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Desarrollo de métodos de preconcentración y de especiación funcional y química multielemental en materia orgánica disuelta y proteínas en agua de mar

TESIS DOCTORAL

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Departamento de Química Analítica, Nutrición y Bromatología

Desarrollo de métodos de preconcentración y de especiación funcional y química multielemental en materia orgánica disuelta y proteínas en agua de mar

Memoria que para optar al Grado de Doctora en Química presenta

Natalia García Otero

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autorizan,

a la licenciada **Dña. Natalia García Otero** a la presentación del trabajo recogido en la memoria titulada "Desarrollo de métodos de preconcentración y de especiación funcional y química multielemental en materia orgánica disuelta y proteínas en agua de mar", que ha realizado bajo su dirección en el Departamento de Química Analítica, Nutrición y Bromatología de la Universidad de Santiago de Compostela, para optar al Grado de Doctora en Química.

Y para que así conste, firmamos la presente autorización en Santiago de Compostela, octubre de 2013.

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Dña. María del Pilar Bermejo Barrera

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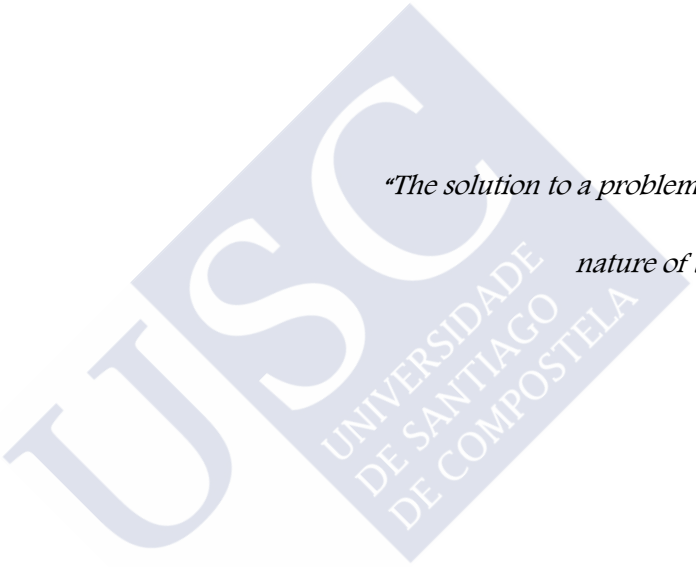
"The solution to a problem changes the

nature of the problem"



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Resumen

Los elementos traza se encuentran de manera natural en el medio marino; sin embargo, en los últimos años la contaminación por metales pesados en agua de mar se ha convertido en un problema a nivel mundial. Algunos de los elementos traza presentes en el medio marino son tóxicos, mientras que otros se consideran esenciales aunque pueden ser tóxicos a determinadas concentraciones. No obstante, la biodisponibilidad, movilidad, distribución y toxicidad de los elementos traza en el medio ambiente no solo depende de la concentración de los mismos, sino también de otros factores como la forma química en que dichos elementos se encuentran, y la naturaleza y tipo de partícula a la que están asociados. Por lo tanto, este trabajo se centra en la especiación funcional y química multielemental en materia orgánica disuelta (DOM) y proteínas en el agua de mar. Por una parte, se han desarrollado diferentes métodos de preconcentración de DOM en agua de mar empleando nanotubos de carbono como adsorbentes de extracción en fase sólida, y la técnica de ultrafiltración en flujo tangencial. Estos métodos se han empleado para la caracterización y determinación de metales asociados a DOM mediante el empleo de la cromatografía líquida de alta resolución con detección UV y detección por espectrometría de masas con plasma de acoplamiento inductivo. Por otra parte, se ha llevado a cabo la detección y caracterización de proteínas disueltas en el agua de mar y proteínas extraídas de plancton marino mediante diferentes técnicas electroforéticas (electroforesis en gel bidimensional y electroforesis bidimiensional OFFGEL-Lab-on-a chip), así como su identificación mediante espectrometría de masas de desorción/ionización por láser asistida por matriz y tiempo de vuelo (MALDI-TOF-MS), y la determinación de los metales asociados a las mismas por espectrometría de masas por ablación láser acoplada a plasma de acoplamiento inductivo (LA-ICP-MS), espectroscopia de emisión óptica por plasma acoplado inductivamente (ICP-OES) y espectroscopia de absorción atómica con atomización electrotérmica (ETAAS).

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I. INTRODUCCIÓN

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1. Introducción a la especiación funcional y química multielemental

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Introducción a la especiación funcional y química multielemental

1.1. Especiación química y funcional de elementos traza

En los últimos años, la contaminación del medio marino por metales pesados se ha convertido en un problema mundial. Los metales pesados se encuentran naturalmente en el medio marino, pero también entran en los sistemas acuáticos a través de las grandes cantidades de agua, tanto residuales como industriales, descargadas en los estuarios y zonas costeras. A diferencia de los contaminantes orgánicos, los metales pesados no son biodegradables y tienden a acumularse en los organismos vivos.

Algunos elementos traza son esenciales para el crecimiento normal y el desarrollo de organismos, pero algunos son tóxicos. Un elemento puede ser considerado esencial solo si el organismo no puede crecer ni completar su ciclo de vida en su ausencia, si el elemento no puede ser reemplazado por cualquier otro elemento o si el elemento ejerce una influencia directa sobre el organismo y su metabolismo. Se incluyen en este grupo: Cu, Co, Fe, Mn, Mo, Zn, Cr, Ni y Se. Del mismo modo, un elemento puede considerarse como tóxico si daña el crecimiento o el metabolismo de un organismo por encima de una determinada concentración. Se incluyen en este grupo: Cd, Hg, Pb, Sb, Bi, Sn y Tl [1].

Por lo tanto, los elementos traza desempeñan un papel importante en el funcionamiento de la vida en nuestro planeta. Algunos de estos elementos pueden ser altamente tóxicos para las diversas formas de vida, mientras que otros se consideran esenciales, pero pueden llegar a ser tóxicos en dosis más altas. Muchos de estos efectos dependen en gran medida de la forma en la cual el elemento está presente en el medioambiente. Por lo tanto, la biodisponibilidad, movilidad, distribución y toxicidad de los elementos traza en el medio ambiente no solo dependen de su concentración, sino también de las formas químicas en que dichos elementos se encuentran, determinando éstas sus asociaciones

fisicoquímicas en el medio natural. La concentración total de un elemento sirve para evaluar su impacto medioambiental solamente cuando dicho elemento está presente en el medio ambiente como una única especie o forma. Cuando diferentes especies de un elemento están presentes en el medio ambiente, para determinar la toxicidad y el comportamiento medioambiental de este elemento es necesario conocer la concentración de las diferentes especies [2]. Debido a que los elementos traza pueden presentarse en los sistemas acuáticos naturales como iones libres bajo diferentes estados de oxidación, formando complejos con otras sustancias o asociados a coloides y partículas, surge la necesidad de hablar del concepto de especiación.

Ure [3] define el término de especiación como:

- el proceso de identificación y cuantificación de las diferentes especies, formas o fases de un elemento presentes en un material o
- la descripción de las cantidades y tipos de especies, formas o fases presentes en el material.

En ambos casos, se pueden definir las especies, formas o fases funcionalmente, operacionalmente y químicamente [3, 4]. En base a esta definición tenemos tres tipos de especiación:

- *Especiación funcional*, que se refiere al impacto biológico y a la función del elemento. Por ejemplo, la especiación funcional nos permite hacer distinción entre los términos esencial y tóxico.
- *Especiación operacional*, que se refiere al procedimiento aplicado para extraer/aislar un elemento de una muestra. Por ejemplo, la distinción entre especies solubles e insolubles en los medios acuáticos naturales se realiza mediante filtros de 0,45 µm y se considera especiación operacional.
- *Especiación química*, que se refiere a los diferentes compuestos químicos específicos o estados de oxidación de un elemento. Por ejemplo, Cr (III) vs Cr (VI),

En el año 2000, la IUPAC [5] presentó la definición de los términos *análisis de especiación* y *especie química* relacionados con la *especiación de los elementos*, diferenciando el *fraccionamiento* del *análisis de especiación*.

- *Especie química*: forma específica de un elemento definida como la composición isotópica, estado electrónico o de oxidación, y/o la estructura o complejo molecular.
- *Análisis de especiación*: proceso analítico para la identificación y/o medida de las cantidades de una o más especies en una muestra.
- *Especiación de los elementos, especiación*: implica conocer la distribución de las especies químicas definidas de un elemento en un sistema.

De acuerdo con la restricción del uso del término *especiación* para definir las *especies químicas*, el término *fraccionamiento* ha sido definido como sigue,

- *Fraccionamiento*: clasificación de un analito o grupos de analitos de una muestra de acuerdo con sus propiedades físicas (por ejemplo, tamaño, solubilidad) o químicas (por ejemplo, reactividad).

Por lo tanto, la especiación química de los elementos traza es uno de los temas más importantes dentro del campo de la Química Analítica, y es esencial para determinar la toxicidad, biodisponibilidad, movilidad y distribución de estos elementos en el medio ambiente.

1.2. Coloides y nanopartículas

El estudio de la naturaleza y tipo de partícula a la que se encuentra asociada una especie química es de suma importancia para conocer el impacto que tiene en su biodisponibilidad, toxicidad y movilidad.

En un sistema acuoso la diferencia entre la fracción disuelta y la fracción particulada se basa en la filtración a través de filtros de 0,2 µm o 0,45 µm. De esta forma, conseguimos disminuir la complejidad de la muestra mediante la eliminación de la mayoría de microorganismos. Si se realizan filtraciones

adicionales a través de membranas con diferentes tamaños nominales de poro podemos definir las fases disuelta, coloidal y particulada [6]. Las partículas se definen como materiales que tienen un tamaño >1 µm, mientras que los coloides son compuestos que tienen un rango de tamaños que varía entre 1 nm y 1 µm. Las nanopartículas son consideradas sustancias con un tamaño entre 1 nm y 100 nm, por lo tanto las nanopartículas constituyen una sub-fracción de los coloides tal y como se muestra en la figura 1 [7].

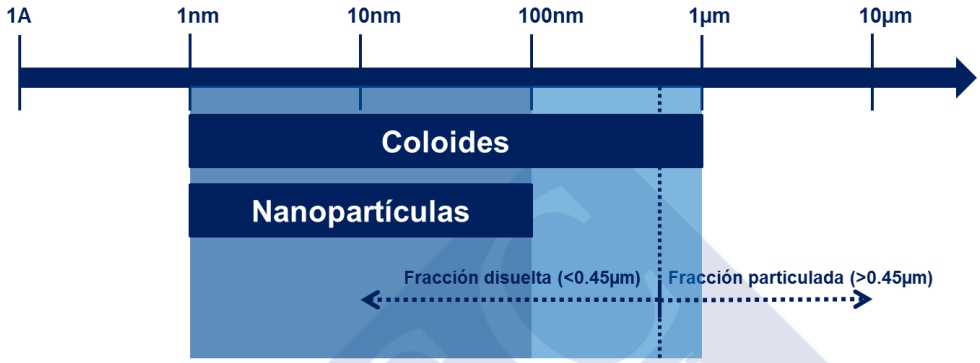

Figura 1: Definición de tamaños para nanopartículas y coloides.

Los coloides en los medios naturales acuáticos pueden ser de origen natural, de origen antropogénico o de origen sintético; por lo tanto, se pueden dividir en tres grupos [8, 9]:

- Coloides naturales: Dentro de los coloides naturales podemos diferenciar entre coloides inorgánicos y coloides orgánicos en función de su composición.
    - Coloides inorgánicos: silicatos, óxidos/hidróxidos, carbonatos, fosfatos y sulfuros.
    - Coloides orgánicos: están formados por macromoléculas (ácidos húmicos, ácidos fúlvicos, proteínas, polisacáridos, etc.) y biocoloides (bacterias, virus, etc.) principalmente.

- Coloides antropogénicos: Los coloides antropogénicos son producidos inadvertidamentes como subproducto de un proceso, como por ejemplo los productos resultado de una combustión.
- Nanopartículas sintéticas: Las nanopartículas sintéticas son producidas intencionalmente debido a sus características particulares. Ejemplos de nanopartículas sintéticas son los fullerenos, nanotubos de carbono de pared simple y de pared multiple, "*quantum dots"*, polímeros, surfactantes, óxidos metálicos (de Ti, Zn, Zr, Ce…), etc.

Los coloides son lo suficientemente pequeños para comportarse de manera similar a los compuestos solubles, y son lo suficientemente grandes para participar en procesos como el transporte de compuestos inorgánicos y orgánicos, vitales o perjudiciales, en los sistemas acuáticos debido a que presentan numerosos sitios de unión [7]. Por lo tanto, los coloides juegan un papel muy importante en procesos como la especiación, el transporte y la biodisponibilidad de contaminantes como pueden ser los metales traza en los medios naturales acuáticos [10].

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2. Materia orgánica disuelta en el agua de mar

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Materia orgánica disuelta en el agua de mar

2.1. Características de la materia orgánica disuelta

La materia orgánica natural (NOM, *natural organic matter*) en el agua de mar es de gran interés en estudios medioambientales debido a que está relacionada con los diferentes ciclos biogeoquímicos en el medio marino como pueden ser el ciclo de nutrientes y la disponibilidad y el transporte de contaminantes [1].

El origen de la NOM en el medio marino se puede deber a diferentes fuentes. Por una parte, la materia orgánica de origen terrestre y que es transportada al mar a través de los ríos; y por otra, la NOM de origen marino, como la materia orgánica producida por la descomposición de organismos muertos, y la materia orgánica debida a las excreciones de los compuestos extracelulares producidos por algas y a las excreciones de los animales marinos [2].

La NOM marina se puede clasificar en materia orgánica particulada (POM, *particulate organic matter*) y materia orgánica disuelta (DOM, *disolved organic matter*). La POM es la fracción de materia orgánica que se puede separar con un filtro de un diámetro nominal de poro de aproximadamente 0,45 µm, y la DOM es la fracción de materia orgánica que no se retiene y pasa a través de un filtro con un diámetro nominal de poro, por lo general de 0,2 µm o 0,45 µm [3].

La DOM forma parte del ciclo global del carbono y representa la mayor reserva de carbono reducido en los océanos. Es una fuente de alimento para los organismos oceánicos, formando parte del ciclo de nutrientes, y tiene la capacidad de formar complejos con metales traza, controlando así su destino en el medio marino [4].

2.1.1. Composición de la DOM

La DOM está formada por una mezcla compleja de compuestos orgánicos con un amplio rango de tamaños moleculares [5]. La fracción de DOM de bajo peso molecular (LMW, *low molecular weight*), menor de 1 kDa, representa aproximadamente el 65-80% del carbono orgánico disuelto (DOC, *dissolved*

organic carbon) en el océano, mientras que la fracción de alto peso molecular (HMW, *high molecular weight*) representa una pequeña porción de DOC (20-35% para DOM > 1 kDa y 2 – 7 % para DOM > 10 kDa). La fracción HMW es más abundante en aguas superficiales (30 – 35 % para DOM > 1 kDa y 5 – 7 % para DOM > 10 kDa) en comparación con aguas profundas (20 – 25 % para DOM > 1 kDa y 2 – 4 % para DOM > 10 kDa). Esto nos sugiere que la HMW-DOM es relativamente reactiva mientras que la LMW-DOM forma la mayor parte de la DOM refractaria en el océano [6]. Entre los compuestos que forman parten de la DOM se encuentran substancias húmicas, hidratos de carbono, proteínas y lípidos.

Las substancias húmicas (HS, *humic substances*) son el mayor componente de la DOM en el agua de mar [7]. Las HS están compuestas por una mezcla de compuestos con estructuras complejas y no repetitivas, mayoritariamente estructuras carboxiladas y estructuras alicíclicas condensadas que pueden complejar metales y contaminantes orgánicos [8] . Los tamaños moleculares de las HS son muy heterogéneos, desde unos cientos a 300000 Da. Químicamente las HS se pueden clasificar en ácidos húmicos (HA, *humic acid*), ácidos fúlvicos (HF, *fulvic acid*) y humina en función de su solubilidad en disoluciones acuosas ácidas y básicas [9]. Los HA se producen a partir de la degradación de plantas y residuos animales.

Una gran fracción de la DOM caracterizada en el agua de mar está formada por hidratos de carbono, como por ejemplo los azúcares que se liberan durante la hidrolisis de polisacáridos y otros biopolímeros. Entre los hidratos de carbono que se pueden encontrar en el agua de mar están los azúcares neutros (glucosa, galactosa, manosa, arabinosa, xilosa, ribosa y fucosa), amino azúcares (galactosamina y glucosamina), azúcares ácidos (ácido glucurónico y ácido galacturónico), y azúcares fosforilados y sulfatados [10]. Los hidratos de carbono representan el 50 – 70 % de la HMW-DOM en el agua de mar superficial, mientras que las proteínas representan el 25 – 50 % y los lípidos representan menos del 1 % de la HMW-DOM [11].

Por último, dentro de la DOM refractaria en las profundidades del océano están las moléculas alicíclicas ricas en grupos carboxilo (CRAM, *carboxyl-rich alicyclic molecules*) [12].

2.1.2. DOM y bomba biológica de carbono en el océano

La DOM marina forma parte del ciclo global del carbono y representa la mayor reserva de carbono reducido en los océanos. La exportación del DOC a través del océano es un factor importante en la bomba biológica de carbono, mediante la cual el carbono de las aguas superficiales es exportado a la profundidad del océano como se puede ver la figura 2 [13].

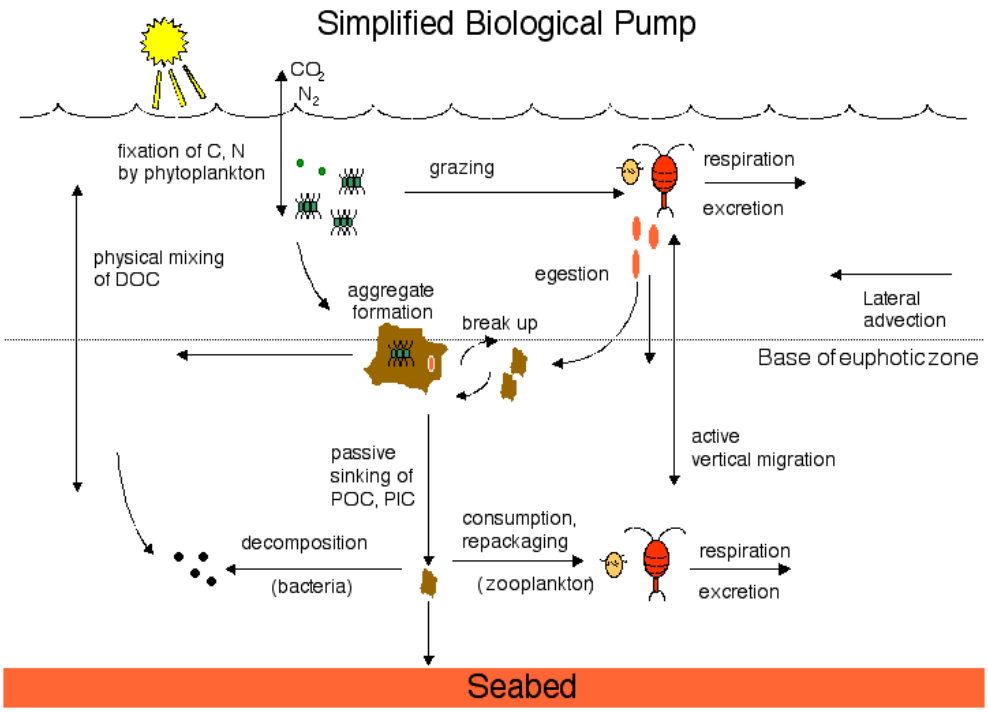

Figura 1: Esquema de la bomba biológica de carbono en el océano [15].

La bomba biológica de carbono se compone principalmente por los procesos de fotosíntesis, alimentación, respiración y descomposición. La producción de DOM se debe principalmente a la actividad fotosintética realizada por el fitoplancton en el agua de mar superficial. En este proceso, el CO_2 disuelto y los nutrientes inorgánicos son absorbidos para sintetizar materia orgánica con la ayuda de la energía de la luz solar. La mayor parte del carbono incorporado en esta materia orgánica es devuelto a la atmósfera rápidamente (en unos pocos días o semanas) a través de la respiración, y una pequeña parte del carbono es exportado hacia el fondo. La materia orgánica producida por el fitoplancton sirve de alimento para los organismos heterótrofos y como fuente de nitrógeno y fosforo para los organismos autótrofos. Pero el funcionamiento de la bomba biológica no depende únicamente de la actividad fotosintética, sino también de la alimentación del zooplancton. Una parte del carbono orgánico producido por el fitoplancton es transformado en materia fecal por el zooplancton, la cual se hunde al océano profundo. La DOM es consumida parcialmente por las bacterias y la respiración; la restante DOM refractaria es desplazada horizontalmente y mezclada en las profundidades del océano. La DOM exportada al fondo del mar es consumida y respirada, transformando así el carbono orgánico en carbono inorgánico disuelto (DIC, *dissolved inorganic carbon*). Aproximadamente solo el 1% de las partículas producidas en la superficie del océano alcanzan el fondo del mar y se consumen, respiran o depositan como sedimentos. El carbono se almacena durante millones de años en las profundidades del océano [14, 15].

2.1.3. Interacciones metal-DOM

La DOM está formada por una mezcla compleja de compuestos orgánicos con un amplio rango de tamaños moleculares, y posee una variedad de grupos funcionales que pueden actuar como bases de Lewis para la formación de complejos con iones metálicos [16]. Por lo tanto, la DOM es capaz de:

- Complejar metales e incrementar su solubilidad.
- Cambiar el estado de oxidación de las especies metálicas.

- Reducir la toxicidad y alterar la biodisponibilidad de los metales en la biota.
- Influir en la adsorción de los metales por la materia suspendida.
- Afectar a la movilidad y estabilidad de los metales que están asociados a coloides.

Las asociaciones metal-DOM son complejas, no solo dependen del metal y la materia orgánica, sino también de las condiciones fisicoquímicas del sistema: la fuerza iónica, el pH, y la competencia con otros elementos traza [17].

Por lo tanto, como la DOM tiene la capacidad de formar complejos con metales traza, va a afectar a su movilidad, toxicidad y biodisponibilidad en el medio marino. De esta forma, la DOM juega un papel importante en el control de la concentración de metales libres a un nivel óptimo para el crecimiento de los microorganismos marinos (fitoplancton y bacterias) [18]. Por ejemplo, la reducción de la toxicidad de Cu para las algas y bacterias en el agua de mar se debe a la formación de complejos con DOM, y también se reduce la toxicidad de Cu para los peces al disminuir la cantidad de iones de Cu libres por complejación [19].

2.2. Aislamiento y concentración de la DOM

La DOM está presente a bajas concentraciones (~1 mg L^{-1}) en el agua de mar, mientras que las sales están a concentraciones mucho más altas (~35 g L^{-1}), por lo que es necesario concentrarla y aislarla de las sales para su caracterización [4]. Las técnicas más usadas para pre-concentrar / separar la DOM del agua de mar son la ultrafiltración en flujo tangencial (UF, *ultrafiltration*), la extracción en fase sólida (*SPE, solid phase extraction*) y la osmosis inversa/electrodiálisis (RO / ED, *reverse osmosis / electrodyalysis*) [3].

2.2.1. Extracción en fase sólida

La SPE es una técnica en la cual la DOM es concentrada en un extractante en fase sólida. En la SPE, el analito (DOM) es separado de las sales y de la matriz de

la muestra al mismo tiempo, y con un disolvente adecuado se puede eluir la DOM concentrada y desalada de la fase sólida.

2.2.1.1. Empleo de soportes convencionales para SPE

Las fases sólidas más utilizadas para separar la DOM del agua de mar son las resinas XAD y los adsorbentes de C18 [3].

Las resinas *Amberlite* XAD son adsorbentes poliméricos con estructuras porosas que se diferencian en el tamaño de poro, área superficial y polaridad. Debido a su gran área superficial específica y a su adsorción reversible de compuestos orgánicos en disoluciones acuosas, estas resinas son adecuadas para el aislamiento de la DOM presente en aguas naturales. Las resinas XAD fueron ampliamente utilizadas por varios autores desde finales de 1970 para aislar la DOM de agua de mar [20-23]. El procedimiento más usado consiste en acidificar las muestras de agua a pH 2, y pasarlas a través del cartucho con la resina XAD; de esta forma los compuestos hidrofóbicos quedan retenidos. Se lava la resina con disoluciones de HCl y posteriormente los compuestos retenidos son eluidos con disoluciones de NaOH o metanol. Slauenwhite et al. [24] emplearon la resina XAD-2 para extraer materia orgánica en muestras de agua de mar obteniendo recuperaciones inferiores al 15 % de la DOM. Lara et al. [25] evaluaron la combinación de resinas XAD-2, XAD-4 y XAD-7 para la extracción de DOM procedente de fitoplancton marino. La recuperación de DOM para cada resina fue 32, 21 y 2 % en XAD-2, XAD-4 y XAD-7, respectivamente. Con la combinación secuencial de XAD-7/2/4 la recuperación obtenida fue del 65 %. Recientemente, Esteves et al. [26] utilizaron dos resinas de XAD-8 y XAD-4 en serie para aislar substancias húmicas del agua de mar. Usaron disoluciones de NaOH como eluyente para ambas resinas, y obtuvieron recuperaciones del 35,5 % de DOM.

Otros adsorbentes muy utilizados para aislar la DOM del agua de mar son C18. Estos adsorbentes son altamente hidrofóbicos, y como los ácidos orgánicos en el agua de mar están protonados en un rango de pH 2 - 3, bajo estas condiciones una fracción significante de DOM en el agua de mar puede ser aislada en este

tipo de adsorbentes. Normalmente para eluir la DOM adsorbida en C18 se utiliza metanol. Las condiciones de adsorción de C18 son muy similares a las de resinas XAD. Mills et al. [27] fueron de los primeros autores en emplear C18 para aislar DOM en muestras de agua de estuarios, consiguiendo aislar entre un 10 – 30 % de la DOM. Más recientemente, Simjouw et al. [28] utilizaron la SPE con C18 para aislar DOM de bajo peso molecular en muestras que habían sido previamente ultrafiltradas a través de una membrana de 1000 Da, obteniendo una recuperación del 30 % de DOM. Boris et al. [29] aislaron un 24 % de DOM en muestras de agua de mar empleando también C18 como soporte de SPE.

Por lo tanto, las resinas XAD y C18 como soportes de SPE para aislar DOM en muestras de agua de mar ofrecen unas bajas recuperaciones. Otro tipo de adsorbentes como Nexus o PPL también fueron empleados para aislar DOM pero las recuperaciones obtenidas son similares a las obtenidas con XAD y C18 [30, 31].

2.2.1.2. Empleo de nuevos soportes para SPE

Los nanotubos de carbono (CNTs, *carbon nanotubes*) son un nuevo nanomaterial de carbono, y debido a su gran área superficial han sido propuestos como un nuevo material adsorbente para un amplio rango de contaminantes, tanto inorgánicos como orgánicos en muestras acuosas [32].

Estructura y propiedades de los nanotubos de carbono

Desde su descubrimiento en el año 1991 por Simio Ijima [33], los CNTs han recibido mucha atención debido a sus excelentes propiedades y su tan pequeño tamaño.

Dentro de las diferentes formas alotrópicas del carbono, los CNTs pertenecen a la familia del fullereno. Los CNTs tienen una estructura que consiste en láminas de grafito, con los átomos de carbono enlazados covalentemente, que se pliegan de manera helicoidal dando lugar a un microtubo [34]. Se pueden clasificar en función de su estructura, y básicamente hay dos tipos de CNTs [35] :

- Nanotubos de carbono de pared simple (SWCNTs, *single-walled carbon nanotubes*): están formados por una única capa de grafito plegada alrededor de un núcleo central formando un cilindro como se puede ver en la figura 3.

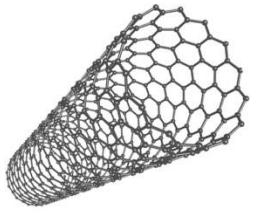

Figura 2: Estructura de un nanotubo de carbono de pared simple.

- Nanotubos de carbono de pared múltiple (MWCNTs, *multi-walled carbon nanotubes*): están formados por dos o más cilindros concéntricos de capas de grafito unidos coaxialmente a un núcleo central como se puede ver en la figura 4.

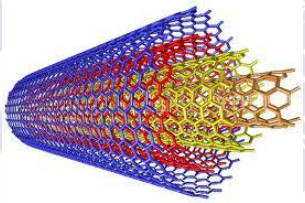

Figura 3: Estructura de un nanotubo de carbono de pared múltiple.

Entre las propiedades más destacadas de los CNTs se encuentran [34]:

- Son insolubles en agua debido a que poseen enlaces no polares y una alta relación longitud a diámetro. Se pueden solubilizar en un medio acuoso modificándolos químicamente con un surfactante.
- Pueden ser funcionalizados covalentemente. En general no son reactivos pero pueden reaccionar bajo condiciones químicas fuertes. También pueden ser inmovilizados covalentemente en soportes sólidos.

- Exhiben una excelente estabilidad térmica en atmosfera inerte, siendo estables hasta los 1200ºC. Esta propiedad es la base de su uso como fases estacionarias en cromatografía de gases.
- Poseen una elevada área superficial, por lo que se pueden utilizar como adsorbentes de SPE para separar los analitos de sus matrices.
- Son unos excelentes conductores eléctricos, esto permite su uso como sensores electroquímicos.
- Pueden resistir una fuerte flexión y deformación, y los cambios resultantes son completamente reversibles.

Nanotubos de carbono como soportes de SPE

Los CNTs tienen una gran capacidad de adsorción, la cual se debe a sus interacciones electrostáticas y a sus grandes áreas superficiales. Esto los convierte en unos excelentes adsorbentes para SPE, facilitando así que la adsorción de los analitos sea selectiva y reproducible. Por norma general, los MWCNTs debido a su estructura poseen una mayor capacidad de adsorción que los SWCNTs, y han sido utilizados por muchos autores como soportes sólidos para la extracción de diversos compuestos orgánicos en muestras de aguas naturales.

Cai et al. [36] desarrollaron un método rápido y fiable para la extracción en fase sólida de bisfenol A, 4-n-nonilfenol y 4-tert-octilfenol en diferentes muestras de agua (agua de grifo, agua de río, agua de mar y aguas residuales) con MWCNTs empaquetados en un cartucho. Los tres analitos son adsorbidos cuantitativamente en los MWCNTs empaquetados y son eluidos fácilmente de forma cuantitativa con la cantidad adecuada de metanol. Parámetros como el volumen de muestra, pH de la muestra y el volumen de eluyente fueron optimizados para obtener una mayor eficacia en la extracción, y las recuperaciones analíticas obtenidas oscilan entre 89,8 - 104,2 %. Los resultados mostraron que los MWCNTs son igual de efectivos que las resinas de C18 para la extracción 4-n-nonilfenol y 4-tert-octilfenol, pero en

cambio para el bisfenol A la extracción es mucho más efeciente con MWCNTs que con resinas de C18.

Estos mismos autores [37] también desarrollaron otro método para la extracción en fase sólida de di-etil-ftalato, di-n-propil-ftalato, di-iso-butil-ftalato y di-ciclohexil-ftalato en disoluciones acuosas con MWCNTs empaquetados en cartuchos. Este método también fue aplicado a muestras de agua de grifo, agua de mar, agua de rio y aguas residuales. Las recuperaciones de SPE con MWCNTs (80,3 - 104,5 %) fueron comparadas con las recuperaciones obtenidas con otro tipo adsorbentes como C18, C8 y PS-DVB, y los resultados mostraron que la extracción con MWCNTs es más eficiente o tan eficiente como la extracción con estos otros tipos de adsorbentes para los cuatro analitos analizados.

Zhou et al. [38-42] desarrollaron varios métodos de SPE con MWCNTs para la extracción de diferentes contaminantes orgánicos de muestras de aguas naturales. Uno de los métodos se desarrolló para la determinación de diclorodifeniltricloroetano (DDT) y sus productos de degradación (1,1-dicloro-2,2-bis-(4'-clorofenil)etano (DDD) y 1,1-dicloro-2,2-bis-(4'-clorofenil)etileno (DDE)) en muestras de agua [38]. Bajo las condiciones óptimas, el procedimiento mostró un buen rango lineal y reproducibilidad, y unos límites de detección de orden de ng L^{-1} tanto para el DDT como para el DDD y DDE. El método fue aplicado a muestras de agua de grifo y aguas subterráneas. Las recuperaciones analíticas obtenidas para el DDT, DDD y DDE están en el rango de 89,7 - 115,5 %. Estas recuperaciones fueron comparadas con las obtenidas empleando como adsorbente C18, y en general, los MWCNTs ofrecen una mayor capacidad de extracción tanto para el DDT como para sus derivados.

Estos autores también desarrollaron un método de SPE con MWCNTs para la determinación de cianacina, clorotoluron y clorobenzuron en muestras de agua de grifo, aguas subterráneas, aguas residuales y agua de nieve [39]. El método presenta una buena precisión, buen rango lineal y unos límites de detección en el rango de 0,012 - 0,034 ng mL^{-1}. Las recuperaciones obtenidas para estos tres

compuestos aplicando el método a muestras reales están en el rango 87,8 - 110,1 %. Para cianina y clorotoluron las recuperaciones obtenidas con MWCNTs son muy similares a las obtenidas con C18; en el caso del clorobenzuron la extracción es más efectiva con MWCNTs que con C18, ya que las recuperaciones obtenidas son más elevadas con MWCNTs.

Zhou et al. también desarrollaron métodos de SPE con MWCNTs para la determinación de tiametoxam, imidacloprid y acetamiprid [40], y para la determinación de simacina y atracina [41]. Ambos métodos fueron aplicados a muestras de aguas naturales y presentan una buena sensibilidad, reproducibilidad, buenos límites de detección (6.1, 5.4 and 6.7 ng L^{-1} para tiametoxam, imidacloprid y acetamiprid; y 33 and 9 ng L^{-1} para simacina y atracina) y buenas recuperaciones.

Asensio-Ramos et al. [43] estudiaron la aplicación de un método de extracción en fase sólida dispersiva (DSPE, *dispersive solid-phase extraction*) con MWCNTs para el análisis de doce pesticidas (carbaril, fensulfotion, mecoprop, fenamifos, haloxifop, diclofop, fipronil, profenofos, fonofos, disulfoton, nitrofen y terbufos) en muestras de agua. Las recuperaciones obtenidas mediante este método están entre el 71 y el 102 % para carbaril, fensulfotion, fenamifos, fipronil y fonofos; y entre el 36 y el 64 % para profenofos, disulfoton, nitrofen y terbufos. Para intentar mejorar las bajas recuperaciones para algunos de los pesticidas se evaluaron diferentes eluyentes, así como mezclas de ellos, pero los resultados fueron similares, por lo tanto, estas bajas recuperaciones se pueden deber a una extracción incompleta con este tipo de CNTs. Las recuperaciones obtenidas utilizando C18 como adsorbente para algunos de estos pesticidas están entre el 36 y el 100 %, por lo tanto, la eficacia de la extracción es similar a la obtenida con los MWCNTs.

Finalmente, Ma et al. [44] emplearon también los MWCNTs como soportes de SPE para la determinación de dieciséis hidrocarburos aromáticos plicíclicos (PAHs) en muestras de agua de grifo, agua de río y agua de mar. El método presenta una

buena precisión y unos buenos límites de detección. Las recuperaciones obtenidas están dentro del rango 76,0 - 125,0 % para muestras de agua de río, 74,5 - 127,0 % para muestras de agua de grifo y 70,0 - 122,0 % para muestras de agua de mar. En la mayoría de los casos, las bajas recuperaciones obtenidas corresponden a los PAHs con mayor número de anillos aromáticos. Esto es debido a que cuanto mayor sea la cantidad de anillos aromáticos mayor es la interacción de estos compuestos con los MWNCTs y, por tanto, su elución es más difícil. El método también fue aplicado utilizando C18 como adsorbente y los resultados mostraron que los PAHs se adsorben más fácilmente en MWCNTs que en C18.

En resumen, los MWCNTs tienen un gran potencial como soportes de SPE para la extracción de un amplio un grupo de compuestos orgánicos en muestras acuosas. Sin embargo, los MWCNTs apenas han sido utilizados para llevar a cabo la determinación de DOM. Una de las pocas aplicaciones son las de Lu et al. [45], en las cuales se emplearon los MWCNTs como adsorbentes para la extracción de DOM en disoluciones acuosas. Los MWCNTs fueron sometidos a un tratamiento térmico (400ºC durante 60 min) para la eliminación del carbono amorfo, modificando así sus propiedades fisicoquímicas y aumentando su capacidad de adsorción. Los experimentos de adsorción fueron llevados a cabo en modo discontinuo con 30 mg de MWCNTs y 200 mL de disolución de DOM. Los resultados de estos experimentos muestran que la cantidad de DOM adsorbida se incrementa al aumentar la concentración inicial de DOM y la fuerza iónica, pero disminuye al aumentar el pH de la disolución.

En otro trabajo encontrado en la bibliografía, Su et al. [46] también trataron los MWCNTs térmicamente para utilizarlos como adsorbentes para el estudio cinético y termodinámico de la adsorción de DOM en disoluciones acuosas. La cinética de adsorción sigue la ley de velocidad de primer orden, mientras que la termodinámica de adsorción indica que es de naturaleza espontánea y exotérmica. Los experimentos de adsorción fueron llevados a cabo en modo discontinuo con 30 mg de MWCNTs y 200 mL de disolución de DOM. El estudio del efecto del tiempo de contacto y temperatura mostró que para todos los experimentos la

cantidad de DOM adsorbida incrementaba rápidamente con el tiempo hasta llegar al equilibrio, y la cantidad de DOM adsorbida una vez alcanzado el equilibrio disminuía al aumentar la temperatura. La comparación entre las propiedades de adsorción de MWCNTs y carbon activado granular (GAC, *granular activated carbon*) reveló que los nanotubos de carbono tienen un mejor rendimiento de adsorción.

En ambos trabajos se realizó un estudio comparativo entre la adsorción de DOM sobre MWCNTs y sobre GAC. En las mismas condiciones, los MWCNTs tratados térmicamente tienen el mayor rendimiento de adsorción de DOM, seguidos de los MWCNTs en bruto y luego el GAC. Esto sugiere que los MWCNTs son adsorbentes muy eficientes para DOM.

2.2.2. Ultrafiltración en flujo tangencial

La ultrafiltración en flujo tangencial es una técnica de separación en la cual las substancias son separadas en función de su peso y tamaño molecular usando unas membranas con un tamaño de poro determinado. Hay una variedad de materiales, tamaños de membrana, y tamaños de poro comercialmente disponibles, por lo que la selección de la membrana es un factor muy importante a la hora de realizar estudios de UF. Materiales como celulosa regenerada, acetato de celulosa, polietersulfonas y fluoropolímeros son usados para la fabricación de membranas de UF, y cada uno posee diferentes propiedades de absorción y estabilidad. La selectividad de una membrana de UF se caracteriza por el peso molecular de corte (MWCO, *molecular-weight cut-off*), el cual es operacionalmente definido como el peso molecular más pequeño que será retenido un 95% por la membrana durante el proceso de UF. La UF no garantiza la eliminación de sales, que pueden ser retenidas por las membranas, y por lo tanto es necesaria una etapa de eliminación de sales posterior a la UF. La diafiltración o la cromatografía de intercambio ionico son usadas comúnmente para eliminar residuos de iones inorgánicos del retentate (fracción de muestra que no atraviesa la membrana) [3].

La ultrafiltración es una técnica que nos permite concentrar y recuperar simultáneamente un analito a partir de grandes volúmenes de muestras acuosas diluidas. Esto hace que la UF sea una excelente elección para la recuperación y concentración de biomoléculas de muestras tanto de agua dulce como de agua de mar. Debido a esta función dual la UF ha sido ampliamente utilizada para aislar y concentrar la DOM marina.

Benner et al. [47] usaron una membrana de UF en flujo tangencial con un MWCO de 1000 Da para aislar DOM marina de muestras de agua de mar recogidas a tres profundidades diferentes (10 m, 765 m y 4000 m) en el norte del Océano Pacífico. Obtuvieron recuperaciones de DOC entre el 33 y 22 %, disminuyendo estas al aumentar la profundidad. Posteriormente, Benner et al. [48] emplearon el método de UF para aislar la DOM de muestras de agua de mar recogidas a diferentes profundidades y en diferentes regiones geográficas del Océano Pacífico y del Océano Atlántico. Las recuperación media obtenida de DOC de las 24 muestras de DOM marina fue 26 ± 5 %. Los resultados del estudio de la abundancia y la distribución de tamaños tanto de la POM como de la DOM indicaron que el 75 % del carbono orgánico marino forma parte de la fracción de DOM de bajo peso molecular (<1 nm), el 24 % procede de la fracción de DOM de alto peso molecular (1-100 nm), y el 1 % se corresponde con la fracción de POM (>100 nm).

Varios grupos de investigación han empleado la UF en flujo tangencial para aislar DOM de muestras de agua con un amplio rango de salinidad. Por ejemplo, Benner et al. [49] recogieron 31 muestras de agua en cuatro temporadas diferentes con un amplio gradiente de salinidad desde el río Mississippi hasta el Golfo de México para la determinación del DOC y aislar la DOM por UF (membrana 1000Da). Las recuperaciones de DOC obtenidas mediante UF varian entre un 49 % correspondiente al agua de río y un 22 % correspondiente al agua de mar. Los resultados mostraron que los porcentajes de recuperación de DOC disminuyen linealmente a través del gradiente de salinidad. En otro trabajo, Benner et al. [50] también usaron la UF para aislar la DOM de 67 muestras de agua de río, estuario y de agua de mar del Ártico. Los porcentajes de recuperación de DOC aislado de

aguas de río y estuario varían entre el 52 % y el 64 %; y los porcentajes correspondientes a DOC aislado de muestras de agua de mar, tanto superficiales como profundas, varían entre el 21 % y el 32 %. Guo et al. [51] también emplearon la UF para aislar la DOM de muestras recogidas en el Golfo de México y en el océano Atlántico con diferentes salinidades. Los resultados mostraron recuperaciones de DOC entre 31 % y 68 % para la fracción de DOM aislada con una membrana de UF de 1000 Da.

Tanoue et al. [52] y Powel et al. [53] emplean la UF en flujo tangencial para la concentración de proteínas disueltas en el agua de mar empleando para ello una membrana de UF con un tamaño de poro de 10000 Da. Powel et al. realizaron un estudio de los parámetros y procesos que afectan al rendimiento de recuperación utilizando la UF en flujo tangencial para la concentración y purificación de una mezcla de proteínas disueltas en agua de mar artificial. Para ello optimizaron las condiciones para minimizar la adsorción de proteínas en los componentes del sistema de UF, añadieron dodecilsulfato sódico (SDS, *sodium dodecyl sulfate*) a las muestras de agua y realizaron un tratamiento con un agente bloqueante de superficie (SBA, *surface blocking agent*) para saturar los sitios de adsorción no específicos. Para este estudio emplearon una muestra de proteínas procedentes de células *E. coli* marcadas radiactivamente que se concentraron a partir de 60 y 20 L de agua de mar artificial. Los resultados mostraron que el pre-tratamiento con SBA dio lugar a porcentajes de recuperación más fiables y reproducibles (55 – 60 %) en comparación con la recuperación inicial (46 %). También investigaron los efectos de la concentración de proteína en la muestra en la recuperación. A baja concentración de proteínas (1,0 µg proteína / L) el porcentaje de recuperación se redujo, mientras que a concentraciones superiores de 2.0 µg proteína / L, el porcentaje de recuperación fue casi constante.

Hay estudios en los que se realiza la comparación entre la UF y la SPE para el aislamiento y concentración de la DOM. Simjouw et al. [28] realizan la comparación entre la UF y la SPE con C18 como métodos para aislar la DOM en muestras de agua recogidas en estuarios. Las recuperaciones obtenidas mediante

UF tienen un valor alrededor del 51 % de DOC y las recuperaciones obtenidas mediante SPE con C18 fueron entre un 10 – 20 % más pequeñas que las obtenidas por UF. Hay que decir que la SPE con C18 se impone una separación química, mientras que en la UF se impone una separación por tamaños. Para el aislamiento y la concentración de la mayor proporción de DOM en aguas costeras y en estuarios la UF ofrece unas recuperaciones mucho mejores, esto se debe a que esta fracción de DOM parece ser rica en hidratos de carbono y proteínas.

2.2.3. Osmosis inversa/electrodiálisis

La osmosis inversa (RO) es un procedimiento utilizado para concentrar la DOM presente en el agua de mar mientras que, la electrodiálisis (ED) es usada para eliminar las sales. El acoplamiento RO/ED fue desarrollado inicialmente por Koprivnjak et al. [54] para concentrar y purificar NOM en muestras de aguas de ríos contaminadas con ácido sulfúrico y acido silícico. Con este procedimiento consiguieron eliminar casi todo el H_2SO_4 y H_4SiO_4 de las muestras con un 84 % de recuperación promedio del carbono orgánico total (TOC, *total organic carbon*).

El procedimiento RO/ED para aislar DOM del agua de mar fue desarrollado por Vetter et al. [55], y más adelante este proceso fue descrito detalladamente por Gurtler et al. [56]. En general, el proceso RO/ED se puede dividir en tres fases operacionales. En la fase inicial ED, la salinidad de la muestra se reduce de 50 a 15 mS cm^{-1}, lo cual causa que la presión osmótica de la muestra disminuya de 379 psi a 114 psi. Durante la fase de RO/ED, se elimina el agua por RO a una presión entre 200-250 psi hasta que el volumen de la muestra se reduce a 10 L o menos y las sales se eliminan por ED para mantener la conductividad a 15 mS cm^{-1}. En última fase, la conductividad de la muestra de DOM concentrada se reduce hasta el valor final deseado. Normalmente, muestras de 200 L de agua de mar con una conductividad de 50 mS cm^{-1} son procesadas en 6 - 8 h para obtener 6 L de muestra con una conductividad de 0,5 µS cm^{-1}. Este procedimiento permite obtener recuperaciones entre el 60 – 90 % de DOC, mientras que se elimina el 99 % de las sales.

Recientemente, Koprivnjak et al. [57] emplearon la RO/ED para aislar la DOM de 16 muestras de agua de mar. Las recuperaciones de DOC obtenidas para las 16 muestras están entre el 61 % y el 95 %, con una recuperación media del 75 ± 12 %.

En resumen, las recuperaciones obtenidas con este método de RO/ED son mucho mayores que las obtenidas mediante SPE con adsorbentes como XAD o C18.

2.3. Caracterización de la DOM

La masa molecular es una de las propiedades utilizada habitualmente para caracterizar la materia orgánica disuelta. La cromatografía de alta resolución por exclusión de tamaños (HPSEC, *high-performance size exclusion chromatography*) con múltiples detectores en línea (UVA, TOC y fluorescencia) ha sido ampliamente utilizada para la caracterización de la materia orgánica disuelta en función de su masa molecular.

Minor et al. [4] emplearon la HPSEC y espectrometría de masas resuelta por temperatura (DT-MS, *direct temperature-resolved mass spectrometry*) para estudiar la distribución de pesos moleculares y las características a nivel molecular de una muestra de materia orgánica disuelta en agua de mar concentrada mediante UF (membrana 1000 Da). Tanto los cromatogramas de IR como los cromatogramas de UV/Vis muestran una separación básica de la DOM en fracciones de bajo y alto peso molecular. Estas fracciones de tamaños moleculares fueron analizadas por DT-MS. Los resultados mostraron que los componentes de alto peso molecular de DOM son ricos en aminoazúcares, desoxiazúcares y azúcares metilados, mientras que los componentes de bajo peso molecular son ricos en hexosas.

Her et al. [5] optimizaron un método para la detección y caracterización de NOM mediante HPSEC con detección UV y detección TOC en línea. Con solo unas pocas modificaciones un analizador de TOC disponible comercialmente sirvió como detector de DOC, integrado con HPSEC para medir TOC junto con UV. Her

et al. [58] emplearon la HPSEC con detección de absorbancia ultravioleta (UV) para estimar el peso molecular y la distribución de los pesos moleculares de la materia orgánica natural (NOM), y compararon estos resultados con los obtenidos mediante HPSEC con un detector TOC. La comparación de un cromatograma obtenido por HPSEC-TOC muestra diferencias significativas con el correspondiente cromatograma obtenido por HPSEC-UV, obteniendo diferentes valores de pesos moleculares. La NOM de diferentes aguas naturales fue caracterizada para demostrar los diferentes MWs obtenidos con los detectores. Los cromatogramas obtenidos por HPSEC-TOC mostraron picos de NOM que no fueron detectados por UVA, por lo tanto empleando una detección TOC en línea se puede obtener una mejor representación de los pesos moleculares de NOM.

Müller et al. [59] emplearon la HPSEC para fraccionar en función del peso molecular una muestra de materia orgánica natural acuática que fue concentrada por UF. Realizaron un estudio de la estabilidad en el tiempo de las fracciones y no se observaron cambios significativos en los cromatogramas después de cinco semanas, lo que sugiere una alta estabilidad de las fracciones ya que casi no se produjeron alteraciones en la distribución de pesos moleculares durante este tiempo.

Her et al. [60] utilizaron HPSEC con detectores en línea secuenciales de UV, TOC y fluorescencia para estudiar la composición de la DOM en función de sus tamaños moleculares. Este sistema proporciona información cuantitativa y cualitativa sobre los componentes de pesos moleculares de DOM, incluyendo la proporción de DOC (por medición de TOC), aromaticidad (por comparación de mediciones UV y TOC) y propiedades químicas (por medición de fluorescencia). Se analizaron tres tipos de muestras agua diferentes (subterránea, de lago y agua residual) utilizando el sistema de HPSEC-UV-fluorescencia-TOC. Los resultados mostraron que la fracción de DOM de alto peso molecular aparente está formada por polisacáridos en el caso de agua subterránea, y por una mezcla de polisacáridos y proteínas en el caso del agua de lago y residual. Las fracciones de DOM en el intervalo de pesos moleculares medios aparentes mostraron una

mayor aromaticidad, lo cual indica un mayor carácter fúlvico. Para la fracción de DOM en el rango de bajo peso molecular aparente se encontró materia orgánica de carácter alifático.

Recientemente, Yan et al. [61] emplearon la HPSEC acoplada con un detector de absorbancia de longitud de onda múltiple (200 – 445 nm) para estudiar la distribución de pesos moleculares aparentes de la DOM. Se utilizaron estándares de DOM (ácidos húmicos, ácidos fúlvicos y ácidos hidrófilos) para determinar el rendimiento y la sensibilidad del método. Los cromatogramas obtenidos para los ácidos húmicos y fúlvicos exhiben cuatro picos con máximos encontrados a 16 kDa (P1), 11 kDa (P2), 5 kDa (P3) y 3 kDa (P4), mientras que el perfil cromatográfico para los ácidos hidrófilos solo muestra tres picos que se corresponden con P1, P2 y P4. Las intensidades relativas de cada pico en las tres muestras fueron significativamente diferentes, esto se debe a que tanto los ácidos húmicos como los ácidos fúlvicos tienen un alto contenido en compuestos aromáticos con elevado peso molecular. Como resultado de esto, los picos P1 y P2 tienen intensidades relativas altas. Por el contrario, los ácidos hidrófilos están compuestos por una fracción hidrófila con pesos moleculares bajos y la contribución a la fracción de bajo peso molecular (P4) fue mayor. Las intensidades de absorción a diferentes tiempos de elución disminuyeron cuando la longitud de onda aumentaba.

La HPSEC también ha sido utilizada para el estudio de metales traza asociados a las diferentes fracciones de tamaños moleculares de DOM mediante el acoplamiento con la espectrofometría de absorción UV-Vis y la espectrometría de masas por plasma de acoplamiento inductivo (ICP-MS, *inductively coupled plasma mass spectrometry*).

Wu et al. [62] investigaron la formación de complejos de los metales Fe, V, Ce, Th, U, Mo, Cu, Ni, Co, Cr, Zn, Pb y Cd con diferentes fracciones de pesos moleculares de DOM en muestras de aguas naturales. Para evaluar la distribución de los pesos moleculares de los complejos metal-DOM las muestras fueron

concentradas por osmosis inversa y analizadas por HPSEC-UV/Vis-ICP-MS. Los resultados mostraron que los pesos moleculares promedio de los complejos metal-DOM siguieron el siguiente orden: Cu > Ni > (Co, Cr, Zn) > Pb > Cd, lo que concuerda con la serie de Irving – Williams relativa a la estabilidad de los complejos formados entre metales divalentes y ligandos, ácidos fúlvicos y proteínas. Los pesos moleculares de complejos metal-DOM para otros metales siguió este orden: (Fe, V, Ce) > Th > U > Mo. Los resultados sugieren que la distribución de metales en las diferentes fracciones de pesos moleculares está estrechamente relacionada con la fuerza de unión del metal. Metales con alta fuerza de unión se distribuyeron entre las fracciones de elevado peso molecular, mientras que los metales con una fuerza de unión más baja se distribuyeron entre las fracciones de menor peso molecular.

Park et al. [7] emplearon la HPSEC con detección UV, fluorescencia e ICP-MS para la caracterización de DOM y sus interacciones con metales en tres tipos de aguas superficiales (arroyo de un bosque, pantano y río urbano). Los cromatogramas obtenidos con detección UV y fluorescencia a 337/423 nm (Ex/Em) para la detección de fúlvicos (FFL, *fulvic-like fluorescence*) muestran patrones de elución similares para las fracciones que van desde los 208 a 1360 Da, lo que sugiere que estas fracciones están compuestas principalmente por sustancias húmicas ricas en compuestos aromáticos. El perfil cromatográfico obtenido con detección fluorescente a 278/353 nm para la detección de proteínas (PFL*, protein-like fluorescence*) muestra once fracciones de pesos moleculares, tanto de alto como de bajo peso molecular, así como las fracciones húmicas detectadas anteriormente por UV y fluorescencia. Los cambios temporales en las concentraciones de Al, Cr, Li, Ni y Fe están correlacionados con las intensidades de las fracciones de bajo peso molecular detectadas por PFL, mientras que variaciones en las concentraciones de As, Co, Mn y Pb fueron relacionados con las intensidades de las fracciones húmicas detectadas por UV y PFL.

Laborda et al. [63, 64] emplearon HPSEC con detección de absorción UV para la detección de la materia orgánica y con detección ICP-MS para la detección

elemental, en el estudio de la movilización de metales en compost en función del pH y del peso molecular de sus complejos con DOM. Para diferenciar entre las contribuciones de ácidos húmicos y ácidos fúlvicos a la DOM movilizada en el intervalo de pH 5 - 10 llevaron a cabo la deconvolución de los perfiles cromatográficos detectados por absorción UV. Los resultados mostraron una mayor contribución de ácidos fúlvicos a valores de pH bajos, y un aumento de la contribución de ácidos húmicos a medida que el pH se hacía más básico. Se aplicó también un procedimiento similar de deconvolución a los cromatogramas obtenidos mediante ICP-MS para los metales Co, Cu, Pb y Bi. En general, se observó que tanto los ácidos húmicos como los ácidos fúlvicos contribuyen a la movilización de metales divalentes, como Co y Cu, mientras que el Bi y el Pb se asocian preferiblemente con ácidos húmicos. Además de los ácidos hidrofóbicos (ácidos húmicos y ácidos fúlvicos), los compuestos hidrofílicos también contribuyen a la movilización de metales en DOM de muestras de compost. En general, los compuestos hidrofílicos forman complejos con hasta un 50 % de los metales lixiviados.

Además de HPSEC, el fraccionamiento en flujo por campo de flujo asimétrico (AsFlFFF, *asymmetric flow field-flow fractionation*) también ha sido empleada para la caracterización de complejos metal-DOM. Bolea et al. [65] utilizaron AsFlFFF acoplado a un sistema ICP-MS para estudiar la contribución de tres fracciones de DOM procedente de compost a la movilización de metales traza. Las tres fracciones de DOM estudiadas son mircropartículas mayores de 1 µm, nanocoloides desde 1 µm hasta 15 nm, y macromoléculas menores de 1000 kDa. En las muestras estudiadas observaron que las partículas y macromoléculas contribuyen en mayor medida a la movilización de la mayoría de los metales estudiados. Pb y Zn se movilizan principalmente asociados a partículas en comparación con el Cu que se moviliza principalmente asociado a macromoléculas. Por el contrario, los coloides solo contribuyen significativamente a la movilización de algún elemento como en el caso de la Ag y el Si. Bolea et al. [66] también emplearon la UF, HPSEC y AsFlFFF acopladas a un sistema de

detección UV-Vis y a un sistema de detección ICP-MS para el estudio de las posibles interacciones de 30 elementos con fracciones de diferentes tamaños de sustancias húmicas en un lixiviado de compost. La materia orgánica está constituida por una mezcla de sustancias húmicas que van desde pesos moleculares bajos (alrededor de 1 kDa) hasta pesos moleculares significativamente más grandes. Los resultados mostraron que los elementos se pueden clasificar en tres grandes grupos de acuerdo con su asociación a sustancias húmicas de diferente tamaño y peso molecular. El primer grupo está constituido principalmente por iones metálicos alcalinos monovalentes como B, W, Mo y As existentes como oxoaniones y que no están significativamente asociados a sustancias húmicas. El segundo grupo está formado por cationes divalentes (Ni, Cu, Cr y Co) que muestran una tendencia evidente a estar asociados a las fracciones orgánicas más pequeñas. Por último un tercer grupo formado por cationes tri- y tetravalentes como Al, Fe, los lántanidos, Sn y Th que están asociados a las fracciones de mayor tamaño. La información obtenida de las tres técnicas de fraccionamiento proporcionan una clasificación en función del tamaño y distribución de sustancias húmicas bastante consistente a pesar de que se observaron algunas discrepancias.

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3. Proteínas marinas

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Proteínas marinas

3.1. Introducción: metaloproteínas y metalómica

La metalómica es el estudio del metaloma, es decir, de las interacciones y conexiones funcionales de iones metálicos y sus especies con genes, proteínas, metabolitos y otras biomoléculas dentro de un organismo y ecosistema. El término metaloma se definió inicialmente como la distribución de un elemento, como las concentraciones en equilibrio de los iones metálicos libres, o como el contenido libre de un elemento en un compartimento celular, célula u organismo. En la actualidad, el término metaloma se refiere a la totalidad de las especies de metal o metaloides presentes en una célula o tipo de tejido, y a su identidad, cantidad y localización [1].

La metalómica o estudio del metaloma nos proporcionará información de como un elemento (metal o metaloide) está distribuido en un compartimento celular; su coordinación medioambiental, en que biomolécula se incorpora o porque un bioligando fue complejado; y la concentración de las diferentes especies metálicas presentes [2].

Elementos en baja concentración, como los iones metálicos, juegan un papel importante en la actividad biológica. El estudio de estas especies metálicas es esencial para comprender su toxicología y su impacto bioquímico en sistemas biológicos. Muchos de estos iones metálicos están unidos a proteínas o enzimas, y ejercen sus efectos como centros activos o estructurales de las proteínas. Las metaloproteínas y los complejos metal-proteínas representan una gran parte de la totalidad de proteínas. Alrededor del 40 % de todas las proteínas y enzimas contienen iones metálicos en sus estructuras. Cuando el metal se une a la proteína mediante interacciones de alta afinidad, es decir, que no se pierden durante la manipulación de la muestra, reciben el nombre de metaloproteínas; por el contrario, cuando el metal se une a la proteína mediante interacciones de baja afinidad y se rompen fácilmente, se llaman complejos metal-proteína [3]. Una

metaloproteína se define como una proteína a la cual un metal le confiere una determinada función. Esta función puede ser, por ejemplo, el desarrollo de una actividad catalítica, la implicación en una reacción de transferencia electrónica, o la estabilización de su propia estructura terciaria o cuaternaria. Generalmente, el metal está unido a la proteína como un ion hidratado o como un cofactor que contiene el metal. Por el contrario, en las proteínas que complejan un metal debido a un equilibrio termodinámico existente en el sistema, el metal no tiene una función determinada [3].

Los principales grupos de proteínas de interés incluyen *metaloenzimas*, *proteínas transportadoras de metales* y *proteínas de estrés metálico* [2].

- *Metaloenzimas*. Los iones metálicos son cofactores esenciales para la expresión funcional de muchas proteínas en los organismos vivos. En las células los elementos traza son necesarios para activar y estabilizar las enzimas. Estos metales generalmente son iones metálicos de transición con radios atómicos pequeños (Cu, Fe, Zn, Mn, Mg, Ni), e interactúan con la proteína a través de interacciones electromagnéticas y electrostáticas.
- *Proteínas transportadoras de metales*. Son proteínas ricas en cisteína, como la albúmina y transferrina, que aseguran el transporte de elementos pesados y esenciales en los organismos.
- *Proteínas de estrés metálico.* Algunos organismos se defienden del estrés provocado por metales pesados mediante la síntesis de proteínas capaces de complejar el exceso de metal. Un grupo de estas proteínas son las metalotioneínas, proteínas de bajo peso molecular ricas en cisteína, y resistentes a la termocoagulación y precipitación ácida.

Algunos de los metales unidos a proteínas y enzimas son el Co, Ni, Cu, Zn, Mo, Cr, Mn, Fe y Pb. En la tabla 1 se muestran algunos ejemplos de metaloproteínas [4].

Tabla 1: Ejemplos de algunas metaloproteínas.

Elemento	Proteína
Se	*Glutatión peroxidasa*: una enzima que cataliza la reducción de los peróxidos y protege las células del daño oxidativo.
Cr	*Transferrina* (proteína plasmática): transporta el Cr(III) por todo el cuerpo humano en las células sanguíneas.
Cu	*Ceruloplasmina* (proteína del suero humano), *ascorbato oxidasa* (plantas y bacterias), *plastocianina* (plantas y cianobacterias), *superoxido dismutasa, tirosinasa, citocromo oxidasa y hemocupreína*.
Pb	Aproximadamente el 95% del plomo total en la sangre humana se une a los eritrocitos. En la mayoría de los eritrocitos, el plomo está unido dentro de la célula en la *hemoglobina*. En los fluidos extracelulares el plomo se une a la albúmina y a algunas proteínas de alto peso molecular (*globulinas*).
Zn	El zinc forma parte de más de 200 enzimas y proteínas. Los principales ejemplos son la *insulina* y *carboxipeptidasa A*.
Mn	Este metal se encuentra en una variedad de enzimas como pueden ser la *piruvato carboxilasa* y *oxalacetato descarboxilasa*. También se puede encontrar en proteínas como la g*lutamina sintetasa, β-globulina* y *albúmina*.
Fe	Las proteínas que contienen hierro se clasifican en dos categorías. Las proteínas con Fe hemo, donde el metal es quelado por porfirinas (un ligando insoluble en agua). Ejemplos de proteínas hemo son la *hemoglobina*, la *mioglobina* y los *citocromos*. Las proteínas formadas por Fe no hemo como por ejemplo la *transferrina, ferritina, ovotrasferrina, caseína, albúmina* y *hemosiderina.*

Los metales representan un vínculo entre la química de la atmósfera y océanos, donde la vida ha evolucionado, con la genómica y proteómica de los seres vivos. Un aspecto de la evolución se debe a la adaptación de los organismos a los cambios en el entorno con el fin de proteger su química citoplasmática y obtener los elementos necesarios de su entorno [5]. Para conocer por completo el metabolismo de los metales de transición es necesario entender como los

organismos detectan, adaptan y usan estos metales dentro de la biodiversidad característica de cada ecosistema. La contaminación medioambiental con metales provoca la evolución de la expresión de proteínas y estimula la asignación de algunas proteínas como biomarcadores de contaminación medioambiental [6].

La especificidad de los estudios de metaloproteínas exige la necesidad de describir los sitios de unión metal-proteína, la estequiometría del metal y los cambios estructurales requeridos para la formación del enlace metal-proteína. Por lo tanto, en el estudio de metaloproteínas es necesario conservar el enlace entre el metal y la proteína, desarrollar metodologías que permitan la detección específica de metaloproteínas en la muestra, y la purificación y/o preconcentración de las proteínas que contienen metales [7].

Debido a que las metaloproteínas están presentes en las muestras biológicas con concentraciones a niveles traza se requiere la combinación de técnicas con una alta resolución para la separación de proteínas con técnicas lo suficiente sensibles para la detección de metales. Las técnicas con alta resolución más empleadas para la separación de proteínas son la electroforesis en gel, electroforesis capilar, y la cromatografía liquida de alta resolución [3]. La espectrometría de masas con plasma acoplado inductivamente (ICP-MS) es la técnica analítica más empleada en metalómica para la detección de los metales en las asociaciones metal-proteína. Su ventaja indiscutible es la capacidad de discriminación entre el metal ligado a la proteína y el metal libre en HPLC o electroforesis capilar, o mediante ablación laser en electroforesis en gel bidimensional [1].

En paralelo a la detección de metales asociados a proteínas son necesarias también técnicas para la identificación de estas proteínas que actúan como ligandos. La espectrometría de masas (MS) es una de las herramientas más importantes en el campo de la bioquímica. Permite determinar el peso molecular exacto de los compuestos mediante la separación de los iones moleculares en función de su relación masa-carga (m / z) y, adicionalmente, nos da información estructural significativa. Los iones moleculares se forman mediante la inducción de

una ganancia o pérdida de carga (por ejemplo, pérdida de electrones, desprotonación o protonación). Las técnicas de ionización más comunes en el análisis de biomoléculas son la desorción/ionización láser asistida por matriz (MALDI, *matrix-assisted laser desorption/ionization*) y la ionización por electroespray (ESI, *electrospray ionization*). Del acoplamiento de la espectrometría de masas con las técnicas de ionización resultan la MALDI-TOF-MS (*matrix-assisted laser desorption/ionization - time of flight mass spectrometry*) y la ESI-MS [3].

3.2. Proteínas disueltas en el agua de mar

Las proteínas son polímeros y están formadas por una mezcla de 20 aminoácidos codificados genéticamente que son los monómeros. La composición y la secuencia de una proteína pueden ser específicas para un organismo determinado y/o función celular. Las proteínas tienen muchas funciones importantes incluyendo la integridad estructural, la transferencia de energía, y la muerte celular. La expresión de la proteína es un indicador importante del estado celular y puede proporcionar información sobre la activación de varias vías celulares. En el medio marino, ya sea en la fracción de materia orgánica disuelta o en la fracción de materia orgánica particulada, las proteínas pueden proporcionar información sobre los mecanismos que controlan la degradación de la materia orgánica. Las proteínas y sus precursores, los aminoácidos, están presentes en el medio marino y son considerados un importante contribuyente a los reservorios de carbono y nitrógeno en el océano [8].

3.3. Plancton marino

El plancton marino está formado por un conjunto de organismos, tanto vegetales como animales, adultos y larvarios, que viven en el agua de mar, flotando o dotados de escasos elementos de locomoción; generalmente presentan tamaño microscópico y para medirlos se utiliza la micra (la milésima parte de un milímetro) [9].

Tradicionalmente, el plancton se puede clasificar en dos grupos principales, fitoplancton (plancton vegetal) y zooplancton (plancton animal) [10]. El fitoplancton está formado principalmente por cianobacterias fotosintéticas y algas unicelulares, y se encuentra flotando en las capas superficiales del océano iluminadas por el sol. El fitoplancton es el principal alimento del zooplancton, por lo que constituye el primer eslabón de la cadena alimentaria. El zooplancton está formado por distintas especies, generalmente de tamaño microscópico, tales como medusas, esponjas y pequeños animales marinos como larvas de peces y moluscos. Los organismos que forman parte del zooplancton pueden pasar toda su vida formando parte del plancton, llamándose holoplanctónicos, como es el caso de diminutos crustáceos denominados copépodos. En otros casos, un mismo organismo pasa la época juvenil de su vida formando parte del plancton y cuando es adulto vive en el fondo del mar, como por ejemplo las estrellas de mar y las medusas; a estos organismos se les llama meroplanctónicos.

Otra forma de clasificar plancton es en función de su tamaño como se puede ver en la tabla 2.

Tabla 2: Clasificación del plancton en función de su tamaño.

Tipo de plancton	Tamaño
Femtoplancton	<0,2 µm
Picoplancton	0,2-2 µm
Nanoplancton	2-20 µm
Microplancton	20-200 µm
Mesoplancton	200 µm – 2 mm
Macroplancton	2-20 mm
Megaloplankton	>20 mm

El fitoplancton oceánico es responsable de casi la mitad de la fijación fotosintética de carbono en la tierra. El CO_2 se convierte en materia orgánica a través de la

fotosíntesis en el fitoplancton. De esta manera, el plancton forma parte del ciclo de carbono y de la bomba biológica de carbono (véase sección 2.1.2).

Además de los diversos macro- y micro-nutrientes, el agua de mar contiene metales tanto esenciales como tóxicos [11]. Una docena de elementos con masa superior a 50 son conocidos por desarrollar un papel biológico, a menudo como cofactores en enzimas y como elementos estructurales en proteínas. De estos, metales traza como el Mn, Fe, Co, Ni, Cu y Zn han sido ampliamente estudiados. Estos metales son requeridos por el fitoplancton para realizar funciones metabólicas. Una deficiencia de estos metales bioactivos puede limitar la producción de plancton en el océano, y un exceso de algunos de estos metales puede inhibir su crecimiento [12]. El Cu forma parte de proteínas y oxidasas en la respiración. Sin embargo, una elevada concentración de Cu^{2+} reduce las tasas de división celular en el fitoplancton. La toxicidad del Cu como está determinada por la concentración del ion metálico libre, se reduce mediante la complejación del metal libre por compuestos quelatantes. La toxicidad del Cu también depende de la especie de microorganismo marino, siendo las cianobacterias uno de los más sensibles a la toxicidad de Cu. Otro metal representativo en el medio marino es el Fe, el cual es uno de los elementos bioactivos más importantes para el crecimiento del fitoplancton. El Fe es un nutriente limitante para la producción de fitoplancton en las zonas con altas concentraciones de nutrientes pero bajas concentraciones de clorofila, siendo el océano abierto la región con mayor deficiencia de Fe [13].

El fitoplancton afecta a la química de metales traza en las aguas naturales y oceánicas no sólo por las reacciones de superficie, sino también por la absorción de metales y por la producción de materia orgánica extracelular con propiedades para complejar metales. La liberación de materia orgánica extracelular por parte del fitoplancton marino es la fuente más importante de sustrato lábil para la DOM en el océano. Tanto los productos directos de exudación extracelular como los productos secundarios tienen la capacidad de complejar metales traza después de la modificación bioquímica. La producción de compuestos orgánicos extracelulares

por el fitoplancton depende del estado fisiológico de las células, así como de factores ambientales como la temperatura, concentración de nutrientes, intensidad de luz, del zooplancton, y de la presencia de compuestos tóxicos en el medio. La producción extracelular de ligandos orgánicos por el fitoplancton es uno de los factores más importantes para controlar los efectos biológicos producidos por las interacciones metal-DOM [14].

3.4. Detección y caracterización de proteínas disueltas en agua de mar

Entre los trabajos presentes en la bibliografía referentes al estudio de proteínas disueltas en agua de mar destacan los realizados por Tanoue [15-22]. Este autor fue uno de los primeros en poner de manifiesto la existencia de proteínas disueltas en el agua de mar mediante electroforesis en gel de poliacrilamida con dodecilsulfato sódico monodimensional (1D-SDS-PAGE, *one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis*) y bidimensional (2D-SDS-PAGE).

Uno de los primeros trabajos realizados por Tanoue [18] fue el desarrollo de un método para la extracción y detección de proteínas disueltas en aguas oceánicas. El procedimiento consta de tres etapas diferentes: (1) concentración de las proteínas disueltas en el agua de mar mediante ultrafiltración en flujo tangencial (10 kDa), (2) una mayor concentración y purificación de las proteínas disueltas mediante precipitación con ácido tricloroacético, y (3) la separación y detección de las proteínas disueltas mediante 1D-SDS-PAGE. Con esta técnica se visualizaron en los geles al menos 30 bandas correspondientes a presentes en muestras de agua de mar recolectadas en estaciones situadas en el océano Pacífico. La mayoría de las proteínas tenían un peso molecular entre 14 y 66 KDa. En los geles de las muestras de la estación situada en la región subartica (estación A), las principales bandas de proteínas observadas se corresponden con pesos moleculares de 48, 40, 37 y 34 kDa a profundidades entre 8-200 m, siendo la correspondiente a 48 kDa la más destacada. El perfil proteico de los geles

correspondientes a las muestras de la estación situada en el Pacífico tropical (estación B) es diferente al de la estación A. El número de bandas de proteínas incrementa entre los 75 y 211 m, siendo los componentes principales las proteínas con pesos moleculares aparentes de 48 y 37 kDa. Proteínas con pesos moleculares aparentes de 66 y 34 kDa fueron predominantes a profundidades entre 462 y 2000 m. La proteína de 66 kDa no aparece en aguas superficiales, pero si aparece en aguas tanto intermedias como profundas. La proteína de 48 kDa encontrada en aguas superficiales como componente principal, también fue encontrada en aguas intermedias. A mayores profundidades, 4000 y 5000 m, los niveles de proteínas son menores y apenas se visualizan proteínas en los geles. La proteína de 37 kDa fue la predominante por debajo de los 4000 m y la de 48 kDa también se encontró en aguas profundas.

