VNIVERSITAT DE VALÈNCIA

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Advanced liquid chromatographic techniques applied to industrial quality control of cleaning products

Técnicas avanzadas en cromatografía líquida aplicadas al control de calidad industrial en formulados de productos de limpieza

Memoria para alcanzar el grado de Doctor en Química dentro del Programa de Doctorado en Química (RD 1393/2007) presentada por:

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Certifican

Que la presente memoria, que lleva por título "*Advanced liquid chromatograpic techniques applied to industrial quality control of cleaning products (Técnicas avanzadas en cromatografía líquida aplicadas al control de calidad industrial en formulados de productos de limpieza)*" constituye la Tesis Doctoral de D. Aarón Escrig Doménech

Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que así conste a los efectos oportunos y a petición del interesado, firmamos la presente en Burjassot, a 26 de mayo de 2017.

Guillermo Ramis Ramos Ernesto Francisco Simó Alfonso

Preface

This Thesis is presented as "a compendium of publications", as it is regulated by the University of Valencia (Reglamento 29/11/2011, ACGUV 266/2011). Accordingly, the first part of the thesis contains a general introduction where all the articles are presented, with justification of the subject and also explaining the original contribution of the PhD candidate. The PhD student has contributed substantially in all stages of development of all the articles, from the development of the idea, literature search, experimental realization, analysis and interpretation of data, drafting and preparation of the manuscript, and monitoring and final correction thereof according to the recommendations of the referees. Then the published articles are included. These correspond entirely to indexed journals. All articles have been written by Aarón Escrig Doménech (identified as first author), with corrections and final review by the supervisors of this Thesis. According to the regulation quoted above, the last part of the thesis contains a comprehensive summary of results, discussion and conclusions.

Esta Tesis se acoge a la modalidad “compendio de publicaciones”, contemplada en el Reglamento de la Universidad de Valencia de 29/11/2011 (ACGUV 266/2011). De acuerdo con dicha normativa, la primera parte de la Tesis contiene una introducción general, donde se presentan los trabajos compendiados, justificando su temática y explicando la aportación original del doctorando. El doctorando ha contribuido sustancialmente en todas las etapas de desarrollo de todos los artículos, desde la elaboración de la idea, búsqueda bibliográfica, realización experimental, análisis e interpretación de los datos, redacción y preparación del manuscrito, y seguimiento y corrección final del mismo de acuerdo con las recomendaciones de los evaluadores. A continuación, se incluyen los artículos ya publicados, los cuales corresponden en su totalidad a revistas indexadas. Todos los artículos han sido escritos por Aarón Escrig Doménech (identificado como primer autor), con correcciones y revisión final por parte de los supervisores de esta Tesis. De acuerdo con la normativa citada, la última parte de la Tesis contiene un resumen global de resultados, discusión y conclusiones.

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A mi familia

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Por último, agradecer a mis padres y hermanos, por su comprensión consejos y ánimos durante todos estos años. Sin su apoyo esta tesis no hubiera sido posible. Gracias.

ABBREVIATIONS

1,4-BuOH	1,4-Butanediol
2D-HPLC	Two-dimensional liquid chromatography
ABS	Alkyl benzene sulfonates
ACN	Acetonitrile
AES	Alkyl ether sulfates
AIBN	α,α'-azobisisobutyronitrile
AOS	α-olefin sulfonates
APCI	Atmospheric pressure chemical ionization
APE	Alkyl phenol ethoxylate
APES	Alkyl phenol ether sulfate
APG	Alkylpolyglucoside
AS	Alkyl sulfates
BDDA	1,3-Butanediol diacrylate
BGE	Background electrolyte
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
DAD	Diode array detector
EDTA	Ethylenediaminetetraacetate
ELSD	Evaporative light scattering detector
EO	Ethylene oxide
EOF	Electroosmotic flow
ESI	Electrospray ionization
FAE	Fatty alcohol ethoxylate
HAcO	Acetic acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
GC	Gas chromatography
LA	Lauryl acrylate
LAB	Linear alkylbenzene

LAS	Linear alkylbenzene sulfonates
LEAC	Liquid exclusion-adsorption chromatography
LC	Liquid chromatography
LCCC	Liquid chromatography under critical conditions
LPO	Lauroyl peroxide
LOD	Limit of detection
LOQ	Limit of quantitation
MDLC	Multidimensional liquid chromatography
MeOH	Methanol
META	[2-(Methacryloyloxy)ethyl] trimethyl ammonium chloride
MS	Mass spectrometry
NH_4AcO	Ammonium acetate
NP	Normal phase
NPE	Nonylphenol ethoxylate
OBA	Optical brightener agents
ODA	Octadecyl acrylate
OPE	Octylphenol ethoxylate
PAH	Polycyclic aromatic hydrocarbon
PVA	Polyvinyl alcohol
PVP	Polyvynyl pyrrolidone
RID	Refractive index detector
RP	Reverse phase
RSD	Relative standard deviation
SEM	Scanning electron microscopy
SCX	Strong anion exchanger
STTP	Sodium tripolyphosphate
TEMED	N,N,N’,N’-tetramethylethylenediamine
UV	Ultraviolet

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ABSTRACT

La presente memoria se enmarca dentro de una de las líneas de investigación del grupo, y cuyo objeto general es el desarrollo de métodos analíticos para el control industrial de componentes de productos de limpieza. Los objetivos responden a las exigencias de continuidad de dicha línea, conjugando el interés, calidad y actualidad desde ambos puntos de vista, el científico y el de transferencia tecnológica. De hecho, en esta misma línea de investigación, el grupo ha venido trabajando durante bastantes años en el marco de sucesivos convenios de colaboración con una empresa, realizando actividades periódicas de transferencia tecnológica. En consecuencia, el objetivo principal de los trabajos presentados en esta memoria es la puesta a punto de métodos de análisis rápidos y fiables para diferentes analitos de la industria de la detergencia. Otro de los aspectos descritos en esta tesis, relacionado también con otra de las actuales líneas de investigación del grupo, es el desarrollo de columnas monolíticas poliméricas y su aplicación para la electrocromatografía capilar (CEC, capillary electrophoresis). Estas columnas permiten poder seleccionar las condiciones de polimerización, ya sean tipo de iniciación, iniciador, monómeros y disolventes porogénicos, con el fin de obtener diferentes prestaciones de las mismas caracterizándose mediante un sistema de analitos estándar.

Esta memoria está dividida en cinco grandes bloques. El primer bloque, constituido por los capítulos 1-3, contiene una introducción general que describe las generalidades de los detergentes, su composición y métodos de análisis de los surfactantes más utilizados en su fabricación (capítulo 1), una breve descripción de la cromatografía líquida de alta resolución (HPLC, high performance liquid chromatography), principal técnica de análisis empleada en el desarrollo de esta tesis (capítulo 2) así como un capítulo dedicado a la electrocromatografía capilar y la síntesis de columnas monolíticas (capítulo 3).

El segundo bloque está dedicado a la revisión de reacciones de derivatización comúnmente empleadas con el fin de mejorar la señal analítica en HPLC y electroforesis capilar (CE, capillary electrophoresis) en la determinación de compuestos con grupos funcionales hidroxilo (capítulo 4).

