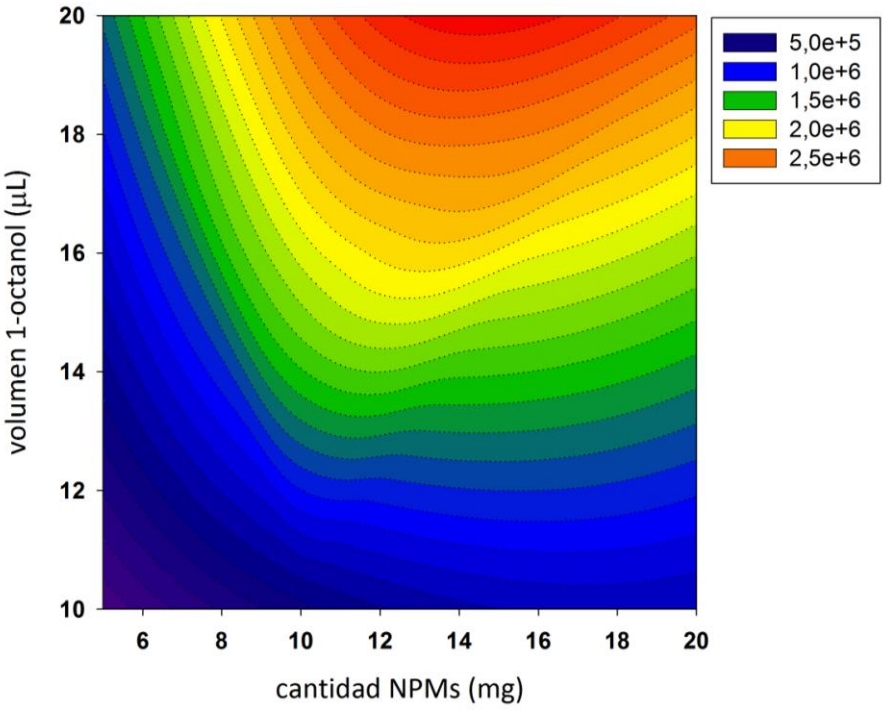

Figura 7: Efecto bivariante del volumen de extractante y cantidad de NPMs para el terbumetón

El estudio combinado de ambas variables permite observar como, para un volumen concreto de disolvente, existe una cantidad de NPMs óptima para retener y recuperarlo. Esto significa que, con el sistema de introducción adecuado de mezcla extractante, podemos analizar elevados volúmenes de muestra siempre y cuando los precursores efervescentes mantengan su relación estequiométrica. Sin embargo, volúmenes elevados de muestra incrementarían el consumo de disolventes y NPMs. Por esta razón, el volumen de muestra analizable no se optimizó y se fijó en 20 mL dentro de un vial de cristal tal y como detalla la Figura 8.

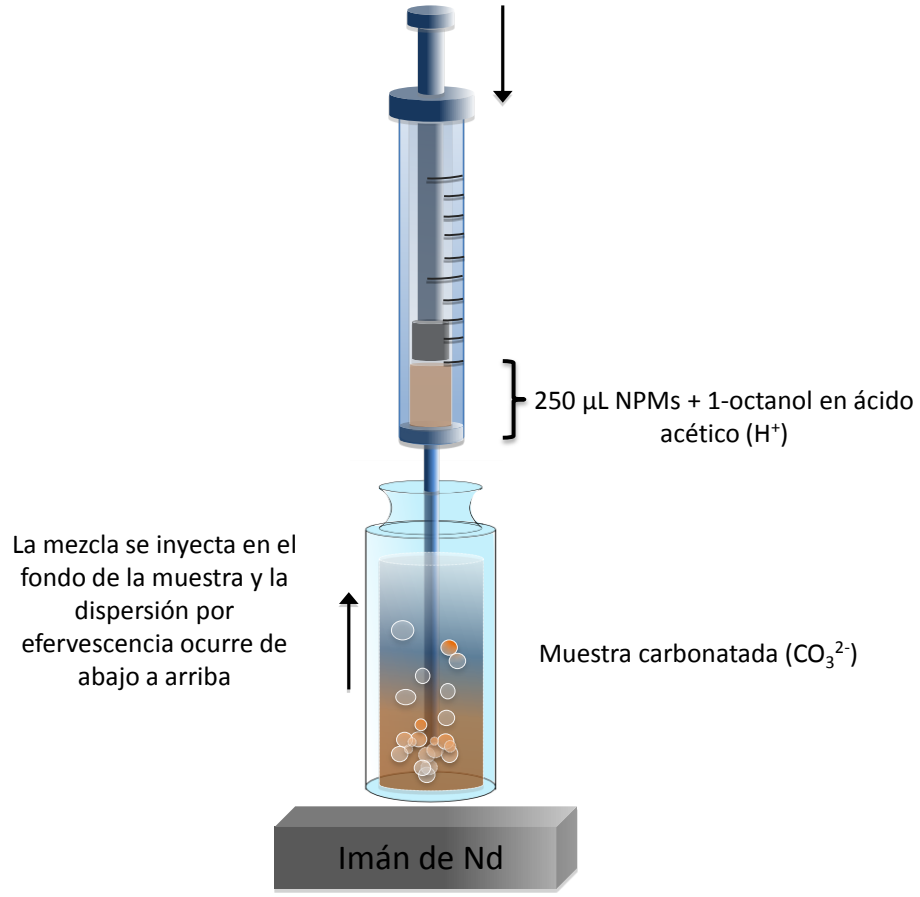

Figura 8: Esquema del procedimiento de EA-DLLME. El imán de neodimio se sitúa tras finalizar la reacción efervescente. Las NPMs se separan gracias al campo magnético y con ellas el 1-octanol con los analitos extraídos.

El empleo de estas NPMs permite simplificar y agilizar los procedimientos de microextracción. Durante el desarrollo de esta Tesis Doctoral se han empleado para separar una fase extractante gracias a su interacción con esta. Sin embargo, como se ha especificado al comienzo de este apartado, es posible emplearlas directamente como fases sorbentes una vez funcionalizadas. El uso de un imán externo para conseguir la separación efectiva de la fase extractante evita una etapa de centrifugación,

permitiendo que este procedimiento pueda utilizarse fácilmente en la misma zona de muestreo.

3.4 Procedimiento de elución y análisis por GC/MS

A pesar de que el 1-octanol es un excelente disolvente para DLLME, no es apropiado para su introducción en el cromatografía de gases. Por este motivo, se realizó una etapa de elución con un disolvente orgánico compatible previa al análisis. Se seleccionó el metanol (MeOH) en función de los resultados previos para los mismos analitos.

Considerando el material empleado y el reducido volumen de extractante empleado se valoró la posibilidad de emplear ultrasonidos como mecanismo para lograr la elución. Sin embargo, la radiación de ultrasonidos parecía provocar la disociación del 1-octanol de la superficie de las NPMs, dando lugar a bajas señales analíticas y un comportamiento cromatográfico inadecuado. En este sentido, se observaba la aparición de un pico adicional derivado del 1-octanol, así como el desdoblamiento de los picos correspondientes a los analitos derivado del reparto ineficiente entre el disolvente y la fase estacionaria. Finalmente se optó por realizar la elución mediante la resuspensión lenta con 100 µL de MeOH mediante micropipeta del material separado con el imán de neodimio en el fondo del vial. El volumen fue fijado en 100 µL, ya que con este volumen todo el material separado quedaba cubierto por disolvente y la dilución del extracto era mínima.

El procedimiento optimizado se caracterizó mediante la extracción de triazinas en muestras acuosas. Las principales características analíticas del método se muestran en la Tabla 3. No obstante, en la seccion 7.2 se abordara el problema analítico seleccionado en el caso de muestras ambientales.

Tabla 3: Principales características analíticas de EA-DLLME con recuperación mediante NPMS

extractante	Analitos	Volumen de muestra (mL)	LdD[a] (µg/L)	DER[b] (%)	FE[c]
1-Octanol/MNPs	Triazinas	20	0.02-0.06	7.8-11.7	21-185

[a] LdD: límite de detección, [b] DER: desviación estándar relativa, [c] FE: factor de enriquecimiento

4. Empleo de CO_2 como mediador de la solubilización de la fase extractante. Disolventes de hidrofilicidad variable

El concepto de disolventes conmutables (*switchable solvents*, SS) fue introducido por Canter en 2006 en el marco de los procesos industriales. Inicialmente, se buscaban disolventes que pudieran alternar entre un estado polar y apolar, con el objetivo de minimizar el número de disolventes empleados en determinados procesos y facilitar su posterior procesado como residuos [34]. Posteriormente, el grupo del Prof. Jessop ha buscado ampliar el campo de aplicación de estos novedosos disolventes a procesos económicamente más asequibles y más respetuosos con el medio.

Jessop y colaboradores desarrollaron un método que permitió alternar las dos formas mencionadas del disolvente empleando dióxido de carbono en disolución como reactivo. El CO_2 presenta características muy interesantes para este tipo de procesos, ya que su coste es reducido, es fácilmente accesible y no es tóxico. Al poder presentarse como gas, su eliminación de la disolución es sencilla, permitiendo alternar las formas del disolvente. Los disolventes que presentaban este comportamiento se denominaron disolventes de hidrofilicidad conmutable (*switchable hydrophilicity*

solvents, SHS). En este caso, consisten en un disolvente orgánico inmiscible con agua (hidrofóbico) que en contacto con CO_2 disuelto (agua carbonatada) alterna con una forma soluble en agua (hidrofílico) dando lugar a mezclas homogéneas acuosas. El SHS puede recuperar su forma hidrofóbica cuando se retira el CO_2 de la disolución. Los SHS se emplearon por primera vez como sustitutos del hexano en la extracción de aceite de soja evitando el uso de destilación. El empleo de este tipo de disolventes en sustitución de los disolventes orgánicos "*tradicionales*" evita riesgos ambientales y además reduce el coste del proceso, al eliminar la etapa de destilación, que implica una inversión energética elevada.

Se han identificado distintos disolventes que presentan este comportamiento. Las amidinas y las aminas terciarias son mayoritarias [35,36], incluso se han identificado líquidos iónicos que pueden presentar este comportamiento [37]. En el caso del IL *tetra-butilfosfonio N-tri-fluoro-metano-sulfonil-leucine* el comportamiento es el inverso al de las amidas y amidinas, ya que la introducción de CO_2 en la disolución lo hace inmiscible con la fase acuosa.

El mecanismo general de interconversión se muestra en la ecuación 1. El cambio de miscibilidad en agua se debe a una reacción ácido-base entre el CO_2 hidratado o el ácido carbónico del agua carbonatada y el SHS. Como resultado, se forma la sal carbonatada del SHS soluble en agua.

$$NR_3 + H_2O + CO_2 \Leftrightarrow NR_3H^+ + HCO_3^- \qquad \text{(ec.1)}$$

Este proceso se ha aplicado con éxito a la retirada de disolventes de productos tan variados como aceites derivados de algas para la producción de biofuel [38,39], bitúmenes [40], polvo de poliestireno de alta densidad [36] o aceite de soja [35].

La Tabla 4 muestra los ejemplos más característicos de SHS estudiados hasta la fecha, resaltando el disolvente empleado durante ésta Tesis Doctoral.

Tabla 4. Principales aminas con carácter conmutable.

Compuesto	Ratio en agua (v:v)	Log K_{ow} [a]	pK_{aH}
Trietilamina	1:1	1.47	10.68
N,N-Dimetilbutilamina	1:1	1.60	10.02
N,N-etilpiperidina	1:1	1.75	10.45
N-metildipropilamina	1:1	1.96	10.40
N,N-dimetilciclohexilamina	**1:1**	**2.04**	**10.48**
N-butilpirrolidina	1:1	2.15	10.36
N,N-dietilbutilamina	1:1	2.37	10.51
N,N-dimetilhexilamina	1:1	2.51	10.18

[a]Predicciones usando el software ALOGPS 2.1

Las propiedades fundamentales que deben cumplir para ser considerados SHSs son:

- *Valores de Log K_{ow} 1.2-2.5:* aquellos disolventes con valores inferiores son considerados demasiado hidrofílicos y forman sistemas monofásicos en su forma neutra. El caso contrario ocurre con aquellos que tienen valores superiores, que forman sistemas bifásicos incluso en presencia de CO_2.
- *Valores de pKa superiores a 9.5*: aquellos disolventes con una basicidad baja no reaccionan con el CO_2 y no alternan las formas hidrofílica e hidrofóbica.

Además de estas condiciones, es interesante que los SHS sean sustancias no volátiles, generalmente de elevado peso molecular. Esto es debido a que la forma más común de eliminar el CO_2 es mediante burbujeo de otro gas (como N_2 o Ar) en la disolución y podría provocar pérdidas por evaporación.

4.1 Selección del SHS y obtención de la forma hidrofílica

Durante la realización de esta Tesis Doctoral, se seleccionó la N,N-dimetilciclohexilamina como candidato de SHS para adaptarlo a una técnica de microextracción. En este sentido, y aprovechando la capacidad para ser totalmente miscible con agua, se optó por una ***microextracción líquido-líquido homogénea***

(homogeneous liquid-liquid microextraction, ***HLLE***). La HLLE puede ser considerada una adaptación de las técnicas dispersivas en fase líquida, con la ventaja de que prescinden del disolvente orgánico dispersante [41]. Este tipo de técnicas requieren una operativa especial para la recuperación de la fase extractante, generalmente una etapa de centrifugación o disolventes solidificables [42]. En este sentido, los SHS presentan una doble ventaja; por una parte, una de sus formas es totalmente miscible en fase acuosa, y por otra, pueden ser fácilmente separados de la muestra mediante su conversión a su forma hidrófoba.

La primera etapa del proceso propuesto consistió en la obtención de la forma hidrofílica del SHS. Con el objetivo de evitar el uso de gas presurizado y complejos sistemas de tuberías, se optó por administrar el CO_2 en disolución mediante hielo seco. El hielo seco se presenta en forma de "pellets" de 3 mm de diámetro, es seguro, económico y no tóxico a los niveles de trabajo. El SHS adquirido comercialmente se presenta en forma nativa hidrofóbica. En nuestro caso, se mezcló con agua para obtener el sistema bifásico (1:1) en una botella de vidrio con tapón roscado. El volumen total de 200 mL ocupaba el 20 % del recipiente, dejando suficiente espacio libre para albergar el CO_2 no disuelto y mantener la presión de éste. A continuación se añadieron en etapas sucesivas 10 g de hielo seco y se mantuvo el recipiente herméticamente cerrado. El proceso se repitió hasta que se observó la desaparición de la interfase y un sistema monofásico. El procedimiento se detalla en la Figura 9. El resultado del proceso es una mezcla 1:1 de la forma hidrofílica del SHS en agua, que se empleará posteriormente en el procedimiento de microextracción.

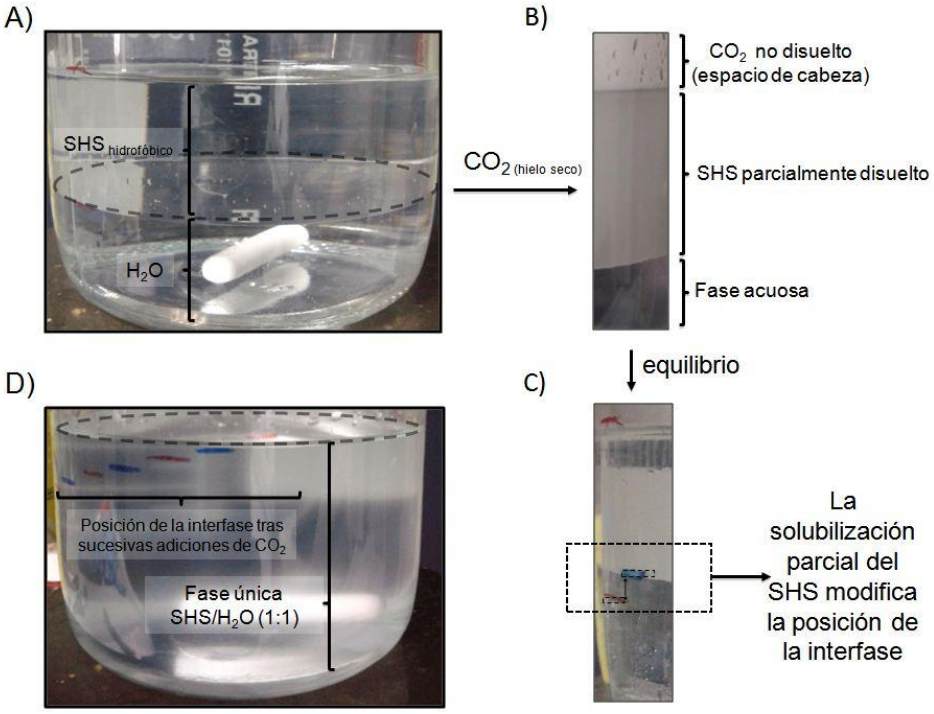

Figura 9: Proceso de solubilización de SHS. (A) estado inicial con dos fases inmiscibles; (B) detalle del recipiente tras la adición de 10 g de hielo seco con tres fases (dos líquidas y una gaseosa); (C) detalle del recipiente tras la solubilización del CO_2. Se observa un cambio en la posición de la interfase; (D) aspecto final tras sucesivas adiciones de hielo seco, se observa una única fase.

4.2 Proceso de separación de fases

En general, la aplicación de SHS a procesos industriales emplea corrientes de CO_2 para solubilizar el disolvente y de N_2 o Ar para insolubilizarlo [R]. Cuando los volúmenes implicados en el proceso son elevados, esto es factible. Sin embargo, en una aplicación para microextracción esto no es posible. Una corriente de N_2 o Ar para eliminar el CO_2 y separar la fase extractante puede provocar pérdidas por proyecciones del disolvente o incluso por evaporación. En nuestro caso, se estudiaron diversos mecanismos físicos y químicos para inducir la separación de fases por conversión del disolvente en su forma hidrofílica (Figura 10).

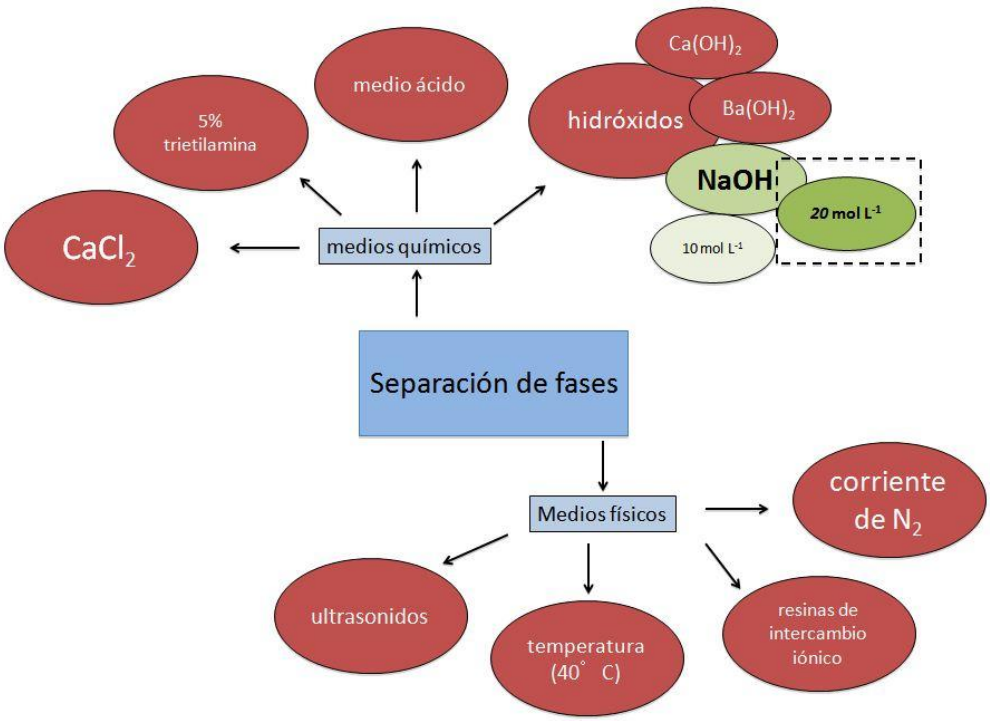

Figura 10: Alternativas estudiadas para la separacion de fases del sistema homogéneo SHS/muestra acuosa

Los medios físicos utilizados con objeto de separar las fases de SHS y acuosa, pretenden esencialmente liberar el CO_2 disuelto, eliminando la posibilidad de formación de la sal carbónica responsable de la solubilización del SHS. Como se ha mencionado, el empleo de corrientes de N_2 generaba pérdidas por proyecciones fuera del recipiente donde las fases se encontraban y al igual que ocurría con la aplicación de calor, se desprendía un fuerte olor característico del SHS, por lo que estaba perdiéndose de la disolución.

Los medios químicos empleados pueden actuar mediante distintos mecanismos, pero en todos los casos se busca la inestabilización de la sal carbónica formada (ver ec.1 anterior) para revertir el disolvente a su estado nativo. De todas las

alternativas evaluadas, únicamente la adición de hidróxidos, mediante un cambio en la carga del disolvente, permitieron la separación cuantitativa de las fases. El empleo de hidróxido de sódio a alta concentración (20 M) proporcionó las separaciones más efectivas en menos tiempo, por tanto fue el método seleccionado.

4.3 Microextracción líquido-líquido homogénea con disolventes de hidrofilicidad variable

Durante la realización de esta Tesis Doctoral se ha abordado por primera vez el empleo de SHSs en técnicas de microextracción. Las dos aproximaciones propuestas comparten la misma fase extractante, adaptándose el procedimiento a cada uno de los problemas analíticos seleccionados..

La primera aplicación desarrollada se optimizó para la determinación de un hidrocarburo policíclico aromático (PAH) como es el benz[a]antraceno (BA), ya que el objetivo principal de la investigación fue evaluar la viabilidad de emplear este tipo de disolventes en microextracción en fase líquida. El empleo de un único analito que además presenta fluorescencia nativa simplifica significativamente el proceso de optimización de la técnica de microextracción.

Como etapa previa a la optimización del procedimiento se evaluó la influencia del medio en la fluorescencia del analito. Se observó un fenómeno de *quenching* de fluorescencia en presencia del SHS, que disminuía la sensibilidad de la medida. Se optó por realizar una dilución en ácido débil (ácido acético) del extracto para minimizar este efecto. Se evaluó la dilución con ácido acético de 0 a 100%, en mezclas conteniendo 1 $\mu g\ L^{-1}$ de analito. Como se observa en la Figura 11, los mejores resultados se obtuvieron para la dilución al 50% en ácido. En el fondo de la imagen se puede observar cada uno de los extractos analizados fotografiados bajo luz UV. La pequeña divergencia entre el vial que manifiesta mayor intensidad y los valores de fluorescencia se debe a que la

lámpara (longitud de onda fija a 365 nm) no permite la medida en las condiciones óptimas (289 nm para excitación y 388 nm para emisión).

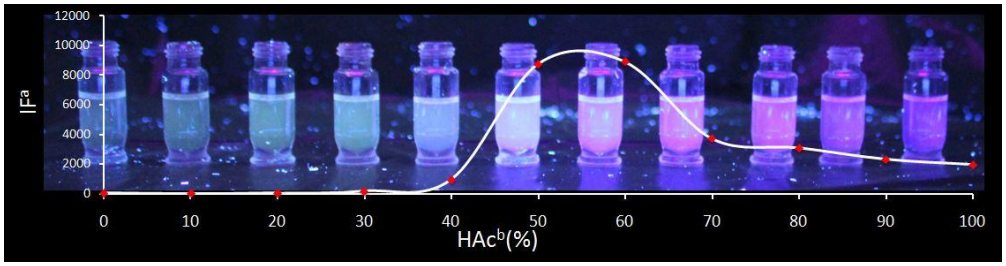

Figura. 11: Influencia de la relación SHS/ácido acético en la fluorescencia de BA. [a]IF: intensidad de fluorescencia, [b]HAc: ácido acético

Desde el punto de vista operacional, se emplearon 10 mL de muestra acuosa que se situaron en un tubo de ensayo. El volumen se fijo en 10 mL para simplificar la manipulación de las muestras y facilitar el empleo de tubos de ensayo convencionales para realizar la extracción.

Se estudiaron tres volúmenes (1000, 750 y 500 µL) de amina acuosa como fase extractante realizandose las medidas por triplicado. Para ello se tomaron los volúmenes correspondientes y fueron homogeneizados en una muestra acuosa que contenía BA a una concentración de 10 µg L^{-1}. Posteriormente, se indujo la separación de fase mediante la adición de 1 mL de NaOH 20 mol L^{-1}y se tomó la fase orgánica para realizar la medida de fluorescencia. El procedimiento es considerablemente rápido. Una vez añadido el hidróxido sódico, bastan 5 minutos para obtener una separación completa de las fases. Cabe destacar que gracias a la geometría del tubo de ensayo, la fase separada genera una columna que puede ser retirada mediante una micropipeta ajustada al volumen correspondiente. Como norma, se optó siempre por recoger el 80% de la fase separada, con el fin de evitar arrastrar parte de la fase acuosa. Volúmenes inferiores a 500 µL de extractante (250 µL de SHS final) resultan muy difíciles de retirar, generando una considerable imprecisión en las medidas. La Figura

12 muestra el resultado de las medidas en términos de intensidad de fluorescencia, así como la desviación asociada a las mismas. Finalmente se seleccionó un volumen de 750 µL de SHS acuoso como compromiso entre la capacidad de extracción y la precisión.