Yamada y Tanoue [17] demostraron que la mayoría de las proteínas disueltas separadas por 1D-SDS-PAGE estaban glicosiladas, con diferentes azúcares y diferentes tipos de unión entre el azúcar y la cadena polipeptídica, a pesar de que las glicoproteínas formaran bandas únicas en los geles. El hecho de que se distingan menos de 30 bandas de proteínas por 1D-SDS-PAGE sugiere que estas bandas de proteínas son una mezcla de proteínas con los mismos pesos moleculares pero que están unidas a azúcares con características diferentes, y la resolución de 1D-SDS-PAGE es insuficiente para la separación de proteínas disueltas. Es evidente que se necesita utilizar un método con mayor resolución que la 1D-SDS-PAGE para la separación de proteínas disueltas y su posterior caracterización. Por esta razón, estos mismos autores [22] aplicaron la electroforesis bidimensional para la separación de proteínas disueltas aisladas de muestras de agua de mar recogidas en el océano Pacífico. Los resultados mostraron que se detectaron un total de 412 spots de proteínas en 10 muestras de agua de mar procedentes de cinco puntos diferentes del océano Pacífico. Se detectaron proteínas en un amplio rango de tamaños moleculares (11 - 95 kDa) y en un amplio rango de puntos isoeléctricos (pI, *isoelectric point*) (3,7 - 9,2). En 1D-SDS-PAGE las proteínas de 48, 39 y 34 kDa fueron las principales bandas

detectadas. Las bandas correspondientes a 39 y 34 kDa en 2D-SDS-PAGE dieron lugar a proteínas con el mismo peso molecular pero diferente pIs. Por ejemplo, en una de las muestras, la proteína de 34 kDa se resolvió en 14 spots (pIs 5,9 - 8,4), y la proteínas de 39 kDa en 17 spots (pIs 6,3 - 8,4). Por el contrario, la banda de proteína correspondiente a 48 kDa observada en SDS-PAGE se mantuvo como un único spot en los geles 2-DE.

Posteriormente,Yamada y Tanoue [21] realizaron un estudio de similitud entre las proteínas disueltas en agua de mar de diferentes zonas. Utilizando SDS-PAGE, se detectaron de cuatro a nueve bandas de proteínas con un rango de tamaños moleculares aparentes entre 12 y 49 kDa, e independientemente del punto y la profundidad del muestreo se detectaron las bandas proteínas correspondientes a 48, 39 y 34 kDa. Mientras que mediante 2-DE se distinguieron de 10 a 46 spots con pesos moleculares aparentes de 12 a 63 kDa y con pIs entre 4,3 y 9,2. Se encontraron tres características en los geles 2-DE. La primera es la aparición de un único spot con un peso molecular de 48 kDa (pI 5,3) en todas las muestras. La segunda la aparición de varios spots de proteínas con un peso molecular de 39 kDa, y la tercera es la aparición de varios spots de proteínas a 34 kDa. Los resultados obtenidos concuerdan con los obtenidos en trabajos anteriores, y se concluye que los patrones electroforéticos son similares entre las muestras de agua de mar costeras y las muestras de agua de mar pelágicas.

Mediante el conocimiento de la secuencia amino terminal (degradación de Edman) y los pesos moleculares aparentes obtenidos mediante SDS-PAGE y 2D-PAGE, Tanoue et al. han conseguido identificar algunas de las principales proteínas disueltas en el agua de mar. La proteína disuelta con peso molecular aparente de 48 kDa, observada en todas las muestras analizadas por Tanoue et al., se identificó como la proteína homóloga a la proteína de membrana externa Porina P procedente de uno de los principales géneros de bacterias representado en el océano, las bacterias Gram-negativas *Pseudomonas aeruginosa PA01* [15]. La proteína disuelta correpondiente con un peso molecular aparente de 40 kDa ha sido identificada como una proteína OmpA, proteína homologa a la proteína de

membrana externa A, procedente de las bacterias Gram-negativas *Acinetobacter sp.* [17]. Las glicoformas de la proteína con peso molecular aparente de 39 kDa fueron identificadas como proteínas homólogas a una fosfatasa alcalina de bajo peso molecular (L-AP) procedente de *Pseudomonas aeruginosa PA01* [21, 22]. La identificación de estas proteínas indica que las proteínas de membrana liberadas por las bacterias son la principal fuente de proteínas disueltas en el agua de mar.

En un estudio realizado por Powell et al. [23] para la determinación de proteínas disueltas en agua de mar, los autores utilizaron dos métodos de separación. En el primero, las proteínas son separadas mediante SDS-PAGE y digeridas enzimáticamente en el gel, y en el segundo, los péptidos resultantes de la digestión proteolítica del *pellet* de proteínas fueron separados por HPLC capilar. En ambos métodos, la separación cromatográfica final se acopló en línea con un espectrómetro de masas mediante una interfaz de electrospray, y el espectro de disociación inducida por colisión (CID, *collision-induced dissociation*) de péptidos fue obtenido por espectrometría de masas en tándem. Mediante la secuenciación de novo se generaron fragmentos cortos de péptidos (huellas peptídicas) que se utilizan para buscar en las bases de datos la clase de proteína. Los resultados obtenidos mostraron secuencias conservadas de dos clases de proteínas diferentes, proteínas de membrana y enzimas. Mediante las huellas peptídicas se identificaron proteínas disueltas como proteínas homólogas a las siguientes: acil-CoA sintetasa para ácidos grasos de cadena larga, antralinato sintasa, ribulosa bisfosfato carboxilasa (RUBISCO), y proteína de unión luminal. Esto demuestra la existencia de otras clases de proteínas disueltas en el agua de mar además de las proteínas de membrana reportadas por Tanoue et al. Por lo tanto, las proteínas disueltas en el agua de mar proceden de una variedad de localizaciones celulares.

3.5. Detección y caracterización de proteínas en plancton marino

Tanoue [24, 25] y Tanoue et al. [26] realizaron la caracterización de proteínas presentes en la POM en el agua de mar mediante el uso de 1D-SDS-PAGE. Se identificaron dos grupos de proteínas a partir de los cambios verticales en su

distribución molecular. El primer grupo está formado por las proteínas de fondo, proteínas que derivan de organismos vivos (principalmente fitoplancton), que no se resuelven mediante 1D-SDS-PAGE, lo que sugiere que se componen de un gran número de proteínas con una distribución de tamaños moleculares en un amplio rango y presentes a muy bajos niveles. La concentración de proteínas en este grupo disminuye rápidamente con la profundidad, lo que indica que estas proteínas son muy susceptibles a la degradación biológica. El segundo grupo está formado por proteínas específicas, un pequeño número de proteínas individuales distribuidas en un intervalo de tamaños moleculares entre 45 y 66 kDa, y que se superponen a las proteínas de fondo. Las proteínas específicas son frecuentes en las aguas intermedias y profundas, una indicación de que pueden ser resistentes al ataque biológico y ser acumuladas en el océano. La inadecuada resolución de 1D-SDS-PAGE impidió tanto la identificación de las proteínas específicas como el estudio de las características químicas de las proteínas de fondo. Por esta razón, estos mismos autores, Tanoue et al. [27], aplicaron la electroforesis bidimensional para el estudio de la características de las proteínas particuladas en el agua de mar. En los geles 2-DE aparece una zona sin resolver correspondiente a un amplio rango de pesos moleculares y pIs ácidos. Estos materiales que no se resuelven fueron considerados péptidos conjugados con ácidos sacáridos y productos de degradación (péptidos) de proteínas, lo que indica que las proteínas procedentes de organismos vivos no sobreviven en la POM detrítica. Sin embargo, 23 proteínas fueron separadas y distinguidas por 2-DE y los patrones electroforéticos indicaron que formaban parte de la POM detrítica. Tres de estas proteínas fueron sometidas a análisis de secuenciación amino terminal, pero sólo se consiguió determinar la secuencia de una de ellas desde el extremo N-terminal hasta el noveno residuo de aminoácidos. Esta secuencia fue homóloga a la proteína de choque térmico de 70 kDa (HSP70) derivada de organismos fotosintéticos. La HSP70 es un miembro importante de las chaperonas moleculares que protegen o reparan las proteínas bajo estrés medioambiental. La identificación de HSP70 en estudio demuestra que el fitoplancton fue capaz de inducir una chaperona molecular.

Moncheva et al [28] realizaron un estudio sobre la influencia de los cambios estacionales en las proteínas del fitoplancton en el Mar Negro, empleando para ello Fluorescencia y SDS-PAGE. Las muestras de fitoplancton fueron recogidas durante la primavera y durante el verano. Tras la separación de las proteínas por SDS-PAGE se observaron diferencias en la cantidad e intensidad de las bandas de proteínas entre las muestras recogidas en verano y en primavera. En los geles correspondientes a las muestras recogidas en verano se observaron bandas de proteínas de intensidad media con pesos moleculares aparentes de 14, 29, 37, 48 y 70 kDa, Mientras que en los geles correspondientes a las muestras recogidas en primavera se observaron bandas de proteínas muy débiles y difusas con pesos moleculares aparentes entre 38 y 48 kDa. Los resultados obtenidos fueron similares a los obtenidos por Tanoue [24, 25] y Tanoue et al. [26] que reportaron dos grupos diferentes de proteínas con pesos moleculares entre 45 y 66 kDa.

3.6. Determinación de metales enlazados a proteínas mediante electroforesis en gel con detección por LA-ICP-MS

La ablación laser (LA, *laser ablation*) acoplada a un sistema de espectrometría de masas con plasma acoplado inductivamente (ICP-MS) es una técnica ampliamente utilizada para la determinación tanto de elementos mayoritarios, como elementos minoritarios y elementos traza en muestras sólidas, así como para realizar medidas de relaciones isotópicas [29]. La técnica ICP-MS nos permite realizar determinaciones multielementales y nos proporciona una alta sensibilidad, un amplio rango dinámico, y espectros relativamente simples para la realización de análisis mediante LA.

La combinación de LA-ICP-MS con técnicas de electroforesis en gel (GE, *gel electrophoresis*) ha sido ampliamente utilizada en proteómica y metalómica para la detección y/o identificación de heteroelementos y metales unidos a proteínas. Los pioneros en esta técnica fueron Nielsen et al. [30], quienes describieron un método de separación de proteínas mediante electroforesis acoplado a LA-ICP-MS para la identificación y cuantificación de metales unidos a proteínas. Los autores utilizaron

muestras de suero humano enriquecidas con Co, las cuales fueron sometidas a una inmunoelectroforesis para la separación de las proteínas. A continuación, los geles correspondientes tanto a la primera como a la segunda dimensión fueron analizados por LA-ICP-MS para la detección de Co. La comparación del mapa de distribución de Co con el mapa de distribución de proteína (gel teñido con Coomassie Brilliant Blue) permitió la identificación de proteínas presentes en el suero sanguíneo que contenían Co. La cuantificación del metal se realizó con geles enriquecidos con cantidades crecientes de Co.

La combinación de técnicas GE y LA-ICP-MS ha sido ampliamente utilizada por muchos grupos de investigación para la detección y/o identificación de heteroelementos y metales unidos a proteínas. Estos estudios se pueden dividir en dos categorías. La primera incluye la determinación de heteroelementos, como el P y el Se, unidos covalentemente a proteínas, y la segunda categoría incluye el estudio de metaloproteínas y complejos metal-proteína en los cuales el metal está unido a la proteína mediante enlaces más débiles.

Fan et al. [31] fueron los primeros en emplear SDS-PAGE-LA-ICP-MS para la identificación de especies de Se en extractos de proteínas de ovarios de peces y embriones de aves acuáticas recolectados en aguas ricas en Se. La detección se llevó a cabo empleando una celda de reacción dinámica (DRC, *dynamic reaction cell*) en el ICP-MS para eliminar las interferencias del Ar^{2+}, lo que permitió el uso de los isótopos más abundantes del Se (m / z de 80 y 78). Los análisis revelaron 8 y 5 bandas de proteínas que contienen Se respectivamente, de las cuales cuatro de ellas (> 100, 60, 50, and 48 kDa) estaban presentes en ambos organismos. Más tarde este método fue utilizado para la detección de selenoproteínas tanto en extractos de proteínas de sangre empleando 1D y 2D-PAGE [32], para estudiar la distrubición de Se en proteínas extraídas de diferentes orgános y tejidos de peces con una dieta enriquecida en Se [33, 34], y para la identificación de proteínas que contienen Se en un extracto de hojas de girasol empleando 2D-PAGE [35]. La SDS-PAGE-LA-ICP-MS es la técnica más adecuada para la separación y detección de selenoproteínas y proteínas que contienen Se. Sin embargo este

método no puede ser aplicado para detectar bajos niveles de Se. Balliuhat et al [36] desarrollaron un método más sensible para la detección e identificación de selenoproteínas en plasma humano. La combinación de una mejor preparación de la muestra, mediante la concentración de las selenoproteínas en membranas de fluoruro de polivinilideno (PVDF, polyvinylidene difluoride) después de su separación por SDS-PAGE, y el aumento de la sensibilidad de la detección de Se por humidificación del ICP con una disolución orgánica, permitió desarrollar un método 80 veces más sensible en comparación con los métodos de LA-ICP-MS utilizados anteriormente. Estos autores, Balliuhat et al. [37], también realizaron la optimización del sistema LA-ICP-MS para mejorar la sensibilidad en la detección de selenoproteínas extraídas de células humanas tras su separación mediante SDS-PAGE. Para ello emplearon tanto un láser de nanosegundos como un láser de femtosegundos, y se obtuvieron relaciones S/N 12 veces superiores con el láser de femtosegundos. El uso de un láser de femtosegundos aumentó la sensibilidad de la detección debido al aumento de la cantidad de materia ablacionada y transportada al ICP, y permitió la detección específica de la formiato deshidrogenasa en *Escherichia coli* y de selenoproteínas en *Desulfococcus multivorans* [38].

Marshall et al. [39] fueron los primeros en presentar la detección de P en β-caseína mediante LA-ICP-MS tras la separación de las proteínas por SDS-PAGE. El elevado ruido de fondo producido por la contaminación hace muy difícil la detección de las proteínas que contienen P, pero la sensibilidad se mejoró mediante la transferencia de la muestra a una membrana de PVDF. Becker et al. [40] utilizaron LA-ICP-MS para la detección y cuantificación de P y S mediante análisis microlocal en el centro de los spots de proteínas en geles 2D. Ellos aplicaron este método para el análisis de la proteína tau humana (proteína clave en la enfermedad del Alzheimer) y consiguieron identificar 17 sitios de fosforilación de esta proteína. La calibración se llevó a cabo introduciendo disoluciones estándar mediante un nebulizador ultrasónico acoplado a la cámara de LA, y emplearon Co como patrón interno adicionándolo al gel. Posteriormente, Becker et

al. [41], aplicaron este método para la determinación de P, S y metales en proteínas del cerebro humano. Wind et al. [42] aplicaron la LA-ICP-MS para la detección de proteínas fosforiladas separadas por SDS-PAGE y transferidas a una membrana. Mediante el análisis de una mezcla de mioglobina, α-caseína y fibrinógeno reducido demostraron que las fosfoproteínas son especialmente reconocidas por este método, siendo necesaria una etapa de lavado para eliminar el fosfato unido a las proteínas no covalentemente. Recientemente, Krüger et al. [43] estudiaron el estado de fosforilación del proteoma citoplasmático de células bacterianas y eucariotas, empleando para ello dos estrategias analíticas basadas en ICP-MS. Una de ellas, emplea la combinación de 1D-PAGE, digestión del gel y finalmente análisis por cromatografía líquida capilar acoplada a ICP-MS, y la otra estrategia, combina la 1D-PAGE, transferencia de membrana y análisis por LA-ICP-MS. Ambas estrategias analíticas proporcionaron resultados cuantitativos consistentes, y mostraron que el proteoma de las células eucariotas presenta significativamente un grado mayor de fosforilación en comparación con el proteoma de las células bacterianas. Feldmann et al. [44] desarrollaron y optimizaron una nueva celda de ablación laser que proporciona una intensidad de señal alta y constante en toda su geometría y con un tiempo de lavado corto. Esta nueva metodología fue aplicada para la detección cualitativa y cuantitativa de proteínas fosforiladas, pepsina y β-caseína, en membranas después de su separación mediante SDS-PAGE [45]. Con la configuración laser que se utiliza en esta investigación, las membranas de nitrocelulosa (NC, *nitro cellulose*) muestran unas señales con mayor intensidad en comparación con las membranas de PVDF.

Además de la determinación de hereroátomos asociados a proteínas, también se han aplicado técnicas de GE-LA-ICP-MS para la detección de metales asociados a proteínas con enlaces más débiles.

Becker et al. han realizado varias aplicaciones de técnicas GE-LA-ICP-MS para la detección de metales asociados a proteínas cerebrales relacionadas con el Alzheimer. En una de estas aplicaciones, Becker et al. [46] realizaron la identificación de proteínas que contenían Cu, Zn y Fe mediante LA-ICP-MS tras la

separación de las proteínas mediante 2D-SDS-PAGE. Los geles se enriquecieron con trazadores enriquecidos isotópicamente y se midieron las relaciones isotópicas $^{54}Fe/^{56}Fe$, $^{65}Cu/^{63}Cu$ y $^{67}Zn/^{64}Zn$ mediante LA-ICP-MS. De esta manera fue posible demostrar la existencia de proteínas que contienen Cu y Zn, las cuales son lo suficientemente estables para soportar las condiciones reductoras durante la electroforesis en gel. Mediante el empleo de isótopos enriquecidos, Benner et al. [47] estudiaron la unión de Cu y Zn a las isoformas de la proteína tau (proteína diana en la enfermedad de Alzheimer) tras la separación de las mismas mediante una electroforesis SDS-PAGE y posterior detección de metales mediante LA-ICP-MS. Con estos experimentos se pudo demostrar que las isoformas de la proteína tau son capaces de unirse a Cu y a Zn. La identificación de las isoformas de la proteína tau se llevó a cabo por espectrometría de masas de desorción/ionización por láser asistida por matriz con un analizador de resonancia iónica en ciclotrón con transformada de Fourier (MALDI-FTICR-MS, *Matrix-assisted laser desorption/ionization - Fourier transform ion cyclotron resonance mass spectrometry*) después de la escisión y la disgestión tríptica de las bandas del gel 1-D. En otros trabajos, Benner et al. [48, 49] emplearon LA-ICP-MS y MALDI-FTICR-MS para la caracterización de proteínas del cerebro en cuanto a su estructura, secuencia, estado de fosforilación y contenido de metal. Para ello realizaron la separación de las proteínas mediante electroforesis en gel 2-D con posterior análisis por LA-ICP-MS para estudiar la presencia de P, S, Cu, Zn y Fe. Las proteínas seleccionadas en el gel que contenían estos elementos fueron investigadas después de una digestión tríptica por MALDI - FTICR – MS. Esto permitió la identificación de proteínas fosforiladas y proteínas que contenían metales.

Benner et al. [50] también emplearon la LA-ICP-MS para estudiar la distribución de Zn en una muestra de babosas, que son de especial interés como organismos monitores para contaminaciones ambientales. Para detectar las proteínas que contenían Zn realizaron la separación de las mismas mediante una electroforesis nativa en presencia del colorante azul de Coomassie (BN-PAGE, *blue native*

polyacrylamide gel electrophoresis) con posterior detección de Zn mediante LA-ICP-MS. Los resultados mostraron que las bandas de proteínas asociadas a Zn fueron las correspondientes a 75, 100 y 150 kDa. Estas bandas de proteínas fueron analizadas por MALDI-TOF-MS tras una digestión tríptica, pero las bases de datos públicas no identificaron proteínas dentro de las bandas que contenían Zn.

Polatajko et al. [51] investigaron la presencia de Cd en extractos de hojas de *Spinacia oleracea L.*, una planta alimenticia típica con una alta susceptibilidad a la absorción de Cd. Para ello llevaron a cabo la determinación de proteínas que contenían Cd tras la separación de las mismas mediante una electroforesis nativa monodimensional y posterior detección de Cd mediante LA-ICP-MS. Los resultados mostraron la detección de tres bandas de proteínas que contenían niveles de Cd más altos entre las cuales una también contiene Zn.

La GE-LA-ICP-MS no solo se ha aplicado a la detección de metales y heteroátomos asociados a proteínas, también hay trabajos en la bibliografía en los que se emplea la GE-LA-ICP-MS para la especiación de metales ligados a materia orgánica disuelta y ácidos húmicos.

Jiménez et al. [52, 53] desarrollaron una nueva metodología de especiación basada en el uso de 1D-PAGE, isoelectroenfoque (IEF, *isoelectric focusing*) y 2D-PAGE como técnicas de separación y LA-ICP-MS como sistema de detección para el estudio de la distribución de complejos metal-acidos húmicos (metal-HA) en muestras medioambientales, tomando como modelo una muestra de compost para estudiar la contribución de DOM a la movilización de metales. Mediante el empleo de 1D-Tris-Tricina-PAGE el perfil de metales (^{63}Cu, ^{65}Cu y ^{55}Mn) obtenido después de LA-ICP-MS no se corresponde con el pefil de HS. Los autores atribuyen este hecho a la ruptura de los enlaces metal-DOM en las condiciones electroforéticas usadas. Por el contrario, el empleo del método 1D-Tris-Borato-PAGE para la separación de HA y posterior detección de metales mediante LA-ICP-MS permitió obtener la distribución de metal-HA, manteniendo la estabilidad de los complejos.

Los resultados obtenidos mostraron que el Mn y Zn están asociados a la fracción de bajos pesos moleculares (aproximadamente 3 kDa). Los perfiles IEF-LA-ICP-MS indican que hay dos regiones principales: una con pIs entre 3 - 4,5 y otra con pIs mayores de 5. Las mayores señales de metales (Cu, Zn y Mn) se corresponde a la región con pIs entre 3 y 4,5, y tan solo el Mn parece estar asociado a HA con pI>5. Finalmente, aplicaron un método de 2D-PAGE seguido de LA-ICP-MS que confirmó los resultados obtenidos anteriormente con 1D-PAGE e IEF. Se obtuvieron señales bastante intensas de Cu, Mn y Zn en el pico correspondiente a 3,7 kDa (pI 3 - 3,5), y además se observaron señales más débiles de estos metales en la zona correspondiente a 79,5 kDa y pI en torno a 5,5. También se observó una señal muy intensa de Mn correspondiente al pico de 20 kDa (pI 3,5 - 4). Estos resultados sugieren que la 2D-PAGE-LA-ICP-MS permite una completa caracterización de complejos metal-HA mediante la combinación obtenida por las técnicas de 1D-PAGE e IEF.

Jiménez et al. [54] también llevaron a cabo un estudio de las limitaciones de PAGE-LA-ICP-MS para la determinación de metales asociados a proteínas, estudiaron la influencia de diferentes condiciones electroforéticas en la estabilidad de las interacciones metal-proteína. Para ello utilizaron dos proteínas con diferentes asociaciones metal-proteína, la superóxido dismutasa (SOD, *superoxide dismutase*), que contiene Cu y Zn, y la alcohol deshidrogenasa (ADH, *alcohol dehydrogenase*), que contiene Zn. Aplicaron dos métodos electroforéticos desnaturalizantes, SDS-Tris-Tricina-PAGE y SDS-Tris-Glicina-PAGE, y otros dos métodos electroforéticos no desnaturalizantes, una electroforesis nativa anódica (AN-PAGE, *anodal native polyacrylamide gel electrophoresis*) y una electroforesis BN-PAGE. En los métodos desnaturalizantes, la ticina parece una mejor opción como ión de arrastre debido a que parece tener menor afinidad por Cu y Zn que la glicina y se mantiene mejor el enlace metal-proteína. En el caso de la ADH, las condiciones no desnaturalizantes, BN-PAGE empleando tricina como ión de arrastre, resultaron ser las más adecuadas para la determinación Zn unido a proteínas de forma no covalente, como es el caso de la ADH. En el caso de la

SOD se pueden usar tanto condiciones desnaturalizantes empleando tricina como ión de arrastre como condiciones no desnaturalizantes para la determinación de Cu y Zn debido a que los enlaces metal-proteína son más fuertes. Además, los resultados también mostraron que cuanto mayor es la intensidad aplicada, mayor es la posibilidad de la ruptura del enlace metal-proteína. En todos los casos es mejor evitar los procedimientos de tinción porque pueden producir la pérdida del enlace metal-proteína.

La electroforesis en gel, tanto en modo monodimensional como bidimensional, ha sido ampliamente utilizada para caracterizar tanto proteínas disueltas en el agua de mar como proteínas en plancton, pero apenas se ha utilizado para la detección e identificación de metales asociados a proteínas en el medio marino.

El único trabajo presente en la bibliografía que utiliza GE-LA-ICP-MS para la determinación de metales asociados a proteínas en plancton es el desarrollado por Jimenez et al. [55]. En este estudio, desarrollaron métodos electroforéticos para la separación de proteínas en una muestra de plancton certificada (BCR 414), y posterior detección de Cu y Zn asociados a las proteínas mediante LA-ICP-MS. Para la separación de proteínas emplearon cuatro métodos diferentes de 1D-PAGE: dos métodos electroforéticos desnaturalizantes, uno basado en un sistema Tris-Glicina (SDS-Tris-Glicina-PAGE) y otro basado en un sistema Tris-Tricina (SDS-Tris-Tricina-PAGE), y dos métodos electroforéticos no desnaturalizantes, un AN-PAGE basado en un sistema que utiliza glicina como ion de arrastre, y un BN-PAGE basado en un sistema que utiliza tricina. Mediante la aplicación de los métodos electroforéticos desnaturalizantes determinaron un gran número de bandas de proteínas con pesos moleculares entre 18 y 75 kDa, aunque la resolución para bandas de 25 a 42 kDa es peor en el caso de SDS-Tris-Tricina-PAGE. La detección de metales por LA-ICP-MS tras la separación mediante SDS-Tris-Glicina-PAGE y AN-PAGE puso de manifiesto que no son métodos electroforéticos adecuados para la determinación de metales asociados a proteínas en plancton. El enlace metal-proteína se rompe durante el proceso electroforético debido a que forman complejos con la glicina, y los metales

avanzan con el frente de electroforesis. Sin embargo, mediante la separación no desnaturalizante BN-PAGE detectaron Cu y Zn en las bandas de proteínas correspondientes a pesos moleculares de 60 y 35-15 kDa, lo que confirmó los resultados obtenidos para el Cu mediante SDS-Tris-Tricina-PAGE, en la que obtuvieron señales de Cu asociadas a bandas de proteínas con pesos moleculares entre 26 y 58 kDa,

Que nosotros conozcamos, no hay trabajos presentes en la bibliografía que utilicen la electroforesis en gel acoplada a LA-ICP-MS para la determinación de metales asociados a proteínas disueltas en el agua de mar.

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4. Preparative and semi-preparative OFFGEL electrophoresis for multidimensional protein / peptide assesment

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Preparative and semi-preparative OFFGEL electrophoresis for multidimensional protein/peptide assessment

Abstract: *An increasing attention on the potentiality of mass spectrometry (MS) for completely identifying the entire protein complement of a cell has occurred during the past ten years. MS techniques coupled to two-dimensional gel electrophoresis as separation methods, have been used to facilitate further analysis of complex protein mixtures after applying few experiments. The possibility of performing proteins/peptides separation based on the differences on their molecular weights (MWs) by chromatographic techniques hyphenated with MS, after a previous fractionation according to their isoelectric point (pIs), has led to time-consuming procedures for preparing the gels. The usefulness of gel-free proteins separations before MS-based strategies has therefore been a topic of interest in recent years that has which resulted in the development of different chromatographic-based approaches, (first and second dimensional separation), and also, in the commercialization of various gel-free electrophoresis devices for performing the fractionation according to the pI differences. This review describes the current state of isoelectric focusing (IEF) gel-free electrophoresis based on the Agilent OFFGEL 3100 fractionator. The review includes therefore (i) an overview on IEF as well as other previous IEF gel-free electrophoresis developments; (ii) OFFGEL fundamentals and future trends; (iii) advantages and disadvantages of current OFFGEL procedures; (iv) requirements of isolated proteins pellet for further OFFGEL fractionation; (v) OFFGEL fractions requirements to perform the second dimensional analysis by advance electrophoresis and chromatographic techniques; and (vi) effect of the OFFGEL operating conditions on the stability of metal-protein complexes.*

Keywords: *OFFGEL electrophoresis, isoelectric focusing, proteins, peptides.*

4.1. Introduction

Proteomics can be considered as the study of proteins, particularly the study their structures and functions [1], as well the analysis of protein's complement the protein complement present in a cell, organ, or organism at any given time. Difficulties on protein similarities in complex specimens as well as the assessment of low abundance proteins, have conventionally been overcome by applying two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for isolation/separation purposes. First dimension separation, proteins separation on the basis of

differences in their isoelectric points (pI), is commonly performed by isoelectric focusing (IEF) techniques; whereas, further proteins separation according to their molecular mass values is achieved by sodium dodecylsulfate (SDS)-PAGE. Despite the potential of traditional 2D-PAGE (IEF and SDS-PAGE) for protein separation, as shown in different reviews [2,3], some drawbacks with respect to automation, sensitivity, and throughput of these methodologies have continuously been reported [4]. In this sense, the development and application of gel-free separation techniques have gained importance when separating proteins according to with their molecular masses by mean of size exclusion (SE) and reverse phase (RP) high performance liquid chromatography (HPLC) techniques, mainly hyphenated with tandem mass spectrometry (MS/MS) [5,6]. In addition, efforts have also been performed for replacing conventional gel-based IEF techniques, (first dimension separations according to proteins' pIs), by gel-free methodologies that allow an easier and more reliable coupling with chromatography techniques, (second dimension separations according to proteins' molecular mass). Recently, the introduction of preparative IPG IEF where proteins can be recovered from liquid phase, (OFFGEL electrophoresis), avoids drawbacks associated with IPG gels, allowing direct sampling of the isolated protein/peptides into HPLC instruments [7].

The aim of the current review is the critical discussion of the possibilities of IEF based gel-free electrophoresis methods, (formally OFFGEL electrophoresis), as a preparative stage, (protein/peptides separation according to their pIs), before further protein separations based on their molecular weights in modern proteomic studies. General IEF fundamentals as well as an OFFGEL overview including trends have been introduced. Procedures for treating OFFGEL fractions before coupling with chromatographic and advance electrophoresis techniques, and conditions for ensuring the integrity of metal-protein complexes have also been fully discussed.

4.2. Overview of IEF techniques

As mentioned above, IEF approaches offer extremely high resolution and make possible fractionation into separate bands of proteins differing by a single charge. IEF techniques can be performed using buffer-free solutions containing carrier amphoteric components (CAs), formally natural pH gradients, or using immobilized pH gradient (IPG) gels [8]. IPGs are a special type of artificial pH gradients in which non-amphoteric substances (acids, bases, or salts) are able to withstand the electric current indefinitely without alteration/distortion by the electric field [9]. IPG gels consist of a mixture of acid and basic compounds (named as immobilines, which exhibit certain pK_a values) covalently grafted to an acrylamide gel matrix or acrylamide gel matrix co-polymerized (IPG gels). The continuous change in ratio of immbolines along the gel leads to specific buffering capacities over the pH span, and an immobilized pH gradient is therefore obtained. Focusing occurs into sharp stationary bands where each protein is positioned at a point in the pH gradient corresponding to its pI. This is the consequence of protein migrations towards the anode (negative), when the protein is positively charged (the protein is in a pH region below its Ip); or towards the cathode (positive), when the protein is negatively charged (the protein is in a pH region above its Ip). Protein charge decreases/increases during migration through the gradient of increasing/decreasing pH until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and protein migration therefore ceases. This behaviour is the specific characteristic of the IEF process in contrast to conventional electrophoresis techniques in which proteins continue to move through the gel until the electric field is removed. IEF can therefore be considered as a steady-state mechanism regarding to pH: proteins migrate at different rates until reaching their respective pIs and remain fixed at those pH values for long periods of time. The steady-state mechanism of the process, offers, as an additional advantage, the fact that proteins migrate from anywhere to their Ip positions, and sample can therefore be loaded throughout the entire separation system.

Resolution between two adjacent protein bands in IEF (ΔpI) is directly proportional to the square root of the pH gradient (*dpH/dx*) and inversely proportional to the square root of the voltage gradient (*dq/dx*) at the position of the bands (equation 1) [10].

$$\Delta pI \ \alpha \sqrt{\frac{dpH \ / \ dx}{dq \ / \ dx}} \quad \text{(equation 1)}$$

In this equation the proportionality constant depends on parameters such as the protein diffusion coefficient and electrophoretic mobility, as well as of the conductivity and the electric current density [9]. From equation 1, high resolution, (small ΔpI), is achieved for narrow pH ranges when applying high voltages. High voltages usage results also in shortened run times, nevertheless due to the large amounts of generated heat, the value of applied voltage is dependent on the ionic strength of the solution used during the IEF process.

Despite the formal classification between buffer-free solutions containing CAs, (natural pH gradients), and IPGs based on non-amphoteric compounds, current IEF application uses hybrid IPG-CA pH gradients [11]. Typically, dried IPG gels must be rehydrated before use with solutions containing CAs at variable concentrations. CAs' role during IEF is variable [10]. First, they contribute to the ionic strength of the solution and can help to counteract lack of salts in the sample. This provides a more uniform conductivity background during the separation process. Second, CAs help to overcome protein precipitation at their pI values. In these cases, non-ionic surfactants and urea are highly recommended. Finally, CAs also help to increase protein solubility. This is especially important when fractionating membrane proteins. Non-ionic surfactants such as octylglucoside, and zwiterionic detergents such as 2-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) and the hydroxyl analog (CHAPSO) are commonly used for these purposes.

4.3. Free flow electrophoresis. OFFGEL electrophoresis fundamentals

The first dimension of two-dimensional gel electrophoresis is conventionally performed by in-gel IEF of proteins [12,13]. However, a major limitation of this method is the tedious post-IEF sample processing and/or its risk for protein recovery before protein analysis by microfluidic chip-based electrophoresis, (formally Lab-on-chip electrophoresis, LOC electrophoresis), and also, before analysis and characterization by MS/MS techniques. The IPG gel strips must be cut into small sections containing the different proteins/peptides that have been focused, and further extraction and cleaning up stages are needed before performing the second dimension by MS/MS [14] and LOC electrophoresis [15]. Inherent characteristics of LOC electrophoresis such as miniaturization, portability, fastness, and low analyte consumption [16], as well as the high resolution and peptide sequence identification provided by MS/MS, are appealing features that have imposed progressively these techniques in modern proteomics studies. The development of gel-free IEF approaches that allows a more convenient coupling has therefore been a matter of interest.

4.3.1. Early developments

Since first descriptions on free zone electrophoresis by Hjertén in 1967 [17], the use of free-flow electrophoresis for proteins fractionation has been fully explored [18-22]. The development of a multicompartment electrolyzer device for electrophoretic separations in absence of gels by Jonsson and Rilbe [23] resulted in the commercialization of the Rotofor (and mini-Rotofor) cell system, the first equipment for gel-free electrophoresis [24]. The system consists of a cylindrical focusing chamber into which it fits a plastic core that divides the chamber into 20 compartments separated by polyester screens; whereas, ionic exchange membranes are placed against the anodic (cation-exchange membrane) and cathodic (anion-exchange membrane) compartments for avoiding electrolytes diffusion within the sample chambers. Fractions collection is simultaneously vacuum-performed through needles connected to septum at the chambers' bottom

when finishing the fractionation process [25]. Although IEF in Rotofor cell has been described as well suited for its use at any stage of a purification scheme [26], poor pI accuracy (from ±0.65 to ±1.73 pI units) [25], as well as important limitations when resolving complex mixtures of proteins [27] have been reported.

Other attempts, such as those based on using multicompartment electrolysers in which proteins are isolated into different compartments separated by Immobiline membranes (IPG technology) [28-30], have been proved to offer better pI resolution. This is mainly because the use of IPGs leads to proteins focusing with hybrid IPG-CA pH gradients [11] instead of conventional CA-IEF used by the Rotofor device [25]. Based on the use of Immobiline membranes, Ros et al. [31] developed an IEF procedure consisting of the placement of the sample in a thin chamber where one wall consists of an IPG strip. Because of the thinness of the chamber, proteins in solution will be charged according to the pH imposed by the IPG strip. Furthermore, the same research group demonstrated that ampholyte was also protoned in the thin layer of the solution-immobilized gel interface [32]. The application of an electric field perpendicularly to the liquid chamber promotes the extraction of charged species, (species exhibiting pIs different than the pH of the IPG gel), from the solution into the gel. After separation, the proteins with a pI close to gel's pH in contact with the chamber remain in solution. This approach provides therefore, proteins separation based on their pIs, and allows protein recovery in liquid phase (preparative OFFGEL electrophoresis).

4.3.2. Current OFFGEL technology

A further modification of the multicompartment electrolysers and Inmobiline membranes combination, also developed by Girault and co-workers, consisted of adapting the OFFGEL IEF to a multiwell format [13,33]. The multiwell device is composed of different compartments of 100 or 300 µL in volume which were opened at top and bottom extremities, and which were placed on an IPG gel conditioned with a thin layer of solution containing buffers. Similarly to the early development [31,32], proteins acquire the charge according to their pI and to the

pH imposed by the gel after loading the sample in each well. Because there is no fluidic connection between the wells, the charged proteins (pI protein ≠ pH gel) migrate through the gel from well to well under the applied electric field until they reach the well where they are neutral (pI protein = pH gel). At this point, protein will be in solution and can be recovered in the liquid phase. Better resolution is achieved with this new format because only those proteins with pIs similar to the pH range defined by the small portion of the IPG gel segment forming the bottom of a certain well will be recovered in that well. The length of the IPG gel, as well as the pH range defined by the portion of the IPG gel in contact with the analyte solution and the number of wells under which the gel is placed, will therefore affect the resolution of the separation.

The first instrument for preparative OFFGEL IEF based on using multicompartments was commercialized in 2006, and the first application was reported by Hörth et al. [7] for fractionating peptides within the 5 – 8 pI range from protein digests from *Escherichia coli*. The OFFGEL instrument uses IPG gel strips in a tray underneath a 12- or 24-well frame that is placed on top of the strips. After sample dilution with the focusing buffer solution and loading (typically 150 µL) in each well (Figure 1a), charged proteins separation by migration through the gel from well to well occurs in a two phase system formed by the upper liquid phase constrained within a well, and, the rehydrated IPG gel strip (Figure 1b), until charged targets reach the well on top the IPG gel strip which imposes the pH close to the protein' pI (Figure 1c). Focusing is typically performed with a maximum current of 50 µA (200 mW) and voltage step gradients can be programmed from 500 V h^{-1} to 1200 kV h^{-1}.

Finally, some authors have proposed OFFGEL well rinsing after withdrawing the liquid fractions to enhance targets yield. For this purpose, water/methanol/formic acid (FA) (49:50:1) mixtures (100 µL) can be added to each well, allowing incubation without voltage for 90 min [34,35]. On other occasions, wells rinsing has been performed with 10 % FA (50 µL) after 20 min incubation [36], or by adding

100 µL of 80 % methanol and 0.5 % trifluoroacetic acid TFA and rinsing retrieval after 5 min [37].

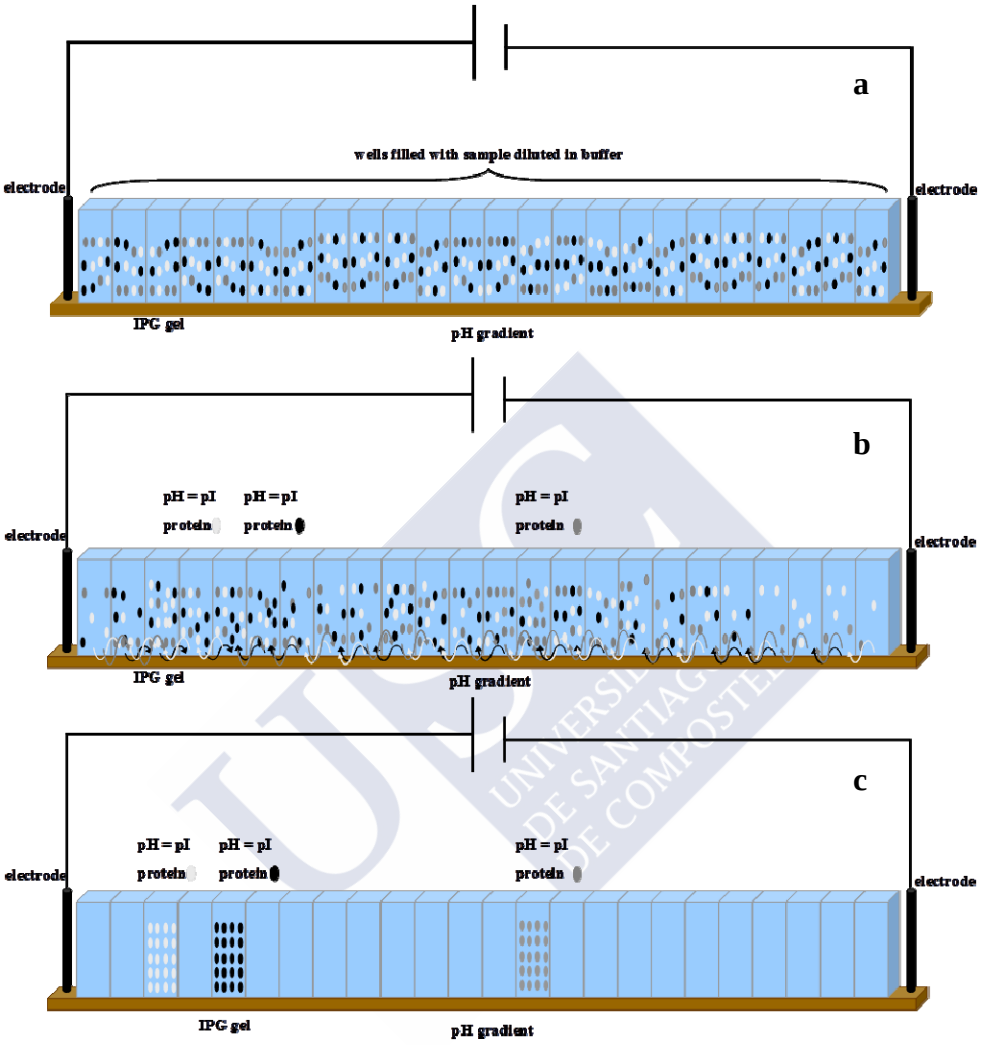

Figure 1: pI-based fractionation: OFFGEL principle.

4.3.3. Advantages and disadvantages of OFFGEL technology

The following advantages can be considered:

(1) Protein/peptide recovery in liquid phase. As commented, the main advantage of the OFFGEL electrophoresis is the possibility of protein recovery in liquid phase, which allows a direct analysis of the isolated protein fractions by advance separation techniques such as MS/MS.

(2) Improved pI resolution: In contrast to other free-flow electrophoresis systems, current OFFGEL electrophoresis devices perform protein/peptide fractionation through the IPG strip, and advantages of using the IPG technology are therefore added; among other, high resolution, high sample loading, full control of a well-defined pH range, buffering capacity, ionic strength and the flexibility of choosing the desired pH gradient [8]. Narrow pI ranges are obtained for all 12 or 24 OFFGEL fractions, ranges that imply that proteins fractionated within the same well offer pIs varying from ±0.26 to ±0.27 pI units [7].

(3) Small influence of OFFGEL reagents on the performance of further separation techniques: Manadas et al. [38] have reported the absence of adverse effects as a consequence of pH gradients and peptide interactions when performing OFFGEL fractionation, which offers additional advantages over HPLC methods based on strong cation exchange (SCX) for performing first dimension separation strategies. In addition, exhaustive desalting procedures are needed when performing SCX HPLC, hydrophilic interaction liquid chromatography (HILIC), and alkaline- and acid-RP HPLC fractionation in shotgun proteomics [38,39].

(4) Physicochemical information: A common feature of electrophoretic techniques, OFFGEL approaches included, is to provide physicochemical information (pI value) for the different focused proteins/peptides. This is also an advantage over HPLC procedures when performing the first dimensional fractionation, allowing further validation/filtering when using MS/MS techniques for the second dimensional separation [14].

OFFGEL disadvantages are as follows:

(1) Long separation time: OFFGEL fractionation approaches show as a disadvantage the long time required for completing the fractionation, typically from some few hours to 2-4 days (in fact, OFFGEL instruments can be programmed for continuous work up to 100 hours). These long times contrast with those required for conventional in-gel electrophoresis and HPLC separation methods. Sample composition, mainly the presence of salts, significantly affects the time required to complete OFFGEL fractionation. In addition, as reported by García-Otero et al. [15], high amounts of salts can even lead to problems in adjusting the electrical current that makes impossible OFFGEL electrophoresis initialization. In this way, an efficient proteins pellet conditioning by salts removal allowed IEF OFFGEL fractionation of dissolved proteins from 20 mL of retentate from seawater after 36 hours. Authors have reported times of approximately 72 hours for untreated retentates (1:4 dilution); whereas, OFFGEL could not be initialized when working with the undiluted retentate [15].

However, the long times required for OFFGEL fractionation can be balanced with the fact that 2 trays (8 different channels each tray) of the OFFGEL device allows up to 16 samples to be fractionated in parallel. Fractionation times can therefore be similar to other fractionation techniques when coping with large number of samples.

(2) Moderate number of identified proteins: When comparing OFFGEL with other fractionation methods such as SCX HPLC, HILIC, and alkaline- and acid- RP HPLC, in shotgun proteomics, SCX and RP-HPLC have provided superior results [38,39], however exhaustive desalting procedures are needed, mainly for SCX fractionation [39]. As an example, Manadas et al. [38] have reported that RP HPLC operating at high pH allows a higher number of membranes proteins identification (1677 peptides) than those provided by OFFGEL (1064 peptides).

4.3.4. Current OFFGEL electrophoresis trends

Despite OFFGEL electrophoresis is a relative new technique, some recent modifications have been proposed mainly for improving even more the pIs resolution. In this sense, Keidel et al. [40] modified the commercially available 24 well frame (Agilent 3100 Fractionator) by inserting 1 mm polymethylmethacrylat plates in the middle of each of the 24 wells of the tray. In this way a 48-well frame per IPG-strip is obtained, and a resolution twice as high as the commercially available 24-wells was obtained. This proposal allowed the separation of proteins with small pI differences and was successfully applied for resolving isoforms and post-translational modification (PTM) isoforms from *E. coli* cell cultures.

More recently, Girault's group has proposed multielectrode setups (segmented field OFFGEL® electrophoresis) for achieving a higher and more homogeneous electric field across the whole system and for improving protein resolution [41]. One of the developments (*multielectrode setup*) was performed by using a 7-well frame and inserting one platinum electrode inside each well. These electrodes were individually powered but connected in series with six power suppliers. The electrode placed in the first well of the multielectrode setup was connected to the ground (0 V) output of each power supply, acting as the more negative electrode in the whole system. The second electrode was connected to the high-tension output of the first power supply and placed inside the second well, while the third and subsequent electrodes were connected to the high tension terminals of their respective power supply and placed in the succeeding wells. In this way the potential difference along the system increases additively, and a more positive electrode each time at the right side is provided. The second development (*two-electrode setup*) consisted of placing the anode and cathode platinum electrodes outside each opposite border of the plastic frame (Agilent 3100 Fractionator), and inserting the two-electrode setup at the extremes of the multiwell frame using one of the power supplies from the multielectrode system.

Numerical simulations were performed to describe both electric field distribution and protein migration inside multi- and two-electrode OFFGEL separations, and results showed a more uniform electric field when using the multielectrode setup, which allowed improved protein IEF separations but also faster separations (3 h when using both two- and multi-electrode setups against 15 h when using conventional OFFGEL electrophoresis with the commercial Agilent 3100 Fractionator). These findings were proved when analysing ideal (β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin, and RNase A) and complex (proteins from *E coli* cell culture) samples.

4.4. Protein pellet treatments before OFFGEL electrophoresis fractionation

4.4.1. Requirements for protein loading

Some OFFGEL electrophoresis fractionation methods have been reported being performed by direct protein extract loading after re-dissolution in the OFFGEL Protein sample solution (Table 1) [33,34,36,42-57]. On other occasions, proteins have been first reduced and alkylated before direct OFFGEL loading [58], buffer exchanged into the OFFGEL running buffer using gel filtration Zeba spin columns [59], or subjected to exhaustive protein pellet purification stages by acetone precipitation [42]. As previously commented (section 3.3), the presence of salts can lead to long OFFGEL fractionation times or even avoid the OFFGEL program initialization [15]. Protein pellet treatments based on methanol- and, mainly, water-soluble species removal by extraction with methanol/chloroform/water mixtures has been proposed when assessing dissolved proteins from seawater [15].

4.4.2. Requirements for peptides loading: Removal of trypsin digestion and labelling reagents

OFFGEL electrophoresis fractionation can be performed for peptides fractionation according to their pIs. In addition to conventional trypsin digestion, peptides labelling techniques, such as isobaric tags for relative and absolute quantitation (iTRAQ), differential $^{16}O/^{18}O$-labeling, and isobaric tandem mass tags (TMT), can also be performed before OFFGEL fractionation. Un-reacted compounds used for both enzymatic digestion and labelling processes, as well as reagents and products originated during these treatments, can alter and/or diminish peptides focusing by OFFGEL fractionation. Peptide extracts conditioning has therefore been recommended by several authors (Table 1), although several reported OFFGEL fractionation methods directly handle the sample (peptides after trypsin digestion) by dilution with the OFFGEL Peptide sample solution [7,34-36,39,43,60-70]. On other occasions the tryptic digests are more conveniently diluted in water (without the addition of ampholytes) before sample loading into each OFFGEL well [71-73].

4.4.2.1. Offline desalting treatments

As previously mentioned, most of the developments require a certain treatment procedure for concomitants removal, which improves OFFGEL fractionation performance and also that allows the implementation of more advanced proteomic schemes. These procedures (Table 1), mainly based SPE methods, are usually offline performed after tryptic digestion and/or iTRAQ/TMM labelling.

Collins et al. [63] have proposed FA acidification (room temperature, 4 h) before centrifugation (13,000 × g, 10 min) for removing Rapigest hydrolysis products (1.3% (w/v) Rapigest, 50 mM NH_4HCO_3, trypsin). The authors, however, have reported a direct OFFGEL performance when using trypsin dissolved in 5 % (w/v) TFA and 50 mM NH_4HCO_3 [63]. After conventional trypsin digestion, salts and detergents removal before OFFGEL peptides fractionation has also been proposed

by using mainly C18 cartridges [37,74-82], although applications of SCX SPE [83], multiple-cation-exchange (MCX) [84], and Oasis HBL [85,86] cartridges have been also found useful. After desalting, clean extracts were vacuum dried and redissolved in the OFFGEL Peptide sample solution. Methods based on combining two different desalting modes can also be found in the literature. Warren at al. [87] have proposed iTRAQ labelled peptides loading onto SCX cartridges and further elution (10 mM KH_2PO_4, 350 mM KCl, 25% ACN, and pH 3.0), followed by loading onto Vydac C18 4.6 × 150 mm RP columns for clean up the unreacted iTRAQ reagents and other contaminants before OFFGEL fractionation.

On other occasions, approaches based on stop-and-go-extraction tips (StageTips) have been proposed. StageTips are ordinary pipette tips containing small disks made of beads with reversed phase, cation-exchange or anion-exchange surfaces embedded in a Teflon mesh [88]. Under different operating conditions, desalting can be performed in approximately 5 min; whereas, pre-concentration processes can take approximately 30 min. Based on Rappsilber et al. protocol [88], Barbhuiya et al. [89] have performed salts removal from tryptic digests from human bile; nevertheless, Fraterman et al. [90] have used m-C18 Ziptips (peptides elution using acid-free buffers) for salts removal from tryptic digests from extraocular muscle from rats.

Finally, offline workflows in which samples are desalted before trypsin digestion and further peptides OFFGEL fractionation have also been proposed. Therefore, Wong et al. [91] have performed a first protein extract desalting (rapid chromatography on a 4.6 × 100 mm mRP-C18 column) using a gradient of 0.1 % TFA in water and 0.08 % TFA in acetonitrile (ACN) before tryptic digestion.

4.4.2.2. Integrated desalting treatments

The introduction of the filter aided sample preparation (FASP) technique by Wiśniewski et al. [92] has allowed biological samples solubilization, including detergent removal, buffer exchange, chemical modification (denaturation,

reduction, and alkylation), tryptic digestion, and peptides separation from undigested material at once. The method has been made commercially available through a kit, and it has been found convenient mainly for in-solution processing of samples solubilised in detergents like SDS [93-96]. The system uses ultra filter devices (molecular weight cut-off, MWCO, of 30 and 50 kDa) for removing interfering compounds associated with the sample during protein digestion, and further peptides recovery after centrifugation (typically 14,000 x g for 15 min). Therefore, excess of urea dissolved in 0.1 M Tris/HCl buffer (pH 8.5) and iodoacetamide (IAA) used for proteins denaturation and alkylation can be removed by centrifugation (treated protein remains inside the filter). When finishing the tryptic digestion, peptides can be recovered after new centrifugation stages (NH_4HCO_3 rinsing included) since un-reacted material remains inside the filter.

Addressing the need for robust and reliable sample processing for quantitative proteomics, trends in FASP developments have recently been focused on combining isobaric mass tagging with the FASP method. The resulting iFASP provides a quick, simple and effective method for obtaining clean samples, ensuring efficient digestion and providing excellent labelling yields for quantitative proteomics experiments [97].

Based on FASP technology, Abdallah et al. [98] have performed in-filter digestion (MWCO of 10 kDa) of microsomal proteins of *Medicago truncatula* roots with trypsin for further offline peptides labelling by the iTRAQ approach before OFFGEL fractionation and HPLC-MS/MS characterization. The same authors have pointed out the need for performing a desalting stage (use of C18 cartridges for solid phase extraction, SPE) for removing iTRAQ reagents excess, which interfere target peptides OFFGEL focusing [98]. Similarly, Ma et al [99] have used 30 kDa filtration devices for FASP peptides isolation from human blood serum. After N-linked glycopeptides enrichment and just before OFFGEL fractionation, in-solution digested samples were desalted with ZIC-HILIC tips.

4.5. OFFGEL electrophoresis fractions treatment for further protein/peptide separations

4.5.1. OFFGEL followed by electrophoresis techniques

High-ionic strength buffers containing urea, thiourea and 1,4-dithiothreitol (DTT), as well as detergents such as SDS and glycerol, are commonly used when performing in-gel and also OFFGEL electrophoresis proteins fractionation. The presence of these reagents is compatible with further SDS-PAGE when performing 2D-SDS-PAGE, and OFFGEL fractions must only be dissolved with sample loading buffer before heating at temperatures within the 30 – 100 °C range and loading onto the gel. Applications based on these schemes can be found in the literature when characterizing a wide variety of proteins (Table 1) [31,33,40,46,50-52,54,100-102].

However, other authors have reported the need for removing salts from the OFFGEL fractions before SDS-PAGE (Table 1). Gannon and Ohlendieck [44] have reported urea interference when isolating the pre-fractionated proteins by acetone precipitation before 2D-SDS-PAGE, and they have indicated the dialysis convenience for OFFGEL fraction purification. Similarly, other authors such as Konečná et al. [103] have reported the use of HiTrap desalting columns for removing OFFGEL buffer components prior to SDS-PAGE for proteins characterization in beer samples. Other authors such as O'Cualain et al. [47] have performed acetone precipitation for desalting OFFGEL fractions before peptide characterization by SDS-PAGE followed by in-gel trypsin digestion and HPLC-MS/MS.

Similarly, OFFGEL components are also adequate for performing further LOC electrophoresis of OFFGEL fractionated proteins. OFFGEL fractions treatment consisted of dilution with few microliters of the denaturing solution (β-mercaptoethanol, among other reagents) and heating at 95 °C for 5 min. These conditions have been used for assessing dissolved proteins from seawater [15] and also proteins from marine plankton [48,49]. Furthermore, descriptions of using

capillary electrophoresis (CE) and capillary zone electrophoresis (CZE) as a second dimension to OFFGEL have not also showed the need for OFFGEL fractions treatment [60,61].

4.5.2. OFFGEL followed by based-MS/MS techniques

There are described some OFFGEL electrophoresis – MS/MS methods, novel solid-phase extraction−gradient elution−mass spectrometry (SPE-GEMS) included [67], that have not performed OFFGEL fractions treatment (Table 1), except vacuum drying or lyophillization in some cases [33-35,38,41,42,56-58,63,66,68-73,78,81,91]. However, reagents used for IEF such as detergents and salts have been reported to damage further separation by HPLC techniques and/or MALDI analysis.

4.5.2.1. OFFGEL reagents composition changes

The presence of glycerol during OFFGEL IEF is needed for avoiding solvent evaporation, mainly in the outer wells. This is because, as previously commented, proteins/peptides separation occurs in solution and since no connection exists between the wells, targets are forced to migrate through the IPG gel which results of excessive water transport during focusing. Therefore, the presence of high glycerol concentrations (typically 12% (v/v)) in the protein/peptide OFFGEL buffers is recommended. However, high glycerol concentrations in the OFFGEL fractions have been reported to interfere with RP HPLC techniques, and also for further SpeedVac concentration (increased viscosity) and MALDI analysis (crystallization) [45,90]. Despite some authors have not reported problems when using high glycerol concentrations for performing OFFGEL fractionation [14,104], other workers have proposed reduced amounts of glycerol for IEF OFFGEL (within the 4.8 – 6.0 % (v/v) range, which implies a 50 % glycerol reduction) [55,75,77,83,87,90,105]. However, Abdallah et al. [98] have reported that a 50 % reduction on the glycerol concentration is not enough for avoiding pre-column clogging or for solving crystallization problems on a MALDI target, and a glycerol concentration of 0.3 % (v/v) has been proposed for OFFGEL fractionation. In

addition, Arrey et al. [45] have proposed glycerol-free OFFGEL IEF fractionation. To prevent the outer wells from running dry through excessive water transport, in addition to the sample volume added to each well (150 µL), supplemental 50-100 µL of 1 % IPG solution was added [45]. Other authors have proposed a 25 % reduction of the standard ampholyte concentration during OFFGEL for avoiding target ionization suppression, attributed to the presence of sample constituents and reagents after enzymatic digestion and iTRAQ labelling peptide [106]. Finally, Warren et al. [87] have reported the need for clarifying OFFGEL fractions with 0.65 µm low protein/peptide binding spin filters for preventing column clogging.

4.5.2.2. OFFGEL fractions desalting

In analogy to the use of IPGs for IEF as a first dimension before SCX-HPLC and MS/MS (shotgun proteomics) [107], salt removal from OFFGEL fractions before HPLC-MS/MS has also been recommended (Table 1). Desalting can be performed after exhaustive OFFGEL fractions washing, as reported by Elschenbroich et al [74] who have proposed at least five wash steps with 5 % ACN and 0.1 % TFA for removing residual reagents from the OFFGEL separation that interfered with subsequent LC-MS/MS analysis. However, desalting has commonly been performed by SPE with C18 SPE cartridges or with StageTip format-based SPE. Salts and other compounds are retained onto the adsorbent when using SPE cartridges/tip format-based, and under different operation conditions the desalted OFFGEL fractions are collected.

C18 SPE cartridges/columns have been used by several authors for OFFGEL fractions desalting purposes. In some cases, OFFGEL fractions can directly be loaded for desalting [39,62,74,82,85]; whereas, other authors have proposed OFFGEL fractions acidification with TFA [37,80,83,84,106], with FA [79], or with TFA/FA mixtures [76] before SPE. Some reported applications involve desalted OFFGEL fractions vacuum drying before TFA acidification for further concentration on RP C-18 cartridges [62]. In some cases, vacuum centrifugation at 35 °C for organic solvents removal before C18 SPE desalting has also been proposed [37].

StageTip approaches for OFFGEL fractions desalting have been demonstrated to be useful [64,65,89,99,105]. Other applications have involved the use of OMIX C18 tips [77] and SCX TopTips [86], or combined C18 ZipTip and C4 ZipTip for peptides OFFGEL fractions (after trypsin digestion) and proteins OFFGEL fractions desalting, respectively [43].

4.5.2.3. OFFGEL fractions treatments before trypsin digestion

Desalting procedures can also be applied before proteins fractionation by OFFGEL and just before in-solution tryptic digestion for MS/MS analysis. For these purposes, Kuhn et al. [59] have used gel filtration Zeba spin columns for buffer exchanged into 50 mM NH_4HCO_3; whereas, Setandreu et al. [101] have proposed the use of Zip-tips (20 µL OFFGEL fractions acidified with TFA, and elution with 20 µL of a solution containing 70 % ACN and 30 % 0.1 M TFA). On other occasions, however, no OFFGEL fractions treatment was performed before typsin digestion [53].

4.6. OFFGEL applicability for metal-protein complexes assessment

Denaturing or non-denaturing SDS-PAGE conditions, as well as applied current and post-separation gel treatment, have been reported to affect the stability of metal-protein complexes. Jiménez et al. [108] have shown that gel staining can alter the stability of the metal–protein complexes and prevent detection of metals bound to proteins. Therefore, staining should be avoided, and OFFGEL electrophoresis could be an advantageous technique for pre-fractionating metal-protein complexes as staining is not needed. García-Otero et al. [15] have studied the stability of two metal-binding proteins with different metal−protein affinities such as superoxide dismutase, SOD (two units of molecular weight of 16.2 kDa for each one and the presence of Cu and Zn), and alcohol dehydrogenase, ADH (tetramer of molecular weight of 141 kDa containing four equal subunits of 32.5 kDa and the presence of Zn) after OFFGEL electrophoresis (denaturing conditions) and further LOC electrophoresis. Results showed that Zn-binding SOD/ADH stability is ensured after OFFGEL electrophoresis separation because the presence of Zn

was detected in the OFFGEL fractions containing the SOD and ADH units (detected by LOC electrophoresis). These results contrast to those reported by Jiménez et al [108] for the same metal-protein complexes that were pI-fractionated by SDS-PAGE. These authors have found that on-denaturing conditions are more suitable for ensuring the integrity of metals non-covalently complexes to proteins, such Zn-SOD and Zn-ADH. Regarding Cu-SOD (weak metal−protein affinity), denaturing OFFGEL conditions led to Cu-SOD breakdown, and Cu was detected in OFFGEL fractions different than those fractions containing SOD units, mainly in OFFGEL fractions 22 and 23 which exhibit the highest alkalinity [15].

García-Otero et al. have also explored the stability of the metal-binding dissolved proteins isolated from surface and deep seawater during OFFGEL electrophoresis fractionation. Analysis of OFFGEL fractions 22 and 23 (the most alkaline fractions) for elements such as Cu, Fe, Mn, Ni, and Zn, gave negligible concentrations, which means that the used OFFGEL operating conditions appear therefore to guarantee the stability of the metal binding dissolved proteins (the presence of these elements was proved in some OFFGEL fractions for which LOC electrophoresis showed the presence of proteins) [15]. Similarly, studies regarding metal-protein complexes from marine plankton have also shown good capabilities of denaturing OFFGEL for ensuring the stability of Cd-, Fe- and Zn-proteins, although, lack of stability was proved for Cu-, Cr, Mn-, and Ni-protein complexes [49].

Although assuming the negative effect of the staining process when performing SDS-PAGE, the assessment of metal-protein complexes from seawater and marine plankton by conventional SDS-PAGE showed that the number of elements bound to the isolated proteins, as well as the concentrations, was lower than that obtained when analysing similar seawater and plankton samples by OFFGEL-LOC electrophoresis, and metals such as Fe, Mn and Ni were not found associated with any isolated protein (dissolved proteins in seawater and isolated proteins from marine plankton) [109]. In conclusion, OFFGEL electrophoresis conditions, in general, appear to be less drastic than those inherent to conventional SDS-PAGE and at least the integrity of some high-affinity metal-protein complexes can be

guaranteed even under denaturing conditions. However, to ensure metal-protein complexes integrity during OFFGEL electrophoresis, a careful selection of fractionating conditions must be performed. Recent studies by Mena et al. [52] have shown that the careful selection of OFFGEL solutions can avoid metal-protein complexes breakdown. These authors have proposed the use of denaturing solutions without thiourea and without DTT for guaranteeing the integrity of platinum-binding proteins during OFFGEL-IEF.

4.7. Conclusions

OFFGEL electrophoresis for further MS-based proteomic tools has been demonstrated to be a useful technique for modern proteomic analysis. Advances on using 48-well frames per IPG-strip, which allowed a resolution twice as high as the commercially available 24-wells devices, as well as the introduction of two- and multi-electrode setups (segmented field OFFGEL® electrophoresis), will improve the separation of proteins with small pI differences. Fractionation of isoforms, PTM isoforms, and in general, complex protein mixtures, is therefore possible, what increases and enhances the performance of IEF (first dimensional separation) in proteomics. Current developments based on the FASP approach for treating samples before OFFGEL electrophoresis are promising for shortening the sample treatment, which provides therefore fast proteomic workflows. Inherent OFFGEL electrophoresis conditions appear to be less drastic than those commonly used for conventional SDS-PAGE, and OFFGEL electrophoresis can therefore be an appealing methodology for assessing metal-proteins. However, further studies must be performed for ensuring the integrity of low affinity metal-protein complexes, which can definitely give additional advantages to this technique over other proposed fractionation techniques in modern proteomics.

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS.