El tercer bloque, detalla el desarrollo de técnicas avanzadas de cromatografía líquida para el control de calidad de detergentes y materias primas. En esta sección se describe un método de 2D-HPLC para la determinación de surfactantes no-iónicos en materias primas (capítulo 5) así como un método para la determinación conjunta de las principales familias de surfactantes presentes en detergentes (capítulo 6).

El cuarto bloque describe la preparación y caracterización de columnas monolíticas para electrocromatografía capilar centrándose en las diferencias entre diversos modos de iniciación de la polimerización (capítulo 7). Por último, el quinto bloque presenta un resumen de los resultados y conclusiones más relevantes procedentes de los tres bloques previos.

En esta sección de la tesis doctoral, y como lo exige la citada normativa de la Universidad de Valencia, se presenta un resumen de la tesis indicando los objetivos, metodología y conclusiones de la tesis.

En las últimas décadas se han producido avances muy significativos en el desarrollo de la cromatografía líquida de alta eficacia y en las técnicas de detección acopladas. Todo ello ha permitido atender la demanda de un mejor control de la calidad industrial y de la evaluación de su impacto ambiental. Concretamente, estos avances son de especial interés en el sector de los detergentes y otros productos químicos utilizados en las formulaciones de productos de limpieza y cuidado personal. Dada la complejidad de estos formulados desde el punto de vista analítico, en muchos casos, no se dispone de métodos suficientemente rápidos y selectivos para el control de calidad, o bien, los métodos descritos exigen

inversiones excesivas para su implementación por parte de la pequeña y mediana industria. El trabajo llevado a cabo durante la realización de esta tesis ha consistido principalmente en el desarrollo de métodos cromatográficos avanzados para la determinación de surfactantes en materias primas y detergentes y productos de limpieza mediante cromatografía líquida. En la composición química de productos de limpieza, los surfactantes constituyen la principal materia activa. En general, los formulados de limpieza no están constituidos únicamente por una clase de surfactantes sino por la combinación de diferentes clases y en diferentes proporciones de forma que se complementan sinérgicamente. La cromatografía líquida, especialmente en fase reversa (RP-LC, reverse phase liquid chromatography) es la técnica preferida de análisis para la caracterización y determinación de las diferentes clases de surfactantes. Los métodos desarrollados a lo largo de la tesis, tratan de cubrir la demanda por parte de las industrias de disponer de métodos de control de calidad de confianza para la verificación de materias primas, así como la de sus productos, para poder asegurar que la producción se realiza de acuerdo a las leyes que rigen su manufactura.

La ausencia de grupos cromóforos en muchos compuestos ha implicado la necesidad del empleo de sistemas alternativos a la detección ultravioleta visible. Como sustitución de este, se han empleado sistemas de derivatización. Especialmente, las reacciones de derivatización se han llevado a cabo en surfactantes de tipo alcohol polietoxilado. Dada la facilidad de derivatización del grupo OH, estos han sido sometidos a diversos sistemas de derivatización, tanto oxidando el grupo OH como el anclaje sobre él de grupos cromóforos. Dichas derivatizaciones en algunos casos han dado lugar a desplazamientos de la distribución de homólogos, dadas las posibilidades de ruptura de las cadenas polietoxiladas. Por ello se ha estudiado la separación de dicha familia en ausencia de reacciones de derivatización. Esto requiere de sistemas de detección que sean o

presenten una mayor inespecificidad. El empleo de detector evaporativo de luz dispersada (ELSD, evaporative light scattering detector) es una posible alternativa a los planteamientos anteriormente expuestos. El inconveniente del ELSD estriba en la falta de una respuesta lineal con la concentración, asignándole una respuesta exponencial. La linearización de la señal se ha llevado a cabo mediante la logaritmización de la respuesta. Sin embargo, la falta del cero químico, el cero de la concentración, ha llevado al ajuste de la respuesta a polinomios de grado 2. Estos sistemas se pueden emplear para concentraciones relativamente bajas, y permitiendo además la inclusión del cero de concentración. La respuesta en ELSD, para cada una de las familias estudiadas en función de su naturaleza, ha sido objeto de la presente tesis. Se realizaron curvas de calibrado, estudiándose la influencia de la eficacia del pico en la respuesta analítica. Si bien estos estudios no se han incluido en la tesis por encontrarse en fase de redacción, si se desea hacer constancia de los mismos

A nivel de estudios de familias de compuestos, hasta la actualidad se han desarrollado trabajos relacionados con la carcaterización individual de cada una de las familias de tensioactivos. Desde nuestro conocimiento, estudios incluyendo tanto tensioactivos aniónicos como neutros, y dentro de los aniónicos cada una de las familias más comúnmente empleadas en formulados de detergentes nunca se han llevado a cabo. En la presente tesis se plantea la posibilidad, mediante una sola inyección, de realizar el análisis de una muestra de detergente determinando cada uno de los surfactantes que lo componen. Además, se llevará a cabo la caracterización de los mismos, determinándose su distribución tanto en función del número de átomos de carbono que componen la cadena alquílica, la parte hidrofóbica del mismo, como del número de moles de óxido de etileno que constituyen la parte hidrofílica. Esta caracterización es fundamental dado que las características de detergencia, mojabilidad y espuma, están relacionados

íntimamente con su distribución hidrofílica e hidrofóbica, en definitiva, de su relación entre ambas partes y de la naturaleza de estas.

Estos estudios se compararán con los resultados obtenidos mediante técnicas inespecíficas como es la tritración de los tensioactivos anionicos llevada a cabo a dos pHs (ácido y básico) con el fin de determinar tensioactivos aniónicos totales y oleínas. Estos estudios se encuentran en fase de redacción por lo que no han sido incluidos en la Memoria de esta Tesis Doctoral. Este procedimiento publicado como norma UNE, y empleado como sistema de referencia contiene una serie de fallos. Las aminas empleadas comúnmente como valorantes han de ser estandarizadas a los dos pHs de estudio, dado que no son productos puros y su concentración varía dependiendo del pH de trabajo. Además, otros parámetros como son la naturaleza del emulsificante y su concentración, volúmenes empleados de detergente y disolvente orgánico, temperatura de trabajo y velocidad de adquisición también requieren de un proceso de optimización.

Objetivos

I. Revisión de reacciones de derivatización de compuestos con grupos hidroxilo

La búsqueda bibliográfica sobre información y métodos de análisis de surfactantes condujo a la recopilación de una gran cantidad de datos sobre la derivatización de compuestos con grupos funcionales hidroxilo, por lo que se pretende ampliar la búsqueda para la redacción de un artículo de revisión centrado en el empleo de estas reacciones para su posterior análisis mediante técnicas de separación cromatográficas. Este artículo de revisión puede resultar de tremenda utilidad como punto de partida para otros grupos de investigación o empresas, pues presenta de forma ordenada una relación actualizada de métodos de derivatización de analitos con grupos hidroxilo.