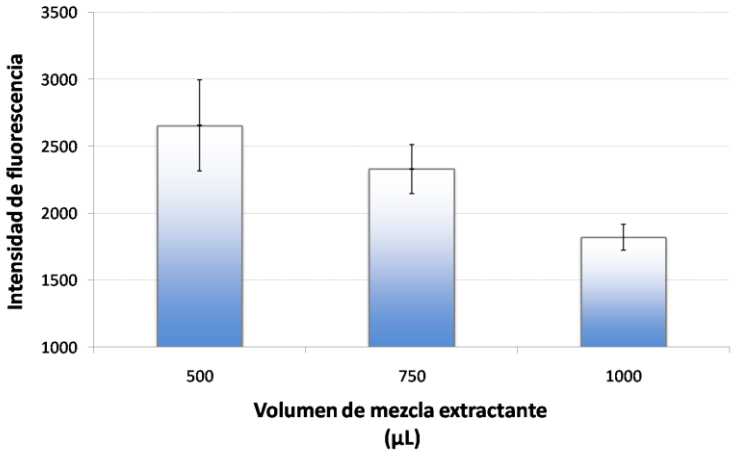

Figura 12: Efecto del volumen de mezcla extractante en la extracción de BA. Cada volumen se corresponde al 50% de volumen final de SHS.

Una vez estudiado este sistema extractante, se llevó una determinación multianalito, en este caso triazinas en muestras de agua. Se optó por modificar el dispositivo de extracción, con el fin de emplear volúmenes inferiores de fase extractante. En esta ocasión, se emplearon matraces aforados de 10 mL para llevar a cabo la extracción. Gracias a este tipo de material, cuyo cuello presenta un diámetro inferior al de los tubos de ensayo anteriormente empleados, podemos recuperar fácilmente volúmenes inferiores. El SHS dará lugar a una columna de líquido de una altura mayor tras la separación de fases. En este caso, la Figura 13 muestra el efecto del volumen de fase extractante para cada uno de los analitos estudiados. Al igual que en el caso anterior, se recupera el 80 % de fase extractante, con el fin de evitar el arrastre de fase acuosa con la fase de SHS. Para el análisis de los extractos se empleó la cromatografía de gases, que había sido previamente utilizada para este tipo de

compuestos. Los extractos de SHS fueron diluidos previamente al análisis cromatográfico con metanol, con el objetivo de mejorar el comportamiento cromatográfico. A partir de los resultados obtenidos se seleccionó 250 µL como volumen óptimo de mezcla extractante, lo cual implica 125 µL de SHS finales como fase aceptora.

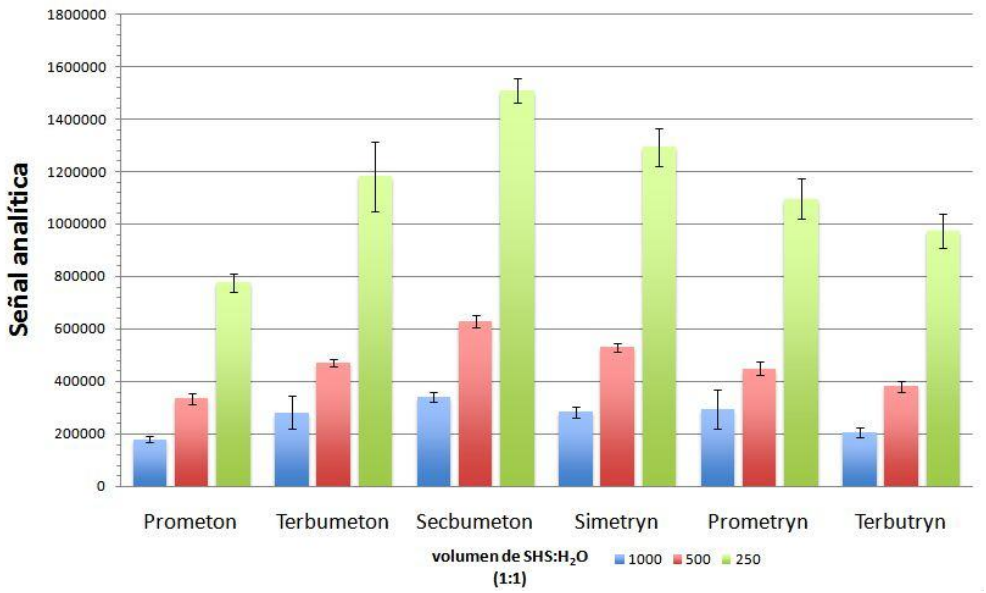

Figura 13: Efecto del volumen de fase extractante en la señal analítica para el procedimiento de microextracción.

Los dos métodos propuestos se han empleado para la determinación de compuestos apolares (como los hidrocarburos policíclicos aromáticos) y moderadamente polares (como las triazinas). Para el caso del BA, al tratarse de un único analito, las medidas de intensidad de fluorescencia aportan información analítica suficiente para la determinación. Sin embargo, en el caso de las triazinas, al tratarse de varios analitos es necesario recurrir a una técnica cromatográfica para obtener la informacion discriminada para cada compuesto (en este caso, GC/MS). Es interesante

destacar que durante el desarrollo inicial de esta herramienta se evaluó la posibilidad de determinar más de un analito de la familia de los hidrocarburos policíclicos aromáticos con resultados satisfactorios. En ese caso, la dilución con HAc no sería necesaria, ya que se recurre a analizar el extracto mediante una técnica cromatográfica. Las características analíticas principales de los métodos propuestos se muestran en la Tabla 5.

Tabla 5: Principales características analíticas de la HLLME mediante SHSs

Analitos	Volumen de muestra (mL)	LdD[a] (µg/L)	DER[b] (%)
BA[c]	10	0.08	6.7
Triazinas	10	0.1-0.37	3.1-12.5

[a] LdD: límite de detección, [b] DER: desviación estándar relativa, [c] benz[a]antraceno

Aunque los resultados en términos de sensibilidad y precisión son comparables, es cierto que cuando se emplea el SHS para extraer compuestos apolares, como los PAHs, la capacidad de extracción es mayor. Las triazinas son compuestos con una polaridad moderada que pueden presentar diferentes grupos funcionales que dificulten la extracción mediante un disolvente tan hidrofóbico como el seleccionado en esta Tesis Doctoral. No obstante, en la seccion 7.3 se abordara el problema analítico seleccionado en el caso de muestras ambientales

5. Evaluación de los métodos propuestos para el tratamiento de muestras de agua.

El objetivo de esta Tesis Doctoral es el diseño de herramientas de microextracción que emplean el CO_2 como sustituto de aparatos y/o disolventes. Una vez desarrolladas las herramientas, estas se aplicaron a la resolución o estudio de un problema analítico concreto. Por ello, todos los métodos presentados fueron evaluados para la extracción y preconcentración de los analitos mencionados en muestras de

agua de distinta naturaleza. Con el objetivo de conocer el potencial de las metodologías propuestas para el análisis *in situ* de las muestras, ninguna de estas se sometió a ningún tratamiento previo al proceso de microextracción. Esto implica la toma de muestra y su almacenamiento sin eliminar ningún posible interferente derivado de la matriz.

5.1 Microextracción en fase sólida dispersiva asistida por efervescencia.

La EA-DμSPE para sorbentes poliméricos se evaluó para la extracción de compuestos nitroromáticos (4-nitrotolueno, 1,4-dinitrobenceno, nitrobenceno, 2,4-dinitrotolueno y 4-nitrobenzaldehido). Este tipo de compuestos están presentes en la mayoría de explosivos utilizados en la industria de la minería o maniobras militares. En muchas ocasiones pueden alcanzar acuíferos o reservas naturales de agua por degradación del material pirotécnico o por un manejo inadecuado. En este sentido, la US-EPA (Environmental Protection Agency de Estados Unidos) impone estrictos niveles de control de sus niveles en aguas y suelos. Para evaluar la capacidad de extraer y preconcentrar este tipo de compuestos en agua de río, se realizo un estudio de recuperación de muestras fortificadas a dos niveles de concentración. Los resultados se recogen en la Tabla 6.

Tabla 6: Análisis de muestras de agua fortificadas con compuestos nitroaromáticos (n=5).

Analito	Concentración ($\mu g\ mL^{-1}$)	R%[a]±DE[b]	Concentración ($\mu g\ mL^{-1}$)	R%[a]±DE[b]
4-nitrotolueno	50	101±4	200	103±4
1,4-dinitrobenzeno	50	100±6	200	100±2
nitrobenzeno	50	96±4	200	100±2
2,4-dininitrotoluen	50	97±4	200	97±2
4-nitrobenzaldehído	50	96±5	200	94±5

[a]R%: Porcentaje de recuperación, [b]DE: desviación estándar

Como se observa, el porcentaje de recuperación es prácticamente del 100 % para todos los analitos independientemente de la concentración añadida.

De igual forma, la metodología propuesta se comparó con otras alternativas similares presentes en la bibliografía. En términos de sensibilidad es comparable con algunas de éstas [43,44] pero permite realizar en análisis en un tiempo muy inferior (5 min frente a 1 h). De igual manera, aunque existan alternativas más sensibles [45], requieren un volumen de muestra muy superior (500 mL frente a los 10 mL de la alternativa propuesta) y una cantidad de sorbente muy elevada (500 mg frente a los 5 mg propuestos).

Por otra parte, la EA-DμSPE para sorbentes nanoestructurados se caracterizó para la extracción y preconcentración de triazinas (simazina, simetrin, atrazina, secbumeton, prometon, terbumeton, propazina, prometrin y terbutrin) en muestras de agua ambientales. Se realizó el estudio de recuperación para tres tipos de muestras de agua (río, grifo y pozo) a un nivel de concentración de 10 μg mL^{-1} (Tabla 7). En este caso, los valores de recuperación estuvieron próximos al 100 % para todos los analitos en el caso de las muestras de río y pozo, sin embargo se observaron valores inferiores para el caso de la muestra de agua de grifo, especialmente en el caso de propazina, atrazina y simazina. Este fenómeno está asociado a la presencia de cloro en este tipo de muestra agua, cuyo efecto ha sido descrito por otros autores [46].

Tabla 7: Análisis de muestras de agua fortificadas con triazinas a una concentración de 10 μg L^{-1}.

Analito	Muestra (R%[a]±DE[b])		
	Río	Grifo	Pozo
Simazina	92 ± 7	77 ± 3	93 ± 9
Simetrin	101 ± 9	91 ± 7	106 ± 11
Atrazina	101 ± 13	82 ± 6	97 ± 7
Secbumeton	109 ± 12	97 ± 12	98 ± 13
Prometon	108 ± 7	86 ± 8	98 ± 17
Terbumeton	89 ± 10	89 ± 4	94 ± 10
Propazin	85 ± 7	63 ± 5	94 ± 12
Prometrin	92 ± 9	72 ± 6	101 ± 9
Terbutrin	87 ± 15	72 ± 7	93 ± 11

[a]R%: Porcentaje de recuperación, [b]DE: desviación estándar

En comparación con otras técnicas empleadas para la determinación de este tipo de compuestos, la EA-DμSPE es una de las más rápidas (3 minutos de tiempo de extracción, frente a alternativas de un tiempo medio de 30 minutos). En términos de precisión es comparable con otros métodos disponibles [47-49], pero presenta una sensibilidad inferior si se compara con aquellos que emplean cromatografía de gases/espectrometría de masas [48-50] o cromatografía de líquidos/espectrometría de masas [51] como técnica instrumental. Sin embargo, es comparable a otras metodologías que emplean detector de diodos en fila [52] y muy superior a otras que emplean fotometría UV [53].

5.2 Microextracción líquido-líquido dispersiva con recuperación mediante NPMs

La EA-DLLME asistida por NPMs se evaluó para la extracción y preconcentración de triazinas (simetrin, secbumeton, prometon, terbumeton, prometrin y terbutrin) en muestras de agua ambientales. Al igual que en el caso anterior, se realizó el estudio de recuperación de muestras de río grifo y pozo. Todas

las muestras fueron fortificadas con los analitos de interés a un nivel de concentración de 10 µg mL^{-1} obteniéndose los resultados que se muestran en la Tabla 8.

Tabla 8: Análisis de muestras de agua fortificadas con triazinas a una concentracion de 10 µg L^{-1}.

Analito	Muestra (R%[a]±DE[b])		
	Río	Grifo	Pozo
Prometón	91± 16	100± 8	88± 3
Terbumetón	91± 11	107 ± 4	99± 3
Secbumetón	101 ±13	98± 8	94 ± 12
Simetrín	113± 9	97 ± 15	99± 2
Prometrín	110± 6	84± 3	92± 5
Terbutrín	125± 12	94± 1	107± 6

[a]R%: Porcentaje de recuperación, [b]DE: desviación estándar

Como puede observarse, el porcentaje de recuperación esta próximo al 100 % en todos los casos, incluso en el caso de las muestras de grifo. Esta alternativa, para el mismo conjunto de analitos que la EA-DµSPE-MWCNT, presenta claras ventajas. En primer lugar el volumen de muestra empleado es muy inferior (20 mL frente a 100 mL). Además, gracias al empleo de NPMs la recuperación del extractante es muy rápida y simple, convirtiéndola en una alternativa mucho más eficiente para el pretratamiento *in situ* de las muestras de agua. La combinación del reducido volumen de extractante y la técnica instrumental seleccionada, hace que el método presentado sea una de las alternativas más sensibles, para un volumen de muestra relativamente pequeño y empleando un extractante líquido más barato y asequible [48, 49, 50, 53].

5.3 Microextracción líquido-líquido homogénea con disolventes conmutables.

Los métodos de SHS-HLLE se evaluaron para la extracción y preconcentración de un hidrocarburo policíclico aromático (benz[a]antraceno) y triazinas (simetrin, secbumeton, prometon, terbumeton, prometrin y terbutrin) en muestras de agua

ambientales de grifo y río y una muestra de agua embotellada comercial. Para cada uno de los casos se realizó un estudio de recuperación. Los resultados para benz[a]antraceno se recogen en la Tabla 9.

Tabla 9: Análisis de muestras de agua fortificadas con BA a una concentración de 1 µg L^{-1}.

Analito	Muestra (R%[a]±DE[b])		
	Río	Grifo	Embotellada
benz[a]antraceno	100 ± 9	72 ± 10	87 ± 10

[a]R%: Porcentaje de recuperación, [b]DE: desviación estándar

Como se observa, todos los valores de recuperación se encuentran próximos al 100 %, sin embargo, el valor de recuperación obtenido para las muestras de grifo es sensiblemente inferior al resto. Este hecho es debido a la presencia de cloro en este tipo de aguas, cuya presencia da lugar a un fenómeno de *quenching* de la fluorescencia [54]. Gracias a la combinación de esta técnica de microextracción con la detección fluorimétrica, podemos alcanzar unos límites de detección comparables a otras técnicas que implican un filtrado previo de la muestra y con mayor consumo de disolvente orgánico [55]. No obstante, existen otras alternativas en fase sólida más sensibles, pero implican un volumen de muestra superior, un procedimiento operacional más laborioso (tiempo de tratamiento superior a 30 min, con participación de agitadores magnéticos, generadores de ultrasonidos y desecador de corriente de N_2) y el empleo de una técnica cromatográfica [56].

En el caso de las triazinas, el estudio de recuperación se llevó a cabo con muestras de agua de rio, grifo y embotellada. Los resultados se muestran en la Tabla 10.

Tabla 10: Análisis de muestras de agua fortificadas con triazinas a una concentración de 10 µg L^{-1} en presencia y ausencia de EDTA.

Analito	Muestra (R%[a]± DE[b])					
	Grifo		Rio		Embotellada	
	sin EDTA	con EDTA	sin EDTA	con EDTA	con EDTA	sin EDTA
Prometon	35±4	65±3	39±7	80±1	57±3	77±6
Terbumeton	35±7	66±3	42±3	77±1	54±8	77±7
Secbumeton	37±2	75±3	45±6	73±6	56±4	73±7
Simetrin	43±7	66±12	36±7	72±2	49±8	67±5
Prometrin	37±6	61±1	45±6	69±1	56±15	93±1
Terbutrin	35±15	74±3	41±14	71±1	54±12	75±5

[a]R%: Porcentaje de recuperación, [b]DE: desviación estándar

Para este procedimiento, se empleó un volumen de SHS inferior, que requiere un tiempo de separación de fases un 50% mayor, ya que es necesario que se forme perfectamente la fase de SHS para poder separarla de forma efectiva. Durante el tratamiento de muestras de agua naturales se observó la aparición al poco tiempo de un precipitado globular blanco que dificultaba la recuperación de la fase extractante tras la adición de hidróxido sódico. El análisis posterior de estas muestras dio como resultado unos porcentajes de recuperación muy bajos. En principio, puede deberse a la precipitación de hidróxidos metálicos a los valores de pH tan alcalinos de trabajo. Para corroborar este hecho, se preparó un estándar de triazinas al que se añadió una sal de calcio de manera que la concentración final de calcio fuera de 80 mg L^{-1} y se trató siguiendo el procedimiento optimizado. En esas condiciones, apareció un precipitado similar al que aparece en las muestras, lo que corrobora la hipótesis planteada. El análisis cromatográfico de esos estándares dio unos valores área de pico inferiores al el estándar equivalente en ausencia de la sal de calcio. Para eliminar esta interferencia se optó por añadir un agente quelante como la sal sódica del acido etilendiaminotetraacetico (AEDT) a la muestra antes de proceder a la extracción. En esas condiciones, los valores de recuperación aumentaron considerablemente, ya que se redujo la precipitación de metales y por tanto se pudo recuperar más fácilmente la

fase extractante. La adición de este agente quelante mejoró también la precisión de la medida.

En términos de sensibilidad y precisión es la alternativa más pobre de las presentadas en esta Tesis Doctoral, para la microextracción de triazinas de muestras acuosas. Este hecho se debe principalmente a que estamos empleando un disolvente muy hidrofóbico para la preconcentración de compuestos moderadamente polares. A pesar de estar en las condiciones menos favorables para la extracción, los limites de detección alcanzados son comparables a los de otras alternativas mencionadas [48, 49, 53, 50], con un consumo de muestra y disolventes muy reducido. Al igual que la alternativa basada en SHS para PAH, es un método rápido y sencillo que no requiere ningún aparato adicional para la separación de fases.

6. Evaluación final de las técnicas presentadas en función de los 12 principios de la Química Analítica Verde.

Las metodologías propuestas en esta Tesis Doctoral pueden evaluarse de acuerdo a los 12 principios básicos de la Química Analítica Verde. La escala arbitraria que hemos fijado establece una calificación de 1 a 5, siendo 5 la calificación más verde. Como quedo patente en la introducción de esta Memoria, la implantación de estos principios puede tener consecuencias negativas sobre el rendimiento del método en términos de sensibilidad y precisión. El objetivo principal de esta investigación ha sido el desarrollo de nuevas metodologías de pretratamiento de muestra y no el desarrollo de metodologías directas de medida, por tanto, el primer y tercer principio de la Química Analítica Verde, que hace énfasis en este punto no se ha incluido en la evaluación. Las calificaciones concedidas a los métodos presentados se presentan en la Tabla 11.

Tabla 11: Calificación de los métodos presentados en función de los principios de la Química Analítica Verde

	Técnica de microextracción propuesta			
Principio de la QAV	EA-DμSPE (OASIS-HLB)	EA-DμSPE (MWCNT)	EA-DLLME	SHS-HLLE
metodologías directas	-	-	-	-
mínima cantidad de muestra necesaria	5	2	5	5
medidas *in situ*	-	-	-	-
mínimo el consumo de reactivos	4	4	5	3
métodos miniaturizados y/o automatizados	5	5	5	4
evitar la derivatización	5	5	5	5
mínima generación de residuo y gestión de los mismos	3	3	5	3
determinación multianalito	5	5	5	5
consumo energético debe ser el mínimo	5	3	5	5
reactivos de fuentes renovables	3	3	3	3
evitar reactivos tóxicos	3	2	5	2
seguridad del operador	4	3	5	3
NOTA MEDIA	4.4	3.5	4.8	3.8

El muestreo es un punto crítico en el conjunto de estos principios. Los métodos permiten realizar la extracción y preconcentración de los analitos en el mismo punto de la toma de muestra, a excepción de la estrategia con nanotubos de carbono que requiere un sistema de filtración a vacío. La simplicidad de estas alternativas permite además procesar un gran número de muestras, para asegurar la representatividad. Como se ha puesto de manifiesto con anterioridad, el residuo derivado de estas técnicas es mínimo y en su mayor parte inocuo. Esto es especialmente relevante para la seguridad del medio y el operador. En este aspecto, los métodos basados en nanotubos de carbono y disolventes conmutables son los peor evaluados. Esto es debido por una parte a la toxicidad moderada que presentan las nanoestructuras, que actualmente representan una linea de investigación relevante en química y toxicología; y por otra los disolventes conmutables, aunque presentan baja toxicidad, en su mayoría son moderadamente corrosivos.

Una de las mayores ventajas del empleo de los métodos propuestos es la escasa dependencia de otros dispositivos durante el desarrollo del método. La efervescencia por una parte, evita el uso de disolventes orgánicos, pero también de agitadores o dispositivos de ultrasonidos para generar las dispersiones. Únicamente en la etapa de elución asociada a los nanotubos de carbono es necesaria una breve irradiación con ultrasonidos, lo cual podría evitarse aumentando el tiempo de contacto entre el eluyente y el sorbente retenido en el filtro de teflón. Gracias a esto, el consumo energético de estas técnicas es mínimo.

Con estas consideraciones, se deduce que la metodología que mejor se adapta a los doce principios de la Química Analítica verde es la EA-DLLME con recuperación asistida por NPMs. El empleo conjunto de un reducido volumen de extractante capaz de interaccionar con el nanomaterial magnético da lugar a una estrategia muy rápida y versátil. El empleo de imanes para recuperar la fase extractante no requiere consumo energético y tiene un coste muy reducido, puesto que actualmente es fácil encontrar potentes imanes de neodimio en el mercado por un coste muy reducido. En el futuro, sería interesante ampliar la selectividad de esta técnica mediante la modificación de la fase sorbente con otro tipo de moléculas con especiales capacidades de reconocimiento.

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Bloque IV:

Perspectivas futuras

La miniaturización hasta la nano-escala de los distintos materiales y dispositivos es una de las tendencias más actuales en Química Analítica. A día de hoy, se han sintetizado un gran número de materiales desde nanopartículas inorgánicas o poliméricas, polímeros biológicos o películas nanoestructuradas con un amplio abanico de propiedades electrónicas, magnéticas, ópticas y (bio)químicas. El progreso de la nanotecnología, sin embargo, no se centra únicamente en la obtención de nuevos materiales miniaturizados, sino también en materiales más complejos con nuevas propiedades. En este sentido, no se trata únicamente de una cuestión de estructura geométrica del material, sino más bien de un conjunto de funciones distribuidas especificamente en determinadas posiciones del material, confiriéndole propiedades excepcionales.

La naturaleza posee dos clases de materiales capaces de llevar a cabo la mayoría de procesos relacionados con la vida, desde el metabolismo celular al reconocimiento específico de señales físico-químicas que rigen todos los procesos. Estos materiales son los polipéptidos (que dan lugar a las proteínas) y los ácidos nucléicos (que son los elementos estructurales del ADN y ARN). Estas biomacromoléculas han dado origen a una nueva generación de materiales y dispositivos bio-nanotecnológicos. Este tipo de materiales han ganado un considerable protagonismo en campos como la ingeniería computacional, la propia nanotecnología analítica, la medicina diagnóstica o como sistemas de administración de drogas *in vivo* [1,2].

La sinergia entre las biomacromoléculas y los nanomateriales es, en esencia, el producto de sus propiedades naturales combinadas. Mientras que las estructuras naturales derivadas de los ácidos nucléicos o las proteínas son responsables de las capacidades de reconocimiento molecular con una elevada especificidad, las propiedades de las nanopartículas confieren al material hibrido excelentes propiedades ópticas, electrónicas o magnéticas [3].

La Química Analítica, por su parte, hace uso de los anticuerpos (de naturaleza proteinica) como elemento de reconocimiento en multitud de técnicas. Las herramientas que emplean anticuerpos como elemento de reconocimiento se denominan inmunoensayos, y estos han sido ampliamente conjugados con variedad de nanoestructuras, especialmente Quantum Dots (QDs) como sondas en diversos tipos de ensayos [5]. Las nanopartículas magnéticas, por su parte, han recibido gran atencion como materiales nanoestructurados para aplicaciones en biosensores. Este tipo de materiales presentan grandes ventajas frente a otros debido a su bajo coste de producción, estabilidad y biocompatibilidad. Además, son calificados como materiales ambientalmente seguros. Son especialmente apropiados para el tratamiento de muestras biológicas, ya que estas apenas magnetismo y pueden por tanto emplearse en muestras turbias o con matrices muy complejas proporcionando medidas sensibles [6]. Las NPMs han dado lugar a materiales híbridos con multitud de moléculas biológicas para un gran número de aplicaciones [6-10].