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins extract from *E. coli* cultures	Enzymatic digestion: Proteins were reduced with 50% TFE with 200 mM DDT (95°C, 20 min) followed by IAA alkylation (1 h, room temperature) before trypsin digestion (trypsin/sample ratio of 1:20, 37°C, overnight) and vacuum dried.	None	HPLC-Chip/MS	7
Digested proteins from human neuroblastoma SH-SY5Y cell line	None	iTRAQ labelling	MALDI-MS-MS	14
Dissolved protein in seawater	Water- and methanol- soluble compounds removal by washing with a water/methanol/chloroform mixtures: 1 mL of retentate plus 4 mL of methanol, 1 mL of chloroform, and 3 mL of water (vortex mixing after adding each solvent). The mixtures were then centrifuged (4000 rpm, 10 min), and the lower chloroform layer recovered.	None	LOC electrophoresis	15
Proteins extract from *E. coli* cultures	None	None	SDS-PAGE	31
Proteins extract from *E. coli* cultures	None	None	Polyimide microchips-MS; LOC	33
Proteins from human plasma	Enzymatic digestion: Proteins were diluted in 50 mM bicarbonate buffer (pH 8.0) containing 5 mM DTT. Digestion was performed with trypsin (protein-to-enzyme ratio of 25/1 w/w) overnight at room temperature.	None	HPLC-MS/MS	34
Proteins from gastrocnemius muscle from Wistar rats	None	Dialysis for urea removal before trypsin digestion	2D-SDS-PAGE, HPLC-MS	44

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from human platelet	Enzymatic digestion: Proteins were reduced (5 mM TCEP, 37°C, 1 h) and alkylated (10 mM IAA, 37 °C, 1 h). Endoproteinase Lys-C was added to a final 1:500 w/w enzyme to substrate ratio, and the reaction was allowed to proceed for 2 h at 37°C. The partial digest was then diluted four-fold in water before trypsin addition (1:25 w/w enzyme to substrate ratio) for digestion overnight at 37°C.	None	HPLC-MS/MS	35
Serum proteins	None	None	HPLC-MS/MS	36
Proteins from liver from mice	(1) Enzymatic digestion: Protein extract was treated with 2% (w/v) Rapigest, before reduction with 3.5 mM DTT (60°C, 10 min) and alkylation with 11.25 mM IAA (room temperature, 30 min in the dark). After 10-fold dilution with 50 mM Tris (pH 8.5), trypsin digestion (1:20 enzyme/protein) was performed at 37°C for 18 h. Afterwards, Rapigest was hydrolyzed by lowering the pH below 2 for 30 min on ice. (2) Desalting: Peptides extract were purified using PepClean C18 spin columns according to manufacturer's instructions.	Desalting: OFFGEL fractions were vacuum centrifuged at 35 °C for 45 min for organic solvents removal before desalting with PepClean C18 spin columns (manufacturer's instructions)	SCX-HPLC; RP-HPLC-MS/MS	37
Cortical brain tissue from mice	None	None	HPLC-MS/MS	38
Proteins extract from *E. coli* cultures	None	None	SDS-PAGE	40

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from human hepatoma cell line PLC/PRF/5	Enzymatic digestion: Protein pellet was reduced with 200 mM DTT in 100 mM NH_4HCO_3 (95°C, 5 min) followed by alkylation (1 M IAA in 100 mM NH_4HCO_3, 45 min, room temperature) before trypsin digestion (trypsin/sample ratio of 1:50, 37°C, 16 h).	Desalting: use of C18 spin columns followed by SpeedVac concentration	HPLC-MS/MS	39
Solutions containing β-lactoglobulin, α-lactalbumin, cytochromeC, myoglobin, and RNase A. Proteins extract from *E. coli* cultures	None	None	MALDI-TOF-MS	41
Proteins from adult worms (A. caninum) from dogs' intestine	Enzymatic digestion: proteins (240 µg) were reduced with 20 mM DTT and SDS (10 to 2% (w/v)) at 65°C for 1 h, before alkylation (50 mM IAA, 22°C, 40 min) and trypsin digestion (1 µL of 1 µg μL^{-1} trypsin plus 9 volumes of methanol at -20°C). After incubation overnight at -20°C, the sample was resuspended in 25 mM NH_4HCO_3 and incubated at 37°C for 5 h with the addition of an additional 1 µg of trypsin after 3 h.	Desalting: Peptide OFFGEL fractions were desalted with C18 ZipTips before vacuum drying, redissolution in 25 mM NH_4HCO_3, and drying down again before mass spectral analysis. Protein OFFGEL fractions were treated with 50 mM IAA and incubated at 22°C for 40 min before desalted with C4 ZipTips. After lyophilization and resuspension in 25 mM NH_4HCO_3, fractionated proteins were digested by incubation with 1 µg of trypsin for 5 h before lyophilization.	HPLC-MS/MS	43

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Bovine saliva proteins	None	Enzymatic digestion: OFFGEL fractions were acetone precipitated and re-suspended in 100 µL of 50 mM Tri-HCl, pH 8.0 before digestion with trypsin.	HPLC-MS/MS	42
Proteins from *Halobacterium salinarium* purple membranes (PMs) with bacteriorhodopsin and from *Corynebacterium glutamicum* DM1730 membranes (CGMs)	None	Glycerol-free focusing.	RP-HPLC-MALDI-MS/MS	45
Antigenic proteins of *Helicobacter pylori* from blood from duodenal ulcer patients	None	None	MALDI-TOF-TOF after in-gel digestion, Western blotting	46
Proteins from marine plankton	The protein pellet was washed once with 100 % methanol and once with 80 % acetone. Then, it was the dried before redissolution in the Protein OFFGEL the buffer solution.	None	LOC electrophoresis	48, 49
Proteins whose expression is reduced in the taxol-resistant stathmin-overexpressing cell lines	None	None	SDS-PAGE	50

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from *Plasmodium falciparum* parasites released from infected erythrocytes	None	Desalting: OFFGEL fractions alkylated (1 -50 M IAA, 22°C in darkness, 20 min), diluted with two volumes of deionized water, and desalting by acetone precipitation with five volumes of ice-cold acetone.	SDS-PAGE, HPLC-MS/MS	47
Proteins from human cerebrospinal fluid	Enzymatic digestion: as reported in reference 34	None	SDS-PAGE followed by HPLC-MS/MS	51
Platinum-binding proteins (proteins from kidney from rats treated with cisplatin).	None	None	SDS-PAGE; MALDI-TOF-MS	52
Proteins from human plasma and amniotic fluid	None	Before trypsin digestion, OFFGEL fractions were diluted five times in 50 mM NH_4HCO_3 buffer (pH 8.0) containing 5 mM DTT (urea dilution below 2 M to allow for trypsin digestion). Digestion was performed overnight at room temperature with a protein: trypsin ratio of 25:1	HPLC-MS/MS	53
Proteins from plasma samples from breast cancer patients	None	None	SDS-PAGE; HPLC-MS/MS	54
Endogenous cardiac sarcomeric proteins	None	Use of 6.0 %(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation	HPLC-MS/MS	55

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Protein biomarkers associated with pro-inflammatory high-density lipoprotein (HDL) in patients with active rheumatoid arthritis	None	None	HPLV-MALDI-TOF-MS	56
Low-abundance proteins in human urinary exosomes	Enzymatic digestion: Sample was reduced and denatured using 50 % TFE with 200 mM DTT at 95°C for 20 min, followed by alkylation with IAA at room temperature for 1 h. After 1:10 dilution, trypsin digestion (1:20 enzyme: substrate, 37°C, overnight) was performed.	None	HPLC-MALDI-MS/MS	57
Proteins from RAW 264.7 cells (anthrax lethal toxin-treated murine macrophages)	Proteins were buffer exchanged into the OFFGEL running buffer using gel filtration Zeba spin columns	Before trypsin digestion, OFFGEL fractions were diluted with 50 mL 100 mM NH_4HCO_3 and alkylated (50 mM IAA, 30 min). Non-reacted IAA was quenched by addition of 50 mM DTT. The fractions were buffer exchanged into 50 mM NH_4HCO_3 using Zeba spin columns. Digestion was performed overnight at 37°C with a protein: trypsin ratio of 30:1.	HPLC-MS/MS	59

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from the MDA-MB-435 cell line isolated from the pleural effusions of a patient with breast carcinoma	Proteins were reduced with TBP and alkylated with acrylamide using a reduction/alkylation kit	None	HPLC-MS/MS	58
Solutions containing bovine serum albumin, β-lactoglobulin, horse myoglobin, and cytochrome C	Each protein (1-3 $mg.mL^{-1}$) was dissolved in a 75 mM NH_4HCO_3 buffer (pH 7.7) and heated for 5 min. Trypsin digestion (enzyme to protein ratio 1:100) was carried out overnight at 37°C.	None	CE	60
Solutions containing bovine serum albumin, β-lactoglobulin, horse myoglobin, and cytochrome C	Each protein was dissolved in a 50 mM NH_4HCO_3 buffer (pH 8.2) and heated at 100°C for 5 min. Trypsin digestion (enzyme to protein ratio 1:100) was carried out overnight at 37°C.	None	CZE	61
Proteins of the molecular machinery that control the Golgi disassembly and reassembly processes in the cell cycle	(1) Enzymatic digestion: Golgi membrane pellets were solubilized on ice in 50 µL buffer containing 0.5 M triethyl ammonium bicarbonate, 8 M urea and 0.4 % SDS before reduction with 5 mM TCEP (37°C, 1 h) and cysteines blocking with 10mM MMTS (room temperature, 20 min). The protein solution was diluted four times with 0.5 M TEAB buffer containing 3 µg of trypsin (1:10 w/w) and digestion was performed at 37°C overnight. (2) iTRAQ labelling	(1) Desalting: OFFGEL fractions were desalted using Pierce C-18 spin columns (2) Concentration: Desalted OFFGEL fractions were vacuum-dried and redissolved in 40 µL 0.1 % TFA for concentration by loading on Zorbax RP C18 cartridges.	HPLC-MALDI-MS/MS	62
Proteins from human follicular fluid	Enzymatic digestion: Protein (500 µg) to reduction and alkylation (details not given) before tryptic digestion (37°C, 16 h).	Desalting: OFFGEL fractions were acidified with 1 % TFA followed by Stage-tip desalting according to reference 88	HPLC-MS/MS	65

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from Wistar rats liver	Enzymatic digestion: Protein extract (200 µg) was reduced (5 mM TCEP, 20 min, room temperature) and alkylated (10 mM IAA, 30 min room temperature in the dark) before precipitation with six volumes of acetone for 2 h at -20°C. The samples were centrifuged for 10 min at 5000 × g and 4°C, and the pellets were resolubilized in Rapigest 1.3 % (w/v), 50 mM NH_4HCO_3 or 5 % (w/v) TFA, 50 mM NH_4HCO_3, and incubated with 2 µg trypsin (overnight, 37°C). Rapigest containing samples were acidified with 2% (v/v) FA (4 h, room temperature) and centrifuged (13,000 × g, 10 min) to remove the Rapigest hydrolysis products. The samples were transferred to clean tubes, evaporated to dryness in a centrifugal evaporator (60°C, 150 × g), and resuspended in 100 µL of 0.1% (v/v) FA, 3% (v/v) ACN.	None	HPLC-Chip-TOF-MS	63
Urinary proteins	Enzymatic digestion: After high-abundance proteins depletion, urinary proteins (200 µg) was loaded on a 10 % Bis-Tris gel in accordance with manufacturer's instructions. The gel lane was then cut into 18 pieces and performed in-gel tryptic digestion. After DTT reduction and IAA alkylation, proteins were digested by trypsin (37 °C, overnight).	None	HPLC-Chip-MS/MS	68

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Membrane proteins in adipose tissue of mice	(1) Enzymatic digestion: Protein pellets were suspended in 100 µL of 0.1 M TEABC, pH 8.5, 6 M urea, 5 mM EDTA, and 2% (w/v) SDS, followed by cysteines reduction (5 mM TCEP, 37°C, 30 min) and alkylation (2 mM MMTS, room temperature, 30 min in the dark). Acrylamide/bisacrylamide, 10% (w/v) ammonium persulfate, and TEMED were then added (sample: acrylamide/bisacrylamide: ammonium persulfate: TEMED = 14:5:0.7:0.3, v/v/v/v), and the solutions were allowed to polymerize directly in the microcentrifuge tubes. The gels were cut into small pieces, washed several times with 1 mL of 50 mM TEABC, pH 8.5/ 50% ACN, and dehydrated (1 mL of ACN and SpeedVac drying). Trypsin digestion (protein: trypsin = 10:1, w/w) was performed in 25 mM TEABC, pH 8.5 (overnight, 37 °C). Peptides were sequentially extracted from the gels with 25 mM TEABC, pH 8.5, 0.1% (v/ v) TFA/water, 0.1% TFA/50% ACN, and 100% ACN. The solutions were finally combined and SpeedVac concentrated. (2) iTRAQ labelling	Desalting: OFFGEL fractions were SpeedVac dried and redissolved in 50 µL of 0.1 % (v/v) TFA before desalting and concentration through C18 ZipTips	HPLC-MS/MS	64
Proteins from HCT-116 cells	Enzymatic digestion: Proteins pellet was suspended in a buffer containing 8 M urea and 400 mM NH_4HCO_3 before reduction (10 mM DTT, room temperature, 30 min) and alkylation (20 mM IAA, 30 min in dark). Trypsin digestion (enzyme to substrate ratio of 1:100 (w:w)) was performed at 37 °C for 16 h.	None	HPLC-MS/MS	69

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Myoglobin	(1) Enzymatic digestion: Myoglobin (5 mg mL^{-1}) was first denatured (100°C, 5 min) before trypsin digested (0.17 mg mL^{-1} in 25 mM NH_4HCO_3 buffer, 37 °C, 18 h).	None	SPE-GEMS	67
Proteins from hydatid cyst fluid (HCF) of *Echinococcus granulosus* from sheep, cattle and humans	Enzymatic digestion: as reported in reference 43	None	HPLC-MS/MS-MS	71
Proteins from *Schistosoma japonicum*	Enzymatic digestion: as reported in reference 43	None	HPLC-MS/MS-MS	72
Proteins from carcinogenic human liver fluke *Opisthorchis viverrini*	Enzymatic digestion: as reported in reference 43	None	HPLC-MS/MS-MS	73
Proteins from he human mantle cell lymphoma cell line Granta-519	(1) Enzymatic digestion: Proteins (100 µg) were reduced in 3 M urea with 10 mM DTT (56 °C, 1 h) before alkylation (55 mM IAA, room temperature, 45 min, RT). DTT was then added to remove IAA excess. Trypsin digestion (protein/enzyme ratio of 50:1 in 100 mM NH_4HCO_3, 1 M urea) was performed overnight at 37 °C. (2) Desalting: Trypsin digests were desalted with UltraMacroSpin C18 columns by washing at least five times with water and eluting with 60 % ACN before SpeedVac concentration.	Desalting: OFFGEL fractions were acidified with 1 % TFA and 0.1 % FA before desalted by loading onto UltraMicroSpin C18 columns, and eluting with 50 % ACN, 0.1 % FA. Eluates were then SpeedVac dried and resuspended in 8 µL of 0.1% FA.	RP-HPLC-MS/MS	76

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Urinary proteins	Enzymatic digestion: Dried purified/depleted protein was dissolved in 100 µL with 100 mM TEAB (pH 8.5) before trypsin digestion (4 µL of 1 mg mL^{-1} trypsin to obtain a ratio of 1:50 w/w, 37°C, overnight).	None	HPLC-MS/MS	70
Proteins from membrane-enriched fraction from C2C12 mouse skeletal muscle cells	(1) Enzymatic digestion: The dried protein pellet was resuspended in 50 µL of freshly prepared resuspension buffer (8 M urea in 100 mM Tris, pH 8.5, 2 mM DTT) and placed in an incubator at 37°C for 30 min. After alkylation (8 mM IA, 37°C, 2 h) and dilution (1.5 M urea, 100 mM NH_4HCO_3 buffer, pH 8.5, and 2 mM $CaCl_2$), proteins were digested with a 1:40 ratio of trypsin at 37°C overnight (2) Desalting: Use of Varian OMIX SPE cartridges. Eluted peptide mixtures were vacuum-dried and reconstituted with 18 mL of OFFGEL Peptide sample solution.	Desalting: Use of C18 MiniSpin columns. After loading, five wash steps with 5 % ACN and 0.1 % TFA were performed. Eluted peptides were vacuum-dried and reconstituted with 40 µL of 5 % ACN and 0.1 % FA.	RP-HPLC-MS/MS	74
Proteins from D. melanogaster Kc167 cells	(1) Enzymatic digestion: Disulfide bonds reduction with 12.5mM TCEP (37°C, 1 h) followed by alkylation (40mM IAA, room temperature, 1 h) and dilution with 20 mM Tris–HCl (pH 8.3), 1.0 M urea. Digestion was performed with 20 µg trypsin per mg of protein overnight at 37°C. (2) Desalting: Use of C18 Sep-Pak cartridges, and vacuum drying	Use of 6.25 %(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation Desalting: use of C18 spin columns according to the manufacturer') instructions	RP-HPLC-MS/MS	75
Proteins from *Saccharomyces cerevisiae* and workhorse prostate cancer cell line LNCaP	(1) Enzymatic digestion: Typsin digestion (details not given) (2) Desalting: MCX cartridges (details not given)	Desalting: details not given	HPLC-MS/MS	84

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from HepG2 and SK-N-MC human cell lines	(1) Enzymatic digestion: Unseparated protein bands after conventional SDS-PAGE were in-gel digested (60 ng μL^{-1} trypsin at 5:1 protein: trypsin (w/w) ratio in 50 mM NH_4HCO_3, pH 8.8 containing 10% (v/v) ACN and 0.01% (w/v) 5-cyclohexyl-1-pentyl-β-D-maltoside, 37°C, overnight). The resulting tryptic peptides were extracted by 1 h incubation in 12 mM NH_4HCO_3 (pH 8.8). (2) Desalting 1: Peptide digests were acidified with 1 % TFA before desalted onto C18 OASIS cartridges and dried-down. (3) Differential $^{16}O/^{18}O$-labeling: dried peptides were subjected to differential $^{16}O/^{18}O$-labeling in 100 mM ammonium acetate (pH 6.0), 20 % CAN, at a 1:200 (v:w) immobilized trypsin/protein ratio. (4) Desalting 2: The two labelled samples were mixed, diluted to 2% ACN and pH adjusted to 3 with 1 M ammonium formate, pH 3, desalted onto C18 Oasis cartridges using as elution solution 50% ACN in 5 mM ammonium formate pH 3, and dried down.	Use of 5 %(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation Desalting: OFFGEL fractions were acidified with 20 µL of 1 M ammonium formate (pH 3.0) before peptides desalting with OMIX C18 tips. After elution with 50 % ACN in 5 mM ammonium formate (pH 3.0), the peptides were dried-down.	RP HPLC-MS/MS	77
Proteins from beer	Protein samples (5 mg of whole protein, representing about 22 mL of raw beer) were solubilized in a Protein OFFGEL fractionation buffer, after degassing, desalting, and complete evaporation of water.	Desalting: use of HiTrap desalting columns to remove OFFGEL buffer components	SDS-PAGE; MALDI-MS/MS	103

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from human colorectal tissue	(1) Enzymatic digestion: proteins extract was incubated with 5 µL of trypsin at 37°C for 1 h with vigorous shaking for 30 s at 20 min intervals, then further incubated overnight at 37°C followed by heating at 95°C for 5 min. After centrifugation (10,000× g), and SpeedVac drying, peptides were resuspended in 100 µl of 0.5 % TFA in 5 % ACN, desalted via PepClean C-18 spin columns, and dried for iTRAQ processing. (2) Desalting: Labelled peptides were desalted onto Sep-Pak C18 cartridges before dilution with OFFGEL Peptide sample solution.	None	HPLC-MALDI-TOF-MS/MS	78
Cardiac sarcomeric proteins from rats	(1) Enzymatic digestion: proteins (125 µg) were reduced with 10 mM DTT (45 min at room temperature), and then treated with 40 mM IAA (45 min at room temperature). The sample was diluted to 1 M urea final concentration with 100 mM triethylammonium bicarbonate pH 8.5, and digested with trypsin (1:20 trypsin: protein ratio) at 37°C for 16 h. (2) iTRAQ labelling: The digest was centrifugal vacuum concentrated to less than 50 µL before iTRAQ labelling using either 114 or 117 following the manufacturer's recommendations. (3) iTRAQ labelled peptides (100 µL) are diluted with in 900 µl of 10 mM KH_2PO_4, 25% ACN, pH 3.0, and syringe loaded onto SCX cartridges, and further eluted by passing 10 mM KH_2PO_4, 350 mM KCl, 25% ACN, and pH 3.0. Eluted peptides are then loaded on a Vydac C18 4.6 ×1 50 mm RP column for complete removal of contaminants before OFFGEL fractionation	Use of 4.8%(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation.	HPLC-MS/MS	87

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from human plasma	(1) Enzymatic digestion: Protein pellets were thawed briefly and resuspended in 100 mM NH_4HCO_3, 8 M urea buffer, pH 8.5 before reduction (5.7 mM TCEP, 37°C, 1 h) and alkylation (30 mM cysteine, 37°C, 15 min). A two-step proteolytic digestion was performed. First, sample was diluted with 25 mM NH_4HCO_3 to 4 M urea, digested with trypsin (enzyme/protein: 1/100) for 4 h at 37°C, and then diluted with 25 mM NH_4HCO_3 to 2 M urea and digested with trypsin (enzyme/protein: 1/50) overnight at 37°C. (2) Desalting: Labelled peptides were desalted onto Sep-Pak C18 cartridges before dilution with OFFGEL Peptide sample solution.	Desalting: OFFGEL fractions were acidified with 10 % FA (final pH 3.0) and desalted Ultra-MicroSpin SPE columns. Desalted fractions were SpeedVac dried before redissolution in 0.1 % FA.	RP-HPLC-MS/MS	79
Protein from human cerebrospinal fluid	(1) Enzymatic digestion: Proteins were denatured with 9.0 M urea, then reduced (10 mM DTT, 37°C in the dark, 1 h) and alkylated (50 mM IAA, 37°C in the dark, 30 min). The sample was diluted to 1.85 M urea concentration by addition of 0.2 M NH_4HCO_3 (pH of 8.5) for protein digestion (20 µg trypsin, 37°C in the dark, overnight). (2) Desalting: Tryptic peptides were then isolated via with a C-18 Sep-Pak Vac cartridges according to manufacturer's instructions. Peptides were eluted with 70 % ACN/0.1 % FA, and were dried using vacuum centrifugation.	None	RP HPLC-MS/MS	82

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from human plasma	(1) Enzymatic digestion: 100 µg of protein solutions, at 5 mg mL^{-1} in 0.5 M TEAB (pH 8.5) were reduced (5 mM TCEP, 60°C, 1 h) and reduced (10 mM MMTS, room temperature, 10 minutes). The proteins were then digested for 40 h at 37°C with10 µg of trypsin. (2) iTRAQ labelling (3) Desalting: Peptides digests were SPE using C18 MacroSpin Columns according to the manufacturer's instructions.	None	HPLC-MALDI-MS/MS	81
Proteins from *Pyrococcus furiosus*	(1) Desalting: Proteins extract was denatured by dissolution in 6 M urea, 1 % acetic acid solution. Denatured proteins (250 µg) were loaded on a 4.6 × 100 mm mRP-C18 column for chromatographic separation with a gradient of 0.1 % TFA in water (A) and 0.08% TFA in ACN (B): 3 min hold at 10% B, followed by a gradient ramp to 70% B at 5 min, then 100% B at 6 min. After holding at 100% B for 2 min, the gradient returned to initial conditions. The fractions eluting between 6 and 9 min were combined and dried. (2) Enzymatic digestion: Dried and desalted sample (250 µg protein) was dissolved in 50% TFE in a 50 mM NH_4HCO_3 buffer, reduced with 4 mM DTT (95°C, 20 min), and alkylated with 16 mM IAA (room temperature, 1 h). Unreacted IAA was quenched by the addition of DTT. After a 1:10 dilution with 25 mM NH_4HCO_3 buffer, trypsin (1:50 enzyme/substrate ratio) was added and digestion was performed overnight at 37°C. The digest was quenched by the addition of FA, then dried and stored at −20°C until fractionation.	None	RP-HPLC-MALDI-TOF-MS	91

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from human bile	(1) Enzymatic digestion: proteins were reduced (5 mM DTT, 60°C, 45 min) and alkylated (20 mM IAA, room temperature, 10 min in dark) before trypsin digestion (trypsin-toprotein ratio of 1:20 w/w, 37°C, 16 h). (2) Desalting: Tryptic digests were desalted using Stage-tips according to reference 88.	Desalting: OFFGEL fractions were acidified (1% TFA) before desalting using Stage-tips according to reference 88.	HPLC-MS/MS	89
Proteins from chicken meat specimens	None	(1) Desalting: each OFFGEL fraction (20 µL) acidified with 20 µL of 0.1% TFA, followed by salts removal using Zip-tips: protein elution was made with 20 µL of 70% ACN/30% 0.1 TFA. The solvent was SpeedVac evaporated and the residue reconstituted with 15 µL of 50 mM ammonium bicarbonate (pH 8.0). (2) Enzymatic digestion: addition of 15 µL of 12.5 ng μL^{-1} trypsin dissolved in the same buffer (37°C overnight), followed by SpeedVac concentration and redissolution with 0.1% (v/v) TFA	MALDI-TOF-MS; HPLC-MS/MS	101

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from chemo-naive ascitic fluid samples	(1) Enzymatic digestion: After high-abundance proteins depletion, 50 µL of freshly prepared buffer was added (8 M urea in 100 mM Tris pH 8.5 containing 2 mM DTT) and samples were reduced at 37°C for 30 min. Alkylation was performed with 8 mM IAA (37°C, 2 h). The sample was then diluted to a urea concentration of ~1.5 M with 100 mM NH_4HCO_3 buffer (pH 8.5) and 2 mM $CaCl_2$. Proteins were digested with a 1:40 ratio of trypsin at 37°C overnight. Protein digestion was stopped by addition of 50 µL of 2.5% TFA (2) Desalting: Peptides digests were SPE using C18 MacroSpin Columns according to the manufacturer's instructions.	Desalting: OFFGEL fractions were desalted with C18 miniSpin columns according to the manufacturer's instructions. Peptide mixtures were eluted, vacuum-dried and reconstituted with 40 µL 5 % ACN, 0.1 % FA.	HPLC-MS/MS; MS/MS	80
Proteins form Drosophila Kc167 cell line	(1) Enzymatic digestion: Proteins (2 mg) were reduced by 5mM TBP and alkylated by 10 mM IAA. After 8-fold dilution with water, trypsin was added (enzyme: protein ratio of 1:50 w/w) and digestion was performed at 37°C overnight. (2) Desalting: Protein digest was acidified to pH 2.8 by 1 % formic acid, and then loaded onto a SCX SPE cartridge. After extensive washing with 4 mL of 0.1% (v/v) FA and 2 mL of 25 % (v/v) ACN in 0.1% (v/v) FA, peptides were eluted with 1.5 mL of 10% (v/v) NH_4OH solution containing 25% (v/v) ACN. The purified peptides were vacuum-dried and redissolved in OFFGEL buffer.	Use of 5.0 %(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation. Desalting: OFFGEL fractions were diluted with 1mL 0.1 % (v/v) TFA and acidified to pH 3.0 by addition of 1% (v/v) TFA, before loading onto a Sep-Pak C18 cartridge Desalted fractions were then evaporated to dryness and redissolved in 20 µL of 0.4% (v/v) acetic acid.	RP HPLC-MS/MS	83
Proteins from chicken meat specimens	None	None	SDS-PAGE plus HPLC-MS/MS	100

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Protein from human non-small cell lung adenocarcinoma cell line H358 of bronchi alveolar origin (ATCC CRL5807)	(1) Enzymatic digestion: Use of trysin (1:20 ratio (w/w)) and incubation at 37ºC for 20h. Protein digests were dried by vacuum centrifugation and suspended in 30 µL of dissolution buffer (5MTEAB, 0,1% SDS) (2) iTRAQ labelling	None	HPLC-MALDI-MS/MS	66
Proteins from the hHSC cell line LX-2 and the hPaSC cell line RLT-PSC (human pancreatic and hepatic stellate cell lines)	(1) Enzymatic digestion: Proteins were diluted to a concentration of 150 µg of total protein per 100 µL of 100 mM TEAB buffer. Protein digests were performed as specified in the manufacturer's instructions for the Thermo Scientific's TMTsixplex Isobaric Mass Tagging Kit. (2) TMT labelling. (3) Desalting: Peptide digests were desalted using OASIS HLB RP cartridges in accordance with the manufacturer's instructions. The methanol-eluted peptides were vacuum centrifuged to dryness.	Deasliting: OFFGEL fractions were desalted in TopTip SCX columns using a binding solution containing 0.1 % FA and 20% ACN. Elution was performed with a solution containing 5% NH_4OH and 30% methanol. The eluent was vacuum centrifuged to dryness	HPLC-MS/MS	86
Microsomal proteins from leaves and roots from plants	None	None	SDS-PAGE	102

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins in cerebrospinal fluid	(1) Enzymatic digestion: Protein samples and also peptide standard mixtures were dissolved in 100 µL of 100 mM TEAB, and pH was adjusted to 8 with diluted HCl. 1 µL of 1% SDS and 2 µL 50 mM TCEP were for reduction (60°C, 1 h). Alkylation was performed by adding 1 µL of 400 mM IAA (30 min in the dark). An amount of 10 µL of trypsin 0.2 µg μL^{-1} freshly prepared in the TEAB solution was added, and the digestion was performed overnight at 37°C. (2) Isobaric tandem mass tags (TMT) labelling. (3) Desalting: Tryptic digests were desalted with Oasis HLB (30 mg) extraction cartridges.	Desalting: OFFGEL fractions were dried and cleaned with C18 ultramicrospin column.	HPLC-MALDI-TOF/TOF-MS	85
Proteins from yeast strain YAL6B	Enzymatic digestion: Proteins (1 mg) were digested with 20 mg of LysC overnight, then diluted with water to 1.5 M urea/0.5 M thiourea and digested with 20 mg trypsin overnight.	Use of 5.0 %(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation. Desalting: use of Stage Tips by eluting with 20 µL of 80% ACN, 0.5% acetic acid (twice). The eluate was speed vacuum reduced to 4 µL. Finally, 2 µL of a 2% ACN and 1% TFA were added to acidify the sample.	HPLC-MS/MS	105
Human serum proteins	(1) FASP: Protein pellet was mixed with 200 µL of UA (8 M urea in 0.1 M Tris/HCl, pH 8.5). The mixed sample was loaded into a 30kDa Microcon filtration device and centrifuged at 14 000 × g for 20 min so that the remaining volume was less than 10 µL. The concentrates were then diluted in the	Desalting: OFFGEL fractions were purified on ZipTips C18 (details not given), and were dried via vacuum centrifugation.	HPLC-MS/MS	99

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Human serum proteins	devices with 200 µg UA solution and centrifuged twice. After centrifugation, the concentrates were alkylated (100 µL 50 mM IAA in UA solution, room temperature in darkness, 30 min), and then centrifuged for 20 min. The concentrate was diluted with 200 µL UA and concentrated again. This step was repeated twice. The samples were then diluted with 100 µL 40 mM NH_4HCO_3 and concentrated twice. After concentrating, 8 µg trypsin or 8 µg Glu-C in 100 µL 40 mM NH_4HCO_3 (pH 7.8) or 100 µL 50 mM NaH_2PO_4 (pH 7.5) was added for digestion overnight at 37°C. The resulting peptides were collected by centrifugation of the filter units with 50 µL binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.3) for 20 min. This step was repeated six times (2) N-linked glycopeptides enrichment. (3) Desalting: 100 µg in-solution digested samples were redissolved in 80% ACN 0.5% FA). The ZIC-HILIC tip was washed with 100 µL 80% ACN, 1% FA, and 19% H_2O for five times, and the bound peptides were eluted with 80 µL elution buffer (99% H_2O, 1% FA) for three times.		HPLC-MS/MS	99

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from extraocular muscle from rats	(1) Enzymatic digestion: protein extract (2 mg lysate from 6 mg wet muscle tissue) was redissolved in 30 mM ammonium bicarbonate and ultra-sonicated for 2 min. The lysates were reduced in 200 mM DTT, alkylated in 500 mM IAA and digested with trypsin overnight at 37°C. After digestion, the lysate was cleared at 5000 rpm for 5min, lyophilized, redissolved in 30 µL 2% formic acid. (2) Desalting: use of m-C18 ZipTips (elution with acid-free buffers).	Use of 6.0%(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation.	RP HPLC-MS/MS	90
Microsomal proteins of *Medicago truncatula* roots	(1) FASP: (a) 10 mM DTT (reduction of protein disulphide bonds). (b) 50 mM IAA (carbamidomethylation of thiols). All the reagents added during these steps were easily removed by centrifugation. (c) Trypsin protein digestion (room temperature, overnight). Peptide fraction free of interfering compounds is obtained by centrifugation. (2) iTRAQ labelling (3) Desalting: use of C18 cartridges for removing iTRAQ reagents	Use of 0.3%(v/v) glycerol in the Protein OFFGEL stock solution during OFFGEL fractionation.	HPLC-MS/MS	98
Peptides from secretome and plasma	iTRAQ labelling	None	MALDI-TOF-TOF	104

Table 1: Protein pellet and OFFGEL fractions treatments when performing OFFGEL electrophoresis hyphenated with SDS-PAGE, LOC electrophoresis, and MS/MS. (Continuación)

Sample type	Protein pellet treatment	OFFGEL treatment	Second dimension analysis	Reference
Proteins from potato tubers	(1) Enzymatic digestion: (a) Lys-C (enzyme to substrate ratio of 1/80 (w/w)) in 3 M urea/0.2% SDS/ 0.5M TABC buffer (pH 8.5) at 33°C overnight. (b) The digests were then diluted to 1 M urea/0.07% SDS with 0.5 M TABC before further trypsin digestion (enzyme to substrate ratio of 1/25 (w/w)) at 33°C overnight. (2) iTRAQ labelling: Digests were SpeedVac dried and reconstituted in 30 µL of 0.5 M TABC buffer, and labelled with iTRAQ reagents following the manufacturer's instruction.	(1) Use of 25% reduction of the standard ampholyte during OFFGEL fractionation (2) Desalting: use of Sep-Pak SPE cartridges after acidifying OFFGEL fractions (iTRAQ labelled peptides) with TFA. Desalted fractions are then SpeedVac concentrated and redissolved with a solution containing 2% acetonitrile (ACN) and 0.5% formic acid (FA).	HPLC-MS/MS	106

ACN, acetonitrile; DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FA, formic acid; HILIC, hydrophilic interaction liquid chromatography; IAA, iodoacetamide; iTRAQ, isobaric tags for relative and absolute quantitation; MMTS, methyl methanthiosulphonate; SDS, sodium dodecyl sulfate; TBP, tri-N-butylphosphine; TCEP, tris(2-carboxyethyl)phosphine; TEAB, triethylammonium bicarbonate; TEMED, N,N,N'N'-tetramethylethylenediamine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TMT, isobaric tandem mass tags

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II. OBJETIVOS

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Objetivos

La biodisponibilidad, movilidad, distribución y toxicidad de los elementos traza en el medio ambiente no solo depende de su concentración, sino también de las formas químicas en que dichos elementos se encuentran, determinando éstas sus asociaciones fisicoquímicas en el medio natural. Por lo tanto, los objetivos generales de este trabajo son los siguientes:

- El desarrollo de métodos de preconcentración para materia orgánica disuelta en agua de mar (DOM).
- La especiación de metales asociados a DOM en agua de mar.
- La especiación de metales asociados a proteínas disueltas en agua de mar.
- La especiación de metales asociados a proteínas extraídas de plancton marino.

Más concretamente, se pueden enumerar los siguientes objetivos específicos:

1. El desarrollo de un método para preconcentrar materia orgánica disuelta (DOM) en agua de mar empleando nanotubos de carbono de pared múltiple (MWCNTs) como adsorbentes de extracción en fase sólida (SPE).

2. La aplicación del método de SPE con MWCNTs para la caracterización de DOM en función de su tamaño molecular mediante cromatografía líquida de alta resolución (HPLC) en el modo de exclusión por tamaños (cromatografía de exclusión por tamaño, SEC).

3. La evaluación de la ultrafiltración (UF) en flujo tangencial como método de preconcentración de DOM de alto peso molecular, empleando para ello membranas con diferentes tamaños de poro (3,0 y 10 kDa de tamaño de corte molecular).

4. El desarrollo y la optimización de un método para la caracterización y determinación de metales unidos a DOM ultrafiltrada. Para este objetivo se emplearán técnicas cromatográficas bidimensionales, en modo de exclusión por tamaños y en modo de cambio aniónico, con detección UV y detección por espectrometría de masas con plasma de acoplamiento inductivo (ICP-MS).

5. El aislamiento y preconcentración de las proteínas disueltas en el agua de mar para su separación y caracterización/determinación bidimensional por electroforesis OFFGEL y electroforesis microfluídica Lab-on-a-chip (LOC). Del mismo modo, se abordará la determinación del contenido de metales asociados a las proteínas en las fracciones líquidas recogidas tras la separación mediante electroforesis OFFGEL.

6. El estudio de diferentes procedimientos para la extracción de proteínas en plancton marino previa a la separación y caracterización de las mismas por electroforesis OFFGEL seguida de electroforesis LOC, y determinación del contenido de metales asociados a las proteínas en las fracciones líquidas recogidas tras la separación mediante electroforesis OFFGEL.

7. La determinación de los metales asociados a proteínas disueltas y a proteínas extraídas del plancton marino mediante Ablación Láser acoplada a Plasma de Acoplamiento Inductivo – Espectrometría de Masas (LA-ICP-MS) tras su separación mediante electroforesis bidimensional en gel de poliacrilamida.

8. La identificación de proteínas disueltas y proteínas extraídas de plancton marino mediante Espectrometría de Masas de desorción/ionización por láser asistida por matriz y tiempo de vuelo (MALDI-TOF-MS) tras su separación mediante electroforesis bidimensional en gel de poliacrilamida.

III. PARTE EXPERIMENTAL

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1. Multi-walled carbon nanotubes – solid phase extraction for isolating marine dissolved organic matter before characterization by size exclusion chromatography

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Multi-walled carbon nanotubes – solid phase extraction for isolating marine dissolved organic matter before characterization by size exclusion chromatography

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Abstract: *The feasibility of multi-walled carbon nanotubes (MWCNTs) for extracting dissolved organic matter (DOM) from seawater has been investigated. Solid phase extraction (SPE) operating in column and batch modes was used as an extractive technique, and the latter mode offered better performance. DOM adsorption was achieved using 60 mg of MWCNTs per each 250 mL of seawater subsample, working at pH 1.0 and under an orbital-horizontal shaking at 180 rpm and 25°C for 4 hours. DOM desorption from the MWCNTs support implied an orbital–horizontal shaking (180 rpm and 25°C) for 2 hours using 10 mL of a 0.1 M sodium hydroxide solution (DOM determination), or using 20 mL of alkaline methanol (pH 10.0) for DOM fractionation by size exclusion chromatography (SEC) with UV detection at 205 nm. A Total Organic Carbon (TOC) analyzer with wet DOM oxidation (30 % (m/v) potassium peroxodisulphate in 6.0 M phosphoric acid) under super critical conditions and with Non-Dispersive Infra Red (NDIR) for CO_2 detection was used for DOM measurements. Calibration covered TOC concentrations within the 0.13 – 1000 mg L-1 range. The percentage of DOM retained in the MWCNTs support was dependent on the seawater sample, and it varied from 34 to 81 %. This means that only the DOM of certain molecular weights is capable to interact with the SPE support. Various SEC columns, ranging from 100 to 7000 and from 500 to 15000 Da (optimum separation range for peptides), were therefore used for DOM fractionation. Results have shown that DOM of low molecular weight is retained in the MWCNTs (fractions within the ranges of 125 – 1102 Da and 6.88 – 125 Da).*

Keywords: *Dissolved organic matter, seawater, carbon nanotubes, solid-phase extraction, TOC analyzer, size exclusion chromatography*

1.1. Introduction

In addition to dissolved inorganic carbon (DIC), which is the largest and best understood group of reactive carbon in the ocean [1], the global carbon cycle in seawater is also affected by the dissolved organic carbon (DOC) fraction [2]. DOC,

also referred to dissolved organic matter (DOM), is the second largest class of carbon in seawater, and comprises various kinds of compounds exhibiting different molecular weights. Some of these compounds can easily be indentified, such as several biochemical substances (sugars, amino acids, proteins) formed by marine algae in the euphotic zone [2], but other substances are more coarsely identifiable such as the case of humic compounds [1]. DOM is operationally defined as the fraction that passes through 0.1 to 1.0 μm filters [3], and it is differenced from undissolved organic matter present in seawater as particulate organic matter (POM) [4]. However, there are some material sizes, such as the colloidal matter and small-sized plankton, which do not fit nearly into either category [5]. The importance of marine DOM derives from its essential participation in the global carbon cycle, but also because the bioavailable fraction of DOM influences the marine microbial loop [3]. In addition, DOM has also been recognized as an important factor for controlling metal toxicity and metal bioavailability due to its chelation properties [3,5].

Improvements in analytical instruments have allowed the characterization of marine DOM as well as a better understanding of marine DOM and the global carbon cycle [1]. Continuous progress has demonstrated important differences in the DOM composition of surface and deep seawater. In the latter, the presence of biologically refractory compounds of limited bioavailability and bioreactivity has been reported. This DOM fraction from the deep ocean are marine-derived polymeric materials [6], and it has recently been characterized as carboxyl-rich alicyclic molecules (CRAMs) [7]. Otherwise, DOM found in surface seawater consists of biochemically active substances released by zooplankton grazing, microbial exudation and cellular lysis [3], and comprises hence a very labile DOM fraction with a high turnover rate [6]. These reactive substances, mainly proteins, are the key factors in the direct effect of DOM in the global carbon cycle, and they provide the clues for a long-term preservation of carbon and nitrogen in the ocean [8].

Marine DOM occurs at very low concentrations while inorganic salts are present at very high levels. Most of the current analytical techniques to assess/characterize DOM require the absence (or a low proportion) of these matrix concomitants, and thus pre–concentration/separation methods for DOM are needed. In addition to the inorganic salts removal, a pre–concentration/separation method must afford a quantitative DOM recovery, a minimal physical/chemical alteration of the sample, and the feasibility for processing large volumes of samples in short time periods [3]. Ultrafiltration and solid phase extraction (SPE) techniques are the most commonly used pre-concentration/separation methods for isolating DOM from seawater. Literature about this topic has recently been summarized in the reviews by Mopper et al [3] and Mecozzi et al. [4]. Different drawbacks are commonly associated to both DOM isolation procedures. In general, yields lower than 100% of DOM are typically reported after ultrafiltration and SPE methods [3]. In addition, ultrafiltration does not guarantee salts removal, and a desalting stage after ultrafiltration must be performed before DOM analysis/characterization by several analytical techniques. When dealing with DOM of high molecular weight (HMW) some of these desalting methods, such as diafiltration produce DOM losses within the 10-35% range [3,9]. Other problems linked to the ultrafiltration techniques are referred to membrane fouling [3] and to the variable rejecting efficiencies of the different commercially available membranes [3,10].

SPE procedures using different XAD resins, mainly XAD8, activated carbon, and C18 have been proposed for isolating DOM from water [3,4]. The latter material has been shown to be more efficient for retaining DOM than XAD resins [9]. In general, SPE methodologies are time-consuming techniques because they require the previous preparation and functionalization of the solid supports for an effective DOM retention, and also because the pH and the salinity of the samples must be fixed at adequate values for an efficient solid support-DOM interaction [3]. In addition, other disadvantages such as contamination due to bleeding and strong retention of some compounds are commonly reported for XAD resins. However, SPE methods are inexpensive procedures, and this is the main the advantage over

ultrafiltration procedures. Applications of C18 cartridges for SPE of DOM have been reported for surface and deep seawater [11,12], and the pore size of this material influences the C18 retention properties as well as the type of retained DOM [12]. Therefore, DOM recoveries of approximately 45% were found when using C18 (60Å pore size), while DOM percentages with the 20-35% range were obtained when using C18 of pore size of 500Å [13]. Different styrene divinyl benzene polymers (formally referred to PPL and ENV) have also been proposed for DOM isolation from seawater, and extraction recoveries from approximately 60-75% and from 40-60% have been reported for PPL and ENV supports, respectively [13]. Other materials such as diethylaminoethyl-cellulose and certain anion exchange resins (MIEX) have recently been proposed for removing DOM from freshwater [14,15]. In addition, different adsorption kinetics, thermodynamics and desorption studies using multiwalled carbon nanotubes (MWCNTs) for removing DOM from freshwater have also been performed [16,17]. Results (DOM recoveries close to 100%) suggested that CNTs are promising supports for the removal of DOM from freshwater [17]. Extensive literature concerning applications of carbon nanostructures, and specifically CNTs, in Analytical Chemistry can be found in the reviews by Valcárcel et al. [18,19]. CNTs as solid supports for SPE procedures have been applied for extracting organic substances and ions [18,19] from different liquid samples. However, the possibilities of using CNTs for the SPE of DOM from complex samples have not been explored. One of the goals of the current work is therefore evaluating the feasibility of MWCNTs as an SPE support for DOM isolation from surface seawater. Operating conditions affecting the SPE in column and batch modes were fully evaluated. A second objective of the current work is the assessment of the molecular weight distribution of the extracted DOM by MWCNTs-based SPE. High performance liquid chromatography (HPLC) using the size exclusion mode (size exclusion chromatography, SEC) has been used to achieve this goal.

1.2. Experimental

1.2.1. Apparatus

A Sievers Innovox Laboratory TOC analyzer from General Electric Analytical Instruments (Boulder, CO, USA) was used for DOM quantification. The analyzer uses a super critical water oxidation (SCWO) technique to achieve superior wet DOM oxidation (persulfate in acid medium), and a Non-Dispersive Infra Red (NDIR) for CO_2 detection. The chromatographic system consisted of a Dionex P680 HPLC pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne 4097 (Cotati, CA, USA) injector with a loop volume of 50 µL, and with a Dionex UVD170U absorbance detector (Dionex). DOM fractions were separated with a Superdex peptide 10/300GL SEC column (30 cm × 10 mm I.D., optimum separation range for peptides between 100 and 7000 Da) from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and a TSK-gel G2000SW (30 cm × 8 mm I.D., optimum separation range for peptides between 500 and 15000 Da) from TosoHaas (Tokyo, Japan). For all cases a TSK-gel SW glass guard column (10 cm × 8 mm I.D.) from TosoHaas was used. A Boxcult incubator situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain) was used to perform batch mode sorption/desorption experiments. SPE support (MWCNTs) were separated by vacuum filtration with 0.22 µm Durapore membrane filters (Millipore Co., Bedford, MA, USA). An 8-way Gilson peristaltic pump (Villiers, France) equipped with 3.18 mm I.D. Tygon tubes (Gilson) was used for column mode sorption/desorption experiments. SPE support (MWCNTs) (70 mg) was placed into empty Sep–Pak® cartridges (plus short) from Waters (Milford, MA, USA) between 20 µm porosity Teflon frits (Supelco, Bellefonte, PA, USA). Glass chromatographic columns (35 cm length and 15 mm diameter) with integral sintered disc and PTFE stopcock (Selecta) were used for sample desalination before column SPE experiments. Other laboratory devices were a muffle furnace L3/11B-180 from J.P. Selecta; a rotavapor® Büchi R-210 equipped with a heating bath Büchi B-491 and a vacuum pump Büchi V-740 (Büchi Laboryechnik AG,

Flawil, Switzerland); an ORION 720A plus pH–meter with a glass–calomel electrode (ORION, Cambridge, UK), 0.45 μm polycarbonate membrane Nucleopore filters (Millipore), and Albet®LabScience 0.20 μm cellulose acetate syringe filters (25 mm diameter) from Albet-Hahnemuehle (Dassel, Germany).

1.2.2. Reagents

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, USA). Multi-walled carbon nanotubes (MWCNTs), industrial grade, 85% purity, 10-30 nm in diameter, and 5-20 μm in length, were purchased from NanoLab Inc. (Waltham, MA, USA). MWCNTs were previously heat-conditioned at 400°C for 60 min (amorphous carbon removal [20]). Potassium hydrogen phthalate stock standard solution (1000 mg L^{-1}) was prepared from 99.5 % potassium hydrogen phthalate supplied by Panreac (Barcelona, Spain). ReadyCalkit PEO containing polyethylene oxides (molecular weights of 232, 600, 2000, 6000 and 18000 Da) was from PSS Polymer Standard Services GmbH (Mainz, Germany). Blue dextran 2000 (molecular weight 2000000 Da) was from Pharmacia Biotech (Piscataway, NJ, USA). Ribonuclease A (molecular weight 13700 Da), vitamin B12 (molecular weight 1855 Da), lysine (molecular weight 146 Da), and glycine (molecular weight 75 Da) were from GE Healthcare (Bucks, UK). Potassium peroxodisulphate solution (30 % (m/v)) was prepared from 99 % potassium peroxodisulphate (Panreac). Phosphoric acid solution (6.0 M) was prepared from 85 % phosphoric acid (Panreac). High purity 69 % nitric acid and high purity 37 % hydrochloride acid were from Panreac, while high purity 25% ammonia and HPLC grade methanol were purchased from Merck (Darmstad, Germany). Sodium hydroxide and potassium dihydrogenphosphate were from Merck. Disodium hydrogenphosphate was from J.T. Baker (Deventer, Holland), while diammonium sulphate was from Panreac, and diammonium hydrogenphosphate was from BDH (Poole, UK). Cation exchange AG-MP5 and anion exchange AG1-X8 resins were from Bio-Rad (Richmond, CA, USA).

1.2.3. Seawater sample collection

Surface seawater samples (1L) were collected from different Galician estuaries (north-western Spain) in pre-cleaned high density polyethylene bottles. After collection, seawater samples were filtered (0.45 µm) and then stored at low temperature until use.

1.2.4. MWCNT cartridge preparation

Empty Sep–Pak® cartridges were filled with 70 mg of heat-conditioned MWCNTs (heating at 400°C for 1 hour) between two Teflon frits. Once prepared, volumes of 50 mL of Milli-Q water and acidified Milli-Q water (pH 1.0) were sequentially passed through the cartridges at a flow rate of 1.5 mL min-1.

1.2.5. Column desalinization procedure

According to manufacturer's recommendations, 5 g of anion (AG1-X8), and cation (AG-MP5) exchange resins were introduced in separate glass chromatographic columns (1 g per 100 mL of sample). Anion exchange resin was conditioned by passing 10 mL of 0.1 M sodium hydroxide; whereas, the cation exchange resin was regenerated with 10 ml of 0.1 M hydrochloric acid. The desalination procedure was performed by passing first the seawater sample (500 mL) through the anion exchange column (drop to drop elution by gravity), adjusting the pH to 6.0 of the seawater percolated through the anion exchange column (0.1 M hydrochloric acid solution), and by passing the sample through the cation exchange column (drop to drop elution by gravity). After this treatment, the pH of the desalinated seawater was adjusted to 1.0 by adding small volumes of a 6.0 M hydrochloric acid solution before SPE (column or cartridge mode).

1.2.6. MWCNT cartridge-solid phase extraction

A volume of 250 mL of desalinated and acidified seawater sample (pH 2.0) was passed through conditioned MWCNT cartridges at a fixed flow rate of

1.7 mL min^{-1} by using a peristaltic pump. The cartridges were then rinsed with 10 mL of acidified Milli-Q water (pH 2.0), and the retained DOM was eluted by pumping 10 mL of 0.1 M sodium hydroxide (flow rate of 1.7 mL min^{-1}). After elution, the MWCNT cartridges were conditioned with 20 mL of acidified Milli-Q water (pH 1.0). DOM was determined in the sample before the SPE procedure, and also determined directly in the waste solution and in the eluate (mixed with 10 mL of 0.1M hydrochloric acid solution). Dilution/neutralization of the alkaline extract with a hydrochloric acid solution is needed for a proper performance of the TOC analyzer. A pre–concentration factor of 12.5 was then achieved under these operating conditions. Blanks (Milli-Q water) were also subjected to this procedure for controlling possible contamination.

1.2.7. MWCNT batch mode-solid phase extraction

Volumes of 250 mL of acidified seawater samples (pH 1.0) together with 60 mg of heated-conditioned MWCNTs (heated at 400°C for 1 hour) were placed into Erlenmeyer flasks, and DOM adsorption onto the MWCNTs SPE support was then performed at 25ºC under orbital–horizontal shaking at 180 rpm for 4 hours. The mixture was then vacuum filtered through 0.22 µm filters. The separated MWCNTs were placed into clean Erlenmenyer flasks and 10 mL of 0.1 M sodium hydroxide or 10 mL of methanol at pH 10 (pH adjusted by adding a small volume of 2.0 M sodium hydroxide solution) were added. DOM desorption was performed at 25°C under orbital–horizontal shaking at 180 rpm for 2 hours. After vacuum filtration (0.22 µm filters) DOM in the eluate (0.1 M sodium hydroxide solution) was measured by TOC analyzer after mixing with 10 ml of 0.1 M hydrochloric acid solution (pre-concentration factor of 12.5). DOM in the seawater (before SPE) and in the waste solution (after SPE) was also measured by TOC analyzer. When performing fractionation by SEC-HPLC-UV, the eluate (alkaline methanol at pH 10) was concentrated to dryness by rotary evaporation (water bath at 25°C, vacuum pressure of 10 mm Hg) and the residue was re-dissolved with 10mL of mobile phase (5 mM / 5 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5). Blanks (Milli-Q water) were also subjected to these

procedures (TOC analysis and SEC-HPLC-UV measurement) for controlling contamination.

1.2.8. TOC measurements

DOM in the treated and untreated seawater, as well as in the eluate (0.1 M sodium hydroxide solution neutralized with 10 mL of 0.1 hydrochloric acid) was assessed as TOC by super critical water oxidation (30 % (m/v) potassium peroxodisulphate in 6.0 M phosphoric acid), and CO^2 NDIR detection with the Sievers Innovox Laboratory TOC analyzer. The analyzer offers advantages over other devices based on wet chemical oxidation methods [21] because it works with supercritical water. Under these conditions the chloride interference (chloride competes with DOM for peroxidisulphate) [22] is avoided, and the wet oxidation is highly efficient. The TOC analyzer operated in the non-purgeable organic carbon (NPOC) measurement mode by mixing the acid (set at 1 %) and the oxidant (set at 15 %) with the sample in the sparger (sparge time of 0.8 min). Under these conditions, any inorganic carbon (IC) present is converted to CO_2 and is removed. A portion of the sparger solution is then pumped to the reactor module where the temperature is rapidly increased to 375°C under a pressure of 22.1 MPa (conditions above the critical point of water). Organic carbon in the solution is then oxidized to CO2 which is subsequently measured in the NDIR detector. Calibration covered TOC concentrations (potassium hydrogen phthalate as a calibrant) within the 0.13 –1000 mg L^{-1} range (concentrations referred to carbon). Four aliquots of each sample and a blank were analyzed.

1.2.9. SEC-HPLC-UV measurements

SEC-HPLC separation (Superdex peptide 10/300GL SEC column, optimum separation range for peptides between 100 and 7000 Da) was performed under isocratic elution conditions (flow rate set at 0.8 mL min^{-1}) with a mobile phase consisting of a 5 mM / 5 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5. The injection volume was 50 µL, and detection

was performed by monitoring the 205 nm wavelength. Column calibration was performed with ribonuclease A, vitamin B12, lysine and glycine standards (molecular weights of 13700, 1855, 146 and 75 Da, respectively). The exclusion volume (V_0) was determined using ribonuclease A (16.6 mL), which implies a retention time of 20.7 min; while the permeation volume (V_p) was fixed by injecting Milli-Q water (24.0 mL), which corresponds to a retention time of 30 min.

1.3. Results and discussion

1.3.1. Preliminary evaluation of MWCNT-based SPE (column mode)

Preliminary experiments were performed by subjecting in duplicate 250 mL of surface seawater (previously acidified to pH 2.0) [23] to a cartridge-based SPE procedure (details are given in section 1.2.6). Similarly, a blank (Milli-Q water acidified to pH 2.0) was also prepared. TOC measurements in triplicate were directly performed in the acidified seawater samples (and in the acidified blanks), in the samples (and in the blanks) after passing through the MWCNT cartridge, and in the 0.1 M sodium hydroxide eluates (after neutralization with 0.1 M hydrochloric acid). Negligible TOC concentrations were found when measuring blanks (mean TOC concentrations lower than 0.13 mg L^{-1}, the LOQ of the method). Values of 1.1 ± 0.072 and 0.89 ± 0.030 mg L^{-1} were found for TOC measurements in the seawater sample before and after MWCNT-cartridge SPE, which implies a low DOM retention percentage (19 ± 7 %). TOC concentration in the eluate was 3.0 ± 0.21 mg L^{-1} (0.24 ± 0.016 mg L^{-1} referred to the seawater sample taking into account the pre-concentration factor of 12.5). This TOC concentration represents 22 ± 2 % of retained/eluted DOM in the CNT support, which is close to the value of 19 ± 7 % (percentage obtained by the difference between TOC concentrations in the seawater sample before and after passing through the cartridge). After varying different operational parameters such as loading flow rate, retained DOM .percentages lower than 25 % were obtained. This result contrasts to those obtained by Su and Lu [17] when using MWCNTs for retaining DOM from freshwater (retained DOM percentages close to 100 %). Our bad results can be

attributed to the high salt content in seawater and also to the SPE operating mode (cartridge-based SPE instead of a batch-SPE mode used by Su and Lu [17]).

To test the effect of the saline matrix on the DOM retention onto the MWCNTs under a cartridge-based SPE, samples were previously desalinated (section 1.2.5) by using sequentially two ionic exchange resins (AG-1X8 and AG-MP-5). Experiments in duplicate gave a retained DOM percentage of 34 ± 5 % (1.8 ± 0.031 and 1.2 ± 0.076 mg L^{-1} of TOC in the sample before and after passing through the cartridges, respectively). The analysis of the alkaline eluate showed a TOC concentration of 6.2 ± 0.17 mg L^{-1} (TOC concentration of 0.50 ± 0.014 mg L^{-1} taking into account a pre-concentration factor of 12.5). The retained DOM percentage by using the TOC concentration found in the eluate is 28 ± 1 %. Although DOM retention was increased after salt removal, the retained DOM percentage is still low. Therefore, insufficient DOM retention onto MWCNTs support must mainly be attributed to the SPE mode rather than to the saline matrix of the seawater sample, and a batch mode SPE was further tried.

Concerning DOM elution from MWCNTs, methanol, commonly used for eluting DOM from octadecyl bonded phase silica-based (C18) [11,13] and styrene divynil benzene polymer [13] supports, was tried. Although this solvent, and especially alkaline methanol (pH 10), was found as useful eluting solution (see section 1.3.3), it resulted useless when performing TOC determinations. Several attempts for removing methanol by rotatory evaporation and re-dissolution of the residue in a 0.1 M hydrochloric acid solution were made, but high TOC values due to traces of methanol were obtained, even after performing the rotatory evaporation to dryness procedure twice. As previously mentioned, since DOM adsorption onto MWCNTs is favored at acid pHs, a 0.1 M sodium hydroxide solution was found adequate as a eluting solution. However, MWCNTs' adsorption properties were reduced after DOM elution with sodium hydroxide solutions, and the support must be again heated-conditioned before use. Thus, previous applications of MWCNTs as SPE support for DOM from freshwater do not elute the retained DOM after adsorption,

and the adsorbed DOM is removed by heating the support at high temperatures [17].

Since the problems occurred when using dilute sodium hydroxide solutions for DOM elution, retained DOM percentages were further calculated by determining the TOC concentrations in the seawater samples before and after the MWCNT-based SPE procedure.

1.3.2. Optimization of loading conditions (batch mode)

Different stirring times (3.0, 4.0, 5.0 and 6.0 hours) were tried in duplicate by treating 250 mL of acidified seawater samples (pH 2.0) together with 30 mg of heated-conditioned MWCNTs under an orbital–horizontal shaking at 180 rpm and 25°C. The seawater volume/MWCNT mass ratio is close to that proposed by Su and Lu [17] when using MWCNTs for removing DOM from freshwater (30 mg of MWCNTs for 200 mL of freshwater). Results for retained DOM percentages after TOC determinations in seawater before and after batch-SPE (in triplicate) are plotted in Figure 1(a). It can be seen that DOM retention percentage remains constant when performing the batch-SPE experiments for times higher than 4.0 hours (retained DOM ratios within the 31 – 33 % range). This result contrasts to that previously reported by Su and Lu [17] when removing DOM from freshwater with MWCNTs. These authors concluded that contact times lower than 120 min (2.0 hours) are enough for reaching the equilibrium between DOM and MWCNTs in batch experiments. The equilibrium time is dependent on the temperature. Under a controlled temperature of 25°C the equilibrium time is 90 min [17].

Batch experiments were also performed at different pHs (1.0, 2.0 and 3.0) for 250 mL subsample and 30 mg of sorbent, and by fixing a contact time of 4.0 hours and under constant shaking (180 rpm, 25°C). Results (batch-SPE in duplicate and TOC determination in seawater before and after batch-SPE treatment in triplicate) are plotted in Figure 1(b). It can be seen that the DOM retention is improved when

working at the lowest pH, and further experiments were then performed by acidifying the seawater samples at a pH of 1.0.

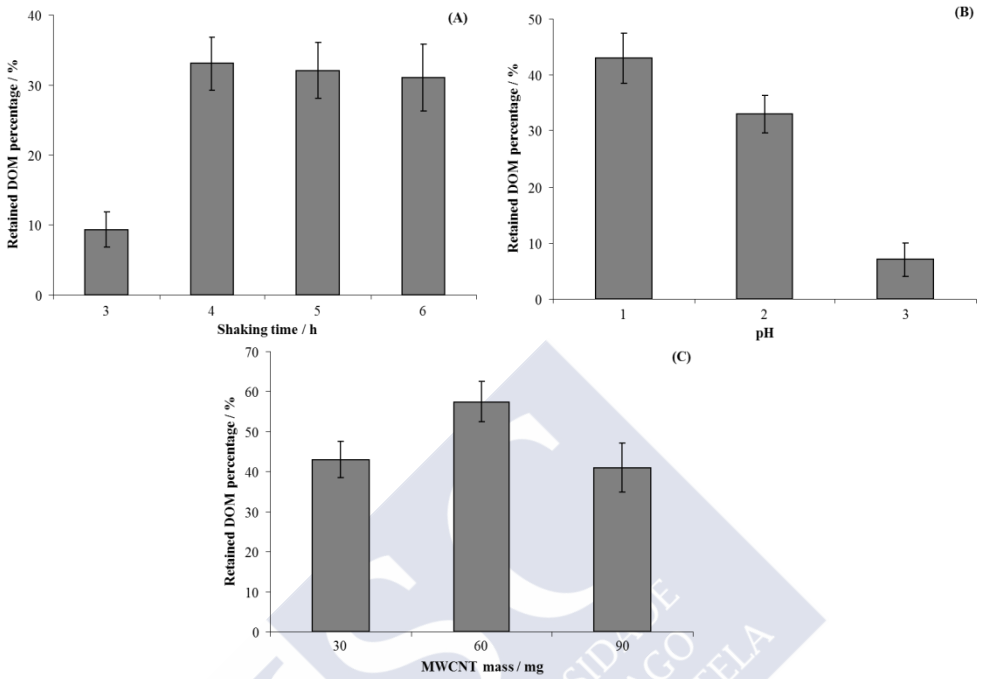

Figure 1: Effect of the load (shaking) time (A), pH (B), and amount of solid support (C) on the marine DOM retention onto MWCNTs.

The amount of sorbent was also investigated within the 30 – 90 mg range. Fixed volumes of seawater of 250 mL were treated in duplicate under controlled shaking (180 rpm, 25°C and 4.0 hours), and after acidifying sample aliquots at pH 1.0. Results (TOC determinations in triplicate) show an increase of the retained DOM from 43 % to 58 % when increasing the MWCNTs mass from 30 mg to 60 mg (Figure 1(c)). However, retained DOM percentage fell to 4 1% when performing the experiment with 90 mg of MWCNTs. This finding can be attributed to interaction among MWCNT particles which facilitates DOM desorption. A similar finding was also observed when performing experiments at high orbital–horizontal shaking rates, and collisions among MWCNT particles diminish DOM retention.

Therefore, loading conditions were set to 60 mg of MWCNTs for 250 mL of acidified seawater (pH 1.0), and controlled shaking (180 rpm and 25°C) for 4.0 hours. The repeatability of the over–all procedure was assessed by subjecting a surface seawater sample to the MWCNT-batch SPE procedure five times. After TOC determinations in the seawater subsamples before and after the SPE process (quadruplicate analysis), a DOM retention percentage of 56 ± 4 % (RSD value of 7 %) was found. The developed procedure was also applied in triplicate to four other surface seawater samples collected from different Galician estuaries (north-western Spain). Retained DOM percentages varied from 34 ± 3 % (Ría de Vigo estuary) to 81 ± 5 % (Ría de Pontevedra estuary), while values in surface seawater from the Ría de Arousa estuary were 60 ± 4 and 77 ± 7 %. These isolated DOM percentage values are similar to those reported by other authors when isolating DOM from seawater (salinity higher than 20 ‰) using other solid supports for SPE such as activated charcoal (from 25 to 72%) [24,35], Amberlite XAD-2 (from 5 to 88 %) [23,26-28], Amberlite XAD-8 (from 7.2 to 82 %) [29,30], C18 (from 38 to 68 %) [9] and styrene divinyl benzene polymers (from 40 to 75 %) [13]. The non-quantitative DOM recoveries from seawater by SPE procedures as well as for ultrafiltration methods [3] must be attributed to the heterogeneity of the DOM in seawater, and only certain DOM types are able to interact with the solid support. One of the factors that control the type of DOM which interact with a certain SPE support must be the molecular weight. A further study for determining the molecular weight of retained DOM onto MWCNTs was therefore performed.

1.3.3. SEC fractionation

A TSK-gel G2000SW SEC column allowing molecular weights separation within the 6.5 – 150 kDa range was first evaluated. A mobile phase consisting of an ammonium sulphate / diammonium hydrogenphosphate (50 mM / 50 mM) buffer solution at pH 6.5 [31] was tried under isocratic conditions at a flow rate of 0.6 mL min^{-1}. For these preliminary experiments, retained DOM was eluted from the MWCNT using 20 mL of methanol [11,13] under orbital–horizontal shaking at 180 rpm for 1 hour. UV detection at 205 nm (the chromatogram is not given)

showed a broad chromatographic peak (retention time of 30 min) which corresponds with the retention time obtained when fixing the permeation volume by injecting Milli-Q water (permeation time of 29 min, permeation volume of 17.4 mL). Therefore, retained DOM onto the MWCNTs consists of substances of molecular weights lower than 6.5 kDa. Because retained DOM percentages up to 80 % have been found in some analyzed surface seawater, it can be concluded that most of the marine DOM in the surface seawater samples analyzed in this study is of low molecular weight. This result agrees with reported data in the literature which affirm that only between 25 and 35 % of marine DOM in surface seawater is in a high molecular weight fraction [2].

A second SEC column (Superdex peptide 10/300GL) with an optimum separation range between 0.1 and 7.0 kDa was further tested. The first experiments (methanol eluate), performed with ammonium sulphate / diammonium hydrogenphosphate (50 mM / 50 mM) buffer solution at pH 6.5 as a mobile phase (isocratic mode, flow rate of 0.8 mL min^{-1}) led to a broad chromatographic peak within the 26.2 – 29.6 min range (Figure 2). This chromatographic signal is between the void volume (9.28 mL, retention time of 11.6 min, measured by injecting blue dextran), and the permeable volume (24.0 mL, retention time of 30.0 min, measured by injecting Milli-Q water).

1.3.3.1. Optimization of the DOM elution procedure

Under the chromatographic conditions listed above, different parameters affecting the elution process were investigated. After DOM retention (batch mode according to section 1.2.7), different eluting solutions subjected to two different stirring times (1 and 2 hours) were tested. Other parameters affecting the eluting procedure such temperature and orbital–horizontal shaking were fixed at 25°C and 180 rpm, respectively. The eluting solutions investigated consisted of 20 mL of methanol (A); 20 mL of alkaline methanol at pH 10 (B), pH adjusted by adding some drops of an aqueous 2.0 M sodium hydroxide solution; 20 mL of a methanol / 0.2 M sodium hydroxide mixture (C); and 20 mL of an aqueous 1.0 M sodium hydroxide

solution (D). When using methanol as an eluting solution, this solvent was removed by rotatory evaporation to dryness (A and B) and to approximately 10 mL of the eluate (C). The residues (A and B) were then re-dissolved with 20 mL of the mobile phase solution (50 mM / 50 mM ammonium sulphate / diammonium hydrogenphosphate, pH 6.5); whereas the aqueous phase after methanol removal (C) was diluted up to 20 mL with the mobile phase. When using 1.0 M sodium hydroxide solution (D) as an eluting solution the pH of the eluate was adjusted to 6.5 by adding small volumes of a 6.0 M hydrochloric solution.

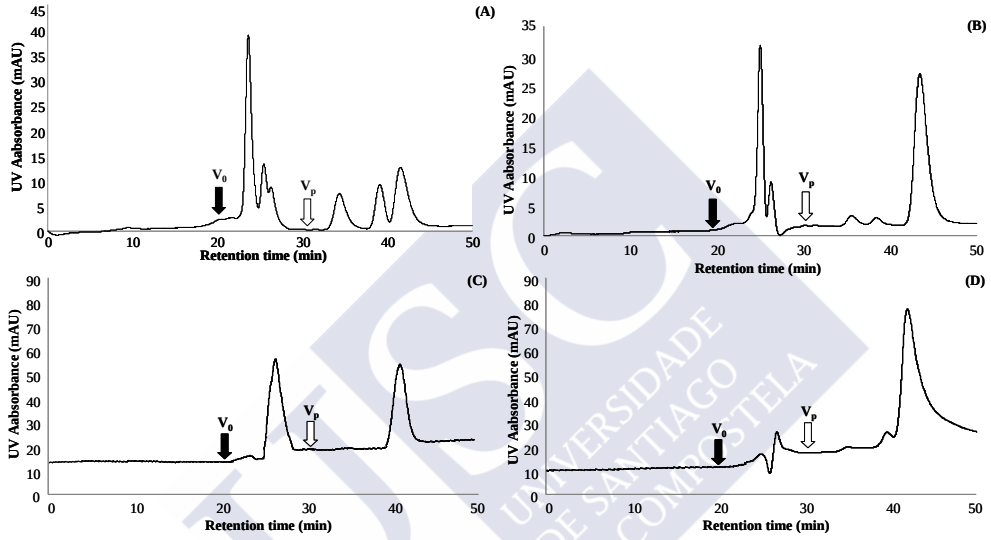

Figure 2: SEC chromatograms (UV detection at 205 nm) of marine DOM eluted with methanol (A), alkaline methanol at pH 10 (B), methanol/0.1M sodium hydroxide (C), and 1.0M sodium hydroxide (D): V0 and Vp are the void volume and the permeation volume, respectively.

SEC chromatograms when using each eluting solution (A to D) and for a shaking time of 2 hours are shown in Figure 2(A-D). It can be seen that the highest chromatographic signals within the resolution range of the column are obtained when using methanol (Figure 2A) and alkaline methanol at pH 10 (Figure 2B). The second chromatographic signal (retention time of approximately 42 min) is attributed to trace amounts of methanol (as shown in Figure 2D, this signal is not observed when using an aqueous sodium hydroxide solution for DOM elution).

Higher chromatographic signals were obtained after an elution time of 2 h (Figure 2), while the heights of these signals were approximately half the heights found when shaking for 1 hour. After selecting alkaline methanol (pH 10) as an eluting solution and an eluting time of 2 hours, experiments when varying the shaking speed showed that similar results were reached for shaking speeds up to 150 rpm. Therefore, this operating parameter was fixed at 180 rpm.

Further experiments based on rotary evaporation were then performed for a more efficient methanol removal. After the first rotary evaporation to dryness, the residue was re-dissolved in 5 mL of ultrapure water, and it was again subjected to rotary evaporation to dryness. Although the chromatographic signal after the permeate volume (retention time of 42 min) attributed to methanol was smaller, TOC analysis of the eluate gave TOC concentrations much higher than the theoretical DOM concentration taking into account the pre-concentration factor of 12.5. Therefore, the DOM determination in the eluate was not possible by TOC measurements. To ensure a quantitative DOM elution from the MWCNTs by the above described method, after a first alkaline methanol elution (20 mL) a second elution with fresh alkaline methanol (20 mL) was performed. SEC analysis of this second eluate gave negligible chromatographic signals within the resolution range of the SEC column (between 11.6 and 30 min).

1.3.3.2. Optimization of the mobile phase composition

The effect of the ionic strength and pH of the mobile phase on the DOM separation by SEC was investigated. Both variables have been reported to change sensitivity of SEC separations [31] as well as causing conformational changes in the DOM (from linear polymer-like to spherocolloid-like) [32]. Ammonium sulphate / ammonium dihydrogenphosphate solutions (pH fixed at 6.5) of four ionic strengths (5.0, 25, 50 and 100 mM) were tested by injecting eluted marine DOM with alkaline methanol, rotary evaporation (section 1.3.3.1.), and re-dissolution in the mobile phase solution of fixed ionic strength. As shown in Figure 3, better separation of DOM fractions is achieved when working at lower ionic strengths (mobile phase

solutions at 5.0 and 25 mM, Figure 3(A-B)), and the substances are shifted toward a slightly higher retention time at the highest ionic strength (100 mM, Figure 3D). The shift of DOM toward higher retention times when using high ionic strengths has also been reported by Minor et al. [31] when separating marine ultrafiltered DOM by SEC. The explanation for these phenomena is attributed to the increased hydrophobic interactions of DOM with the SEC column at higher ionic strengths [31,33].

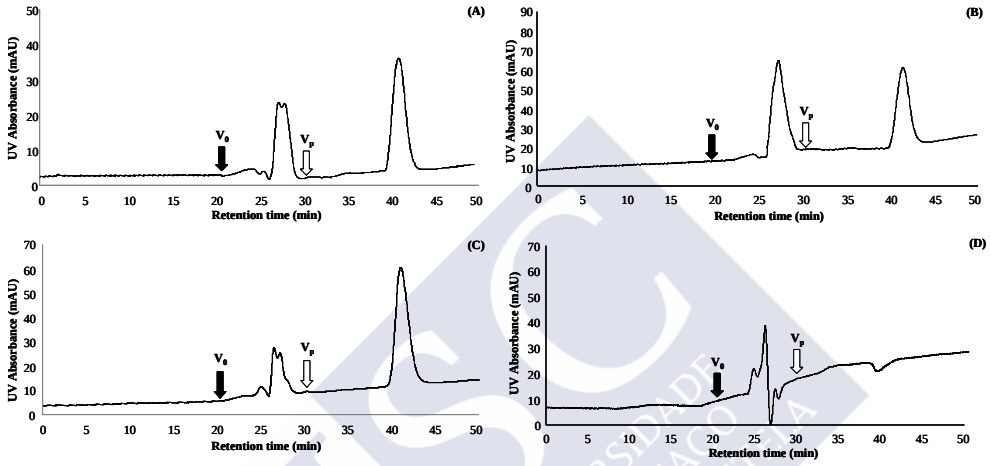

Figure 3: SEC chromatograms (UV detection at 205 nm) of marine DOM eluted with alkaline methanol (pH 10) using ammonium sulphate/ammonium dihydrogenphosphate (pH 6.5) at 5.0mM/5.0mM (A), 25mM/25mM (B), 50mM/50mM (C), and 100mM/100mM (D): V0 and Vp are the void volume and the permeation volume, respectively.

Concerning pH, slight variations on the pH of a 5.0 mM / 5.0 mM ammonium sulphate / ammonium dihydrogenphosphate solution (pHs within the 6.0 – 7.5 range) did not affect DOM separation. A pH of 6.5 (close to seawater pH) was therefore selected.

1.3.3.3. Molecular weight assessment of the retained marine DOM onto MWCNTs

Different polyethylene oxides (PEOs) and polyethylene glycols (PEGs) of molecular weights of 232, 600, 2000, 6000 and 18000 Da were tested for

calibrating the Superdex peptide 10/300GL SEC column. However, the PEOs / PEGs standards were retained in the SEC column under the elution conditions previously optimized for resolving marine DOM. Her et al. [34] have reported a similar behaviour of sodium polystyrene sulfonates (PSSs) standards when using the TSK 50S column, and the strong interaction of PSSs with the SEC column was attributed to the presence of sodium sulphate in the eluent. Possibly, the presence of ammonium sulphate in the mobile phase used in the current application leads to a similar interaction of PEOs and PEGs standards with the stationary phase of the column. This fact was confirmed by using the mobile phase recommended by the manufacturer (aqueous 0.2 % (m/v) sodium azide), which allowed a convenient elution of the PEOs / PEGs standards. Therefore, different standards such as ribonuclease A (molecular weight 13700 Da), vitamin B12 (molecular weight 1855 Da), lysine (molecular weight 146 Da) and glycine (molecular weight 75 Da) were used for column calibration. These standards (final concentration of 1000 mg L^{-1}) were dissolved in the optimized mobile phase, and were chromatographied under the same operating conditions used for resolving marine DOM. Retention time (R_t) and the logarithm of the molecular weight (Log(MW)) data were adjusted to a lineal model, and good lineal relationship was achieved (regression coefficient, r2, of 0.9905 for a calibration equation Log (MW) = – 0.4216 R_t + 12.6). As shown in Figure 4, DOM in fraction 1 (F1) from 22.7 to 24.9 min is formed by molecular weights substances within the 125 – 1102Da range; whereas DOM in fraction 2 (F2) from 24.9 to 28.8 min is formed by substances of molecular weights from 6.88 to 125 Da, most of then out of the resolution range of the column (approximately 100 Da).

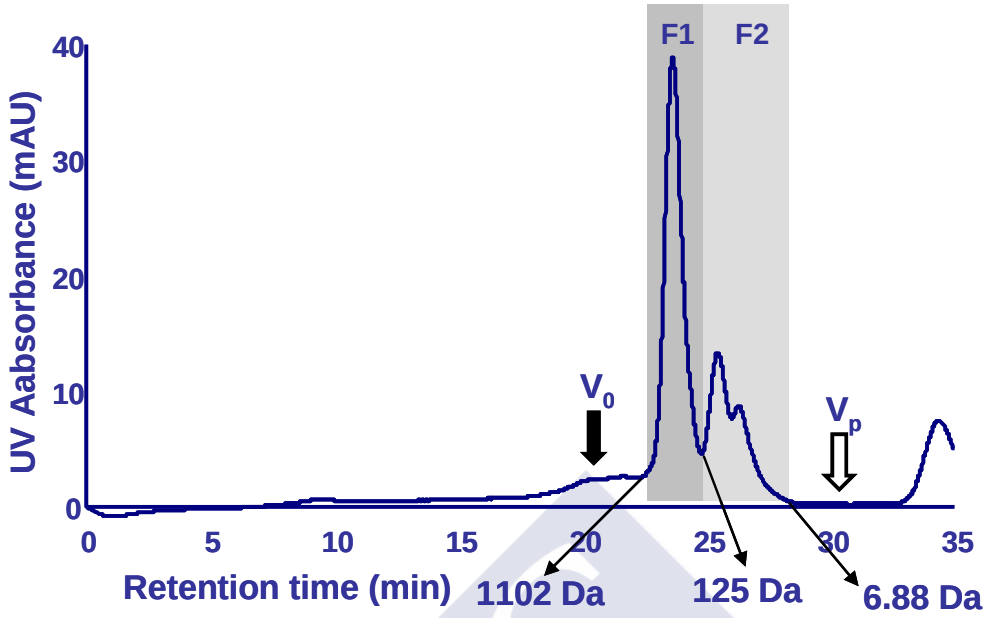

Figure 4: SEC chromatogram (UV detection at 205 nm) of marine DOM showing molecular weights of the two separated fractions (F1 and F2).