II. Métodos cromatográficos de análisis de surfactantes

II.1. 2D-HPLC aplicado a la determinación de alcoholes grasos etoxilados

En trabajos anteriores del grupo de investigación en el seno del cual se ha realizado la presente Tesis, y en el marco de una de sus líneas de investigación, se desarrolló un montaje de 2D-HPLC para la determinación de alcoholes grasos etoxilados (FAE, fatty alcohol ethoxylates). Los FAE son los surfactantes no iónicos actualmente más empleados. Se producen por condensación de óxido de etileno (EO, ethylene oxide) con alcoholes alifáticos de cadena larga. Esta condensación da lugar a complejas mezclas de oligómeros con distinto número de unidades de EO. En la presente tesis, se pretende la puesta a punto de un montaje automatizado para 2D-HPLC, basado en la impulsión con una sola bomba y juego de dos válvulas, con mejoras significativas respecto a un montaje anterior. El montaje y procedimiento establecidos se aplicarán a la caracterización y determinación de alcoholes grasos en muestras de materias primas utilizados en la fabricación de detergentes y productos cosméticos.

II.2. Determinación de las cuatro grandes clases de surfactantes en productos de limpieza mediante cromatografía líquida en fase reversa con detección UV seguida de detección evaporativa de luz dispersada

Como se ha comentado anteriormente, un detergente es un tipo de muestra de extremada complejidad desde el punto de vista analítico. En este sentido, se desarrollará un método de HPLC capaz de determinar en un solo análisis los cuatro tipos de surfactantes comúnmente presentes en productos de limpieza: FAE, y surfactantes aniónicos, incluyendo sulfonatos de alquilbenceno lineares (LAS, linear alkylbenzene), alkyl eter sulfatos (AES, alkyl ether sulfates) y jabones. LAS y AES eluyen antes que los surfactantes no iónicos (FAE) y los jabones. No se encontró ningún método RP-LC capaz de separar LAS y AES. La estrategia que

se seguirá para su determinación será usar la detección UV conectada en serie con ELSD. Dado que únicamente el LAS presenta grupos cromóforos, podrá llevarse a cabo su cuantificación mediante la curva de calibrado de UV. La concentración de AES se obtendrá por sustracción de la concentración de LAS de la concentración total de LAS y AES obtenida de la interpolación en la curva de calibrado efectuada con la señal total en ELSD de ambos surfactantes. El método se aplicará a la determinación de las cuatro clases mayoritarias de surfactantes en productos de limpieza.

III. Fases estacionarias monolíticas

III.1. Preparación y caracterización de monolitos de acrilato de octadecilo para electrocromatografía capilar mediante iniciación fotoquímica, térmica y química

Se pretende describir la preparación de columnas monolíticas para CEC, usando acrilato de octadecilo (ODA, octadecyl acrylate) como base para sintetizar el polímero. La polimerización se llevará a cabo mediante radiación UV e iniciación térmica utilizando peróxido de lauroilo (LPO, lauroyl peroxide) como iniciador, o mediante iniciación química utilizando N,N,N’,N’-tetrametiletildiamina (TEMED, N,N,N’,N’-tetramethylethyldiamine). Para obtener resultados satisfactorios se optimizará la composición de la mezcla de polimerización, es decir, relaciones de monómeros/porógenos y monómeros/agente entrelazante, así como la composición de los disolventes porogénicos). La caracterización morfológica de las columnas se llevará a cabo mediante fotografías de micoscopía electrónica de barrido, (SEM, scanning electron microscopy). Las prestaciones de estas columnas desde el punto de vista de CEC se realizará mediante medida de los factores de retención y eficacias de una serie de analitos no cargados. Además, se compararán las fases estacionarias

obtenidas mediante fotopolimerización con las obtenidas con iniciación térmica y química.

Metodología y conclusiones

I. Revisión de reacciones de derivatización de compuestos con grupos hidroxilo

Las reacciones de derivatización se usan frecuentemente para introducir un cromóforo o un fluoróforo a un analito con el fin de permitir o mejorar la señal mediante detección UV, fluorescencia o espectrometría de masas (MS, mass spectrometry). Esta revisión, se centró en la derivatización de moléculas que presentan grupos funcionales hidroxilo para su análisis por cromatografía líquida y electrocromatografía capilar.

Los métodos de derivatización han sido clasificados según el grupo reactivo del agente derivatizante, incluyendo cloruros de acilo, anhídridos orgánicos, isocianatos y otros reactivos. Las reacciones y métodos se ordenaron convenientemente en tablas para obtener una visión global de las principales características de cada método.

La mayoría de las reacciones revisadas se usan para introducir un cromóforo o un fluoróforo en analitos que tienen un grupo alcohol alifático, con el fin de permitir su detección o para aumentar la sensibilidad. Otras reacciones se dirigen a introducir un grupo ionizable o una carga permanente para realizar separaciones mediante CE, mejorar la sensibilidad en MS o disminuir la volatilidad, aumentando así la sensibilidad de los analitos volátiles en los detectores evaporativos.

En cuanto a las aplicaciones, una gran cantidad de las reacciones revisadas implican la derivatización de analitos con grupos funcionales hidroxilo, como tensioactivos alifáticos no iónicos (principalmente FAE) y otros compuestos de

interés industrial como etilenglicol y sus polímeros condensados como el polietilenglicol (PEG, polyethylen glycol) o alcoholes alifáticos de cadena corta. Las matrices estudiadas en todos estos métodos comprenden desde muestras ambientales típicas como agua dulce, aguas residuales, sedimentos y lodos hasta matrices biológicas como cultivos celulares, fluidos biológicos o tejidos, incluyendo también varias muestras industriales como materias primas, alimentos y bebidas.

II. Métodos cromatográficos de análisis de surfactantes

II.1. 2D-HPLC aplicado a la determinación de alcoholes grasos etoxilados

En este trabajo, se diseñó un sistema de cromatografía líquida bidimensional de corte medular, impulsado por una sola bomba y provisto de una válvula de 6-puertos y 2 posiciones ($V_{6/2}$) y una válvula selectora de columnas (V_{CS}), para la determinación de alcoholes grasos etoxilados en materias primas.

En primer lugar, se seleccionaron tanto las fases estacionarias como las fases móviles que se emplearían en la primera y la segunda dimensión de acuerdo a las propiedades de los oligómeros de FAE. Para la separación en la primera dimensión, se probaron columnas con diferentes sustituyentes alquílicos. El uso de gradientes de ACN/agua sobre columnas C8 no permitían la separación de las series hidrocarbonadas sin separar los oligómeros entre sí. Sin embargo, el uso de gradientes MeOH/agua sí que consiguió la elución de cada serie hidrocarbonada como picos aislados. Llevando a cabo la separación a 60 ºC se mejoró la resolución entre las series hidrocarbonadas y se consiguieron tiempos de análisis más cortos. La separación de los oligómeros de FAE de cada serie hidrocarbonada se consigue de forma satisfactoria mediante columnas C8 y mezclas ACN/agua, por lo que se escogieron estas condiciones para la elución en la segunda dimensión. Se optimizaron también las concentraciones iniciales y finales del gradiente de ACN

para la elución de los oligómeros de cada serie hidrocarbonada. Mediante estas condiciones, se obtuvieron buenas separaciones a lo largo de la primera dimensión de mezclas de FAE que contenían series hidrocarbonadas pares e impares, mientras que los oligómeros dentro de cada serie se separaron satisfactoriamente en la segunda dimensión.