Recientemente, los ácidos nucléicos, han ganado protagonismo en el campo de la Química Analítica, especialmente gracias a los *aptámeros.* Los aptámeros son moléculas sintéticas de ADN o ARN diseñadas específicamente para interaccionar con un determinado analito [11]. Formalmente presentan un mecanismo de reconocimiento similar a los anticuerpos, pero con afinidades muy superiores y estabilidad física y química muy superior [12]. Pueden ser fácilmente hibridados con nanomateriales de muy distinta naturaleza, como QDs [13], nanomateriales de carbono [14], nanopartículas de oro [15], sílice [16] o magnéticas [17] para funcionar como elementos de reconocimiento en ensayos homogéneos [18] o biosensores [19].

Durante el desarrollo de esta Tesis Doctoral, se ha realizado un estudio detallado y sistemático de las posibilidades de este tipo de materiales híbridos, especialmente de los aptámeros. El siguiente capítulo detalla las propiedades de las

nanopartículas disponibles en el ámbito del análisis (bio)químico y la capacidad de los aptámeros como ligando de analitos de muy diferente naturaleza en Química Analítica.

Esta investigación trasversal pretender ser el inicio del trabajo postdoctoral del doctorando, explotando su formación bioquímica. De hecho, ya se ha iniciado con éxito esta nueva línea de trabajo en el grupo de investigación.

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Capítulo VII:

Nanoparticle-based microextraction techniques in bioanalysis

REVIEW
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Nanoparticle-based microextraction techniques in bioanalysis

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Abstract

Nanoparticles (NPs) have attracted a great deal of attention in the last decade due to their exceptional mechanical, optical and electronic properties. This article deals with the use of NPs as probes for the extraction of biomolecules from biological samples. In this context, NPs present some advantages compared with conventional sorbents. Their high surface-to-volume ratio, easy synthetic (especially for inorganic NPs) and derivatization procedures, and their biocompatibility make them a powerful alternative. In order to provide a systematic approach to the topic, NPs have been divided into two general groups attending to their chemical nature. Carbon-based (e.g., fullerene and nanotubes) and inorganic NPs (e.g., gold and magnetic NPs) are considered in depth, explaining their main properties and applications. After these critical considerations, the most important conclusions and essential trends in this field are also outlined.

Key terms: ***Microextraction:*** *Those extraction techniques which involve the use of a low amount of extractant phase (liquid or solid) for the isolation and/or preconcentration of the target analytes from a given sample.* ***Bioanalysis:*** *Development and application of chemical measurements to solve analytical problems of biological concern.* ***Nanoparticles:*** *Those particles that present at least one dimension in the nanometer range which gives them novel and special properties.*

1. Introduction

Sample treatment is an important step in many analytical processes since it adapts the samples to the selected instrumental technique. It becomes critical when complex (e.g., biological) matrices are analyzed. In this context, direct analysis is not practical due to the lack of selectivity and sensitivity. The large amount of potential interferents and the low concentration of the target analytes make necessary a previous extraction procedure that usually involves isolation and preconcentration of the analytes. In this sense, the ideal sample treatment should be: simple, automatic, miniaturized, rapid, safe to operators, environmental friendly and economic. These main characteristics have directly influenced the evolution of extraction techniques in recent years, allowing the develop- ment of solid-phase microextraction (SPME) and liquid-phase microextraction (LPME) techniques. Both miniaturized approaches are very versatile and their acronyms involve a large number of different subtechniques that may be adapted to the given bioanalytical problem. The main features of SPME [1,2] and LPME [3–5] in bioanalysis have been extensively reviewed.

The development of new techniques, and the improvement of the existing ones by using novel extracting materials, can be considered as the main trends in the current research in this field. Regarding the new materials, ionic liquids and nanoparticles (NPs) are promising tools. Ionic liquids have been successfully employed in LPME (as solvents) and SPME (as solid polymeric coatings or elution solvents). NPs, covering different types, shapes and sizes, have also been employed [6].

This article deals with the use of NP-based microextraction techniques in bioanalysis. This is a challenging topic due to the great variety of NPs, the large number of bio-related substances that may be considered (from simple drugs to biopolymers) and the high number of potential instrumental techniques involved in the bioanalytical methods presented in the literature. This article is be divided into different sections according to the different NPs used.

2. Nanoparticles: definitions and general properties as probes

The definition of NPs is a complex task due to the great variety of existing NPs with different properties and covering a wide variety of application fields. The first nuance refers to dimensions. In fact, a NP is a particle that has one or more dimensions in the nm range, considering 100 nm as an arbitrary reference [7]. The second nuance of the NPs definition is related to their characteristics. In this sense, a NP presents novel properties in the nanoscale compared with those observed in the bulk material. Taking into account both nuances, NPs can be defined as *"those particles that present at least one dimension in the nm range that gives them novel and special properties"*.
The binomial NPs bioanalysis can be con- sidered from different perspectives [8]. Within all the possible applications, the use of NPs as extraction probes of target analytes from biological samples is very interesting due to the following properties:

- NPs are commercially available with characterized dimensions and properties. Moreover, some of them are easily synthesized in the laboratory using conventional reagents. The properties of the laboratory-made NPs can be selected by controlling the synthesis variables;
- NPs can be easily derivatized in order to promote their selective interaction with the target analytes. This derivatization may involve the introduction of biorecognition molecules on the NPs surface;
- NPs present a high surface area. This is very useful in SPME as the efficiency of these processes is surface-dependent [9];
- Some NPs are biocompatible, which is essential in the extraction of proteins since it means that protein structure is not modified as a consequence of its interaction with the NP;

- NPs have been proven as exceptional substrates in MALDI. In fact, MALDI [10] in combination with MS (MALDI–MS) is a common instrumental technique in bioanalysis [11];
- NPs allow the efficient isolation, and even preconcentration, of different analytes in a complex environment;
- The use of NPs simplifies extraction procedures. Magnetic NPs (MNPs), which can be easily isolated by using an external magnetic field, are a clear paradigm in this sense.

NPs can be classified according to different criteria, such as properties or composition. In this article, the second approach was selected, classifying them into carbon-based and inorganic NPs.

3. Carbon based nanoparticles

Carbon exists in different allotropic forms with different structures and properties. In this section, the main allotropes used in the development of microextraction procedures in bioanalysis are considered. Their structures are depicted in Figure 1.

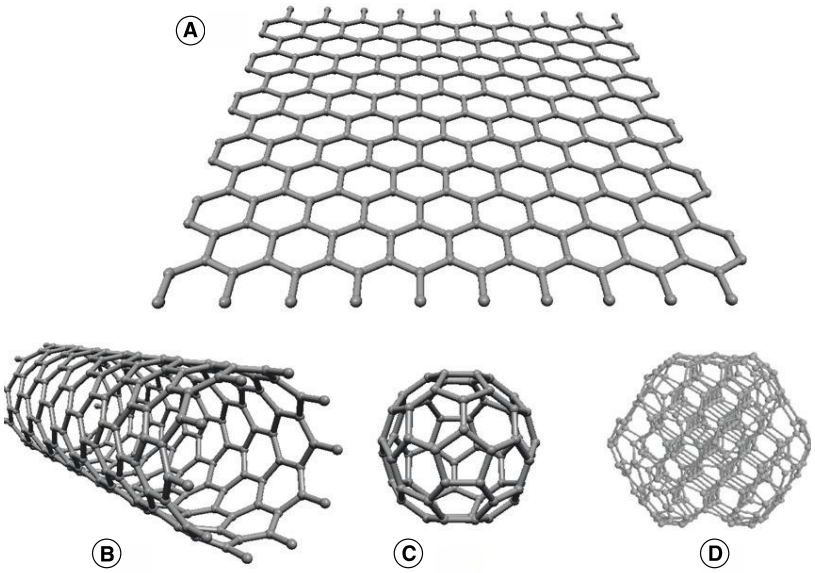

Figure 1: General structures of (A) graphene, (B) nanotubes,(C) fullerene and (D) nanodiamonds. Structures (A) and (B) designed with nanotube modeler. Structure (D) reproduced with permission from [30] © Elsevier Inc.

3.1 Carbon Nanotubes

Since their discovery in 1991 by Ijima [12], carbon nanotubes (CNTs) have attracted much attention due to their exceptional properties [13], such as high thermal stability, excellent mechanical and electric properties and high tensile strength. Moreover, their sorption capabilities make them an excellent material for extraction and microextraction procedures under the analytical chemistry framework [14]. Within these general characteristics, some of them are highlighted. First, CNTs present a high surface-to-length ratio, which is essential in any microextraction procedure. Moreover, CNTs can be functionalized, including carboxylic groups that can be further derivatized in order to incorporate special functional groups on the CNT surface, or to immobilize CNTs in different materials. Moreover, CNTs can establish non-covalent interactions with analytes that include hydrogen bonding, π–π stacking, dispersion forces, dipole–

dipole interactions and hydrophobic effects [15]. The main limitation of CNTs is their aggregation tendency, which results in a dramatic decrease in the active surface, and therefore their extraction capability [16]. The immobilization of CNTs in an inert support, such as inert glass or membranes, has been proposed in order to avoid this limitation.

In the bioanalytical context, CNTs have been used as efficient extraction materials for the isolation of drugs, toxics and proteins from biological samples. Until now, CNTs have been the most used carbon-based nanostructures in a bioanalytical context. However, the appearance of new promising materials (e.g., nanodiamonds or graphene) may modify this tendency.

3.1.1 Carbon nanotube extraction of simple molecules

Suarez *et al.* proposed the use of CNTs for the extraction of non-steroidal anti-inflammatory drugs from urine samples for their subsequent determination by capillary electrophoresis/mass spectrometry [17]. A special mini-column containing the sorbent material was inserted in a flow injection manifold in order to automate the sample treatment. As CNTs tend to aggregate, providing high back-pressures in flow manifolds, the authors immobilized the CNTs (previously derivatized to their carboxylic form) in the surface of an inert solid (controlled pore glass). This methodology allows the determination of non-steroidal anti-inflammatory drugs in urine samples with limits of detection in the range of 1.6 to 2.6 μg L^{-1}.

Despite this aggregation tendency, CNTs have also been used in conventional SPE cartridges. In this sense, Cruz-Vera *et al.* employed CNTs for the extraction of nine antidepressants in urine samples [18]. After the extraction, the analytes were separated by LC, assisted with ionic liquids and determined by UV detection. The limits of detection, in the range of 12.3 to 90.1 ng mL^{-1}, allow the therapeutic monitoring of these drugs in urine samples. Huang *et al.* have also proposed the use of CNTs in

conventional manifolds for the determination of thiol compounds (glutathione, cysteine and *N*-acetylcysteine) in urine and plasma samples [19]. In this case, the analytes are previously derivatized with a fluorescent promoter, in order to enhance the sensitivity of the final determination. The limits of detection were in the low pM range.

Apart from their good extraction capabilities in SPE, CNTs are also good matrices in MALDI. Therefore, the combined use of CNTs as probes and matrices in MALDI-MS, has also been proposed for the determination of drugs (propanolol, quinine and cinchonine) in urine samples [20]. In the general procedure, the CNTs are incubated with the sample and recovered by centrifugation. Finally, the CNTs are suspended in methanol for their subsequent MALDI-MS analysis.

SPME fibers containing CNTs have been proven as useful extraction tools in many fields of application. The application of SPME in biological samples is a challenging issue due to the complexity of the matrices and the low amount of extractant. SPME, working under the headspace mode, has been proposed for the determination of some gasoline additives (methyl *tert*-butyl ether, ethyl *tert*-butyl ether and methyl *tert*-amyl ether) in human urine of people exposed to this type of compound [21]. Headspace modality reduces the sample matrix effect, as only the volatile compounds are in close contact with the CNT fiber. Excellent performance, with detections limits of 10 ng L^{-1} for all the analytes, was obtained.

3.1.2 Carbon nanotube extraction of biomolecules

The selective extraction of proteins from complex samples is a key and challenging step in proteomics. It is usually achieved by means of immunosorbents, which are expensive. CNTs are promising materials in this context, thanks to their high superficial area and potential extractability toward different molecules. Due to their hydrophobic surface, CNTs may extract proteins and peptides via a nonspecific

interaction [22], which is interesting when the whole proteome is analyzed [23]. However, hydrophobic interactions may induce conformational changes on the extracted proteins. This limitation may be overcome by surface modification of CNTs by chemical derivatization, which may or may not involve covalent and stable binding. Chen *et al.* proposed the functionalization of CNTs with hyperbranched polyethyleneimine, an amino-rich cationic polyelectrolyte, to allow the extraction of bovine serum albumin by more selective electrostatic interactions [24]. For this purpose, CNTs were previously oxidized to introduce carboxylic groups that confer a negative charge to the surface. The sign of the superficial charge is changed by the polyethyleneimine coating, which allows the extraction of acidic proteins by electrostatic interactions. Although bovine serum albumin was selected as the model analyte, the methodology was qualitatively evaluated by the determination of human serum albumin in whole blood. In this way, Du *et al.* proposed the wrapping of CNTs with polydiallyldimethylammonium chloride to produce a hydrophilic surface, with a high cationic density, to favor the extraction of acidic proteins from whole blood [25]. The composite polydiallyldimethylammonium-CNT was immobilized in a filter since CNT-based filters [26] improve the extraction recovery (by increasing the contact surface between the analytes and the solid) and the flow conditions (allowing the flow of the sample through the extractant).

3.2 Fullerenes

Vallant *et al.* studied the potential of fullerenes as extraction probes towards different types of biomolecules [27] for their subsequent MALDI-TOF-MS analysis [28]. In this approach, C60 fullerene is immobilized on the surface of silica particles of different porosity in order to enhance the extractability, thus reducing the aggregation of the NPs. The silica particles are firstly derivatized, including amine groups on their surface, which are finally reacted with two different C60 compounds, namely:

epoxyfullerene and fullerene acetic acid. Both silica-C60 sorbents were applied for the extraction of peptides and proteins with very good results. Moreover, they were evaluated for the extraction of phosphopeptides, which are extracted with difficulty using conventional reversed-phase sorbents. The results show that silica-C60 provides superior features in the extraction of hydrophilic peptides. In the same way, Böddi *et al.* evaluated the SPE performance of silica-C60 compared with ocatadecyl (silica- C18) and triaconthyl (silica-C30) silicas. The results demonstrated that silica-C60 and silica C-30 provided the best extraction efficiency at low peptide concentrations [29].

3.3 Nanodiamonds

Carbon nanodiamonds (CNDs) present great applicability on the extraction and determination of proteins, peptides and DNA. Their structure [30] is characterized by a large surface (a property that they share with other NPs), which allows interaction via hydrophobic forces with different targets. Since hydrophobic interactions are less selective, the derivatization of CNDs is required when biomolecules are intended to be extracted. The functionalization of CNDs usually starts with oxidation of the NPs with a sulphuric and nitric acid mixture at high temperature [31]. This functionalization produces oxygen-containing groups (mainly carboxylic groups) on the surface, which gives a potential negative charge (depending on the working pH) to the nanostructures. This negative charge allows the selective interaction of CNDs with proteins, based mainly on electrostatic forces between the carboxylate groups ($–COO^-$) and the protonated amine groups ($-NH_3^+$) of the peptides. Although the usefulness of carboxylated CNDs for biopolymers extraction has been demonstrated [32], this approach presents two limitations. On the one hand, the low number of carboxylic groups on the CND surface does not provide the best extraction capacity. On the other hand, hydrophobic interaction with the bare CND surface is still possible. The coating of carboxylated NDs with poly-L-lysine has been proposed in order to overcome this

limitation [33]. Poly-L-lysine is coated to the CNDs in an extended configuration by electrostatic forces, leaving a high number of amino-groups available on the surface. The aminated surface can interact with negatively charged groups of bio-polymers. Kong *et al.* proposed polyaminated oxidized CNDs for the extraction of DNA oligonucleotides for their subsequent analysis by MALDI-TOF. This procedure allows the selective extraction of DNA in the presence of proteins by the appropriate selection of the working pH [34].

The extracted biomolecules can be directly analyzed by MALDI-MS on the CND surface, without a previous elution, due to the small particle size, inertness and optical transparency of the NPs. Chen *et al.* exploited these characteristics in the so-called technique solid phase extraction and elution on diamond (SPEED) [35]. In this platform, carboxylated CNDs are employed for the extraction of proteins from urine samples allowing their subsequent analysis by SDS-PAGE [36] or MALDI-MS. The proteins are attached to the CND's surface by electrostatic forces with the $–COO^-$ groups, by hydrogen bonding with the C=O and/or by hydrophobic interactions with the bare CND surface. Moreover, the intense interaction of proteins with CNDs allows us to perform crucial steps on proteomics (e.g., reduction of disulfide bounds or proteolysis) on the particle surface without a previous elution. Sabu *et al.* extended the usefulness of SPEED in peptide analysis combining the technique with atmospheric pressure MALDI analysis [37]. As it can be deduced, the SPEED technique critically depends on the exhaustive recovery of CNDs after their incubation with the sample matrix. This recuperation, usually performed by centrifugation, may be problematic when low amounts of NPs are employed. Recently, Kong and Sahadevan proposed filtration as a suitable alternative to centrifugation [38]. The CNDs may be retained in polyvinylidene fluoride filters, which can be directly analyzed by MALDI.

3.4 Graphene

Graphene (G) is a strictly 2D carbon allotrope that exhibits high crystal and electronic quality. Its structure consists of one-atom-thick planar sheets of sp^2-bonded carbon atoms that are densely packed in a honeycomb crystal lattice [39]. As with other allotropes of carbon previously described, it has exceptional mechanical, electric, thermal and optical properties. Moreover, its high specific area (2630 m^2 g^{-1}, theoretical value) and its easy derivatization make it a promising material in extraction procedures. In the usual synthesis path, G is obtained by oxidation of graphite following the Hummers and Offeman procedure [40]. As a result, graphene oxide (GO) is obtained, which can be reduced to G using hydrazine. Both structures, G and GO, show different potentialities in the extraction context, as they present hydrophobic and hydrophilic surfaces respectively. In fact, their properties render them as exceptional reversed- phase and normal-phase sorbents [41].

Huang *et al.* have recently proposed the use of G for the SPE of glutathione in human plasma for its subsequent fluorescence determination [42]. Before the extraction, the target analyte is derivatized to include a fluorophore that makes the final determination easier. This reaction makes the analyte more hydrophobic, favoring its retention in G. In fact, G provides better performance than more usual sorbents such as C_{18}-silica, graphitic carbon and CNT.

The hydrophobicity of G may result in a non-selective interaction with the target analytes, which is essential in a biological medium. For this reason, GO has been employed as an alternative, as it presents polar moieties such as hydroxyl, carboxylic and epoxy groups. These functional groups allow hydrogen bonding and also electrostatic interactions with charged analytes, increasing the versatility of the material. Liu *et al.* proposed the use of GO for the isolation of hemoglobin from whole blood samples for its subsequent determination by SDS-PAGE [43]. For this purpose, the authors immobilized GO on the surface of silica particles. The resulting composite

presents a higher particles size (which makes the handling of the NPs easier) while maintaining the exceptional sorption properties of GO.

The introduction of a bio-recognition element, such as an aptamer, on the surface of GO clearly improves the selectivity of the extraction. Gulbakan *et al.* employed this approach for the determination of cocaine in human plasma. A dual platform, in which GO is used as probe and matrix, is proposed for the extraction of the target analyte and its subsequent analysis by MALDI-MS [44].

3. Carbon based nanoparticles

3.1 Gold nanoparticles

Metallic gold NPs (AuNPs) have been broadly used in recent years for the improvement of analytical methodologies due to their inherent properties [45,46]. On the one hand, they can be synthesized in the laboratory, controlling not only the shape (nanospheres or nanorods), but also their size (which dramatically affects their properties). On the other hand, AuNPs present long-term stability in different solvents and pH conditions [47]. Moreover, the surface of AuNPs can be easily functionalized and these surfaces present a high affinity for different biomolecules, which makes AuNPs so attractive in the bioanalytical context.

Although there are several methods for AuNPs synthesis, the typical procedure consists of the reduction of Au(III), usually in the form of hydrogen tetrachloroaurate, with sodium citrate in an aqueous media under heating and vigorous stirring. As a result, citrate-capped AuNPs (AuNPs–citrate) are obtained with a specific size depending on the stoichiometry of the reagents. The NP size can be directly measured according to their UV/visible spectrum, since the maximum absorption wavelength depends on the particle size. The obtained NPs tend to aggregate and sediment in water and chemical modifiers, especially surfactants, are required to stabilize the NPs in solution.

AuNPs have a great affinity towards thiol functional groups, as a result of the forma- tion of Au–S bonds [48]. This characteristic has been exploited in the development of µSPE approaches focused on the extraction of aminothiols, which are considered biomarkers of some diseases (e.g., Alzheimer's disease), in serum and urine samples. In their raw state, AuNPs–citrate present a residual negative charge on their surface that is responsible for nonselective extraction of some proteins by electrostatic interaction. To reduce this effect, the AuNPs–citrate are capped with surfactants that improve the dispersion of the NPs in aqueous media, but minimize their extraction surfaces. The general scheme for aminothiols extraction is depicted in Figure 2. First of all, the AuNPs–citrate are incubated with the surfactant, which is deposited onto the AuNPs surface. Later on, the sample is added to the extraction mixture and incubated for 2 h. Then, the NPs are recovered using centrifugation, and the analytes eluted using a thiol compound that acts as a displacement reagent. Li *et al.* proposed this extraction procedure for the isolation of cysteine, homocysteine and glutathione from urine samples. The final determination by capillary electrophoresis with UV detection provided limits of detection in the µM range [49]. Following a similar procedure and including a final derivatization of the aminothiols, Shen *et al.* have achieved the extraction of homocysteine, glutathione and g-glutamycysteine from urine samples [50]. Better limits of detection were obtained in the interval of nM to several pM, due to the use of laser-induced fluorescence as the instrumental technique. In both approaches, only free-reduced aminothiols can be determined. However, in biological samples, aminothiols can be found in three different formats, namely: free reduced, free oxidized or bonded to proteins. The determination of total free (reduced and oxidized) and protein-bounded aminothiols can be achieved using a modification of the general extraction procedure. This procedure (Figure 2) involves the use of a reducing agent that is able to reduce the oxidized aminothiols and break their bonds with plasmatic proteins [51]. Total aminothiols are extracted by being added directly to the

reducing agent and extracting the free-reduced aminothiols generated by the general procedure. On the other hand, free aminothiols are obtained by adding the reducing agent after a filtration, which removes all the proteins (and the bonded aminothiols) present in the sample.

AuNPs are a very versatile material in sample preparation, since their easy derivatization allows the development of different chemistries (hydrophobic, electrostatic and immunological) on the extraction of several substances in biological samples. This aspect is clearly demonstrated in the extraction of five indoleamines in a urine sample using AuNPs–citrate as the extractant [52]. The target analytes are extracted from the sample by two different interaction mechanisms depending on the nature of the substance. On the one hand, 5-hydroxytryptophan, tryptophan and 5-hydroxyindoleacetic acid are adsorbed onto the AuNPs by van der Waals interactions between the indole group of the analytes and the hydro- phobic surface of the AuNPs-citrate. On the other hand, tryptamine and 5-hydroxytrypta- mine are extracted by electrostatic interactions with the partial negative charge of the NPs. In the µSPE scenario, AuNPs–citrate has also been used to simplify the determination of monohydroxy-polycyclic aromatic hydrocarbons in urine samples [53]. The hydrophobicity of AuNPs– citrate can also be boosted for special applications, such as the determination of neutral steroids in urine samples [54]. In this case, the AuNP surface is derivatized with 1-octadecanethiol and self-assembled in silica gel.

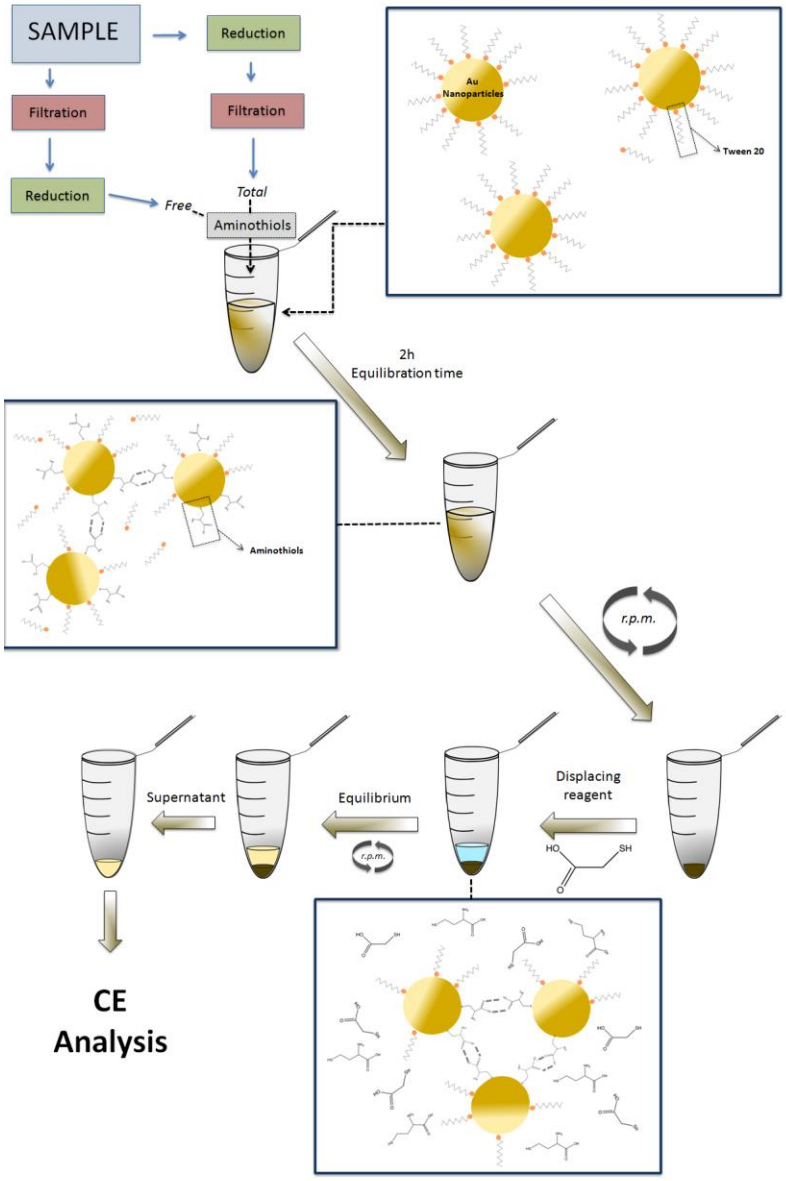

Figure 2: General procedure for the extraction and determination of thiols (free and protein-bound) on biological fluids using metallic gold nanoparticles as selective probes.