1.4. Conclusions

The use of MWCNTs as a solid support for batch-SPE procedures has been found useful for retaining marine DOM. Percentages of retained DOM varied from 30 % to 80 %, values lower than those reported when working with freshwater (100 % retention). The lower percentages obtained for seawater when comparing with results for freshwater can be attributed to the large variety of marine DOM, mainly due to the presence of substances of high molecular weights (commonly isolated by ultrafiltration procedures) which does not interact with the support material. In general, percentages of marine DOM retained onto MWCNTs are slightly higher than those reported for other SPE supports for retaining marine DOM, such as Amberlite XAD, C18, PPL and ENV.

Studies conducted to characterize the retained marine DOM by assessing the molecular weights range have revealed that MWCNTs are able to retain marine DOM of low molecular weight, which is the major DOM fraction in surface

seawater. The main marine DOM fraction comprises molecular weights within the 125 – 1102 Da range, while another important DOM fraction (molecular weights between 6.88 and 125 Da) is also retained onto MWCNTs. This latter fraction is, however, close to the permeable volume of the SEC column, and most of the substances included in this fraction are in molecular weights outside of the resolution range of the used SEC column (lower than 100 Da).

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2. Size exclusion and anion exchange high performance liquid chromatography for characterizing metals bound to marine dissolved organic matter

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Size exclusion and anion exchange high performance liquid chromatography for characterizing metals bound to marine dissolved organic matter

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Abstract: *A speciation procedure based on the use of size exclusion chromatography (SEC) followed by anion exchange chromatography (AEC) hyphenated with inductively coupled plasma – mass spectrometry (ICP-MS) was developed for assessing metals bound to marine dissolved organic matter (DOM). Surface seawater samples (100 L) were subjected to tangential flow ultrafiltration (10000 Da cut off) for isolating and pre-concentrating dissolved large molecules. The isolated fraction (retentate) consisted of 1 L, which was further freeze-dried and re-dissolved to 250 mL with ultrapure water. After HI Trap desalting of the re-dissolved retentate, SEC with UV detection showed marine DOM ranging from 6.5 kDa (lower than the permeable volume of the SEC column) to 16 kDa. A further characterization of this fraction by AEC with UV detection revealed the existence of four groups of macromolecules exhibiting retention times of 2.3, 2.8, 4.5 and 14.0 min. AEC hyphenated with ICP-MS showed the presence of strontium and zinc in the first AE fraction isolated from the SEC fraction; while manganese was found to be bound to the second AE fraction. Cobalt was found to be bound to molecules comprising the third AE fraction.*

Keywords: *Metal bound to marine dissolved organic matter, tangential flow ultrafiltration, size exclusion chromatography, anion exchange chromatography, inductively coupled plasma - mass spectrometry.*

2.1. Introduction

Dissolved organic matter (DOM) comprises various kinds of compounds exhibiting different molecular weights which originated from both natural and anthropogenic sources. Humic substances are the major fraction of natural organic matter (NOM) present in surface waters and groundwaters, and they are the best characterized NOM fraction [1]. Reported data shows complex macromolecular substances derived from biological and chemical degradation processes from biota, such as proteins and carbohydrates [2]. The importance of the assessment and the

understanding of NOM derives from the different environmental processes dependent on NOM, such as the fate and transport of micropollutants and their impacts on potable water treatment unit operations [3]. Similarly, marine DOM is considered to be as the second largest source of dissolved carbon in oceans [4,5], and plays an important role for controlling the transport of toxic contaminants and nutrients, and influencing toxicity and bioavailability of those compounds in the marine environment [6,7]. The several highly reactive functional groups present in NOM and also in marine DOM are responsible for binding contaminants such as organic pollutants and trace metals [6,7,8].

In addition to the large types of bio-molecules present in NOM and DOM, a myriad of degraded and reworked products derived from these large bio-molecules are also present in these dissolved organic carbon fractions. According to Woods et al. [9] the complexity of these mixtures requires the use of multiple techniques (hyphenated into a single analytical run or conducted off-line) for a complete assessment. Continuous progress in analytical instrumentation, mainly based on mass spectrometry and nuclear magnetic resonance spectroscopy techniques [6], has allowed a better knowledge of the composition and reactivity of NOM [1,3,8-17] and marine DOM [18-32]. The assessment and characterization of proteins has recently received special attention. This is because proteins are the most reactive substances present in marine DOM and can persist as discrete units in the environment [33].

In addition to the type of the analytical techniques used for assessment/characterization purposes, the sample pre-treatment procedure for isolating marine DOM will condition the type of marine DOM under study. Therefore, solid phase extraction (SPE) procedures have been reported to isolate marine DOM of low molecular weight [32], and they are appealing approaches because marine DOM of low molecular weight accounts for 65 to 75% of DOM in surface seawater [5]. However, the assessment of marine DOM of high molecular weight, mainly marine proteins, usually requires tangential flow ultrafiltration (UF) as a sample pre-treatment [6,21,33]. In addition, UF techniques offer additional

advantages for high molecular weight DOM, such as the higher pre-concentration factors, which results suitable because the low proportion of marine high molecular weight DOM [5].

As previously mentioned, DOM plays a crucial role in the transport and fate of carbon, nutrients and trace micropollutants in surface waters and seawater [6-8]. Concerning trace metals, there is a large amount of literature discussing metal complexation by NOM [8,34], as well as the assessment of metal binding NOM in natural waters by hyphenated size exclusion chromatography (SEC) and inductively coupled plasma – mass spectrometry (ICP-MS) [10,35-37]. Results showed that metals with high binding strength tend to be associated to organic compounds of high molecular weight; whereas, metals exhibiting low strength were mainly bound to compounds of low molecular weights [8,37]. More recently, Laborda et al. have also applied SEC-ICP-MS to assess metal-dissolved organic matter complexes in water soluble fractions of compost samples [38,39]. The same research group developed methods based on asymmetrical flow field flow fractionation (FFF) hyphenated with ICP-MS for the same purpose [40,41].

Although some developments have been reported for assessing metal binding dissolved organic matter, the studies have been mainly performed for natural waters [8,10,34-37]. However, the assessment of metal binding marine DOM has not been addressed. The main difficulty when analysing seawater samples is the low concentration of marine DOM; hence, the low concentration of metals binding marine DOM. In addition, the high salts content in seawater also hinders the application of the analytical techniques commonly used in the analysis of freshwater. One of the goals of the current work is therefore evaluating the feasibility of a UF procedure combined with a HI Trap desalting procedure to isolate high molecular weight marine DOM. Methods based on SEC with UV and ICP-MS have been optimized to assess large marine DOM and metals bound to marine DOM. A further fractionation of the SEC fractions by anion exchange chromatography (AEC) with UV and ICP-MS detection was also applied for a better evaluation of marine DOM and those metals associated with it.

2.2. Experimental

2.2.1. Apparatus

The tangential flow ultrafiltration (UF) system consisted of a Masterflex I/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane (nominal MW cut-off 10 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge. A Sievers Innovox Laboratory TOC analyzer from General Electric Analytical Instruments (Boulder, CO, USA) was used for DOM quantification. The analyzer uses a super critical water oxidation (SCWO) technique to achieve superior wet DOM oxidation (persulfate in acid medium), and a Non-Dispersive Infra Red (NDIR) for CO_2 detection. A Dionex P680 HPLC pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne 4097 injector (Cotati, CA, USA) with a 50 µL injection loop, and a Dionex UVD170U absorbance detector was used for HPLC-UV determinations. A 250 µL Hamilton Gastight 1725 syringe (Bonaduz, Switzerland) was used for manual injection. A Dionex UltiMateO 3000 LC HPLC (Dionex), equipped with a GP50 gradient pump (Dionex), an AS50 thermal compartment (Dionex) and an AS50 autosampler (Dionex) was coupled to an ICP-MS Thermo Finnigan X Series (Thermo Fisher Scientific Inc., Waltham, MA, USA) for assessing metals bound to DOM. The ICP-MS Thermo Finnigan X Series was also used for total metal determination. DOM fractions were separated with a TSK-G4000 SW SEC column (30 cm × 8 mm I.D., optimum separation range for Polyethylene Glycol (PEGs) between 2 and 250 kDa, and for proteins between 20 and 7000 kDa) from TosoHaas (Tokyo, Japan) coupled to a 10 cm × 8 mm i.d. TSK-gel SW glass guard column (TosoHaas). A PRP-X100 anion-exchange column (250 mm × 4.1 mm i.d. × 10 µm) from Hamilton (Reno, NV, USA) coupled to a 25 × 2.3 mm I.D. PRP-X100 guard column (Hamilton) was also used. A 77530 LYPH-LOCK 6-L freeze-dry system from Labconco Corp. (Kansas City, MO, USA) was used to freeze-dry the ultrafiltrate sample. An ORION 720A plus pH–meter with a glass–calomel electrode (ORION, Cambridge, UK) was used for pH measurements. Conductivity/salinity was measured by using a MultiLine P4 set equipped with a TetraCon 325 cell (WTW,

Weilheim, Germany). Hi-Trap Desalting 5.0 mL columns containing Sephadex G-25 Superfine, cross-linked dextran (fractionation range between 1 and 5 kDa) from GE Healthcare (Buks, UK) were used for ultrafiltrate desalting. A Harvard Pump 11 Plus Single Syringe (Harvard Apparatus, Holliston, MA, USA) was used for ultrafiltrate loading into the Hi-Trap Desalting columns by using 5 mL BD DiscarditTM II syringes (Becton, Dickinson and Company, Fraga, Huesca, Spain). HAWP14250 Millipore 0.45 µm mixed esters of cellulose membrane filters (140mm diameter) were used for seawater filtration. Albet®LabScience 0.20µm cellulose acetate syringe filters (25 mm diameter) from Albet-Hahnemuehle (Dassel, Germany) were used for extracts filtration before HPLC experiments.

2.2.2. Reagents

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore). Potassium hydrogen phthalate stock standard solution (1000 mg L^{-1}) was prepared from 99.5 % potassium hydrogen phthalate supplied by Panreac (Barcelona, Spain). Potassium peroxodisulphate solution (30 % (m/v)) and phosphoric acid solution (6.0 M) were prepared from 99 % potassium peroxodisulphate and from 85 % phosphoric acid (Panreac), respectively. ReadyCalkit PEO containing polyethylene oxides (molecular weights of 6.55, 17.9, 40.1, 87.8, and 220 kDa) were from PSS Polymer Standard Services GmbH (Mainz, Germany). Blue dextran 2000 (molecular weight 2000 kDa) was from Pharmacia Biotech (Piscataway, NJ, USA). Aprotinin (6.5 kDa MW), ribonuclease A (13.7 kDa MW), ovalbumin (43 kDa MW), conalbumin (75 kDa MW), aldolase (158 kDa MW) and ferritin (440 kDa MW) were from GE Healthcare. Mixed exchange AG 501-X8 resin was from Bio-Rad (Richmond, CA, USA). Other reagents were high purity 69 % nitric acid (Panreac), high purity 25 % ammonia (Merck, Darmstad, Germany), sodium hydroxide (Merck), diammonium sulphate (Panreac), and diammonium hydrogenphosphate (BDH, Poole, UK). Multi-element standard solutions were prepared by combining stock standard solutions (1.000 g L^{-1}) supplied by Merck (Poole, Dorset, UK).

2.2.3. Seawater sample collection

Surface seawater samples (100 L) were collected from the Ría de Arousa estuary (north-western Spain) in pre-cleaned 12 L non-metallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection, seawater samples were filtered (0.45 μm) and immediately subjected to the ultrafiltration procedure.

2.2.4. Seawater tangential flow ultrafiltration

Before use, the ultrafiltration (UF) system was cleaned with 2 L of 0.1 M NaOH at 45 ± 5ºC (this solution was re-circulated for 60 minutes). The system was then rinsed with 9 L of Milli-Q water, also at 45 ± 5ºC, according to manufacturer's instructions. Filtered seawater samples (100 L) were then concentrated by tangential flow UF through a polyethersulfone membrane (size 0.6 m^2, nominal molecular mass cut-off of 10 kDa) until obtaining a volume of retentate (ultrafiltrate containing substances of molecular weight higher than 10 kDa) of approximately 1 L. The remaining sample (permeate) contained substances of molecular weight lower than 10 kDa and was reserved for mass balance studies. The retentate was freeze-dried and re-dissolved in 250 mL of Milli-Q water.

2.2.5. Retentate desalting procedure by using HI Trap desalting columns

Hi Trap Desalting columns were used to remove the salts from the lyophilized retentate. These columns are made of polypropylene, which is biocompatible and non-interactive with bio-molecules. The fractionation range for globular proteins is between 1 and 5 kDa, with an exclusion limit of approximately 5000 Da. This ensures the separation of bio-molecules exhibiting a molecular weight larger than 5 kDa from those molecules with molecular weights less than 1 kDa. The mobile phase used for desalting consisted of a 25 mM / 25mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5. The column was connected to a chromatographic system with UV detection, and was first equilibrated by passing 25 mL of a buffer solution at a flow rate of 2 mL min^{-1}. Once

equilibrated, 1.5 mL of re-dissolved retentate was loaded into the column using a 5 mL disposable syringe connected to a Harvard Pump 11 Plus Single Syringe for sample infusion which worked at a flow rate of 2 mL min^{-1}. After loading, the column was again connected to the chromatographic system for sample elution (pH 6.5 buffer solution at a flow rate of 2 mL min^{-1}). The excluded fraction was finally collected for further SEC-HPLC characterization. The Hi Trap column was equilibrated with approximately 10 mL of the pH 6.5 buffer solution before desalting the following 1.5 mL retentate sample.

2.2.6. Seawater and permeate desalting procedure

A mixed exchange resin (AG 501-X8) was used for salts removal from seawater samples and for the permeate fractions. According with manufacturer's instructions, 5 g of fresh resin for every 100 mL of seawater sample / permeate were used (batch mode). The mixture was shaken for 1 hour to achieve deionization. Finally, the desalted seawater sample / permeate was separated from the resin by filtration.

2.2.7. TOC measurements

Concentrations of dissolved organic carbon in the untreated seawater, retentate and permeate were assessed as TOC by supercritical water oxidation (30 % (m/v) potassium peroxodisulphate in 6.0 M phosphoric acid), and CO_2 NDIR detection. The use of supercritical water oxidation (SCWO) conditions diminishes the chloride interference, and the organic matter oxidation is therefore more efficient. The TOC analyzer was operated in the non-purgeable organic carbon (NPOC) measurement mode by mixing the acid (set at 1 %) and the oxidant (set at 15 %) with the sample in the sparger (sparge time of 0.8 min). Any inorganic carbon (IC) present is converted to CO_2 by the acid and is removed. The solution is then pumped from the sparger by the peristaltic pump in the reactor module where the temperature is rapidly increased to 375°C under a pressure of 22.1 MPa (conditions above the critical point of water). Organic carbon in the solution is then oxidized to CO_2 which

is subsequently measured in the NDIR detector. The analyzer was calibrated using standard solutions of potassium hydrogen phthalate ranging from 0.1 to 1000 mg L^{-1} (concentrations referred to carbon). Each sample (seawater, retentate and permeate) was analysed by quadruplicate. In addition, different blanks were also analysed for contamination control. The TOC procedure offers a LOD (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) of 0.13 mg L^{-1}; whereas, the LOQ (10 SD criterion) is 0.43 mg L^{-1}.

2.2.8. ICP-MS measurements

The ICP-MS Thermo Finnigan X Series was used for total metal determination (operating conditions listed in Table 1) in the desalted seawater, permeate, retentate and also in the fractions collected by SEC-HPLC. The standard addition technique (8.5 mL of desalted seawater, permeate or retentate diluted to 10 mL) covering metal concentrations between 0 and 200 µg L^{-1} (Co, Mn, and Zn) and between 0 and 20 µg L^{-1} (Ba and Sr) was used. A mixture of different internal standards (Sc, Ge, Y, and In) were added to the standards and samples at constant concentrations of 50 µg L^{-1} for Sc, 10 µg L^{-1} for Ge, and 5.0 µg L^{-1} for Y and In. Metals bound to marine DOM (SEC fraction) were assessed by using an aqueous calibration matched with the mobile phase (25 mM / 25 mM diammonium sulphate / diammonium hydrogen phosphate, pH 6.5). Similarly, Co, Mn and Zn concentrations were covered from 0 to 200 µg L^{-1}, while Ba and Sr concentrations were varied between 0 and 20 µg L^{-1}. The SEC fractions (8.5 mL) were diluted to 10 mL with the mobile phase, and the same internal standards (at the same concentrations listed above) were used for calibration. For all cases, analysis were performed in triplicate and different blanks were also analysed for contamination control.

The LODs (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) expressed as ng L^{-1} were 1.76, 23.5, 21.2, 2.20, and 225.9 ng L^{-1} for Ba, Co, Mn, Sr and Zn, respectively. Similarly, the LOQs (10 SD criterion) were 5.87, 78.3, 70.7, 7.33, and 753 ng L^{-1} for Ba, Co, Mn, Sr and Zn, respectively.

Table 1: Operating ICP–MS conditions

General		
	Radiofrequency power / W	1400
	Sample uptake rate / r.p.m.	3.0
	Stabilization delay / s	35
	Number of replicates	3
Nebulizer type	Beat impact (cooled spray chamber)	
Gas flows / L min^{-1}	Plasma	13.0
	Auxiliary	0.80
	Nebulizer	0.90
Ion optics / V		
	Extraction	-125
	Lens 1	-1000
	Lens 2	-80
	Lens 3	-195.3
	Hexapole Bias	-4.0
	Pole Bias	0.2
	D1	-40.8
	D2	-140
	Hexapole Bias	-4.0
	Pole Bias	0.2
Torch alignment / mm		
	Horizontal	80
	Vertical	405
	Sampling death	150
Mass-to-ratio		
	Ba	137
	Co	59
	Mn	55
	Sr	88
	Zn	66
	Sc (internal standard)	45
	Ge (internal standard)	72
	Y (internal standard)	89
	In (internal standard)	115

2.2.9. SEC-HPLC-UV/ICP-MS

SEC-HPLC separation (TSK-G4000 SW column, 30 cm × 8 mm i.d.) was performed under isocratic elution conditions (flow rate set at 1.0 mL min^{-1}) with a mobile phase consisting of a 25 mM / 25 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5. UV detection was performed at 205 nm, and the injection volume was set at 50 µL. Column calibration was carried out with aprotinin, ribonuclease A, ovalbumin, conalbumin, aldolase and ferritin standards (molecular weights of 6.5, 13.7, 43, 75, 158 and 440 kDa, respectively). The exclusion volume (V_0) was determined using blue dextran (2000 kDa) and the permeation volume (V_p) was fixed by injecting aprotinin (6.5 kDa). For ICP-MS detection, the outlet of the chromatographic column was directly coupled to the nebulizer of the ICP-MS (operating ICP-MS conditions are also listed in Table 2).

2.2.10. AEC-HPLC-UV/ICP-MS

DOM fractions collected by SEC were then subjected to Anion Exchange Chromatography (AEC) with UV detection and also hyphenated with ICP-MS. Separation was performed with a PRP-X100 column under isocratic conditions (25 mM / 25mM $(NH_4)_2SO_4/(NH_4)_2HPO_4$, pH 6.5, as a mobile phase, flow rate of 1 mL min^{-1}). When using UV detection, absorbance was monitored at 205 nm. When coupling the AEC-HPLC with ICP-MS for metal detection, the operating ICP-MS conditions listed in Table 2 were used.

Table 2: SEC/AEC-UV/ICP-MS operating conditions.

SEC	TSK-G4000 SW (30 cm × 8 mm I.D.) coupled to a TSK-gel SW glass guard column (10 cm x 8 mm I.D.)	
	Injection volume (µL)	50
	Column temperature (ºC)	25
	Mobil phases flow rate/mL min^{-1}	1.00
Mobile phase	25 mM/25 mM ammonium sulphate/diammonium hydrogen phosphate, pH 6.5	
	UV detection (nm)	205
AEC	PRP-X100 (250 mm × 4.1 mm I.D.) to a PRP-X100 guard column (25 × 2.3 mm I.D.)	
	Injection volume (µL)	50
	Column temperature (ºC)	25
	Mobil phases flow rate/mL min-1	1.00
Mobile phase	25 mM/25 mM ammonium sulphate/diammonium hydrogen phosphate, pH 6.5	
	UV detection (nm)	205
ICP-MS	Radiofrequency power/W	1400
	Peristaltic pump speed/r.p.m.	2.5
Nebulizer type	Beat impact (cooled spray chamber)	
Gas flows/L min^{-1}	Plasma	13.0
	Auxiliary	0.8
	Nebulizer	0.90
Ion optics/V	Extraction	-125
	Lens 1	-1000
	Lens 2	-80
	Lens3	-195.3
	Focus	11
	D1	-40.8
	D2	-140
	Pole bias	0.2
	Hexapole bias	-4
Torch alignment/mm	Horizontal	80
	Vertical	405
	Sampling death	150
Mass-to-ratio	Ba	137
	Co	59
	Mn	55
	Sr	88
	Zn	66

2.3. Results

2.3.1. Ultrafiltration and TOC measurements

The UF procedure described in section 2.2.4 was applied to a surface seawater sample (100 L), and the TOC concentrations were measured in the permeate fraction (99 L) containing marine DOM of molecular weight lower than 10 kDa, and also in the retentate fraction (1 L, subjected to freeze-drying and redisololution to 250 mL) containing marine DOM of molecular weight higher than 10 kDa. The TOC concentration was also measured in the untreated seawater sample, and a mass balance was performed to test the amount of marine DOM lost by absorption onto the membrane throughout the UF procedure. Analysis in quadruplicate (Table 3) showed a mean TOC concentration in the untreated seawater of 0.867 ± 0.006 mg L^{-1}; whereas, the TOC concentration in the permeate was 0.737 ± 0.015 mg L^{-1}. The mean TOC concentration in the retentate (marine DOM exhibiting molecular weights higher than 10 kDa) was 0.0081 ± 0.0006 mg L^{-1} (8.1 ± 0.6 µg L^{-1}). This value was obtained by taking into account the pre-concentration factor of the ultrafiltration method (400). Therefore, TOC concentration as a sum of the TOC concentrations in the permeate and in the retentate was 0.745 ± 0.015 mg L^{-1}, which implies a loss of TOC throughout the UF procedure of 0.122 ± 0.016 mg L^{-1} (14.1 ± 2 %). This result agrees with those reported by Minor et al. [19] who have found losses by adsorption onto the UF membrane of 11 % when monitoring solutions containing polysaccharide dextran (molecular weight of 10 kDa) by SEC, and 16 % when performing experiments with bovine albumin (molecular weight of 66 kDa).

2.3.2. Retentate desalting

Although UF allows an effective separation of organic compounds according to their molecular weight, this procedure does not guarantee salts removal, and a desalting stage after UF must be performed before DOM analysis/characterization by several analytical techniques. This is particularly important when using SEC

with UV detection because the presence of salts can damage the chromatographic resolution, and also because most dissolved salts offer UV absorption, and chromatographic signals from salts are observed in the chromatograms. According to the published literature, diafiltration procedures commonly performed by using the UF devices are used for desalting purposes [6,19,26]. A first attempt consisted of subjecting the retentate to diafiltration at different stages, and measuring the salinity and the TOC concentration after each step. Results showed that salinity was efficiently reduced from 15.9 ‰ (un-diafiltrated retentate) to 0.9 ‰ (diafiltrated retentate after five diafiltration steps). However, a significant marine DOM loss was observed after each diafiltration step. These results agree with those of other authors who have reported DOM losses within the 10 – 35 % range, and have concluded that these losses are more important when dealing with marine DOM of high molecular weight [6,26]. Other desalting procedures based on using mixed exchange resins, such as AG 501-X8 (section 2.2.6), were unsuccessful because organic matter contamination [32].

Therefore, an approach based on HI Trap Desalting columns was implemented for removing salts content in the retentate fraction. An aliquot of a retentate and a solution of bovine albumin (0.025 g) dissolved in 200 mL of synthetic seawater (32 g of sodium chloride, 14 g of magnesium sulphate, and 0.15 g of sodium hydrogencarbonate) were subjected to desalting procedure described in section 2.2.5. UV monitorization showed that after loading 1.5 mL of retentate (Figure 1) compounds of high molecular weight elute from the column within the first 1 minute (elution flow rate of 2 mL min^{-1}), and an efficient separation of marine DOM from salts is obtained. As a consequence of the proposed desalting treatment, the sample (retentate) is diluted by a factor of 1.3 (1.5 mL of the original solution to 2 mL).

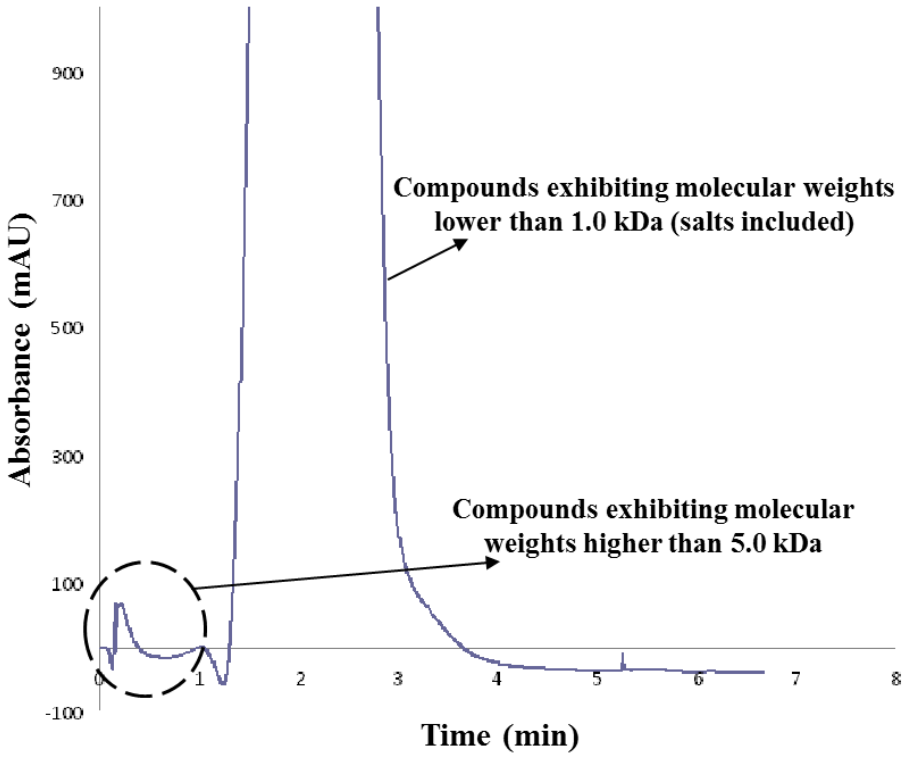

Figure 1: Size exclusion chromatogram when using the HI Trap desalting columns for treating a freeze-dried retentate (UV detection at 205 nm).

2.3.3. Size exclusion chromatography (SEC) fractionation

Preliminary experiments were performed by injecting 50µL of the desalted retentate as well as standards of aprotinin (MW of 6.5 kDa) and blue dextran (MW of 2000 kDa) on a TSK-G4000 SW SEC column. Detection was performed by UV monitoring at wavelengths of 205, 210, 220, and 280 nm. Different mobile phases were tested under isocratic conditions (1 mL min^{-1}): ultrapure water [8,10,35], 0.5 M / 0.5 M potassium dihydrogen phosphate / disodium hydrogen phosphate (pH 6.7) [8], and 0.1 M / 0.1 M diammonium sulphate / diammonium hydrogen phosphate (pH 6.5) [8,19,32]. Ultrapure water as a mobile phase did not offer satisfactory results because blue dextran and aprotinin showed similar elution volumes (retention times of 13.03 and 14.5 min, respectively). The use of

potassium dihydrogen phosphate / disodium hydrogen phosphate (0.5 M / 0.5 M) offered small chromatographic signals when injecting the desalted retentate, and also a high column pressure. However, satisfactory results were achieved when using diammonium sulphate / diammonium hydrogen phosphate (0.1 M / 0.1 M) as a mobile phase. Retention times for aprotinin and blue dextran were of 13.67 and 28.65 min, respectively, and the chromatographic signal when analysing the desalted retentate was between the void volume, obtained when injecting blue dextran, and the permeate volume, approximately 30 min (injection of ultrapure water). A buffer solution consisting of 0.1 M / 0.1 M diammonium sulphate / diammonium hydrogen phosphate (pH 6.5) was therefore chosen for further optimization.

2.3.3.1. Optimization of the mobile phase composition

As shown by several authors [8,19], DOM separation by SEC is largely affected by the ionic strength of the mobile phase which can change the sensitivity of SEC separations [19] and also cause conformational changes in the DOM (from linear polymer-like to spherocolloid-like) [42]. In general, mobile phases of low ionic strengths offer better separation than those achieved when increasing the ionic strength of the mobile phases [8]. Therefore, different mobile phases consisting of ammonium sulphate / ammonium dihydrogen phosphate solutions (pH fixed at 6.5) were prepared at different ionic strengths (concentrations of 5.0 / 5.0, 10 / 10, 25 / 25, 50 / 50 and 100 / 100 mM), and were tested for marine DOM fractionation in desalted retentates. In these cases, the ultrafiltrated sample was desalted (section 2.2.5) by using the ammonium sulphate / ammonium dihydrogen phosphate buffer (pH 6.5) at the studied ionic strength. As shown in Figure 2, better separation of DOM fractions is achieved when working at lower ionic strengths (within 10 / 10 to 50 / 50 mM, Figure 2(B-D)). Chromatograms when using the lowest mobile phase composition (5.0 / 5.0 mM), as that shown in Figure 3(A), gave negative signals quite similar to those obtained when using ultrapure water as a mobile phase. Finally, the use of mobile phases at the highest ionic strength (Figure 2 (E)) led to low chromatographic signals and shifts toward slightly

higher retention times. This phenomena has been attributed to the increased hydrophobic interactions of DOM with the SEC column at higher ionic strengths [19,43], and has been reported by several authors when fractionating DOM by SEC [8,19,32,44].

As shown in Figure 2 (C), a second chromatographic signal (retention time of 15.9 min) was observed. A similar result has been reported by Schmitt et al. [36] when analysing natural DOM by SEC using a 28 mM phosphate buffer as a mobile phase. According to these authors, this sharp peak (elution volume of 15.9 mL) can be attributed to a salt boundary peak which was produced by a gradient in the ionic strength between the sample and the mobile phase. This assumption will be further proved when applying AEC with UV detection and also when hyphenating SEC and AEC with ICP-MS.

As a result, the composition of the ammonium sulphate / ammonium dihydrogen phosphate was set at 25 / 25 mM (Figure 2(C)), and the pH of the mobile phase, within 6.5 to 8.0, was further investigated. The pH of the 25 / 25 mM ammonium sulphate / ammonium dihydrogen phosphate was increased from 6.5 to 7.0, 7.5 and 8.0 by adding small volumes of a 0.1 M sodium hydroxide solution. Results showed that slight variations on the pH of the mobile phase did not affect DOM separation, and a pH of 6.5 (close to seawater pH) was finally selected.

2.3.3.2. Molecular weight assessment of marine DOM

First attempts for calibrating the TSK-G4000 SW SEC column were performed by using a mixture of polyethylene oxides (PEOs) and polyethylene glycols (PEGs) standards (molecular weights of 6.55, 17.9, 40.1, 87.8, and 220 kDa). However, the PEOs / PEGs standards elution was not achieved under the isocratic conditions optimized when using the diammonium sulphate / diammonium hydrogen phosphate buffer (pH 6.5) as a mobile phase. Similar results have been previously reported when using PEOs and PEGs [32], and also for polystyrene sulfonates (PSSs) standards [45]. In the latter case, this phenomena was attributed

to the strong interaction of PSSs with the SEC column in the presence of sodium sulphate in the eluent. In the current application, an effective PEOs and PEGs elution was achieved under chromatographic conditions recommended by the manufacturer (aqueous 0.2 % (m/v) sodium azide).

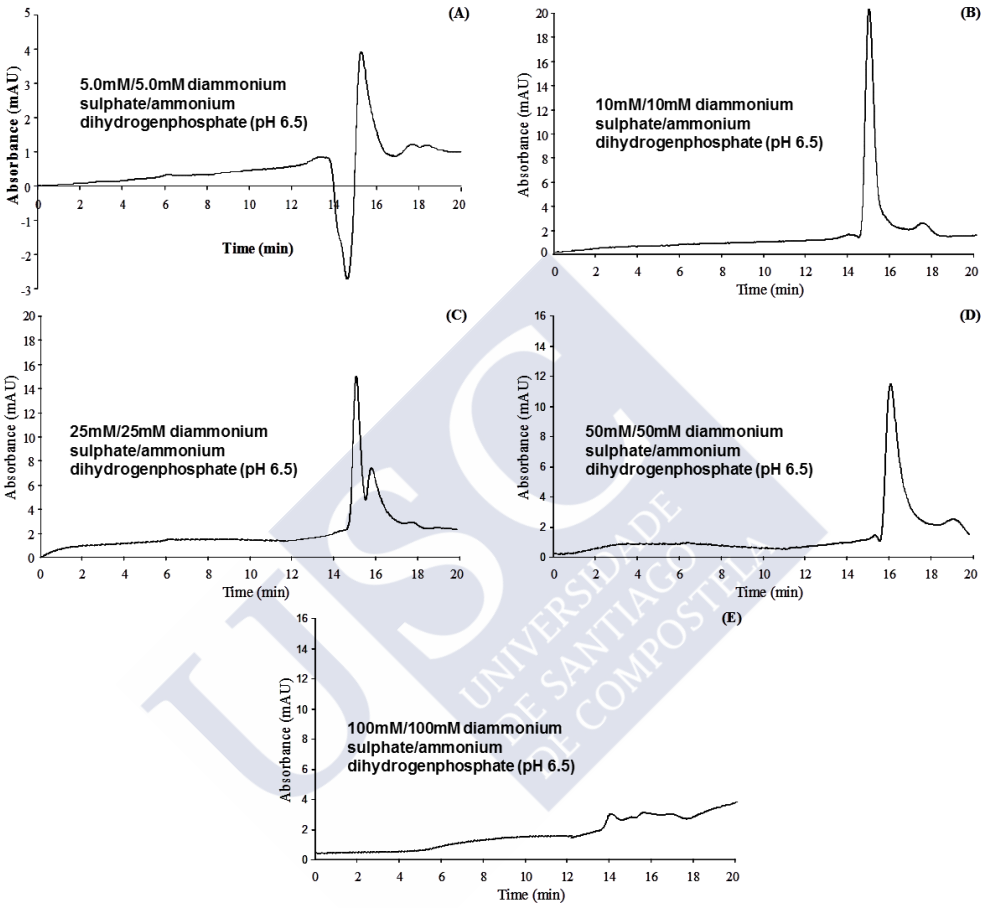

Figure 2: SEC chromatograms (UV detection at 205 nm) of marine DOM when using diammonium sulphate / ammonium dihydrogenphosphate (pH 6.5) as a mobile phase at different concentrations: 5.0 mM / 5.0 mM (A), 10 mM / 10 mM (B), 25 mM / 25 mM (C), 50 mM / 50 mM (D), and 100 mM / 100 mM (E).

A further calibration was then performed by using proteins of different molecular weights as standards (1000 mg L^{-1}, dissolved in mobile phase): aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), aldolase

(158 kDa), and ferritin (440 kDa). The regression of the retention time (R_t) and the logarithm of the molecular weight (Log(MW)) was adjusted to a lineal model (regression coefficient, r^2, of 0.9975), and the calibration equation can be then expressed as Log (MW) = – 0.358 R_t + 9.43). The exclusion volume (V_0) was determined using blue dextran (2000 kDa), and it was 6.15 mL (retention time of 6.15 min); while the permeation volume (V_p) was fixed by using the aprotinin standard (volume of 15.7 mL, retention time of 15.7 min). From chromatograms plotted in Figure 2(B-C), marine DOM exhibits a variety of molecular weights ranging from 16 to 1.7 kDa (times between 14.6 min, which is the beginning of the first SEC signal; and 17.3 min, which is the ending of the second SEC signal). It is shown that a part of the isolated marine DOM fell out the range of molecular weights fixed by aprotinin (higher than 15.7 min). Marine DOM consists of a complex mixture of polydisperse molecules and the molecular weight identifications should be considered apparent molecular weights only because the SEC column used offers a very different molecular weight resolution when calibrating with globular proteins (within the 20 – 7000 kDa range) or when using PEGs (from 2 to 250 kDa). Therefore and according with Rottmann and Heumann [10], conclusions from SEC experiments can only be used to determine molecular weight distribution profiles rather than the exact molecular weight of the substances.

2.3.4. Anion exchange chromatography (AEC) fractionation

A further characterization by using AEC was performed. The whole SEC fraction (retention times from 14.5 to 17.3 min) or both separated SEC fractions (from 14.5 to 15.6 and 15.6 to 17.3) were chromatographied by using the same mobile phase as that used for SEC (25 mM / 25 mM diammonium hydrogenphosphate / diammonium sulphate (pH 6.5)), the same flow rate (1 mL min^{-1}) and UV detection. Figure 3(A-B) shows the AE chromatograms when analysing the first and the second SEC fractions, respectively. Four different chromatographic signals can be seen for both cases (analysis of both SEC fractions) at retention times of 2.3, 2.8, 4.5 and 14.0 min (chromatogram peaks for the analysis of the first SEC fraction offer higher heights than those obtained when analysing the second SEC fraction).

The same AEC pattern obtained for both SEC peaks suggests that the two chromatographic SEC signals observed in Figure 2(C) are attributed to the same group of organic substances. The presence of the sharp peak at 15.9 mL (retention times from 15.6 to 17.3 min) may be caused by an additional phenomena (as a salt boundary peak produced by a gradient in the ionic strength between the sample and the mobile phase [36]) in addition to the size exclusion mechanism. This fact was verified after injecting the whole SEC fraction (retention times from 14.5 to 17.3 min), and also by injecting the SEC fraction isolated when using 10 mM / 10mM diammonium hydrogenphosphate / diammonium sulphate (pH 6.5). These experiments led to the same AE chromatogram, with four chromatographic signals at the same retention times as shown in Figure 3. Finally, the variation of the pH of the mobile phase for AEC separations did not offer any differences in the AE chromatograms.

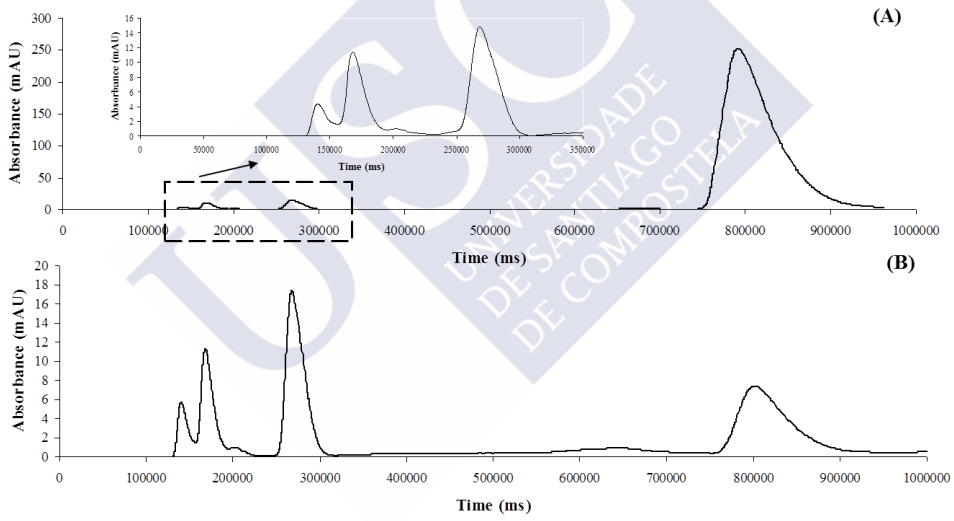

Figure 3: AEC chromatograms (UV detection at 205 nm) of marine DOM using 25 mM / 25 mM diammonium sulphate / ammonium dihydrogenphosphate (pH 6.5) as a mobile phase when analysing the first SEC fraction, elution volume from 14.5 to 15.6 mL (A), and the second SEC fraction, elution volume from 15.6 to 17.3 mL (B).

2.3.5. Metal patterns

SEC was coupled with ICP-MS to assess metals bound to marine DOM. In addition, the SEC fractions were also resolved by AEC hyphenated with ICP-MS to assess metal patterns. ICP-MS operating conditions as well as SEC/AEC parameters are listed in Table 2. Metal patterns when coupling SEC-ICP-MS are plotted in Figure 4, where coelution with an identical distribution pattern with marine DOM (SEC-UV fractionation) can be seen for Mn, Sr and Zn. Nevertheless, Ba showed coelution without an identical distribution pattern that marine DOM. It must be pointed out that the chromatographic signals for Mn, Sr and Zn (Figure 4) match the UV signal and encompass both SEC peaks. This proves that the sharp SEC peak (15.9 mL) is an artifact caused by differences between the ionic strength of the samples and the mobile phase. Finally, negligible signals (coelution with or without identical distribution pattern with marine DOM) were recorded for other elements such as Al, Cd, Co, Cr, Cu, Fe, Mo, Ni, Pb and V.

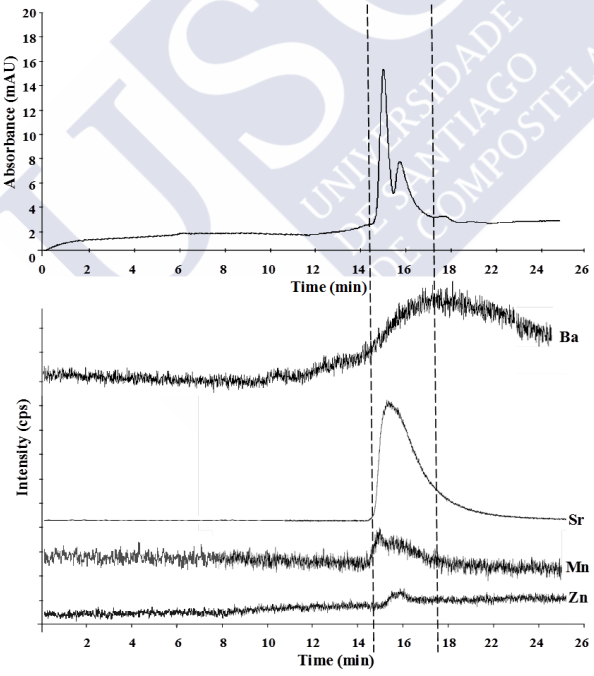

Figure 4: SEC chromatograms (UV and ICP-MS detection) of marine DOM.

Figure 5 shows AEC-ICP-MS chromatograms when monitoring metals such as Ba, Co, Mn, Sr and Zn. It can be seen that Sr and Zn coelute with an identical distribution pattern as that of the first AE chromatographic signal; whereas, Ba and Mn coelute with an identical distribution pattern as that of the second AE chromatographic signal. Co was also found to coelute with a distribution pattern quite close to the third AE chromatographic signal. It must be mentioned that a negligible signal for this element was found after SEC-ICP-MS experiments. This fact can be attributed to a low concentration of this element and to the characteristic broadening of the SEC chromatographic signals, while AEC provides narrow chromatographic signals with greater heights (Figures 2 and 3). Finally, any studied metal was found to be bound to the fourth AE chromatographic signal.

In addition, high signals were observed for metals such as Cd and Ni (chromatograms are not given), although no specific coelution with marine DOM monitored with UV detection was observed. A similar finding for Ni has been reported by Rottmann and Heumann [10] when assessing DOM in natural water samples by SEC-ICP-MS.

Similar AEC-ICP-MS chromatograms when injecting the “first SEC fraction” (retention time between 14.5 and 15.6), the “second SEC fraction” (between 15.6 and 17.3 min), and the whole SEC fraction (between 14.5 and 17.3 min) were obtained, which proved the artifact nature of the sharp SEC peak at 15.9 mL.

Metal patterns in marine DOM after SEC/AEC-ICP-MS are different than those reported by other authors when analysing freshwater (natural DOM). As an example, Ba, Mn, and Sr bound to natural DOM after SEC-ICP-MS have not been reported in sewage, groundwater, brown water, and seepage water from soils [35], neither in bog [10,36], lake [10,37] and river [10] waters. Metal reported to be associated to natural DOM in bog, lake and river waters were Cu, Mo and Zn [10,36,37], and also Cd, Co, Cr, Fe, Ni, V, and Zn [10,37]. Wu et al. [37] have also reported the association of Ce, Th, and U to natural DOM in lake waters. Finally,

Al, Fe, Ni and Pb were also found to be bound to natural DOM in sewage, groundwater, brown water, and seepage water from soils [35].

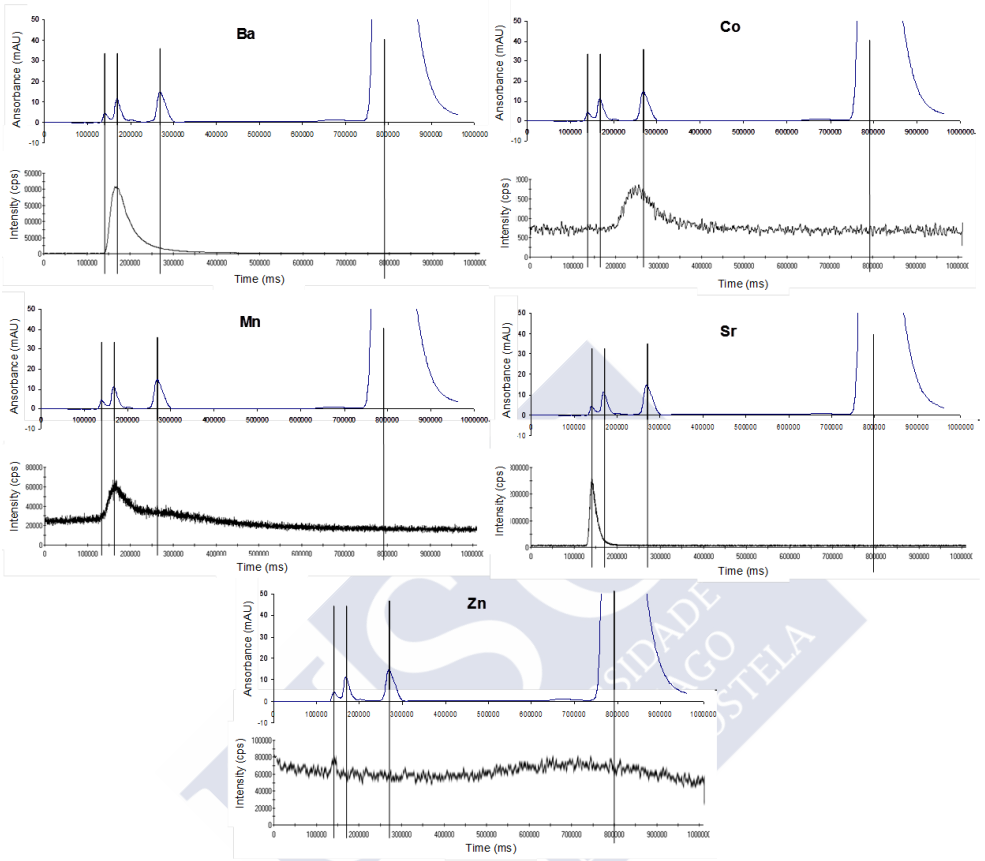

Figure 5: AEC chromatograms (UV and ICP-MS detection) of marine DOM fractionated by SEC.

2.3.6. Metal quantification. Mass balance studies

The amount of metals bound to the isolated marine DOM (Ba, Co, Mn, Sr, and Zn) was assessed by combining three whole SEC fractions (times from 14.4 to 17.4 min, which implies 3.0 mL each run) from the same desalted retentate; and by direct ICP-MS analysis (8.5 mL of the collected / combined fraction diluted to 10 mL) against an aqueous calibration matched with mobile phase (25 mM / 25 mM

diammonium hydrogenphosphate / diammonium sulphate, pH 6.5) and by using ^{45}Sc, ^{72}Ge, ^{89}Y, and ^{115}In as internal standards. Analysis was performed in triplicate and also different blanks were analysed for contamination control. Similarly, total concentrations of the selected metals were determined in the seawater sample and also in the permeate fraction after the desalting procedure shown in section 2.2.6; and also in the retentate fraction after HI-Trap desalting (section 2.2.5). The standard addition technique plus the addition of a mixture of internal standards was used for calibration. Table 3 lists the found metal concentrations in the studied surface seawater, in the desalted permeate and retentate after the UF procedure, and also in the isolated SEC fraction. TOC concentrations are also listed. It can be seen that metal concentrations as a sum of the metal concentration in the permeate and the metal concentration in the retentate is lower (between 30 and 60 %) than the metal concentrations in the seawater. This can be attributed to the efficient desalting procedure applied to the retentate fraction (use of HI Trap desalting columns, section 2.2.5) that remove dissolved metal ions in addition to the major inorganic salts. The use of the mixed exchange resin AG 501-X8 for desalting the surface seawater and the permeate fraction (section 2.2.6) only removes major inorganic salts (monovalent ions such as Na^+ and Cl^-). However, contamination was detected when assessing Zn: the concentration of this element in the permeate fraction is quite higher than that found in the untreated seawater, and hence the concentration of Zn in the retentate fraction could also be higher than expected. Assuming the total concentration of the studied metals in the untreated seawater, percentages of metals bound to marine DOM range from 0.24 % (Co) to 0.67 % (Zn). However, a lower percentage was found for Sr, approximately 2.7 10^{-3} %, which indicates lower binding properties of this element.

Table 3: Metal concentrations (n = 3) in surface seawater, permeate and retentate fractions, and concentrations of metal bound to marine DOM.

SSW 01	Concentration (µg L^{-1})		Concentration (ng L^{-1})	
	Surface seawater	Permeate fraction[a]	Retentate fraction[b]	Metal binding marine DOM[b]
Co	4.5 ± 0.06	4.4 ± 0.1	$9.7 \pm 7 \times 10^{-2}$	$11 \pm 3 \times 10^{-2}$
Mn	2.6 ± 0.08	0.95 ± 0.1	32 ± 0.6	$38 \pm 3 \times 10^{-2}$
Sr	2282 ± 13	1554 ± 57	9560 ± 58	179 ± 3.85
Zn	218 ± 10	279 ± 21	8.0 ± 0.1	$4.2 \pm 4 \times 10^{-3}$
TOC[c]	0.87 ± 0.006[d]	0.74 ± 0.015[d]	8.1 ± 0.6[e]	

[a] Dilution factor of 0.9975, [b] Preconcentration factor of 400, [c] n=4, [d] Concentration expressed as mg L^{-1}, e Concentration expresses as µg L^{-1}.

2.4. Conclusions

Studies for the assessment of marine DOM as well as metals bound to marine DOM has been performed by combining two different chromatographic modes (SEC and AEC), and using UV and ICP-MS as detection systems. Isolation of large marine DOM from surface seawater was achieved by tangential ultrafiltration (molecular cut off of 100 kDa), and the use of HI Trap columns for desalting purposes was found adequate for preparing the sample before SEC/AEC analysis. Results from SEC-UV showed a group of organic substances comprising a variety of molecular weights ranging from 16 to 1 kDa. Experiments after SEC hyphenation with ICP-MS revealed that metals such as Ba, Mn, Sr and Zn form complexes with the isolated marine DOM. A further characterization by AEC-UV showed four different groups of organic substances, and AEC-ICP-MS analysis showed that those metals are associated only to certain AE fractions (the largest AE fraction, retention time of 14.0 min, was not found to complex any metal). Percentages of metals bound to the isolated marine DOM were 0.24, 0.50, and 0.67 % for Co, Mn, and Zn, respectively. However, percentages lower than 0.1 % were found for Sr binding isolated marine DOM ($2.7\ 10^{-3}$ %).

Acknowledgements

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3. Evaluation of tangential flow ultrafiltration procedures to assess trace metals bound to marine dissolved organic matter

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Evaluation of tangential flow ultrafiltration procedures to assess trace metals bound to marine dissolved organic matter

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Abstract: *A procedure based on two-dimensional size exclusion chromatography (SEC) and anion exchange chromatography (AEC) with UV (205 nm) and ICP-MS detection was used to assess dissolved organic matter (DOM) and trace metals associated to DOM in surface seawater. Marine DOM was isolated by tangential ultrafiltration (UF) using two different polyethersulfone membranes exhibiting different molecular weight cut-off (MWCO), 3 and 10 kDa. The procedures require a volume of seawater sample of 100 L, and marine DOM of molecular weight higher than 3 and 10 kDa (UF membranes of 3 and 10 kDa MWCO) were pre-concentrated in 0.5 L (retentate), which implies a pre-concentration factor of 200. Retentate fractions obtained after the different UF procedures were further desalted by using HI Trap desalting mini-columns before two-dimensional SEC/AEC. Aparent molecular weights of isolated compounds after SEC with UV detection ranged from 1.5 kDa (close to the permeable volume of the SEC column fixed by injecting vitamine B12) to 16 and 22 kDa (UF membranes of 3 and 10 kDa MWCO, respectively). Further AEC/UV characterization of SEC fractions showed a large group of macromolecules eluted at 4.5 min, and small signals at shorter retention times (2.5 and 3.5 min). In addition, AEC experiments of the isolated SEC fractions when using 10 kDa MWCO UF membranes showed a group of substances eluted at high retention times (13 min). SEC hyphenation with ICP-MS proved the existence of several trace elements (Ni, Co, Cu, Zn, Mn, Mo and Sr) bound to the isolated marine DOM. Mass balance studies after analyzing the retentate and permeate fractions for trace elements indicates good recoveries (close to 100 %) for elements such as Mo, Sr, Ba and Zn when performing the UF with both 3 and 10 kDa MWCO membranes. However, recoveries from 36 to 81 % were obtained for the remaining studied elements after either UF procedure. SEC-ICP-MS experiments showed percentages of metals bound to the isolated marine DOM ranging from 0.055 and 0.077 % (Zn) to 4.1 10^{-4} and 1.4 10^{-4} % (Sr).*

Keywords: *Metal bound to marine dissolved organic matter, tangential flow ultrafiltration, size exclusion chromatography, anion exchange chromatography, inductively coupled plasma - mass spectrometry.*

3.1. Introduction

Marine dissolved organic matter (DOM) is a complex mixture of very different organic molecules from natural and anthropogenic sources. Marine DOM is one of the planet's most important reservoirs of carbon (approximately 700 × 10^{15} g), an amount similar to the carbon content in atmospheric carbon dioxide (750 × 10^{15} g) [1,2]. The ocean is an important sink for anthropogenic carbon dioxide because it can absorb one third of the carbon dioxide emissions from combustion of fossil fuels and from tropical deforestation by fire. Marine DOM presents, therefore, a considerable contribution to the global carbon cycle [3,4]. On the other hand, the presence of various highly reactive functional groups in marine DOM is responsible for the binding properties of many different types of contaminants, such as organic compounds and trace metals. Marine DOM plays, therefore, an important role for controlling the transport of toxic contaminants and nutrients and influencing toxicity and bioavailability of those compounds in the marine environment [5-7]. Among the different compounds contained in marine DOM, proteins are important components of high molecular weight. The study of marine proteins has increased because they are the most reactive substances present in marine DOM [8]. Trace metals can be bound to marine proteins and thus affect metal toxicity and availability.

The assessment of marine DOM of high molecular weight is difficult because this fraction occurs at low concentrations, while a large amount of inorganic salts are present in seawater. Some solid phase extraction (SPE) procedures have been applied for pre-concentrating marine DOM. These procedures have been shown to be sucessful when separating DOM of low molecular weight [9], a fraction which can account for 65 – 75 % of DOM in surface seawater [4]. On the contrary, DOM of high molecular weight, such as marine proteins, is more easily isolated by tangential flow ultrafiltration (UF) procedures [5,8]. This technique offers the advantage of high pre-concentration factors and the possibility of using large sample volumes. UF procedures usually require membranes with a nominal molecular weight cut-off (MWCO) of 10 kDa, which guarantees the isolation of

marine DOM of high molecular weight (marine proteins included) [10-20]. Nevertheless, marine DOM can be lost by adsorption onto the UF membranes [14,15], and this problem is especially important when dealing with marine proteins due to their highly adsorptive nature. Different reagents such as sodium azide and sodium dodecylsulphate (SDS) are commonly added to the filtered seawater before UF to prevent protein adsorption onto the ultrafiltration membrane [15-20]. On other occasions, UF procedures based on membranes with nominal MWCO of < 10kDa have been proposed, and 1 kDa MWCO membranes have been used in several studies [11,21-25]. UF procedures by using 5 kDa MWCO membranes have also been reported [20].

The aim of this work is the development of pre-concentration procedures for marine DOM of high molecular weight using tangential UF procedures. Isolation was performed by using two membranes with different MWCO (3 and 10 kDa). Differences of the isolated marine DOM were established by two-dimentional size exclusion chromatography (SEC) and anion exchange chromatography (AEC) with UV detection. Inductively coupled plasma-mass spectrometry (ICP-MS) was also used as a detector for assessing trace elements bound to the isolated marine DOM.

3.2. Material and methods

3.2.1. Apparatus

The tangential UF system consisted of a Masterflex I/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with two polyethersulfone membranes (nominal MWCO 10 kDa and 3 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge.

A Sievers Innovox Laboratory TOC analyzer from General Electric Analytical Instruments (Boulder, CO, USA) was used for DOM quantification. The analyzer uses a super critical water oxidation (SCWO) technique to achieve superior wet

DOM oxidation (persulfate in acid medium), and a Non-Dispersive Infra Red (NDIR) for CO_2 detection.

A Dionex P680 HPLC pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne 4097 injector (Cotati, CA, USA) with a 50 µL injection loop, and a Dionex UVD170U absorbance detector was used for HPLC-UV determinations. A 250 µL Hamilton Gastight 1725 syringe (Bonaduz, Switzerland) was used for manual injection.

A Dionex UltiMateO 3000 LC HPLC (Dionex), equipped with a GP50 gradient pump (Dionex), an AS50 thermal compartment (Dionex) and an AS50 autosampler (Dionex) was coupled to an ICP-MS Thermo Finnigan X Series (Thermo Fisher Scientific Inc., Waltham, MA, USA) for assessing metals bound to DOM. DOM fractions were separated with a TSK-G4000 SWXL SEC column (30 cm × 7,8 mm I.D., 8 µm particle size, optimum separation range for Polyethylene Glycol (PEGs) between 2 and 250 kDa, and for proteins between 20 and 10000 kDa) from Tosoh Bioscience (Tokyo, Japan) coupled to a 10 cm × 8 mm i.d. TSK-gel SW glass guard column (TosoHaas, Tokyo, Japan). A PRP-X100 anion-exchange column (250 mm × 4.1 mm i.d. × 10 µm) from Hamilton (Reno, NV, USA) coupled to a 25 × 2.3 mm I.D. PRP-X100 guard column (Hamilton) was also used.

Hi-Trap Desalting 5.0 mL columns containing Sephadex G-25 Superfine, cross-linked dextran (fractionation range between 1 and 5 kDa) from GE Healthcare (Buks, UK) were used for ultrafiltrate desalting. A Harvard Pump 11 Plus Single Syringe (Harvard Apparatus, Holliston, MA, USA) was used for ultrafiltrate loading into the Hi-Trap Desalting columns by using 5 mL BD DiscarditTM II syringes (Becton, Dickinson and Company, Fraga, Huesca, Spain).

Other materials were an ORION 720A plus pH–meter with a glass–calomel electrode (ORION, Cambridge, UK), HAWP14250 Millipore 0.45 µm mixed esters of cellulose membrane filters (140mm diameter), and Albet®LabScience 0.20 µm

cellulose acetate syringe filters (25 mm diameter) from Albet-Hahnemuehle (Dassel, Germany).

3.2.2. Reagents

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore). Potassium hydrogen phthalate stock standard solution (1000 mg L^{-1}) was prepared from 99.5 % potassium hydrogen phthalate supplied by Panreac (Barcelona, Spain). Potassium peroxodisulphate solution (30 % (m/v)) and phosphoric acid solution (6.0 M) were prepared from 99 % potassium peroxodisulphate and from 85 % phosphoric acid (Panreac), respectively. Ready Calkit PEO/PEG (PSS) containing polyethylene oxides (molecular weights between 6.7 - 478 kDa) were from PSS Polymer Standard Services GmbH (Mainz, Germany). Blue dextran 2000 (molecular weight 2000 kDa) was from Pharmacia Biotech (Piscataway, NJ, USA). Carbonic Anhidrase (29 kDa MW), ribonuclease A (13.7 kDa MW), ovalbumin (43 kDa MW), conalbumin (75 kDa MW), aldolase (158 kDa MW) and ferritin (440 kDa MW) were from GE Healthcare.

Mixed exchange AG 501-X8 resin was from Bio-Rad (Richmond, CA, USA). Other reagents were high purity 69 % nitric acid (Panreac), high purity 25 % ammonia (Merck, Darmstad, Germany), sodium hydroxide (Merck), diammonium sulphate (Panreac), and diammonium hydrogenphosphate (BDH, Poole, UK). Multi-element standard solutions were prepared by combining stock standard solutions (1.000 g L^{-1}) supplied by Merck (Poole, Dorset, UK).

3.2.3. Seawater sample collection

Surface seawater samples (100 L) were collected from the Ría de Arousa estuary (north-western Spain) in pre-cleaned 12 L non-metallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection, seawater samples were filtered (0.45 µm) and inmediately subjected to the ultrafiltration procedure.

3.2.4. Seawater tangential flow ultrafiltration

According to manufacturer's instructions, the UF system was cleaned before use by re-circulating 2 L of 0.1 M NaOH at 45 ± 5ºC for 60 minutes; and rinsing with 9 L of Milli-Q water, also at 45 ± 5 ºC. The seawater (100 L) was then concentrated by tangential flow UF through two different polyethersulfone membranes (size 0.6 m^2, nominal MWCO of 10 kDa and 3 kDa) until obtaining a volume of retentate (ultrafiltrate containing substances of molecular weight higher than 10 and 3 kDa, respectively) of approximately 500 mL. The remaining sample (permeate, ~99.5 L) contained substances of molecular weight lower than 10 and 3 kDa (membranes of nominal MWCO of 10 kDa and 3 kDa, respectively), and it was reserved for further mass balance studies. No prerservatives for avoiding marine DOM adsorption onto the UF membrane was added, because mass balance studies for marine DOM were performed through TOC determinations.

3.2.5. Retentate desalting procedure by using HI Trap desalting columns

Hi Trap Desalting columns were used for removing the salts from the retentate. The Sephadex G-25 Superfine containted in the columns offer a fractionation range for globular proteins between 1 and 5 kDa, with an exclusion limit of approximately 5 kDa. This ensures the separation of bio-molecules exhibiting a molecular weight higher than 5 kDa from those molecules with molecular weights less than 1 kDa. The mobile phase used for desalting consisted of a 25 mM / 25 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5. The column was connected to the chromatographic system with UV detection, and was first equilibrated by passing 25 mL of a buffer solution at a flow rate of 2 mL min^{-1}. Once equilibrated, 1.5 mL of re-dissolved retentate was loaded into the column using a 5 mL disposable syringe connected to a Harvard Pump 11 Plus Single Syringe for sample infusion, which worked at a flow rate of 2 mL min^{-1}. After loading, the column was again connected to the chromatographic system for sample elution (pH 6.5 buffer solution at a flow rate of 2 mL min^{-1}). The excluded fraction was finally collected for further SEC-HPLC characterization. The

Hi Trap column was equilibrated with approximately 10 mL of the pH 6.5 buffer solution before desalting the following 1.5 mL retentate sample.

3.2.6. Seawater and permeate desalting procedure

A mixed exchange resin (AG 501-X8) was used for salt removal from seawater samples and for the permeate fractions. According with manufacturer's instructions, 5 g of fresh resin for every 100 mL of seawater sample/permeate were used (batch mode). The mixture was shaken for 1 hour to achieve deionization. Finally, the desalted seawater sample/permeate was separated from the resin by filtration.

3.2.7. TOC measurements

Concentrations of dissolved organic carbon in the untreated seawater, retentate and permeate were assessed as TOC by supercritical water oxidation (30 % (m/v) potassium peroxodisulphate in 6.0 M phosphoric acid), and CO_2 NDIR detection. The TOC analyzer was operated in the non-purgeable organic carbon (NPOC) measurement mode by mixing the acid (set at 1 %) and the oxidant (set at 15 %) with the sample in the sparger for 0.8 min (inorganic carbon removal). The solution is then pumped from the sparger by the peristaltic pump in the reactor module, where the mixture is subjected to conditions above the critical point of water (375°C, 22.1 Mpa) for organic carbon oxidation to CO_2, and subsequent NDIR detection. The analyzer was calibrated using standard solutions of potassium hydrogen phthalate ranging from 0.1 to 1000 mg L^{-1} (concentrations referred to carbon). Each sample (seawater, retentate and permeate), as well as different blanks, was analysed in quadruplicate. LOD (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) of the procedure was 0.13 mg L^{-1}; whereas, the LOQ (10 SD criterion) was 0.43 mg L^{-1}.

Table 1: Operating ICP–MS conditions.

General		
	Radiofrequency power / W	1400
	Sample uptake rate / r.p.m.	3.0
	Stabilization delay / s	35
	Number of replicates	3
Nebulizer type	Beat impact (cooled spray chamber)	
Gas flows / L min^{-1}	Plasma	13.0
	Auxiliary	0.80
	Nebulizer	0.90
Ion optics / V		
	Extraction	-125
	Lens 1	-1000
	Lens 2	-80
	Lens 3	-195.3
	Hexapole Bias	-4.0
	Pole Bias	0.2
	D1	-40.8
	D2	-140
	Hexapole Bias	-4.0
	Pole Bias	0.2
Torch alignment / mm		
	Horizontal	80
	Vertical	405
	Sampling death	150
Mass-to-ratio		
	^{51}V, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{58}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{77}Se, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{127}Al, ^{137}Ba	
	Internal standards	
	^{54}Sc, ^{72}Ge, ^{89}Y, ^{115}In	

3.2.8. ICP-MS measurements

Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Mo, Cd, Ba, and Pb were assessed in desalted seawater, permeate, retentate, and also in the SEC fractions ICP-MS under the operating conditions listed in Table 1. The standard addition technique (8.5 mL of desalted seawater, permeate or retentate diluted to 10 mL) ranging in metal concentrations from 0 to 200 µg L^{-1} for all elements except for Sr (within the 0 - 20 µg L^{-1} range). A mixture of different internal standards (Sc, Ge, Y, and In) were added to the standards and samples at constant concentrations of 50 µg L^{-1} for Sc, 10 µg L^{-1} for Ge, and 5.0 µg L^{-1} for Y and In. Metals bound to marine DOM (SEC fraction) were assessed by using an aqueous calibration matched with the mobile phase (25 mM / 25 mM diammonium sulphate / diammonium hydrogen phosphate, pH 6.5). Similarly, trace metal concentrations ranged from 0 to 200 µg L^{-1}, except for Sr (from 0 to 20 µg L^{-1}). The SEC fractions (8.5 mL) were

diluted to 10 mL with the mobile phase, and the same internal standards (at the same concentrations listed above) were used for calibration. For all cases, analysis were performed in triplicate. Different blanks were also analyzed for contamination control. Table 2 lists the limits of detection (LODs) and quantification (LOQs) taking into account the pre-concentration factor of 200 when analyzing the retentate and 150 when assessing trace elements bound to SEC fractions.

Table 2: Limits of detection and quantification for the different studied samples.

	SEC procedure (ng L^{-1})[a]		Seawater and permeate fraction (µg L^{-1})		Retentate fraction (ng L^{-1})[b]	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Cu	1.3	4.5	0.20	0.67	1.0	3.4
Mn	0.38	1.3	0.057	0.19	0.29	0.95
Mo	0.30	1.0	0.045	0.15	0.23	0.75
Sr	0.13	0.45	0.019	0.067	0.095	0.34
Zn	6.9	23	1.04	3.45	5.2	17
As	0.73	2.7	0.11	0.40	0.55	2.0
Ba	0.13	0.45	0.020	0.067	0.10	0.34
Rb	0.067	0.22	0.010	0.033	0.05	0.17
V	0.02	0.067	0.0030	0.010	0.015	0.050

(a) Pre-concentration factor 150; (b) pre-concentration factor 200

3.2.9. SEC-HPLC-UV/ICP-MS measurements

SEC-HPLC separation (TSK-G4000 SWXL column, 30 cm × 8 mm i.d.) was performed under isocratic elution conditions (flow rate set at 1.0 mL min^{-1}) with a mobile phase consisting of a 25 mM / 25 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5. UV detection was performed at 205 nm, and the injection volume was set at 50 µL. Column calibration was performed with globular proteins of different molecular weight such as ribonuclease A (13.7 kDa), carbonic anhidrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), and aldolase (158 kDa). In addition, calibration was also obtained by using PEO/PEG standards with molecular weights within the 6.7 – 478 kDa range. When calibrating with globular proteins, the exclusion volume (V_0) was determined using blue dextran (2000 kDa) and the permeation volume (V_p) was fixed by injecting

vitamin B12 (1.9 kDa). When using PEO/PEG standards, V_0 and V_p were fixed with polymers of molecular weight of 1015 and 0.232 kDa, respectively. For ICP-MS detection (operating conditions in Table 3), the outlet of the chromatographic column was directly coupled to the nebulizer of the ICP-MS.

Table 3: SEC/AEC-UV/ICP-MS operating conditions.

SEC	TSK-G4000 SW_{XL} (30 cm × 8 mm I.D.) coupled to a TSK-gel SW glass guard column (10 cm × 8 mm I.D.)	
	Injection volume (µL)	50
	Column temperature (°C)	25
	Mobile phases flow rate /mL min^{-1}	1.00
Mobile phase	25mM/25mM ammonium sulphate/diammonium hydrogen phosphate,	pH 6.5
	UV detection (nm)	205
AEC	PRP-X100 (250 mm × 4.1 mm I.D.) to a PRP-X100 guard column (25 × 2.3 mm I.D.)	
	Injection volume (µL)	50
	Column temperature (°C)	25
	Mobile phases flow rate /mL min^{-1}	1.00
Mobile phase	25mM/25mM ammonium sulphate/diammonium hydrogen phosphate,	pH 6.5
	UV detection (nm)	205
ICP-MS		
	Radiofrequency power / W	1400
	Peristaltic pump speed / r.p.m.	2.5
Nebulizer type	Beat impact (cooled spray chamber)	
Gas flows / L min^{-1}		
	Plasma	13.0
	Auxiliary	0.8
	Nebulizer	0.90
Ion optics / V		
	Extraction	-125
	Lens 1	-1000
	Lens 2	-80
	Lens 3	-195.3
	Focus	11
	D1	-40.8
	D2	-140
	Pole Bias	0.2
	Hexapole Bias	-4
Torch alignment / mm		
	Horizontal	80
	Vertical	405
	Sampling death	150
Mass-to-ratio	^{52}Cr, ^{55}Mn, ^{57}Fe, ^{58}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{77}Se, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{127}Al, ^{137}Ba	

3.2.10. AEC-HPLC-UV/ICP-MS measurements

DOM fractions collected by SEC were then subjected to Anion Exchange Chromatography (AEC) with UV and ICP-MS detection. Separation was performed with a PRP-X100 column under isocratic conditions (25 mM / 25 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution, pH 6.5, as a mobile phase, flow rate of 1 mL min^{-1}). Since the composition of the mobile phase when using AEC was the same as that used for SEC, collected SEC fractions were neither pre-treated nor pre-concentrated before AEC. When using UV detection, absorbance was monitored at 205 nm. When coupling the AEC-HPLC with ICP-MS for metal detection, the operating ICP-MS conditions listed in Table 3 were used.

A diagram showing the complete procedure is provided in Figure 1.