El método optimizado de separación en ambas dimensiones, se empleó para la determinación de FAEs y su proporción en función de sus series hidrocarbonadas en muestras industriales. Para ello, se propuso un factor de respuesta medio para cada serie. Estos factores tienen en cuenta los diferentes factores de respuesta UV-vis de los diferentes oligómeros etoxilados, y se tabularon en función de la longitud de la cadena hidrocarbonada (*n*) y la longitud media de la cadena etoxilada ($\overline{m}$). Estos factores de respuesta promedio, se utilizaron para corregir el área de pico de las series hidrocarbonadas en la primera dimensión. También se demostró que la dependencia del factor de respuesta promedio con respecto a $\overline{m}$ es pequeña cuando $\overline{m}>5$. Esto permite el uso de valores inexactos de $\overline{m}$ sin que ello afecte considerablemente a las determinaciones de cada serie hidrocarbonada.

En conclusión, se ha demostrado que se puede utilizar un sistema de cromatografía líquida bidimensional de corte medular, constituido por una sola bomba, una válvula de 6 puertos y 2 posiciones y una válvula selectora de columnas para la caracterización de FAEs en materias primas. En la primera dimensión los FAE se separaron de acuerdo a su cadena hidrocarbonada, mientras que en la segunda dimensión cada serie de FAE se separó de acuerdo a la longitud de la cadena etoxilada. La ortogonalidad en la separación se consiguió mediante el uso de fases móviles complementarias en cada dimensión. También se propuso un factor de respuesta promedio para corregir el área de pico correspondiente a cada serie hidrocarbonada.

II.2. Determinación de las cuatro grandes clases de surfactantes en productos de limpieza mediante cromatografía líquida en fase reversa con detección UV seguida de detección evaporativa de luz dispersada

Se desarrolló un método para la determinación simultanea de LAS, AES, FAEs y oleínas o jabones (sales de ácidos grasos) en productos de limpieza mediante una única inyección cromatográfica. Las fases estacionarias de fase reversa comúnmente empleadas (C8, pentafluorofenil y bifenilo), no fueron capaces de separar los surfactantes aniónicos LAS y AES; sin embargo, dado que solamente el LAS absorbe en el UV, estas dos clases se pudieron cuantificar independientemente usando una columna C8 y un detector UV conectado en serie con un ELSD.

Para encontrar las mejores condiciones de separación se probaron diferentes columnas de fase reversa, así como combinaciones de todas ellas unidas en serie. En relación a las fases móviles, se probaron tanto combinaciones de ACN/agua con MeOH/agua. Los mejores resultados para resolver las cuatro clases de surfactantes y los oligómeros dentro de cada clase se consiguió empleando una columna C8 y gradientes ACN/agua. Para mejorar la retención de los surfactantes aniónicos, se añadió acetato amónico, compatible con la detección ELSD, a la fase móvil como agente formador de pares iónicos. La presencia de este aditivo, no modificó la elución de los FAE y las oleínas. La presencia de ácido acético en la fase móvil también se estudió, y se observó que afectaba principalmente a la retención de las oleínas respecto a los oligómeros de FAE. Con estas observaciones en mente, se decidió implementar la modulación de la fase móvil durante la separación, utilizando acetato de amonio durante la elución de LAS y AES y ácido acético tras la elución de estos, aumentando la retención de LAS y AES y desplazando de forma independiente los picos de la oleína en función del pH de la fase móvil. Por último, se estudió el efecto de la temperatura sobre la separación,

siendo significante para la resolución de los oligómeros, pero manteniendo prácticamente invariable la separación del resto de surfactantes. El método cromatográfico optimizado consistió en la separación sobre una columna C8 a 15 ºC, mediante un gradiente de 40 a 90% de ACN en 40 min en presencia de 10 mM acetato amónico hasta t = 12 min, sustituyéndolo por 17,5 mM de ácido acético a partir de ese tiempo hasta el final de la elución.

La cuantificación de LAS y AES, se consiguió utilizando un detector UV para cuantificar LAS y el ELSD para determinar la concentración de AES por diferencia de la concentración de LAS sobre la concentración total de LAS y AES. Sin embargo, la sensibilidad está influenciada por el ratio LAS/AES. Para superar esta dificultad, se probaron dos estrategias. La primera aproximación fue utilizar un modelo de calibración linear simple para calcular la concentración de AES considerando tanto la concentración de AES como la señal conjunta de LAS y AES proporcionada por el ELSD. Por otro lado, se observó que para mezclas de LAS y AES, cuando el porcentaje de LAS se encontraba entre 0,1 y 0,5 %, la sensibilidad dependía poco del porcentaje de LAS. Para evitar esta desviación, la alternativa fue medir los patrones y las muestras únicamente en este intervalo. Para ello, las muestras se adicionaron con una cantidad elevada de AES. Las concentraciones de AES en las muestras se obtuvieron restando a la concentración predicha por la calibración la cantidad adicionada. Los LODs y LOQ fueron 2 y 6 mg L^{-1} para el LAS y 20 y 60 mg L^{-1} para AES respectivamente.

Por otro lado, la determinación de FAE y oleína se puede llevar a cabo fácilmente mediante sus rectas de calibrado individuales. El área de los picos de FAE que se solapan con los picos de oleína se obtuvieron por interpolación con los picos de FAE vecinos. Las correcciones aumentaron ligeramente los errores en la determinación de FAE y los redujeron en el caso de la oleína. Los LODs y LOQ fueron 50 y 150 mg L^{-1} para FAEs y 10 y 30 mg L^{-1} para AES respectivamente.

En conclusión, se desarrolló un método cromatográfico para la determinación simultánea de las cuatro grandes clases de surfactantes empleadas en la preparación de productos de limpieza. La separación se llevó a cabo mediante modulación de la fase móvil, consistiendo en un cambio de los aditivos de la fase móvil, esto es, acetato amónico en una primera fase de elución y ácido acético a continuación. Además, la falta de un método apropiado para resolver los oligómeros de LAS y AES, y por tanto su cuantificación adecuada, se superó gracias al uso de un detector UV conectado en serie con un ELSD. Por último, el problema de la variación de la sensibilidad de la respuesta del ELSD respecto al ratio LAS/AES se rectificó aumentando la concentración de AES en las muestras.

III. Fases estacionarias monolíticas

III.1. Preparación y caracterización de monolitos de acrilato de octadecilo para electrocromatografía capilar mediante iniciación fotoquímica, térmica y química

En este trabajo, se compararon las propiedades cromatográficas de columnas monolíticas basadas en ODA para CEC, sintetizadas mediante diferentes sistemas de iniciación (iniciación por irradiación UV, térmica y química) utilizando LPO como iniciador de la polimerización. Para cada sistema de iniciación se evaluó la influencia de la composición de disolventes porogénicos (1,4-butanodiol/1-propanol) sobre las propiedades morfológicas y elctrocomatográficas. Las cualidades electrocromatográficas de las diferentes columnas se evaluaron midiendo el factor de retención y la eficacia de una mezcla test de hidrocarburos aromáticos policiclicos (PAHs, polyciclic aromatic hydrocarbons) y sus características morfológicas se evaluaron a partir de imágenes de los monolitos obtenidos mediante SEM.