The synergic combination of AuNPs with MALDI-MS has been demonstrated in the literature [55]. In this context, AuNPs can play two different but complementary roles. AuNPs can act as probes in order to isolate the target analytes from biological samples, reducing to a large extent the suppression effect of the sample matrix in MALDI analysis. On the other hand, AuNPs are themselves excellent matrices in MALDI analysis, due to their high surface and high UV absorptivity.

AuNPs have been used as probes for the extraction of peptides and proteins through a double mechanism that involves electrostatic and hydrophobic interactions. In fact, at pHs lower than their isoelectric points, peptides present a positive charge that facilitates their adsorption. Moreover, the hydrophobic nature of the citrate capping favors the interaction with the lipophilic part of these macromolecules. The combination of AuNPs and MNPs has allowed the extraction of low amounts of peptide residues from the tryptic digest products of cytochrome, which can be finally determined by MALDI-MS [56]. Sudhir *et al.* have proposed a single-drop microextraction approach for the extraction of peptides from urine samples. In this case, AuNPs are dispersed in toluene and a drop of the solvent is immersed directly in an extract of the treated sample. After the extraction, the microdrop is retracted and finally deposited in the MALDI matrix for its subsequent analysis [57].

Although MALDI has been successfully employed for the analysis of proteins, it lacks the sensitivity for determination of low-molecular weight compounds due to the high back- ground signal of the typical MALDI matrices. This problem limits the use of MALDI when small drugs are the target analytes. Surface-assisted laser desorption/ionization (SALDI) using NPs overcomes this limitation by opening up a new application perspective. Nile red- adsorbed AuNPs have been used as probes (for the extraction) and matrices (for the SALDI analysis) for the determination of three amino-thiols (glutathione, cysteine and homocysteine) in blood [58]. SALDI-MS using AuNPs

has also been proposed for the selective determination of ATP and glutathione in culture cells. In this case, AuNPs coated with an aptamer are synthesized as selective probes of ATP. After the extraction, the AuNPs–aptamer are mixed with AuNPs that act as a matrix for SALDI analysis. In this application, ATP can be determined in the low µM range [59].

Finally, the versatility of AuNPs has been clearly demonstrated by Ho *et al.* who proposed microscopy image is obtained. The images obtained for a specific bacterium, *Staphylococcus saprophyticus*, are presented in Figure 3. First of all, an interaction between IgG and the bacteria is observed since the AuNPs–IgG cover the surface of the bacteria. For comparative purposes, raw AuNPs and AuNPs decorated with human serum albumin were also synthesized and evaluated. As it is observed, these NPs do not present any interaction, which also indicates the selectivity of AuNPs–IgG. The authors proposed the use of MNPs, based on magnetite, decorated with IgG (Fe_3O_4-IgG) for the isolation of bacteria from urine samples. After the incubation between the sample and NPs, Fe_3O_4–IgG are isolated by applying an external magnet. The NPs with isolated bacteria are finally mixed with AuNPs that act as a matrix for the subsequent MALDI-MS analysis.

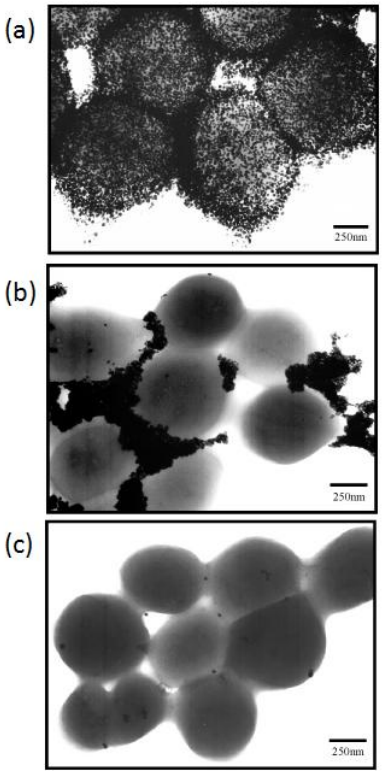

Figure 3: TEM images of *Staphylococcus saprophyticus* obtained after incubating these bacteria with (A) Au–IgG nanoparticles, (B) unmodified metallic gold nanoparticles and (C) Au–bovine serum albumin nanoparticles. Reproduced with permission from [60] © American Chemical Society.

3.2 Silver nanoparticles

Silver NPs (AgNPs), which present sizes in the interval from 1 to 100 nm, have demonstrated a wide applicability in different fields [61], such as catalysis or bactericide. From the analytical point of view, AgNPs are attractive material since they can be easily synthesized and derivatized for different purposes. Although there are several methods for their synthesis, the easiest consists of the reduction of silver nitrate with sodium borohydride in an aqueous media [62].

AgNPs have been reported as excellent probes for the LPME of proteins and peptides. In these procedures, the AgNPs are derivatized with functional groups that promote the interaction with proteins by hydrophobic or electrostatic interactions. Figure 4 shows the typical extraction manifolds used in these cases. In the usual approach (Figure 4A), the modified NPs are dispersed in toluene and a few millilitres of the dispersion are directly immersed in the treated sample. In such conditions, the proteins and peptides diffuse from the bulk solution to the acceptor phase, allowing their isolation and preconcentration. Once finished, the organic microdrop is mixed with α-cyano-4-hydroxycinnamic acid for the subsequent analysis by AP-MALDI-MS. Following this general procedure, hydrophobic AgNPs (obtained by surface reaction with octadecanethiol) have been employed for the extraction of gramicidin D from urine and plasma samples [63], providing limits of detection of 0.13 and 0.16 µM, respectively. These values involve a signal enhancement factor in the range of 266 to 388, compared with the direct AP-MALDI-MS analysis. Moreover, the methodology can be extended to other proteins such as myoglobin, ubiquitin and bovine serum albumin. In this approach, the weak hydrophobic interaction between AgNPs and proteins makes the extraction kinetics too slow, requiring long extraction times (2 h). This shortcoming may be overcome by modifying the chemistry of the interaction. Sudhir *et al.* have proposed the use of AgNPs as electrostatic probes for peptide analysis under a single-drop microextraction format (Figure 4B) [64]. For this purpose, tetraalkylammonium bromide-coated AgNPs are synthesized and dispersed in toluene. These AgNPs, which present a residual positive charge on their surface, are able to extract proteins at pH values above their isoelectric points in only a few minutes. In the optimal conditions, this procedure allows the determination of *Met*-enkephalin and *Leu*-enkephalin with detection limits of 160 and 210 nM, respectively.

Kailasa and Wu have proposed the use of modified silver selinide NPs (AgSeNPs) under a LPME scheme, similar to that described in Figure 4A for the

extraction of valinomcyn and gramicidin D [65]. In this sense, AgSeNPs are derivatized on surface with octadecanethiol or 11-mercaptoundecanoic acid in order to promote the hydrophobic interaction with the proteins. In the procedure, 900 µL of sample are incubated with 100 µL of a dispersion of modified AgSeNPs in toluene under continuous stirring. As it was previously outlined, long extraction times (1 h) are required, due to the hydrophobic nature of the interaction. After the extraction, 2 µL of the extract is mixed with the appropriate matrix for MALDI analysis. The limit of detection, which depends on the type of modified NPs, is in the range from 20 to 126 nM.

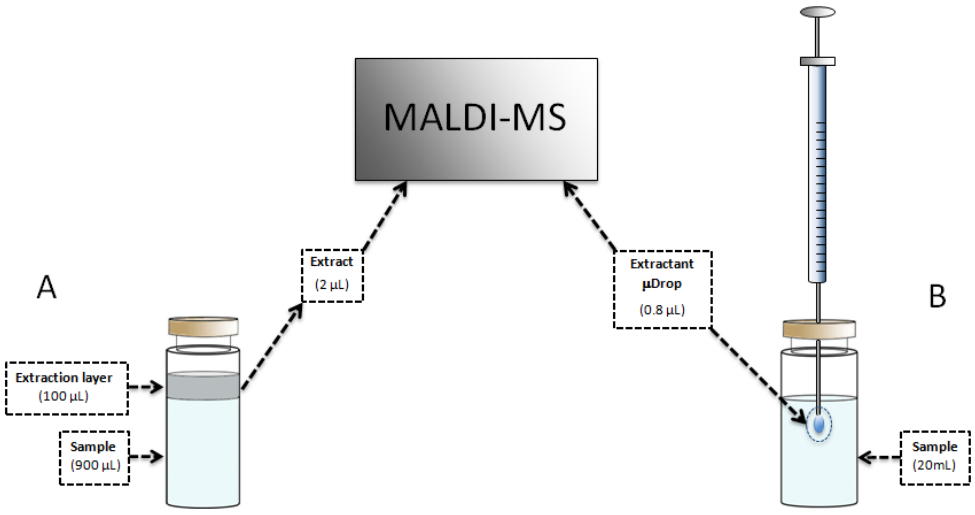

Figure 4: Use of silver nanoparticles as probes for the extraction of different analytes under (A) general liquid–liquid and (B) single-drop microextraction procedures.

AgNPs can also be used as affinity probes in SALDI for the determination of biothiols in urine [66] since thiols are easily attached to the NPs surface by covalent bonding. In fact, bare NPs provide better results on the extraction in comparison with modified NPs (with citrate or cetyltrimethylammonium bromide) as the coating avoids

the covalent bond formation. In the general procedure, the sample is incubated with the bare NPs for a defined time allowing the extraction of the biothiols. Once extracted, the analytes and NPs are recovered by centrifugation, with the analytes (cysteine and homocysteine) finally being determined by SALDI-MS. The authors outlined the potential applicability of AgNPs as affinity probes for sulfur drugs. AgNPs have also been employed in the μSPE context for the extraction of manganese (Mn) from biological samples [67]. For this purpose, the NPs are modified with 1-(2-pyridylazo)-2-naphthol. The sample is incubated with the AgNPs for a fixed period of time, allowing the extraction of Mn. After the extraction, Mn is eluted with a mixture of dimethylsulfoxide and nitric acid for subsequent analysis with inductively coupled plasma optical emission spectrometry. The technique provides a limit of detection of 0.08 μg L^{-1}.

4. Magnetic nanoparticles

Magnetic nanoparticles (MNPs), based on magnetite (Fe_3O_4), have been proven as useful tools in microextraction [68] due to their inherent characteristics. MNPs can be synthesized by different procedures [69], but the usual one consists of the co-precipitation from aqueous Fe^{2+}/Fe^{3+} salt solutions, by the addition of an alkali (sodium or ammonium hydroxide) under inert atmosphere (a nitrogen stream) at room or elevated temperature [70]. The characteristics (particle size, shape and composition) of the obtained MNPs depend directly on the experimental conditions of synthesis employed and they mark the magnetic behavior of the particles. In fact, only those MNPs that present a particle size lower than 100 nm are superparamagnetic. Superparamagnetism, which involves MNPs presenting a magnetic behavior under the influence of an external magnetic field, results essential in the performance of these NPs in microextraction.

Figure 5 presents the general scheme of a MNP. Although bare MNPs can be used as sorbent or support in microextraction procedures, it is usual to protect them with an inorganic shell. The shell can be made from different materials and it stabilizes the MNPs in an aqueous medium and it may also act as a support for a subsequent functionalization. The coating shell, as well as the possible surface functionalization of the MNPs, makes them versatile probes in microextraction. In fact, MNPs can be task-specifically synthesized, taking into account the peculiarities of the analytical problem (sample, analyte) to be solved.

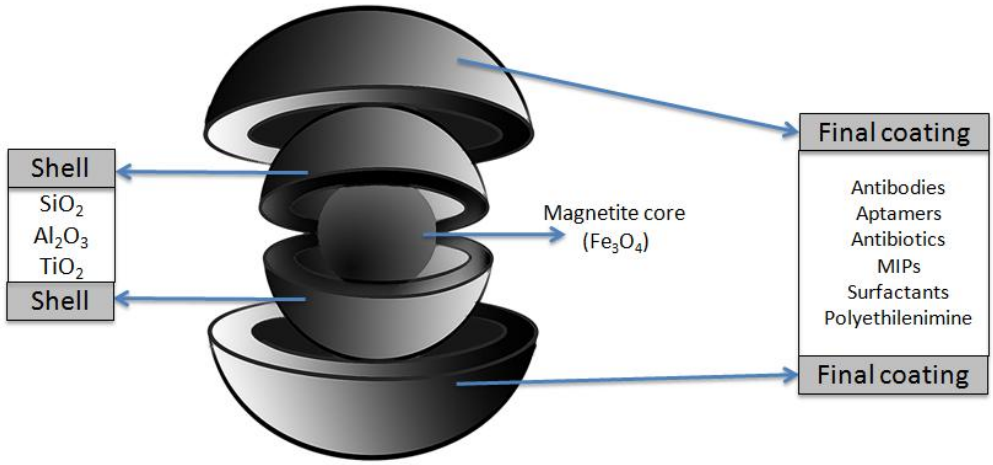

Figure 5: General scheme of a magnetic nanoparticle. The usual components (core, shell, coating) and materials are highlighted.

The general extraction procedure employed with MNPs is schematically presented in Figure 6. First, the sample is incubated with the appropriate MNPs inducing the isolation and extraction of the target analytes. After an incubation time, a magnet is applied to the outside wall of the extraction vessel allowing the separation of the MNPs from the sample, which is discarded. Later on, after a washing step with an

appropriate solvent, the MNPs are recovered. These MNPs can be directly analyzed (e.g., in MALDI analysis) or the analytes can be eluted for their subsequent determination (by chromatographic or electrophoretic techniques). The main objective of these procedures is the joint improvement of sensitivity and selectivity, although some approaches are only focused on selectivity enhancement [71].

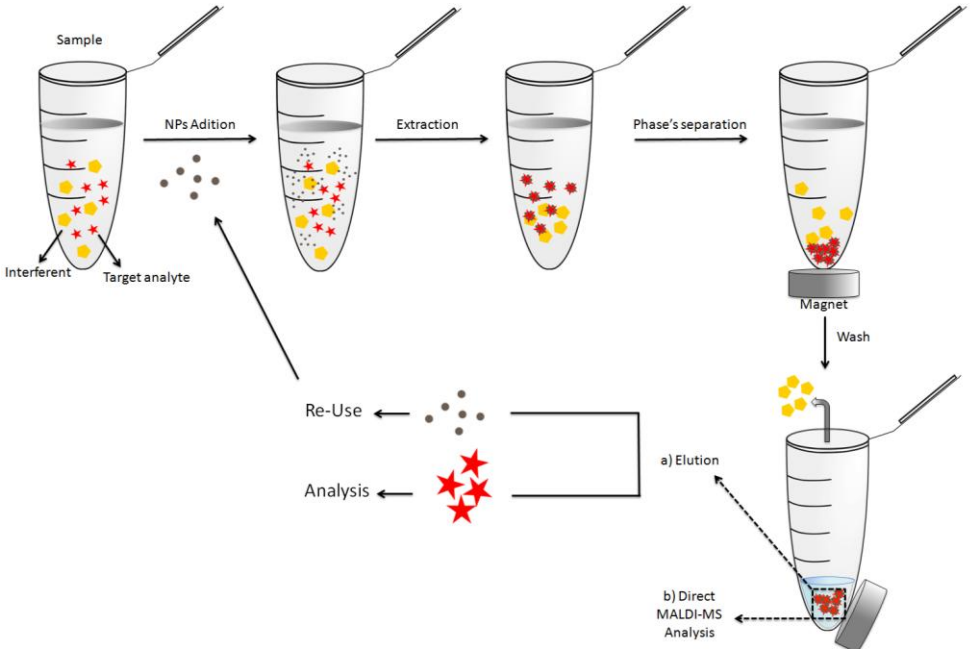

Figure 6: General extraction procedure using magnetic nanoparticles as probes.

In bioanalysis, MNPs have been used extensively for the extraction of small drugs, proteins, nucleic acids and even cells. The main issue to be considered in these applications is the selectivity, taking into account the complexity of biological matrices. In some cases, the inherent interaction of the MNPs towards particular analytes is exploited, but in other cases an additional derivatization (including more selective functional groups) is required.

The direct use of bare MNPs has been proposed for the extraction of salicilate from plasma samples after its previous reaction with Fe (III) [72]. The complex Fe–salicilate, which presents a characteristic purple/violet color, is efficiently extracted (recoveries near to 100%) on the surface of magnetite, due to the absorption affinity that the hydrated surface of ferric oxides presents at acidic pH. The elution, performed by a pH exchange, provides a colored solution that is photometrically measured.

However, bare MNPs are not usually employed for complex analysis due to selectivity problems, a functionalization being necessary. In the simplest case, a straightforward coating with appropriate characteristics may be employed. In this sense, Lin *et al.* took advantage of the great affinity that metal oxides present towards phosphate for the selective extraction of phosphopeptides from complex samples such as human serum, milk and the digest of cell lysates [73]. MNPs (10 nm in size) were subsequently covered with silica and tantalum oxide for this purpose, providing functionalizated MNPs with a particle size of 25 nm. After the extraction, the isolated MNPs can be directly analyzed by MALDI-MS. A simple but selective derivatization was also proposed by Dou *et al.* for the extraction of riboflavin from urine samples and its subsequent analysis by capillary electrophoresis [74]. In this case, MNPs with a particle size of 60 nm were coated with a poly-3-aminophenylboronic acid, since boronic acid residues tend to be very selective towards 1,2- and 1,3-*cis*-diol-based analytes, even in the presence of non-*cis*-diol molecules. Apart from the selectivity, MNPs present a high extraction capacity (0.32 ng of riboflavin per mg of NPs). In the above-mentioned examples, laboratory made MNPs are employed for the extraction of target analytes, due to their easy synthesis and derivatization. However, MNPs are also commercially available for analytical purposes. Bansal *et al.* used commercial MNPs with weak cation exchange groups in their surface for the extraction of hepcidin, a peptide hormone involved in the iron homeostasis, from urine samples [75]. Exceptional results

(recoveries in the 80% level, accuracy better than 10% and precision better than 10%) were obtained in the LC–MS analysis of urine samples where no matrix effect was observed.

The extraction of complex analytes is also possible with simple MNPs. This is the case with DNA, which has some chemical characteristics that may be exploited for its selective extraction. DNA presents a 3D structure with a large number of phosphate groups. In fact, DNA is a poly- anionic molecule that has a tendency to interact with positively charged species by electrostatic interactions. However, bare MNPs have a net negative charge on their surface that avoids any interaction with DNA. Chiang *et al.* proposed the coating of MNPs with polyethylenimine, in order to change the sign of the superficial charge and, therefore, to promote the extraction of DNA [76]. For DNA extraction, the use of carboxylated magnetite NPs, obtained by poly-(methacrylic acid) coating has also been proposed [77–78]. The immobilization of biorecognition molecules on the surface of MNPs is the preferred method for the extraction of target compounds from biological samples. Immunomagnetic separation, which consists of the immobilization of an antibody toward a target on the surface of MNPs, has been extensively used in this context. Immunomagnetic separation has been proposed even for the extraction of pathogens from biological samples. In this sense, microbeads of magnetite (with particle sizes in the interval 2–5 μm), in combination with polyclonal antibodies against all serotypes of *Salmonella* have been used for the isolation and pre-concentration of *Salmonella choleraesuis* from urine samples [79] that are finally analyzed by MALDI-MS. The main shortcoming of the proposal is the sensitivity obtained, which can be improved by including an amplification step after bacteria isolation. The same authors presented an intensification procedure based on bacteriophage amplification (usually called phage typing) for the sensitive determination of *Escherichia coli* [80]. As a consequence of this amplification, a 100-fold improvement of the limits of detection was achieved. The great selectivity (near to

specificity) of immunosorbents may also be employed for the extraction of simple analytes as biomarkers [81] or endogenous compounds [82].

Antibodies provide great selective interaction, although they can present cross-reactivity. They are obtained by inoculating the target antigen in a host animal, a complex isolation and purification processes being necessary. This complex process is the main limitation of antibodies and therefore alternative bio-recognition substances have been investigated. In this sense, Lin *et al.* used vancomycin as an affinity probe [83]. Vancomycin is a selective glycopeptide antibiotic towards Gram-positive bacteria, and therefore it can be employed for the selective isolation and pre-concentration of this type of bacteria. The authors successfully immobilized vancomycin in the surface of MNPs (11 nm in size) providing a final coverage rate of 218 molecules of antibiotic for each MNP. Vancomycin-MNPs interact with Gram-positive bacteria through hydrogen bonds with the D-Ala-D-Ala moieties of the peptide units of the cell walls of this pathogen. This approach has been successfully employed for the isolation of *S. saprophyticus* and *Staphylococcus aureus* from urine samples, and their subsequent analysis by MALDI-MS. Aptamers can also be used as biorecognition elements in MNPs. Smith et al. used aptamer-conjugated NPs for the selective extraction and imaging detection of different cancer cells [84]. This approach allows the fractionation of a mixture that contains three different types of cells.

Molecularly imprinted polymers provide a selectivity enhancement based on chemical recognition. These polymers present cavities in their structures that act as selective hosts towards particular analytes. Lee et al. proposed the use of magnetic molecularly imprinted particles using poly(ethylene-co-vinyl alcohol) for the extraction of different analytes such as albumin or creatine [85].

The methodologies described above use MNPs as the extraction tools for different analytes in biological matrices. MNPs may also be used as support in hemi-micelles SPE, which takes advantage of the extractability of cationic surfactants and

their ability to be retained on the surface of metal oxides [86]. This retention is pH-dependent, since cationic surfactants are immobilized due to electrostatic interaction with the negative charge of the MNPs. MNPs only render a negative charge at a pH below theirs (i.e., 6.5 for magnetite and 3.3 for silica-coated MNPs). The type of surfactant–metal oxide aggregate also depends on the concentration of the former, as can be seen in Figure 7. At low concentrations, a monolayer of cationic surfactant is retained leaving their hydrophobic bones face up to the liquid solution. These aggregates, termed hemimicelles, have a hydrophobic nature. At higher concentrations (always below the critical micellar concentration), a double layer is formed as a consequence of the retention of a second layer of surfactant in the first layer by hydrophobic interactions. These aggregates, called admicelles, have a hydrophilic nature since the ionic groups are face up to the solution. Moreover, at intermediate concentrations, a mixed behavior between hemimicelles and admicelles is observed. Zhu et al. proposed a methodology based on the use of silica-coated MNPs in combination with cetyltrimethylammonium bromide for the extraction of herbal bioactive compounds from serum and urine with excellent recovery values (92.8–109.9%) [87].

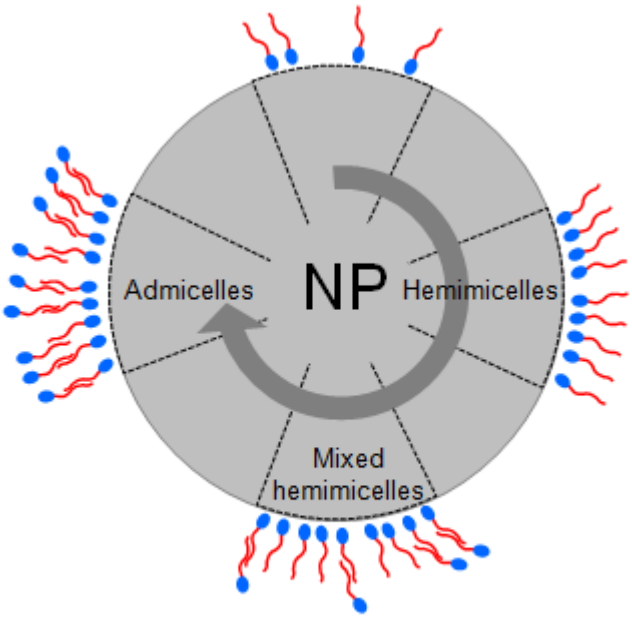

Figure 7: Adsorption of ionic surfactants on the surface of the mineral oxides. The arrow indicates an increase in the surfactant concentration. Reproduced with permission from [6] © Elsevier Inc.

5. Conclusion and future perspectives

In recent years, the use of NPs in bioanalysis has been the focus of intensive research. NPs, including carbon-based and inorganic ones, have been shown to be exceptional probes for the microextraction of different targets from biological samples. Compared with conventional microextraction sorbents, NPs present remarkable advantages, namely: a high surface-to-volume ratio, easy synthetic and derivatization procedures and biocompatibility. Moreover, in the bioanalytical context, the double role (as probes and matrix) that NPs may play on MALDI analysis should also be highlighted.