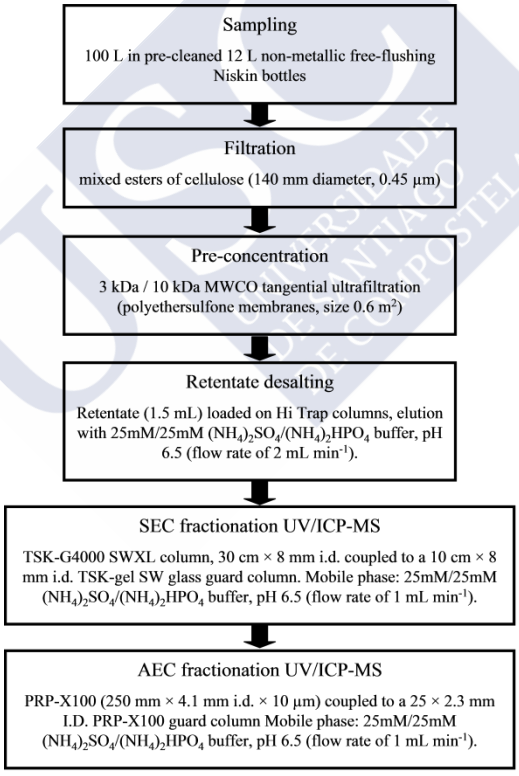

Figure 1: Sampling and analytical scheme used for marine DOM characterization.

3.3. Results and discussion

3.3.1. Marine DOM isolation by UF procedures

UF by using membranes with MWCO of 3 and 10 kDa was performed as described in section 3.2.4. Filtered seawater samples (100 L) were subjected to the UF procedure until obtaining a volume of retentate of approximately 0.5 L. The procedure implied ultrafiltration times of 2 hours when using the membrane of 10 kDa MWCO; whereas, a time of approximately 12 hours was required when performing UF with the membrane of 3 kDa MWCO.

The total organic carbon (TOC) concentration was previously assessed in the unultrafiltrated seawater sample, and also in the permeate fraction (99.5 L), which contains marine DOM of molecular weight lower than 3 or 10 kDa; and in the retentate fraction (0.5 L), which contains marine DOM of molecular weight higher than 3 or 10 kDa. A TOC value of 0.84 ± 0.08 mg L^{-1} was obtained in the unultrafiltrated seawater sample (five replicates). Regarding UF with 3 kDa MWCO membranes, TOC concentration in the retentate (preconcentration factor of 200) was 0.17 ± 0.02 mg L^{-1}; whereas, TOC concentration in the permeate fraction was 0.65 ± 0.03 mg L^{-1}. TOC concentrations as a sum of TOC concentrations in the permeate and in the retentate (0.82 ± 0.04 mg L^{-1}) implies a marine DOM retention on the 3 kDa MWCO membrane of 2.4 ± 0.26 %. Similarly, the retentate and permeate fractions after UF with 10 kDa MWCO membrane showed TOC values of 0.004 ± 0.0003 mg L-1 and 0.80 ± 0.06 mg L^{-1}, respectively. A mass balance study shows a TOC value of 0.804 ± 0.060 mg L^{-1} as a sum of TOC concentrations in the permeate and retentate fractions, which implies marine DOM losses by adsorption onto the UF membrane accounting for 4.3 ± 0.52 %. Therefore, similar marine DOM losses onto the UF membranes are observed when using either membranes of low and high MWCO. Losses by adsorption onto the UF membrane of 5 % have been reported by Schmitt et al. [26] when assessing NOM. However, marine DOM retention onto the UF membranes are also probably dependent on the nature of the dissolved DOM because other authors have reported marine DOM losses

within the 11 – 16 % range for seawater UF with a 10 kDa MWCO membrane [14,15]. In addition, higher loss rates have been reported when combining a diafiltration stage before UF for salt removal. Hertkorn et al. [21] have therefore reported marine DOM losses within the 20 – 26 % range when using 1 kDa MWCO UF membranes. Similarly, losses close to 50 % have also been observed by Aluwihare et al. [24].

3.3.2. Separation of the preconcentrate marine DOM by size exclusion chromatography

As previously mentioned, large DOM losses by retention onto the UF membranes are observed when using the tangential UF system for diafiltrating the retentate. Low DOM losses and efficient salt removal can be obtained using preparative HI Trap columns for retentate desalting (section 3.2.5) [14]. The delivering solution used for desalting consists of a 25 mM / 25 mM ammonium sulphate / diammonium hydrogen phosphate buffer solution at pH 6.5, which is the solution chosen as a mobile phase for the TSK4000SW$_{XL}$ SEC. Therefore, desalted retentates (UF with 3 and 10 kDa MWCO membranes) was characterized by SEC with UV detection under operating conditions listed in Table 3.

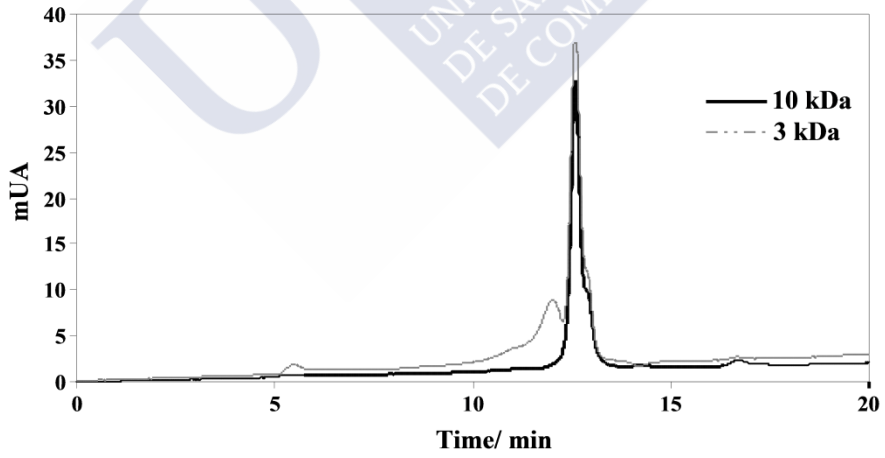

Figure 2: SEC chromatograms (UV detection at 205 nm) of marine DOM isolated by tangential UF with 3 and 10 kDa MWCO membranes.

Figure 2 shows typical chromatograms for retentates obtained after UF with 3 kDa (dashed line) and 10 kDa (bold line) MWCO membranes. In both cases an important peak was observed at a retention time of 12.6 min; whereas, elution of pre-concentrate marine DOM by 3 kDa UF membrane shows other small chromatographic peaks at a shorter retention time (12 min). As previously reported for SEC experiments when analysing natural DOM [26] and marine DOM [14], sharp peaks (retention time of 12 min for UF preconcentration with 3 kDa MWCO membranes) can be attributed to a salt boundary peak produced by a gradient in the ionic strength between the sample and the mobile phase.

To obtain the molecular weight of the isolated fractions, the TSK4000SW$_{XL}$ column was calibrated using two different sets of standards: a mixture of polyethylene oxides (PEOs) and polyethylene glycols (PEGs) standards (molecular weights within the 6.7 - 478 kDa range), and a mixture of globular proteins (ribonuclease A, 13.7 kDa; carbonic anhidrase, 29 kDa; ovalbumine, 43 kDa; conalbumine, 75 kDa; and aldolase, 158 kDa). In the first case, the calibration equation obtained was Log MW = - 0.302 t_r + 7.5679 (R^2 = 0.9845). The exclusion volume (V_0) and the permeation volume (V_p) were assessed using two polymers with molecular weights of 1015 and 0.232 kDa, respectively. Assessed V_0 was 6.2 mL (retention time of 6.2 min), while V_p was 12.7 mL (retention time of 12.7 min). Regarding calibration with globular proteins, the equation was Log MW = -0.6317 t_r + 11.725 (R^2 = 0.9601). In this case V_0 was determined by injecting blue dextran (2000 kDa), and V0 was fixed at 6.2 mL (retention time of 6.2 min). Similarly, V_p was fixed by using vitamin B12 as a standard (1.9 kDa) at a volume of 12.7 mL (retention time of 12.7 min). As shown, part of the isolated marine DOM contained in the large fraction (retention time of 12.6 min), obtained for retentate isolation by both 3 and 10 kDa UF membranes) fell out of the range of molecular weights fixed by either vitamin B12 or PEO of 0.232 kDa (12.7 min). Similarly, the small chromatographic signal at a retention time of 6.0 min (3 kDa UF membrane) is out of the exclusion volume of the column (6.2 min). However, it must be pointed out that conclusions from SEC experiments can only be used to determine molecular weight distribution

profiles rather than the exact molecular weight of the substances [27]. Table 4 lists therefore the apparent molecular weights obtained for the different SEC fractions when analyzing the 3 and 10 kDa retentates. It can be observed that the molecular weight range obtained is quite different when using the PEOs/PEGs or the globular proteins standard sets for column calibration. However, molecular weights of the isolated compounds obtained when using globular proteins as standards for SEC calibration (within the 1.6 – 16.1 kDa and 1.2 – 21.6 kDa ranges when using 10 and 3 kDa MWCO membranes for UF, respectively) are more realistic than those offered when calibrating with PEOs/PEGs mixtures (molecular weights lower than 10 kDa for all cases).

Table 4: Molecular weights obtained for the chromatographic peaks separated by SEC for the retentate fractions after UF with 3 kDa (R3) and 10 kDa (R10) MWCO membranes.

		t_R (min)	PEGs (kDa)	Globular Proteins (kDa)
R3	FI	11.7-12.3	10.8-7.1	21.6-9.1
	FII	12.3-13.7	7.1-2.7	9.1-1.2
R10		11.9-13.5	9.4-3.1	16.1-1.6

3.3.3. Second dimension anion exchange chromatography

AEC with UV detection (25 mM / 25mM diammonium hydrogenphosphate / diammonium sulphate, pH 6.5; 1 mL min^{-1} flow rate) was performed by injecting the isolated SEC fractions after UF with 3 and 10 kDa MWCO membranes. Regarding SEC fractions obtained when pre-concentrating with 3 kDa MWCO membranes, both fractions (eluted compounds from 11.7 to 12.3 min, and from 12.3 to 13.7 min) were chromatographed. Chromatograms in Figure 3(a) show the same AEC pattern for both SEC peaks (a large chromatographic peak at 4.5 min, and small signals at 3.5 and 9.0 min). This fact suggests that the two chromatographic SEC signals observed in Figure 2 can be attributed to the same group of organic substances, and as previously reported [26], the sharp peak (retention time of

12 min) can be therefore attributed to the ionic strength gradient between the sample and the mobile phase. AEC analysis of the SEC fraction obtained when using UF membranes of 10 kDa MWCO (Figure 3(b)) shows two different chromatographic signals at retention times of 4.5 and 13 min, and small chromatographic peaks at 2.5 and 3.5 min.

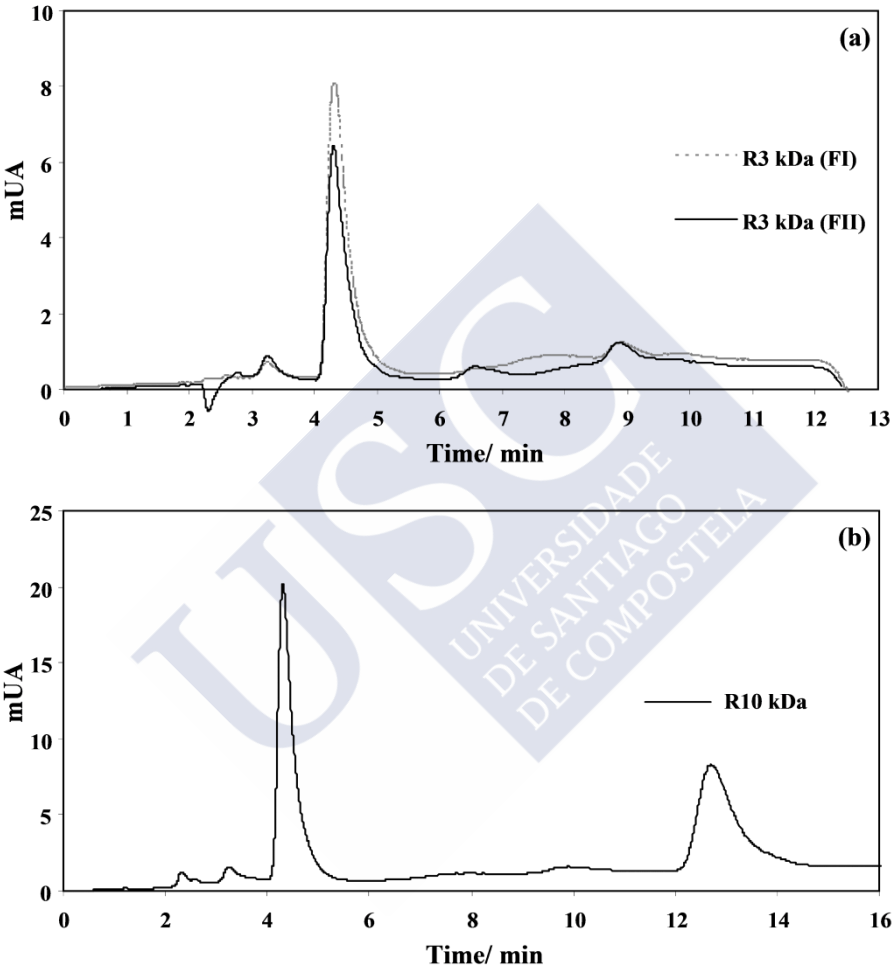

Figure 3: AEC chromatograms (UV detection at 205 nm) of SEC fractions isolated by tangential UF with 3 (a) and 10 kDa (b) MWCO membranes.

3.3.4. Size exclusion chromatography / anion exchange chromatography – inductively coupled plasma – mass spectrometry

Trace metal patterns (Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Mo, Cd, Ba, and Pb) were obtained after preparative SEC, and also after AEC of the SEC fractions, by coupling both chromatographic modes with ICP-MS. Figure 4 shows the SEC-ICP-MS chromatograms where the maximum m/z signals were found to be close to the maximum of the UV signals for trace elements such as Ni, Zn, Mn and Sr when isolating marine DOM by UF with 3 and 10 kDa MWCO membranes. Significant chromatographic signals can also be observed for Cu and Mo for SEC of the isolated material by 3 kDa MWCO UF; whereas, the signals are less intense when analyzing marine DOM after UF with 10 kDa MWCO membranes. Finally, small signals were obtained when monitoring Co, and no significant differences in the elution volumes of trace elements such as Al, V, Fe, As Cd, Ba and Pb were observed.

AEC and ICP-MS coupling shows negligible signals for all trace metals found to be associated to SEC fractions, except for Sr (Figure 5); although no specific coelution with marine DOM monitored with UV detection was observed. As shown in Figure 5, broad Sr signals encompass the small AE chromatographic peaks at 2.5 and 3.5 min, and part of the large AE signal at 4.5 min. These findings agree with data previously reported for Ni in natural DOM [27], and for Ni and Cd in marine DOM [14].

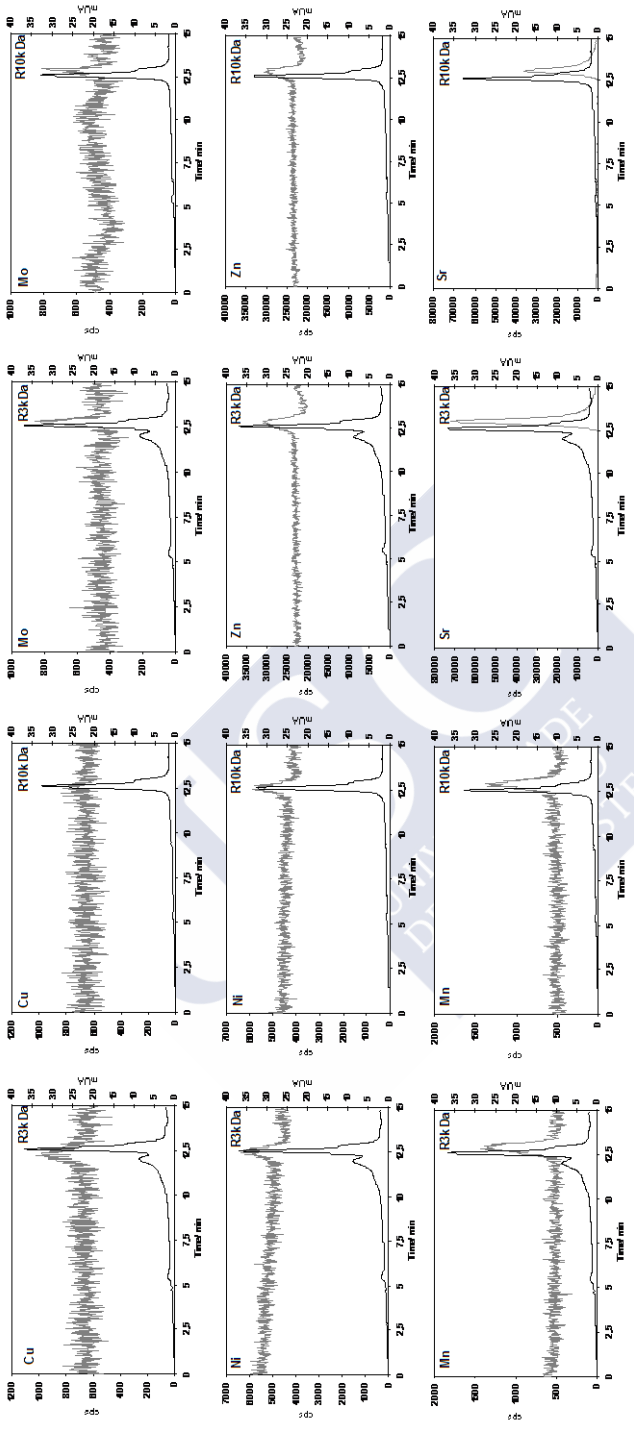

Figure 4: SEC chromatograms (UV and ICP-MS detection) of marine DOM isolated by tangential UF with 3 (R3 kDa) and 10 kDa (R10 kDa) MWCO membranes.

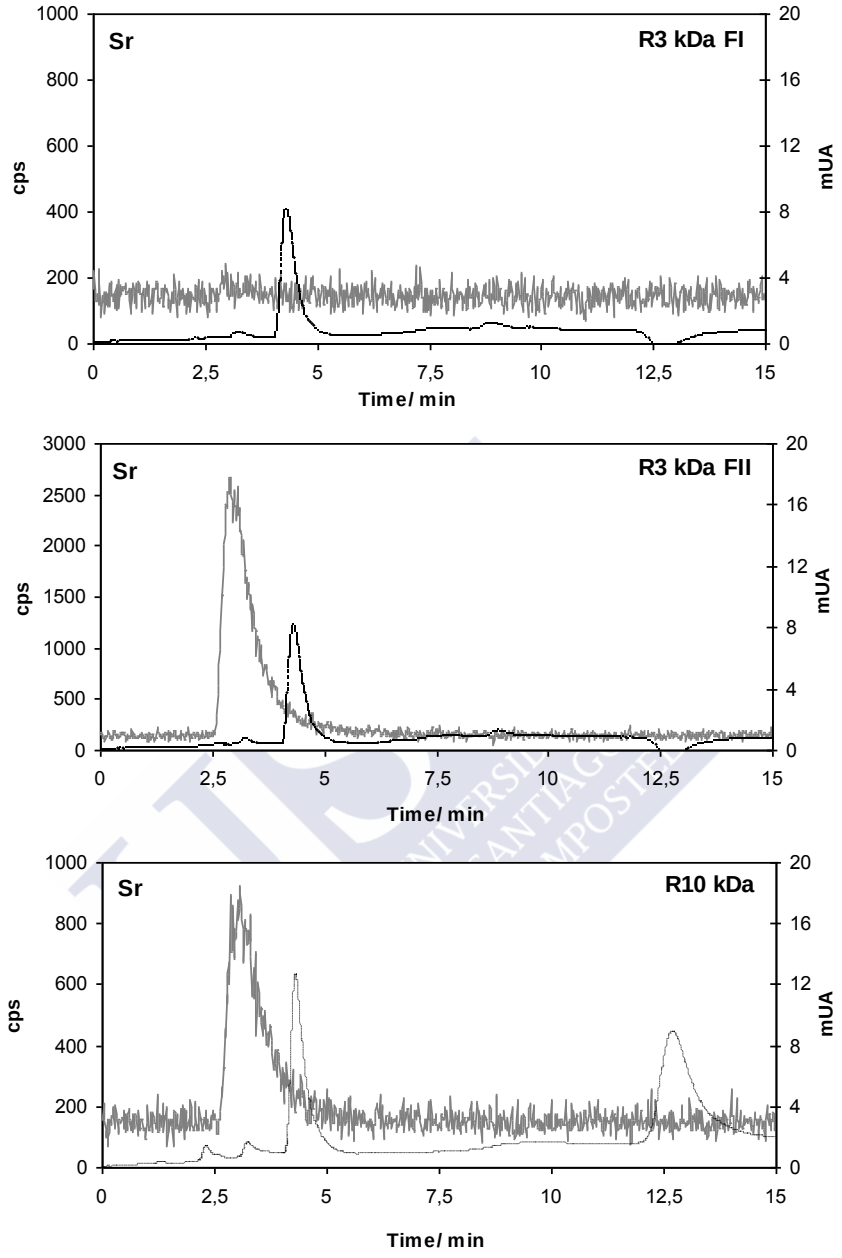

Figure 5: AEC chromatograms (UV and ICP-MS detection for Sr) of marine DOM fractionated by SEC and isolated by tangential UF with 3 (R3 FI and R3 FII) and 10 kDa (R10) MWCO membranes.

3.3.5. Mass balance studies for trace elements bound to marine DOM

A mass balance study was performed for those trace elements found to be associated to marine DOM after SEC (Cu, Mn, Mo, Sr, and Zn), and also for As, Ba, Rb and V. First, total concentrations of trace elements were determined in the seawater sample and also in the permeate and retentate fractions after the AG-501-X8 desalting procedure (section 3.2.6). The retentate fraction was also desalted by using HI-Trap desalting as described in section 3.2.5. Measurements were performed by using the standard addition technique plus the addition of a mixture of internal standards according to section 3.2.8.

Selected trace elements were also measured in the SEC fractions (from 11.7 to 13.7 min, and from 11.9 to 13.5 min for desalted retentates, by UF with 3 and 10 kDa MWCO membranes), after combining collected fractions from four runs. This implies eluted volumes of 2 and 1.6 mL (3 and 10 kDa MWCO membranes, respectively) for each run, and total collected volumes of 8 and 6.4 mL for 3 and 10 kDa MWCO membranes, respectively. Direct ICP-MS analysis of the combined SEC fractions was performed after dilution to 10 mL with the mobile phase and the addition of internal standards as shown in section 3.2.8. For all cases, analysis was performed in triplicate and also different blanks were analyzed for contamination control.

Table 5 lists the found metal concentrations in the desalted surface seawater, and in the desalted permeate and retentate fractions after both UF procedures (3 and 10 kDa MWCO membranes). Regarding metal concentrations in the retentate fraction, both desalting procedures (AG 501-X8 resin and HI Trap) offer similar results for Zn and Ba. However, higher values were found in the desalted retentate with AG 501-X8 resin for the remaining elements. This can be attributed to the fact that the mixed exchange resin only retains monovalent ions, while monovalent and divalent ions are separated by size exclusion when performing HI Trap desalting. It must be pointed out that the use of the mixed exchange resin for retentate

desalting before SEC characterization was not performed due to organic matter contamination from the resin.

A mass balance study was therefore established by assessing trace elements in the surface seawater and in the retentate and permeate fractions after AG 501-X8 resin desalting. Percentages were calculated as a sum of the metal concentration in the permeate and the metal concentration in the retentate with respect to the metal concentration found in the untreated seawater sample. It can be seen that percentages vary from 34 % for Mn to 95 % for Sr and Rb when performing UF with membranes of 3 kDa MWCO; whereas, percentages vary within the 36 – 105 % range after UF with membranes of 10 kDa MWCO. This implies quantitative mass balances for some elements such Mo, Sr, Ba and Rb. It can also be observed that recovered percentages are quite similar after both UF procedures for all studied elements except for Zn when using the 10 kDa MWCO membrane, or for Mn when using the 3 kDa MWCO membrane. Conclusions could not be made for other trace elements such as Al, Fe and Ni due to the existence of contamination throughout the procedure. The source of this contamination can be attributed to the UF membranes because high Al, Fe and Ni concentrations were found in the retentate and permeate fractions when using both 3 and 10 kDa MWCO membranes. Other trace elements such as Cd, Cr, Pb, Sb, Se and Sn were found at concentrations lower than the LODs of the method.

Table 6 lists the metal concentrations found in the SEC fractions as well as the percentages of metals bound to marine DOM, taking into account the total concentrations of the studied metals in the desalted surface seawater. Similar values were found after UF with both membranes. Regarding 3 kDa MWCO membranes, percentages account for 0.022 and 0.057 % for Mn and Zn, respectively, and lower values were found for the remaining elements associated to the isolated marine DOM ($2.0\ 10^{-3}$, $3.2\ 10^{-3}$ and $4.1\ 10^{-4}$ % for Cu, Mo, and Sr, respectively). UF isolation with 10 kDa MWCO membranes gave percentages of 0.019 % for Mn, and 0.077 % for Zn; whereas, Mo and Sr percentages were $1.8\ 10^{-3}$ and $1.4\ 10^{-4}$ %, respectively.

Table 5: Metal concentrations (n = 3) in surface seawater and the permeate fraction after AG501-X8 resin desalting, and in the retentate fractions after AG501-X8 resin and HI Trap desalting procedures.

	Concentration (µg L^{-1})								
		UF 3 kDa MWCO			UF 10 kDa MWCO				
		Permeate fraction[a]	Retentate fraction[b]		Permeate fraction[a]	Retentate fraction[b]		Percentage (%)[c]	
	Surface seawater		AG 501-X8 exchange resin	HI Trap desalting		AG 501-X8 exchange resin	HI Trap desalting	3 kDa MWCO	10 kDa MWCO
Cu	239±6	148±4	1.3±0.2	0.07±0.002	134±6	1.2±0.09	48±0.4[d]	53±2	48±1
Mn	1.6±0.2	0.52±0.02	4.4±0.8[d]	19±0.1[d]	0.77±0.12	10±0.9[d]	4.2±0.1[d]	34±2	50±3
Mo	13±0.2	12±0.1	71±1[d]	0.67±0.2[d]	13±0.2	59±0.8[d]	2.9±0.01[d]	93±1	101±2
Sr	9261±44	8707±91	52±0.5	2.7±0.01	9535±79	44±0.2	1.4±0.01	95±3	103±1
Zn	102±4	62±7	0.26±0.02	0.40±0.004	36±1	0.42±0.02	0.23±0.004	61±2	36±2
As	3.9±0.1	2.43±0.17	21±1[d]	7.4±0.4[d]	2.99±0.30	18±1[d]	6.0±0.3[d]	64±3	78±3
Ba	4.2±0.06	3.7±0.27	22±2[d]	37±1[d]	4.1±0.21	19±1[d]	33±1[d]	88±4	96±4
Rb	103±1	97±2	0.55±0.004	6.8±0.05[d]	108±2	0.49±0.01	3.4±0.02[d]	95±2	105±3
V	5.7±0.3	4.1±0.03	27±2[d]	1.1±0.1[d]	4.6±0.3	26±2[d]	1.0±0.1[d]	72±3	81±2

(a) dilution factor of 0.995; (b) pre-concentration factor of 200; (c) percentage calculated as the sum of metal concentrations in the permeate and retentate fractions (AG 501-X8 desalting procedure) with respect to the metal concentration in the untreated seawater; (d) expressed in ng L^{-1}

Table 6: Metal concentrations (n = 3) in surface seawater and the permeate fraction after AG501-X8 resin desalting, and in the retentate fractions after AG501-X8 resin and HI Trap desalting procedures.

		SEC fraction (ng L^{-1})[a]		Percentage of metal bound to marine DOM (%)[b]	
	Surface seawater (µg L^{-1})	UF 3 kDa MWCO	UF 10 kDa MWCO	UF 3 kDa MWCO	UF 10 kDa MWCO
Cu	239±6	5.8±0.1	< 1.3	$2.0\ 10^{-3}$	----
Mn	1.6±0.2	0.35±0.01	0.3±0.01	0.022	0.019
Mo	13±0.2	0.42±0.03	3.1±0.1	$3.2\ 10^{-3}$	$1.8\ 10^{-3}$
Sr	9261±44	38±0.1	13±0.1	$4.1\ 10^{-4}$	$1.4\ 10^{-4}$
Zn	102±4	57±4	79±1	0.057	0.077

(a) pre-concentration factor of 200; (b) percentage calculated as the metal concentrations in SEC fraction after desalting the retentate by the HI Trap desalting procedure respect to the metal concentration in the untreated seawater

3.4. Conclusions

Studies regarding the use of UF membranes of 3 and 10 kDa MWCO during marine DOM pre-concentration showed that both membranes offer a similar behavior in terms of marine DOM losses by adsorption onto the membranes (2.4 and 4.3 % for 3 and 10 kDa MWCO membranes, respectively), and also for trace elements bound to the isolated marine DOM. Mass balance studies for trace elements such as Mo, Sr, Ba and Rb have revealed recoveries close to 100 % when using either UF membranes; whereas, recoveries within the 34 – 61 % range were found for Cu, Mn and Zn. Results from SEC-UV experiments showed a group of organic substances comprising a variety of molecular weights ranging from 16.1 to 1.6 kDa (isolated material with 10 kDa MWCO membranes), and from 21.6 to 1.2 kDa (3 kDa MWCO membranes). Direct SEC-ICP-MS hyphenation showed that metals such as Mn, Mo, Sr and Zn form complexes with the isolated marine DOM. However, the presence of Cu-DOM complexes was only verified when ultrafiltrating with 3 kDa MWCO membranes. Percentages of metals bound to marine DOM were low, and they ranged from 4.1 10^{-4} and 1.4 10^{-4} % for Sr to 0.055 and 0.77 % for Zn. A further SEC fractions characterization by AEC-UV showed two main groups of organic substances for the isolated material with 3 kDa MWCO membranes. In contrast, organic substances contained in the SEC fraction after UF with 10 kDa MWCO membranes were separated in four different groups of substances after AEC. Dilutions made when coupling AEC with ICP-MS only allowed us to observe the presence of Sr. However, no specific co-elution with marine DOM monitored with UV detection was observed.

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4. Two-dimensional isoelectric focusing OFFGEL and microfluidic lab-on-chip electrophoresis for assessing dissolved proteins in seawater

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Two-dimensional isoelectric focusing OFFGEL and microfluidic lab-on-chip electrophoresis for assessing dissolved proteins in seawater

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Abstract: *Dissolved proteins were assessed in surface and deep seawater by two-dimensional isoelectric focusing (IEF) OFFGEL – lab-on-chip (LOC) electrophoresis after tangential flow ultrafiltration followed by centrifugal ultrafiltration (pre-concentration factor of 3000). Dissolved proteins isolation was performed by treating the ultrafiltrated retentate with cold acetone and also with chloroform as precipitating reagents. The best electrophoretic behaviour of the isolated proteins was obtained after protein precipitation with chloroform before different rinsing stages for removing methanol and water interferences. Metals bound to proteins in the different OFFGEL fractions were assessed by inductively coupled plasma – optical emission spectrometry (ICP-OES) and electrothermal atomic absorption spectrometry (ETAAS) under optimized operating conditions. Experiments regarding stability of the metal-binding proteins (superoxide dismutase, SOD, and alcohol dehydrogenase, ADH, as protein models) showed the integrity of the Zn-binding SOD / ADH under the OFFGEL electrophoretic conditions. However, stability of Cu bound to SOD is not guaranteed. The first electrophoretic dimension (IEF OFFGEL) showed that dissolved proteins in surface seawater exhibit alkaline isoelectric points (pIs of 8.10 and 8.37), and also acid Ips (4.82, 5.13, 5.43, and 5.73), while LOC showed that the isolated proteins exhibit a spread molecular weight range (within 15 – 63 kDa); although, high molecular weights were the most commonly found. Regarding deep seawater, isolated proteins were of acid Ips (from 3.30 to 4.22) and low molecular weight (within the 21 – 24 kDa range). Elements such as Cd, Cu, Mn and Ni were mainly associated to dissolved proteins of alkaline pIs in surface seawater, while Zn is mainly associated to proteins of acid pIs. However, only Cu and Mn were found to be bound to dissolved proteins of higher pIs in deep seawater, and the amount of Mn (from 68 to 84 µg L^{-1}) was higher than that found in dissolved proteins in surface seawater (22.4 µg L^{-1}).*

Keywords: *Dissolved proteins, seawater, tangential flow ultrafiltration, OFFGEL electrophoresis, microfluidic lab-on chip electrophoresis.*

4.1. Introduction

The global carbon cycle is affected by the dissolved inorganic carbon (DIC) fraction and also, by the fraction of dissolved organic carbon [DOC, or dissolved organic matter (DOM)] [1]. DIC is the largest and best understood group of reactive carbon in the oceans [2]. Nevertheless, little is known about DOM, mainly because this fraction comprises a large group of coarsely identifiable substances which exhibit very different molecular weights [1,2]. Complex macromolecular compounds from biological and chemical degradation processes from biota are included in this latter fraction, which also encompass large bio-molecules and degraded and reworked products derived from them [3]. Sugars, lipids, amino acids and proteins are biochemical substances identified in seawater, and their presence is attributed to marine algae metabolism in the euphotic zone, as well as to microbial exudation and cellular lysis [2]. This class of substances, mostly found in surface seawater, show a labile nature which explains the important role of these reactive substances in influencing toxicity and bioavailability of nutrients and toxic contaminants [4,5]. However, some of these marine-derived polymeric materials (such as dissolved proteins) in deep seawater constitute the largest DOM biorefractory fraction, as explained by Powell et al. [6] on the basis of two theories (physical protection and selective preservation), which were previously proposed by Hedges et al. [7]. Dissolved proteins affect therefore the global carbon cycle through the long-term preservation of carbon and nitrogen in the ocean [8]. In addition, dissolved proteins have also been found to persist as discrete units in the environment [6,8] and are probably the most characterizable component of refractory marine DOM at the molecular level [6].

Although there is some literature regarding marine DOM in surface and deep waters, as reviewed by Mecozzi et al. [3] and by Mopper et al. [4] data concerning dissolved proteins are scarce. Pioneering works were developed by Tanoue who demonstrated the occurrence of proteins associated to the particulate organic matter (POM) fraction from intermediate and deep waters [9-11] and also as discrete dissolved units in oceanic waters [11,12]. Further studies suggested that

bacterial porins (β barrel proteins) [13] and other major outer membrane proteins, such as OmpA-like proteins, of most pathogenic gram-negative bacteria [14] are the major source of dissolved proteins in seawater. Recent literature encompass both particulate [15] and dissolved proteins [6,16-18], as well as degradation products (peptides) dissolved in deep waters [19] or associated to POM.

As in the case of other types of dissolved marine DOM, the assessment of dissolved proteins in seawater is difficult due to their inherent low concentration and the presence of large amount of inorganic salts. Tangential flow ultrafiltration (UF) rather than solid phase extraction (SPE) techniques are preferable when isolating marine DOM of high molecular weight [4,21,22], resulting in a more appealing approach when dealing with dissolved proteins [8]. It must be pointed out that losses of marine DOM by adsorption onto the UF membranes within the 11 – 16% range commonly occur [22,23], and as reported by Powell and Timperman [16], this phenomena is especially important for dissolved proteins because of their highly adsorptive nature. Nevertheless, all the recent developments for dissolved proteins assessment are based on UF as a sample pre-treatment [6,11,14,16-19]

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [9-14,16] and a two-dimensional electrophoresis (2-DE) [15,17,18] have been used for characterizing dissolved proteins. In general, 2D gels methods are time-consuming and require tedious procedures, and other 2D approaches for assessing proteins have been developed. With Regard to marine proteomics, Powell et al. [6] have therefore applied a 2D (SCX and RPC) high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) approach for dissolved proteins in seawater. Additionally, two-dimensional approaches based on the combination of IPG IEF (first dimensional separation according to the proteins' pIs) and RPC (second dimensional separation according to the proteins' molecular weights) have also been developed [24,25]. Recently, the introduction of preparative IPG IEF where proteins can be recovered from liquid phase (OFFGEL electrophoresis) avoids drawbacks associated with IPG gels, allowing the direct

sampling of the isolated protein (1D separation) into HPLC instruments [26]. Further developments are focused on coupling OFFGEL electrophoresis to microfluidic chip-based liquid chromatography [27], on increasing protein recoveries by cup loading instead of conventional in-tray rehydration [28], and on using 48-wells setup instead of 24-wells setup for improving the separation of proteins with small pI differences [28].

Although the high degree of automation of OFFGEL-lab on chip (LOC) electrophoresis and the avoidance of gel cutting and further protein extraction and cleaning up stages when performing 2D approaches, some limitations must be reported. First, once the OFFGEL fractions are loaded into the chips, they can not be recovered, which limits further analysis for protein identification and for elucidating metals bound to each separated protein. In these cases 2--DE are more advantageous because direct MS/MS protein identification after tryptic digestion of in-gel proteins can be performed, as well as metals can be assessed by direct laser ablation (LA)-ICP-MS analysis of the dehydrated gels. In addition, the low volume of the OFFGEL fractions, and also the high salt content, can lead problems when combining OFFGEL electrophoresis with chromatographic methods.

As previously reported, natural organic matter (NOM) plays a crucial role on the adsorption behaviour and transport of trace metals in surface and deep water, as well as in soils and sediments, which conditions metals toxicity / bioavailability towards micro- and macro-organisms [29,30]. Several studies have therefore been performed to understand and characterize its interactions with metals in fresh waters [29,31,32], and recent papers have also shown the association of bioactive elements (Cu, Fe and Zn) [22,33] and other trace elements such as Ba, Co, Mn, and Sr [22] with marine DOM. As dissolved proteins are an important fraction of DOM, the characterization of metal-dissolved proteins complexes in marine ecosystems is a topic of interest.

One of the aims of the current work has been the development and application of an IPG IEF OFFGEL electrophoresis method followed by microfluidic lab-on-chip (LOC) electrophoresis for assessing dissolved proteins in surface and deep seawater. Because proteins are recovered from liquid phase after IPG IEF OFFGEL electrophoresis, microfluidic devices can be used for a direct sampling of the isolated proteins (first dimensional separation). As shown by recent reviews [34-37], LOC electrophoresis has gained popularity, driven by the increased need to create portable, fast, and low analyte consumption devices [35]. Therefore, the improved sensitivity of this 2D technique in addition to the minimal sample consumption and the reduction of the number of handling steps inherent to conventional SDS-PAGE, make this combination an appealing approach for the assessment of low abundance proteins, such as dissolved proteins. In addition, since the electrophoretic operating parameters mainly condition the sample pre-treatment, different strategies were tested and optimized to achieve an adequate protein pellet before two-dimensional IEF OFFGEL-LOC electrophoresis analysis. Finally, an additional goal of the current work has been the identification and quantification of trace metals bound to dissolved proteins. To the best of our knowledge, these two latter objectives have not yet been addressed.

4.2. Experimental

4.2.1. Apparatus

Protein pI-based fractionation with liquid-phase recovery in 24-well (first-dimensional separation) was achieved by using the Agilent 3100 OFFGEL fractionator (Agilent Technologies, Santa Clara, CA, USA). 2D electrophoresis was performed by using the Agilent 2100 Bioanalyzer (Agilent Technologies) with electrode cartridge for on-chip electrophoresis and fluorescence detection. A Perkin Elmer Model 1100B (Perkin Elmer, Norwalk, CT, USA) atomic absorption spectrometer equipped with an HGA-700 graphite furnace atomizer, deuterium background correction and an AS-70 auto-sampler was used for determining cadmium, cobalt, chromium, copper, manganese, nickel and lead bound to

proteins of similar pIs (OFFGEL fractions). The sources of radiation were monoelement hollow cathode lamps (HCLs): chromium, copper, manganese and nickel HCLs were from Cathodeon (Cambridge, U.K), while cobalt and lead HCLs were LuminaTM type from Perkin Elmer. In all cases, HCLs were operated at the recommended manufacturer operating conditions. An Optima 3300 DV inductively coupled plasma (ICP) atomic emission spectrometer (Perkin Elmer) equipped with an autosampler AS 91 (Perkin Elmer) and a Gem-Cone cross-flow nebulizer type (Perkin Elmer) was used for iron and zinc determinations. The tangential flow ultrafiltration (UF) system consisted of a Masterflex I/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane (nominal MW cut-off 10 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge. Centrifugal ultrafiltration was performed with an Alresa Digtor centrifuge (Madrid, Spain). Other laboratory devices were an ultracentrifuge Laborzentrifugen model 2K15 (Sigma, Osterode, Germany), a Heidoph shaker type Reax 2000 (Gemini B.V., Apeldoorn, Netherlands), and an ORION 720A plus pH–meter with a glass–calomel electrode (ORION, Cambridge, UK).

4.2.2. Reagents and material

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore). IPG Dry Strips (pH 3 - 10), OFFGEL Buffer 3 - 10, and Plus One DryStrip cover fluid used for pI protein fractionation were supplied by GE Healthcare Life Science (Uppsala, Sweden). The Protein OFFGEL stock solution was prepared by dissolving 25.2 g of urea (electrophoresis grade, Sigma Aldrich, St. Louis, MO, USA), 9.1 g of thiourea (analytical grade, Sigma Aldrich), 600 mg of 1,4-dithiothreitol (DTT, electrophoresis grade, Merck, Darmstadt, Germany), 6 mL of glycerol solution (Merck), and 600 µL of OFFGEL Buffer (pH 3 - 10) in ultrapure water (final volume of 50 mL). This solution was used for proteins pellet re-dissolution, and it allowed an adequate isoelectric focusing for OFFGEL electrophoresis separation. Sizing and analysis of proteins by LOC electrophoresis was performed with the Agilent protein 80 Kit Agilent Technologies

(Waldbronn, Germany). This supply consists of: protein chips containing an interconnected set of gel filled microchannels that sieves proteins by size within the 5 – 80 kDa range (electrophoresis separation); Protein 80 Gel-Matrix and Protein 80 Dye Concentrate, which are used as a filling gel for chips; and Protein 80 Sample Buffer, which is used for preparing the denaturing solution. The kit also contains the Protein 80 Ladder, which consists of standard proteins with molecular weights of 6.5, 15, 28, 46 and 63 kDa (concentration of 600 ng μL^{-1} as a sum of all standard proteins) used for molecular weight assessment; and also, standard proteins coded as lower marker and upper marker, at concentrations of 1 ng μL^{-1} and 60 ng μL^{-1}, respectively, used for protein quantification. Centrifugal ultrafiltration was performed with Vivaspin 20 ultrafiltration tubes (polyethersulfone membrane of 10 kDa molecular cut-off) from Sartorius Stedin Biotech (Goettingen, Germany). Seawater filtration was achieved by using Millipore HAWP14250 0.45 µm mixed esters of cellulose membrane filters (140 mm diameter). Superoxide dismutase from bovine erythrocytes (SOD), and alcohol dehydrogenase from Saccharomyces cerevisiae (ADH) were from Sigma Aldrich, and they were used when studying metal-proteins stability during the OFFGEL electrophoresis procedure. Sodium azide (used for avoiding dissolved protein degradation), acetone, and methanol plus chloroform (used for obtaining protein pellet), were from Panreac (Barcelona, Spain); while 2-mercaptoethanol (BME), used when preparing the denaturing solution, was from Fluka (Vancouver, Canada). Sodium dodecyl sulphate, SDS, was used for avoiding protein adsorption onto the UF membranes, was purchased from AppliChem (Darmstadt, Germany). Other reagents were high purity 69 % nitric acid (Panreac), used for treating the OFFGEL fractions before ETAAS/ICP-OES measurements; sodium hydroxide (Merck), for UF membrane cleaning; and ammonium hydrogencarbonate (BDH, Poole, UK), for salt removal after retentate preparation by centrifugal UF. Element standard solutions (used for metal quantification) were prepared from cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, and zinc stock standard solutions (1.000 g L^{-1}) from Scharlau (Barcelona, Spain). Bovine serum albumin

(BSA) standard (2 mg mL^{-1}), from Thermo Scientific (Rockford, IL, USA), was used when performing analytical recovery studies.

4.2.3. Seawater sample collection

Three surface seawater samples (1 - 2 m depth, 60L), coded as SS1, SS2 and SS3; and one deep sea water sample (50 m depth, 60L), coded as DS4, were collected from the Ría de Arousa estuary (north-western Spain) in pre-cleaned 12 L non-metallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection, seawater samples were filtered (0.45 μm) and immediately subjected to the tangential flow ultrafiltration procedure.

4.2.4. Seawater tangential flow ultrafiltration

According to manufacturer's instructions, the ultrafiltration system was cleaned by passing 2 L of 0.1 M NaOH at 45 ± 5ºC (this solution was re-circulated for 60 minutes), and then by rinsing with 9 L of Milli-Q water, also at 45 ± 5 ºC. Operating conditions for the tangential ultrafiltration process were described elsewhere [22], although with slight modifications. Before ultrafiltration, filtered seawater samples (60 L) were treated with 19.5 g of sodium azide (concentration of 5.0 mM in the 60 L of seawater) and 6.0 g of SDS (concentration of 0.01 % (m/v) in the 60 L of seawater) [14,15,17-19] to prevent proteins adsorption onto the ultrafiltration membrane [16]. The preserved seawater sample was then concentrated through a polyethersulfone membrane (size 0.6 m^2, nominal molecular mass cut-off of 10 kDa) until obtaining a volume of retentate (ultrafiltrate containing substances of molecular weight higher than 10 kDa) within the 400 – 600 mL range.

4.2.5. Retentate centrifugal ultrafiltration

The retentate fraction (volumes ranging from 400 to 600 mL) was further ultrafiltrated by centrifugal ultrafiltration (ultrafiltration tubes with polyethersulfone membrane of 10 kDa molecular cut-off). Before use, polyethersulfone membranes were rinsed by loading 20 mL of ultrapure water and centrifuging at 4000 rpm for

10 min. This treatment allows the removal of trace amounts of glycerine adsorbed onto the polyethersulfone membrane. The centrifugal ultrafiltration procedure consisted of subjecting successive 20 mL aliquots of retentate at 4000 rpm for centrifugation times within the 10 – 30 min range. Because of the high volume of retentate (400 to 600 mL) four different centrifugation tubes were needed for treating each sample (retentate). Centrifugation was performed until obtaining a volume of 5 mL of retentate in each membrane tube. Each 5 mL retentate was finally rinsed by loading 15 mL of a cleaning solution containing 0.01 % (m/v) SDS and 35 mM ammonium hydrogencarbonate and centrifuging (4000 rpm) until obtaining 5 mL of clean retentate in each membrane tube. The four retained and clean 5 mL retentate aliquots were combined, and a retentate of 20 mL was finally obtained (pre-concentration factor of 3000).

4.2.6. Protein pellet precipitation

Twenty different aliquots (1 mL) from the whole retentate (20 mL) were mixed with methanol (4 mL), chloroform (1 mL) and water (3 mL), inserting a vortex mixing stage after adding each solvent. The mixtures were then centrifuged at 4000 rpm for 10 min, and the upper methanol-water layer was discarded. The chloroform layer containing proteins was then vortexed with 4 mL of methanol, and after centrifugation (4000 rpm, 10 min), the liquid phase was discarded, and the different precipitates were combined (protein pellet). Protein pellets were re-dissolved in 3 mL of Protein OFFGEL Stock solution (see section 4.2.2) plus 0.75 mL of ultrapure water before OFFGEL electrophoresis analysis.

4.2.7. Isoelectric focusing OFFGEL electrophoresis

IPG Dry Strips (24 cm pH 3 - 10) were first rehydrated for 15 min by loading in each 24 well 40 µL of OFFGEL Rehydration solution (prepared by mixing 960 µL of Protein OFFGEL Stock solution and 240 µL of ultrapure water). Isoelectric focusing electrophoresis separation was then performed by loading 150 µL aliquots of the proteins solution (protein pellet re-dissolved in Protein OFFGEL Stock solution – ultrapure water mixtures, as shown at section 4.2.6.) in each well.

Proteins separation according with their pIs was performed by focusing with a maximum current of 50 µA (200 mW) and typical voltages ranging from 500 to 4500 V (voltage to time ratio of 64 kV h^{-1}). Protein separation zones were maintained at a constant voltage of 500 V (20 µA and 50 mW).

4.2.8 Lab-on-chip electrophoresis

After pIs proteins separation (first dimension), the recovered fractions were further separated according to their molecular weights by Lab-on-a-chip (LOC) electrophoresis (second dimension). OFFGEL fractions (4 µL) were combined with 2 µL of the Denaturing solution (1 µL of BME plus 28.6 µL of Protein 80 Sample Buffer) in 0.5 mL Eppendorf tubes. The latter solution contains a lower (1.6 kDa) and an upper (95 kDa) marker, which allows the correct alignment of each lane. Sample tubes were then heated at 95ºC in a thermostated water bath for 5 minutes and after cool down, 84 µL of ultrapure water were added before vortexing. A similar procedure (6 µL of Protein 80 Ladder solution) was performed when preparing the ladder (mixture of proteins exhibiting different molecular weights). The standard protein ladder is loaded on each chip (commonly coded as “lane L”), and allows an estimation of the appropriate molecular weight of the separated proteins. This solution also contains the lower marker and the upper marker, proteins of known molecular weight at concentrations of 1 ng μL^{-1} and 60 ng μL^{-1}, respectively, used for proteins quantification. Before loading the samples (6 µL) and the ladder (6 µL) into the chips, they were filled with 12 µL of the Gel-Dye Mix (G/D) and Destaining solution (DS). The G/D and DS solutions were prepared following manufacturer’s recommendations: the DS solution consists of 650 µL of Protein 80 Gel-Matrix previously filtered and centrifuged; whereas, the G/D solution is a mixture of 650µL of Protein 80 Gel-Matrix (also filtered and centrifuged) with 25 µL of Protein 80 Dye concentrate. LOC electrophoresis (proteins detection by laser-induced fluorescence) was performed under recommended manufacturer’s conditions: the system automatically fixes parameters such as voltage (a value within the 50 – 1500 V range), current (within -20 – +20 µA range), and LED wavelength (a value from 458 to 482 nm) in accordance with the chip type (protein

80 kit in the current application). The Agilent 2100 Bioanalyzer software controls data collection, reporting and interpretation functions, and proteins quantification is performed taking into account the upper marker concentration of 60 ng μL^{-1}.

4.2.9. ETAAS measurements

Cadmium, cobalt, chromium, copper, manganese, nickel and lead were determined in the OFFGEL fractions containing proteins. These elements were assessed by ETAAS under optimized graphite furnace temperature programmes (Table 1). OFFGEL fractions (100 µL) were first made up to 500 µL with 2 % (v/v) nitric acid. ETAAS determinations involved a further dilution by mixing 50 µL of the acidified OFFGEL fraction, 25 µL of 500 mg L^{-1} palladium nitrate and 25 µL of 200 mg L^{-1} magnesium nitrate, as chemical modifiers, and 100 µL of ultrapure water. Different reagent blanks were also analysed, and negligible background signals were recorded. Determinations were performed by using aqueous standards matched with Protein 80 OFFGEL standard solution (100 µL) plus 2 % (v/v) nitric acid (400 µL). Calibrations for Co, Cr, Cu, Ni and Pb covered concentrations up till 20 µg L^{-1}, while calibrations were performed up till 10 µg L^{-1} and 4 µg L^{-1}, for Mn and Cd determinations, respectively. The LODs (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) referred to the OFFGEL fraction (1:5 dilution) and expressed as µg L^{-1} were 0.33, 2.9, 12, 2.6, 4.8, 11, and 12 µg L^{-1} for Cd, Co, Cr, Cu, Mn, Ni, and Pb, respectively. Similarly, the LOQs (10 SD criterion) were 1.1, 9.8, 41, 8.8, 16, 35, and 41 µg L^{-1} for Cd, Co, Cr, Cu, Mn, Ni, and Pb, respectively.

Table 1: ETAAS operating conditions and graphite furnace programs for the determination of Cd, Co, Cr, Cu, Mn, Ni, and Pb in acidified protein fractions (OFFGEL fractions) from seawater.

Step	Temperature (ºC)	Ramp time (s)	Hold Time (s)	Ar flow (mL min^{-1})
Drying 1	110	1	20	300
Drying 2	150	5	30	300
Pyrolisis	600 (Cd), 800 (Pb), 1400 (Cu, Mn), 1600 (Ni), 1500 (Co, Cr)	10	20	300
Atomization	1800 (Cd, Pb), 2000 (Mn), 2200 (Co, Cu), 2400 (Ni), 2500 (Cr)	0	2 (Cd), 5 (Co, Cr, Cu, Mn, Ni)	0 (READ)
Cleaning	2600	1	2	300
Wavelength / nm: 228.8 (Cd), 242.5 (Co), 357.9 (Cr), 324.8 (Cu), 249.5 (Mn), 232.0 (Ni), 283.3 (Pb).				
Slit width / nm: 0.7 (Cd, Cr, Cu, Pb), 0.2 (Co, Mn, Ni)				
Injection volume / µL: 20				
Deuterium background correction system: Cd, Co, Mn, Ni, Pb.				
Zeeman effect background correction system: Cr				
Tubes: Pyrolytic coated graphite tubes with L'vov platforms				
Integrated absorbance measurement				
Chemical modifier: $Mg(NO_3)_2$ / $Pd(NO_3)_2$ (25/62.5 mg L^{-1})				

4.2.10. ICP-OES measurements

Iron and zinc in the OFFGEL fractions from seawater, and also copper and zinc when performing metal-binding protein stability studies, were determined by ICP–OES (axial configuration) using the operating parameters listed in Table 2. Determinations were performed by using aqueous standards matched with Protein 80 OFFGEL standard solution (0.5 mL) made up to 5 mL with 1 % (v/v) nitric acid after adding the adequate standard volumes when analysing OFFGEL fractions from seawater. Calibrations covered Fe and Zn concentrations within the 0 - 2 mg L^{-1} range. Volumes of 200 µL of OFFGEL fractions were mixed with

1800 µL of 1 % (v/v) nitric acid, and directly nebulised in the ICP torch. Regarding metal-proteins stability studies, matched calibrations with Protein 80 OFFGEL standard solution (500 µL), variable volumes of standard solutions (covering concentrations from 0 to 2 mg L^{-1}) and dilution to 10 mL with 1 % (v/v) nitric acid. Metal-binding proteins were analysed after dilution of 100 µL of each OFFGEL fraction with 1900 µL of 1 % (v/v) nitric acid. The LODs (3 SD criterion) expressed as µg L^{-1} were 0.11, 0.006, and 0.015 mg L^{-1} for Fe, Cu and Zn, respectively. Similarly, the LOQs (10 SD criterion) were 0.37, 0.02, and 0.05 mg L^{-1} for Fe, Cu, and Zn, respectively.

Table 2: ICP–OES operating conditions for the determination of Cu, fe, and Zn in acidified protein fractions (OFFGEL fractions) from seawater.

General	Radiofrequency power / W	1300
	Sample uptake rate / mL min^{-1}	1.5
	Stabilization delay / s	45
	Number of replicates	4
	Integration time / s	5
	Nebulizer type	Cross flow
	Axial view	
Gas flows / L min^{-1}	Plasma	15.0
	Auxiliary	0.5
	Nebulizer	0.8
Detection wavelengths / nm	Cu	327.393
	Fe	238.205
	Zn	213.857

4.3. Results and discussion

4.3.1. Preliminary studies for dissolved marine protein precipitation

Dissolved proteins were isolated by tangential ultrafiltration (as described in section 4.2.4) followed by centrifugal ultrafiltration of the retentate fraction (section 4.2.5). A general protocol for protein precipitation based on ice-cold acetone (protein pellet formation at –20°C for 2 h) [38] was first applied (4 mL of precipitating solution for every 1 mL of retentate). Preliminary experiments by loading small amounts of the obtained protein pellet (commonly from 5 mL of ultrafiltrated retentate) directly re-dissolved with 3.8 mL of Protein OFFGEL stock solution, showed long separation times (approximately 72 h) for completing the IEF OFFGEL electrophoresis (first dimension). These problems can be attributed to the presence of other compounds in the re-dissolved protein pellet. This fact was confirmed when loading the whole re-dissolved pellet (from 20 mL of retentate), which led to problems for adjusting the electrical current, and the IEF OFFGEL electrophoresis could not be initialized.

To obtain a purified and SDS-free protein pellet an alternative method consisting of removing methanol and water soluble interferences was tested [6]. The method requires the addition of methanol, water and chloroform and the final protein precipitation in the separated chloroform layer (section 4.2.6). The protein pellet re-dissolved in the Protein OFFGEL stock solution led to free-interference IEF OFFGEL separation after 36 hours, even when obtaining the protein pellet from the whole retentate (20 mL).

4.3.2. Two-dimensional IEF OFFGEL-LOC electrophoresis

4.3.2.1. Molecular weight repeatability

The repeatability of the molecular weight assessment was studied by loading the ladder solution (containing standard proteins with molecular weights of 6.5, 15, 28, 46 and 63 kDa) eight times. After LOC electrophoresis, the calculated molecular

weights were 6.5 ± 0.1, 15 ± 0.1, 28 ± 0.2, 46 ± 0.2, and 63 ±0 .08, which offer RSD values of 2, 0.7, 0.6, 0.4, and 0.1 % for standard proteins of molecular weights of 6.5, 15, 28, 46 and 63 kDa, respectively. Good repeatability when assessing the molecular weights of the isolated proteins (OFFGEL fractions F7 to F10, F17 and F18 in surface seawater, and F2 to F5 in deep seawater) is therefore expected.

4.3.2.2. IEF OFFGEL-LOC analytical recovery

Because certified reference material for proteins, and especially for dissolved proteins, are not available, accuracy of the overall IEF OFFGEL-LOC electrophoresis method was assessed by the analytical recovery approach. Therefore, the IEF OFFGEL of an albumin aqueous standard solution (0.20 mg of albumin) was run twice. After LOC electrophoresis, albumin was indentified in the OFFGEL fraction 10 (pI of 5.73). Because the separation process was performed under reductive conditions (use of DTT) the molecular weight found for albumin (66.5 kDa) was shifted to 72.4 kDa. This fact can be attributed to disulphide bridges breakdown which leads to a different migration behavior [39]. By performing LOC electrophoresis in triplicate, a mean molecular weight of 72.4 ± 0.19 kDa (n=6) was measured for albumin; whereas, the albumin mass measured was 0.22 ± 0.011 mg (n=6), offering a mean analytical recovery of 109 ± 6% (n=6). Therefore, albumin is not partially retained onto the IPG dry strips and remains in the liquid fraction. Good analytical recovery can therefore be expected for dissolved proteins.

4.3.3. Metal binding proteins stability studies

Data reported by Jiménez et al. [40] show the influence of different PAGE conditions when assessing two metal-binding proteins with different metal–protein affinities such as superoxide dismutase, SOD (two units of molecular weight of 16.2 kDa each one, and presence of Cu and Zn); and alcohol dehydrogenase, ADH (tetramer of molecular weight of 141 kDa containing four equal subunits of 32.5 kDa, and presence of Zn).

To test the effect of the denaturing OFFGEL conditions on the stability of the metal-protein bindings, experiments by loading SOD and ADH aqueous solutions (2.8 and 3.1 mg, respectively, in 4.0 mL of Protein OFFGEL stock solution) were performed. Regarding SOD, LOC electrophoresis showed the presence of this protein in OFFGEL fractions 9 to 12 (pIs of 5.43, 5.73, 6.04, and 6.34), with a molecular weight of 18.6 kDa quite similar to the theoretical value (16.2 kDa). The determination of Zn in all 24 OFFGEL fractions proved the presence of Zn in the OFFGEL fractions containing SOD (fractions 9 to 12). The Zn concentrations in these four fractions were 6.9 ± 0.2, 26.6 ± 1.1, 3.0 ± 0.1 and 0.7 ± 0.1 mgL^{-1}, respectively, and negligible Zn concentrations were found in the remaining OFFGEL fractions. A mass balance study implying the Zn concentration in the SOD solution (2.2 ± 0.08 mg g^{-1}), and the Zn concentration as a sum of Zn concentrations in fractions 9 to 12 (2.4 ± 0.05 mg g^{-1}) shows a Zn percentage of 109 %. However, Cu was not found in the OFFGEL fractions containing SOD (fractions 9 to 12), but it was found in fractions 22 (11.4 ± 0.4mgL^{-1}) and 23 (6.10 ± 0.03 mgL^{-1}). Similarly, the Cu content in a SOD solution (2.3 ± 0.1 mg g^{-1}), the negligible Cu concentrations in the OFFGEL fractions 9 to 12 (presence of SOD), and the presence of Cu in fractions 22 and 23 (absence of SOD, 0.80 ± 0.02 and 0.40 ± 0.002 mg g^{-1}, respectively) suggest that Cu binding SOD is completely broken down under the used OFFGEL conditions. In addition, a Cu percentage of 53 % is found in fractions 22 plus 23, which means that the remaining Cu released from SOD must be spread among other OFFGEL fractions. Electropherograms when subjecting the OFFGEL fractions 22 and 23 to LOC electrophoresis showed a signal for low molecular weights (6.5 kDa). The proteinaceous and/or amphoteric nature of these compounds can be responsible for complexing the major amount of released Cu. In addition, the presence of Cu is not attributed to metal contamination because analysis of the OFFGEL stock standard solution and OFFGEL fractions from ultrapure water subjected to the whole process gave negligible metal concentrations.

OFFGEL results when analyzing ADH showed the presence of this protein in the fractions 9, 10, 11, 12, 13 and 14 (pIs of 5.43, 5.73, 6.04, 6.34, 6.74 and 7.1); whereas, LOC electrophoresis gave a molecular weight of 39.8 kDa, quite similar to the theoretical value (32.5 kDa). The determination of Zn showed the presence of this metal in the OFFGEL fractions 9, 10 and 11 (5.2 ± 0.06, 22.2 ± 0.3 y 2.30 ± 0.07 mgL^{-1} respectively), fractions which contain the ADH protein. In addition, negligible Zn concentrations were found in the remaining OFFGEL fractions. Results were confirmed by a mass balance study after analyzing an ADH solution for Zn (2.10 ± 0.06 mg g^{-1}) and comparing to the Zn concentrations as a sum of Zn concentrations in fractions 9, 10, and 11 (2.10 ± 0.08 mg g^{-1}), which offered a Zn percentage of 100 %.

Therefore, Zn-binding SOD / ADH appears to offer high affinity; whereas, Cu-SOD offers a weak metal–protein affinity. In general, OFFGEL conditions are less drastic than those inherent to conventional PAGE [40] and at least the integrity of the Zn-binding SOD / ADH appears to be guaranteed.

4.3.4. Application

4.3.4.1. Dissolved proteins assessment

Three surface seawater samples and one deep seawater sample were analysed for dissolved proteins by two-dimensional IEF OFFGEL – LOC electrophoresis after tangential ultrafiltation and centrifugal ultrafiltration, and marine protein precipitation. Proteins were detected in OFFGEL fractions 7, 8, 9, 10, 17, and 18 (pIs of 4.82, 5.13, 5.43, 5.73, 8.10, and 8.37) in surface seawater SS1 (Figure 1); whereas, deep seawater DS4 analysis (Figure 1) showed the presence of dissolved proteins in OFFGEL fractions 2, 3, 4 and 5 (pIs of 3.30, 3.61, 3.91, and 4.22). The obtained pIs of dissolved proteins in surface seawater are similar to those offered by Yamada and Tanoue [17], after conventional 2D electrophoresis, who has reported dissolved proteins of pIs within the 5.0 – 8.0 range. However, the pIs of the isolated proteins in the deep seawater are lower than those reported by the same authors for deep seawaters (from 5.1 to 8.7) [18]. A possible explanation

must be different depth of the sampled seawaters, between 150 to 1000 m depth in Yamada and Tonoue's studies [18], and 50 m depth in the current study.

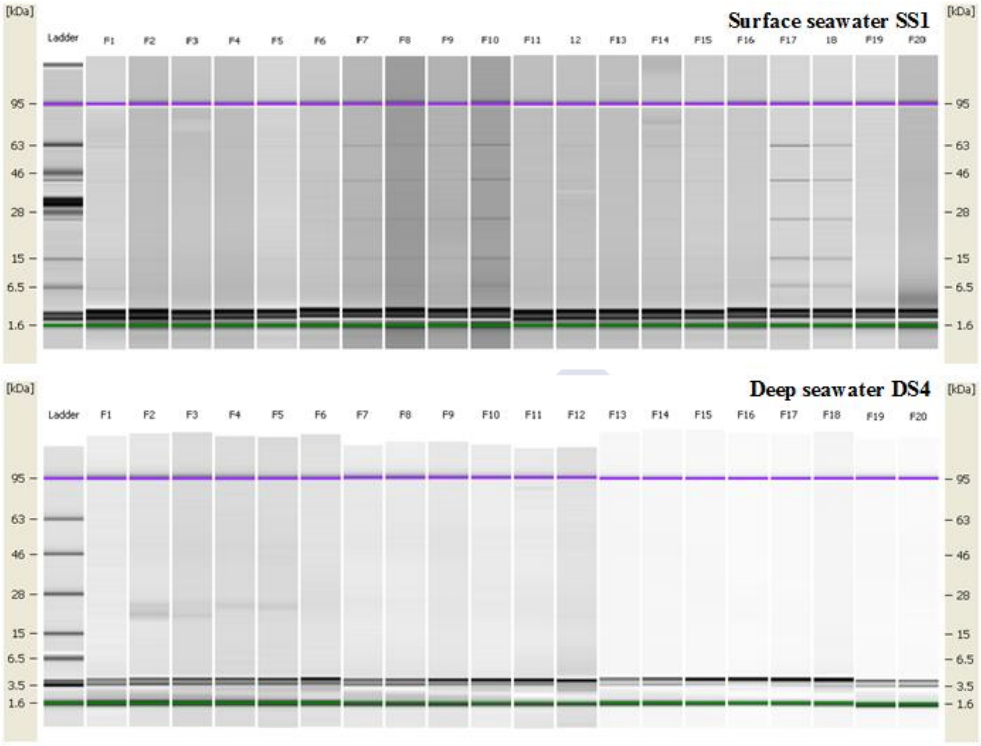

Figure 1: Gel-like images (fractions from 1 to 20) for dissolved proteins in surface and deep seawater samples: Ladder lane corresponds to standard proteins of known molecular weight; F lanes correspond to OFFGEL fractions 1 to 20.

Proteins were not detected in any OFFGEL fractions obtained from surface seawater samples coded as SS2 and SS3. After LOC electrophoresis of OFFGEL fractions from seawater sample SS1, several proteins exhibiting different molecular weights were measured (values are listed in Table 3). As an example, Figure 2 shows the electropherograms when analysing the OFFGEL fractions 10 and 17 (surface seawater SS1) and OFFGEL fraction 5 (deep seawater DS4).

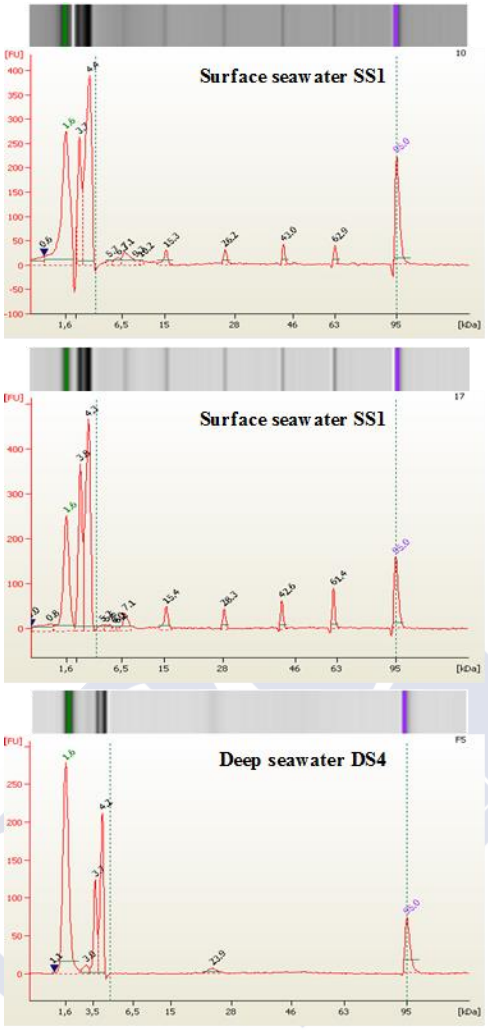

Figure 2: Electropherograms of selected OFFGEL fractions for dissolved proteins in surface (F10 and F17) and deep (F5) seawater samples.

The isolated proteins offered molecular weights ranging from 15 to 63 kDa in most of the OFFGEL fractions, values similar to than reported by Yamada and Tanoue, molecular weights from 20 to 48 kDa [17], and between 16 and 48 kDa [18]. Regarding dissolved proteins in OFFGEL fractions from the deep seawater sample DS4, molecular weights ranged from 21 to 24 kDa. Although a small number of samples have been analyzed, dissolved proteins in deep seawater samples are of

lower molecular weight (and also different pIs) that those determined in surface seawater. The molecular weights of the isolated proteins are also lower that those reported by Yamada and Tanoue when analyzing dissolved proteins of deep seawater, between 31 and 48 kDa [18]. Similarly, it must be pointed out that the deep seawater analyzed in the current work (50 m depth) is quite different than those deep seawater samples analysed by Yamada and Tanoue [18] of depths within the 150 – 1000 m range.

Table 3: pI values, molecular weights and relative concentrations of dissolved proteins in surface and deep seawater.

Sample	OFFGEL fraction	pI	MW (kDa)	Relative concentration (ng μL^{-1})
SS1	F7	4.82	15 - 63	119
	F8	5.13	15 – 63	4.4
	F9	5.43	15 – 63	5.9
	F10	5.73	15 – 63	103
	F17	8.10	15 – 63	188
	F18	8.37	15 – 63	53.5
DS4	F2	3.30	21 – 24	58.3
	F3	3.61	21 – 24	19.1
	F4	3.91	21 – 24	13.7
	F5	4.22	21 – 24	15.9

Table 3 also lists the relative protein concentrations of each OFFGEL fraction. Regarding surface seawater sample (SS1), high concentration was measured for proteins content in the OFFGEL fraction 17 (relative concentration of 188.2 ng μL^{-1}). Other OFFGEL fractions showed protein concentrations from 4.4 ng μL^{-1} (OFFGEL fraction 8) to 120 ng μL^{-1} (OFFGEL fraction 7). Protein concentrations in the deep seawater (DS4) varied from 13.7 ng μL^{-1} (OFFGEL fraction 4) to 58.3 ng μL^{-1} (OFFGEL fraction 2).

4.3.4.2. Quantification of metals bound to dissolved proteins

The concentrations of trace elements (Table 4) were assessed in all the OFFGEL fractions (sections 4.2.10 and 4.2.11). Regarding surface seawater SS1, cadmium have been found to be associated to some of the proteins content in OFFGEL fractions 10, 17 and 18; whereas, copper was mainly associated to some of the proteins in OFFGEL fractions 17 and 18 (alkaline pIs). Manganese and nickel were mainly found to be bound to certain proteins in OFFGEL fraction 18 (alkaline region), while zinc is associated to certain proteins of acid pIs (OFFGEL fraction 9 and 10). Finally, iron was detected in all OFFGEL fractions. Regarding deep seawater DS4, Cu was found to be bound to proteins content in OFFGEL fraction 5; whereas, Mn was quantified in proteins of OFFGEL fractions 4 and 5. Therefore, dissolved proteins in surface seawater appear to offer a larger binding capacity for trace elements than dissolved proteins in deep seawaters. In general, elements such as Cu, Fe, Mn, Ni and Zn have been found to be bound to dissolved proteins. These elements are considered to be essential for life, and they are integrated in different biological macromolecules [41]. However, it must point out that certain dissolved proteins in surface seawater are also associated to toxic elements such as cadmium.

With regard to stability of the metal binding dissolved proteins, OFFGEL fractions 22 and 23 were analyzed for Cu, Fe, Mn, Ni and Zn, and negligible concentrations of these elements were found. Therefore, the used OFFGEL operating conditions appears to guarantee the stability of the metal binding dissolved proteins. Experiments that could show the nature of the metal-binding proteins (metal-proteins complexes or metalloproteins) have not been developed in the current work. However, because of the metal ions found are not usually integrated in the amino acids present in proteins, the isolated metal-binding proteins must be metal-protein complexes [42].

Table 4: Concentrations of metal binding dissolved proteins in surface and deep seawater.