Se llevó a cabo una comparación de los tres modos de iniciación en términos de eficacia, permeabilidad y reproducibilidad para las condiciones óptimas de polimerización. Se observó que los monolitos polimerizados por iniciación química y fotoquímica proporcionaban mejores eficacias (alturas de plato mínimas de 6,9-10,7 y 6,5-12,6 µm, respectivamente), mayores permeabilidades y tiempos de análisis más cortos que las iniciadas térmicamente. Las reproducibilidades columna-a-columna y lote-a-lote también se evaluaron bajo las condiciones óptimas y dieron valores de RSD por debajo de 9,2, 10,6 y 9,8 % para las columnas UV, térmica o químicamente iniciadas respectivamente. Además, por un lado, la iniciación UV proporciona una forma más rápida de preparación del monolito, mientras que los monolitos iniciados químicamente no necesitarían de equipamiento adicional (baño de agua o lámparas UV).

En conclusión, se prepararon fases estacionarias monolíticas basadas en ODA para su uso en CEC utilizando diferentes sistemas de iniciación (iniciación por irradiación UV, térmica y química). El estudio de sus propiedades electrocromatográficas y su morfología, reveló que las columnas monolíticas iniciadas por radiación UV y químicamente procuraban los mejores resultados, haciendo de ambos sistemas de iniciación excelentes candidatos para la preparación de columnas monolíticas más largas y dispositivos miniaturizados.

SECTION I.

INTRODUCTION

CHAPTER 1.

Detergents and surfactants

1.1. Detergents

Since ancient times people has been concerned to be neat and live in a clean environment. Soaps are some of the oldest chemical products of mankind. They were obtained by saponification of plant or animal fats and vegetal ashes or natural soda (alkaline carbonates). The original formula was already known by Egyptians, Babylonians and Phoenicians; the first records of the preparation of soap were found on Sumerian clay tablets (2500 BC) on which the amounts of the necessary raw materials, the manufacture procedure as well as its application to clean textile pieces were described in detail.

The use of soaps became more important around the II century, when the Greek physician Galen recommended the use of soaps not only for textile cleaning and bathing but also by its medicinal and cleansing properties. With the evolution of the Roman society so did bathing. After the fall of the Roman Empire, the decline in bathing habits led to unsanitary living conditions contributing severely to the great plagues of the Middle Ages. The use of soap became appreciated again in the 17^{th} century, when cleanliness and bathing came back into fashion when doctors realized of the importance of hygiene on health.

Soap became a more accessible product thanks to research conducted by Chevreul, about the structure of oils and fats, and Leblanc who patented a process for synthesizing sodium carbonate, commonly used combined with fat to form soap.

These scientific discoveries, together with the development of power to operate factories, made fabrication of soaps easier and helped to broad its availability, and becoming an item of everyday necessity. Soap manufacturing did not advance much until 1916, when the first synthetic detergent was developed in Germany to overcome the shortage of soap during World War I. Detergents are

mixtures of different compounds whose purpose is to support the removal of dirt from a surface [1].

1.1.1. Typical detergent composition

Nowadays detergents and other cleaning products can be composed by 20 or more ingredients depending on the dirt to be removed and the washing conditions. Also their combination in different proportions is important either to seek a specific use of the detergent or a more general use. The main active ingredients of a cleaning product formulation are surfactants, which provide the basis of the cleaning power. Many other components are added to the formulation to complement and enhance the effect of surfactants, in order to obtain the best blending for a specific use. The following is a general overview of the more common ingredients in detergent formulations and their roles.

1.1.2.1. Surfactants

Surfactants wet surfaces and reduce the interfacial tension between dirt and water, removing the dirt from the surface to be cleaned and dispersing it in the aqueous phase. [1].

1.1.2.2. Builders and quelants

The purpose of using builders is to enhance the cleaning performance of the surfactants. The primary function of builders is to reduce the amount of free cations responsible for the hardness of water (mainly calcium and magnesium) to avoid their interaction with anionic surfactants leading to their precipitation and thus reducing the effective concentration available for cleaning. Cation removal is accomplished by chelation, precipitation or by ion exchange. A very well-known chelating agent is sodium tripolyphosphate (STPP, $Na_5P_3O_{10}$) but its effects on the environment has led to the use of a number of substitutes. Other commonly used

chelants include ethylenediaminetetraacetate (EDTA), citric acid, which can also be employed for the removal of transition metals such as copper, zinc or iron ions, and sodium nitrilotriacetate. Sodium carbonate (Na_2CO_3) and noncrystalline sodium silicates (xNa_2O-$ySiO_2$) form insoluble salts in the presence of calcium and magnesium ions. Abstraction of divalent cations by ionic exchange is often accomplished by the use of insoluble inorganic compounds such as zeolites or sodium aluminium silicates ($Na_2O – Al_3O_3 – 2SiO_2 – xH_2O$) [1]. Builders can also provide and maintain alkalinity, which assists cleaning especially of acid stains, helps on keeping the removed dirt from being redeposited during washing by maintaining negative charged surfaces, and stimulates the emulsification of oily and greasy stains.

1.1.2.3. Bleaching agents

For the removal of persistent stains bleaching agents are often used. Their action arises by either oxidatively modifying the stain in such a way that it becomes more water soluble and easier to remove, or by decolorizing the stain such that it is no longer visible. The most common bleaching agents are peroxygenated inorganic salts, such as sodium perborate tetrahydrate ($NaBO_3 \cdot 4H_2O$), sodium percarbonate or peroxyhydrated sodium carbonate ($2Na_2CO_3 \cdot 3H_2O_2$). Their action mode consists in their decomposition in water, liberating hydrogen peroxide which is actually theo xidizing agent. Sodium hypochlorite is more active and aggressive than perborate, thus being particularly effective on the oxidation of substances containing nitrogen. Apart from having a bleaching action (even at low temperature) hydrogen peroxide is an effective bactericide. Due to its action on nitrogenated substances, it cannot be formulated as granular detergents or as liquid in detergent formulation that contain ammonium salts, amine, amides, etc. That is why it is commonly used as an aside ingredient. Perborate and similar salts exhibit

a less aggressive bleaching power, but as they are solids they are compatible with most of the powder detergents [1-3].

1.1.2.4. Optical brighteners

Whiteness of bleaching agents can be enlarged using optical brightener agents (OBA). These substances are fluorescent polyaromantic organic dyes that absorb ultraviolet light and emit back visible blue light. At daylight, they add a blue tone which conceales any yellowing that may be present in the fabric, thus improving whiteness and intensifying the colours. Depending on its final purpose, these dyes possess more or less amounts of polar groups to adsorb onto hydrophilic fabrics such as those based on cotton or more hydrophobics such as polyester. Several types of compounds can be used as optical brighteners including coumarins, naphthotriazolylstilbenes, benzoxazolyl, benzimidazoyl, naphthylimide and diaminostilbene [2,4].