In this article, the state of the art of the use of NPs as extraction probes in bioanalysis has been presented. Bioanalysis research into binomial NPs is continuously evolving and different clear trends may be highlighted and anticipated:

- The evaluation of new NPs. In this sense, different materials have been proposed recently, such as zirconia [88–90] or zinc-based NPs [91,92];
- The complete characterization (chemical and physical) of new and existing materials;
- The evaluation of hybrid NPs, obtained for combinations of different ones.

The economical viability of NPs will be a key aspect for broadening their use in a bioanalytical context. Although NPs are economically attainable for research purposes, their use in routine laboratories is still limited. It is difficult to rank NPs according to their prices, since this depends on their nature (carbon-based or inorganic ones), purity and even size distribution. As has been previously described, inorganic NPs can be easily synthesized in the laboratory using simple and cheap materials. However, laboratory-made NPs require final characterization (in terms of size, properties and purity), which usually involves an extra expense. This expense is usually included in the price of commercial NPs, which are usually acquired with a complete characterization report. Moreover, some NPs, require modifications (in order to promote the selective interaction with the targets analytes), which makes the product more expensive. The final consolidation of the use of NPs will depend on the availability of cheap commercial task-specific NPs.

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Capítulo VIII: Aptamers in Analytical Chemistry

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Aptamers in Analytical Chemistry

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Abstract

Aptamers have gained importance in the recent years. They can be defined as specific nucleic acid sequences that are able to bind to target molecules with high affinity and specificity. They can be synthesized using combinatorial libraries of nucleic acids in an iterative process, each cycle comprising several sequential steps (selection, partition, and amplification). Their exceptional selectivity has been exploited in different areas such as proteomics or drug discovery. Analytical chemistry has also made use of the exceptional binding capacity of aptamers for the development of more selective analytical devices. In this context, they have appeared as an alternative to antibodies, thanks to their versatility, stability, reusability, and better batch-to-batch reproducibility and to their in vitro synthesis. Concerning the analytical applications of aptamers, their use as recognition element in biosensors is the most reported in literature. Indeed, a specific term, aptasensors, has been coined to refer to such devices. Aptamers can be immobilized in a variety of supports, which can be coupled to different transducers in order to generate the most appropriate analytical signal. This article first presents an overview of the synthetic selection process of aptamers. Next, their advantages over antibodies are discussed. Finally, their main uses in biosensors development and fruitful combination with nanoparticles are outlined to conclude with future perspectives in the analytical context.

1. Introduction

Analytical chemistry is an informative discipline as its main objective is to obtain the highest quality (bio)chemical information from the systems under study. The quality of the results is directly related to the analytical properties of the method, the most important of them being representativeness and accuracy. However, these capital analytical features are supported by others of a lower hierarchical rank but not less important, as they influence the quality level that can be reached by representativeness and accuracy. Basic analytical properties (sensitivity, selectivity, and precision) are attributes of the analytical processes, and they affect the accuracy of the result because it is impossible to achieve accurate data if the method is not sensitive and selective enough. Productivity-related properties (expeditiousness, cost-effectiveness, and personnel-related factors) are also of relevance. In the framework of analytical properties, it is clear that they cannot be dealt with in an isolated manner, as the contradictory and complementary relationships among them are more important than the properties themselves [1].

Most of the research efforts are focused on the development of very sensitive and precise analytical methodologies. However, the selectivity of the measurement, i.e. obtaining a signal free from the influence of other species present in the sample, is crucial to deliver high- quality information, especially when complex samples are processed.

Selectivity can be defined as *the capacity of an analytical process to generate signals that depend almost exclusively on the target analyte present in the sample* [2]. This means that selectivity expresses the degree of interferences in the sample matrix affecting the analytical signal and that selectivity is a property that can be graded, being thus the methods highly acceptable or poorly selective. Specificity is the highest level of selectivity, being thus an absolute term in such a way that only a method that is perfectly selective for an analyte or group of them is said to be specific.

A difference can also be established on the concept depending on the application field, which also conditioned the preferential use of selectivity and specificity. The following sections discuss the use of the two terms in the analytical and biochemical contexts.

1.1 Selectivity in Analytical Chemistry

It was previously set that the main objective of an analytical chemist is to obtain the highest quality information from a given sample. In the past two decades, this quality mainly refers to the sensitivity and precision of the analytical methods. However, especial attention should be paid to the selectivity of the procedures, which is even more relevant at the very low analyte concentration level, where the higher concentration of the interferences would affect the analytical signal of the target compound to a larger extent.

Moreover, the continuous development of the analytical procedures and the tools and instruments involved in each one has also made feasible the distinction between very closely related substances. This evolution helps in the development of more selective analytical tools that can be easily adapted to the given analytical problem, thanks to the more detailed information obtained.

The selectivity of the analytical process depends on the degree of this basic analytical property that can be reached in the sequential operations involved in it. The main achievements in this context are briefly commented on.

Sample treatment is an unavoidable step in any analytical procedure. It usually includes the isolation of the analytes of interest aimed at increasing not only the sensitivity but also the selectivity of the measurement. The use of immunoaffinity sorbents or molecularly imprinted polymers has raised as powerful tools to successfully separate a group of compounds.

The chromatographic separations are also considered as a milestone in the field of selectivity, as they permit the discrimination among the components of a family of compounds on the basis of the stationary phase selection. Chiral stationary or mobile phases are available to separate enantiomers.

Selectivity is also affected by the detection system selected, either if it is used as stand-alone instruments or coupled to a chromatographic technique. Such is the case with the spectrophotometric, fluorimetric, and chemilunimiscent detectors. Fluorescence is more selective than molecular absorption spectrometry, thanks to the reduced number of fluorescent compounds. Chemiluminiscence is the most selective of the three techniques owing to the nature of the chemical reaction responsible for the analytical response. In gas chromatography, the electron capture and nitrogen phosphorous flame ionization detectors are typical examples of selective detectors in comparison with the thermal conductivity of flame ionization ones.

Especial attention should be paid to hyphenated techniques, which mainly refers to the coupling between chromatographic separations and mass spectrometry or tandem mass spectrometry. It is currently considered the most selective instrumentation available.

Selectivity can also be enhanced by the mathematical treatment of the analytical signal obtained. In this regard, chemometrics, Kalman filter, or artificial neuronal networks permit selective information to be extracted from nonselective data.

1.2 Selectivity in Biochemistry

The concepts selectivity and specificity are almost equally used in biochemistry because of the especial interactions that are involved in biochemical reactions, which can be occasionally specific. However, the frequent reference to cross-reactivity in

these methods points out that specificity is far to be reached. The methodologies developed in the biochemical field are based on three types of interactions: antigen – antibody, enzymatic, and substrate – receptor. Immunochemical reactions are based on the specific interaction between an antigen and an antibody. Although, in general, it can be said that this reaction is specific, some authors use the term polyvalent or polyspecific antibodies, which clearly reflects the absence of specificity, as the interaction with a variety of antibodies is feasible. Vessman recommends the term *group selective* to refer to these polyvalent antibodies [3].

As far as enzymatic reactions are concerned, these reactions have always been proposed as the specific coupling ‘‘key-lock’’. However, it can also be said that the enzyme interacts preferably with a given substrate, having less affinity to closely related substances that present a worse equilibrium constant. Again, the advances in instrumentation revealed this potential interaction after the more detailed analysis of the enzymatically-catalyzed reactions in the analytical context.

Finally, the substrate–receptor interactions are similar to the antigen–antibody interactions. In this case, a given receptor is exposed to a variety of potential analytes, being possible the interaction with them but with a different affinity. This fact results in a different selectivity toward a given drug. A paradigmatic example is the affinity assay for drugs that act as receptor antagonists by exhibiting different selective interaction toward the receptor [4].

Aptamers appeared in this context as a new class of ligands with outstanding binding constants. Their high selectivity has been exploited in different application fields, including analytical sciences. This article presents the state of the art of aptamers in analytical chemistry, pointing out their main applications in this discipline and their positive effect on the selectivity of the (bio)chemical measurement processes based on their use.

2. Generalities of aptamers

2.1 Aptamer definition

Aptamers are small, artificial biological molecules (nucleic acids or peptides) that can bind target molecules with high affinity and specificity [5]. In early 1990s, Tuerk and Gold [6] announces the development of a technique that allows obtaining high-specific nucleic acids ligands specifically designed for a target of interest. These synthetic ligands or aptamers are defined in the *Encyclopedia of Analytical Chemistry* as *the artificial nucleic acid ligands that can be generated against aminoacids, drugs, proteins, and other molecules. They are isolated from complex libraries of synthetic nucleic acid by an iterative process of adsorption, recovery, and reamplification. They have potential applications in analytical devices, including biosensors, and as therapeutic agents* [7]. The first described aptamers consisted of unmodified RNA [5,6] and later on, single-stranded DNA (ssDNA) was used against different targets [8]. In 1995, Green et al. [9] and Gold et al. [10] described the first chemically modified aptamers with enhanced properties.

2.2 Aptamers versus antibodies in bioanalysis

Aptamers have been stated as ‘‘nucleic acid antibodies’’. However, there are clear differences between both selective biomolecules, which have been exhaustively discussed by Jaysena et al. [11]. The use of antibodies as screening tool dates back to 1950 and was strengthened in 1970 with the use of polyclonal sera from immunized animals. The demand for polyclonal sera increased until the discovery of monoclonal antibodies by Kohler and Milstein, allowing the production of unique antibodies in large quantities. The monoclonal antibodies are produced by cells in sufficient quantities to optimize further immunoassays, but this technology presents some disadvantages. The antibodies identification process always requires a living animal to

trigger an immunological response to the target molecule, which limits the field of applicability since some molecules presents lethal toxicity at immunogenic dose or the opposite, poor immunogenic response. In addition to the identification, production is time consuming and expensive and usually requires the scrutiny of a large number of colonies. Once the best production bacterial colony is identified, its conservation is critical. Generally, the cell line is conserved frozen in liquid N_2 or especial containers at temperature below −80°C. To overcome the accidental loss or death of the cell lines, multiple containers are required, which results expensive. The production of antibodies may depend on the genotypic characteristics of each cell line, but it can vary even within the same line depending on the lot. For this reason, the characteristics of the antibody need to be evaluated and re-optimized with each new batch. Antibodies are produced *in vivo*; thus, the optimal conditions for their functions are physiological. This restriction limits their use in target recognition in non-physiological conditions. Concerning their use as (bio)analytical tool, their best performance is achieved under physiological conditions. Moreover, taking into account their high sensitivity to environmental condition, they may suffer an irreversible denaturation process, losing their functionality.

The aptamers are completely different molecules and display a number of features that overcome these limitations in analytically equivalent systems. Aptamers are selected through an *in vitro* iterative process, independent of living systems. Their properties do not depend on physiological conditions, and their kinetic and thermodynamic properties can be adjusted according to the final purpose. For these reasons, aptamers can be used in non-physiological medium and temperature for *in vitro* applications. The independence from biological systems allows the selection of aptamers against virtually any analyte, even those that show a high toxicity (biotoxins, heavy metals, etc.) or poor immunogenicity. Aptamer are chemically synthesized with very accurate and reproducible techniques with little or no batch-to-batch variations.

Their nucleic acid nature permits to be denatured and renatured easily at room temperature, without altering their functionality. Currently, a large number of modifications are available, which can be added to the aptamers during synthesis depending on the ultimate objective. This expands their range of application as diagnostic or therapeutic tools. The aptamers are much smaller than antibodies (80–100 nucleotides versus 110–130 aminoacids of the variable region), but the amount available in a single experiment is unlimited, thanks to the polymerase chain reaction (PCR) amplification step. Their small size also allows them to be used as cell-penetrating molecules, which permits several *in vivo* applications.

3. The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process

The discovery of aptamers is intrinsically linked to the development of techniques for screening large libraries of DNA. In this sense, the systematization of the selection procedure is essential.

3.1. General principle

The SELEX (Systematic Evolution of Ligands by Exponential Enrichment) is based on combinatorial chemistry by the screening of large random DNA sequence pools with a vast array of structural (and conformational) possibilities [12]. This technology is an excellent tool to find nucleic acid molecules with high affinity for a particular analyte, thanks to the secondary and tertiary structures of the DNA and RNA that can potentially bind any target of interest. The selection process involves three general steps: (i) binding of the sequences with affinity for the target; (ii) partition of bound sequences by different methods; and (iii) amplification of the selected sequences. The SELEX starts with an initial complex library (1013–1016 sequence space, which is the maximum number of different sequences for a given length and variety of

nucleotides) until one (or more) molecule (6–40kDa) displaying the desired characteristic is finally isolated. A schematic representation of the SELEX process is depicted in Figure 1 and the main steps are commented on in the following subsections.

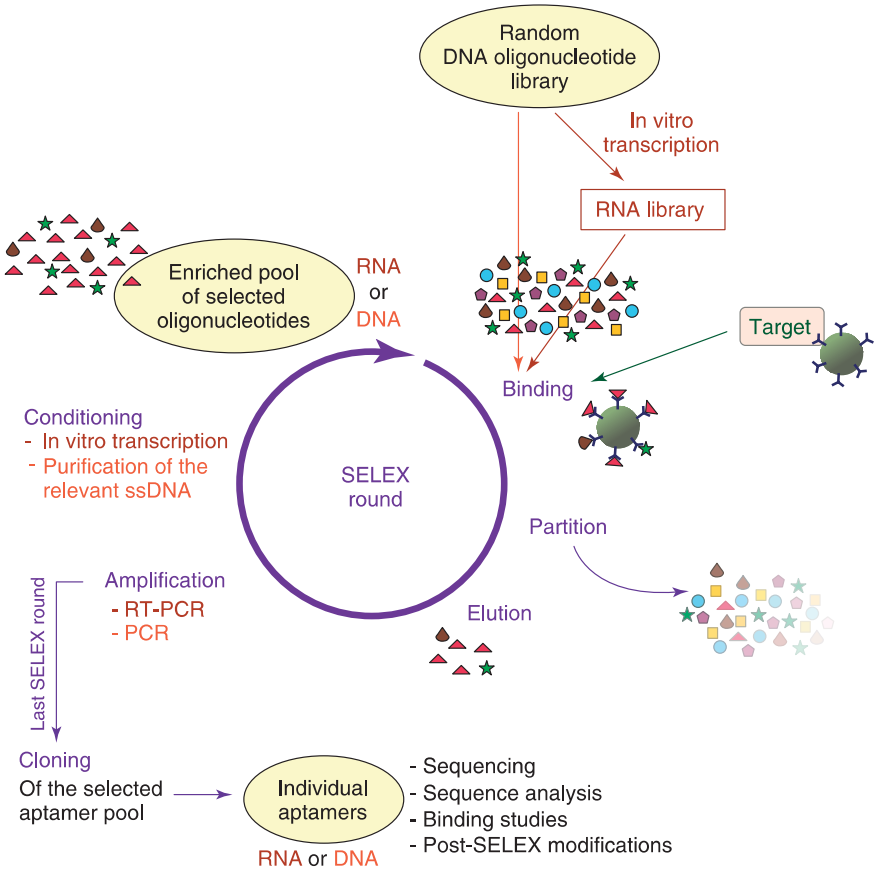

Figure 1: General outline of the SELEX process. (Reproduced with permission from Reference 12. Copyright 2007, Elsevier.)

3.1.1 Binding

The first step of the process is aimed at obtaining the sequences with high affinity and specificity for the target compound. At this stage, the target and the library

of oligonucleotides interact for a period of time, either in solution or fixed on a support.(13) The potential aptamers bind, thanks to its spatial conformation. Target in solution is, a priori, preferable, as binding to a solid support hinders the conjugated side of the loss of some potential high-affinity aptamers. Also, the presence of additional molecules used in the attachment process could generate cross-reaction of non-target-specific aptamers. The selection conditions vary along the different rounds. The first rounds of selection require longer incubation times and conditions that favor general interactions, whereas final rounds require more stringent conditions, specific binding buffer modifications, and shorter incubation times, favoring the interaction with the target of the higher specificity molecules. If required, counter-selection can be used to select certain aptamers sequences and exclude others that do not bind to the target. The target is switched with a non-desired molecule during one selection round, and the sequences bound to that molecule are removed from the pool prior to the next selection rounds. This reduces the sequence space of the remaining pool and increases the specificity for the target of interest.

3.1.2 Partitioning

The separation of target-bound and unbound nucleic acid sequences is crucial in the characteristics of the final product [13]. This separation is usually carried out using affinity columns or nitrocellulose membranes. However, these techniques have low separation efficiency, and the resulting aptamers possess worst K_d values. For that reason, several improvements have been proposed in recent years, as discussed extensively by Gopinath [14].

3.1.2.1 Affinity immobilization and separation

The use of affinity techniques usually reduces the number of selection cycles required to obtain the final product compared with other techniques such as membrane filtration methods.

The most obvious improvement is the immobilization of the target on a solid support, to develop affinity surfaces. Ellington and Szostak used different types of organic dyes attached to agarose to select RNA ligands [5]. In this procedure, the dyes are attached to the agarose in a column and the RNA sequences are applied to the column for binding. After binding, the column is washed with buffer and the bound sequence eluted with water in the first rounds of the process and 2 mmol L^{-1} EDTA (ethylenediaminetetraacetic acid) in the final ones (when sequences are tightly bound). After five rounds, the aptamers obtained have 50% binding efficiency compared to 0.1% of the original pool.

Another interesting approach is the immobilization of the target to magnetic beads, to improve the separation step. Stoltenberg et al. [15] developed the FluMag-SELEX procedure, using streptavidin-coated magnetic beads for the isolation of specific DNA aptamers. After the selection, four aptamers were isolated, with K_d in the low nanomolar range, which are better than other previously published streptavidin-specific RNA aptamers. Magnetic separation permits easy handling and efficient separation of bound and unbound sequences, thanks to target immobilization. More recently, Bruno and Kiel used magnetic beads for selection of aptamers against biotoxins conjugated with tosyl-activated magnetic beads to improve separation with novel PCR in-bead amplification [16].

Other immobilization approaches employ affinity tags. For example, affinity tags fused to N-terminus or C-terminus of protein targets allow the separation of the RNA–target complex by affinity beads for the tag. The GST (glutathione S-transferase) tag was first used by Dobbelstein et al. in 1995 to obtain high-specific RNA aptamers

against L22 ribosomal protein of Epstein-Barr virus [17]. A construction of GST-tagged L22 protein was used during incubation with RNA library. After four rounds of selection using GST affinity beads for separation, the RNA aptamer obtained was able to bind L22 under native conditions.

3.1.2.2 UV-Photocrosslinking

In 1995, Jensen et al. developed a novel strategy based on photo-cross-linking to obtain single-stranded RNA (ssRNA) aptamers against HIV-1 Rev protein [18]. This technique allows stabilizing RNA–protein complexes in tough conditions, minimizing unspecific interactions [19]. The basis of the photo-cross-linking requires the substitution of the RNA with a fluorophore (5-iodouracil or 5-bromodeoxyuridine) in the original pool and incubation with the target (generally proteic) during irradiation with UV light (308nm for bromide-modified and 325 nm for iodide-modified nucleotides), which permits covalent cross-linking of the aptamer and the target [20]. The potential amino acids able to generate photo-cross-linkage are tryptophan, tyrosine, histidine, phenylalanine, and cysteine. The proximity of these amino acids to the target surface with the correct spatial arrangement to a modified nucleotide residue is a necessary condition, as it increases the specificity of the aptamer–protein interaction.

The UV photo-cross-linking technology is especially useful in the microarray fabrication, and it has been applied to create a chip of ssDNA for detecting up to 17 different proteins with limits of detection (LODs) below 10 fmol L^{-1} [21]. Despite the wide range of applications in proteomics and direct analysis of biological fluids, photo-cross-linking is limited to target with the ability to create covalent linkage with nucleic acids, so it is restricted to small targets with no functional groups.

3.1.2.3 Electrophoretic separations techniques

Poly-acrylamide gel electrophoresis (PAGE) has also been successfully used to separate aptamers. The analytical properties of aptamers obtained by this technique are substantially better, with K_d values in the picomolar range, high binding affinity, and stable complex formation. In the case of HIV-1 Rev protein, the aptamer shows no binding with related proteins or serum components. Golden et al. developed the aptamer against bFGF (basic fibroblastic growth factor) protein, with a LOD of 0.058 ng L^{-1} , similar to monoclonal antibody. This technique has also been successfully applied to obtain an aptamer against integration host factor (IHF) of bacterial protein. The RNA–protein complex is separated during SELEX using native PAGE conditions in electrophoretic mobility shift assay (EMSA) [22]. The selected band was obtained and purified directly from the gel and used in PCR amplification [23].

Aptamer–protein complexes are separated from free oligonucleotides very efficiently using capillary electrophoresis (CE), requiring only two to four rounds of selection for high-affinity ssDNA [13].

The major disadvantage of CE-SELEX methods is the limited quantity of DNA library that can be injected in the capillary in a single run. The normal injection volume is in the range of nanoliter, without the risk of damaging the column. In terms of library complexity, it means that the sequences used are 10^{13} instead of 10^{14}–10^{15} of the conventional SELEX [24].

3.1.2.4 Surface plasmon resonance

In general, the characterization of the aptamer–target interaction is performed after each cycle of SELEX, which is a considerable expenditure of time and effort. The application of the surface plasmon resonance (SPR) technique allows obtaining real-time information about the interaction of two or more molecules [25,26]. The aptamer against the H3N2 virus (human influenza virus A) was isolated and characterized by this

technique as it is shown in Figure 2. The virus HA (Influenza hemagglutinin) protein (1.2 µg) was immobilized on a chip in a flow cell, and the RNA pool was injected during 2min. After that, the binding buffer is injected into the cell at a constant flow and collected. The fractions are precipitated to recover the bound sequences and amplified for the next cycle [14]. The SPR studies permits to obtain high-affinity aptamers in the first two rounds of selection. Moreover, the great advantage of this technique is that it provides information about binding affinities prior to aptamer selection. With this technique, four high-affinity aptamers were isolated with a best K_d value of 115±23 pmol L^{-1} against HA. The isolated aptamers show a highly conserved recognition motif (5'- GUCGNCNU(N)2−3 GUA-3').

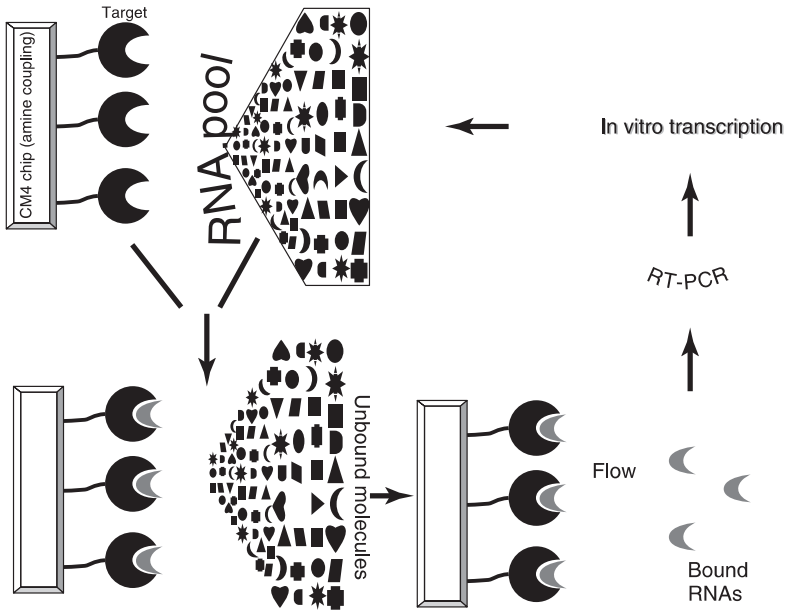

Figure 2: Aptamer selection using surface plasmon resonance. (Reproduced with permission from Reference 14. Copyright 2007, Springer).

3.1.3 Amplification

Among the potential aptamers of the initial library, only a few have affinity for the target. This reduced number of active molecules will be amplified by PCR, in order to restrict the conformational space for the next round of selection. Besides the active molecules enrichment, it is possible to use this step for introducing mutations or especial functionalities through the primers used in PCR.

The amplification step is different for DNA and RNA molecules. RNA requires reverse transcription step from the T7 promoter to obtain cDNA and then amplification by PCR. On the other hand, ssDNA only requires regular PCR amplification. After the amplification step, an enriched library is obtained.

3.1.4 Cloning and characterization

The iterative process of selection (usually 6–20 rounds, depending on the complexity of the target) ends with an enriched library, with no possibilities of increasing the affinity for the target. The characterization of the selected sequences is carried out by bacterial cloning. The selected sequences are inserted in bacterial plasmid, and the individual colonies are sequenced.

The number of aptamers characterized is variable (usually 50 or more) and depends on the stringency of the SELEX conditions. The evaluation of the aptamer sequence is important for grouping aptamers that differ only in a few nucleotides and show highly conserved sequence patterns. The conserved regions, common to most of the isolated aptamers, are usually involved in target recognition and binding.

As it was mentioned before, secondary structure of the aptamers is determinant for target binding. The study of the structure is usually performed *in silico* by calculating the possible configurations of nucleic acid using the minimum energy method with stems, loops, and bulges [27]. The most common target-binding motifs

are stem–loops [28,29], but it is possible to find more complex secondary and tertiary structures (Figure 3).