	Metal concentrations (µg L^{-1})								
	SS1								
	Cd	**Co**	**Cr**	**Cu**	**Fe[a]**	**Mn**	**Ni**	**Pb**	**Zn[a]**
F7	<1.1	<9.8	<41	<8.8	3.1±0.1	<16	<35	<41	<0.05
F8	<1.1	<9.8	<41	<8.8	2.9±0.1	<16	<35	<41	<0.05
F9	<1.1	<9.8	<41	<8.8	3.5±0.1	<16	<35	<41	<0.05
F10	2.8±0.4	<9.8	<41	<8.8	2.8±0.1	<16	<35	<41	1.0±0.1
F17	3.1±0.6	<9.8	<41	12.0±1.0	2.6±0.1	<16	<35	<41	<0.05
F18	2.0±0.3	<9.8	<41	20.5±1.2	2.6±0.1	22.4±1.2	43.6±2.5	<41	<0.05
	DS4								
	Cd	**Co**	**Cr**	**Cu**	**Fe[a]**	**Mn**	**Ni**	**Pb**	**Zn[a]**
F2	<1.1	<9.8	<41	<8.8	<0.37	<16	<35	<41	<0.05
F3	<1.1	<9.8	<41	<8.8	<0.37	<16	<35	<41	<0.05
F4	<1.1	<9.8	<41	<8.8	<0.37	84.1±4.4	<35	<41	<0.05
F5	<1.1	<9.8	<41	20.3±1.2	<0.37	68.1±2.2	<35	<41	<0.05

(a) expressed as mg L^{-1}

4.4. Conclusions

Dissolved proteins were efficiently isolated from surface and deep seawater after successive tangential ultrafiltration and centrifugal ultrafiltration of the retentate fraction (10 kDa cut off). The overall procedure involves a pre-concentration factor of 3000 (60 L of seawater until 20 mL of ultrafiltrated retentate). An improved method based on methanol / chloroform / water was optimised for achieving an adequate protein pellet for IEF OFFGEL and LOC electrophoresis. The optimized procedure guaranteed a successful isoelectric focusing and further proteins' separation in the basis of their different molecular weights. LOC electrophoresis

was demonstrated to be no affected by the incubation time and temperature, and best performances were achieved by subjecting the re-dissolved protein pellet at a temperature of 95°C for only 5 min before analysis. Most of the isolated proteins found in surface seawater offer alkaline pIs (8.10 and 8.37) and molecular weights between 15 and 63 kDa. These proteins were found to be mainly associated several trace elements such as Cd, Cu, Fe, Mn and Ni. Dissolved proteins exhibiting acid pIs, from 4.82 to 5.73 (15 – 63 kDa) were also detected in surface seawaters, and Cd, Fe, and Zn were found to be bound them. Dissolved proteins in deep seawaters offered acid pIs (3.30 – 4.22) and lower molecular weights (within the 21 – 24 kDa range). In addition, low concentrations of essential elements such as Cu and Mn were also found. Limitations of OFFGEL-LOC electrophoresis regarding proteins identification as well as metal bound to dissolved proteins will lead us to develop further studies by 2-DE for protein identification by MS/MS techniques, as well as metal determination in the isolated proteins by LA-ICP-MS.

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Study of extraction procedures for protein analysis in plankton samples by OFFGEL electrophoresis hyphenated with Lab-On-a-Chip technology

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Abstract: *Extraction procedures for protein analysis from plankton samples were studied. OFFGEL Electrophoresis combined with Lab-on-a-chip technology has been applied for protein analysis in plankton samples. BCR-414 (plankton) certified reference material from the European Commission was used to evaluate the protein extraction procedures. Three protein extraction procedures were studied: 1) by using Tris-HCl buffer containing a protease inhibitor cocktail, 2) Urea/Triton X-100 buffer extraction, 3) using the phenol/Sodium dodecyl sulphate method after different washing steps with 10 % trichloroacetic acid /acetone solution and methanol. The pellet of proteins obtained was dried and then dissolved in the OFFGEL buffer. Proteins were separated according to their ioselectric point by OFFGEL electrophoresis. This separation was performed using 24 cm, pH 3 - 10 IPG Dry Strips. The proteins present in each liquid fraction (24 fractions) were separated according to their molecular weight using a microfluidic Lab-on-a chip electrophoresis with the Protein 80 LabChip kit. This kit allows for the separation of proteins with a molecular weight ranging from 5 - 80 kDa. Taking into account the intensity and the number of the protein bands obtained, the protein extraction procedure using the phenol/Sodium dodecyl sulphate after different wash steps with 10 % trichloroacetic acid /acetone solution was selected.*

The developed method was applied for protein determination in a fresh marine plankton sample. The proteins found in this sample have a molecular weight ranging from 6.4 to 57.3 kDa, and the proteins with highest molecular weight were in the OFFGEL fractions with an isoelectric point ranging from 4.40 to 8.60. The concentration of proteins were calculated using external calibration with Bovine Serum Albumin, and the protein concentrations varied from 50.0 to 925.9 ng μL^{-1}.

Keywords: *Protein extraction, plankton samples, OFFGEL electrophoresis, Lab-on-a-chip Technology.*

5.1. Introduction

Plankton in the marine environment can be classified in two main groups, zooplankton (animal plankton) and phytoplankton (vegetal plankton) [1]. Plankton contains all the components (proteins, amino acids, lipids, carbohydrates mineral and vitamins) necessary for marine life [2,3]. Plankton can be considered as a primary source of marine proteins dissolved in open seawater. They are some of the most reactive substances and the key factor in the direct effect of dissolved organic matter in the global carbon cycle [4]. Therefore, the analysis of proteins in plankton samples is important for understanding the processes in the marine ecosystem.

Sample preparation is one of the most important steps in an analytical procedure. When protein analysis is performed, this step includes the protein extraction procedure as well as the elimination of matrix interferences such as pigments, polysaccharides, high levels of salts, etc. The presence of these substances can affect the protein resolution in the separation procedure. Furthermore, the protein extraction efficiency and the presence of matrix interferences can influence analytical sensitivity.

Different protein extraction procedures have been reported in the literature as a first step in proteomics studies. The protein extraction protocol must be selected in function of the sample matrix and must be optimized for each type of sample to obtain the highest extraction efficiency. Moncheva et al. [5] extracted the proteins in homogenised phytoplankton by using 20 % (w/v) trichloroacetic acid (TCA) in acetone containing 0.1 % 2-mercaptoethanol and 1 % sodium dodecyl sulphate (SDS). This extraction procedure was repeated three times. The remaining pellet was then lyophilised and dissolved in phosphate buffer before the analysis. Barbarino et al. [6] evaluated different extraction procedures for extraction and quantification of proteins from marine macro and microalgae. The protein extraction protocol selected by these authors involve the immersion of samples in 4.0 mL ultra-pure water for 12 h, followed by complete grinding of the samples with a Potter homogeniser and re-extraction with 1.0 mL 0.1 N NaOH with 0.5 % β-

mercaptoethanol (v/v). Protein precipitation was performed with 2.5:1 25 % TCA:homogenate (v/v). Carpentier et al. [7] evaluated four extraction protocols for protein extraction from plant tissues (banana, potato and apple): 1) TCA precipitation, 2) TCA / acetone precipitation and fractionation, 3) no precipitation fractionation, and 4) phenol extraction methanol / ammonium acetate precipitation. The authors concluded that TCA / acetone precipitation and phenol extraction methanol / ammonium acetate precipitation were useful as standard methods for plant tissues. Wang et al. [8] proposed a simple and universal protocol for extracting proteins from recalcitrant plant tissues such as, leaves from bamboo, grapes, lemon, pine etc. and they concluded that the protein extraction efficiency depends on the sample disruption. Therefore, in the first step of the proposed protocol, the plant tissues are pulverized to a fine powder in a mortar under liquid nitrogen to minimize photolytic and other modes of degradation. The sample was first washed with TCA / acetone, followed by a second washing step with methanol and acetone to remove residual TCA and contaminants. Finally, an extraction with phenol / SDS mixture to extract proteins from the dry pellets was performed. Recently, Pains Rodrigues et al. [9] proposed a simplified protocol (based on the Wang et al. [6] protocol) for protein studies of soybean roots. In this new procedure the three preliminary washing steps and the vacuum-drying step were eliminated, reducing reagents and time consumption. Maldonado et al. [10] compared three precipitation protocols of protein extraction for 2-DE proteomic analysis using *Arabidopsis* leaf tissue: TCA-acetone, phenol, and TCA-acetone-phenol. The protocols were compared taking into account the number of spots, image quality and reproducibility obtained in the analysis by two dimensional gel electrophoresis (2-DE). The authors concluded that TCA-acetone-phenol protocol provided the best results in terms of spot focussing, resolved spots, spot intensity and reproducibility. Da-Zhi Wang et al. [11] compared four protein extraction methods for proteomic study of dinoflagellate *Alexandium sp.* using two-dimensional electrophoresis: 1) Urea / Triton X-100 buffer extraction with TCA / acetone precipitation, 2) TCA / acetone precipitation, 3) 40 mM Tris(hydroxymethyl) aminomethane buffer extraction, 4) 50 mM Tris-HCl / 5 % glycerol buffer extraction.

The 2-DE protein profiles showed that the urea / Triton X-100 buffer extraction with TCA/acetone precipitation method was the best. This method removed all the interfering substances and salts efficiently and also offered a high quality in terms of resolution, number spots and spot intensity.

Recently, Jimenez et al. [12] evaluated different extraction, purification and concentration conditions for protein extraction from plankton, taking into account the protein concentration in the extract, the number of bands obtained by poliacrilamide gel electrophoresis separation (PAGE), as well as metal-protein binding before electrophoretic separation. The extraction procedure with 25 $mmolL^{-1}$ Tris-HCl buffer and a protease inhibitor was the most efficient means of protein extraction. The presence of reducing agents (tris(2-carboxyethyl)phosphine hydrochloride, TCEP) or absorbents (phenylmethylsulfonyl fluoride, PMSF), for phenolic compounds, in the extracting solution did not improve protein and metal extraction efficiency.

Isoelectric foccussing (IEF) of proteins combined to a mass separation technique is the most commonly used method in the proteome analysis [9,13,14]. Although in-gel IEF is used routinely as the first dimension in 2D gel electrophoresis protein separation, this technique presents same drawbacks, such as low sensitivity and tedious sample preparation procedures to recover the protein from the gel for a further analysis (for example, with mass spectrometry). The latter stage increases therefore the risk of protein losses or sample contamination.

An alternative approach for the first dimension separation of proteins is the use of the OFFGEL Isoelectric Focusing. In this technique, proteins or peptides are separated according to their isoelectric point (pI), using immobilized pH-gradient gels (IPG) but working with a liquid phase. Fractionated proteins or peptides are easily recovered from the liquid phase to be separated in the second dimension, for example, by mass spectrometry or MS/MS. In recent years, different applications for protein identification using this fractionation technique combined with LC/MS or LC/MS/MS have been reported [15,16,17]. This methodology has

been applied for protein fractionation and identification in different types of samples, such as extraocular muscle, plasma samples, cortical brain tissues, etc. This fractionation technique presents the same high resolution as IPG gels and offers several advantages such as: (i) low sample volumes; (ii) the procedure is less tedious than IPG gels fractionation because the sample is recoved in a liquid phase; (iii) there are commercial available IPG strips of various pI ranges; (iv) focused component are concentrated; and (v) the system provides information about the pI of proteins and peptides acting as a filter in further protein identification procedures [13,18,19,20].

Regarding quantitative and qualitative sizing of proteins, poliacrilamide gel electrophoresis (PAGE) [9,21,22,23] and high-performance liquid chromatography (HPLC) or HPLC-MS [15,24] are normally used. Capillary Gel electrophoresis by using Lab-on-a-chip technology can be an alternative approach for assessing information concerning the second dimension. This technology provide some advantages such as: it uses small volumes of reagents and samples; it provides real-time data on composition, molecular weight and concentration for ten samples in less than 45 minutes; it consists of an automatic analysis; the resolution, the precision and the sensitivity achieved are superior than those offered by the gel electrophoresis [25]. This methodology has been used by different authors for qualitative and quantitative protein determination in different matrices, such as soybean cultivars [23], tear fluid [26] and human granulocyte-colony [27].

The aim of this work is the study of different protein extraction procedures for protein extraction from plankton samples. The proteins extracted were analysed by the application of the new OFFGEL electrophoresis-Lab-on-a-chip technology.

5.2. Materials and Methods

5.2.1. Reagents

The pH 3 - 10 IPG Dry Strips, IPG Buffer 3 - 10 and Plus One DryStrip cover fluid used for protein fractionation were supplier by GE Healthcare Life Science (Uppsala, Sweden). Urea (electrophoresis grade), thiourea (analytical grade) ammonium acetate and phenol solution, equilibrated with 10 mM Tris-HCl (pH=8.0, 1 mM EDTA) were provided by Sigma Aldrich (St. Louis, MO, USA). 1, 4 Dithiothreitol (DTT, electrophoresis grade), Methanol, Tris-HCl and glycerol solution were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid and acetone were from Panreac (Barcelona, Spain and 2-mercaptoethanol was from Fluka (Vancouver, Canada). Sodium dodecyl sulphate and sucrose were purchased from AppliChem (Darmstad, Germany). β-mercaptoethanol was from Fluka (Vancouver, Canada) and Bovine Serum Albumin from Sigma Aldrich (St. Louis, MO, USA). The Agilent protein 80 Kit containing the protein chips, Protein 80 Gel-Matrix, Protein 80 Dye Concentrate, Protein 80 Sample Buffer and Protein 80 Ladder was supplied by Agilent Technologies (Waldbronn, Germany). BCR-414 (plankton) certified reference material from the European Commission was used to develop this study. Protein inhibitor cocktail (Complete lysis-y, EDTA free) was purchased from Roche Diagnostics (Mannhein, Germany).

5.2.2 Instruments

The 3100 OFFGEl fractionator and the Bioanalyzer 2100 used for the protein analysis were purchased from Agilent Technologies.

A Heidoph shaker, type Reax 2000 (Gemini B.V. Apeldoorn, Netherlands) was used to vortex the samples. A SIGMA 2K15 centrifuge (SIGMA Laborzentrifugen GmbH, Osterode amm Harz, Germany) was used in the protein extraction procedure.

A Branson digital Sonifer was from Branson Ultrasonics S.A. (Geneve, Switzerland) and the ultrasonic bath was from VWR International Eurolab, SL (Barcelona, Spain).

5.2.3. Protein extraction procedures

Different protein extraction procedures for protein extraction from plankton samples were evaluated in this work.

5.2.3.1. Procedure 1

In the first extraction procedure the proteins present in the plankton samples were extracted using a Tris-HCl buffer containing a protease inhibitor cocktail (Roche) (Solution A). Solution A was prepared by mixing 450 µL of stock solution of protease inhibitor cocktail with 2550 µL of Tris-HCl 25 mM (pH=7.5). A volume of 1200 µL of solution A was added to 0.1000 g of dry plankton sample and agitated vigorously for 24 hours. The sample was then centrifuged at 2500 rpm during 30 minutes at 4ºC and the supernatant was then transferred to a new tube. The proteins were precipitated adding 2400 µL of ice cold acetone and stored at -20ºC for 120 minutes. The sample was then centrifuged at 2500 rpm for 5 minutes (4ºC) and the supernatant was discharged. The pellet obtained was then washed several times with ice-cold acetone. The pellet of proteins was dried and then dissolved in the buffer.

5.2.3.2. Procedure 2 (Urea/Triton X-100 buffer extraction)

The extracting solution used in this protein extraction procedure was prepared containing 7 M urea, 2 M thiourea, 2 % SDS (w/v), 2 % triton X-100 (v/v), 1 % DTT (w/v) and 2 % buffer pH 3 - 10. Therefore, 0.1000 g of dry plankton sample were mixed with 1200 µL of the extracting solution and then subjected to ultrasound energy. The uses of an ultrasound bath (for 1, 2 and 4 hours, at room temperature) or an ultrasonic probe (for 30 and 60 seconds, applied at intervals of 10 s) were evaluated in this work. After the ultrasonic treatment, the sample was centrifuged at 4000 rpm for 30 minutes (4ºC) and the supernatant was transferred to a new

tube. The proteins were precipitated adding 2400 µL of ice cold acetone and stored at -20ºC for 120 minutes. The sample was then centrifuged at 2500 rpm for 5 minutes (4ºC) and the supernatant was discharged. The pellet obtained was then washed several times with ice-cold acetone. The pellet of proteins was dried and then dissolved in the buffer solution.

5.2.3.3. Procedure 3 (TCA/acetone wash and phenol precipitation)

Proteins were extracted from the plankton sample using the extraction protocol proposed by Wei Wang et al. [6]. Therefore, 0.3000 g of dry plankton sample were introduced into a 2 mL tube and filled with 10 % TCA / acetone. The tube was vortexed and centrifuged at 10,000 rpm for 3 minutes at 4ºC; and the supernatant was then removed. Afterwards, the tube was filled with 80 % methanol plus 0.1 M ammonium acetate, vortexed and centrifuged again at 10,000 rpm for 3 minutes at 4ºC, to remove the supernatant. The solid residue was then washed with acetone and dried for at least 10 minutes at 50ºC. After this treatment, the proteins were extracted and precipitated by adding 0.8 mL of 1:1 phenol (pH 8.0) / SDS buffer, by mixing thoroughly and incubating 5 minutes before centrifugation at 10000 rpm for 3 minutes. The upper phenol phase was then transferred into a new 2 mL tube, and the tube was filled with methanol containing 0.1 M ammonium acetate and stored at -20ºC from 2 hours to ten hours. The sample was then centrifuged at 2500 rpm for 5 minutes (4ºC) and the supernatant was discharged. The protein pellet obtained was then washed once with 100 % methanol and once with 80 % acetone. The pellet of proteins was dried and then dissolved in the buffer solution.

5.2.4. Procedure of the OFFGEL electrophoresis

The proteins extracted from the sample were separated according to their isoelectric point (pI) using a 3100 OFFGEL fractionator. The separation was performed using 24 cm, pH 3 - 10 IPG Dry Strips following the manufacturer's instructions. The strips were rehydrated for 15 min in the 24-well assembled device with 40 µl of OFFGEL Rehydration solution. This solution was prepared by mixing 960 µL of Protein OFFGEL Stock solution with 240 µL of ultrapure water. The

Protein OFFGEL Stock solution contains 25.2 g of urea, 600 mg of DTT, 6 mL of glycerol solution and 600 µL of OFFGEL Buffer (pH 3 - 10) in a final volume of 50 mL with ultrapure water.

The pellet of proteins obtained following the procedure described in section 5.2.3 was redissolved in 3000 µL of Protein OFFGEL Stock solution mixed with 750 µL of ultrapure water, and 150 µL of this solution was loaded in each well of the OFFGEL Fractionator.

The proteins were then fractionated based on their pI using the conditions detailed in Table 1. The recovered fractions were stored at -20ºC until further analysis.

Table 1: Instrumental conditions for OFFGEL fractionation (Method OG24PR01).

	Volt. Hour [KVh]	*Voltage [V]*	*Current [µA]*	*Power [mW]*	*Time [h]*
Focusing	64.0	4500	50	200	100
Hold		500	20	50	

5.2.5 Procedure for the protein separation and quantification using the Agilent 2100 Bioanalyzer system

The proteins present in each fraction obtained in the fractionation were separated according to their molecular weight using Lab-on-a chip technology.

The Lab-on-a-chip separation was performed on the Agilent 2100 Bioanalyzer system in combination with the Protein 80 LabChip kit. This kit allows sizing and quantification of 10 protein samples in less than 30 min with a size resolution of approximately 10 % or better. All chips were prepared according to the protocol provided with the Protein 80 LabChip kit.

The denaturing solution was prepared adding 1 µL of β-mercaptoethanol (BME) to an aliquot of 28.6 µL of sample buffer (provided in the kit). A portion of 4 µL of protein sample (isolated by electrophoresis) was then combined with 2 µL of

denaturing solution in a 0.5 mL eppendorf tube. The molecular weight standard solution (6 µL) (Ladder provided in the kit) was also pipetted in a 0.5 mL eppendorf tube. The samples tubes and the ladder tube were then heated for 5 minutes at 95 – 100 ºC in a hot water bath and cooled down afterward. Uktrapure water (84 µL) was then added to samples and ladder and vortexed. The Gel-Dye Mix (G/D) and the Destaining solution (DS) were prepared following the provider's instructions. A volume of 6 µL of each sample and ladder were then loaded onto the chip, previously filled with G/D and DS solutions. The chip was placed in the Agilent 2100 bioanalyzer and analyzed immediately. Separated proteins were detected by laser-induced fluorescence. The Agilent 2100 Bioanalyzer was controlled by software that includes data collection, reporting and interpretation functions. Agilent 2100 Expert Software shows the results of the analysis as gel-like images, electropherograms as well as a Table with the molecular weight and the relative concentration of the proteins found.

5.2.6. Statistical analysis

Statistical analysis was performed using the Statgraphics Centurioh XVI Version 16.1.11 program (Stat Point Technology, Inc, Warrenton, Virginia, USA).

5.3. Results and discussion

The protein extracted from the plankton samples were analysed in this work by OFFGEL electrophoresis combined with lab-on-a-chip technology. With this methodology the proteins were separated in the first dimension, using OFFGEL electrophoresis, according to their isoelectric point. In the second dimension, the proteins were separated according to their molecular weight (kDa). This second dimension was performed on a chip and using the Agilent 2100 Bioanalyzer system. The results obtained with this technology are analogous to those obtained by SDS-PAGE [26,28,29].

For protein fractionation using the OFFGEL electrophoresis, the dried pellet obtained using the different extraction procedures proposed in this work was

dissolved in 3000 µL of Protein OFFGEL Stock solution mixed with 750 µL of ultrapure water, prepared as described in section 5.2.4. Portions of the dissolved pellet (150 µL) were then loaded in each well (24 wells) of the OFFGEL electrophoresis system. The proteins were fractionated based on their pI using the procedure detailed in Table 1 and with 24 cm, pH 3 - 10 IPG Dry Strips. This separation was performed in 38 hours and the recovered fractions were stored at -20ºC until further analysis. The pH corresponding to each well of the OFFGEL fractionator is shown in Table 2.

Table 2: pH ranges in each fraction of OFFGEL fractionation.

Fraction	pH range	Fraction	pH range
1	3.35-3.61	**13**	6.50-6.76
2	3.61-3.88	**14**	6.76-7.03
3	3.88-4.14	**15**	7.03-7.29
4	4.14-4.40	**16**	7.29-7.55
5	4.40-4.66	**17**	7.55-7.81
6	4.66-4.93	**18**	7.81-8.08
7	4.93-519	**19**	8.08-8.34
8	5.19-5.45	**20**	8.34-8.60
9	5.45-5.71	**21**	8.60-8.86
10	5.71-5.98	**22**	8.86-9.13
11	5.98-6.24	**23**	9.13-9.39
12	6.24-6.50	**24**	9.39-9.65

The proteins present in each fraction obtained in the OFFGEL fractionation were separated according to their molecular weight using Lab-on-a-chip technology following the procedure described in section 5.2.5. The separation was performed using the Protein 80 LabChip kit. Migration time and fluorescence intensity are the parameters used for sizing and quantification of proteins. Sizing and analysis of proteins by LOC electrophoresis is performed with the Agilent protein 80 Kit Agilent

Technologies (Waldbronn, Germany). This supply consists of: protein chips containing an interconnected set of gel filled microchannels that sieves proteins by size within the 5 – 80 kDa range (electrophoresis separation); Protein 80 Gel-Matrix and Protein 80 Dye Concentrate, which are used as a filling gel for chips; and Protein 80 Sample Buffer, which is used for preparing the denaturing solution. The kit also contains the Protein 80 Ladder (external standard), which consists of standard proteins with molecular weights of 6.5, 15, 28, 46 and 63 kDa (concentration of 600 ng μL^{-1} as a sum of all standard proteins) used for molecular weight assessment; and also, standard proteins coded as lower marker and upper marker (internal standard), at concentrations of 1 ng μL^{-1} and 60 ng μL^{-1}, respectively, used for protein quantification. The internal standard (upper marker) is a protein that is part of the sample buffer and is added to each sample in a defined ratio. Protein sizing and quantification is performed automatically by the Agilent 2100 Expert Software, based on a number of mathematical calculations and algorithms. For protein size determination, the retention times are compared to the ladder retention times. For protein quantification, the peak areas are compared with to the internal standard proteins. One of the advantages of this technique is that the system can analyze and provide the results (molecular weight and concentration) for ten samples in less than 45 minutes. Furthermore, the Agilent 2100 Expert Software shows the results of the analysis as gel-like images and electropherograms.

5.3.1 Study of Protein extraction procedures

As mentioned in the Introduction section, different protein extraction procedures have been reported in the literature as a first step in proteomics studies. In this step, interfering compounds from the sample such as pigments, polysaccharides, high levels of salts, etc., should be eliminated. Therefore, one of the objectives proposed in this study was to compare different protein extraction procedures for protein extraction from marine plankton samples.

The study of these protein extraction procedures was performed using a BCR-414 (plankton) certified reference material for metal determination from the European Commission. The selected protein extraction procedure was then applied for the protein analysis of a plankton sample.

5.3.1.1. Procedure 1:

In the first extraction procedure the proteins present in the plankton samples were extracted using a Tris-HCl buffer containing a protease inhibitor cocktail (Solution A) prepared as described in section 5.2.3.1. This extraction solution was previously used by Jimenez et al.[10] for screening analysis of Zn and Cu-binding proteins in plankton samples.

The gel-like images obtained in the Bioanalyzer system using this protein extraction procedure are shown in Figure 1A. Each line of the gel shows the protein sizing of one liquid fraction. The liquid fractions are named with a number from 1 to 20 and the pH values varies from 3 (fraction 1) to 10 (fraction 24) (Table 2). The gel-like images for off-gel proteins fractions from 21 to 24 are not shown in Figure 1A, because proteins bands were not found in these fractions. As can be seen in Figure 1A, bands of proteins are only detected in the off-gel fractions 2, 3, 4, 5, 6, 7 and 10. The isoelectric point for the proteins present in these fractions varies from 3.61 (in fraction 2) to 5.98 (in fraction 1) (see Table 1). Proteins with a molecular weight of 27.5 kDa were found in fractions 2, 3 and 4, with molecular weight of 26.8 kDa in fractions 5 and 6, and proteins with a low molecular weight (6.5 kDa) were detected in fraction 10.

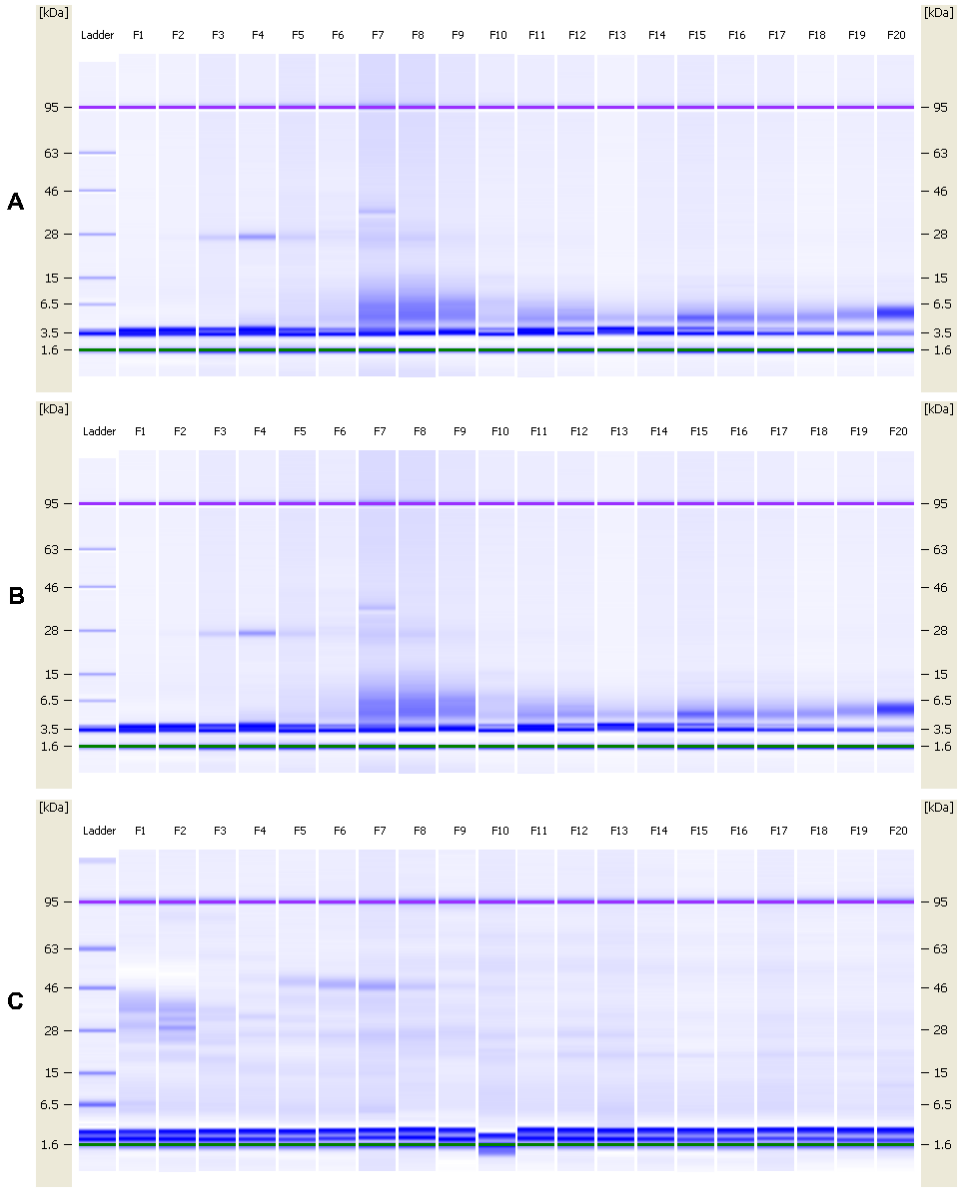

Figure 1: Gel-likes images obtained for offgel fractions from 1 to 20 using 0.1000 g of plankton sample (BCR-414). (A) Procedure 1, (B) procedure 2 and (C) procedure 3.

5.3.1.2. Procedure 2:

The second protein extraction procedure is a modification of the procedure proposed by Da-Zhi Wang et al. [9]. In this case the extracting solution was prepared containing 7 M urea, 2 M thiourea, 2 % SDS (w/v), 2 % triton X-100 (v/v), 1 % DTT (w/v) and 2 % buffer pH 3 - 10. The difference between this procedure and the proposed method is that we used SDS instead of CHAPS. This second extraction procedure is described in section 5.2.3.2.

The first experiments were performed subjecting the sample for 1, 2 and 4 hours to ultrasound energy by using an ultrasonic bath and working at room temperature. The results obtained in the three experiments were comparable and an increment of the intensity at longest times was not observed. With the aim of increasing the extraction efficiency, experiments were then performed using an ultrasonic probe. Sonication times of 30 and 60 seconds (applied at intervals of 10 seconds) were studied, and during this time the sample was introduced into an ice bath to avoid sample heating. The results obtained using an extraction time of 30 seconds are shown in Figure 1B. The results obtained with this extraction procedure were similar to the results obtained with the extraction Procedure 1. The only difference was shown in fraction 4. The protein band with a molecular weight of 28 kDa shows higher intensity using procedure 2 than with procedure 1, and relative concentrations of 146.6 and 174.6 ng μL^{-1} were obtained for this protein band using procedure 1 and 2, respectively. Therefore, we concluded that the extraction efficiency was not improved using this second protein extraction procedure and using ultrasound energy.

5.3.1.3. Procedure 3:

In the third protein extraction procedure applied in this study, proteins were extracted from the plankton sample using the extraction protocol proposed by Wang et al. [6] and described in section 5.2.3, by using TCA / acetone washing and phenol precipitation. These authors proposed this rapid protocol for protein extraction for recalcitrant plant tissues for proteomic analysis, and they applied the

protocol for analysis the proteins present in different plant tissues, such as lemon leaves, aged olive leaves and bamboo leaves.

The protocol proposed by Wang et al. [6] requires a mass sample range from 0.1 - 0.3 g for the extraction procedure. In the first experiments, 0.1000 g of plankton sample were subjected to this protein extraction procedure. The proteins pellet was dried, dissolved in the buffer solution, fractionated by electrophoresis and then separated according to molecular weight of proteins by using the experimental conditions described above. The gel-like images obtained using this third protein extraction procedure is shown in Figure 1C. Results indicate that the intensity of the protein bands was higher with this method (procedure 3) than with the Procedures 1 and 2. Furthermore, with Procedure 3, the number of proteins bands increased and new protein bands were observed in the other fractions such as fraction 8. Therefore, Procedure 3 was selected as the best extraction procedure for protein extraction from plankton samples.

5.3.2. Selection of the sample plankton mass

Taking into account the results obtained with extraction Procedure 3, and with the objective of increasing the intensity of the protein bands, a new experiment was performed using 0.3000 g of plankton sample. The gel-like images obtained using this third protein extraction procedure are shown in Figure 2. The gel-like images and the electropherograms obtained for the OFFGEL fractions 2 and 6, using 0.1 g and 0.3 g of sample are shown in Figure 3. As can be seen in the figures, the intensity was increased with the mass of plankton used in the extraction procedure. As an example, fraction 2 shows proteins with molecular weights of 29.3 kDa and 36.8 kDa, and with relative concentrations of 69.6 ng/μL and 70.5 ng/μL, respectively, using 0.1 g of sample; whereas, relative concentrations of 167.6 and 251.0 ng/μL were obtained when using 0.3 g of sample. In fraction 6, the protein with a molecular weight of 47.4 kDa showed relative concentrations of 79.3 and 265.2 ng/μL when using 0.1 and 0.3 g of sample, respectively. Taking into account

the results obtained in this study, the mass of plankton sample was fixed at 0.3000 g for further experiments.

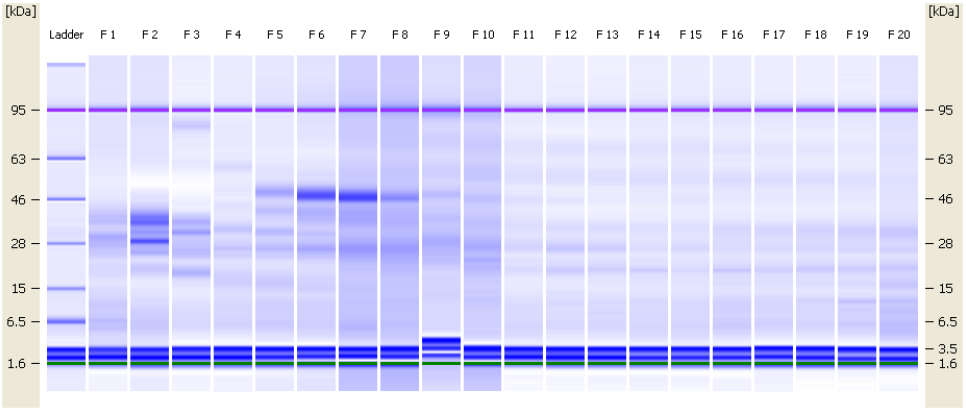

Figure 2: Gel-likes images obtained for off-gel fractions from 1 to 20 using 0.3000 g of plankton sample (BCR-414) and using the protein extraction procedure 3.

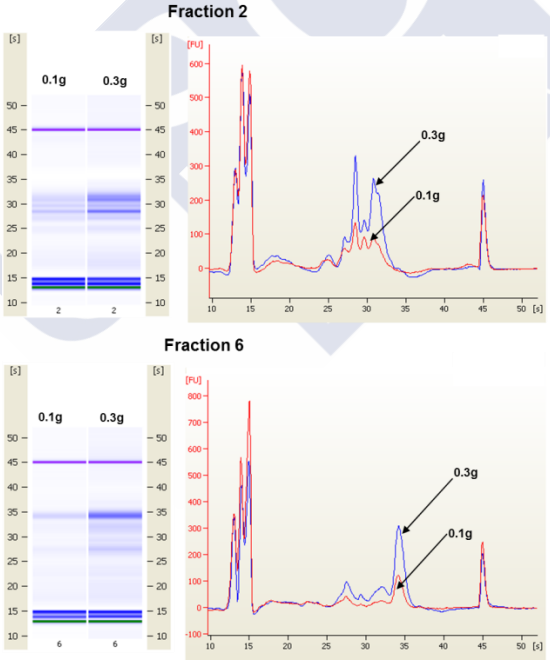

Figure 3: Gel-like images and electropherograms obtained for fraction 2 and fraction 6, using 0.1 and 0.3 g of sample in the protein extraction procedure.

5.3.3. Protein quantification in the plankton sample

The proteins present in the liquid fractions obtained from the plankton sample (extraction procedure 3 with 0.3000 g of sample) were quantified using the internal calibration described above. As can be seen in Figure 2, the proteins with the highest molecular weight are present in the fractions with pI lower than 6, and they offered the highest concentrations in fractions 2, 3, 5, 6, 7 and 8. Fraction 2 presents proteins with relative concentrations from 7.5 to 251.0 ng μL^{-1}. The highest concentration was found for proteins of 36.5 kDa (251.0 ng μL^{-1}), followed by proteins of 28.9 kDa (167.6 ng μL^{-1}). Fraction 3 contains proteins with a molecular size between 6.4 to 36.9 kDa and relative concentrations from 5.9 ng μL^{-1} to 110.4 ng μL^{-1} (19.5 kDa). The molecular weight found in Fraction 5 varied from 6.5 kDa to 48.9 kDa, with relative concentrations lower than 83.5 ng μL^{-1}. Fraction 6 is the fraction with the highest relative concentrations. The proteins sizes varied from 6.3 to 47.6 kDa and the highest relative concentrations are for the sizes of 26.4 kDa (128.0 ng μL^{-1}), 40.4 kDa (105.9 ng μL^{-1}), and 47.6 kDa (265 ng μL^{-1}). The proteins present in this fraction have a pI ranging from 4.66 to 4.93. Fraction 7 also showed high relative concentrations of proteins. The proteins sizes varies in this fraction from 6.0 to 46.7 kDa, with concentrations of 168.7 and 221.9 ng μL^{-1} for proteins with a size of 26.6 and 46.7 kDa, respectively. Proteins with a size between 6.4 to 46.4 kDa were found in Fraction 8, with the highest relative concentration of 133.3, 68.7 and 49.1 ng μL^{-1} for proteins with a size of 26.7, 46.4 and 36.8 kDa, respectively. The concentration of proteins present in the OFFGEL fractions decreased at highest pH.

5.3.4. Precision in the protein sizing analysis

The precision in the analysis using the Agilent 2100 Bioanalyzer system was evaluated. For this purpose, eight replicate analysis of the ladder (standard) were performed using the operating conditions shown in section 5.2.5. This standard contained proteins with molecular weights of 6.5, 15.0, 28.0, 46.0 and 63.0 kDa. The gel-like images obtained in this experiment are shown in Figure 4. The experimental values obtained were 6.6 ± 0.14, 15.1 ± 0.10, 28.01 ± 0.16,

46.05 ± 0.17, and 62.9 ± 0.075 kDa, which offered RSD values of 2.1, 0.7, 0.6, 0.4, and 0.1 %, respectively. Results show that the analysis performed with this system offer good precision.

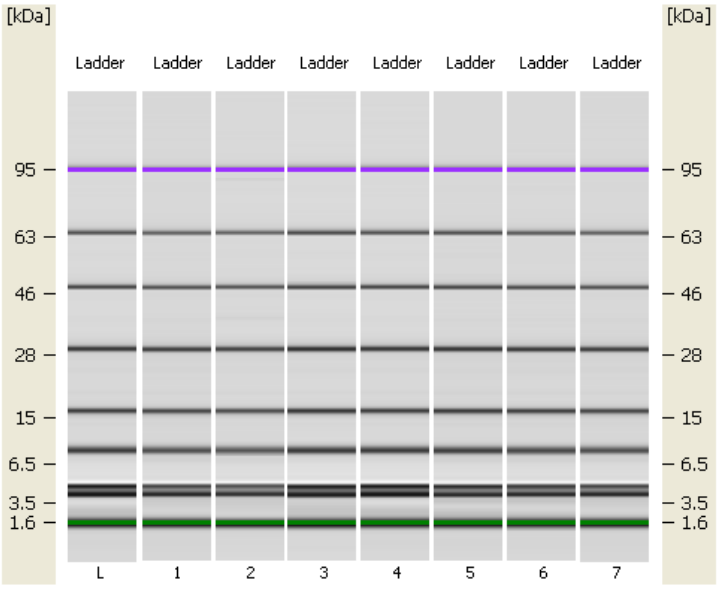

Figure 4: Gel-like images obtained using the Agilent 2100 analyser for the separation of proteins present in the ladder.

5.4. Application to a real fresh marine plankton sample

The method developed in this work was applied for protein sizing and quantification in a fresh marine plankton sample. This marine plankton sample was collected from a feeding tank in a clam hatchery located in the Ria de Arousa (North-western of Spain) in pre-cleaned 5 L bottles. After collection, plankton was concentrated by centrifugation at 4000 rpm for 15 min at 20ºC, and the supernatant was removed. A portion of wet plankton sample was first ground in a mortar under liquid nitrogen; and portions of 1.0800 g, 1.0050 g and 1.0010 g of the ground sample were then subjected to the protein extraction Procedure 3. The proteins pellets obtained were redissolved in 3000 µL of Protein OFFGEL Stock solution mixed with 750 µL of ultrapure water, and 150 µL of these solutions were loaded into each well of the

OFFGEL Fractionator. The proteins were then analysed in each liquid fraction by using the Agilent 2100 Bioanalyzer system in combination with the Protein 80 LabChip kit, which was described in Section 5.2.5. Proteins were quantified using external calibration with Bovine Serum Albumin (BSA) in a concentration ranging from 50 to 1000 ng μL^{-1}. The gel-like image obtained, for one replicate, is shown in Figure 5, and the concentrations of proteins found in each liquid fraction are shown in Table 3.

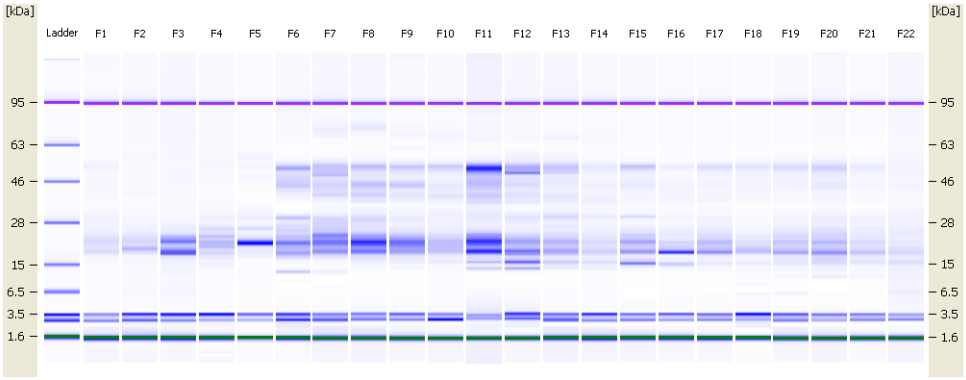

Figure 5: Gel-likes images obtained for off-gel fractions from 1 to 22 using 1.0800 g of fresh plankton sample and using the protein extraction procedure 3.

As can be seen, the proteins molecular weight (MW) varies from 6.4 to 57.3 kDa, and the proteins with the highest MW are present in fractions 5, 6, 7, 8, 9, 10, 11, 12, 13 15, 18, 19 and 20. These fractions contain proteins with pIs from 4.40 to 8.60. The highest protein concentrations were found in fractions 5, 6, 7, 8, 11, 12 and 15, and the proteins with the highest concentration were in fraction 5 (22.0 kDa, 925.9 ng μL^{-1}), fraction 8 (22.2 kDa, 459.9 ng μL^{-1}), fraction 11 (19.4 kDa, 394.0 ng μL^{-1}; 22.5 kDa, 380.0 ng μL^{-1}; 52.7 kDa, 409.9 ng μL^{-1}) and fraction 16 (19.1 kDa, 492.8 ng μL^{-1}). As an example, Figure 6 shows the electropherograms obtained for fractions 5, 6, 11 and 12.

Table 3: Molecular weight and concentration of proteins present in each liquid fraction obtained with the OFFGEL fractionation for a fresh marine plankton sample.

F1		F2		F3		F4		F5		F6	
MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}
19.4	54.1	20.3	121.6	18.9	352.8	18.9	84.4	19.3	59.9	13.1	115.2
22.6	56.5	22.5	66.8	22.6	291.3	21.0	142.3	22.0	925.9	19.0	195.1
						22.2	152.4	26.5	127.4	22.0	304.1
						24.2	129.9	30.6	57.3	25.9	90.5
						26.4	78.9			30.5	128.2
										44.6	108.9
										52.6	196.9

F7		F8		F9		F10		F11		F12	
MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}
19.3	225.0	19.5	284.7	19.6	202.3	19.7	149.8	14.1	72.5	14.1	166.8
22.3	302.1	22.2	459.9	22.3	317.0	21.3	149.5	16.1	114.6	16.1	336.9
24.4	256.9	27.4	50.0	44.8	113.2	22.4	151.2	19.4	394.0	19.5	306.5
27.9	84.3	30.4	71.8	53.3	116.4	39.9	56.9	22.5	380.0	22.6	194.9
40.8	74.9	40.4	90.7			53.3	128.4	40.3	82.3	45.3	62.0
45.1	82.6	44.9	133.0					45.4	113.9	50.4	270.0
49.4	142.5	49.4	48.6					52.7	409.9	53.1	174.0
51.5	110.2	53.3	129.1								
53.5	117.2										

F13		F14		F15		F16		F17		F18	
MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}
16.1	117.0	15.9	77.8	15.6	321.8	15.4	121.7	15.6	51.0	19.4	167.5
19.5	202.9	19.4	101.5	19.3	243.4	19.1	492.8	19.1	268.9	22.3	79.5
22.4	172.1	22.3	78.8	22.3	220.0	22.2	98.0	22.2	159.2	53.7	58.5
31.2	78.3			24.2	109.9			24.2	79.0		
50.8	101.0			31.1	78.5						
53.4	124.8			53.7	139.9						

F19		F20		F21		F22		F23		F24	
MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}	MW kDa	Conc. ng µL^{-1}
15.8	53.7	16.8	89.2	16.1	61.7	16.1	65.4	7.2	590.4	6.4	1724.3
17.2	51.4	19	269.8	19.1	147.0	19.0	87.8	16.4	67.2	13.3	55.7
18.9	224.4	22.1	174.6	22.2	93.5			19.8	69.0		
22.2	141.7	23.9	97.6	24.0	51.7						
24.0	90.0	52.9	100.5								
53.3	86.8										

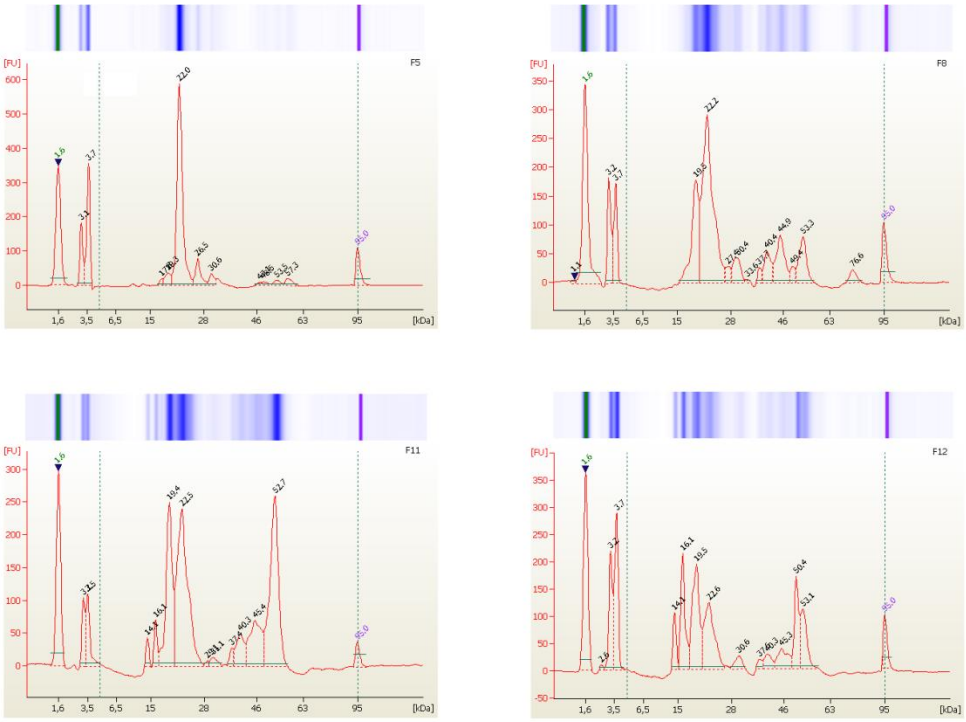

Figure 6: Electropherograms obtained for the OFFGEL fractions 5, 8, 11 and 12 obtained from a fresh marine plankton sample and using the protein extraction procedure 3.

The precision of the method was study taking into account the results obtained for three replicates. As an example, the gel-like images obtained for fractions 6 corresponding to each extraction replicate (6.1, 6.2 and 6.3), are shown in Figure 7. The relative standard deviations for the proteins of Fractions 6, with molecular weight of 13.1, 19.0, 22.0, 30.5 and 52.6 kDa, were 12, 2, 8, 12 and 9 %, respectively. Taking into account the RSDs values obtained and the number of steps on the procedure, we consider that the method is precise.

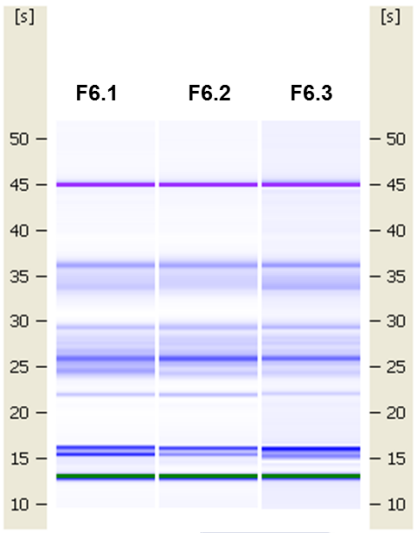

Figure 7: Gel-like images obtained for fractions 6 corresponding to each extraction replicate (6.1, 6.2 and 6.3).

5.5. Conclusion

Three protein extraction procedures for protein extraction from plankton samples were studied. OFFGEL Electrophoresis combined with Lab-on-a-Chip technology was applied for protein analysis in a plankton sample. Taking into account the intensity and the number of the protein bands obtained, the protein extraction procedure using phenol/sodium dodecyl sulphate after different washing steps with 10% trichloroacetic acid /acetone solution, was selected as the best extraction procedure for this type of sample. The method was applied for protein analysis from a fresh plankton sample. The proteins found in this sample had a molecular weight ranging from 6.4 to 57.3 kDa and the proteins with the highest molecular weight were in the OFFGEL fractions with an isoelectric point from 4.40 to 8.60. The protein concentrations varied from 50.0 to 925.9 ng μL^{-1}. The method was precise, obtaining RSDs values lower than 12 %.

The proposed method offers some advantages, such as low sample consumption, less tedious procedures, good repeatability and shorter analysis time. Further

studies will be performed to identify the separated proteins by proteomics techniques.

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6. Integrated OFFGEL and Lab-on-chip electrophoresis for characterizing marine plankton proteins

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Integrated OFFGEL and Lab-on-chip electrophoresis for characterizing marine plankton proteins

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Abstract: *A novel application of two-dimensional OFFGEL and lab-on-a-Chip (LOC) electrophoresis was performed for assessing proteins from marine plankton. Proteins were isolated from plankton samples by using a phenol / SDS mixture as an extracting solution after different washing steps involving 10 % TCA / acetone and methanol. Proteins exhibiting the highest molecular weight MW, within the 52.6 - 57.3 kDa range, were found in different OFFGEL fractions of isoelectric points (pIs) ranging from 4.40 to 8.60; whereas, the highest protein concentrations were found in fraction 5 (925.9 ng μL^{-1} for a protein of pI of 4.5 and MW of 22.0 kDa,), fraction 16 (492.8 ng μL^{-1}, protein of pI / MW of 7.4 / 19.1 kDa,), and fraction 8 (459.9 ng μL^{-1},pI / MW of 5.3 / 22.2 kDa,). Further studies were also developed for determining the amount of trace metals associated to the isolated proteins of similar pIs by electrothermal atomic absorption spectrometry (ETAAS) and inductively coupled plasma optical emission spectrometry (ICP-OES). Preliminary results indicate that Fe, Ni, Mn and Zn were found to be associated to proteins of variable MW (from 14 to 54 kDa) and pIs (from 3.4 to 8.2). Otherwise, Cd was found to be associated to proteins of certain pIs (6.0 - 6.5) and with spread MW (from 14.1 to 53.1 kDa).*

Keywords: *Lab-on-a-chip electrophoresis, marine plankton proteins, OFFGEL electrophoresis, trace metals.*

6.1. Introduction

Marine plankton plays a double role in the marine ecosystem. First, beta barrel proteins as well as other major outer membrane proteins, such as OmpA-like proteins, are considered to be the sources of dissolved protein in seawater [1,2]. Therefore, plankton directly affects the global carbon cycle in oceans [3]. Second, marine plankton takes up nutrients (C, N, P, and Si) and essential micronutrients (Mn, Fe, Co, Ni, Cu, and Zn) [4], and pollutants from seawater, which affects the bioavailability and transport of nutrients and pollutants in the marine ecosystem The presence of these trace elements in the marine environment seems to have influence on the primary productivity. As example, Mn, Fe, Co, Ni, Cu and Zn

deficiency may limit oceanic plankton production, while, an excess of some of these metals may inhibit plankton growth [5]. The importance of these micronutrients is explained taking into account the physiological functions of the different metalloproteins and/or metal-binding proteins, which can act as transporters of essential ions, or can be involved in defence mechanisms of the organism against heavy metal stress [6]. Protein identification/characterization of these protein types should therefore consider the identification of trace elements associated to them [7].

Different protein extraction procedures have been reported in the literature as a first step in proteomic studies for different materials such as plant tissues [8-10], soybean roots [11], and dinoflagellate *Alexandium sp* [12]. The protein extraction protocol depends on the sample matrix and must be optimized for each sample type to obtain the highest extraction efficiency. Regarding proteins isolation from plankton, some reports can be found in the literature [13-15]. Moncheva et al. [13] have proposed the use of 20 % (w/v) TCA in acetone containing 0.1 % 2-mercaptoethanol and 1 % SDS for protein isolation from marine phytoplankton; whereas, Jiménez et al. [14] after comparing different extraction, purification and concentration conditions have proposed the treatment with 25 M Tris-HCl buffer plus a protease inhibitor as the most efficient mixture for protein extraction from a plankton based-certified reference material (BCR-414). Recently, García-Otero et al. [15] have also found the usefulness of a based-phenol/SDS extraction for BCR-414 proteins isolation after different TCA / acetone and methanol washing stages.

Concerning proteins assessment, SDS-PAGE as well as 2-DE have commonly be used for proteome analysis [10,12,16]. In general, two-dimensional gels methods are time-consuming and tedious procedures, mainly because after immobilized pH gradient (IPG) - isoelectric focusing (IEF) when separating proteins of similar isoelectric point (pI), several steps (gel cutting, protein extraction and cleaning up) are needed before protein separation according to their molecular weight (second dimension). Recently, the introduction of preparative IPG IEF where proteins can be recovered from liquid phase (OFFGEL electrophoresis) allows the direct

sampling of the isolated protein (first dimensional separation) into HPLC instruments as well as in microfluidic chip-based liquid chromatography [17,18]. The use of OFFGEL fractionation technique combined with liquid chromatography – mass spectrometry (LC-MS) and – tandem mass spectrometry (LC-MS/MS) have been reported for protein fractionation/identification mainly for clinical purposes [17,19-23]. On other occasions, first fractionation by OFFGEL has been combined with conventional SDS-PAGE [12,24-26] for quantitative and qualitative sizing of proteins. Microfluidic chip-based devices such as capillary gel electrophoresis by using Lab-on-a-chip technology (LOC electrophoresis) can be an alternative approach for assessing information concerning the second dimension (molecular weight sizing). This methodology has been used by different authors for qualitative and quantitative protein determination in different matrices such as soybean cultivars, tear fluid, human granulocyte-colony, and surface and deep seawater [27-30].

In addition to the improved sensitivity inherent to LOC electrophoresis, low sample consumption as well as fast electrophoresis separations are other advantages of this technique [31-34]. The combination of OFFGEL and LOC electrophoresis is therefore an appealing methodology which reduces the number of handling steps inherent to conventional 2-DE. The aim of this work has been the application of an optimized protein extraction procedure from fresh marine plankton samples before OFFGEL-LOC electrophoresis. Since OFFGEL electrophoresis allows recovering proteins exhibiting similar pIs (OFFGEL fractions) in liquid phase, a second goal of work has been the assessment of trace elements bound to the extracted proteins by simple and direct analysis of these fractions by conventional atomic absorption techniques such as ETAAS and ICP-OES. Although some developments have been addressed to protein assessment in fresh marine plankton by SDS-PAGE [13] and in a plankton based-certified reference material (BCR-414) by SDS-PAGE [14] and OFFGEL-LOC [15], to the best of our knowledge, the assessment of marine proteins from fresh marine plankton by OFFGEL-LOC and, mainly, the characterization of trace elements bound to plankton proteins have not yet been

addressed. Therefore, OFFGEL-LOC operating conditions have been set for assessing the pIs and MWs of the isolated proteins, and then, the determination of trace elements bound to proteins of similar pIs was carried out. Because of OFFGEL conditions could alter the metal-protein complexes, a study based on the use of superoxide dismutase (SOD) and alcohol dehydrogenase (ADH), as metal-protein complex models, was also performed.

6.2. Experimental

6.2.1. Reagents

The pH 3 - 10 IPG Dry Strips, IPG Buffer 3 - 10 and Plus One DryStrip cover fluid used for protein fractionation were supplied by GE Healthcare Life Science (Uppsala, Sweden). Urea (electrophoresis grade), thiourea (analytical grade) ammonium acetate and phenol solution, equilibrated with 10 mM Tris-HCl (pH=8.0, 1 mM EDTA) were from Sigma Aldrich (St. Louis, MO, USA). DTT (electrophoresis grade), methanol, Tris-HCl and glycerol solution were purchased from Merck (Darmstadt, Germany). TCA and acetone were from Panreac (Barcelona, Spain), and β-mercaptoethanol was from Fluka (Vancouver, Canada). SDS and sucrose were purchased from AppliChem (Darmstad, Germany). The Agilent protein 80 Kit containing the protein chips, Protein 80 Gel-Matrix, Protein 80 Dye Concentrate, Protein 80 Sample Buffer and Protein 80 Ladder was supplied by Agilent Technologies (Waldbronn, Germany). Total protein assessment was performed with a Bradford protein assay containing Coomassie Brilliant Blue G-250 and BSA from Thermo Scientific (Rockford, IL, USA). Isolated proteins (Bradford assay) were re-dissolved in 2-DE rehydration solution prepared with 5 M urea, 2 M thiourea, 2 mM tributyl-phosphine (Sigma-Aldrich), 65 mM DTT, 65 mM CHAPS (Sigma-Aldrich) , 0.15 M NDSB-256, 1 mM sodium vanadate (Sigma-Aldrich) , 0.1 mM sodium fluoride (Sigma-Aldrich), and 1 mM benzamidine (GE Healthcare Life Science). Element standard solutions were prepared from Cd, Co, Cr, Cu. Fe, Mn, Ni, Pb, and Zn stock standard solutions (1.000 g L^{-1}) from Scharlau (Barcelona, Spain). BSA standard (2 mg mL^{-1}) was from Thermo Scientific (Rockford, IL, USA).

Superoxide dismutase (SOD) from bovine erythrocytes and alcohol dehydrogenase (ADH) from Saccharomyces cerevisiae were from Sigma–Aldrich. Ultrapure water, resistivity 18 MΩcm (25 °C), was obtained from a Milli-Q water-purification system (Millipore).

6.2.2. Instruments

Two dimensional electrophoresis was performed with a 3100 OFFGEL fractionator and a Bioanalyzer 2100 from Agilent Technologies (Santa Clara, CA, USA). A SIGMA 2K15 centrifuge (SIGMA Laborzentrifugen GmbH, Osterode, Germany) was used proteins extraction. A centrifuge Centromix (Selecta, Barcelona, Spain) was used to pre-concentrate plankton samples. A Heidoph shaker, type Reax 2000, used for vortexing samples, was from Gemini B.V. (Apeldoorn, Netherlands). A Perkin Elmer Model 1100B (Perkin Elmer, Norwalk, CT, USA) atomic absorption spectrometer equipped with an HGA-700 graphite furnace atomizer, deuterium background correction and an AS-70 auto-sampler was used for determining Cd, Co, Cr, Cu, Mn, Ni, and Pb in the OFFGEL fractions. The sources of radiation were monoelement hollow cathode lamps (HCLs) from Cathodeon, Cambridge, U.K (Cr, Cu, Mn and Ni HCLs); whereas, Co and Pb HCLs were LuminaTM type from Perkin Elmer. An Optima 3300 DV inductively coupled plasma (ICP) atomic emission spectrometer (Perkin Elmer) equipped with an autosampler AS 91 (Perkin Elmer) and a Gem-Cone cross-flow nebulizer type (Perkin Elmer) was used for Cu, Fe and Zn determinations. A Hitachi double–beam spectrophotometer model U–2010 (Hitachi, Berkshire, UK) equipped with 10 mm quartz cells was used for all UV–visible measurements (Bradford assay).

6.2.3. Plankton sample collection

A fresh marine plankton sample (5 L) was collected in triplicate from a clam hatchery located in the Ría de Arousa estuary (Northwestern of Spain). After collection, each plankton sample was centrifuged at 6400 g for 15 min at 20 ºC. Because centrifuge rotor consists of four 25 mL centrifuge tubes, several

centrifugation stages were needed for completing plankton isolation from the collected plankton sample. After each centrifugation stage, the supernatant was removed, and the isolated plankton fractions were finally combined. Isolated wet plankton masses from each 5 L collected samples were within the 4.0 – 4.5 g range.

6.2.4. Procedure for protein extraction from plankton sample

The protein extraction procedure was optimized in previous studies [15]. The method is based on the extraction protocol proposed by Wang et al. [9] for protein extraction from recalcitrant plant tissues. The whole wet plankton isolated from each sample (three sub-samples) was firstly ground in a mortar under liquid nitrogen. Protein extraction was performed from 1.000 g of the ground material by subjecting ten different portions (0.1000 g each one) to the following process: Each 0.1000 g subsample was first washed with 2 mL of 10 % TCA in acetone by vortexing for a few seconds before centrifugation at 16000 g and 4 ºC for 3 minutes. After discarding the supernatant, the tubes were filled with 2 mL of 0.1 M ammonium acetate in 80 / 20 methanol / water, and the mixtures were again vortexed and centrifuged (same conditions) before removing the supernatant. The solid residue was finally washed with 2 mL of acetone and oven-dried at 50 ºC for at least 10 minutes. After this treatment, the plankton proteins were extracted by adding 0.8 mL of phenol (pH 8.0) plus 0.4 mL of SDS buffer (30 % (m/v) sucrose, 2 % (m/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5 % (v/v) β-mercaptoethanol and protease inhibitor cocktail), and thoroughly mixed and kept at room temperature for 5 minutes before centrifugation (16000 g 4 °C) for 3 minutes. The upper phenol phase containing proteins was then transferred into new 2 mL tubes, and was mixed with 0.1 M ammonium acetate in methanol and kept at -20 ºC for a time of at least 2 hours. Precipitated proteins were then centrifuged (4000 g, 4 °C) for 5 minutes, and the protein pellet was finally washed once with methanol and once with 80 / 20 acetone / water. Protein pellets isolated from each 0.1000 g ground plankton were combined before drying and dissolution in 3000 µL of Protein OFFGEL Stock solution mixed with 750 µL of ultrapure water before OFFGEL

fractionation. The Protein OFFGEL Stock solution contains 25.2 g of urea, 600 mg of DTT, 6 mL of glycerol and 600 µL of OFFGEL Buffer (pH 3 - 10) in a final volume of 50 mL with ultrapure water.

6.2.5. Procedure for proteins separation by OFFGEL electrophoresis

Re-dissolved proteins from each protein pellet were separated according to their isoelectric point (pI) using 24 cm, pH 3 - 10 IPG Dry Strips following the OFFGEL manufacturer's instructions. Because the marine plankton was treated in triplicate, three different replicates of each 24 OFFGEL fractions were obtained. The strips were first rehydrated for 15 min in the 24-well assembled device with 40 µL of OFFGEL Rehydration solution (solution prepared by mixing 960 µL of Protein OFFGEL Stock solution with 240 µL of ultrapure water). Then, 150 µL of the re-dissolved proteins solution were loaded in each well of the OFFGEL Fractionator, and pI-based separation was performed under operating conditions detailed in Table 1. Recovered OFFGEL fractions were stored at -20 ºC until further analysis.

Table 1: Instrumental operating conditions for OFFGEL fractionation.

	Volt. Hour [KV h]	Voltage [V]	Current [µA]	Power [mW]	Time [h]
Focusing	64.0	4500	50	200	100
Hold		500	20	50	

6.2.6. Procedure for the protein separation and quantification by LOC electrophoresis

The proteins present in each OFFGEL fraction (24 OFFGEL fractions in triplicate) were further separated according to their molecular weights by LOC electrophoresis with the Protein 80 LabChip kit which allows proteins sizing and quantification within the 5 - 80 kDa range. Portions of 4 µL of OFFGEL proteins fraction were mixed with 2 µL of denaturing solution (1 µL of β-mercaptoethanol plus 28.6 µL of Protein 80 Sample Buffer) in 0.5 mL eppendorf tubes. The latter

solution contains a lower (1.6 kDa) and an upper (95 kDa) marker, which allows the correct alignment of each lane. Sample tubes were then heated at 95 - 100 ºC in a thermostatic water bath for 5 minutes, and after cool down 84 µL of ultrapure water were added before vortexing. Similarly, 6 µL of Protein 80 Ladder solution (mixture of proteins exhibiting different molecular weights) were treated as above. The standard protein ladder is loaded on each chip (commonly coded as "lane L"), and allows an estimation of the appropriate molecular weight of the separated proteins. This solution also contains the lower marker and the upper marker, proteins of known molecular weight at concentrations of 1 ng μL^{-1} and 60 ng μL^{-1}, respectively, used for proteins quantification. Before loading the samples (6 µL) and the ladder (6 µL) into the chips, they were filled with 12 µL of the Gel-Dye Mix (G/D) and Destaining solution (DS). The G/D and DS solutions were prepared following manufacturer's recommendations: the DS solution consists of 650 µL of Protein 80 Gel-Matrix previously filtered and centrifuged; whereas, the G/D solution is a mixture of 650 µL of Protein 80 Gel-Matrix (also filtered and centrifuged) with 25 µL of Protein 80 Dye concentrate. LOC electrophoresis (proteins detection by laser-induced fluorescence) was performed under recommended manufacturer's conditions. The Agilent 2100 Bioanalyzer was controlled by software that includes data collection, reporting and interpretation functions.

6.2.7. Protein quantification by the Bradford assay

Total protein determination in the re-dissolved protein pellets was assessed by the Bradford method (Coomassie Brilliant Blue G-250 as a chromogenic reagent, and measurement at 595 nm). Calibration was performed with BSA as a protein standard prepared in the 2-DE rehydration solution (see section 6.2.1.). The standards were prepared by diluting the stock standard solution with the 2-DE rehydration solution before mixing with the chromogenic reagent. Preparation of standard was as follows: vial A (1000 µg mL^{-1}) consists of 40 µL of BSA stock solution; vial B (500 µg mL^{-1}), 20 µL of vial A plus 20 µL 2-DE rehydration solution; vial C (250 µg mL^{-1}), 20 µL vial B plus 20 µL 2-DE rehydration solution; vial D (125 µg mL^{-1}), 20 µL vial C plus 20 µL 2-DE rehydration solution; vial E (62.5 µg mL^{-1}),

20 µL vial D plus 20 µL 2-DE rehydration solution; vial F (31.25 µg mL^{-1}), 20 µL vial E plus 20 µL 2-DE rehydration solution; and vial G (0.00 µg mL^{-1}), 20 µL 2-DE rehydration solution. These solutions were mixed with 600 µL of Coomassie Brilliant Blue G-250 (final volume of 620 µL) which offer BSA concentrations of 0.0, 1.0, 2.0, 4.03, 8.06, 16.1, and 32.23 µg mL^{-1}. Samples (re-dissolved protein pellets) were prepared by mixing 1 µL with 19 µL of 2-DE rehydration solution before addition of 600 µL of chromogenic reagent. Finally, both samples and standards were kept at room temperature for at least 5 min before spectrophotometric quantification at 595 nm.

6.2.8. Determination of Cd, Co, Cu, Mn, Ni and Pb by ETAAS

Cd, Co, Cr, Cu, Mn, Ni, and Pb were determined in OFFGEL fractions containing proteins by ETAAS. The graphite furnace programs used for their determination are shown in Table 2. OFFGEL fractions (100 µL) were first made up to 500 µL with 2 % (v/v) nitric acid. A volume of 50 µL of the acidified OFFGEL fraction were then mixed with the chemical modifier (25 µL of 500 mg L^{-1} palladium nitrate and 25 µL of 200 mg L^{-1} magnesium nitrate), and 100 µL of ultrapure water (final volume of 200 µL). A volume of 20 µL of this solution was injected into the graphite furnace tube for metal determination. Determinations were performed by using aqueous standards matched with Protein 80 OFFGEL standard solution (100 µL) plus 2 % (v/v) nitric acid (400 µL). Calibrations for Co, Cr, Cu, Ni and Pb ranged from 0 to 20 µg L^{-1}, while Mn and Cd calibrations were performed within the 0 - 10 µg L^{-1} and 0-4 µg L^{-1} ranges, respectively. The limit of detection (LOD) of the methods (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) referred to the OFFGEL fraction (1:20 dilution) and expressed as µg L^{-1} were 0.90, 4.1, 12.2, 13.5, 4.6, 17.6, and 14.1 µg L^{-1} for Cd, Co, Cr, Cu, Mn, Ni, and Pb, respectively. Similarly, the limit of quantification (LOQ) of the methods (10 SD criterion) were 3.1, 13.7, 40.7, 45.2, 15.5, 58.7, and 47.0 µg L^{-1} for Cd, Co, Cr, Cu, Mn, Ni, and Pb, respectively.

Table 2: Operating conditions for Cd, Co, Cr, Cu, Mn, Ni and Pb determination by ETAAS.

Step	Temperature (ºC)	Ramp time (s)	Hold Time (s)	Ar flow (mL min^{-1})
Drying 1	110	1	20	300
Drying 2	150	5	30	300
Pyrolisis	600 (Cd), 800 (Pb), 1400 (Cu, Mn), 1600 (Ni), 1500 (Co, Cr)	10	20	300
Atomization	1800 (Cd, Pb), 2000 (Mn), 2200 (Co, Cu), 2400 (Ni), 2500 (Cr)	0	2 (Cd), 5 (Co, Cr, Cu, Mn, Ni)	0 (READ)
Cleaning	2600	1	2	300
Wavelength / nm: 228.8 (Cd), 242.5 (Co), 357.9 (Cr), 324.8 (Cu), 249.5 (Mn), 232.0 (Ni), 283.3 (Pb).				
Slit width / nm: 0.7 (Cd, Cr, Cu, Pb), 0.2 (Co, Mn, Ni)				
Injection volume / µL: 20				
Deuterium background correction system: Cd, Co, Mn, Ni, Pb.				
Zeeman effect background correction system: Cr				
Tubes: Pyrolytic coated graphite tubes with L'vov platforms				
Integrated absorbance measurement				
Chemical modifier: $Mg(NO_3)_2$ / $Pd(NO_3)_2$ (25/62.5 mg L^{-1})				

6.2.9. Determination of Cu, Fe and Zn by ICP-OES

Iron and Zn determination in OFFGEL fractions from plankton, and also Cu and Zn determination when performing metal-binding protein stability studies, were performed by ICP–OES (axial configuration) using the instrumental conditions listed in Table 3. Fe and Zn determination volumes of 200 µL of each OFFGEL fraction were mixed with 1800 µL of 2 % (v/v) nitric acid, and directly nebulized into the ICP torch. Calibrations (Fe and Zn ranging from 0 to 2 mg L^{-1}) were performed with aqueous standards matched with Protein 80 OFFGEL standard solution (0.5 mL) made up to 5 mL with 2 % (v/v) nitric acid. Regarding metal-proteins stability studies, matched calibrations (covering concentrations from 0 to 2 mg L^{-1}

Cu and Zn) with Protein 80 OFFGEL standard solution (500 µL) diluted to 10 mL with 1 % (v/v) nitric acid were used. Metal were determined after diluting 100 µL of each OFFGEL fractions with 1900 µL of 1 % (v/v) nitric acid. The LODs (3 SD criterion) were 0.08, 0.006, and 0.09 mg L^{-1} for Fe, Cu and Zn, respectively. Similarly, the LOQs (10 SD criterion) were 0.3, 0.02, and 0.3 mg L^{-1} for Fe, Cu, and Zn, respectively.