1.1.2.5. Polymers

The use of polymers in detergent formulas has increased as detergent formulations have evolved. Polymers started to replace phosphates that lapsed into disuse due to its environmental impact. The focus was on polyacrylate-type polymers with the goal of replacing some of the lost building and dispersancy power of STPP. Carboxymethyl cellulose has been also one of the earlier polymers used for this purpose. Success obtained with the use of polymers boosted the appearance of a great number of polymers for a variety of purposes [5]. Among many other uses, new water-soluble polymers with dispersive and dye transfer inhibition properties, have been broadly commercialized. For every new polymer actually commercialized there are countless ones patented, attesting the level of interest in the area [1,6].

1.1.2.6. Softeners

After washing with synthetic detergents containing quelants, dry fabrics usually present a surface that can result unpleasant in contact with the skin. The adsorbed remnants of the synthetic surfactants enhance the static charge of the fibres, and the absence of lubricating substances makes the fabric relatively rigid. Softeners counteract both occurrences: on the one hand they reduce the static charge and on the other hand they settle a lubricant layer. Overall, softeners improve textile softness, smoothness, hand feel, and also fibre lubricity, flexibility, elasticity and processibility [7]. A lot of cationic surfactants produce these effects, but are incompatible with the anionic surfactants used in most of the commercial formulations, thus they should be used separately. Substituted quaternary ammonium and imidazolium salts are the most extensively used cationic softeners. There is a tendency to produce formulations containing softeners compatible with the cleaning agents. These softeners are surfactants with certain cationic character which adsorb onto the textile fibres, but that are also compatible with the commonly used anionic surfactants. To this end, non-ionic surfactants with a nitrogen moiety, or some amphoteric surfactants usually containing an amine or an amide group are employed [1,2].

1.1.2.7. Anti-redeposition agents

These substances are added to detergents to keep dirt from setting back on the materials that have been washed. Most used anti-redeposition agents are carboxymethylcellulose derivatives, other non-ionic cellulose derivatives and polyethylene glycol. Commercial synthetic polymers such as PVP, PVA and some of their copolymers are also used [2].

1.1.2.8. Foaming and anti-foaming agents

Contrary to the popular believe, foam generation has nothing to do with the cleaning power. In some applications, most notably hand dishwashers and shampoos, it is desirable for the detergent formulation to generate a large-volume of stable foam. While most surfactants are capable of generating and sustaining foam in the absence of dirt, these foams rapidly collapse in the presence of dirt, especially with particulate and fatty stains. In applications where foam must be maintained throughout the course of detergent use, specific boosters may be added, such as sodium lauryl sulfate and non-ionic nitrogenated surfactants [1,2]. Nevertheless, in other applications it is desirable to minimize foam generation. For example, in automatic dishwashing foam generation can interfere with rotation of the spray arm leading to degradation in the performance of the dishwasher. Antifoam agents act to reduce or eliminate foams. They either prevent formation of the foam or accelerate its collapse. Alkyl ethoxylate nonionic surfactants, calcium soaps of long-chain fatty acids and antifoams consisting of colloidal hydrophobic silica particles suspended in a silicone oil like polydimethyl siloxane are particularly effective antifoaming agents [1,2].

1.1.2.9. Enzymes

Used primarily in cleaning formulations, enzymes promote dirt removal by catalytic breakdown of specific soil components. They are also biodegradable, thus, making them a good choice for today's increasingly environmentally conscious consumers. Proteases (enzymes that degrade proteins) are the most common of all the detergent enzymes, but amylases (starch degraders), lipases (lipid degraders), and cellulases (cellulase degraders) are also used [8]. Enzymes are able to fastly degrade stains in alkaline pH and at temperatures up to 60 ºC.

They are designed to be active at room temperature, being widely employed in the formulation of laundry liquid detergents [2].

1.1.2.10. Thickeners

It is often desirable to modify the rheology of a detergent formulation, that is, the consistency (the viscosity) of the detergent in flowing dynamic conditions, to fit a particular application. For example, gel-type automatic dishwashing detergents are thickened to help suspend phosphates and other solids that would otherwise separate out from the liquid phase. Thickening can be achieved through the use of inorganic electrolytes, e.g., NaCl, clays, such as laponite or hectorite, or a high-molecular-weight polymer like carboxymethylcellulose, guar foam, or xanthan gum. The Carbopol® series of polymers from Noveon, homo and copolymers of acrylic acid cross linked with polyalkenyl polyether, are particularly effective thickeners for household cleaning detergent formulations [1].

1.1.2.11. Perfumes

From a technical viewpoint, perfumes add no cleaning power to a detergent. However, from the consumer point of view perfumes have a major impact on the overall impression of how well a detergent works. Odor has been proven to be an important driver for consumer acceptance, and thus, it should be carefully considered when formulating a product. Perfumes are complex mixtures of organic compounds. For example, a detergent perfume may be composed of 30, 50, or even over 100 different organic materials. Given this complex nature, perfumes can have complex interactions with the detergent actives that affect both the perfume character and possibly the actives' performance. In domestic use detergents, particularly for dishwasher and disinfectants, most of the added perfumes are terpenes, whose structure is composed by 5 or 10 linked isoprene units (2-methyl-butadiene) [2,6].

1.1.2.12. Solvents

The selection of solvents to use in detergent formulation depends on the nature of the actives being formulated, the intended application of the detergent, and economics. Water is the dominant solvent in most household and industrial cleaning formulations. However, many common detergent actives have limited solubility in water requiring formulation of a co-solvent and/or a hydrotrope. Typical co-solvents used in household cleaning formulations include ethanol, glycerol, and 1,2-propanediol [1].

1.1.2.13. Hydrotropes

In liquid detergents, it is often necessary to include hydrotropes to guarantee the stability of the detergent in a broad range of temperatures. Hydrotropes are hydrophilic substances with a nonpolar group, whose purpose is to increase the solubility of surfactants in water-based liquid formulations. Hydrotropes have no surfactant properties by themselves, but act as co-solubilizers at high concentrations. Common hydrotropes are toluene, xylene and cumene sodium sulfonates [1,2].

1.1.2.14. Bactericides

Some formulas contain bactericides, which can be amphoteric surfactants that can act also as calcium dispersing agents. Cationic compounds which also present softening properties are also oftently used. Disinfectants may also contain bactericide compounds and substances with deodorant properties [2]. In liquid detergents, especially in the most diluted ones, in which water constitutes the greater part of the total volume of the product, the formulation usually includes biocides, such as isothiazolinones, to prolong the product shelf-life [6, 9].