Among these more complex motifs, it is common to find the ‘‘G-cuadruplex’’ structure [30,31], which appears in G-rich oligonucleotides and forms a four-stranded structure. The structure is maintained by the formation of hydrogen bonds between four guanine residues in a planar arrangement [32]. These structures have considerable van der Waals attraction and can stack with some aromatic planar ligands. The well-known thrombin aptamer forms a G-cuadruplex-type structure [33].

It is possible to study each aptamer individually, in terms of its specificity and affinity for the target, but the final mixture of the ultimate SELEX round can be used for the final recognition purpose. In general terms, it can be compared to a polyclonal antibody serum [34].

Many authors have gone one step further in the optimization of techniques for the selection of aptamers. Currently, there are different approaches for the automation of the techniques outlined above. The expectations generated by automated selection systems involve an increase in flexibility and versatility of the process and can efficiently handle several targets at once, or even different buffers varying in ranges of stringency. Ellington and Cox developed one of the first automated stations [35]. They employed an automated system to generate anti-lysozyme aptamer, which has proved to be very efficient in the inhibition of cell lysis. In 2007, Hybarger et al. [36] created a microfluidic SELEX prototype. This is a microline-based prototype controlled by Labview interface with a PCR thermocycler integrated to generate and verify the aptamers, in this case, anti-lysozyme.

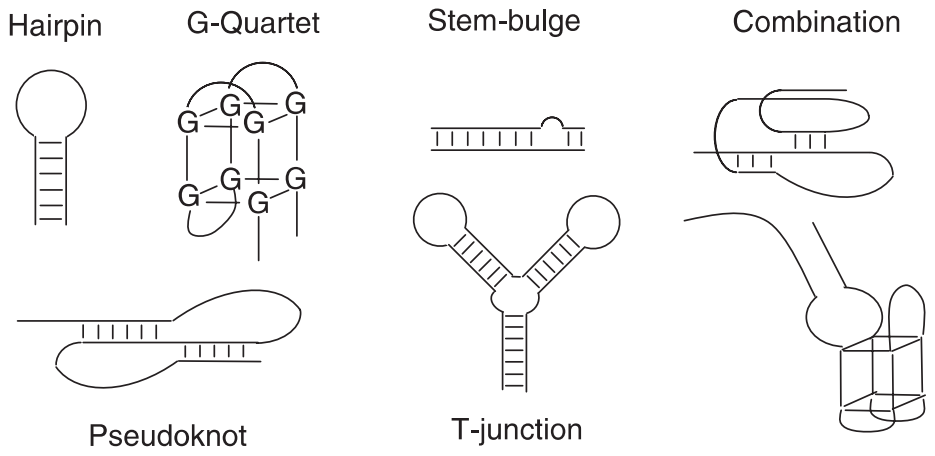

Figure 3: Typical nucleic acid structures in target-bound aptamers. (Reproduced with permission from Reference 30. Copyright 2008, Elsevier.)

3.2 Random libraries

A typical SELEX library is composed of ssRNA or ssDNA, with a central random sequence (20–80 nt) flanked by fixed primer sequences (18–21 nt) for (RT) PCR (reverse transcription polymerase chain reaction) amplification. The random oligonucleotidic library is chemically synthesized and typically contains 10^{13}–10^{15} of sequence space. If the desired aptamer is DNA, the sequences are used directly in the first round of the SELEX procedure, whereas if the aptamer is RNA, a random library of DNA has to be transformed prior to its use. The transformation of a random DNA library in a random RNA library requires the use of T7 RNA polymerase. All the process is schematized in Figure 4.

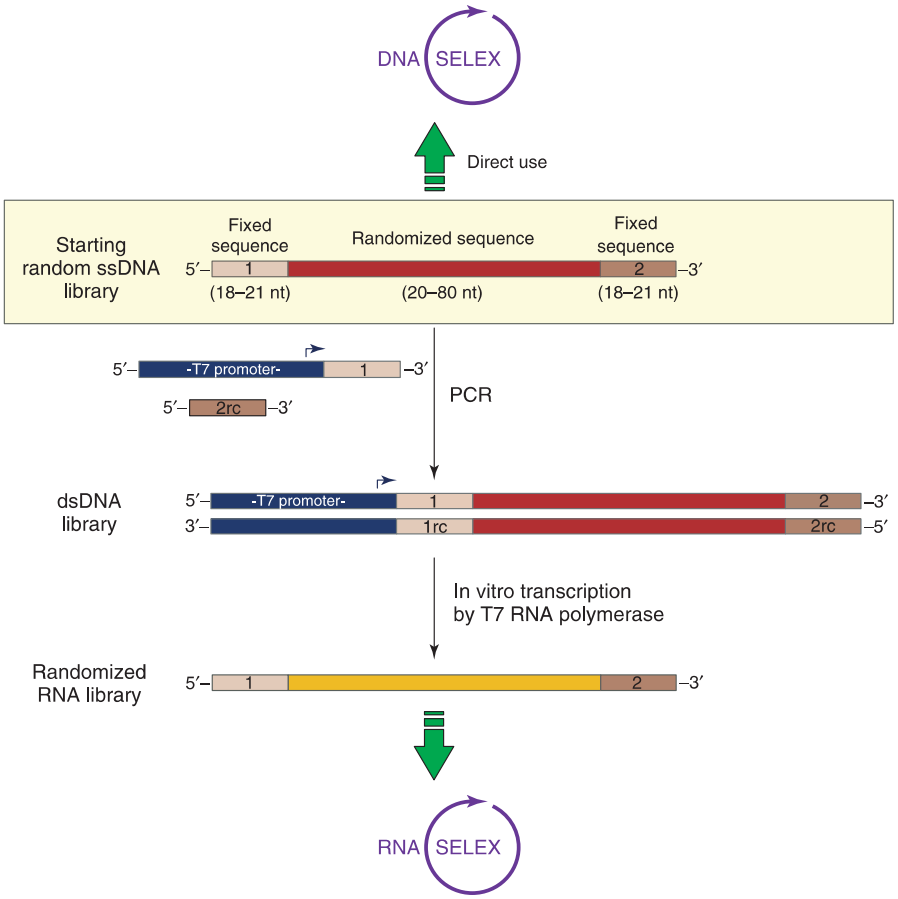

Figure 4: Sequence requirements for nucleic acid amplification and transcription. (Reproduced with permission from Reference 12. Copyright 2007, Elsevier.)

RNA libraries are especially interesting because they usually result in aptamers with higher affinities due to the wider variety of structural conformations than DNA sequences. On the other hand, aptamers that came from DNA libraries have increased stability, thanks to the lack of -OH groups in the ribose residue.

One of the most important aspects in the design of the library is the length of the randomized section. Usually, the sequences used for *in vitro* selection are 20–80 nt, but it is possible to select certain functional sequences even below this limit. For

example, Bock et al. found an aptamer against human thrombin with 96-mer structure and 60-nt random sequence. Further experiments with a 15-mer of consensus region permitted to obtain a shorter but more functional aptamer [37]. This suggests that shorter random regions are enough to obtain functional aptamers and are preferable in terms of handling, cost of chemical synthesis, and usefulness. However, shorter libraries are limited in terms of structural complexity compared to larger ones.

In aptamers discovery, the structure of the target is known, and in many situations, there is a natural DNA/RNA motif with binding affinity for the target. Davis and Szostak use a partially structured RNA library to select a GTP-binding aptamer. This especially designed library consists of a 12-nt stem loop structure in the centre of a 52-nt random region. The stem loop acts as structural anchor for the formation of recognition loops. The introduction of partially structured regions provides a superior source of high-affinity structures in the library [38]. With a previous knowledge of the structure of the nucleic acid random sequence, it is possible to design a strategy for aptamers selection. In this context, Nutiu and Li designed a specific 15 nt region flanked by random sequences and further by primer-binding sequences. The especially designed region was complementary to a biotinylated capture oligonucleotide, and the library was immobilized on an avidin-coated surface by hybridization. Only aptamers that binds the target are released from the surface, thanks to their structural switch. This strategy hinders the immobilization of small molecules and facilitates the formation of the complex.

Besides the natural nucleotides, certain chemical modifications can be introduced to improve stability or even to add some functionality. These aspects are discussed in subsequent sections.

3.3 Targets

An aptamer database collects aptamers against organic and inorganic small molecules, proteins and peptides, nucleic acids, aminoglycoside and carbohydrate targets, among others. Given the large number of targets known to date, it is obvious that the application range of aptamers is extremely wide. The larger the target is, the more surface is available for interaction with nucleic acids, and the higher the probability of finding an aptamer is. One of the pre-requisites for the target is to be stable in SELEX conditions and, if necessary, suitable for modifications to improve partitioning.

Selection of aptamers against small molecules presents some technical disadvantages, due to the small interaction surfaces, but binding the small target to a presenter protein can overrun this limitation. Small targets aptamers shows a K_d values in the range of millimolar or micromolar instead of the nanomolar or picomolar of larger ones. With the approach presented by Plummer et al. [38], it is possible to obtain aptamers against small targets with K_d values below 50 nmol L^{-1}. Among the small targets with especial interest in analytical chemistry, metal ions such as Zn^{2+} [39] or Ni^{2+} [40], small organic dyes such as malachite green [41], or ethanolamine [42] are of especial relevance. Other targets of interest, especially in biochemistry, are the nucleotides such as cAMP (cyclic adenosine monophosphate) [43] and ATP [44], several aminoacids [45−47] and cofactors [48−50].

The aptamers have raised great expectations in the field of biomedicine and applied biotechnology as therapeutic and diagnostic tools [51]. In this sense, the development of SELEX with whole cells as targets has gained importance in scientific research. This approach uses complete cells to select aptamers against cell surface molecules and proteins. Owing to their small size, the aptamers are able to penetrate tissues or even cells, acting as therapeutic agents. The main limitation is the abundance of potential targets during the process, resulting almost impossible to know exactly the

target of the aptamer selected. Usually, the use of specific recombinant protein of the cell population of interest led to well-defined aptamers with K_d between 1 and 70 nmol L^{-1} [52,53].

4. Enhancing aptamers (bio)stability through chemical modifications

Most of the aptamers applications require the contact with biological fluids. One of the problem that this raises is the presence of nucleases, a group of enzymes capable of cleaving the phosphodiesther bond between nucleotides of nucleic acids. There are two categories depending on whether the cleavage occurs within the nucleic acid chain (endonuclease) or from the end (exonuclease). For this reason, the use of modified libraries and/or subsequent amendments represents a key step in aptamers technology development. The most common modifications implemented for this aim may be addressed towards two main objectives, namely, (i) to improve the stability of the aptamers for its use or (ii) to add specific functional groups. Modifications can take place before or after the SELEX and may involve the alteration of the phosphodiesther backbone, the nucleotides, or both.

4.1 Phosphodiesther backbone modification

Altering the backbone may involve the incompatibility with the replication process, as the polymerase may be unable to incorporate these nucleotides during synthesis, which reduces the range of chemical modifications available. However, there is a common backbone modification compatible with the enzymes used in SELEX: the replacement of nonbinding oxygen in phosphodiesther linkage by sulfur [54]. This modification results in the so-called ‘‘thioaptamers’’, a group of aptamers with a phosphorothioate linkage that gives them resistance to nuclease activity. An example of aptamers using phosphorothioate chemistry is obtained by King et al. against NF-IL6, NFκB, p65, and p50 proteins, the latter with a K_d value of 800 pmol L^{-1} [55]

Another way to create more stable aptamers by chemical modification is the use of locked nucleic acids (LNAs). LNAs were first synthesized by Imanishi in 1997 [56] and consist of a 2′-O-4′ -C methylene bridge in the ribose structure, which confers high nuclease resistance and binding affinity for proteins that normally interact with G-quadruplex motifs [57]. Their structures are given in Figure 5. Wengel et al. have demonstrated the compatibility of the LNA with the *in vitro* PCR amplification [58, 59], opening the door to their use as monomers for the generation of libraries for SELEX.

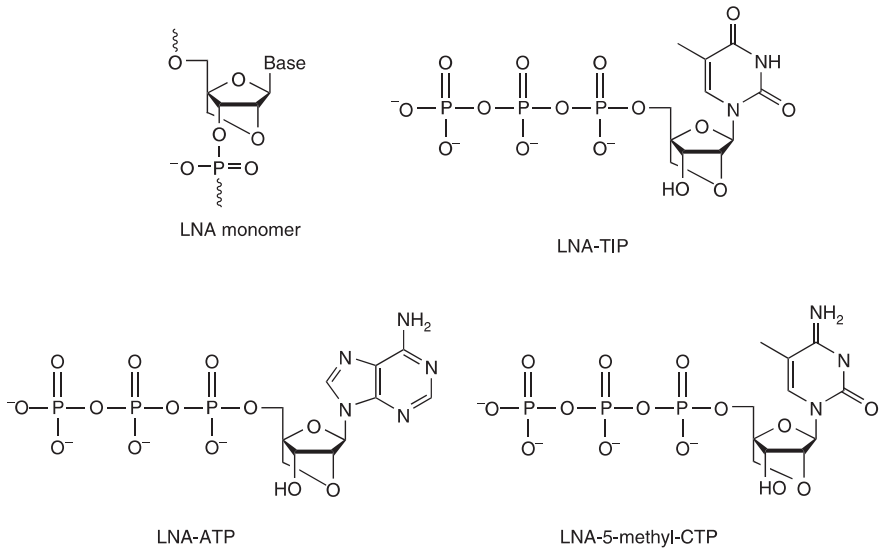

Figure 5: Structural representation of a LNA nucleotide monomer and LNA nucleosides 5′-triphosphate compatible with polymerases. (Reproduced with permission from Reference 60. Copyright 2009, Royal Society of Chemistry.)

4.2 Nucleotides modification

One of the most common pre-SELEX modifications is the substitution of ribose 2′-OH group of pyrimidine nucleotides in RNA pool, which makes the molecule resistant to nuclease activity as most common nucleases in biological fluids are pyrimidine-

specific endonucleases. This modification usually involves -NH2 or -F groups, compatible with the enzymes used in SELEX. Using this strategy, aptamers with improved half-lives in biological fluids are obtained [11]. The 2'-O-methyl substitution used in the discovery of aptamers against vascular endothelial growth factor (VEGF) was evaluated by Burmeister et al., which, thanks to its low toxicity, is one of the first aptamers approved by FDA for age-related macular degeneration (AMD) treatment [60].

The C5 position of pyrimidines can also be used for the development of modified libraries. Substitution in this position does not alter helicoidal structures of the nucleic acids and are also accepted by polymerases. This sort of modifications adds new functionality to and/or increased stability of the aptamers. For example, C5 substituted dUTPs with aminoacids permit *in vitro* selection of aptamers against anionic targets. The use of this aminoacid substitution permits the creation of pseudo peptide/nucleic acid libraries, which is particularly interesting in chemical dressing for

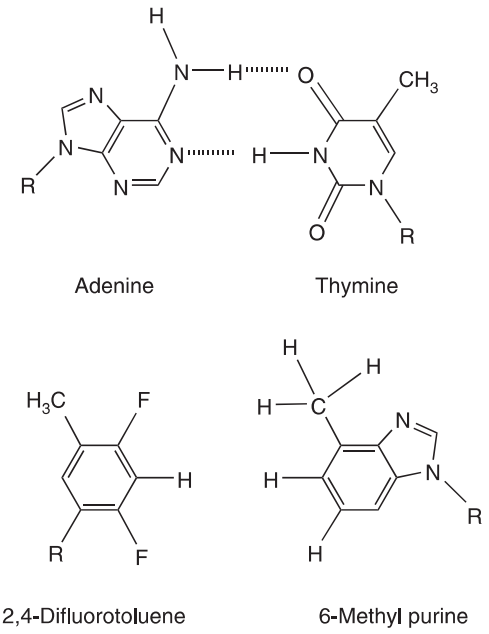

Figure 6: Unnatural base pairing. 6-Methyl purine and 2,4-difluorotoluene mimics adenine–thymine base pair compat- ible with enzymatic replicating system.

A novel alternative to create nuclease-resistant aptamers is the use of the so-called *spiegelmers* (from mirror german term *spiegel*). Spielmers are synthetic oligonucleotides from unnatural enantiomeric forms of L-ribose and L-2′-deoxyribose that can specifically bind to a target but are not recognized by nucleases. Spiegelmers are obtained from an especial SELEX procedure called ‘‘mirror-SELEX’’, in which D-aptamers are selected against synthetic enantiomer of the target of interest and then create an enantiomeric L-aptamer to the selected D-aptamer. This enantiomeric aptamer will bind to the original target and cannot be degraded by nucleases. Several examples of spiegelmers against many targets have been reported, such as GnRH [65], arginine [66], adenosine [67], vasopressin [68] or staphylococcal entero-toxin B [69].

Modifications can also be introduced after the SELEX process, such as the 2′-O substitution or the LNA methylene bridge. However, the use of a modified library is

preferred to direct chemical post-SELEX modification of selected aptamers because of the risk of weakening the efficiency or specificity [51]. In any case, there are modifications that should be introduced after the selection. Such is the case of the end capping, to avoid the action of exonucleases. It is possible to attach an inverted thymidine to the 3′ end (3′-idT) of an aptamer to introduce an additional 5′ end. In this case, exonuclease cannot recognize the 3′ end necessary for its degradative action [70]. Another type of 3′ end capping without nucleotides is the incorporation of biotin, which conjugated with setreptavidin increases the half-life *in vivo*, but probably because of the increase in size of the complex, rather than to the nuclease insensitivity. Although less common, there are also exonucleases that degrade from the 5′ end, and depending on the application, it may be important to protect the aptamers against these enzymes. For this purpose, one of the most relevant modifications is the addition of polyethylene glycol (PEG), cholesterol, and fatty acids. These modifications are mainly designed to increase the lifetime of the aptamers for therapeutic applications, usually through an increase in size that slows its elimination by renal filtration.

5. Aptamers as recognition elements in biosensors

The aptamers can be used in dissolution (homogeneous assays) or can be immobilized on a solid support. The second alternative is the topic of this section, as it compiles the main characteristics of a sensor. However, readers interested in the homogeneous assays are referred to the excellent review of Sassolas et al.[71].

The excellent selectivity presented by the aptamers can be exploited in the development of more efficient biosensors. In this particular case, the resulting device is known as aptasensor. In addition to the selectivity they provide, other properties such as high affinity, easy regeneration, tunability, and thermal stability can be highlighted. Moreover, the small size of the aptamers generates a high density of monolayer of the surface, which clearly enhances their response as compared with other biosensors.

In general, aptasensors can be used with and without aptamer labeling. The inclusion of the label (fluorofore, enzyme, redox, or radioactive groups) is aimed at facilitating or enhancing the binding capacity. However, as it was mentioned before, the position of the label can interfere (i) with the folding of the aptamer and (ii) with the binding group, thus affecting the sensitivity and selectivity of the resulting aptasensor [72]. On the other hand, the immobilization allows the close contact between the receptor (aptamer) and the transducer, which ensures the optimal functioning. The immobilization of the aptamers on a solid support facilitates their recovering and the development of portable and miniaturized devices as well. For this step, the introduction of -SH, -NH2, or biotin moieties at the 3' or 5' ends is required to link the aptamer to the solid support. A variety of substrates have been reported in the literature. The type of solid support used usually depends on the transducer or detection technique selected. Balamurugan et al. [73] have reviewed these alternatives. For example, a metallic surface is required for SPR, acoustic wave, quartz crystal microbalances, and electrochemical detection, while optical measurements need the transparent substrate, and therefore, glass or polymeric supports are preferred. Nanomaterials, such as gold nanoparticles, carbon nanotubes, and quantum dots (QDs), have also been proposed as supporting material.

Concerning the regeneration of the sensing capacity of the aptasensor (i.e. the binding capacity of the aptamer), a great differentiation between aptamers and antibodies has to be made at this point because the former can undergo several regeneration cycles, while the antibodies suffer irreversible degradation. The regeneration of the aptasensor can be achieved following several mechanisms such as temperature, ionic strength, pH changes, or the use of additives (surfactants, chelating agents) [73] Sometimes, a combination of regeneration reagents is needed.

This section presents the most relevant applications of aptasensors, using the nature of the transducer element as classification criteria.

5.1 Optical aptasensors

These aptasensors use fluorescence and SPR as typical detection modes. The fluorescence-based aptasensors require, in the majority of the cases, the immobilization of a labeled aptamer, while the SPR does not need aptamer modification.

Concerning fluorimetric aptasensors, thrombin can be detected by displacement of fluoresceinated thrombin from the aptamer using as low as 140 pL volume of sample, with LODs of 0.7 attomol L^{-1} [74]. An array of optical aptasensor has also been proposed in the proteomic field.

The possibility of using different aptamers for the simul- taneous determination of a variety of targets is one of the main advantages of this aptasensor, which works under the ELISA/ELONA (enzyme-linked immunosorbent assay/enzyme-linked oligonucleotide assay) sandwich format [75].

SPR aptasensors have been used for the determination of the affinity constant of aptamer–target [76]. Other theoretical studies include the determination of the primary binding sites of the aptamer [77] and the evaluation of the affinity of immobilized and free aptamers toward a given target [78].

Especially relevant are the contributions of nanoparticles in the development of miniaturized optical aptasensors: QDs, for fluorimetric detection, and gold nanoparticles in the colorimetric context can be highlighted. They are commented on in a separate section of this article.

5.2 Electrochemical aptasensors

Probably, the immobilization of aptamers in electrochemical devices is one of the most exploited combinations. This is because of the favorable characteristics of the electrochemical response such as sensitivity, expeditious- ness in signal generation,

relatively low cost, and easy miniaturization. Moreover, the wide variety of transducer available increases the type of analytes that can be detected.

The electrochemical aptasensors are usually labelled with an enzyme (e.g., glucose dehydrogenase, horseradish peroxidase) or a redox molecule (e.g., methylene blue, ferrocene). The electrons produced during the reduction of the enzyme substrate are transferred to the electrode surface to obtain an analytical signal. In the case of the redox moieties, the conformational change of the aptamer, which allows or not allows its interaction (or the label) with the electrode surface, is responsible for the electrochemical measurement.

The first reported application is the chronoamperometric anti-thrombin aptasensor [79]. It presents a sandwich format taking into consideration that the thrombin has two different electropositive exosides suitable for aptamer binding. The detection limit was as low as 10 nmol L^{-1}, with a linear range extending up to 100nmol L^{-1}. Similar LODs were achieved by Hianik *et al* [80] by changing the enzyme by methylene blue using chronoamperometry and differential pulse voltammetry techniques for signal transduction.

Sometimes, the aptasensor can be used to perform indirect measurements, considering the influence of external compounds on the aptamer response. For example, it is known that the structural rearrangement of the thrombin aptamer is affected by the presence of K+ and, therefore, it can be used to detect this ion at concentrations as low as 0.3 mmol L^{-1} without interference of Na^{+}, Ca^{2+} , and Mg^{2+} after the addition of EDTA as masking reagent for the divalent cations.

Unlabelled aptamers have also been used for the development of impedance-based aptasensors. Thrombin and IgE were the targets of the detection using ferrocyanide in solution [81,82]. The binding target–aptamer can be corroborated by atomic force microscopy.

Electrochemical aptasensors based on the immobilization of the aptamer on the carbon nanotubes surface are discussed in the section dedicated to the joint use of nanoparticles and aptamers.

5.3 Mass sensitive aptasensors

Quartz crystal microbalance and surface acoustic sensors have also been proposed in combination with aptamers. As an advantage over the optical and electrochemical sensors, they do not require the labelling of the aptamer, which clearly simplifies their fabrication. They have been preferably used for the detection of proteins or high-molecular-weight compounds; their sensitivity toward small molecules is very limited.

This particular case of aptasensors surpasses the antibody biosensors in terms of stability, reusability, and density and orientation of the immobilized active element, which also results in wider linear ranges. The sensitivity was similar in both the cases.

IgE-specific aptamer has been determined in very complex samples without interferences or sensor deactivation [83]. A relevant issue of Love-wave aptasensors (a particular case of surface acoustic wave sensors) is their use in array format to detect multifunctional serine protease, thrombin, and Rev peptide [84].

6. Nanoparticles and aptamers combination

Aptamers have also been used in combination with nanomaterials. These approaches combine the excellent sensing properties of the aptamers with the outstanding characteristics of the nanoparticles. QDs, gold nanoparticles and carbon nanotubes are the most reported in the literature. In each case, the affinity of the aptamer towards the target is measured through a relevant property of the nanoparticle, fluorescence in the case of QDs, colour change due to a different aggregation degree of gold nanoparticles and electronic transference for carbon

nanotubes. General considerations of each binomial are discussed in this section and illustrated with representative examples.