Table 3: Instrumental conditions for Cu, Fe and Zn determination by ICP-OES**.**

General	Radiofrequency power / W	1300
	Sample uptake rate / mL min^{-1}	1.5
	Stabilization delay / s	45
	Number of replicates	4
	Integration time / s	5
	Nebulizer type	Cross flow
	Axial view	
Gas flows / L min^{-1}	Plasma	15.0
	Auxiliary	0.5
	Nebulizer	0.8
Detection wavelengths / nm	Cu	327.393
	Fe	238.205
	Zn	213.857

6.3. Results and discussion

6.3.1. Fractionation and quantification of proteins from fresh plankton samples

Total protein contents were assessed (section 6.2.7) before OFFGEL fractionation. Values of 0.72, 0.65, and 0.56 mg mL^{-1} were calculated in the three replicates (mean concentration of 0.64 ± 0.080 mg mL^{-1}). Proteins were then fractionated based on the pI (OFFGEL operating conditions listed in Table 1), separation

perform in approximately 38 hours. Proteins present in each OFFGEL fraction, except in OFFGEL fraction 23 and 24 in which any protein was found, were further separated according to their molecular weight by LOC (details in section 6.2.6). The migration time or the molecular weight are the parameters used for protein sizing; whereas, the fluorescence intensity is used for quantification purposes. Because the migration times and the fluorescence intensities can vary during the analysis of multiple samples, internal and external standards have to be used for sizing and quantification. The internal standard (upper marker) is a protein that is part of the sample buffer and is added to each sample at a fixed ratio. A lower marker is added to the sample in the same way and it is used for alignment purposes and to define the measurement range. The external standard is a specific protein (ladder) provided in the Protein 80 kit, which is analyzed on-chip before the sample analysis. For protein size determination, the migration times are compared with the ladder migration times.

In the procedure described in section 6.2.6, the sample (4 µL) with the denaturing solution (2 µL) was introduced in a 0.5 mL eppendorf tube and heated for 5 minutes at 95 - 100 ºC in a thermostatic water-bath. In previous studies performed by Schmut et al. [28] the effect of sample treatment on separation profiles of tear fluid proteins using LOC electrophoresis was evaluated. These authors studied the influence of the incubation temperature (20 and 37 ºC) and the time (10, 20, 30 minutes) on the protein profiles. The authors concluded that the incubation time of 30 minutes at 37ºC improved the separation profile as well as increased the sensitivity when detecting tear fluid proteins. Taking into account these results, preliminary experiments were performed to study the influence of the incubation temperature. Therefore, experiments by fixing the incubation time at 5 min were performed at incubation temperatures of 37ºC and 95ºC (temperature indicated in the Protein 80 LabChip kit procedure provided with the kit). As an example, the overlaid electropherograms of proteins present in the OFFGEL fraction 5 (pIs within the 4.40 - 4.66 range) are shown in Figure 1. The best separation profiles and the highest sensitivity were obtained when incubating at 95ºC. This

temperature was therefore selected. Regarding the incubation times, similar protein profiles were obtained when incubating at 95°C for 5, 10 and 30 min. Therefore, this variable was fixed at 5 min to speed up the preparation process.

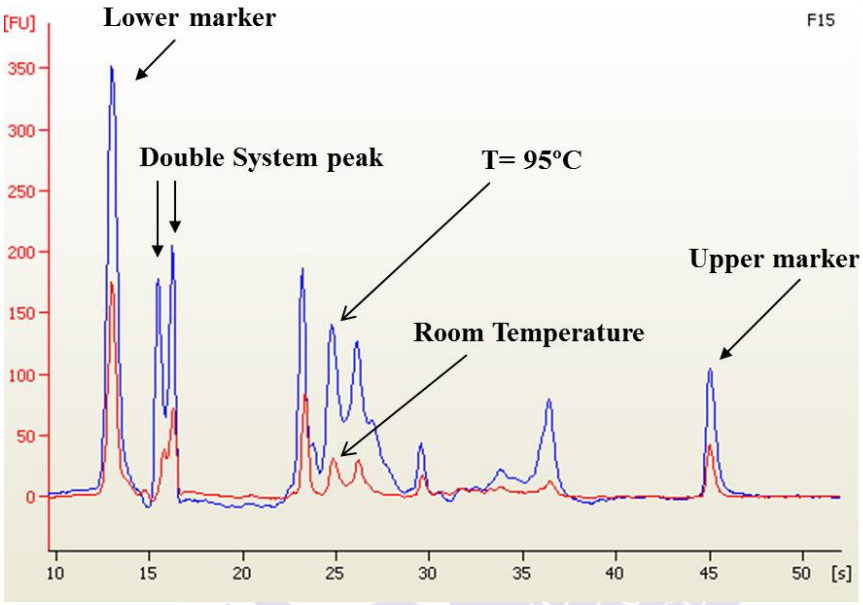

Figure 1: Overlay of electropherograms of protein profiles from plankton separated after incubation at room temperature and 95ºC for 5 min.

Proteins were quantified using external calibration with Bovine Serum Albumin (BSA) in a concentration ranging from 50 to 1000 ng μL^{-1}. The gel-like image obtained is shown in Figure 2 (individual electropherograms for each OFFGEL fraction are given as supplementary information in Annexes I (figures I – XI)); whereas, and the concentrations (mean ± standard deviation for three replicates) as well as the molecular weight (MW) of each separated protein are listed in Table 4. In general, MWs of the isolated proteins vary from 13.1 to 57.3 kDa, and most of the found proteins are of low MW, within the 14-20 kDa range. The proteins exhibiting the highest MW are present in OFFGEL fractions 5-13 15, 18, 19 and 20 (OFFGEL fractions containing proteins with pI from 4.40 to 8.60). It must be mentioned that proteins were not found in OFFGEL fractions 23 and 24,

comprising pIs from 9.13 to 9.39, and from 9.39 to 9.65, respectively. Although the nature of the marine plankton can vary enormously, MWs of the isolated proteins are quite similar to those published data reported by Moncheva et al. [13] for marine phytoplankton proteins assessment by SDS-PAGE. The authors have reported some discrete proteins bands with apparent MWs of 14, 29, 37, 48 and 70 kDa. However, data on pIs of the separated proteins as well as protein concentrations have not given. Our findings show that the proteins which occur at the highest concentration are found in OFFGEL fraction 5 (a protein of MW of 22.0 kDa at 925.9 ng μL^{-1}), fraction 8 (MW of 22.2 kDa, 459.9 ng μL^{-1}), fraction 11 (MW of 19.4 kDa, MW of 394.0 ng μL^{-1}; MW of 22.5 kDa, 380.0 ng μL^{-1}; and MW of 52.7 kDa, 409.9 ng μL^{-1}), and fraction 16 (MW of 19.1 kDa, 492.8 ng μL^{-1}). It must be mentioned that the protein concentrations given are relative concentrations, i.e., concentrations referred to the OFFGEL fraction isolated in accordance to the pIs.

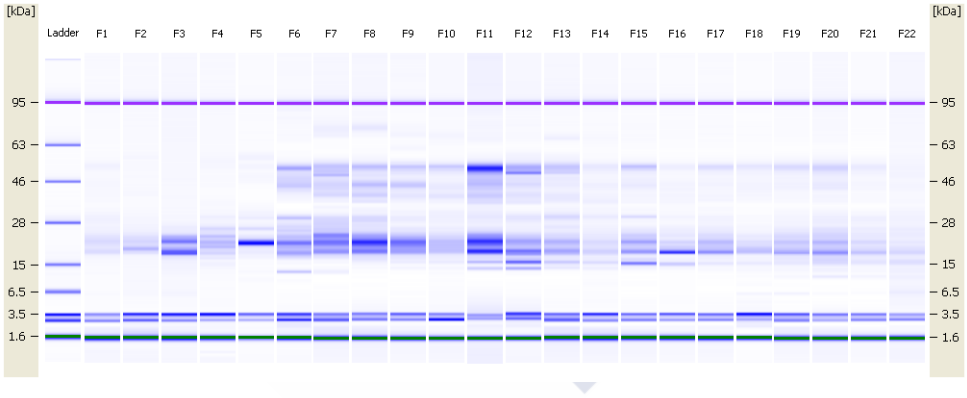

Figure 2: Gel-like images obtained for OFFGEL fractions 1 to 24 from a fresh plankton sample.

Finally, the low standard deviation values listed in Table 4 and Table 5 when assessing protein concentration indicate good repeatability of the whole procedure (plankton collection, protein extraction, and OFFGEL and LOC electrophoresis separation). In general, relative standard deviations (RSDs) were lower than 25 % for proteins quantification.

Table 4: Molecular weight and protein concentrations (n = 3) in OFFGEL fractions (F1 – F12) from a fresh marine plankton sample.

F1			F2			F3			F4			F5			F6		
MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %
19.4 22.6	54.1±11.4 56.5±10.7	21 19	20.3 22.5	121.6±27.3 66.8±15.2	22 23	18.9 22.6	352.8±3.5 291.3±13.1	1 5	18.9 21.0 22.2 24.2 26.4	84.4±4.3 142.3±6.5 152.4±23.5 129.9±4.8 78.9±7.1	5 5 15 4 9	19.3 22.0 26.5 30.6	59.9±0.3 925.9±25.1 127.4±3.3 57.3±6.0	1 3 3 10	13.1 19.0 22.0 25.9 30.5 44.6 52.6	115.2±15.4 195.1±13.3 304.1±52.1 90.5±11.9 128.2±9.6 108.9±9.3 196.9±3.8	14 7 17 13 7 9 2
F7			**F8**			**F9**			**F10**			**F11**			**F12**		
MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %	MW kDa	Conc ng µL^{-1}	CV %
19.3	225.0±33.3	15	19.5	284.7±23.4	8	19.6	202.3±46.2	23	19.7	149.8±3.9	3	14.1	72.5±15.6	22	14.1	166.8±12.2	7
22.3	302.1±42.0	14	22.2	459.9±30.8	7	22.3	317.0±12.6	4	21.3	149.5±34.9	23	16.1	114.6±5.8	5	16.1	336.9±58.7	17
24.4	256.9±36.8	14	27.4	50.0±2.6	5	44.8	113.2±4.7	4	22.4	151.2±4.0	3	19.4	394.0±22.8	6	19.5	306.5±60.1	20
27.9	84.3±7.8	9	30.4	71.8±0.8	1	53.3	116.4±17.1	15	39.9	56.9±13.1	23	22.5	380.0±6.1	2	22.6	194.9±27.1	14
40.8	74.9±13.0	17	40.4	90.7±0.4	1				53.3	128.4±19.8	15	40.3	82.3±17.2	21	45.3	62.0±11-3	18
45.1	82.6±20.6	25	44.9	133.0±0.3	1							45.4	113.9±1.1	1	50.4	270.0±7.0	3
49.4	142.5±37.0	26	49.4	48.6±10.4	21							52.7	409.9±60.5	15	53.1	174.0±20.4	12
51.5	110.2±27.3	25	53.3	129.1±18.6	14												
53.5	117.2																

Table 5: Molecular weight and protein concentrations (n = 3) in OFFGEL fractions (F13 – F22) from a fresh marine plankton sample.

F13			F14			F15			F16			F17			F18		
MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %
16.1	117.0±8.5	7	15.9	77.8±16.5	21	15.6	321.8±31.8	10	15.4	121.7±25.2	21	15.6	51.0±10.4	20	19.4	167.5±45.6	27
19.5	202.9±5.3	3	19.4	101.5±6.1	6	19.3	243.4±53.8	22	19.1	492.8±28.6	6	19.1	268.9±2.3	1			
22.4	172.1±18.7	11	22.3	78.8±17.7	22	22.3	220.0±44.0	20	22.2	98.0±2.5	3	22.2	159.2±6.5	4			
31.2	78.3±2.7	3				24.2	109.9±11.7	11				24.2	79.0±17.6	22			
50.8	101.0±4.5	4				31.1	78.5±17.3	22									
53.4	124.8±2.5	2				53.7	139.9±15.3	11									

F19			F20			F21			F22		
MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %	MW kDa	Conc ng μL^{-1}	CV %
15.8	53.7±2.3	4	16.8	89.2±8.4	9	16.1	61.7±9.4	15	16.1	65.4±2.7	4
17.2	51.4±2.8	6	19	269.8±16.1	6	19.1	147.0±19.6	13	19.0	87.8±20.0	23
18.9	224.4±12.9	6	22.1	174.6±7.3	4	22.2	93.5±0.6	1			
22.2	141.7±12.7	9	23.9	97.6±16.9	17	24.0	51.7±12.2	24			
24.0	90.0±2.4	3	52.9	100.5±19.1	19						
53.3	86.8±2.5	3									

6.3.2. Stability study of metal-binding proteins during the OFFGEL electrophoresis

Recent studies performed by Jiménez et al [35] have evaluated the influence of different SDS-PAGE conditions (denaturing or non-denaturing conditions, applied current, post-separation gel treatment) in the stability of certain metal-binding proteins. The study used two proteins with different metal–protein affinities such as SOD, consisting of two units, each one of MW of 16.2 kDa containing Cu and Zn; and ADH, a tetramer with MW of 141 kDa which contains four equal subunits of 32.5 kDa and active sites containing Zn. PAGE under non denaturing conditions was found to be more suitable for Zn non-covalently complexed to proteins [35]. Similarly, OFFGEL IEF and PAGE conditions were also evaluated when separating of platinum-binding proteins by Mena et al. [36]. The authors concluded that a denaturing solution without thiourea is suitable for separating of platinum-proteins by OFFGEL IEF.

Taking into account the results obtained by these authors, different studies were performed to evaluate the effect of the denaturing OFFGEL conditions on the stability of the metal-binding proteins. Experiments were performed loading SOD (2.8 mg) and ADH (3.1 mg) aqueous solutions and using the OFFGEL operating conditions shown in Table 1. Similarly, a blank was also performed. The proteins present in each fraction after OFFGEL electrophoresis were then analyzed by LOC electrophoresis. The results showed that SOD was found in OFFGEL fractions 9 to 12 (pIs between 5.43 and 6.34); whereas, ADH appears in OFFGEL fractions 9 to 14 (pIs between 5.43 and 7.1). The MWs measured were 18.6 kDa and 39.8 kDa for SOD and ADH, respectively, values quite similar to the theoretical values for these proteins (16.2 kDa for SOD and 32.5 kDa for ADH). Cu and Zn were then analyzed in all 24 OFFGEL fractions to evaluate the possible metal-protein breakdown. Regarding SOD, Zn was found in the OFFGEL fractions containing SOD (fractions 9 - 12) with Zn concentrations of 6.9 ± 0.2, 26.6 ± 1.1, 3.0 ± 0.1 and 0.7 ± 0.1 mg L^{-1} in fractions 9, 10, 11 and 12, respectively. It must be said that

negligible Zn concentrations were detected in the remaining OFFGEL fractions. These findings imply that Zn-binding SOD offers high affinity. However, negligible Cu concentrations were found in OFFGEL fractions containing SOD (OFFGEL fraction 9 to 12), and Cu was found in OFFGEL fractions 22 and 23 (concentrations of 11.4 ± 0.4 and 6.10 ± 0.03 mgL^{-1}, respectively). This indicates Cu-SOD breakdown under the OFFGEL electrophoresis operating conditions used, and hence, weak metal–protein affinity. These results indicate Cu-SOD breakdown under the OFFGEL electrophoresis operating conditions used. These findings imply that Zn-binding SOD offers high affinity; whereas, Cu-SOD offers a weak metal–protein affinity.

For ADH experiments, Zn was only detected in OFFGEL fractions 9, 10 and 11 at concentrations of 5.2 ± 0.06, 22.2 ± 0.3 and 2.30 ± 0.07 mg L^{-1}, respectively. These OFFGEL fractions were those fractions containing ADH. Therefore, Zn-binding ADH appears also to offer high affinity.

Mass balance studies for Zn determination in SOD/ADH OFFGEL fractions were performed. A SOD solution (2.8mg of protein in 4.0mL of Protein OFFGEL stock solution) was analyzed for Zn, and a concentration of 2.2 ± 0.08 mg g^{-1} was obtained. The Zn concentration as a sum of Zn concentrations in fractions 9 - 12 was 2.4 ± 0.05 mg g^{-1}, which implies a Zn recovery percentage of 109 %, and certain stability of Zn-SOD complex during OFFGEL separation. Similar experiments performed with ADH offered a Zn concentration of 2.10 ± 0.06 in a ADH solution prepared from 3.1 mg of ADH in 4.0 mL of Protein OFFGEL stock solution; whereas, a Zn concentrations of 2.10 ± 0.08 mg g^{-1} was found as a sum of Zn concentrations in fractions 9 - 11 after OFFGEL fractionation. Zn recovery percentage was 100 %, which also demonstrates the stability of the Zn-ADH during OFFGEL electrophoresis.

6.3.3 Determination of metal-binding proteins from the plankton sample

Although the extractive conditions for isolating proteins from fresh marine plankton can alter the stability of certain metal-binding proteins, and although OFFGEL electrophoresis conditions can also affect the integrity of some metal-protein complexes, such as in the case of Cu-SOD, different experiments were developed to assess trace elements that could be bound to the isolated proteins. Therefore, the concentrations of Cu, Cr, Cd, Co, Pb, Mn, Ni, Fe and Zn were determined in all OFFGEL fractions to know the existence of certain trace metals associated with proteins of similar pI. Fe and Zn determinations were performed by ICP-OES (section 6.2.9). However, ETAAS, technique which provides low limits of detection, was needed when determining Cu, Cr, Cd, Co, Pb, Mn and Ni (procedure described in section 6.2.8).

Table 6: Cd, Cr, Cu, Fe, Mn, Ni, and Zn concentrations (n=3) in OFFGEL fractions.

Metal concentrations /µg L^{-1}						
Cd	Cr	Cu	Fe[a]	Mn	Ni	Zn[a]
F11: 48.2±0.7	F23: 57.8±9.4	F23: 229.4±4.6	F3: 0.30±0.001	F20: 120.7±2.2	F22: 152.4±7.7	F10: 1.21±0.004
		F24: 88.1±1.2	F4: 0.34±0.004	F21: 179.0±2.9	F23: 561.4±10.3	F11: 2.18±0.002
			F9: 0.30±0.001	F22: 161.9±8.0	F24: 250.5±2.6	F12: 0.98±0.003
			F17: 0.43±0.001	F23: 126.4±7.3		
			F18: 0.40±0.003	F24: 114.0±8.7		

(a) concentrations expressed in mg L^{-1}

Results are summarized in Table 6. Trace elements such as Co and Pb were not detected in any of the 24 OFFGEL fractions. Iron was found in OFFGEL fractions 3, 4, 9, 17 and 18, and the concentrations ranged from 0.30 mg L^{-1} (proteins in OFFGEL fraction 3 and 9) to 0.43 mg L^{-1} (proteins in OFFGEL fraction 17). Therefore, Iron appears to be bound to proteins of acid pIs (from 3.88 to 5.71, OFFGEL fractions 3, 4 and 9) and to proteins of pIs slightly alkaline (from 7.55 to 8.08, OFFGEL fractions 17 and 18). Most of the proteins found in these fractions are of low MW (from 15.6 to 26.4 kDa), although some proteins of higher MW (44.8

and 53.3 kDa in OFFGEL fraction 9, and 53.7 kDa in OFFGEL 18) are also found (Table 4 and Table 5). Zn was detected in OFFGEL fractions 10, 11 and 12 at concentrations ranging from of 0.98 to 2.18 mg L^{-1}. These OFFGEL fractions encompass proteins of acid pIs (from 5.71 to 6.50) and very different MWs (within the 14.1-53.3 kDa range). Cadmium (48.2 ± 0.7 µg L^{-1}) was detected in OFFGEL fraction 11 (pIs 5.98-6.24), in which several proteins of MW between 14.1 and 52.7 kDa are included. Manganese was found in OFFGEL fractions of alkaline pIs (fractions 20, 21, and 22). As shown in Table 5, OFFGEL fraction 20 encompass different proteins of MWs ranging from 19 to 53 kDa; whereas, proteins in OFFGEL fractions 21 and 22 are mainly proteins of low MW (within the 16.1 – 24.0 kDa range). The Mn concentration in these three OFFGEL fractions was between 120.7 ± 2.2 µg L^{-1} (OFFGEL fraction 20) and 179.0 ± 2.9 µg L^{-1} (OFFGEL fraction 21). Nickel was detected in OFFGEL fraction 22 (152.4 ± 7.7 µg L^{-1}), and could be associated to proteins of low MW (16.1 and 19.0 kDa) and alkaline pIs (8.86 - 9.13). It must be mentioned that Mn and Ni, as well as Cr and Cu are detected in OFFGEL fractions 23 and 24, fractions in which any protein was found (Table 5). The presence of these elements in OFFGEL fractions 23 and 24 can be attributed to certain metal-protein breakdown during the OFFGEL fractionation, in a similar way that occurred for Cu-SOD (section 6.3.2). In addition, the presence of Mn, Ni, Cu and Cr in these OFFGEL fractions is not attributed to metal contamination because analysis of the OFFGEL stock standard solution and OFFGEL fractions from ultrapure water subjected to the whole process gave negligible metal concentrations. Electropherograms when subjecting the OFFGEL fractions 23 and 24 to LOC electrophoresis showed a signal for low MW (6.5 kDa). The proteinaceous and/or amphoteric nature of these compounds can be responsible for complexing the major amount of released Mn, Ni, Cu and Cr. Taking into account the results obtained, further experiments are needed for elucidating the influence of the different OFFGEL reagents and OFFGEL conditions on the stability of Cu-, Cr-, Mn- and Ni- proteins complexes.

6.4. Conclusion

Marine plankton proteins were efficiently isolated by an improved method based on phenol/SDS extractions before several washing steps with a 10 % TCA in acetone, and with methanol. LOC electrophoresis was demonstrated to be affected by the incubation temperature, and the best performances were achieved by subjecting the re-dissolved protein pellet at a temperature of 95°C for only 5 min before LOC analysis. Several proteins have been observed after OFFGEL-LOC electrophoresis, most of them exhibiting pIs from 4.40 to 8.60, low MWs (within the 14 – 20 kDa range), and at concentrations from 50.0 to 925.9 ng μL^{-1}. Findings of the current study have revealed the existence of metal-protein complexes in fresh marine plankton. Trace elements such as Cd, Fe, Mn, Ni and Zn have been found to be bound to certain proteins, manly plankton proteins of acid pIs. This is the case of essential trace elements such as Fe and Zn which were associated to proteins of pIs from 3.88 to 4.40, and from 5.71 to 6.50 for Fe and Zn, respectively. Fe was also found to be associated to proteins of higher pIs (within the 5.45 – 5.71 range, and also from 7.55 to 8.08). Cd, a toxic trace element, was also found to be bound to proteins of acid pIs (from 5.98 to 6.24). It was also found that the affinity of the Cd-, Fe- and Zn-protein bounds was high because these three elements were not detected in the OFFGEL fractions 23 and 24, the most alkaline OFFGEL fractions which contain any protein and towards which the free ions migrate. Trace elements such as Mn and Ni were found to be associated to proteins of alkaline pIs (within the 8.34 – 9.13 range for Mn, and from 8.88 to 9.13 for Ni). However, higher concentrations of these two elements were found in OFFGEL fraction 23 and 24, which indicates a low affinity of some of the Mn- and Ni-protein complexes, which are easily broken down during the OFFGEL fractionation. Finally, trace elements such as Cr and Cu were only detected in the OFFGEL fractions 23 and/or 24, which indicates the breakdown of the isolated Cr-/Cu-protein complexes under the OFFGEL operating conditions (low affinity for the Cr-/Cu-protein bounds. Further experiments are therefore needed to evaluate the influence of the OFFGEL electrophoresis conditions in the stability of the isolated metal-protein complexes.

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7. Assessment of metals bound to marine plankton proteins and to dissolved proteins in seawater

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Assessment of metals bound to marine plankton proteins and to dissolved proteins in seawater

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Abstract: *Studies based on laser ablation – inductively coupled plasma – mass spectrometry (LA-ICP-MS) have been performed to assess metal bound to dissolved proteins and proteins from marine plankton after two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Dissolved proteins were pre-concentrated from surface seawater (60 L) by tangential ultrafiltration with 10 kDa molecular weight cut-off (MWCO) membranes and further centrifugal ultrafiltration (10 kDa) before proteins isolation by methanol/chloroform/water precipitation. Proteins isolation from plankton was assessed after different trichloroacetic acid (TCA) / acetone and methanol washing stages, and further proteins extraction with a phenol solution. LA-ICP-MS analysis of the electrophoretic profiles obtained for dissolved proteins shows the presence of Cd, Cr, Cu, and Zn in five spots analyzed. These proteins exhibit quite similar molecular weights (within the 10 – 14 kDa range) and pIs (from 5.8 to 7.3). Cd, Cr, Cu, and Zn have also been found to be associated to proteins isolated from plankton samples. In this case, Cd has been found to be bound to proteins of quite different molecular weight (9, 13 and 22 kDa) and pIs (4.5, 5.2, 5.5, and 10). However, trace elements such as Cr, Cu and Zn appear to be mainly bound to plankton proteins of low molecular weight and variable pI.*

Keywords: *2D polyacrylamide gel electrophoresis, laser ablation inductively coupled plasma mass spectrometry, metal-binding proteins, phytoplankton, dissolved proteins.*

7.1. Introduction

Plankton plays an important role in the ocean's carbon cycle. Phytoplankton in surface seawater, mainly photosynthetic cyanobacteria and single-celled algae [1], is responsible for about half the photosynthetic fixation of carbon (primary production) on Earth, and it is a primary producer of dissolved organic matter (DOM) in oceans [1,2]. DOM assessment in marine ecosystems is important because of the complexing properties of DOM for several dissolved substances

which conditions the fate of trace metals and hydrophobic organic contaminants by marine biota [3].

In addition to various macro- and micro- nutrients, non-essential or toxic metals are present at low concentrations in seawater. Trace metals can be captured by phytoplankton, and introduced into the marine food chain by producing extracellular organic matter with metal complexing properties [4]. Some metals, such as Cu, Zn, Fe, Cr and Co, are essential micronutrients for phytoplankton and act as co-factors in different enzymatic reactions. As an example, Fe is involved in phytoplankton growth; whereas, Cu plays an important role in photosynthesis. Other metals such as Pb and Cd are non-essential or toxic. These elements may displace bioactive trace metals in enzymes and thus they can alter the proper enzymes activity. Obviously, these changes produce significant effects at the different trophic levels in the aquatic food chain [5,6]. Therefore, metal-proteins complexes and metalloproteins are an important fraction of DOM as well as important constituents in marine plankton. Metal-binding proteins with high-affinity interactions are considered as metalloproteins, while metal-binding proteins with low-affinity interactions (easily broken) are referred to metal-protein complexes. Both, metalloproteins and metal-protein complexes, can be involved in complex biochemical reactions and can participate in several biological functions [7,8]. For this reason, metal-binding proteins assessment in phytoplankton, as well as the determination and characterization of DOM and dissolved proteins in water are issues of interest.

One or two-dimensional polyacrylamide gel electrophoresis (1D or 2D-PAGE) with subsequent laser ablation inductively coupled plasma mass spectrometry (LA–ICP–MS) has been increasingly used for the analysis of metalloproteins and metal-protein complexes [9]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used for the separation of protein mixtures on the basis of their molecular size. This technique applied in the one-dimensional mode has also been used for isolating dissolved proteins, but it fails when resolving complex protein mixtures [10-12]. In this case, 2D-PAGE is a

powerful and sensitive technique for separating individual proteins from complex samples. In the first dimension, proteins are separated according to their isoelectric point (pI) by isoelectric focusing (IEF); whereas, separation according to the molecular weight (MW) is achieved in the second dimension. 2D-PAGE has been used for characterizing MWs of both particulate and dissolved proteins in surface seawater, and proteins from marine plankton [13-15].

The choice of adequate sample preparation methods is important for obtaining reliable results by electrophoresis analysis. Different protein extraction procedures from plankton have been reported [16-19]. Jiménez et al. [16] have found that a treatment with a buffer solution consisting of 25 mM Tris–HCl and a commercial cocktail protease inhibitor, followed by protein precipitation with acetone was the most efficient procedure for protein extraction from plankton. Recently, García-Otero et al. [17] have proposed a protein extraction method for plankton which combines different washing stages with trichloroacetic acid (TCA) / acetone, and with methanol, before proteins extraction with phenol. Regarding dissolved proteins, main problems arise from the low concentrations in seawater and from the presence of high levels of dissolved salts. Therefore, pre-concentration methods for isolating the dissolved proteins from the saline matrix are needed. The literature shows that tangential ultrafiltration (UF) procedures are more adequate than solid phase extraction (SPE) methods for isolating dissolved compounds of high molecular weight such as dissolved proteins [10,11,13-15,20,21]. The selection of UF membranes of 10 kDa molecular weight cut-off guarantees the successful isolation of dissolved proteins in a minimum volume of retentate, which offers high pre-concentrations factors [22]. Further salts removal is also needed when performing chromatographic and electrophoresis analysis [22]. When dealing with dissolved protein, the formation of the protein pellets guarantees that most of dissolved salts remains in the liquid phase of the retentate. However, some protein precipitation procedures such as those based on ice-cold acetone (protein pellet formation at –20°C for 2 h) [23] have proved to be unsuccessful for further protein separation by OFFGEL electrophoresis [24]. In these cases, trichloroacetic acid as

a precipitating reagent [10,20], and combined procedures based on water and methanol soluble interferences (salts) removal before protein precipitation by chloroform [20] have offered reliable electrophoresis separations [24].

The assessment of metals bound to proteins is commonly performed by direct analysis of gels by LA-ICP-MS. In addition to the development by Jiménez et al. [16] for assessing metals bound to proteins from plankton, LA-ICP-MS methods after 1D and 2D electrophoresis have been performed for detecting P in standard phosphorylated proteins [25], for assessing Cu-, Zn- and Fe-containing human brain proteins [26], Zn-containing proteins in slug (Genus Arion) tissues [27], Se-containing proteins from sunflower leaves [28], Cd- and Zn-binding proteins in *Spinacia oleracea* [29], and determining Cu, Fe, Zn, Mn and Pb in metalloproteins from rat kidney [30]. Other developments involve the assessment of metal-humic acid complexes [31].

There are few developments regarding metal bound to proteins from plankton. Jiménez et al. [16] have recently applied LA-ICP-MS to assess metal bound to plankton proteins after 1D electrophoresis. However, this application has been performed with a plankton-based certified reference material (BCR-414), and the assessment of metal bound to proteins from fresh marine plankton remains un-reported. In addition, although various published papers on dissolved proteins characterization in seawater by 1D and 2D electrophoresis can be found in the literature, LA-ICP-MS developments focused on determining/identifying metals bound to dissolved proteins have not yet been addressed. The aim of the current work has been the application of LA-ICP-MS for determining metal-containing proteins from fresh marine phytoplankton and dissolved proteins in seawater. Detection/determination of trace metals associated to the isolated proteins has been performed after 2D-PAGE. Possibilities and problems found when applying the proposed methodology for assessing metals binding dissolved proteins and proteins from fresh phytoplankton are fully discussed.

7.2. Experimental

7.2.1. Apparatus

LA-ICP-MS measurements were performed with an UP-213 Nd-YAG LA system operating at 213 nm (New Wave Research, Huntingdon, UK) coupled to an ICP-MS (Elan 6000, Perkin Elmer Sciex, Toronto, Canada). Hitachi double–beam spectrophotometer model U–2010 (Hitachi, Berkshire, UK) equipped with 10 mm quartz cells was used for all UV–visible measurements. Isoelectrofocusing was performed with a Protean IEF System from Bio-Rad (Hercules, CA, USA), and second dimension was run in a Protean XL (BioRad). The gels were vacuum dried before LA-ICP-MS measurements with a model 583 gel drier (Bio-Rad). The tangential flow ultrafiltration (UF) system consisted of a Masterflex l/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane (nominal MW cut-off 10 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge. Centrifugal ultrafiltration was performed with an Alresa Digtor centrifuge (Madrid, Spain). Other laboratory devices were an ultracentrifuge Laborzentrifugen model 2K15 (Sigma, Osterode, Germany), a centrifuge Centromix (Selecta, Barcelona, Spain), a Reax top shaker from Heidoph (Schwabach, Germany), and an Basic 20 pH–meter with a glass–calomel electrode (Crison Instruments S.A., Barcelona, Spain).

7.2.2. Reagents and material

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore). Centrifugal ultrafiltration was performed with Vivaspin 20 ultrafiltration tubes (polyethersulfone membrane of 10 kDa molecular cut-off) from Sartorius Stedin Biotech (Goettingen, Germany). Seawater filtration was achieved by using Millipore HAWP14250 0.45 µm mixed esters of cellulose membrane filters (140mm diameter). Total protein assessment was performed with a Bradford protein assay containing Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) from Thermo Scientific (Rockford, IL, USA). IPG Dry Strips (7

cm, pH 3 - 10; and 10 cm, pH 3 - 10), and Plus One DryStrip cover fluid (GE Healthcare Life Science, Uppsala, Sweden) were used for pI protein fractionation. Buffer solution (10mL) for equilibration after IEF was prepared with 6 M urea, 0.375 M Tris-HCl (pH 8.8), 20 % (v/v) glycerol, 2 % (w/v) sodium dodecyl sulphate (SDS), 0.002 % (w/v) bromophenol blue and 100 mg DTT (all reagents from Sigma Aldrich, St. Louis, MO, USA). SDS gel (10 mL) was prepared with 2.3 mL of water, 5 mL of 30 % acrylamide mix (Bio Rad), 2.5 mL of 1.5 mol L^{-1} Tris-HCl pH 8.8 (Sigma-Aldrich), 0.1 mL of 10 % SDS (Sigma-Aldrich), 0.1 mL of 10 % ammonium persulfate (Bio Rad) and 0.004 mL of tetramethylethylenediamine (TEMED) (Bio Rad). Trichloroacetic acid, chloroform and acetone were from Panreac (Barcelona, Spain). Tris(hyddroxymethyl)aminomethane (Tris), sodium dodecyl sulphate (SDS), glycine, ammonium persulfate, and N,N,N´, N´-tetramethylethylenediamine (TEMED) were from Sigma Aldrich. Phenol solution, equilibrated with 10 mM Tris-HCl (pH 8.0, 1 mM EDTA) was provided by Sigma Aldrich. 2-DE rehydration solution was prepared with 5 M urea, 2 M thiourea, 2 mM tributyl-phosphine (Sigma-Aldrich), 65 mM DTT, 65 mM CHAPS (Sigma-Aldrich) , 0.15 M NDSB-256, 1 mM sodium vanadate (Sigma-Aldrich) , 0.1 mM sodium fluoride (Sigma-Aldrich), and 1 mM benzamidine (GE Healthcare Life Science). The running buffer (4L) was prepared with glycine (576g) (Sigma-Aldrich), Tris-HCl pH 8.8 (121.1g) and SDS (40g). The SDS buffer for protein extraction from plankton was prepared with 30 % (m/v) sucrose (AppliChem (Darmstad, Germany), 2 % (m/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5 % (v/v) 2-mercaptoethanol (Fluka, Vancouver, Canada) and protease inhibitor cocktail (Roche Diagnostics, Mannhein, Germany). Sodium hydroxide (Merck), used for UF membrane cleaning, and ammonium hydrogencarbonate (BDH, Poole, UK), for salt removal after retentate preparation by centrifugal UF, were used when treating seawater. Kit for silver staining polyacrylamide gels was purchased from Bio Rad. Element standard solutions (used for metal quantification) were prepared from cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, and zinc stock standard solutions (1.000 g L^{-1}) from Scharlau (Barcelona, Spain).

7.2.3. Sample collection

Surface seawater samples (1 - 2 m depth, 60L) were collected from the Ría de Arousa estuary (north-western Spain) in pre-cleaned 12 L non-metallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection, samples were filtered (0.45 μm) and immediately subjected to the tangential flow UF.

Fresh plankton samples, consisting of mixture of microalgae (phytoplankton), were collected in a clam hatchery located at the Ría de Arousa estuary in pre-cleaned 5L bottles. After collection, plankton was concentrated by removing the supernatant after centrifugation at 4000 rpm for 15 min at 20ºC.

7.2.4. Protein extraction

7.2.4.1. Extraction of dissolved proteins from seawater samples

Filtered seawater samples (60L) were treated with sodium azide (final concentration referred to 60 L of seawater of 5.0 mM) for avoiding sample degradation, and also with SDS (final concentration referred to 60 L of seawater of 0.01 % (m/v)) for minimizing proteins adsorption onto the UF membrane. Preserved samples were then subjected to tangential flow UF through a polyethersulfone membrane (size 0.6 m^2, nominal molecular mass cut-off of 10 kDa) until recovering approximately 400 – 600mL of retentate (ultrafiltrate containing substances of molecular weight higher than 10 kDa). The UF system was previously cleaned by passing 2 L of 0.1 M NaOH at 45 ± 5 ºC, followed by rinsing with 9 L of Milli-Q water, also at 45 ± 5 ºC. After tangential flow UF, the retentate was further concentrated and desalted with a cleaning solution containing 0.01 % (m/v) SDS and 35 mM ammonium hydrogencarbonate by centrifugal ultrafiltration (ultrafiltration tubes with polyethersulfone membrane of 10 kDa molecular cut-off), until obtaining a concentrated and desalted retentate of 20 mL (pre-concentration factor of 3000). Finally, protein pellets were obtained by precipitation with chloroform after water and methanol soluble interferences

removal [24]. The protein pellet was partially dried under a N_2 steam, and then re-dissolved in 50 µL of the buffer solution for equilibration after IEF.

7.2.4.2. Extraction of proteins from plankton samples [17]

A portion of concentrated plankton sample was first grinded into a fine powder in a mortar and pestle under liquid nitrogen. Six independent portions of 0.1 g of the grinded plankton were introduced into 2 mL tubes, and the sample was subjected to different washing stages before protein extraction. First, 2 mL of 10 % (v/v) TCA in acetone were added, and after vortexing for a few seconds, and further centrifugation at 10000 rpm, 4ºC for 3 minutes, the supernatant containing interfering substances was discarded. Then, washing was performed with 2 mL of 0.1 M ammonium acetate in 80/20 methanol/water mixture (vortex shaking, centrifugation at 10000 rpm, 4ºC for 3 minutes, and supernatant discarding), followed by a final washing with 2mL of a 80/20 acetone/water solution, and final oven-drying at 50ºC for at least 10 minutes. Protein extraction was performed by adding 0.4 mL of phenol (pH 8.0) plus 0.4 mL of SDS buffer (30 % (m/v) sucrose, 2 % (m/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5 % (v/v) 2-mercaptoethanol and protease inhibitor cocktail) into each tube. The mixture was vortexed thoroughly and was kept at room temperature for 5 minutes. The phenol phase was then separated by centrifugation (10000 rpm, 4ºC) for 3 minutes, and the upper phenol phase containing proteins was transferred to a 2 mL tube. The phenol phase was finally treated with 5 volumes of cold methanol containing 0.1 M ammonium acetate (approximately 2 mL), and the mixture was stored at -20ºC for 2 hours to overnight. Precipitated proteins were recovered by centrifugation at 2500 rpm for 5 minutes (4ºC), and successively washed with 100 % methanol and 80/20 acetone/water. Protein pellet obtained was finally dried under a N_2 stream, and re-dissolved in 300 µL of 2-DE rehydration solution.

7.2.5. Protein quantification by the Bradford assay

Total protein determination in the re-dissolved protein pellets was assessed by the Bradford method, which uses Coomassie Brilliant Blue G-250 as a chromogenic reagent (protein-dye complex with maximum absorbance at 595 nm). Calibration was performed with BSA as a protein standard prepared in the 2-DE rehydration solution (stock concentration of 1000 μg mL^{-1}). The standards were prepared by diluting the stock standard solution with the 2-DE rehydration solution before mixing with the chromogenic reagent. Preparation of standard was as follows: vial A (1000 μg mL^{-1}) consists of 40 μL of BSA stock solution; vial B (500 μg mL^{-1}), 20 μL of vial A plus 20 μL 2-DE rehydration solution; vial C (250 μg mL^{-1}), 20 μL vial B plus 20 μL 2-DE rehydration solution; vial D (125 μg mL^{-1}), 20 μL vial C plus 20 μL 2-DE rehydration solution; vial E (62.5 μg mL^{-1}), 20 μL vial D plus 20 μL 2-DE rehydration solution; vial F (31.25 μg mL^{-1}), 20 μL vial E plus 20 μL 2-DE rehydration solution; and vial G (0.00 μg mL^{-1}), 20 μL 2-DE rehydration solution. These solutions, except solution in vial F, from which 20 μL were pipetted and diluted, were mixed with 600 μL of Coomassie Brilliant Blue G-250 (final volume of 620 μL) which offer BSA concentrations of 0.0, 1.0, 2.0, 4.03, 8.06, 16.1, and 32.26 μg mL^{-1}. Samples (re-dissolved protein pellets) were prepared by mixing 1 μL with 19 μL of 2-DE rehydration solution before addition of 600 μL of chromogenic reagent. Finally, both samples and standards were kept at room temperature for at least 5 min before spectrophotometric quantification at 595 nm.

7.2.6. Separation of proteins by 2D PAGE

Aliquots of the re-dissolved protein pellet from plankton samples (300 μg) were applied to IPG strips, previously rehydrated for 12 h, for IEF. The IPG strips (17 cm, pH 3 - 10) were then recovered with DryStrip Cover Fluid, and IEF was performed using a Protean-IEF and applying the following program: voltage at 250 V for 15 min, 2 h linear gradient until 4000 V, 4 h linear gradient until 8000 V, 1 h at 8000 V, and, stop and hold at 500 V. After IEF, the IPG strips were covered with a buffer solution (equilibration buffer described in section 7.2.2) for 15 min.

SDS-PAGE was performed using 15 % polyacrylamide gel by applying an electric current of 15 mA at 10ºC for 6 h. A molecular weight marker containing proteins with the 2 - 250 kDa molecular weight range was used for assessing molecular weights of the separated proteins (Dual Xtra Precision Protein Prestained Standards, BioRad). Finally, the 2D-PAGE gels were silver nitrate stained following standard procedures.

IEF (IPG strips pH 3 - 10, 7 cm) for dissolved proteins from seawater sample (50 µg of re-dissolved protein pellet were loaded) was performed applying the following program: voltage at 250 V for 15 min, 2 h linear gradient until 4000 V, 1 h at 4000 V, and, stop and hold at 500 V. SDS-PAGE when fractionating dissolved proteins was also performed using 15 % polyacrylamide gel under the same operating conditions than those listed above (15 mA, 10ºC) but for 1.5 h.

Before LA-ICP-MS analysis, gels were vacuum dried at 80ºC for 2 h by placing them on chromatography paper and covering with a porous cellophane sheet.

7.2.7. LA-ICP-MS measurements and sample preparation

LA was performed with an Nd-YAG LA system operating at 213 nm. Before LA experiments, the ICP-MS instrument was set for routine multi-element analysis in accordance with the manufacturer´s instructions: the nebulizer gas flow rate, lens voltage, and daily performance of the instrument were optimized by aspirating a solution containing Rh, Mg, Pb, Ba, and Ce (10 µg L^{-1} of each one), while autolens calibration was performed by aspirating a solution of Be, Co, In and U (10 µg L^{-1} of each one). The plasma was extinguished and the spray chamber and nebulizer assembly were replaced with LA transfer line and its adapter. Argon was used to transport sample aerosol to the ICP-MS. The final optimization of lens voltage and Ar carrier gas flow for dry plasma conditions was performed by monitoring the ^{63}Cu intensity of a NIST sample. Dried gels sections corresponding to the protein migration zone were cut with a size to fit in the ablation chamber for LA-ICP-MS analyses. The gels sections were fixed to the support using double-sided adhesive

tape. This operation was required to prevent the gel can shrivel and to maintain a flat surface during the laser ablation process. Each protein spot was ablated in raster mode, and different mass-to-charge ratios for each metal were monitored. Signal drift correction was performed by using ^{13}C as an internal standard. Matrix-matched standards with known metals concentrations were used for metal quantification in gels.

7.3. Results and discussion

7.3.1. 2D-SDS-PAGE

The assessment of total protein contents in protein pellets from surface seawater and plankton samples was firstly performed by the Bradford assay (section 7.2.5). Regarding plankton samples, determinations in duplicate showed total protein concentrations of 9.5 ± 0.5 and 10.4 ± 0.5 μg μL^{-1}. However, very low total protein content was determined in protein pellets from surface seawater, a value of 2.9 ± 0.2 μg μL^{-1} was found (pre-concentration factor of 3000). Therefore, a mass of 300 μg of protein pellet from plankton samples was used for performing the 2D-SDS-PAGE; whereas, the total amount of the isolated protein pellet from seawater (50 μg) was loaded for 2D-SDS-PAGE experiments.

7.3.1.1. Proteins from plankton samples

Examination of silver-stained 2-DE gels obtained when loading pellets from plankton demonstrated the existence of several proteins, around 80 – 100 different protein spots. Some of them offer a low intensity, which means that proteins occur at low concentrations; and also, some of the protein bands are not properly resolved. As an example, 2-DE gel obtained from plankton sample coded as P1 is shown in Figure 1. Well-resolved and intense protein bands are highlighted and coded in different rectangles. These protein spots were further cut and subjected to LA-ICP-MS for assessing metal bound to them. In general, proteins exhibit molecular weights ranging from 5 to 50 kDa with pI between 3 and 10.

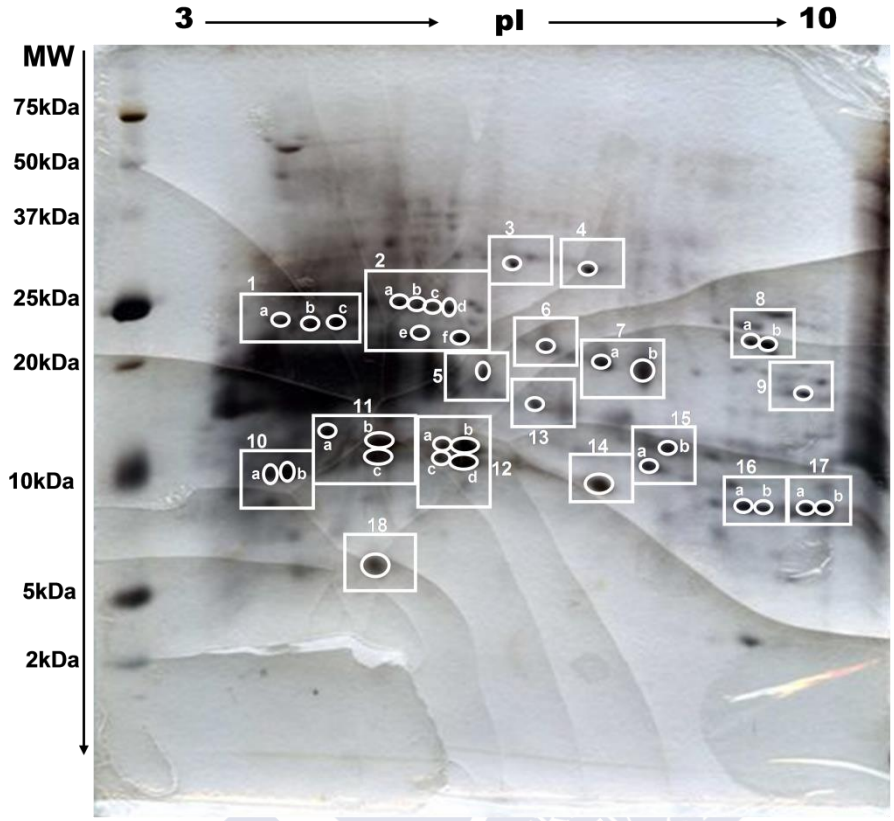

Figure 1: 2DE gel of proteins extracted from marine plankton (plankton sample P1).

Table 1 lists the approximate molecular weights and isoelectric points calculated for each protein band (protein spots in rectangles 1 to 18 in Figure 1 for plankton sample P1), and also for protein spots from plankton sample P2 (2D-PAGE gel not given). The occurrence of single protein spots was detected at MW of 31 kDa for pIs around 6.5 and 7.5 (rectangles 3 and 4 in Figure 1) in both plankton samples. In addition, other single protein spots were also observed for proteins of high MW (18 kDa) and alkaline pI (9.7), as protein spot in rectangle 9; for proteins of low MW (6 kDa) and acid pI (5.0), rectangle 18; and for proteins of variable MW (10, 16, 20 and 22 kDa) and neutral pIs (within the 6.5 – 7.5 range), single protein spots in rectangles 5, 6, 13 and 14. Other proteins were found to exhibit similar MWs and pIs. This is the case of proteins in rectangle 1 (MW of 23 kDa and pIs from 4.0 to

4.6), proteins in rectangle 2 (MW of 25.5 and 22 kDa, and pIs from 5.3 to 6.0), proteins in rectangle 7 (MW/pI ratio of 20-21/7.5-8.0), proteins in rectangle 8 (MW/pI ratio of 22/9.1-9.4), proteins in rectangle 10 (MW/pI ratio of 10.5/3.9-4.1), proteins in rectangles 11 and 12 (MW/pI ratio of 11-13/4.5-6.0), proteins in rectangle 15 (MW/pI ratio of 11-12/8.0-8.2), and proteins in rectangles 16 and 17 (MW/pI ratio of 8.0/9.4-10).

Table 1: MW range and pI range of protein spots on 2-DE for plankton.

Plankton P1			Plankton P2		
Protein spot	MW (kDa)	pI	Protein spot	MW (kDa)	pI
1a	23	4.0			
1b	23	4.3			
1c	23	4.6			
2a	25	5.3	2a	25	6.0
2b	25	5.5	2b	25	6.1
2c	25	5.7			
2d	25	5.7			
2e	22	5.8			
2f	22	5.5			
3	31	6.5	3	31	6.7
4	31	7.4	4	31	7.5
5	20	6.2			
6	22	6.8	6	23	6.9
7a	20	7.5	7a	21	7.6
7b	20	8.0	7b	21	8.0
8a	22	9.1	8a	22	9.0
8b	22	9.4	8b	21	9.1
			8c	21	9.2
			8d	21	9.3
9	18	9.7	9	18	9.7
10a	10	3.9	10a	11	4.1
10b	10	4.1	10b	10	4.3
11a	13	4.5	11a	13	5.2
11b	13	5.0	11b	12	5.3
11c	12				
12a	13	5.7	12a	14	6.0
12b	13	6.0	12b	13	6.0
12c	11	5.7			
12d	11	6.0			
13	16	6.7	13	16	6.9
14	10	7.5			
15a	11	8.0	15a	12	7.7
15b	12	8.2	15b	11	8.0
16a	9	9.0	16a	10	8.8
16b	9	9.4	16b	10	9.1
			16c	9	9.3
17a	9	9.8	17	9	9.8
17b	9	10			
18	6	5.0	18	6	5.3

Most of the results agree with those obtained when analyzing similar plankton samples by OFFGEL-lab-on-chip (LOC) electrophoresis [17,32]. Although the higher resolution of the latter technique allowed the identification of more protein bands, coincidences for several MW/pI ratios, mainly for single protein spots, are observed.

7.3.1.2. Proteins from surface seawater

Examination of silver-stained 2D-PAGE gel from surface seawater (Figure 2) demonstrated the occurrence of few protein spots (denoted as *a* to *e* in Figure 2). Proteins exhibit similar MWs, within the 10 – 14 kDa range; whereas, pIs vary from 5.8 to 7.3 (Table 2).Results agree with those previously reported for surface seawater by 2D-PAGE, MW from 16 to 48 kDa [14,15], and also after OFFGEL–LOC electrophoresis, MWs within the 15 – 63 kDa range [24]. In addition, proteins pIs are also quite similar (from 4.8 to 8.4) [14,15], and most abundant proteins in surface seawater from Ría de Arousa estuary offered pI from 5.7 to 8.1 [24].

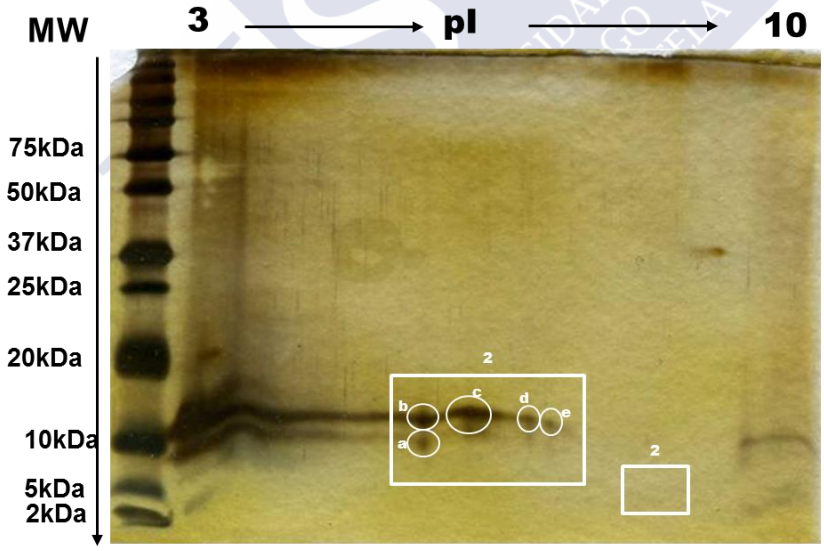

Figure 2: 2DE gel of proteins extracted from surface seawater.

Table 2: MW range and pI range of protein spots on 2-DE for surface seawater.

Protein spot	MW (kDa)	pI
a	10	5.8
b	13	5.8
c	14	6.3
d	13	7.0
e	12	7.3

7.3.2. Detection of metals in gels with LA-ICP-MS

Matrix-matched standards with known metal concentrations were prepared and used for metal quantification by LA-ICP-MS. Standard solutions containing metals at concentrations of 100, 10, 1.0, and 0.1 μg mL^{-1} (20 μL) were loaded on 50 mg portions of gels (15 % polyacrylamide). Similarly, a blank was also prepared by loading 20 μL of a blank solution, which will be used as standard of concentration 0.0 μg mL^{-1}. Once dried, the gel portions were weighed (weights approximately 10 mg), and the metal concentrations were re-calculated and refereed to mass of metal to mass of gel: final metal concentrations of 200, 20, 2.0, 0.2 and 0.0 μg g^{-1}. The different gel portions were fixed to the support using double-sided adhesive tape and were ablated. Carbon-13 was used as the internal standard for correcting plasma instabilities. Table 3 lists the equations for the calibrations curves obtained. Regression coefficients were higher than 0.995 for several targets, although values within the 0.977 – 0.9850 range were obtained for some metals.

The limit of detection (LOD), based on 3 Sd criterion (3 Sd / m, where Sd is the standard deviation of 5 measurements of blank gels, and m is the slope of calibration graph) was established. Table 3 also lists the LODs obtained, which range from 0.0266 μg g^{-1} (Mn detection) to 4.96 μg g^{-1} (Cr detection).

Table 3: Calibration curves and limit of detection of the method.

	Calibration curve	Correlation (R^2)	LOD (µg g^{-1})
^{111}Cd	y = 0.0003 [ ] + 0.0008	0.9999	0.96
^{114}Cd	y = 0.0009 [ ] + 0.0011	0.9988	0.43
^{59}Co	y = 0.0272 [ ] + 0.169	0.9834	0.047
^{63}Cu	y = 0.0127 [ ] + 0.0902	0.9783	0.060
^{65}Cu	y = 0.0062 [ ] + 0.0605	0.9776	1.4
^{57}Fe	y = 0.0006 [ ] + 0.0091	0.9787	2.6
^{55}Mn	y = 0.0391 [ ] + 0.2735	0.9815	0.027
^{60}Ni	y = 0.0053 [ ] + 0.0322	0.9843	0.15
^{62}Ni	y = 0.0008 [ ] + 0.0051	0.9850	0.34
^{206}Pb	y = 0.0276 [ ] + 0.2184	0.9775	0.034
^{207}Pb	y = 0.021 [ ] + 0.1673	0.9772	0.045
^{208}Pb	y = 0.0524 [ ] + 0.4146	0.9774	0.052
^{68}Zn	y = 0.001 [ ] + 0.0204	0.9802	1.6
^{64}Zn	y = 0.0026 [ ] + 0.0341	0.9955	2.3
^{66}Zn	y = 0.0015 [ ] + 0.0143	0.9958	0.94
^{50}Cr	y = 0.0012 [ ] + 0.0361	0.9819	4.9
^{53}Cr	y = 0.0027 [ ] + 0.0274	0.9795	0.21

7.3.3. Detection of metals bound to proteins with LA-ICP-MS

As previously mentioned intense and resolved protein spots, highlighted inside rectangles in Figures 1 and 2, were cut and further analyzed by LA-ICP-MS. In

addition, region number BLK in the gel from surface seawater (Figure 2) was also analyzed as a blank to assess the presence background trace metals in the gels and reagents used for performing 2-DE.

Regarding isolated proteins from plankton, bioactive elements such as Cu and Zn, as well as Cr and Cd, were identified in the protein spot coded as 11a (~13 kDa, pI of ~4.5) in plankton sample P1. This sample also showed the presence of Zn in protein spot coded as 13 (~16 kDa, pI of ~6.7), and Cd in protein spots coded as 2f (~22 kDa, pI of ~5.5) and 17b (~9.0 kDa, pI of ~10). The relative concentration of these elements, expressed as μg g^{-1}, are listed in Table 4, and accounted for low values for Cd, Cu and Zn (7.5, 4.9, and within the 10.7 – 19.2 μg g^{-1} range, respectively), and high levels for Cr (74.4 μg g^{-1}). The presence of Cd, Cr and Zn was also observed in some protein spots from plankton sample P2 (Table 4), although at lower concentrations than in plankton sample P1. Studies based on OFFGEL-LOC electrophoresis when analyzing similar plankton samples [32] showed the association of Zn and Cd with proteins of acid pIs (within the 5.9 – 6.5 range), result similar to that found when analyzing plankton sample P1. In this technique, proteins are separated according to their isoelectric point (pI) by OFFGEL electrophoresis, using immobilized pH-gradient gels (IPG), and the separated components are recovered in liquid fractions to be separated according to their molecular weight using a microfluidic Lab-on-chip electrophoresis. In general, the number of elements bound to the isolated proteins, as well as the concentrations, is lower than that obtained when analyzing similar plankton samples by OFFGEL-LOC electrophoresis [32]. Under OFFGEL conditions, the association between certain proteins and other bioactive trace metals such as Fe and Mn is guaranteed. This fact can be attributed to the more drastic conditions inherent to conventional 2D-PAGE which imply metal-protein breakdown during isoelectric focusing [31,32]. However, parameters affecting the protein extraction process, such as the low temperature and the presence of proteinase inhibitors, can also contribute to minimize metal-protein complexes degradation.

Table 4: Trace metal concentrations in protein spots from plankton and surface seawater samples.

Plankton P1		
Trace metal	Protein spot code	Relative concentration (μg g^{-1})
Cd	2f	0.5
	11a	3.0
	17b	0.6
Cu	11a	4.9
Cr	11a	74.4
Zn	11a	19.2
	13	10.7
Plankton P2		
Cd	8b	0.9
	8c	0.6
	8d	0.7
	15b	3.2
Cr	15b	5.8
Zn	8a	11.0
	8b	4.0
	8d	4.5
	13	3.8
	15a	9.5
	15b	2.9
	16a	14.5
Surface seawater		
Cd	a	4.8
	c	11.7
	d	5.5
	e	1.0
Cu	c	6.7
	d	2.0
Cr	a	16.9
	c	60.0
	d	22.0
Zn	a	6.0
	b	9.4
	c	12.7
	d	7.6

The assessment of trace metal associated to proteins from surface seawater showed the presence of Cd, Cu, Cr and Zn in some proteins spots (Figure 2). Table 4 lists the relative concentrations of these metals in the ablated spots. Proteins in spots coded as *c* (MW/pI of ~14 kDa/~6.3) and *d* (MW/pI of ~13 kDa/~7.0) are associated to Cd, Cu, Cr and Zn; whereas, the occurrence of Cd, Cr and Zn was also found in the protein spot coded as *a* (MW/pI of ~10 kDa/~5.8). Protein spots coded as *b* (MW/pI of ~13 kDa/~5.8) and *e* (MW/pI of ~12 kDa/~7.3) only showed association with Cd (spot *e*) and Zn (spot *b*). The determination of

metals associated to protein fractions of similar pIs (OFFGEL conditions) when analyzing dissolved proteins [24] showed the occurrence of Cd and Zn in proteins of acid pIs (within the 5.2 – 6.0 range), which agree to the current results for protein spots *a* and *d* (Figure 2). As previously mentioned for proteins from plankton, OFFGEL conditions led to the assessment of other bioactive metals such as Fe and Mn bound to certain dissolved proteins [24]. These metal-proteins association were not observed when performing 2D-PAGE and can be attributed to the used 2D-PAGE conditions [31], as well as to conditions inherent to the proteins extraction itself.

7.4. Conclusions

LA-ICP-MS has effectively been used to assess trace metals bound to dissolved proteins and proteins from marine plankton after effective protein isolation methods and conventional 2D-PAGE. The presence of certain metals bound to dissolved proteins in seawater after two-dimensional pI and MW separation has been demonstrated by the first time. These findings confirm results from earlier studies which reported the existence of trace metal – protein complexes in seawater after isolating groups of dissolved proteins of similar pIs [24]. Results showed that bioactive metals such as Cu and Zn are mainly associated to dissolved proteins of neutral pIs (approximately pIs of 6.3 and 7.0), although Zn was also found to be associated to proteins of pIs 6.0 and 7.6. Other metals such Cd and Cr were also detected in some isolated proteins exhibiting variable pIs (from 5.8 to 7.0). The presence of metals in some protein spots from fresh marine plankton was also proved. Elements such as Cd, Cr, Cu and Zn were found to be associated to certain proteins of MW ranging from 9 to 22 kDa and pIs from 5.5 and 10, results which also agree to earlier findings for fresh marine plankton when assessing metals bound to protein fractions of similar pIs by OFFGEL electrophoresis [32]. In general, it can be concluded that the used 2D-PAGE conditions appear to be more drastic than those involved when using OFFGEL electrophoresis. This fact explains certain breaking metal-protein complexes dissociations, mainly Fe- and Mn-proteins, which were found in fresh marine plankton after OFFGEL [17,32], and

which were not observed in the current application. Finally, findings regarding dissolved proteins can not be used to prove correlation between the MW and the pI of the isolated proteins and the type of metal bound to them. This is because the isolated proteins exhibits very close MW (from 10 to 14 kDa) and pI (from 5.8 to 7.3) values. Regarding plankton proteins, no correlation between the type of metal and the protein' pIs have been shown. However, certain essential elements such as Cr, Cu and Zn, appears to be mainly bound to proteins of low MW.

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8. Detection and identification of dissolved proteins in seawater by 2D-SDS-PAGE and MALDI-TOF-MS

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Detection and identification of dissolved proteins in seawater by 2D-SDS-PAGE and MALDI-TOF-MS

Abstract

Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2D-SDS–PAGE) was applied to separate protein molecules in dissolved organic matter (DOM) from seawater. Dissolved proteins were concentrated and purified using tangential flow ultrafiltration, followed by centrifugal ultrafiltration, and protein precipitation by adding a chloroform/water/methanol mixture. After proteins separation by 2D-SDS-PAGE, twelve proteins spots were observed in the Sypro Ruby stained 2D-gels. Isolated proteins exhibited isoelectric points (pIs) varying from 3.5 to 6.0, and molecular weights(MWs) ranging from 10 to 50 kDa. Proteins were enzymatically in gel digested and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Eight of the 12 resolved spots were identified, and 11 different proteins were successfully assigned: Penicillinase repressor, flagelar hook-associated protein FliD, glucose/sorbosone dehydrogenase, carbamoyl-phosphate synthase small subunit, TRAP-T family transporter, L-aspartate oxidase, translation elongation factor Tu, malate synthase A, ATP-dependent DNA helicase RecQ, Glycogen/starch/alpha-glucan phosphorylase, and LysA proteins. Most of identified proteins were enzymes, which were reported to be located in different cellular components. These results demonstrated that bacterial outer membrane proteins are not the only source of dissolved proteins in seawater.

Keywords: *Dissolved proteins, seawater, two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*

8.1. Introduction

Dissolved organic matter (DOM) is a large reactive component of the global carbon cycle and the largest reduced carbon pool in the oceans. DOM plays a crucial role in the transport and fate of carbon, nutrients, and trace toxic contaminants in seawater [1]. DOM is formed by a complex mixture of organic compounds (lipids, humic acids, fulvic acids, proteins, carbohydrates…) with a wide range of molecular sizes. The low molecular weight (LMW) fraction (less than 1 kDa) accounts for approximately 65–80% of the bulk DOC, while the high molecular weight (HMW) fractions are minor portions of DOC (20–35% for >1 kDa, and 2–7% for >10 kDa) [2]. Proteins are an important component of the HMW fraction of DOM and they are

considered to be an important contributor to carbon and nitrogen pools. Proteins in living organisms should be transferred eventually to the dissolved pool via biogeochemical processes, and the survival of particular proteins in the marine environment can provide insight into mechanisms that control the degradation of organic matter [3]. Advances in gel electrophoresis (GE) methodologies and the combination of GE with mass spectrometry techniques have allowed the characterisation of proteins at the macromolecular level. As a result, proteins can represent a potentially characterisable fraction of marine DOM, which may thus provide vital clues on the biogeochemical cycling of the entire nitrogen and carbon pool [4].

One or two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D or 2D-SDS-PAGE) has been widely used for the separation of proteins. 1D-SDS-PAGE has been applied for detection of dissolved proteins in oceanic waters, but SDS-PAGE resolution is insufficient for a complete separation of dissolved proteins [5, 6]. A method with higher resolution that SDS-PAGE is therefore needed for separating dissolved proteins and their further characterization. 2D-SDS-PAGE is a powerful and sensitive technique for separating individual proteins from complex protein mixtures, and some applications for detecting/characterizating dissolved proteins in oceanic waters have been reported [7, 8].

Identification of the proteins present in a spot on a 2D-gel has traditionally been performed by micro-Edman degradation. The N-terminal amino acid sequencing allowed the identification of one of the dissolved proteins as the porin P protein of *Pseudomonas aeruginosa* PAO1 [9]. Porin P is a member of the outer-membrane channel proteins of Gram-negative bacteria. Other outer membrane protein, such as OmpA-like protein from Gram-negative bacterium *Acinetobacter sp*., [5] and a low molecular weight alkaline phosphatase (L-AP) from *Pseudomonas aeruginosa* PAO1 [7] were also identified in oceanic waters. The identification of these proteins indicates that bacterial membrane proteins are an important source of dissolved proteins. Edman degradation methods are limited for providing sequence

information routinely for only 15 to 20 amino acids at the termini of the protein. Due to the limited sensitivity of this technique, alternative methods for proteins identification are needed [10]. Current analytical techniques in protein identification by mass spectrometry are based on the generation of peptide mass maps and / or sequence tags that are idiotypic for the protein sequence. Protein identity is established by correlating such patterns with sequence databases. Protein identification by electrospray ionization tandem mass spectrometry (ESI-MS/MS) is an appealing technique for protein identification [11]. Powell et al. [12] have applied HPLC-ESI-MS/MS for collision-induced dissociation (CID) spectra acquisition before searching in available proteomic and genomic databases for marine proteins identification, and further interpretation by peptide *de novo* sequencing. Peptide tags produced identification of conserved sequences from several protein homologues: long chain fatty acyl CoA synthetase (membrane-associated enzyme), anthranilate synthase (enzyme), ribulose bisphosphate carboxylase (enzyme), and luminal binding protein (membrane associated protein). These results showed evidences for dissolved proteins other than bacterial outer membrane proteins reported by Tanoue et al. [Ref]. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) is the simplest and the most sensitive technique for assessing biological macromolecules extracted from cells and tissues [13]. The inherent ability of this methodology for desorbing thermolabile HMW compounds, as well as the high accuracy and sensitivity, and wide mass range resolution (1–300 kDa), make MALDI-TOF-MS a promising method for the identification of biomolecules (peptides, proteins, oligosaccharides and oligonucleotides) in complex samples. [14].

The objective of this study has been the identification of dissolved proteins from seawater samples by MALDI-TOF-MS. Dissolved proteins from seawater were concentrated and purified using tangential flow ultrafiltration and centrifugal ultrafiltration, followed by protein precipitation by using a chloroform/water/methanol mixture. Proteins were then separated by 2D-SDS–PAGE, and enzymatically in-gel digested before MALDI-TOF-MS assessment.

8.2. Experimental

8.2.1. Apparatus

Protein identification was carried out in a mass spectrometer MALDI-TOF/TOF 4800 Analyzer (Applied Byosistems Foster City, CA, USA) in positive reflectron mode. Gel images were scanned in a Typhoon 9410 (GE Healthcare). A Hitachi double–beam spectrophotometer model U–2010 (Hitachi, Berkshire, UK) equipped with 10 mm quartz cells was used for all UV–visible measurements. Isoelectric focusing was performed with a Protean IEF System from Bio-Rad (Hercules, CA, USA), and second dimension was run in a Protean XL (BioRad). The tangential flow ultrafiltration (UF) system consisted of a Masterflex I/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane of nominal molecular weight cut-off (MWCO) of 10 kDa, and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge. Centrifugal ultrafiltration was performed with an Alresa Digtor centrifuge (Madrid, Spain). Other laboratory devices were a Reax top shaker from Heidoph (Schwabach, Germany), and a Basic 20 pH–meter with a glass–calomel electrode (Crison Instruments S.A., Barcelona, Spain).

8.2.2. Reagents and material

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q® water-purification system (Millipore). Centrifugal ultrafiltration was performed with Vivaspin 20 ultrafiltration tubes (polyethersulfone membrane of MWCO of 10 kDa) from Sartorius Stedin Biotech (Goettingen, Germany). Seawater filtration was achieved by using Millipore HAWP14250 0.45 µm mixed esters of cellulose membrane filters (140 mm diameter). Total protein assessment was performed with a Bradford protein assay containing Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) from Thermo Scientific (Rockford, IL, USA). IPG Dry Strips (7 cm, pH 3-10; and 10 cm, pH 3-10), and Plus One DryStrip cover fluid (GE Healthcare Life Science, Uppsala, Sweden) were used for pI protein fractionation. Buffer solution (10 mL) for equilibration after IEF was prepared with 6 M urea,

0.375 M Tris-HCl (pH 8.8), 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 0.002% (w/v) bromophenol blue, and 100 mg of DTT (all reagents from Sigma Aldrich, St. Louis, MO, USA). SDS gel (10 mL) was prepared with 2.3 mL of water, 5 mL of 30% acrylamide mix (Bio Rad), 2.5 mL of 1.5 mol L^{-1} Tris-HCl pH 8.8 (Sigma-Aldrich), 0.1 mL of 10% SDS (Sigma-Aldrich), 0.1 mL of 10% ammonium persulfate, and 0.004 mL of tetramethylethylenediamine (TEMED) from Bio Rad. Methanol plus chloroform, acetone (used for obtaining protein pellets), and sodium azide (used for avoiding dissolved protein degradation) were from Panreac (Barcelona, Spain). Tris(hyddroxymethyl)aminomethane (Tris), sodium dodecyl sulphate (SDS), glycine, and ammonium persulfate were from Sigma Aldrich. 2-DE rehydration solution was prepared with 5 M urea, 2 M thiourea, 2 mM tributyl-phosphine 65 mM DTT, 65 mM CHAPS, 0.15 M NDSB-256, 1 mM sodium vanadate (Sigma-Aldrich), and 0.1 mM sodium fluoride (reagents from Sigma-Aldrich); and 1 mM benzamidine (GE Healthcare Life Science). The running buffer (4 L) was prepared with 576 g of glycine, 121.1 g of Tris-HCl pH 8.8, and 40 g of SDS (Sigma-Aldrich).. Sodium hydroxide (Merck, Darmstadt, Germany), used for UF membrane cleaning, and ammonium hydrogencarbonate (BDH, Poole, UK), for salt removal after retentate preparation by centrifugal UF, were used when treating seawater. Sypro Ruby stain (Lonza, Rockland, ME, USA) was used for gel stained. Acetonitrile (HPLC grade) was from Scharlau. Trypsin, ammonium bicarbonate, iodoacetamine, trifluoacetic acid, formic acid and alpha-cyano-4-hydroxycinnamic acid were from Sigma Aldrich.

8.2.3. Sample collection

Surface seawater sample (1-2 m depth, 60 L) was collected from the Ría de Arousa estuary (north-western Spain) in pre-cleaned 12 L non-metallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection, the sample was filtered (0.45 µm) and immediately subjected to the tangential flow UF.