1.2. Surfactants

1.2.1. Introduction

The word surfactant derives from the contraction of the terms surface-active agent and covers a group of chemicals which are able to modify the interfacial properties of the liquids in which they are present. The peculiar properties of these molecules are due to their typical molecular structure, essentially linear and asymmetric, and to their amphiphilic character, which stems from the presence of both a hydrophilic part and a hydrophobic (or lipophilic) part. As a result, they concentrate at the interfaces separating immiscible liquid phases, thereby decreasing the interfacial tension. The hydrophobic part is a linear or branched aliphatic chain, usually containing between 10 and 18 carbon atoms. In natural products, such as vegetal oils (mainly palm and coconut oils) or other renewable resources used in the surfactant manufacturing, as well as derivative products from those sources, non-branched chains with an even number of carbon atoms in the aliphatic chain prevail. However, in petroleum derivatives and in synthetic products, a high amount of branched chains, with both odd and even numbers of carbon atoms in the aliphatic chain can be found. The hydrophilic part, which determines the water solubility, can be an acidic polar group such as a sulfate, sulfonate or carboxylate or a basic group such as an amine, a quaternary ammonium salt or the pyridinium ion, although it can also be a non-ionic polar group. These characteristics provide the surfactants with the property of detergency, phenomenon characterized by the following effects (see **Fig. 1.1.1, 2, 3 and 4**):

1) destabilization of the adhesion of hydrophobic particles to surfaces,
2) stabilization of the dispersion of those particles,
3) reduction of their wettability (increasing the contact angle), and
4) micellar solubilization of hydrophobic compounds and particles.

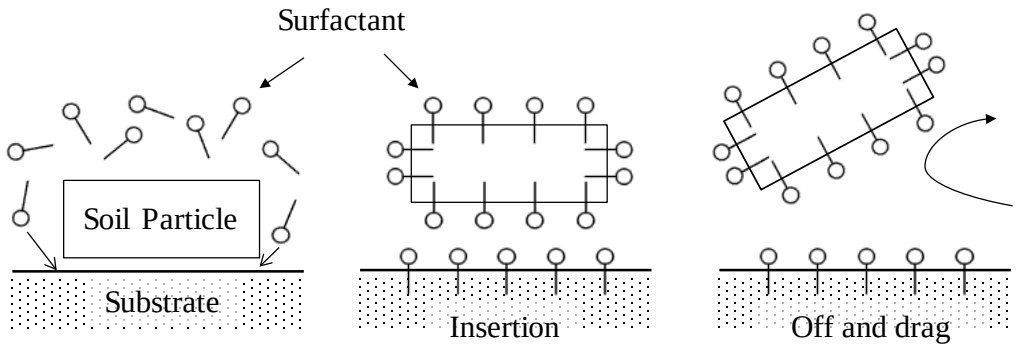


Fig. 1.1.1. Destabilization of the adhesion of a hydrophobic particle to a surface.

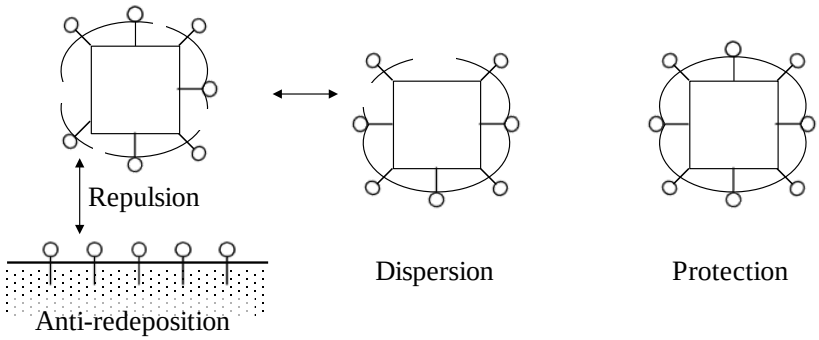


Fig. 1.1.2. Stabilization of a hydrophobic particle dispersion.

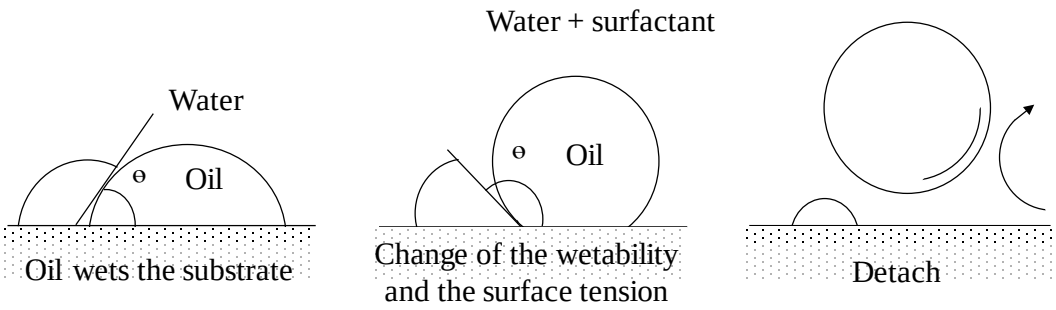


Fig. 1.1.3. Reduction of the wettability of the hydrophobic particles.

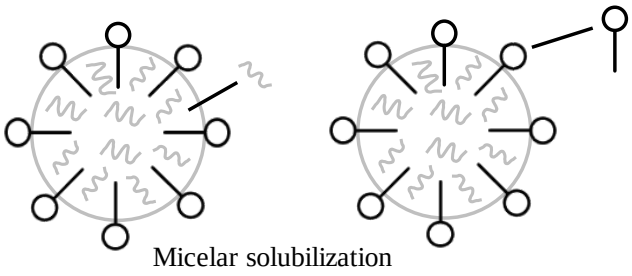


Fig. 1.1.4. Micellar solubilization.

Surfactants, either natural or synthetic, organize the medium in which they are present thanks to the formation of micelles and other nanostructures, changing the solubility and the state of other constituents present in the medium. The surfactant properties of a compound are not only dependent of the nature of the hydrophilic group, but also on the position of the group in the molecule (at the end or in the middle of the chain). There are also surfactants with two or more hydrophobic groups, such as dialkyildimethyl ammonium salts, or with two or more hydrophilic groups, such as betaines or the alkyl sulfosuccinates.

1.2.2. Surfactant classification

According to their charge, surfactants can be divided into four major classes: anionic, cationic, amphoteric and nonionic [10]. Although each surfactant has its own particularities, there are some common characteristics to each class.

1.2.2.1. Anionics

They are characterized by having an acidic hydrophilic group, thus easily forming an anion. They are historically the earliest and the most common surfactants. They are usually considered to be the “workhorse” in the detergency world. Accordingly, they are produced with the highest volumes and most of them are inexpensive. The oldest and most known are soaps and the following families are usually distinguished: linear alkylbenzene sulfonates (LAS), alkyl sulfates (AS), alkyl ether sulfates (AES), alkyl phenol ether sulfates (APES), α-olefin sulfonates (AOS), alkyl sulfonates, α-sulfonate fatty acids (ionic and alkyl esters), mono- and di-alkyl sulfosuccinates and sulfonates petroleum derivatives. (**Fig. 1.2**).

Anionic surfactants are especially beneficial for their excellent detersive action, owing to the fact that many substrates are negatively charged, so they do not get adsorbed onto them, avoiding the redeposition of undesirable soils.

Depending on the nature of the negatively charged head group, they show a variable resistance to hydrolysis. For example, sulfates hydrolyze easily, while sulfonates are very stable. Some anionic surfactants show the property to generate viscous aqueous phases, thereby yielding self-thickened products. A limitation of anionic surfactants is that they have tendency to precipitate in the presence of calcium and magnesium ions, which are present in high concentration in hard water, although AESs are much less sensitive to alkaline-earth ions than ASs. On the other hand, the lower water solubility and the peculiar interfacial properties of sulfonate Mg salts are sometimes positively exploited to optimize the detergent performance.