QDs are semiconductor crystal whose fluorescence efficiency is sensitive to the presence and nature of adsorbates in their surface. Therefore, QDs can be used for the development of fluorescent nanosensors. Several analytical applications of QDs are based on fluorescence quenching/enhancement after the interaction of the target molecule with the QD. In the case of aptamers, this property can also be exploited using the QD as a fluorescent label. Taking into consideration their relative size, several aptamers can be immobilized on the QDs surface, containing either the same or different oligonucleotide sequences. Each aptamer is binded to a fluorescence quencher, which is displaced upon interaction of the QD-aptamer with the target resulting in a fluorescence enhancement. Proteins with histidine tag [85] and campylobacter [86] have been the target for RNA-aptamer-functionalized QDs. In the case of the histidine-tagged protein detection, the authors proposed the use of RNA-based aptamers instead of the anti-histamine antibody selector kit on the basis of its comparable or superior affinity. The authors used amino-modified QDs which are conjugated to thiol-containing RNA aptamers using sulfo-SMCC cross-linker. The resulted system is capable of detecting only the His-tagged protein from a partially purified protein mixture at a concentration as low as 375 ng after an incubation time of 2 h. The anti-histidine antibody kit does not generate any visible signal in a similar incubation time. The proposed methodologies combine the benefits that the QDs and apatamers show separately such as simplicity, expeditiousness and sensitivity. Moreover, it can be developed using a simple handheld UV lamp to illuminate the blot and the fluorescent bands can be captured by means of a high-quality camera. The authors consider the proposed methodology as an alternative to the conventional Western blots.

The combination of gold nanoparticles (AuNPs) with aptamers makes use of two detection modes: colorimetric assays and chemiluminiscence detection. As far as colorimetric detection is concerned, it surpasses fluorescence in terms of simplicity of the measurement as the analyte can be visually detected. On the contrary, the sensitivity is lower than that provided by fluorescence. The foundation of the analytical response can be found in the different aggregation of AuNPs. When dispersed, AuNPs are red and the color shift to blue after aggregation [87]. Nucleic acid sequences, proteins and inorganic ions can be detected using this phenomenon.

Similarly to colorimetry, chemiluminiscence is also simpler than fluorescence and, under some conditions, more selective. An aptasesnor has been proposed for the determination of thrombin at concentrations as low as 26 fM [88]. The proposed label-free aptamer biosensor relies on the catalytic activity of unmodified AuNPs on the luminol-H_2O_2 chemiluminiscence reaction and the interaction if the nanoparticles with the aptamer. Indeed, once the aptamer-target interaction occurs, the AuNP aggregate and the chemiluminiscence is enhanced. The mechanism proposed by the authors comprises several steps, being the unmodified AuNPs the key element. In absence of thrombin, the unfolded aptamer is absorbed on the nanoparticles surface thanks to an electrostatic interaction between the nitrogen atom of the unfolded aptamer and the negatively charged AuNPs. This effect stabilizes the nanopaticles in aqueous solution even in presence of a salting out agent such as NaCl. As a result, a weak chemiluminiscence is induced in the luminol system. In the presence of thrombin, the aptamer is folded into a specific three-dimensional conformation, which is unable to stabilize the nanoparticles in the presence of NaCl. Therefore, an aggregation of the AuNPs occurred and the chemiluminiscence is enhanced. All the process requires less than 30 min to be completed. The precision of the reported aptasensor is better than 4% and the procedure is easy to operate as it is developed in solution. Moreover, an automatization via robotic arm could be also feasible.

Other species such as heavy metals and drugs can also been monitored by chemiluminiscence reaction enhanced by AuNPs.

Carbon nanotubes present several and well-known outstanding properties that have been exploited in Analytical Chemistry. Among them, the higher electric capacity of these nanostructures over other materials has been used for the development of more efficient electrodes. In this context, the immobilization of the aptamers on their surface has been exploited for the proposal of very sensitive and selective electrochemical biosensors. Field-effect transistors modified with single-walled carbon nanotubes have been used for the determination of thrombin [89] and IgE [90], being this one better than that based on antibodies. The main advantage of using DNA or RNA aptamers in FET (Field-Effect transistors) sensors is consequence of the small size of the aptamer compared with the antibodies. It allows the recognition binding to occur inside the electrical double layer of the sensor, which allows the detection of the target. The larger size of the antibodies displaces this event outside the electrical layer, hindering the target detection.

7. Conclusions

Aptamers have been revealed as powerful tools for developing very selective methods. Their usefulness is clear in different application fields, including analytical chemistry. Their high affinity and selectivity are among the most remarkable properties. One of the main limitations, however, is that despite the large number of possibilities theoretically available after the SELEX process, only a reduced number of aptamers have been used for the development of analytical methods. Aptamers can be used in dissolution and immobilized on solid supports and nanoparticles. The second alternative results in the so-called aptasensors, the applicability of which has been extensively reported in the literature. Further work must be aimed at improved SELEX formats which results in functionalized libraries, more stable versus nucleases and label

with fluorescent or redox moieties. This will result in the development of new sensor formats, of reduced size, which facilitates the miniaturization of the devices to the chip dimensions.

An important research field will also be the symbiosis between these improved aptamers and the increasing number of nanoparticles and nanomaterials available. The advances in this field will result in more sensitive, simple and miniaturized analytical devices which will be of high potential in the (bio)chemical field. In this way, high quality information can be obtained using very low sample volumes or even in vivo measurements.

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Conclusiones

Las etapas de tratamiento de muestra son el cuello de botella de la mayoría de procedimientos analíticos. Esto se debe al número de etapas implicadas y a la complejidad de las muestras. La Química Analítica actual, centra gran parte de sus esfuerzos en el diseño y optimización de metodologías más simples, rápidas, baratas y seguras para facilitar esta etapa. Durante el desarrollo de esta Tesis Doctoral, se ha profundizado en el diseño de nuevas alternativas para la microextracción dispersiva (tanto en fase sólida como en fase líquida) reduciendo el número de disolventes implicados, la cantidad de extractante y los residuos derivados de estos procesos. Las alternativas presentadas, además, pueden emplearse para el análisis *in situ* de muestras ambientales, sin necesidad de transportar grandes volúmenes de muestra al laboratorio, ya que son rápidas y no requieren el uso de equipamiento especial para desarrollarse.

Los resultados experimentales derivados de las investigaciones presentadas en esta Memoria han sido evaluados y discutidos desde un punto de vista operacional y analítico. Del mismo modo, este apartado recoge las conclusiones más relevantes derivados de estos.

La dispersión de una fase extractante (sólida o líquida) en una muestra acuosa mediante efervescencia es una alternativa altamente eficiente. La efervescencia mediante pastilla permite dispersar fases extractantes sólidas, bien sean poliméricas o nanoestructuradas. La efervescencia genera dentro de la propia muestra una elevada presión de CO_2 gas que actúa como fuerza dispersante del sorbente. La administración conjunta de sorbente y precursores efervescentes genera una suspensión estable que permite distribuir uniformemente la fase extractante a lo largo de todo el volumen de la muestra. Gracias a la efervescencia evitamos el empleo de disolventes orgánicos (tales como acetonitrilo o metanol) que habitualmente se emplean en este tipo de técnicas. La pastilla efervescente no genera residuos significativos una vez finaliza el procedimiento, el CO_2 es liberado de la disolución y las sales resultantes de la reacción

de intercambio de protones son inocuas. La pastilla puede utilizarse confinada en una jeringa para volúmenes de hasta 10 mL o en vasos de precipitados para volúmenes superiores con cantidades muy reducidas de sólidos sin alterar la composición reactiva de la pastilla (5 mg para sorbentes poliméricos y 7.5 mg para sorbentes nanoestructurados). El sorbente puede ser recuperado por filtración manual o mediante vacío y eluído con un disolvente orgánico compatible con la técnica cromatográfica requerida en función del problema analítico de estudio.

Por su parte, la dispersión de fases líquidas mediante efervescencia, permite dispersar volúmenes muy reducidos de disolvente orgánico extractante (20 µL de 1-octanol) en muestras carbonatadas mediante su introducción mediante una fase ácida. Gracias a la reacción efervescente, el residuo derivado del proceso es mínimo e inocuo, ya que se genera acetato en disolución en muy baja cantidad y el CO_2 generado es liberado de la disolución. Para simplificar la recuperación de la fase extractante se emplearon nanopartículas magnéticas, que mediante interacción con el disolvente orgánico permite separarlo de la fase acuosa mediante un imán de neodimio externo. El empleo de nanopartículas magnéticas agiliza considerablemente el proceso de separación de fases, evitando etapas de centrifugación o tediosos procedimientos de decantación, especialmente complejos en este caso por el reducido volumen de disolvente empleado. En el futuro, sería interesante abordar la combinación de pastillas efervescentes con sorbentes magnéticos tanto nano como microestructurados.

El CO_2 puede ser empleado no solo como agente dispersante, sino como mediador del proceso de solubilización de los llamados *disolventes conmutables*. Los *disolventes conmutables* han sido empleados en distintos procedimientos industriales y a escala de laboratorio. Durante el desarrollo de esta Tesis Doctoral se han utilizado satisfactoriamente en el contexto de la microextracción líquido-líquido. Gracias a la solubilización de CO_2 a partir de hielo seco se ha logrado obtener una fase acuosa

estable de un disolvente de hidrofobicidad variable. Esta fase acuosa que contiene el disolvente se ha empleado en una modalidad de microextracción líquido-líquido homogénea. Cuando el proceso de interconversión de la forma hidrofílica a hidrofóbica de este tipo de disolventes tiene lugar, los analitos de carácter apolar o parcialmente polar pueden extraerse y preconcentrarse. Gracias a esta propiedad, se ha desarrollado una alternativa de microextracción que consume un volumen muy reducido de disolvente (125-375 µL) y que no requiere el empleo de aparatos para generar la mezcla y separar las fases. Además, se ha logrado combinar el uso de estos disolventes especiales con técnicas de detección fluorimétricas y cromatográficas. Puesto que el CO_2 es el responsable del comportamiento conmutable de estos disolventes, sería interesante abordar el empleo de la efervescencia para solubilizar in situ la fase extractante, permitiendo simultáneamente la separación de fases cuando el gas es liberado de la disolución.

Las alternativas desarrolladas durante la realización de esta Tesis Doctoral se han evaluado mediante la aplicación a muestras de agua ambientales con diferentes analitos (compuestos nitroaromáticos, triazinas y hidrocarburos policíclicos aromáticos). En todos los casos se han alcanzado buenos valores en cuanto a los limites de detección y precisión. Así mismo, los resultados para muestras fortificadas de diferente naturaleza (aguas de rio, pozo, grifo y embotellada) analizas en cada caso, cumplieron los requisitos propuestos por la US-EPA (criterio de recuperación de 70-130%) con buenos valores de precisión. Cabe destacar que en ningún caso las muestras fueron filtradas o sometidas a ningún tratamiento distinto de las alternativas propuestas. Precisamente, la validación de estos métodos en estas condiciones, refuerza la idea de poder utilizar estas metodologías para el análisis *in situ* de muestras acuosas de similares características.

Finalmente, para poder explotar todo el potencial de las técnicas presentadas, se debería abordar su aplicaciones a matrices más complejas, como pudieran ser muestras alimentarias o biológicas. Del mismo modo, sería interesante estudiar el comportamiento de nanomateriales híbridos que permitan realizar determinaciones más selectivas.

Actividades de divulgación

INVESTIGACIÓN GRASEQA: MINIATURIZACIÓN E INSTRUMENTACIÓN PORTÁTIL

Microextracción asistida por efervescencia

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1. Introducción

Las técnicas de pretratamiento de muestra han experimentado un rápido y prolífico desarrollo en las últimas décadas. En esencia, buscan dotar al químico analítico de herramientas eficientes para poder aislar analitos de interés de muestras complejas mejorando la selectividad de las determinaciones. Esta mejora suele ir acompañada de una preconcentration de los analitos de interés para adecuarlos a la sensibilidad de la técnica instrumental empleada en su determinación. En la mayoría de los casos, el pretratamiento de muestra es un paso insoslayable del proceso analítico, teniendo una influencia clave, no solo en la sensibilidad y selectividad, sino también en la exactitud y precisión de los resultados.

Las técnicas de pretratamiento de muestra han experimentado una evolución constante en los últimos años hacia la simplificación, miniaturización y automatización. Estas tres tendencias principales han permitido no sólo la mejora de las propiedades analíticas básicas (selectividad y sensibilidad) sino también la de las propiedades productivas (costes, riesgos y tiempo de análisis). Las técnicas de microextracción originadas como consecuencia de esta evolución, siendo la microextracción en fase solida (SPME) y en fase liquida (LPME) sus principales paradigmas, son más baratas (se consumen menos disolventes y materiales), más seguras (y menos contaminantes) y reducen el tiempo de análisis incrementando así el volumen de trabajo de los laboratorios. Estas técnicas se encuentran por tanto bajo el paraguas de la Química

verde [1, 2] y de la Responsabilidad social [3]. A pesar de su relativa juventud, estas técnicas experimentan una continua evolución.

Los aspectos termodinámicos y cinéticos son determinantes para definir la eficacia de una técnica de extracción, de tal forma que los primeros definen la cantidad máxima de analito que puede extraerse mientras que los últimos explican la velocidad a que tiene lugar dicha extracción. El tratamiento de muestra ideal debería alcanzar un balance positivo entre ambos aspectos.

Entre los parámetros cinéticos, la superficie de contacto entre muestra y fase (sólida o líquida) extractante juega un papel primordial. Las técnicas de microextracción dispersiva buscan conseguir una dispersión eficiente, mediante el empleo de agentes químicos (disolventes orgánicos, tensioactivos…) o medios físicos (agitación, ultrasonidos...), del la fase extractante en la muestra maximizando así esta superficie de contacto.

2. **Microextracción en fase solida asistida por efervescencia**

En 2011 nuestro grupo de investigación desarrolló una nueva modalidad de microextracción en fase solida dispersiva que emplea dióxido de carbono como agente dispersante. Esta técnica, denominada microextracción en fase sólida asistida por efervescencia, se fundamenta en la generación in situ de CO_2 mediante una reacción efervescente. Para tal fin, se prepara una pastilla efervescente que contiene una fuente de CO_2 (carbonato sódico), un donador de protones (dihidrogenofosfato sódico) y el sorbente adecuado. En el caso de ser necesario pueden añadirse otros reactivos (electrolitos para aumentar la fuerza iónica) a dicha formulación.

La pastilla se prepara mediante molturación y mezcla de todos sus componentes que son prensados finalmente en una prensa hidráulica. La pastilla resultante es suficientemente estable siempre que se almacene en ausencia de humedad.

2.1 Microextracción en fase sólida asistida por efervescencia usando micropartículas poliméricas como sorbentes

La microextracción en fase sólida asistida por efervescencia fue originalmente evaluada empleando micropartículas poliméricas como material sorbente. Para tal efecto, se empleó un sorbente comercial OASIS-HLB para la extracción de compuestos nitroaromáticos de muestras de agua. En esta aplicación, 5 mg del sorbente se mezclaron con 250 mg de precursores efervescentes en su proporción estequiométrica y se prensaron para formar una pastilla de 120 mm de diámetro interno, la cual se introdujo en una jeringa de vidrio de 10 mL. El procedimiento de extracción consiste en la aspiración de 10 mL de muestra con la jeringa que al entrar en contacto con la pastilla provocan la reacción efervescente y por tanto la eficiente dispersión del extractante en la muestra. Tras la extracción, el sólido se recupera por filtración usando un filtro de jeringa de 0.2 µm de tamaño de poro. La elución final de los analitos se realiza pasando 500 µL de acetonitrilo por el filtro que retiene el sorbente (Figura 1A).

La composición de la pastilla efervescente determina su estabilidad química, la cual puede definirse en base a su higroscopicidad. En términos generales, una baja higroscopicidad permite mantener el poder efervescente hasta que se emplee la pastilla en el proceso de extracción. Se estudiaron distintas combinaciones de precursores efervescentes; carbonato y bicarbonato sódico como fuentes de CO_3^{2-} y acido cítrico, ascórbico y dihidrogenofosfato sódico como fuentes de H^+. De todas ellas, la combinación de carbonato sódico y dihidrogenofosfato sódico presentó la menor higroscopicidad, medida como variación de peso en función del tiempo.

La composición de la pastilla determina el tiempo de efervescencia e influye directamente en el poder dispersante y en el tiempo de extracción. Además, y debido a la naturaleza de la reacción efervescente, es deseable que durante la efervescencia se produzca la menor variación posible de pH, de forma que la técnica sea

suficientemente versátil para familias de analitos que puedan ver influenciada su extracción por esta variable. Los resultados de estas pruebas se resumen en la Tabla 1.

Como se ha comentado, esta aproximación se caracterizó mediante la extracción de compuestos nitroaromáticos de muestras de agua, obteniéndose resultados muy interesantes en términos de sensibilidad y reproducibilidad como se muestran en la Tabla 1. Además la efervescencia produce una preconcentración 2-3 veces mejor que la extracción en fase sólida convencional (usando la misma cantidad de sorbente) y 2 veces mayor que la extracción dispersiva convencional.

El estudio de validez empleando el método para la extracción de muestras ambientales (rio) permitió obtener resultados de recuperación (expresados como % de recuperación) próximos al 100% en todos los casos.

2.2 Microextracción en fase sólida asistida por efervescencia usando nanopartículas de carbono como sorbentes

El empleo de nanomateriales, que se caracterizan por su gran área superficial, en el contexto de la extracción en fase sólida puede considerarse como un hito clave de su desarrollo. A pesar de su potencial, muchos materiales nanoestrucutrados presentan una gran tendencia a la agregación lo que impide que su superficie pueda ser explotada en todo su potencial. La técnica asistida por efervescencia se evaluó en estas condiciones tan desfavorables usando nanotubos de carbono de pared múltiple como material sorbente [4]. En este caso se incorporaron 7.5 mg de este nanomaterial a la composición ya definida de precursores efervescentes para fabrica r una pastilla de 120 mm de diámetro interno.

Considerando una composición fija de pastilla, y aprovechando las características extractivas de los nanotubos de carbono, se desarrolló una estrategia capaz de dispersar la cantidad seleccionada de nanotubos en un volumen de muestra mucho mayor que en la anterior aproximación. El volumen de muestra y la cantidad de

nanotubos son dos variables experimentales intrínsecamente relacionadas, puesto que para poder abarcar un mayor volumen de muestra será necesaria una mayor cantidad de sorbente, siempre y cuando la cantidad de precursores efervescentes se mantenga fija. En este caso, y debido al volumen de muestra (100 mL), la recuperación del sorbente requiere el empleo de un sistema de filtración a vacío, empleando una membrana de teflón (PTFE) comercial (Figura 1B).

El procedimiento de elución de esta alternativa es sensiblemente diferente. La membrana de PTFE con los nanotubos retenidos es introducida en una jeringa de vidrio. Se aspiran 2.5 mL de eluyente, volumen capaz de cubrir por completo la membrana, y se sonica la jeringa para favorecer la transferencia de los analitos al eluyente. Finalmente, el eluido se filtra usando un filtro de jeringa (0.2 μm). Puesto que el volumen de elución es relativamente grande, es necesario introducir un paso de evaporación-redisolución para conseguir una mejora de la sensibilidad en el posterior análisis por cromatografía de líquidos y detector de diodos en fila.

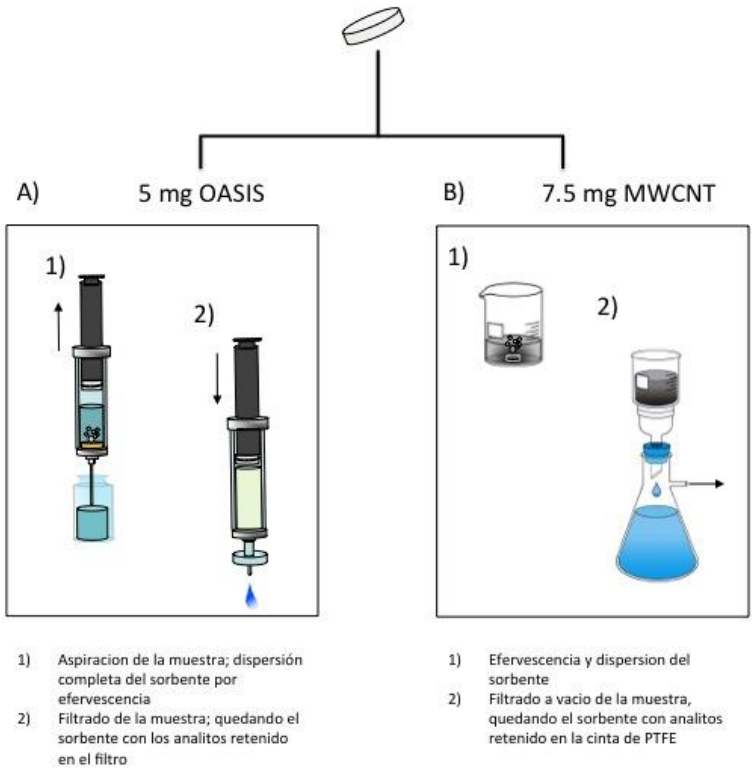

Figura 1: Microextracción en fase sólida asistida por efervescencia en su modalidad de sorbente polimérico (A) y nanoestructurado (B).

El empleo de la efervescencia como método para dispersar nanotubos en muestras acuosas resulto eficaz para alcanzar todo el volumen de muestra dispuesto, y adicionalmente, genero una dispersión estable durante el tiempo de extracción. La figura 2 muestra la comparación entre distintas alternativas de dispersión, (A) la dispersión se genera mediante la disolución de la mezcla que conforma la pastilla sin prensar; (B) mediante la pastilla efervescente; (C) empleando los nanotubos directamente sobre el agua y (D) en dos etapas, primero disolviendo los componentes efervescentes y posteriormente los nanotubos. Todos los viales fueron agitados

mecánicamente durante un minuto y posteriormente dejados en reposo durante otros dos minutos, previa a la toma de la imagen. Como puede observarse, el método que implica el empleo conjunto de los componentes en forma de pastilla genera mejores dispersiones y más estables en el tiempo, siendo la efervescencia la responsable de este hecho (B) y no la fuerza iónica generada (D).

El método propuesto se caracterizó mediante la extracción de triazinas de muestras acuosas como problema analítico modelo. Como se muestra en la tabla 3, se pudieron obtener limites de detección en el rango bajo de los μg L^{-1} con una reproducibilidad (expresada como desviación estándar relativa) inferior al 9.3%. El estudio de validez empleando el método para la extracción de muestras de agua ambientales (rio, grifo y pozo) arrojó resultados de recuperación (expresados como % de recuperación) próximos al 100% en todos los casos.

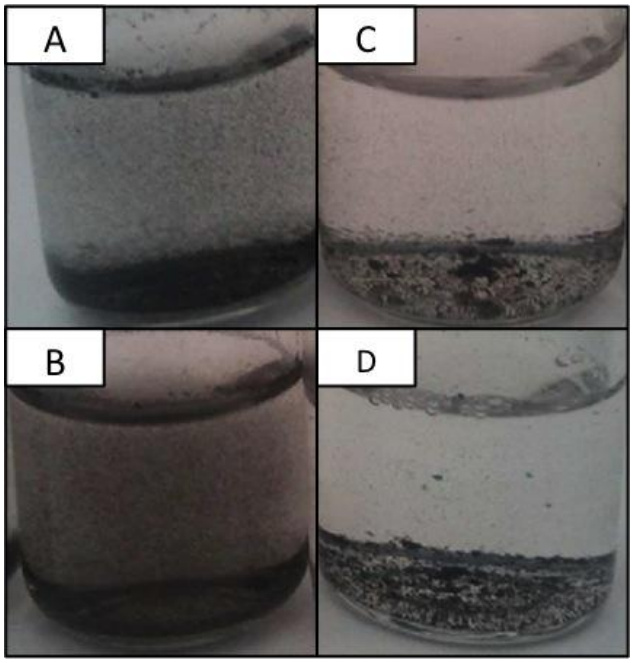

Figura 2 A-D: Dispersión de MWCNTs en las distintas condiciones experimentales. Ver texto para más detalles.

Tabla 1: Principales caracteristicas analíticas de los métodos presentados

	Analitos	Volumen de muestra (mL)	LdD[a] (µg/L)	DER[b] (%)	FE[c]
dSPME					
OASIS HLB	Comp. nitroaromáticos	10	1.8-7	1.7-8.6	13-17
MWCNTs	Triazinas	100	0.15-0.40	3.9-9.3	480-755
dLPME					
1-Octanol/MNPs	Triazinas	20	0.02-0.06	7.8-11.7	21-185

[a] LdD: límite de detección; [b] DER: desviación estándar relativa; [c] FE: factor de enriquecimiento

3. Microextracción en fase liquida asistida por efervescencia y nanopartículas magnéticas

La generación in situ de CO_2 en el seno de una muestra acuosa genera un intenso burbujeo que permite no solo dispersar eficientemente extractantes sólidos

sino también líquidos [5]. Esta última alternativa se ha evaluado recientemente empleando 20 µL de 1-octanol como disolvente para la extracción de triazinas de muestras acuosas. El pequeño volumen de fase orgánica usado permite alcanzar grandes factores de preconcentración pero supone un reto importante a la hora de su recuperación. En este caso, se optó por el empleo de nanopartículas magnéticas de óxido de hierro (Fe_3O_4) para favorecer el proceso de recuperación.

El procedimiento de extracción consiste en la inyección de 250 µL de una suspensión que contiene 20 µL de 1-octanol y 10 mg de nanopartículas magnéticas de ferrita de cobalto en acido acético sobre 20 mL de una muestra previamente carbonatada. La reacción entre el carbonato de la muestra y el acido de la suspensión genera un intenso burbujeo que abarca todo el volumen de muestra dispersando todos los componentes en menos de 5 s. Posteriormente mediante la aplicación de un potente imán de neodimio en el fondo de dispositivo de extracción conseguimos la separación del disolvente y las nanopartículas con los analitos extraídos (Figura 3).

Figura 3: Modalidad de microextracción dispersiva en fase líquida asistida por efervescencia y recuperación asistida por nanopartículas magnéticas.