8.2.4. Preparation of protein sample

Filtered seawater sample (60 L) was treated with sodium azide (final concentration referred to 60 L of seawater of 5.0 mM) for avoiding sample degradation, and also with SDS (final concentration referred to 60 L of seawater of 0.01% (m/v)) for minimizing proteins adsorption onto the UF membrane. Sample was then subjected to tangential flow UF through a polyethersulfone membrane (size 0.6 m^2, nominal MWCO of 10 kDa) until recovering approximately 400–600 mL of retentate (ultrafiltrate containing substances of MW higher than 10 kDa). The UF system was previously cleaned by passing 2 L of 0.1 M NaOH at 45 ± 5ºC, followed by rinsing with 9 L of Milli-Q water, also at 45 ± 5ºC. After tangential flow UF, the retentate was further concentrated and desalted with a cleaning solution containing 0.01% (m/v) SDS and 35 mM ammonium hydrogencarbonate by centrifugal ultrafiltration (ultrafiltration tubes with polyethersulfone membrane of MWCO of 10 kDa), until obtaining a concentrated and desalted retentate of 20 mL (pre-concentration factor of 3000). Finally, protein pellet was obtained by precipitation with chloroform after water and methanol soluble interferences removal [26]. The protein pellet was partially dried under a N_2 stream, and then re-dissolved in 50 µL of the 2D PAGE rehydration solution. Finally, total protein determination in the re-dissolved protein pellet was assessed by the Bradford method.

8.2.5. Two dimensional gel electrophoresis

IEF was performed by applying an aliquot of the re-dissolved protein pellet (50 µg) to IPG strip (7 cm, pH 3-10) which was previously rehydrated for 12 h. The IPG strip was then covered with DryStrip Cover Fluid, and IEF was performed using a Protean-IEF under the following conditions: voltage at 250 V for 15 min, 2 h linear gradient until 4000 V, 1 h at 4000 V, and stop and hold at 500 V. After IEF, the IPG strip was covered with a buffer solution (equilibration buffer described in section 2.2) for 15 min. SDS-PAGE was performed using 15% polyacrylamide gel by applying an electric current of 15 mA at 10 ºC. A molecular weight marker containing proteins within the 2-250 kDa MW range was used for assessing the MWs of the separated proteins (Dual Xtra Precision Protein Prestained Standards,

BioRad). Finally, 2-DE gels were fixed in 10 % methanol/7 % acetic acid for 1 h, and stained overnight with Sypro Ruby fluorescent dye following the manufacturer's recommendations. After staining, gels were washed in 10 % methanol/7 % acetic acid for 1 h, and scanned in a Typhoon 9410.

8.2.6. Sample preparation for mass spectrometry

Protein spots chosen for mass spectrometric analysis were excised from the gels and manually in-gel digested with trypsin following the protocol defined by Shevchenko [15], with some modifications. The excised gel pieces were washed three times using 190 µL washing buffer (50 mM NH_4HCO3, 50% methanol v/v). A centrifugation stage (600 rpm, room temperature) for 20 min was performed for removing the washing solution after each washing steps. The gel pieces were then dehydrated for 10 min by addition of 100 µL acetonitrile, followed by incubation at 56 ºC with 50 µL of a solution containing 10mM dithiotreitol (DTT) dissolved in 50 mM NH_4HCO_3 for 30 min. The DTT solution was then removed and the gel pieces were washed three times with the washing buffer solution, and dehydrated with acetonitrile. The gel pieces were then incubated at room temperature and dark in 50 µL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 20 min. After removal of the iodoacetamide solution, the gel pieces were washed three times with the washing buffer solution, and dehydrated with acetonitrile. The gel pieces were soaked in a digestion buffer containing 20 ng μL^{-1} trypsin in 50 mM NH_4CO_3. Enzymatic digestion was performed overnight at 37ºC. Peptides were extracted thrice by 20 min incubation in 40 µl of 60% acetonitrile in 0.5% HCOOH. The resulting peptide extracts were pooled, concentrated in a SpeedVac and stored at -20ºC.

8.2.7. MALDI-TOF-MS

For MS analysis, dried peptides were dissolved in 4 µL of 0.5 % formic acid. Equal volumes (0.5 µL) of peptide and matrix solution, consisting of 3 mg alpha-cyano-4-hydroxycinnamic acid (α-CHCA) dissolved in 1 mL of 50 % acetonitrile in 0.1 % trifluoroacetic acid, were deposited using the thin layer method, onto a 384 Opti-

TOF MALDI plate (Applied Biosystems). Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). MS spectra were acquired in reflectron positive-ion mode with a Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots and at least three trypsin autolysis peaks were used as internal calibration. All MSMS spectra were performed by selection the precursors with a relative resolution of 300 (FWHM) and metastable suppression. Automated analysis of mass data was achieved using the 4000 Series Explorer Software V3.5. Peptide mass fingerprinting (PMF) and peptide fragmentation spectra data of each sample were combined through the GPS Explorer Software v3.6 using Mascot software v2.1. (Matrix Science) to search against a non-identical protein database (NCBInr release data 20100526), with 30 ppm precursor tolerance, 0.35 Da MSMS fragment tolerance, CAM (carbamydomethyl cystein) as fixed modification, oxidized methionine as variable modification and allowing 1 missed cleavage. All spectra and database results were manually inspected in detail using the previous software. Protein scores greater than 56 were accepted as statistically significant ($P < 0.05$), considering positive the identification when protein score CI (confidence Interval) was above 98%. In case of MSMS spectra, total ion score CI was above 95%.

8.3. Results and discussion

8.3.1. Protein identification assessment

In the present study (2D-SDS–PAGE) was used for dissolved proteins separation. Examination of Sypro Ruby stained 2D-gel demonstrated the occurrence of twelve protein spots (denoted as 1 to 12 in Figure 1). Proteins exhibit pIs varying from 3.5 to 6.0, with MWs within the 10 – 50 kDa range. These results are similar to those previously reported for dissolved proteins in surface seawater after 2D-PAGE [7, 8] (MWs from 16 to 48 kDa and pIs from 4.8 to 8.4), and also after OFFGEL–LOC electrophoresis [16] (MWs within the 15 – 63 kDa range, and pIs between 5.7 and 8.1).

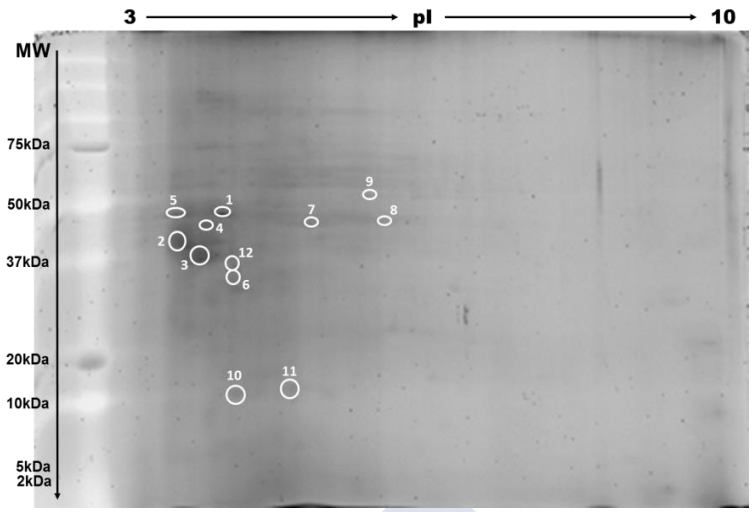

Figure 1: 2DE gel proteins.

The twelve spots from a Sypro Ruby stained 2D-gel were subjected to tryptic digestion for protein identification by MALDI-TOF-MS. The proteins from the different spots were identified by querying NCBInr and SwissProt databases using the Mascot search engine. pBLAST was used in these spots which the identification failed; allowing the identification performing DeNovo Sequencing with the fragmentation spectra availables (between 3 to 10). Results are listed in Table 1, which also shows an assigned protein spot number, the protein identity, the score, and the theoretical and experimental pIs and MWs. Eight of these 12 spots were identified, and 11 different proteins were successfully identified. This was because three spots (denoted as 1, 5 and 12) yielded confident identification for multiple proteins. Sequences for several proteins were identified by similarity searching (Table 1). Penicillinase repressor, flagelar hook-associated protein FliD, glucose/sorbosone dehydrogenase, carbamoyl-phosphate synthase small subunit, TRAP-T family transporter, L-aspartate oxidase, translation elongation factor Tu, malate synthase A, ATP-dependent DNA helicase RecQ, Glycogen/starch/alpha-glucan phosphorylase, and LysA proteins were produced as homologue matches for eleven of peptide tags. Each of these sequences matched returned for different bacterium.

Table 1: List of identified proteins.

Spot no.	Protein name	Peptide tag	Score	matching peptides	MW (kDa)		pI	
					Theo.	Exp.	Theo.	Exp.
1	Penicillinase repressor	AAVMNVLWEAA	69	10/11	13.9	49.0	6.31	4.0
	Flagelar hook-associated protein FliD	ARMDTGTGVDI	68	10/11	70.0	49.0	6.74	4.0
2	Glucose/sorbosone dehydrogenase	TTKYYTSSNRSRNA	69	11/14	51.2	41.0	5.91	3.4
4	Carbamoyl-phosphate synthase small subunit	GTNNNSDESSKLY	68	11/13	43.0	44.0	9.23	3.8
5	TRAP-T family transporter, fused small and large inner membrane subunits	MMADVLMLSMPM	76	10/12	70.7	49.0	9.13	3.5
	L-aspartate oxidase	MKMMGELVMLS	73	9/11	58.9	49.0	6.01	3.5
7	Translation elongation factor Tu	GEELELVGLR	63	10/10	43.1	46.0	5.03	4.9
9	Malate synthase A	HKRGSPAAGGM	62	9/11	59.0	60.0	6.11	5.7
10	ATP-dependent DNA helicase RecQ	DKPDVRFGAHVE	74	11/12	67.5	14.0	5.96	4.2
12	Glycogen/starch/alpha-glucan phosphorylase	RDRVWRNNPG	74	9/10	91.9	35	5.56	4.1
	LysA protein	EKAVERWVRDRMV	74	11/13	25.2	35	10.3	4.1

Most of the identified proteins corresponded to bacterium found in marine environment. For example, the peptide tag for the identified protein Translation elongation factor Tu returned matches for *Proclorococcus marinus* and *Synechococcus.* The peptide tag for ATP-dependent DNA helicase RecQ returned matches for *Marinobacter adhaerens*, *Hahella chejuensis* and *Desulfovibrio desulfuricans*. The peptide tag for TRAP-T returned matches for *Marinobacter;* and the peptide tag for L-aspartate returned matches for *Flavobacteriaceae bacterium. Synechococcus* are marine unicellular cyanobacteria that are responsible for an estimated 20–40% of chlorophyll biomass and carbon fixation in the oceans [17]; *Hahella chejuensis* are marine bacteria responsible for the biosynthesis of a pigment which shows lytic activity against a red-tide dinoflagellate [18]. *Marinobacter adhaerens* induces aggregate formation while interacting with the diatom. *Thalassiosira weissflogii,* and diatom aggregation is substantial for organic carbon flux from the photic zone to deeper waters [19]. *Proclorococcus marinus* are marine cyanobacteria; whereas, *Desulfovibrio desulfuricans* are anaerobic,

sulfate-reducing bacterium in marine sediments [20], and *Flavobacteriaceae* are marine bacterium which are able to degrade hydrocarbons.

From Table 1, different protein spots exhibited remarkable discrepancies between the theoretical and experimental values in MW and/or pI. These discrepancies in could be attributed to isoforms generated by a variety of causes, including post-translational modifications, alternative splicing, and the occurrence of multigene families [21].

8.3.2. Comparison to published data

The production of DOM in the marine environment is mainly due to extracellular DOM releasing by phytoplankton; egestion, excretion, and sloppy feeding by grazers (i.e., protozoa and zooplankton); and DOM releasing due to cellular lysis of bacteria and phytoplankton by viruses. Some available characteristics of the proteins found in the current work are listed in Table 2. Only two membrane proteins (TRAP-T and LysA) have been found as dissolved proteins in surface seawater from Ría de Arousa estuary (North Atlantic). These findings contrast to those found by Yamada and Tanoue for surface and deep seawater from the Pacific, who have reported outer-membrane-channel protein types (porin P and OmpA-like proteins) from Gram-negative bacteria as the main source of dissolved proteins in seawater [5-9]. Among membrane proteins identified by the Tanoue's group, one protein (a low molecular weight alkaline phosphatase from *Pseudomonas aeruginosa*) was of enzymatic nature [7]. However, as pointed out by these authors, all dissolved proteins were associated with membrane vesicles.

In contrast to Tanoue's findings, most of identified proteins in the current work (Tables 1 and 2) are cytoplasmatic enzyme types (penicillinase repressor, glucose/sorbosone dehydrogenase, carbamoyl-phosphate synthase small subunit, L-aspartate oxidase, translation elongation factor Tu, malate synthase A, ATP-dependent DNA helicase RecQ, and glycogen/starch/alpha-glucan phosphorylase). These results agree to those obtained by Powel et al. [12], who have identified enzymes such as long chain fatty acyl synthetase, anthranilate synthase, and

ribulose bisphosphate carboxylase in deep seawater from the Gulf of Mexico. The first of these three proteins (long chain fatty acyl synthetase) is a membrane associated enzyme; whereas, the other two protein are not associated to plankton membranes [12]. Finally, it must be mentioned that these authors have also identified a membrane associated protein (luminal binding protein) [12].

As shown, enzymatic proteins as well membrane proteins identified in the analyzed surface seawater from Ría de Arousa estuary are different that those previously reported [5-9,12]. The great variety and the spread distribution of marine plankton must explain the presence of very different protein types in surface and deep seawater worldwide.

Table 2: Molecular function, biological process and celular component of proteins

Protein name	Molecular function	Biological process	Cellular component
Penicillinase repressor	DNA binding	negative regulation of transcription, DNA-dependent	
Flagelar hook-associated protein FliD		Cell adhesion	bacterial-type flagellum
Glucose/sorbosone dehydrogenase	oxidoreductase activity, acting on the CH-OH group of donors, quinone or similar compound as acceptor	Carbohydrate metabolic process	
Carbamoyl-phosphate synthase small subunit	Ligase	Amino-acid biosynthesis, arginine biosynthesis, and pyrimidine biosynthesis	
TRAP-T	Transporter, fused small and large inner membrane subunits		membrane
L-aspartate oxidase	L-aspartate oxidase activity and L-aspartate:fumarate oxidoreductase activity	NAD biosynthetic process	cytoplasm
Translation elongation factor Tu	GTP binding, GTPase activity and translation elongation factor activity	GTP catabolic process	cytoplasm
Malate synthase A	malate synthase activity	glyoxylate cycle and tricarboxylic acid cycle	
ATP-dependent DNA helicase RecQ	ATP binding, ATP-dependent 3'-5' DNA helicase activity	DNA recombination, DNA repair, and DNA replication	intracellular
Glycogen/starch/alpha-glucan phosphorylase	glycogen phosphorylase activity and pyridoxal phosphate binding	carbohydrate metabolic process	
LysA protein		cell wall macromolecule catabolic process	membrane

8.4. Conclusions

The use of UF techniques (tangential flow UF followed by centrifugal UF) has been shown to be adequate for extracting marine DOM, dissolved proteins included, from seawater. Dissolved proteins isolation from the obtained retentates has successfully achieved by chloroform precipitation after water and methanol soluble

interferences removal. 2D–SDS–PAGE showed the occurrence of several proteins exhibiting pIs within the 3.5-6.0 range, and MWs from 10 to 50 kDa. Molecular-level characterization of dissolved proteins from seawater by MALDI-TOF-MS after tryptic digestion provided different peptide sequences which allowed the identification of most of the fractionated proteins. Specifically, eleven proteins were successfully assessed. Two proteins were found to be membrane associated proteins; whereas, the remaining proteins were of enzymatic nature and were reported to be associated to cell compartments different than cellular membranes. These findings agree therefore to those reported by Powel et al. [12] that hypothesized that dissolved proteins in seawater contains a variety of protein classes from a variety of cellular locations.

Identified proteins are different than those previously reported. Comparisons are therefore difficult mainly because the scarce literature regarding dissolved proteins in seawater. In addition, difficulties are also arisen from the huge variety of marine plankton which is the main source of dissolved proteins in marine ecosystems.

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9. Detection and identification of proteins from plankton by 2D-SDS-PAGE and MALDI-TOF-MS

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Detection and identification of proteins from plankton by 2D-SDS-PAGE and MALDI-TOF-MS

Abstract: *Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2D-SDS–PAGE) was applied to separate proteins from plankton samples. Proteins were extracted from plankton by using the phenol/SDS method with prior washing steps with 10% TCA in Acetone and methanol. After proteins separation by 2D-SDS-PAGE, approximately 150 – 180 different protein spots were observed in the Sypro Ruby stained 2D-gels. In general, proteins exhibit molecular weights (MWs) ranging from 12 to 150 kDa with isolelectric points (pIs) between 3 and 10.* Twenty-four p*rotein spots were in gel trypsin digested and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).* Seven *of these 24 resolved spots were identified, and 4 different proteins were successfully assigned: Ribulose bisphosphate carboxylase small chain, Cytochrome C-550, Photosystem I reaction center subunit II, and Photosystem II (12 kDa extrinsic protein).*

Keywords: *proteins, plankton, two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*

9.1. Introduction

Dissolved organic matter is a large reactive component of the global carbon cycle, and the largest reduced carbon pool in the oceans. Dissolved organic carbon (DOC) export across the ocean is an important factor in the biological carbon pump, whereby carbon from surface waters is exported to the deep ocean. The biological carbon pump is mainly affected by the photosynthesis, feeding, respiration and decomposition processes [1], and marine plankton plays therefore an important role in the biological pump. Plankton can be classified in two main groups, zooplankton (animal plankton) and phytoplankton (vegetal plankton) [2]. Dissolved organic matter (DOM) production is mainly attributed to phytoplankton photosynthetic activity in surface seawater. However, the biological pump not only depends on the photosynthetic activity, but also of zooplankton feeding [3]. Therefore, plankton can be considered as the primary source of marine proteins dissolved in open oceans. Dissolved proteins are some of the most reactive

substances and the key factor in the direct effect of dissolved organic matter (DOM) in the global carbon cycle [4]. The analysis of proteins in plankton samples is therefore important for understanding the processes affecting global carbon cycle in marine ecosystems.

One or two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D or 2D-SDS-PAGE) has been commonly used for the separation of proteins. Tanoue [5, 6] and Tanoue et al. [7] have performed the characterization of proteins present in the particulate organic matter (POM) from seawater using 1D-SDS-PAGE. Results showed two different groups of proteins. The first group is directly derived from marine organisms, mainly phytoplankton, and it consists of a large number of low abundance proteins with a widely MWs range. The second group, however, includes specific proteins, which exhibit a narrow MWs range, between 45 and 66 kDa. Moncheva et al. [8] have performed SDS-PAGE for studying seasonal qualitative and quantitative phytoplankton proteins changes. SDS-PAGE analysis showed different protein patterns for each season. These authors have found that the major protein constituents were of 14, 37, 48 and 70 kDa in summertime; whereas, the sizes ranged between 38 and 48 kDa in the springtime. These results are similar to those reported by of Tanoue et al. [5-7]. However, because of 1D-SDS-PAGE limitations, methods with improved resolution are needed for separating and characterizing dissolved and particulate proteins. Tanoue et al. [9] have therefore applied 2D-SDS-PAGE to study the characteristics of particulate proteins in seawater. Proteins exhibiting a wide MWs range and mainly spots with acid pIs were found in the 2-DE gels. Nevertheless, 23 discrete proteins were distinguished by 2DE. Three discrete proteins were subjected to N-terminal amino acid sequence analysis, but only one protein was determined from the N terminus to the ninth amino acid residue. The N-terminal amino acid sequence of the protein was homologous to the heat shock protein of 70 kDa (HSP70) derived from photosynthetic organisms. HSP70 belongs to 70 kDa heat shock protein family, molecular chaperones with a role in protecting or repairing proteins from damages under environmental stress.

Other characterization methods such as Edman degradation are limited to providing sequence information routinely for only terminal 15 to 20 amino acids of the protein. Due to the limited sensitivity of this technique, alternative methods for proteins identification are needed [10]. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) and matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) are the better ionization techniques for biological macromolecules [11]. The ability for high-molecular-weight thermolabile molecules desorption, the high accuracy and sensitivity, and the allowed resolved mass range (1–300 kDa), make MALDI-TOF-MS an appealing technique for biomolecules identification, peptides, proteins, oligosaccharides and oligonucleotides included, in complex samples [12].

The objective of this study has been the identification of proteins from marine plankton by MALDI-TOF-MS. Proteins were isolated from plankton samples by using a phenol/SDS mixture as an extracting solution after different washing steps involving 10 % TCA/acetone and methanol, followed by protein precipitation using 0.1 M ammonium acetate in methanol. Proteins were then separated by 2D-SDS–PAGE, and enzymatically in-gel digested before MALDI-TOF-MS assessment.

9.2. Experimental

9.2.1. Apparatus

Protein identification was carried out in a mass spectrometer MALDI-TOF/TOF 4800 Analyzer (Applied Byosistems Foster City, CA, USA) in positive reflectron mode. Gel images were scanned in a Typhoon 9410 (GE Healthcare). Hitachi double–beam spectrophotometer model U–2010 (Hitachi, Berkshire, UK) equipped with 10 mm quartz cells was used for all UV–visible measurements. Isoelectrofocusing was performed with a Protean IEF System from Bio-Rad (Hercules, CA, USA), and second dimension was run in a Protean XL (BioRad). Other laboratory devices were an ultracentrifuge Laborzentrifugen model 2K15 (Sigma, Osterode, Germany), a centrifuge Centromix (Selecta, Barcelona, Spain),

a Reax top shaker from Heidoph (Schwabach, Germany), and a Basic 20 pH–meter with a glass–calomel electrode (Crison Instruments S.A., Barcelona, Spain).

9.2.2. Reagents and material

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore). Total protein assessment was performed with a Bradford protein assay containing Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) from Thermo Scientific (Rockford, IL, USA). IPG Dry Strips (7 cm, pH 3-10; and 10 cm, pH 3-10), and Plus One DryStrip cover fluid (GE Healthcare Life Science, Uppsala, Sweden) were used for pI protein fractionation. Buffer solution (10 mL) for equilibration after IEF was prepared with 6 M urea, 0.375 M Tris-HCl (pH 8.8), 20 % (v/v) glycerol, 2 % (w/v) sodium dodecyl sulphate (SDS), 0.002 % (w/v) bromophenol blue, and 100 mg DTT (all reagents from Sigma Aldrich, St. Louis, MO, USA). SDS gel (10 mL) was prepared with 2.3 mL of water, 5 mL of 30 % acrylamide mix (Bio Rad), 2.5 mL of 1.5 mol L-1 Tris-HCl pH 8.8 (Sigma-Aldrich), 0.1 mL of 10 % SDS (Sigma-Aldrich), 0.1 mL of 10 % ammonium persulfate (Bio Rad), and 0.004 mL of tetramethylethylenediamine (TEMED) (Bio Rad). Trichloroacetic acid, chloroform and acetone were from Panreac (Barcelona, Spain). Tris(hyddroxymethyl)aminomethane (Tris), sodium dodecyl sulphate (SDS), glycine, and ammonium persulfate were from Sigma Aldrich. Acetonitrile (HPLC grade) was from Scharlau. Trypsin, ammonium bicarbonate, iodoacetamine, trifluoacetic acid, formic acid and alpha-cyano-4-hydroxycinnamic acid were from Sigma Aldrich. Phenol solution, equilibrated with 10 mM Tris-HCl (pH 8.0, 1 mM EDTA) was provided by Sigma Aldrich. 2-DE rehydration solution was prepared with 5 M urea, 2 M thiourea, 2 mM tributyl-phosphine (Sigma-Aldrich), 65 mM DTT, 65 mM CHAPS (Sigma-Aldrich) , 0.15 M NDSB-256, 1 mM sodium vanadate (Sigma-Aldrich) , 0.1 mM sodium fluoride (Sigma-Aldrich), and 1 mM benzamidine (GE Healthcare Life Science). The running buffer (4 L) was prepared with glycine (576 g) (Sigma-Aldrich), Tris-HCl pH 8.8 (121.1 g) and SDS (40 g). The SDS buffer for protein extraction from plankton was prepared with 30 % (m/v) sucrose (AppliChem, Darmstad, Germany), 2 %

(m/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5 % (v/v) 2-mercaptoethanol (Fluka, Vancouver, Canada), and protease inhibitor cocktail (Roche Diagnostics, Mannhein, Germany). Kit for silver staining polyacrylamide gels was purchased from Bio Rad. Sypro Ruby stain (Lonza, Rockland, ME, USA) was used for gel stained.

9.2.3. Sample collection

Fresh plankton samples, consisting of mixture of microalgae (phytoplankton), were collected in a clam hatchery located at the Ría de Arousa estuary in pre-cleaned 5 L bottles. After collection, plankton was concentrated by removing the supernatant after centrifugation at 4000 rpm for 15 min at 20ºC.

9.2.4. Protein extraction

A portion of the concentrated plankton sample was first grinded into a fine powder in a mortar under liquid nitrogen. Six independent portions of 0.1 g of the grinded plankton were introduced into 2 mL tubes, and the sample was subjected to different washing stages before protein extraction. First, 2 mL of 10 % (v/v) TCA in acetone were added, and after vortexing for a few seconds, and further centrifugation at 10000 rpm, 4 ºC for 3 minutes, the supernatant containing interfering substances was discarded. Then, washing was performed with 2 mL of 0.1 M ammonium acetate in 80/20 methanol/water mixture (vortex shaking, centrifugation at 10000 rpm, 4ºC for 3 minutes, and supernatant discarding), followed by a final washing with 2 mL of a 80/20 acetone/water solution, and final oven-drying at 50 ºC for at least 10 minutes. Protein extraction was performed by adding 0.4 mL of phenol (pH 8.0) plus 0.4 mL of SDS buffer (30 % (m/v) sucrose, 2 % (m/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5 % (v/v) 2-mercaptoethanol and protease inhibitor cocktail) into each tube. The mixture was thoroughly vortexed and kept at room temperature for 5 minutes. The phenol phase was then separated by centrifugation (10000 rpm, 4 ºC) for 3 minutes, and the upper phenol phase containing proteins was transferred to 2 mL tubes. The phenol phase was finally

treated with 5 volumes of cold methanol containing 0.1 M ammonium acetate (approximately 2 mL), and the mixture was stored at -20ºC for 2 hours. Precipitated proteins were recovered by centrifugation at 4 °C and 2500 rpm 5 minutes, and successively washed with 100 % methanol and 80/20 acetone/water. Protein pellet obtained was finally dried under a N_2 stream, and re-dissolved in 300 µL of 2-DE rehydration solution.

9.2.5. Separation of proteins by 2D-SDS-PAGE

Aliquots of the re-dissolved protein pellet from plankton samples (300 µg) were applied to IPG strips, previously rehydrated for 12 h, for IEF. The IPG strips (17 cm, pH 3-10) were then covered with DryStrip Cover Fluid and IEF was performed using a Protean-IEF under the following conditions: voltage at 250 V for 15 min, 2 h linear gradient until 4000 V, 4 h linear gradient until 8000 V, 1 h at 8000 V, and stop and hold at 500 V. After IEF, the IPG strips were covered with a buffer solution (equilibration buffer described in section 9.2.2) for 15 min. SDS-PAGE was performed using 15 % polyacrylamide gel by applying an electric current of 15 mA at 10ºC. A molecular weight marker containing proteins within the 2 - 250 kDa MW range was used for protein MWs assessment (Dual Xtra Precision Protein Prestained Standards, BioRad). Finally, 2-DE gels were fixed in 10 % methanol/7 % acetic acid for 1 h, and stained overnight with Sypro Ruby fluorescent dye following the manufacturer's indications. After staining, gels were washed for 1 h in 10 % methanol/7 % acetic acid, and scanned in a Typhoon 9410.

9.2.6. Sample preparation for mass spectrometry

Protein spots chosen for mass spectrometric analysis were excised from the gels and manually in-gel digested with trypsin following the protocol defined by Shevchenko [13], with some modifications. The excised gel pieces were washed with 190 µL washing buffer (50 mM NH_4HCO_3, 50 % methanol v/v) at room temperature and 600 rpm for 20 min (this procedure was repeated three times). The gel pieces were then dehydrated for 10 min by addition of 100 µL acetonitrile

(ACN), followed by a 30 min incubation step at 56ºC in the presence of 50 µL 10 mM dithiotreitol (DTT) in 50 mM NH_4HCO_3 (enough volume to cover the gel pieces). The DTT solution was removed and the gel pieces were washed three times with washing buffer and dehydrated with ACN. The gel pieces were then incubated in 50 µL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 in the dark for 20 min at room temperature. After removal of the iodoacetamide solution, the gel pieces were washed three times with washing buffer and dehydrated with ACN. The gel pieces were swollen in a digestion buffer containing 20 ng μL^{-1} trypsin in 50 mM NH_4CO_3. Trypsin digestion was performed at 37 °C overnight. Peptides were extracted thrice by 20 min incubation in 40 µl of 60% acetonitrile in 0.5% HCOOH. The resulting peptide extracts were pooled, concentrated in a SpeedVac and stored at -20ºC.

9.2.7. MALDI-TOF-MS

For MS analysis, dried peptides were dissolved in 4 µL of 0.5 % formic acid. Equal volumes (0.5 µL) of peptide and matrix solution, consisting of 3 mg alpha-cyano-4-hydroxycinnamic acid (α-CHCA) dissolved in 1 mL of 50 % acetonitrile in 0.1 % trifluoroacetic acid, were deposited using the thin layer method, onto a 384 Opti-TOF MALDI plate (Applied Biosystems). Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). MS spectra were acquired in reflectron positive-ion mode with a Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots and at least three trypsin autolysis peaks were used as internal calibration. All MSMS spectra were performed by selection the precursors with a relative resolution of 300 (FWHM) and metastable suppression. Automated analysis of mass data was achieved using the 4000 Series Explorer Software V3.5. Peptide mass fingerprinting (PMF) and peptide fragmentation spectra data of each sample were combined through the GPS Explorer Software v3.6 using Mascot software v2.1. (Matrix Science) to search against a non-identical protein database (NCBInr release data 20100526), with 30 ppm precursor tolerance, 0.35 Da MSMS fragment tolerance, CAM (carbamydomethyl cystein) as fixed modification, oxidized methionine as variable

modification and allowing 1 missed cleavage. All spectra and database results were manually inspected in detail using the previous software. Protein scores greater than 56 were accepted as statistically significant (P< 0.05), considering positive the identification when protein score CI (confidence Interval) was above 98%. In case of MSMS spectra, total ion score CI was above 95%.

9.3. Results and discussion

Examination of Sypro Ruby stained 2D-gels demonstrated the existence of several proteins, around 140 different protein spots (Figure 1). Some of them offer low intensities, which means that proteins occur at low concentrations. In general, proteins exhibit MWs ranging from 5 to 150 kDa with pIs between 3 and 10. Most of the results agree with those obtained when analyzing similar plankton samples by OFFGEL-lab-on-chip (LOC) electrophoresis [14]. Although the higher resolution of the latter technique allowed the identification of more protein bands, coincidences for several Mw/pI ratios, mainly for single protein spots, are observed.

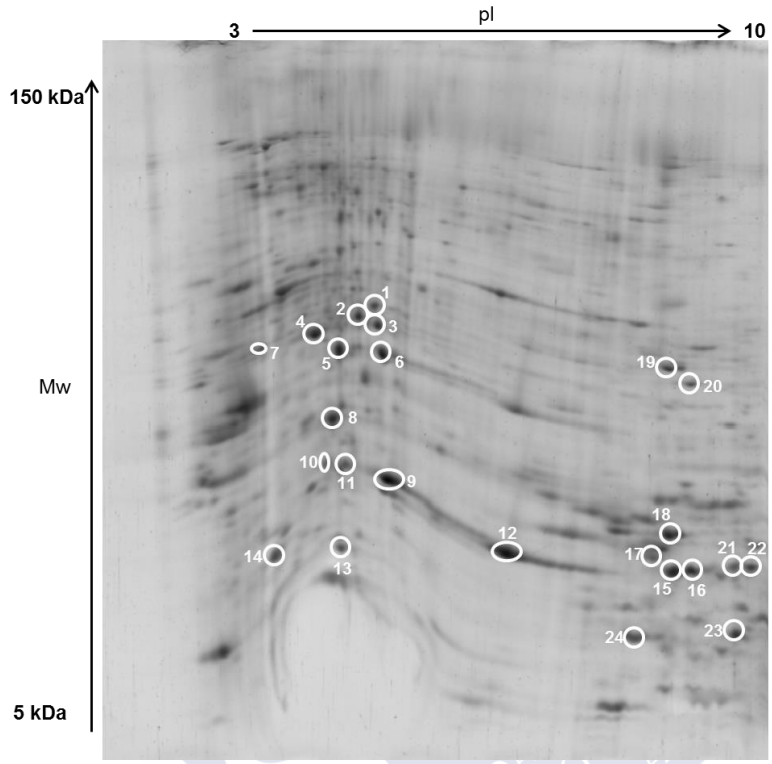

Figure 1: 2-DE gel of proteins.

Twenty-four protein spots (denoted as 1 to 23 in Figure 1) from a Sypro Ruby stained 2D-gel were subjected to tryptic digestion for protein identification by MALDI-TOF-MS. The proteins from the different spots were identified by querying NCBInr and SwissProt databases using the Mascot search engine. pBLAST was used in these spots which the identification failed; allowing the identification performing DeNovo Sequencing with the fragmentation spectra availables (between 3 to 10). Results are listed in Table 1, which also shows an assigned protein spot number, the protein identity, the score, and the theoretical and experimental pIs and MWs. Seven of these 24 spots were identified, and 4 different proteins were successfully identified. This was because some spots yielded confident identification for same protein. Sequences for several proteins were identified by similarity searching (Table 1). Ribulose bisphosphate carboxylase

small chain, Cytochrome C-550, Photosystem I reaction center subunit II and Photosystem II (12 kDa extrinsic protein) proteins were produced as homologue matches for seven of peptide tags. From Table 1, different protein spots exhibited remarkable discrepancies between the theoretical and experimental values in MW and/or pI. These discrepancies could be attributed to isoforms generated by a variety of causes, including post-translational modifications, alternative splicing, and the occurrence of multigene families [15].

Some available characteristics, such as molecular function, biological process and cellular component, of the proteins found in the current work are listed in Table 2. The identified proteins corresponded to different species of marine plankton. The peptide tag sequence for the protein cytochrome c-550 returned matches for *Thalassiosira pseudonana*, *Phaeodactylum tricornutum* and *Synechococcus. Thalassiosira pseudonana* and *Phaeodactylum tricornutum* are marine diatoms, a group of marine unicellular algae (phytoplankton) which play an important role in the marine food chain, and which also significantly contribute to drawdown of atmospheric CO_2 [16]. *Synechococcus* is a marine cyanobacterium and an important component of the autotrophic picoplankton (phytoplankton).

Table 1: List of identified proteins.

Spot no.	Protein name	Peptide tag	Score	matching peptides	MW (kDa)		pI	
					Theo.	Exp.	Theo.	Exp.
9	Ribulose bisphosphate carboxylase oxygenase small chain	VYTIESYAVTSKPEG ERY	109	15/18	16.1	16.0	6.58	5.3
10	Cytochrome C-550	IDPEAISIATPR	77	12/12	17.8	18.0	6.8	4.6
11	Ribulose bisphosphate carboxylase oxygenase small chain	SVKANPEGGRY	70	10/11	15.9	17.0	5.01	4.8
12	Ribulose bisphosphate carboxylase oxygenase small chain	DVAAVMMEINACR	76	11/13	15.9	10.0	6.57	6.6
15	Photosystem I reaction center subunit II	EKIMEMPTSGAAIMK K	79	12/16	15.7	9.0	8.87	8.4
21	Photosystem I reaction center subunit II	NGENIIYIAR	66	10/10	25.3	10.0	5.85	8.9
24	Photosystem II 12 kDa extrinsic protein, choroplastic	DYAGVGYIGGSSTID VNNANLR	108	17/22	16.7	5.0	9.85	8.1

The peptide tag sequence for the protein Photosystem II 12 kDa returned matches for *Karenia brevis*, *Cyanidium caldarium* and *Phaeodactylum tricornutum. Karenia brevis* is a marine toxic dinoflagellate responsible for red tide; whereas, *Cyanidium caldarium* is a microscopic red algae belonging to marine phytoplankton. Enami et al. [17] have reported that the protein cytochrome c-550 is an extrinsic component stoichiometrically associated with photosystem II (PS II) complex from the red alga *Cyanidium caldarium*, and is required for maintaining PS II oxygen-evolving activity and the effective binding of the 12 kDa extrinsic protein to PS II.

The peptide tag sequence for the protein Photosystem I reaction center subunit II returned matches for *Odontella sinensis* (marine diatom), *Chlamydomonas reinhardtii* (single celled green alga), *Skeletonema costatum* (marine diatom) and *Synechococcus*. Photosystem I and photosystem II reaction centers are complex proteins involved in the process of photosynthesis, and they are located in the thylakoid membrane.

Table 2: Molecular function, biological process and cellular component of identified proteins.

Protein name	Molecular function	Biological process	Cellular component
Ribulose bisphosphate carboxylase oxygenase small chain	Lyase, monooxygenase and oxidoreductase	Calvin cycle, carbon dioxide fixation, photorespiration and photosynthesis	Chloroplast and plastid
Cytochrome C-550	electron carrier activity, heme binding and iron ion binding	cytochrome c-heme linkage, photosynthesis (light reaction) and respiratory electron transport chain	Chloroplast thylakoid membrane and photosystem II
Photosystem I reaction center subunit II		Photosynthesis	Chloroplast thylakoid membrane and photosystem I reaction center
L-ribulose-5-phosphate 4-epimerase	L-ribulose-phosphate 4-epimerase activity	L-ascorbic acid catabolic process	
Ribosomal protein S16	Structural constituent of ribosome	Translation	Ribosome
Photosystem II 12 kDa extrinsic protein, choroplastic		Oxidation-reduction process, photosynthesis and photosystem II stabilization	Chloroplast thylakoid membrane, extrinsic to membrane and oxygen evolving complex

The peptide tag sequence for the protein ribulose bisphosphate carboxylase oxygenase (RuBisCO) returned matches for different species of phytoplankton: marine diatoms *Cylindrotheca*, *Denotula confervacea* and *Thalassiosira nordenskioeldii*, and marine phytoflagellate *Olisthodiscus.* RUBISCO is one of the most important enzymes and the most abundant protein in the planet. RuBisCO is a vital enzyme in the photosynthetic cycle, which is responsible for catalyzing the bulk of the conversion of inorganic carbon to organic carbon; therefore, it is responsible for 95 % of marine photosynthesis [18]. This enzyme was detected and identified by Powell et al. [19] as a dissolved protein in seawater. This fact is consistent with the idea that DOM production in the marine environment is mainly due to extracellular DOM releasing by phytoplankton and DOM releasing due to cellular phytoplankton lysis by viruses. RuBisCO has also been found to be assembled in marine polymer microgels, three-dimensional polymers ranging from colloidal to micrometer size (standing at the DOM - POM interface). The polymer gels may account for at least 6.6 x 10^{16} g C in the global organic DOC pool. Thus, microgels might be an important mechanism for transferring proteins and DOM to

the deep ocean, and may protect RuBisCO from degradation in the water column [20].

9.4. Conclusions

Marine plankton proteins were efficiently isolated by an improved method based on phenol/SDS extractions before several washing steps with a 10 % TCA in acetone, and methanol. Proteins from marine plankton were separated by 2D-SDS-PAGE. The results showed the occurrence of several proteins exhibiting pIs and MWs in a wide range (pI between 3 and 10, MWs between 5 and 160 kDa). Molecular-level characterization of dissolved proteins from seawater by MALDI-TOF-MS after tryptic digestion provided different peptide sequences which allowed the identification of most of the fractionated proteins. Specifically, four different proteins were successfully identified. One of the identified proteins was RuBisCO, the most important enzyme and the most abundant protein on Earth. This protein was previously detected and identified by Powel et al. [19] as a dissolved protein in seawater, and this finding supports the idea that the production of DOM in the marine environment is mainly due to DOM produced by phytoplankton.

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IV. CONCLUSIONES

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Conclusiones

Las principales conclusiones de este trabajo se han ido exponiendo al final de cada capítulo de la parte experimental, por lo que a continuación se recogen de forma resumida las conclusiones generales más relevantes de esta tesis.

La materia orgánica natural (NOM) juega un papel muy importante en procesos como el transporte y la biodisponibilidad de metales traza en el medio marino. La NOM marina se puede clasificar en materia orgánica disuelta (DOM), dentro de la cual se encuentran las proteínas disueltas en el agua de mar, y en materia orgánica particulada (POM), dentro de la cual se encuentra el plancton marino. Por lo tanto, las conclusiones de este trabajo las podemos dividir en tres bloques: Un primer bloque centrado en el estudio de la DOM, un segundo bloque centrado en el estudio de las proteínas disueltas, y un tercer bloque centrado en el estudio de las proteínas extraídas de plancton marino.

1. En relación a la caracterización y la determinación de metales unidos a DOM en el agua de mar.

1.1 Se ha desarrollado un método para preconcentrar DOM en agua de mar empleando MWCNTs como adsorbente de extracción en fase sólida. Los porcentajes de retención de DOM obtenidos variaron entre un 34% y un 81% dependiendo de la muestra de agua de mar. Las recuperaciones no cuantitativas pueden ser atribuidas a la heterogeinedad de la DOM en el agua de mar, lo cual significa que solo la DOM de un determinado peso molecular es capaz de interaccionar con el adsorbente. La corroboración de esta premisa se obtuvo tras el desarrollo de métodos de separación cromatográfica (HPLC-SEC) cuya aplicación reveló el aislamiento de sustancias con pesos moleculares comprendidos entre 125 y 1102 Da. Estos resultados nos confirman que los MWCNTs solo son capaces de retener la DOM de bajo peso molecular, fracción que representa el mayor porcentaje de DOM en agua de mar superficial.

1.2. Se ha desarrollado un método para preconcentrar DOM en agua de mar empleando la ultrafiltración en flujo tangencial (membrana con un tamaño de corte molecular de 10 kDa) para abordar el aislamiento de la DOM de alto peso molecular. La determinación del TOC en el agua de mar, *retentate* y *permeate* nos reveló que un 14,1 ± 2 % del TOC se pierde a través del proceso de UF. Para la caracterización de la DOM aislada se ha desarrollado y optimizado un método de cromatografía bidimensional basado en la separación por tamaños (SEC) y la posterior separación de las distintas fracciones por cambio aniónico (AEC). Como técnicas de detección se ha empleado espectrometría UV para la caracterización de DOM, y el detector ICP-MS para la determinación de los metales asociados a DOM. La caracterización de DOM mediante SEC con detección UV mostró la presencia de DOM con tamaños moleculares entre 6,5 y 16 kDa. La caracterización de estas fracciones de SEC mediante AEC mostró la existencia de cuatro grupos diferentes de macromoléculas. Mediante el acoplamiento de AEC con ICP-MS se probó la asociación de elementos traza como el Sr y el Zn a las sustancias correspondientes a la primera fracción de AEC, mientras que el Mn se encontró asociado a la segunda fracción y el Co a la tercera.

1.3. Se ha desarrollado un método para preconcentrar DOM en agua de mar empleando la ultrafiltración en flujo tangencial (membrana con un tamaño de corte molecular de 3,0 kDa) para abordar el aislamiento de la DOM de alto peso molecular. Los resultados tras aplicación de cromatografía bidimensional (SEC/AEC-HPLC-UV/ICP-MS) mostró el aislamiento de substancias con tamaños moleculares (21,6 – 1,2 kDa) similares a los encontrados tras el empleo de membranas de ultrafiltración de mayor corte molecular (16,1 – 1,6 kDa). La posterior caracterización de las fracciones de SEC mediante AEC-UV mostró la existencia de dos grupos diferentes de substancias, lo cual contrasta con los resultados obtenidos tras la ultrafiltración con membranas de 10 kDa (cuatro grupos de substancias). Del mismo modo, el acoplamiento SEC-ICP-

MS reveló la existencia de metales como el Mn, Mo, Sr y Zn asociados a la DOM en el agua de mar.

2. En relación a la caracterización y la determinación de metales asociados a proteínas disueltas en agua de mar.

2.1. Se abordó el aislamiento de proteínas disueltas en agua de mar, fracción importante dentro de la DOM de alto peso molecular. Para ello se desarrolló una separación bidimensional basada electroforesis OFFGEL y electroforesis en microfluidos (Lab-on-chip, LOC). Se encontró que la salinidad de las fracciones de proteínas aisladas condicionaba enormemente la separación electroforética por OFFGEL (separación en función de los puntos isoeléctrico, pIs). El mejor tratamiento del *pellet* de proteínas consistió en la eliminación de compuestos solubles en agua y en metanol durante la precipitación de las proteínas aisladas por UF. La mayoría de las proteínas disueltas en el agua de mar superficial mostraron pIs básicos (8,10 y 8,37) y pesos moleculares entre 15 y 63 kDa, aunque también se detectaron algunas proteínas con pIs ácidos de 4.82 a 5.73 (15 – 63 kDa). Las proteínas disueltas encontradas en agua de mar profunda fueron, sin embargo, de menor peso molecular (entre 21 – 24 kDa) y con pIs ácidos (3.30 – 4.22). Se encontró la asociación de metales como Cd, Cu, Mn y Ni asociados a las proteínas disueltas de pIs alcalino (fracciones OFFGEL), mientras que el Zn se encontró asociado principalmente a proteínas con pIs ácidos. En contrapartida, solamente Cu y Mn se encontraron asociados a proteínas disueltas en el agua de mar profunda. Además, la concentración de Mn (de 68 a 84 µg L^{-1}) fue mucho mayor que en el agua de mar superficial (en torno a 22.4 µg L^{-1}). Finalmente, estudios de estabilidad de complejos metal-proteína (empleando proteínas tipo, tales como superóxido dismutasa, SOD, y alcohol deshidrogenasa, ADH) revelaron que las condiciones desnaturalizantes empleadas en electroforesis OFFGEL eran adecuadas para garantizar la estabilidad de los complejos Zn-SOD y Zn-ADH (complejos metal-proteína de alta afinidad). Por el contrario, las condiciones empleadas durante la separación electroforética OFFGEL no garantizaron la

estabilidad del complejo Cu-SOD. De esta forma, se puede concluir que las condiciones inherentes a la electroforesis OFFGEL permiten la estabilidad de complejos metal-proteína de alta afinidad.

2.2. Se empleó la 2D-SDS-PAGE para la separación de proteínas disueltas en agua de mar seguida de a determinación de metales asociados a estas proteínas por LA-ICP-MS. Los resultados obtenidos revelaron la presencia complejos entre algunas proteínas (pesos moleculares entre 10 y 14 kDa, y pIs entre 5,8 y 7,3) y metales como Cd, Cr, Cu, y Zn. En comparación con los resultados obtenidos tras separación por OFFGEL-LOC, el número de spots conteniendo metales, así como la concentración de metales en los mismos, tras electroforesis bidimensional convencional fue mucho menor. Además, no se detectó la presencia de complejos Mn- y Ni-proteína. La posible explicación puede atribuirse al revelado de los geles para el posterior análisis por LA-ICP-MS, procedimiento que condiciona la estabilidad de algunos complejos metal-proteína; y también, a las condiciones desnaturalizantes más drásticas empleadas en los métodos electroforéticos convencionales.

2.3. Mediante 2D-SDS-PAGE y MALDI-TOF-MS se han conseguido identificar once proteínas diferentes en agua de mar: Penicillinase repressor, flagelar hook-associated protein FliD, glucose/sorbosone dehydrogenase, carbamoyl-phosphate synthase small subunit, TRAP-T family transporter, L-aspartate oxidase, translation elongation factor Tu, malate synthase A, ATP-dependent DNA helicase RecQ, Glycogen/starch/alpha-glucan phosphorylase, y LysA. Excepto dos proteínas de membrana (TRAP-T y LysA), la mayoría de las proteínas identificadas son enzimas, lo cual demuestra que las proteínas de membrana de bacterias no son la única fuente de proteínas disueltas en el agua de mar.

3. En relación a la caracterización y la determinación de metales asociados a proteínas extraídas de plancton marino.

3.1. Se empleó la electroforesis bidimensional OFFGEL-LOC para la evaluación de tres procedimientos diferentes para la extracción de proteínas en un material de referencia certificado BCR-414 (plancton). En función de la intensidad y del número de bandas de proteínas obtenidas el procedimiento de extracción seleccionado fue el que emplea fenol/SDS como extractante tras varias etapas de lavado con TCA/acetona y metanol. Las bandas de proteínas resultantes tras el análisis de muestras de plancton marino tienen pesos moleculares de 6,4 a 57,3 kDa, y las concentraciones de proteína variaron entre 50,0 y 925.9 ng μL^{-1}. La determinación de metales asociados a proteínas extraídas de plancton en las fracciones líquidas recogidas tras la separación mediante electroforesis OFFGEL mostró la presencia de éstos en proteínas con pesos moleculares entre 52.6 y 57.3 kDa y pIs entre 4.40y 8.60. Metales como Cd, Mn, Fe, Ni y Zn se encontraron principalmente asociados a proteínas con pIs ácidos.

3.2. Se empleó la 2D-SDS-PAGE para la separación de proteínas disueltas y proteínas extraídas de plancton seguida de LA-ICP-MS para la determinación de metales asociados a estas proteínas. Los resultados obtenidos revelaron la presencia de Cd, Cr, Cu, y Zn asociados a proteínas extraídas de plancton de pesos moleculares entre 9 y 22 kDa, y pIs entre 4,5 y 10. Igual que en el caso de las proteínas disueltas en agua de mar, el número de spots de proteínas conteniendo metales, y la concentración de los mismos, fue inferior al encontrado tras electroforesis bidimensional OFFGEL-LOC. De la misma forma, tanto el revelado de los geles como las condiciones empleadas en 2D-SDS-PAGE son los factores responsables de la ruptura de ciertos complejos metal-proteína durante el proceso de separación.

3.3. Mediante 2D-SDS-PAGE y MALDI-TOF-MS se han conseguido identificar cuatro proteínas diferentes en el plancton marino: Ribulose bisphosphate

carboxylase small chain, Cytochrome C-550, Photosystem I reaction center subunit II, and Photosystem II (12 kDa). Una de las proteínas identificadas es la RUBISCO, la enzima más importante y abundante de la tierra. Esta enzima fue detectada también en el agua de mar por otros autores, por lo tanto se puede confirmar que el fitoplancton es una de las principales fuentes de DOM en el agua de mar.

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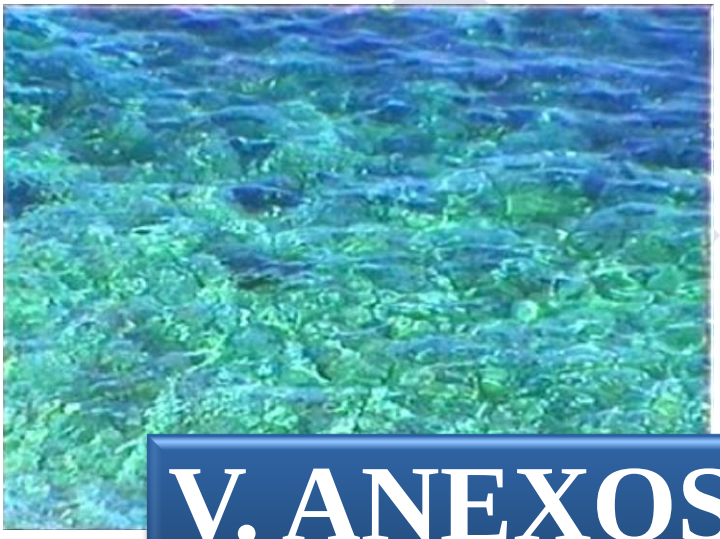

V. ANEXOS

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ANEXO I: INDIVIDUAL ELECTROPHEROGRAMS FOR OFFGEL FRACTION FOR PROTEINS EXTRACTED FROM PLANKTON

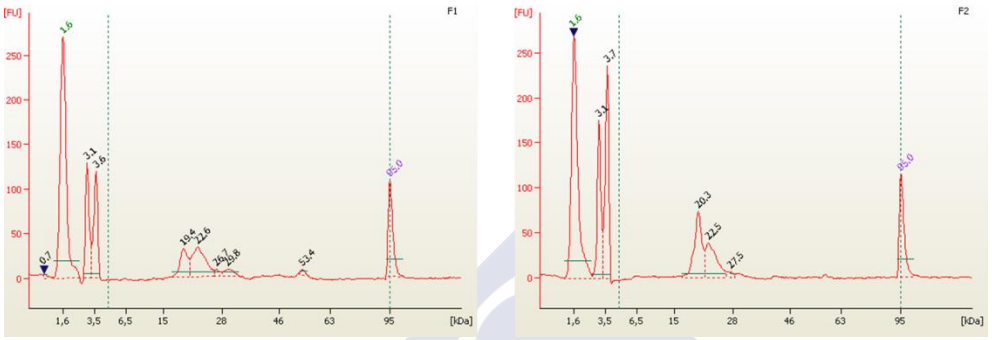

Figure I: Electropherograms correspoding to OFFGEL fractions F1 y F2.

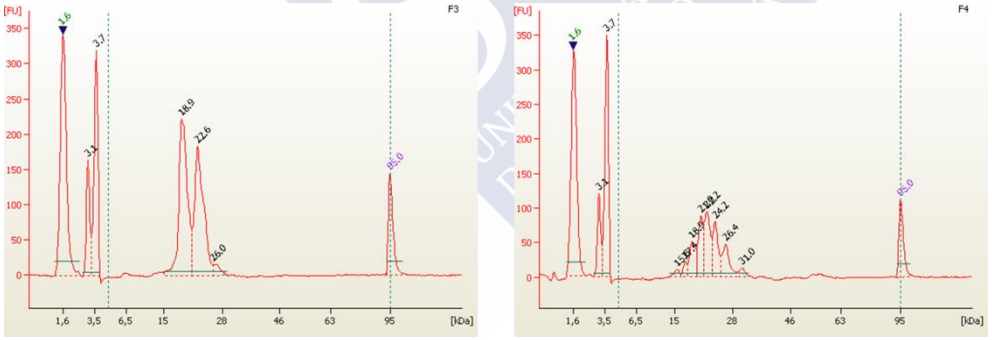

Figure II: Electropherograms correspoding to OFFGEL fractions F3 y F4.

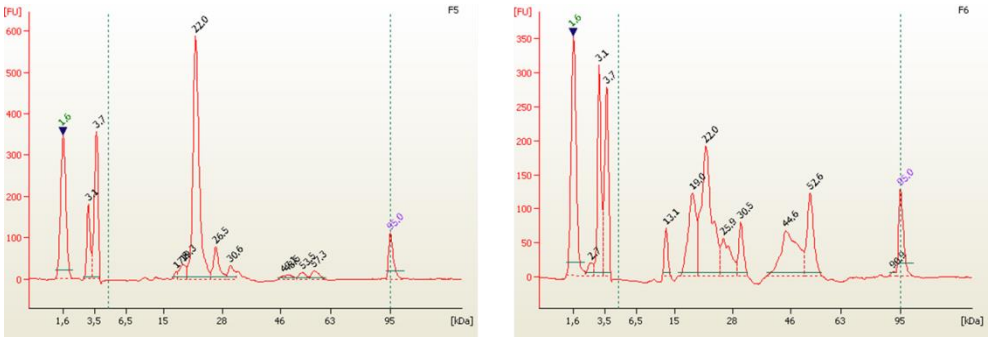

Figure III: Electropherograms correspoding to OFFGEL fractions F5 y F6.

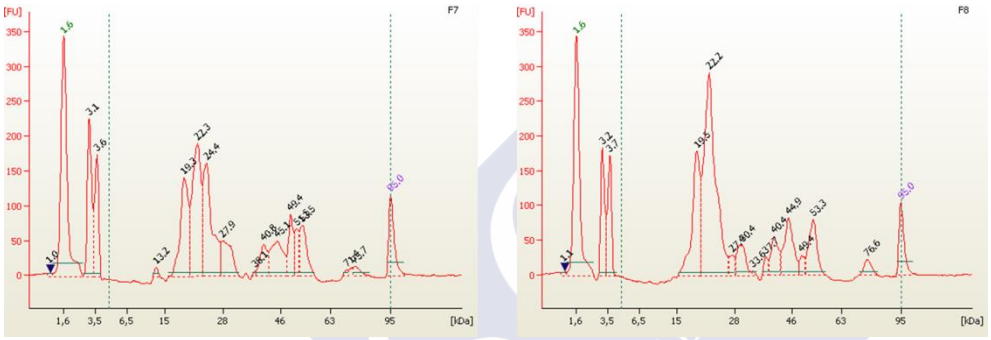

Figure IV: Electropherograms correspoding to OFFGEL fractions F7 y F8.

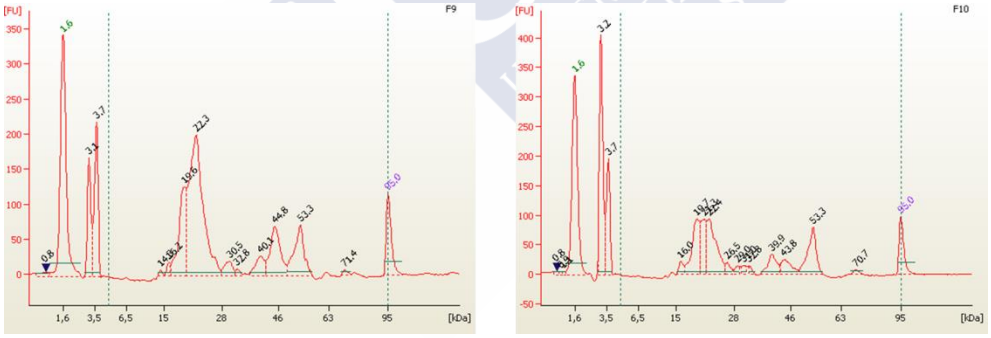

Figure V: Electropherograms correspoding to OFFGEL fractions F9 y F10

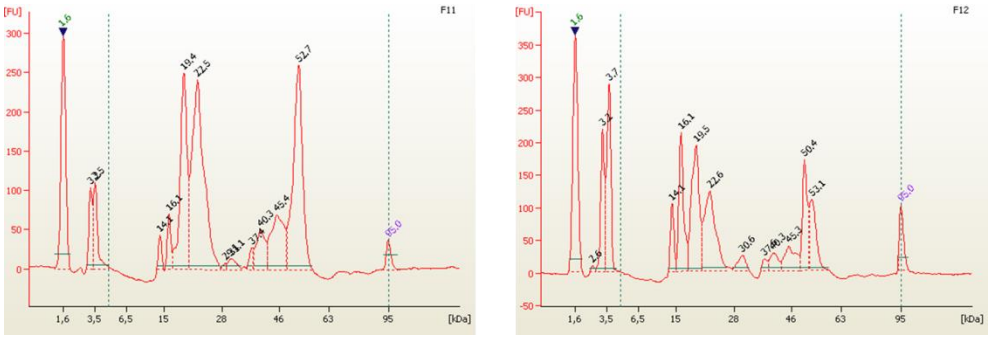

Figure VI: Electropherograms correspoding to OFFGEL fractions F11 y F12.

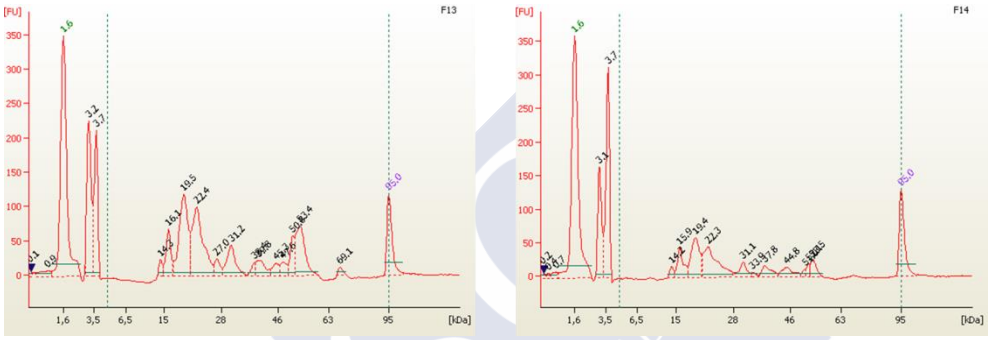

Figure VII: Electropherograms correspoding to OFFGEL fractions F13 y F14.

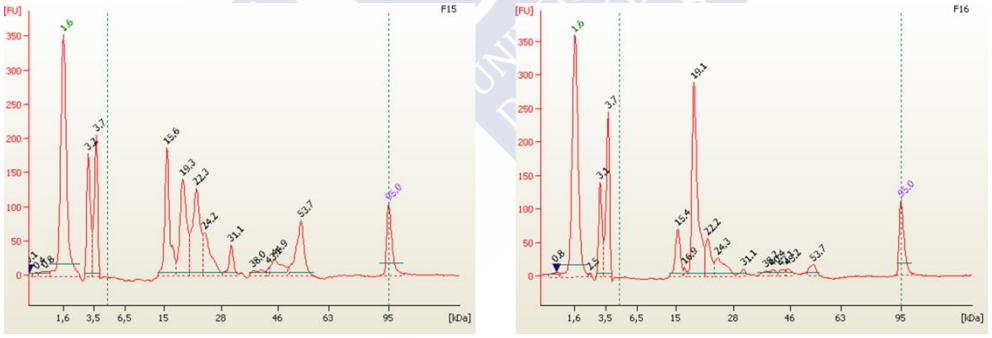

Figure VIII: Electropherograms correspoding to OFFGEL fractions F15 y F16.

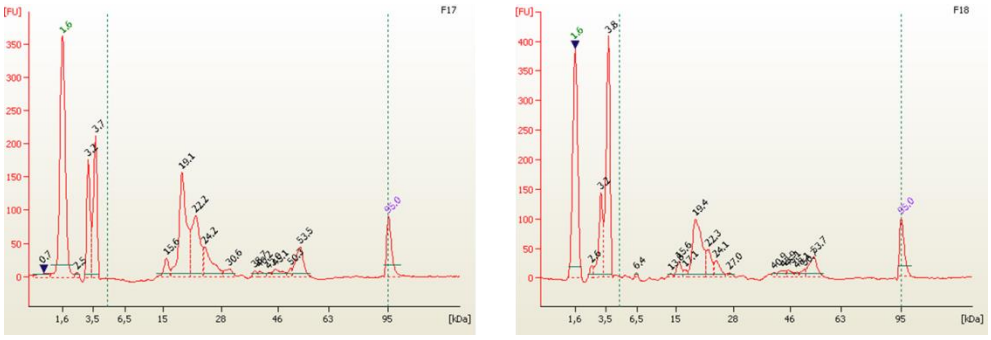

Figure IX: Electropherograms correspoding to OFFGEL fractions F17 y F18.

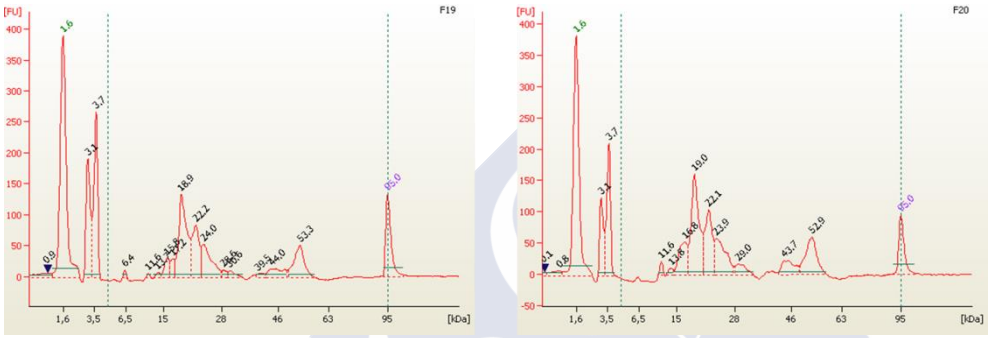

Figure X: Electropherograms correspoding to OFFGEL fractions F19 y F20.

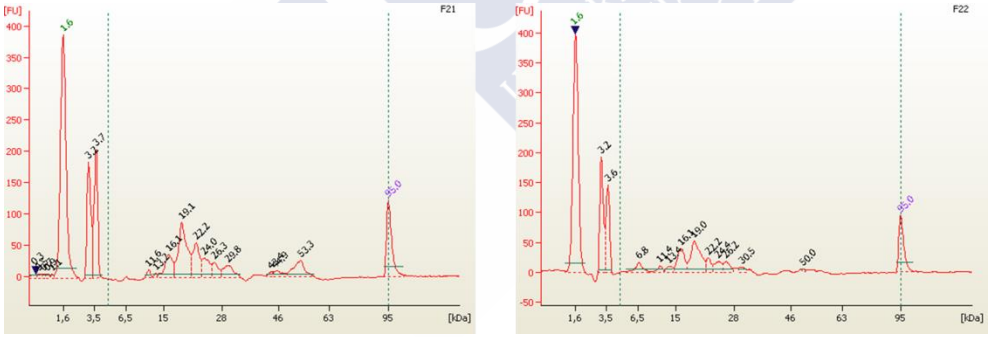

Figure XI: Electropherograms correspoding to OFFGEL fractions F20 y F21.

ANEXO II: ABBREVIATIONS AND ACRONYMS

1D-PAGE	One dimensional polyacrylamide gel electrophoresis
1D-SDS-PAGE	One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2-DE	Two dimensional electrophoresis
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
2D-SDS-PAGE	Two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
A	
ACN	Acetonitrile
AEC	Anion exchange chromatography
AEC-HPLC	Anion exchange chromatography – high performance liquid chromatography
AEC-ICP-MS	Anion exchange chromatography - inductively coupled plasma - mass spectrometry
ADH	Alcohol dehydrogenase
AN-PAGE	Anodal native polyacrylamide gel electrophoresis
APS	Ammonium persulfate
AsFlFFF	Asymetric flow field-flow fractionation
B	
BME	β- mercaptoethanol
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BSA	Bovine serum albumine

C

CAs	Carrier amphoteric components
CE	Capillary electrophoresis
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID	Collision-induce dissociation
CNTs	Carbon nanotubes
CRAM	Carboxyl-rich alicyclic molecules
CZE	Capillary zone electrophoresis

D

DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DIC	Dissolved inorganic carbon
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DRC	Dynamic reaction cell
DS	Denaturing solution
DSPE	Dispersive solid-phase extraction
DT-MS	Direct temperature-resolved mass spectrometry
DTT	1,4-dithiothreitol

E

EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
ETAAS	Electrothermal Atomic Absorption Spectrometry

F

FFL	Fulvic-like fluorescence
FA	Formic acid
FASP	Filter aided sample preparation

G	
GAC	Granular activated carbon
G/D	Gel-Dye Mix
GE	Gel electrophoresis
H	
HA	Humic acid
HF	Fulvic acid
HMW	High molecular weight
HPLC	High performance liquid chromatography
HPSEC	High-performance size exclusion chromatography
HS	Humic substances
I	
IAA	Iodoacetamine
IC	Inorganic carbon
ICP-MS	Inductively coupled plasma – mass spectrometry
ICP-OES	Inductively coupled plasma – optical emission spectrometry
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
L	
LA	Laser ablation
LA-ICP-MS	Laser ablation – inductively coupled plasma – mass spectrometry
LC	Liquid chromatographic
LMW	Low molecular weight
LOC	Lab-on-chip electrophoresis
LOD	Limit of detection
LOQ	Limit of quantification

M	
MALDI	Matrix-assisted laser desorption/ionization
MALDI-FTIR-MS	Matrix assisted laser desoption/ionization - Fourier transform ion cyclotron resonance mass spectrometry
MALDI-TOF-MS	Matrix assisted laser desorption/ionization - time of flight - mass spectrometry
MS	Mass spectrometry
MW	Molecular weight
MWCNTs	Multi-walled carbon nanotubes
MWCO	Molecular-weight cut-off
N	
NC	Nitro cellulose
NDIR	Non-dispersive infra red
NOM	Natural organic matter
NPOC	Non-purgeable organic carbon
P	
PAHs	Polycyclic Aromatic Hydrocarbons
PEGs	Polyethylene glycols
PEOs	Polyethylene oxides
PFL	Protein-like fluorescence
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
POM	Particulate organic matter
PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
R	
RO/ED	Reverse osmosis/electrodyalisis
RPC	Reverse phase chromatography
RUBISCO	Ribulose-1,5-bisphosphate carboxylase oxygenase

S

SBA	Surface blocking agent
SCE	Strong cation exchange chromatography
SCX	Strong cation exchange chromatography
SCWO	Super critical water oxidation
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sultate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEC-HPLC	Size exclusion chromatography – high performance liquid chromatography
SEC-ICP-MS	Size exclusion chromatography – inductively coupled plasma - mass spectrometry
SOD	Superoxide dismutase
SPE	Solid phase extraction
SPE-GEMS	Solid-phase extraction – gradient elution – mass spectrometry
SWCNTs	Single-walled carbon nanotubes

T

TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TOC	Total organic carbon
TMT	Tandem mass tags

U

UF	Ultrafiltration
UV	Ultraviolet
UV-Vis	Ultraviolet-visible

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ANEXO III: PUBLICATIONS

COMPRISED IN THIS MANUSCRIPT:

J. Sánchez-González, N. García-Otero, P. Bermejo-Barrera, A. Moreda-Piñeiro, *Multi-walled carbon nanotubes – Solid phase extraction for isolating marine dissolved organic matter before characterization by size exclusion chromatography*, Microchemical Journal 102 (2012) 75-82. **Parte experimental, Capítulo 1.**

N. García-Otero, P. Bermejo-Barrera, A. Moreda-Piñeiro, *Size exclusion and anion exchange high performance liquid chromatography for characterizing metals bound to marine dissolved organic matter*, Analytica Chimica Acta 760 (2013) 83-92. **Parte experimental, Capítulo 2.**

N. García-Otero, P. Herbello-Hermelo, R. Domínguez-Gonzalez, M. Aboal-Somoza, A. Moreda Piñeiro, P. Bermejo-Barrera, *Evaluation of tangential flow ultrafiltration procedures to assess trace metals bound to marine dissolved organic matter*, Microchemical Journal 110 (2013) 501–509. **Parte experimental, Capítulo 3.**

N. García-Otero, E. Peña-Vázquez, M. C. Barciela-Alonso, P. Bermejo-Barrera, A. Moreda-Piñeiro, *Two-dimensional isoelectric focusing OFFGEL and microfluidic lab-on-chip electrophoresis for assessing marine proteins in seawater*, Analytical Chemistry 85 (12) (2013) 5909–5916. **Parte experimental, Capítulo 4.**

N. García - Otero, M. C. Barciela - Alonso, A. Moreda – Piñeiro, P. Bermejo-Barrera, *Study of extraction procedures for protein analysis in plankton samples by OFFGEL electrophoresis hyphenated with Lab-On-Chip technology*, Talanta 115 (2013) 631–641. **Parte experimental, Capítulo 5.**

N. García-Otero, M. C. Barciela-Alonso, R. Domínguez-González, P. Herbello-Hermelo, A. Moreda-Piñeiro, P. Bermejo-Barrera, *Integrated OFFGEL and Lab-on-chip electrophoresis for characterizing marine plankton proteins*, Send to Electrophoresis. **Parte experimental, Capítulo 6.**

N. García-Otero, M. C. Barciela-Alonso, P. Bermejo-Barrera, A. Moreda-Piñeiro, M. S. Jiménez, M. T. Gómez, J. R. Castillo, *Assessment of metals bound to marine plankton proteins and to dissolved proteins in seawater,* Analytica Chimica Acta (2013) doi:10.1016/j.aca.2013.10.016. **Parte experimental, Capítulo 7.**

OTHER PUBLICATIONS:

N. García-Otero, C. Teijeiro-Valiño, J. Otero-Romaní, E. Peña-Vázquez, A. Moreda-Piñeiro, P. Bermejo-Barrera, *On-line ionic imprinted polymer selective solid phase extraction of Nickel and lead from seawater and their determination by inductively coupled plasma-optical emission spectrometry*, Analytical and Bioanalytical Chemistry 395 (2009) 1107-1115.

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Este trabajo se centra en la especiación funcional y química multielemental en materia orgánica disuelta (DOM) y proteínas marinas. Por una parte se ha realizado la caracterización y determinación de metales asociados a DOM mediante el empleo de la cromatografía líquida de alta resolución con detección UV y detección por espectrometría de masas con plasma de acoplamiento inductivo. Por otra parte, se ha llevado a cabo la detección y caracterización de proteínas disueltas en el agua de mar y proteínas extraídas de plancton marino mediante electroforesis en gel y electroforesis OFFGEL, así como la determinación de los metales asociados a las mismas por LA-ICP-MS, ICP-OES y ETAAS, y su identificación mediante MALDI-TOF-MS.