Como se ha comentado, el empleo de nanopartículas magnéticas es esencial para la eficiencia del proceso de recuperación del disolvente. Como el recubrimiento de estas nanopartículas puede jugar un papel fundamental en dicho proceso, se estudiaron nanopartículas de Fe_3O_4 sin y con recubrimiento de C_{18}. El 1-octanol puede interaccionar con ambos tipos de nanopartículas por dos mecanismos diferentes: uno basado en interacciones apolares con el C18 y otro basado en interacciones polares con los grupos -OH residuales en la superficie de la nanopartículas sin recubrir. Esta última resultó ser la más adecuada al presentar una mejor dispersabilidad y una escasa tendencia a la agregación (Figura 4).

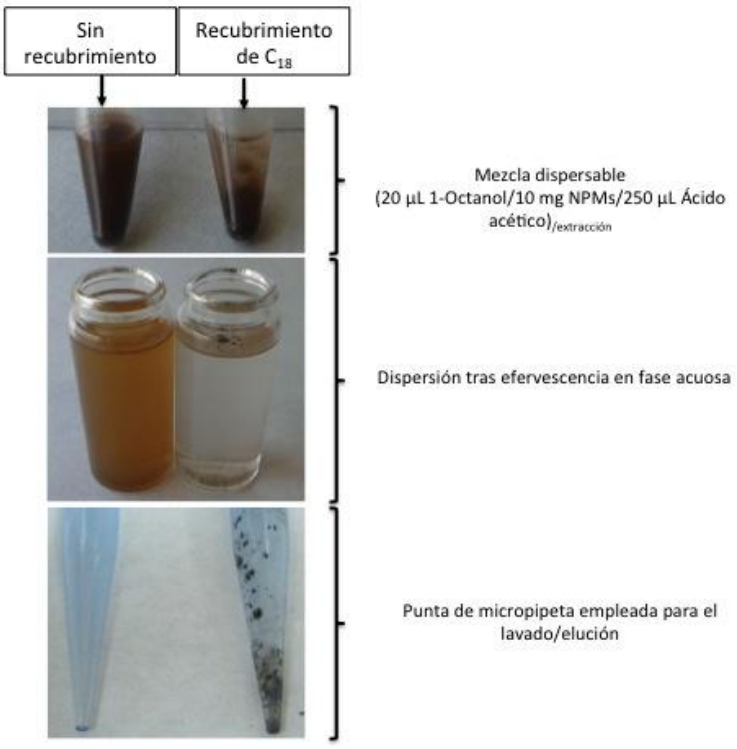

Figura 4: Comparativa entre los recubrimientos de las nanoparticulas magnéticas empleadas en la modalidad de microextracción en fase líquida asistida por efervescencia.

Una vez seleccionado el método de recuperación de 1-octanol se optimizó la relación entre la cantidad de nanopartículas y el 1-octanol. En términos de señal analítica, la combinación de 20 µL de 1-octanol y 10 mg de nanopartículas por extracción resultó la más ventajosa. Es necesario destacar que cantidades mayores de nanopartículas tendían a provocar atascos en la jeringa, impidiendo su dispersión. Puesto que todo el procedimiento tiene lugar en el mismo vial de extracción gracias al empleo del imán, que permite recuperar de forma sencilla la fase extractiva, la elución de los analitos se realizó mediante la adición de 100 µL de metanol al mismo vial y separándolo de nuevo de las nanopartículas magnéticas.

El método propuesto se empleó para la determinación de triazinas en muestras de agua. Como muestra la tabla, se obtuvieron límites de detección muy bajos con una precisión (expresada como desviación estándar relativa) inferior al 12%. La validación del método se llevo a cabo mediante el estudio de recuperación en muestras de agua ambientales (rio, grifo y pozo) obteniendo en todos los casos valores cercanos al 100%.

4. Conclusiones y perspectivas futuras

Las alternativas presentadas representan una clara apuesta por la simplificación de las técnicas de microextracción y su abaratamiento. En el caso de las técnicas en fase solida presentadas se hace uso de pastillas preparadas en el laboratorio a partir de reactivos de bajo coste, evitando el uso de disolventes orgánicos. La dispersión basada en la generación in situ de CO_2 permite la distribución

homogénea de un sorbente polimérico o nanoestructurado en el seno de una muestra acuosa de manera rápida y sencilla empleando materiales comunes de laboratorio. En el caso de la alternativa con sorbentes poliméricos, podemos plantear escenarios en los que la toma de muestra y extracción se realice en el mismo momento, empleando la jeringa para el muestreo y extracción simultanea. Esta alternativa es especialmente útil cuando los analitos de interés pueden sufrir una degradación durante el transporte desde el sistema objeto de estudio al laboratorio de análisis. Del mismo modo, el almacenamiento de un filtro de jeringa que contenga el sorbente con los analitos extraídos es mucho más sencillo que el almacenamiento de contenedores para las muestras acuosas. En el caso de la propuesta con sorbentes nanoestraturados, si no se dispone de sistemas portátiles de filtrado a vacío, puede aportar una ventaja adicional en el procesamiento de numerosas muestras, ya que es factible la preparación y mantenimiento de las pastillas con el sorbente de antemano y su adición sistemática a las muestras en el laboratorio, de forma que conseguimos en un único paso la dispersión del sorbente sin necesidad de agitación de ningún tipo.

En el caso de la extracción dispersiva en fase liquida, es especialmente interesante el empleo de CO_2 como agente dispersante. Este procedimiento evita el uso de disolventes orgánicos dispersantes que además de ser costosos y tóxicos pueden influir en el equilibrio de extracción al incrementar la solubilidad de los analitos en la matriz acuosa. En este caso, el ácido acético usado se convierte como consecuencia de la reacción efervescente en un electrolito (acetato sódico) que puede actuar como agente salting-out. Por otro lado, el empleo de nanopartículas sin capacidad de extracción para los analitos propuestos pero si de interacción con el disolvente, permite, gracias a la co-dispersión de los mismos una extracción extraordinariamente rápida (5 s) y una recuperación total de volúmenes muy bajos de extractantes (20 μL). El empleo de imanes y nanopartículas magnéticas permite el

empleo de un único recipiente para la extracción, recuperación y elución de los analitos, simplificando y agilizando el procedimiento.

Por último, la tabla 1 recoge los aspectos analíticos más relevantes de los métodos presentados.

El empleo de pastillas efervescentes para dispersar sorbentes permite la aplicación de estos procedimientos en muestras acuosas biológicas complejas, ya que, gracias a la limitada variación de pH que provocan en la muestra y la rapidez del proceso de dispersión, las muestras no experimentan ningún tipo de alteración química. Del mismo modo, la composición de las pastillas puede ser ajustada al tipo de muestra o analito, pudiendo introducir sorbentes funcionalizados que aporten selectividad adicional tales como anticuerpos o aptámeros. Así mismo, en función de la proporción de los precursores efervescentes podemos conseguir efervescencia más o menos intensa para variar la velocidad del proceso dispersivo.

Por su parte, las aplicaciones en fase liquida asistida por efervescencia y nanopartículas magnéticas abre un nuevo campo de aplicación, mediante la co-dispersión de disolventes especiales tales como líquidos iónicos y/o nanopartículas magnéticas funcionalizadas, que permitiría aplicar esta técnica a un amplio abanico de muestras acuosas.

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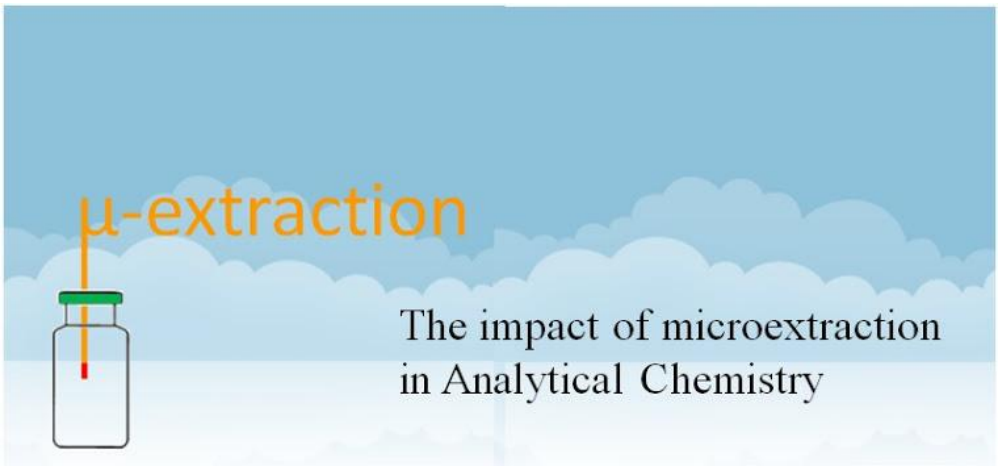


Effervescence-assisted dispersive micro-solid phase extraction

In 2011 a new sample pre-treatment technique, called *Effervescence-assisted dispersive micro-solid phase extraction,* was proposed. The technique was based on the use of an effervescent reaction (reaction between a proton donor and a carbonate source releasing gaseous CO_2) for the efficient dispersion of a sorbent. For this purpose a lab-made effervescent tablet, containing all the reagents necessaries to perform the dispersive extraction (NaH_2PO_4) as proton donor, Na_2CO_3 as carbonate source and the appropriate sorbent) is directly added to the sample. The tablet, which is 250 mg in weight and 102 mm in diameter, is produced by the simple blending of the precursors and their final compression in a hydraulic manual press. The final tablets are stable enough if they are stored under inert atmosphere to preserve them from the environmental humidity, which reduces the CO_2 releasing potential and dispersion efficiency.

The tablet composition is optimized considering the effervescent precursors in one hand and the sorbent in the other. The effervescent precursors must be

stoichiometrically adjusted to produce a complete reaction with a minimum pH variation within the sample. The nature of these compounds determines also the hygroscopicity of the tablet. The selected precursors produce a pH variation of 0.2 pH units during the extraction time (considered as the time required to complete dissolution of the tablet, in this case 4.5 min c.a.) and a hygroscopicity (measured as weight variation in non-inert storage conditions) of 0.55 % in 192 h.

The sorbents employed in the two published investigation are a commercial polymeric sorbent (OASIS HLB from Waters Corp.) and multiwalled carbon nanotubes (MWCNTs). The first approach employs the tablet inside a 10 mL syringe in which sorbent dispersion occurs immediately after sample aspiration. The sorbent with the extracted analytes is retained using a 0.2 µm in-syringe filter and eluted prior to the UPLC-UV analysis. This method was used to the analysis of nitroaromatic compounds in environmental water samples with great values in terms of sensitivity and reproducibility as shown in Table 1. The second option developed introduces a nanostructured sorbent (MWCNTs) in the effervescent tablet. One of the main problems associated to the use of raw carbon nanotubes is the aggregation tendency, especially in aqueous samples. With the effervescent dispersion this problem is avoided. The dispersion generated during extraction, without the use of any organic solvent or surfactant is stable enough to interact with the analytes in the sample, in this case a 100 mL aqueous sample contained in a glass beaker. Due to the sample volume employed the sorbent with extracted analytes is recovered by vacuum filtration using a commercial PTFE tape as filter. To evaluate the dispersion process a simple experiment using different dispersion alternatives is performed. For this purpose different dispersion are prepared (Figure 1); (A) uncompressed effervescent tablet powder, (B) effervescent tablet containing the sorbent, (C) MWCNTs directly added to an aqueous sample and (D) MWCNTs added to a water sample in which an effervescent tablet without sorbent had been dissolved. The vials containing these mixtures are

mechanically agitated and leave at rest for two minutes before taking the pictures. As can be seen the effervescent tablet is the responsible of the efficient dispersion during extraction and not the ionic strength derived from the effervescent precursors. This alternative is evaluated by the extraction of triazine herbicides from environmental water samples by GC-MS with good values of sensitivity and reproducibility as can be seen in Table 1.

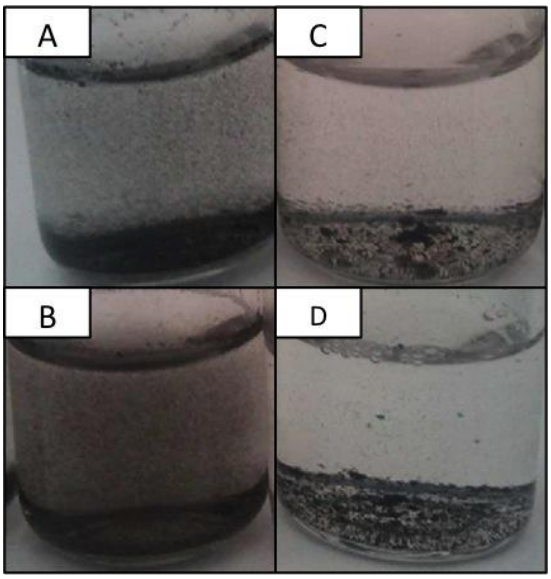


Figure 1: Different dispersions obtained for MWCNTs (for details, read text)

Table 1. Analytical figures of merit of the presented alternatives

	Analytes	Sample volume (mL)	LoD[a] (μg L^{-1})	RSD[b] (%)	EF[c]
dSPME					
OASIS HLB	Nitroaromatic	10	1.8-7	1.7-8.6	13-17
MWCNTs	Triazines	100	0.15-0.40	3.9-9.3	480-755
dLPME					
1-Octanol/MNPs	Triazines	20	0.02-0.06	7.8-11.7	21-185

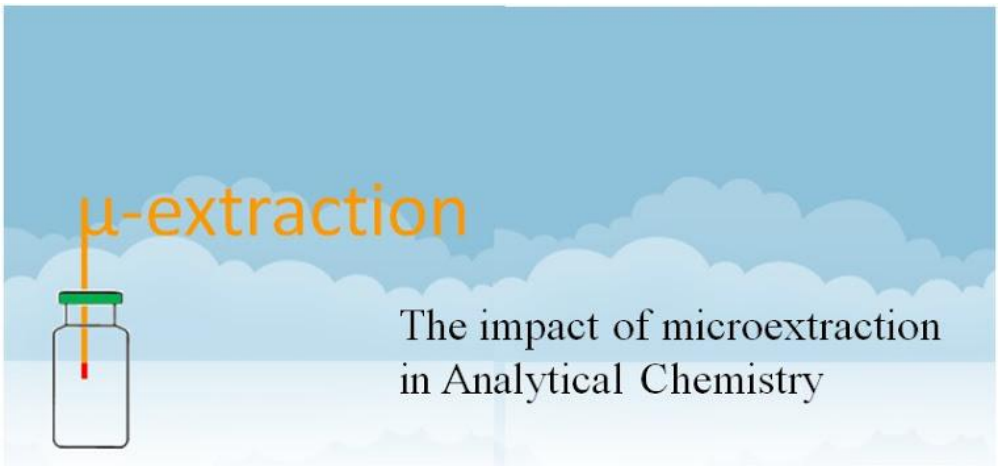
[a]LOD: limit of detection; [b]RSD: relative standard deviation; [c]EF: enrichment factor

Effervescence allows also the dispersion of liquid in a new liquid-liquid micro extraction approach

The most recent effervescent-approach is based on the dispersion of a very low volume (20 µL) of an organic solvent (1-octanol) in aqueous samples, using Fe_3O_4 magnetic nanoparticles co-dispersed with the extractant phase to achieve the extractant phase separation in a very simple way. The dispersion is based in the effervescent reaction between a carbonated aqueous sample and a liquid mixture containing 20 µL of organic solvent and 10 mg of magnetic nanoparticles in acetic acid media. A volume of extractant mixture (250 µL) is injected in the bottom sample and the effervescent reaction disperses both solvent and magnetic nanoparticles. The subsequent application of an external magnet permits the solvent recovery by the interaction of the alcoholic group of the solvent and the hydroxide residues of the MNPs surface. This alternative is evaluated by the extraction of selected triazine herbicides by GC-MS with great values of sensitivity and reproducibility.

Conclusions

The employ of effervescence as dispersion method for solid or liquids reduces the use of organic solvent or apparatus (like vortex or ultrasonic baths) to perform the extraction. Furthermore, the reagents employed are non-toxic and cheaper than other alternatives. The simplicity of the inclusion in a single device (in this case a tablet) permits the employ of these alternatives for the on-site analysis of environmental waters. In the case of the dLLME, the very low volume of extractant used permits high pre-concentration factors. The simplicity of the extractant phase recovery process, using an external magnet avoids the use of complex lab-ware (such a separation funnel) or centrifuges, which probably affects the reproducibility of the method at this very low volume.



Switchable hydrophilicity solvents in the microextraction context

The concept of switchable solvents (SS) was firstly introduced by Canter in 2006, in the framework of industrial processes. The initial idea was to introduce solvents which can switch between a polar and non-polar chemical form, aiming to reduce the number of organic solvent and toxic wastes generated during extraction procedures [1]. Recently, professor Jessop and co-workers expands the application field of this kind of solvents in order to develop cheaper and greener chemical processes.

Jessop proposed gaseous CO_2 as trigger reagent to switch between the two chemical forms of the SS (see equation 1), which is pretty interesting since it is cheap, easily available and non-toxic. Moreover, it can be easily introduced and removed from the solution, thus simplifying the "switching" process. Solvents presenting this behavior were named as "switchable hydrophilicity solvents" (SHS) as they are miscible with water in the presence of CO_2 while become immiscible when CO_2 is removed. This removal can be simply achieved by bubbling the solution with N_2 or Ar.

$$NR_3 + H_2O + CO_2 \Leftrightarrow NR_3H^+ + HCO_3^- \quad \text{(Equation 1)}$$

SHS were firstly used as substitute of hexane in soybean oil extraction. In this case, SHS avoid the traditional separation by distillation, minimizing the environmental impact and reducing the overall economic and energy costs of the process by substitution of the distillation process [2]. Recently, SHS has been successfully employed for removing solvent from products such as algae oil in bio fuel production [3,4], bitumen [5] and high-density polystyrene [6].

Several solvents showing this behavior had been identified, most of them amidines and tertiary amines [7,8]. It is also available a small group of Ionic Liquids (IL) which present a switchable behavior [9]. One of the IL shows exactly the opposite polarity change in presence of CO_2: tetrabutylphosphonium N-tri-fluoro-methanesulfonyl-leucine became hydrophobic when CO_2 is dissolved in an aqueous phase containing the IL.

Table 1 summarizes the main SHS employed and indicates two principal properties, namely the logarithm of the octanol/water portioning coefficient (log Kow) and the negative logarithm of the acid dissociation constant (pKa) [10].

Table 1. Main studied amines presenting switchable behavior

Compound	Ratio in water (v:v)	Log K_{ow} [a]	pK_{aH}
Triethylamine	1:1	1.47	10.68
N,N-Dimethylbutylamine	1:1	1.60	10.02
N,N-ethylpiperidine	1:1	1.75	10.45
N-methyldipropylamine	1:1	1.96	10.40
N,N-dimethylcyclohexylamine	1:1	2.04	10.48
N-buthylpyrrolidine	1:1	2.15	10.36
N,N-Diethylbutylamine	1:1	2.37	10.51
N,N-Dimethylhexylamine	1:1	2.51	10.18

[a]Calculation based on ALOGPS 2.1 software (adapted from [10])

The ideal SHS should present a log K_{ow} value in the range from 1.2 to 2.5. Solvents presenting lower values are too hydrophilic and generate monophasic system in its neutral form. Solvents with higher values generate biphasic systems even in the presence of CO_2. The pKa values should be higher than 9.5. In addition to these requisites, it is desirable for a SHS to be a non-volatile solvent, generally with a high molecular weight. The main reason is that the common way to eliminate CO_2 from the solution is by bubbling another gas (such as N_2 or Ar), and it could lead to partial/complete losses of SHS.

Our research group has recently proposed the adaptation of SHS as solvents in microextraction. In this case, we used N,N-dimethylcyclohexylamine (DMCA) [11] as solvent under a homogeneous liquid-liquid microextraction (HLLE) format. The miscibilitation of DMCA and water in a 1:1 v/v ratio is accomplished using dry ice. The resulting solution is injected in a defined volume of aqueous sample where the DMCA is completely solubilized. For phases´s separation, sodium hydroxide has been proposed instead of N_2 bubbling since the evaporation of SHS can have a dramatic effect when the solvent are used in the microliter range. After the extraction, DMCA with the target analytes is recovered for further analysis.

The SHS-HLLE procedure was evaluated by extracting benz[a]anthracene from water samples. Fluorescence detection was selected as instrumental technique to take advantage of the native photoluminescence of the target analyte. However, during the optimization process, we found that DMCA quenched benz[a]anthracene fluorescence. To solve this issue, a dilution (1:1) with acetic acid (HAc) was necessary (see Figure 1). The dilution avoided quenching and produced a 35 % increase in the fluorescence intensity compared to that obtained in pure methanol.

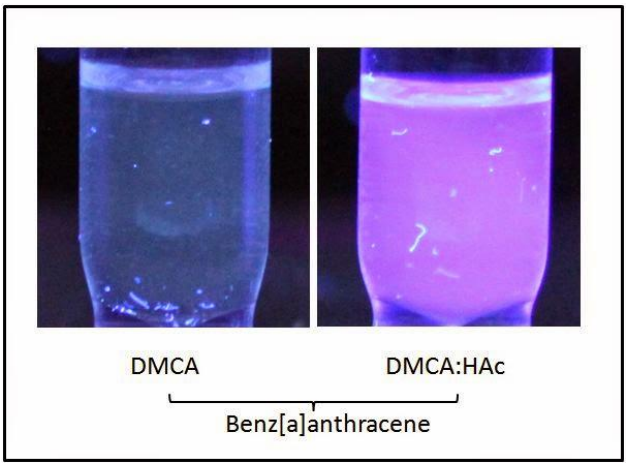


Figure 1: Fluorescence of benz[a]anthracene in DMCA and DMCA:HAc media

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Producción científica derivada de la Tesis Doctoral

Publicaciones científicas

Artículos Científicos

- G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, *Effervescence-assisted dispersive micro-solid phase extraction,* Journal of Chromatography A 1218 (2011) 9128. (Índice de impacto: **4.531**).
- G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, *Nanoparticle-based microextraction techniques in bioanalysis,* Bioanalysis 3 (2011) 2533. (Índice de impacto: 3.223).
- E. M. Reyes-Gallardo, G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, *Hybridization of commercial polymeric microparticles and magnetic nanoparticles for the dispersive micro-solid phase extraction of nitroaromatic hydrocarbons from water,* Journal of Chromatography A 1271 (2013) 50. (Índice de impacto: **4.612**).
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- G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, *Effervescence-assisted dispersive liquid-liquid microextraction with extractant removal by magnetic nanoparticles,* Analytica Chimica Acta 807 (2014) 61. (Índice de impacto: **4.517**).
- G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, *Use of switchable solvents in the microextraction context,* Talanta 131 (2014) 645. (Índice de impacto: **3.511**)
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triazine herbicides from environmental water samples, Journal of separation sciences (Artículo enviado, pendiente de publicación)

Capítulos de libro

- G. Lasarte-Aragonés, R. Lucena, S. Cárdenas*, Aptamers in Analytical Chemistry, Encyclopedia of Analytical Chemistry,* John Wiley & Sons *(2012) 1.*
- G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, *Dispersive micro-solid phase extraction, Microextraction Techniques,* Bentham eBooks (2014) 1.

Comunicaciones a congresos

- Comunicación poster titulada *"Effervescence assisted micro-solid phase extraction"* presentada en las 13ª Jornadas de Análisis Instrumental realizadas en Barcelona en Noviembre de 2011.
- Comunicación oral titulada: *"Effervescence-assisted carbon nanotubes dispersion for the micro-solid-phase extraction of triazine herbicides from environmental waters"* presentada en el IV Nanouco realizado en Córdoba en Febrero de 2013.
- Comunicación oral titulada: *"Hybridization of commercial polymeric microparticles and magnetic nanoparticles for the dispersive micro-solid phase extraction of nitroaromatic hydrocarbons from water "* presentada en el IV Nanouco realizado en Córdoba en Febrero de 2013.
- Comunicación oral y poster titulada: *"Effervescence-assisted carbon nanotubes dispersion for the micro-solid-phase extraction of triazine herbicides from environmental waters"* presentada en VI Workshop on Analytical Nanoscience and Nanotechnology realizado en Alcalá de Henares en Julio de 2013.

- Comunicación poster titulada *"Effervescence assisted dispersive micro-solid phase extraction "* presentada en el XVII Euroanalysis realizado en Varsovia en agosto de 2013.
- Comunicación poster titulada *"Hybridization of commercial polymeric microparticles and magnetic nanoparticles for the dispersive micro-solid phase extraction of nitroaromatic hydrocarbons from water"* presentada en el XVII Euroanalysis realizado en Varsovia en agosto de 2013.

Actividades de divulgación

- Artículo en el Boletín del Grupo Regional Andaluz de Química Analítica (GRASEQA) " Miniaturización e Instrumentación portátil" 8 (2014). G. Lasarte-Aragonés, R. Lucena, S. Cárdenas, M. Valcárcel, "Microextracción asistida por efervescencia"
- Participación en el blog para la divulgación de investigación en Química Analítica http://microextraction.blogspot.com/:
  - Effervescence assisted micro-solid phase extraction (25 de Abril de 2014)
  - Switchable hydrophilicity solvents in the microextraction context (19 de Septiembre de 2014)