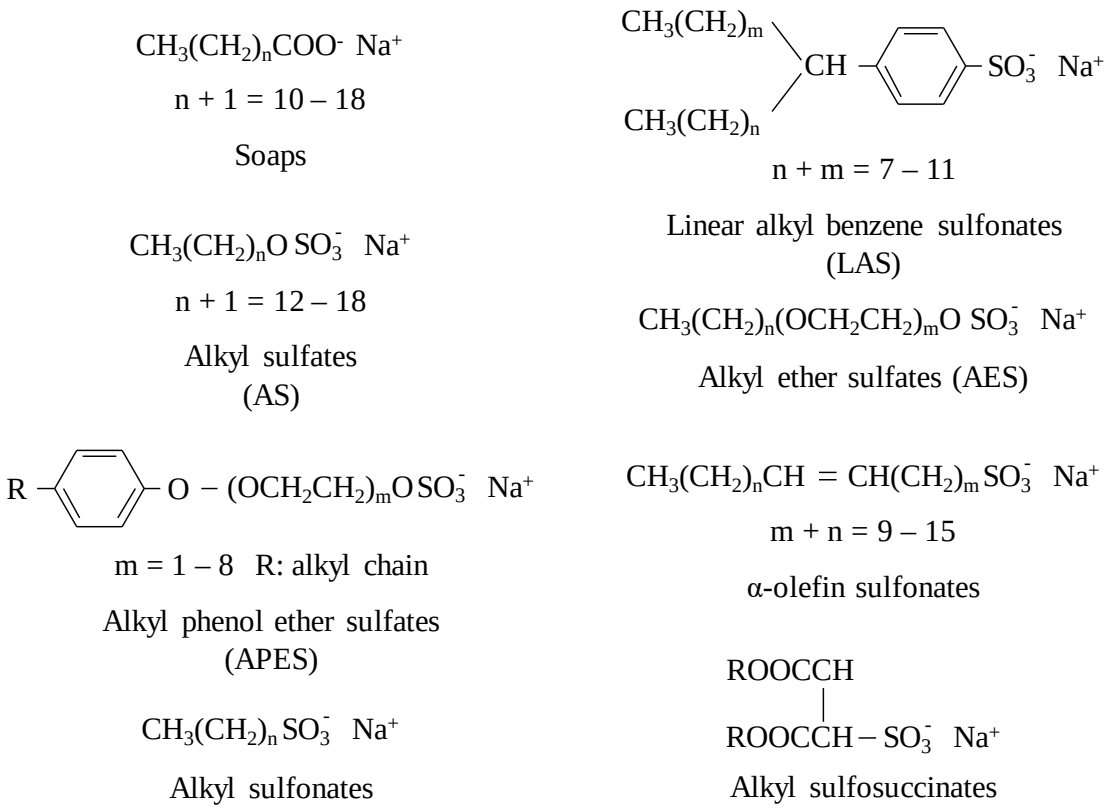
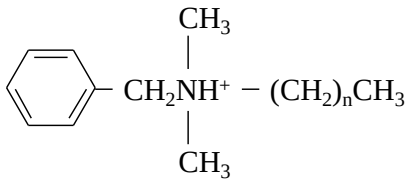


Fig. 1.2. Structure and names of the main anionic surfactant families.

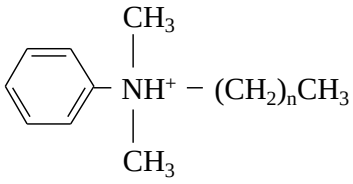
1.2.2.2. Cationics

Cationic surfactants have a basic hydrophilic group, providing them with a positively charged group. They usually are grouped in alkyl amines, amidoamines, alkylimidazolines, tetraalkyl(-aryl) ammonium salts, heterocyclic ammonium salts, alkyl betaines, ethoxylated alkyl amines, cationic polymeric compounds and amine oxides. The structure and the names of the main cationic surfactant families are indicated in **Fig. 1.3**.



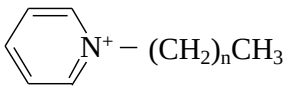
n + 1 = 7 – 18

Alkylbenzildimethylammonium salts



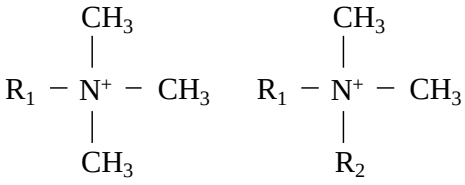
n + 1 = 7 – 18

Alkylphenyldimethylammonium salts



n + 1 = 12 – 14

Alkyl pyridinium salts



R_1, R_2: alkylic chains

Alkyl trimethyl and dialkyldimethyl ammonium salts

Fig. 1.3. Structure and names of the main cationic surfactant families.

From an industrial point of view, the most important cationic surfactants are long-chain fatty amines, and particularly compounds bearing quaternary nitrogen [11]. Generally, they are of little use in cleaning considering that most of the substrates have a negative charge, thus cations get retained over them instead of solubilizing the adhered soil. However, due to the same properties they have many other particular applications. For example, amine and quaternary compounds prevent microorganism and algae growth. Besides, primary fatty amines and fatty

aminopropylamines are used as corrosion inhibitors and in the cleaning process of metals when HCl is used to dissolve rust. The amine is orientated in the interface between the metal and the acidic solution, with the hydrophobic tails compressed against themselves, establishing a protecting layer of one or two molecules width. This layer is so tight that avoids the attack of the acid to the clean underlying metal surface. Other application of cationic surfactants that depends on the surface activity and the orientation of the surfactant ions, is the softening of textiles. The cationic surfactant adsorbs and orientates at the interface formed between the fabric and the water. Likewise, they have affinity for the hair surface, thus being used as hair softeners and conditioners products which are usually applied after the hair wash, countering the matting effect of anionic surfactants.

1.2.2.3. Nonionics

In this class of surfactants, the hydrophilic group is incapable of hydrolysing and forming salts. Nonionics are especially useful because of their low sensitivity to water hardness and pH. Since they are compatible with charged molecules, they are easily used in mixtures with other ionic surfactants, which often results in beneficial associations. For instance, nonionics can help to solubilize calcium or magnesium salts of anionic surfactants. The hydrophilic-lipophilic balance in these surfactants can be adjusted properly, balancing the amount and the nature of the polar units and the hydrophobic part of the molecule (carbon chain length). The hydrophilic part of these molecules is frequently an ethylene oxide chain. Ether groups may also be used to provide the required polarity to assure their solubility in water by hydrogen bond formation. Fatty alcohol ethoxylates are the most employed nonionics in cleaning products, cosmetics, herbicides, etc. Alkyl phenol ethoxylates (APEs), mostly octylphenol ethoxylates (OPEs) and nonylphenol ethoxylates (NPEs), also have a chain with EO units, but unlike FAE, they absorb

in the UV region. Application of APEs in detergents is legally restricted, due to their low biodegradability of the most hydrophobic metabolites, specifically non ethoxylated alkylphenols and monoethoxylates. Further, they were banned in most countries due to their ability of mimeting some hormones, thus disrupting the endocrine systems of mammals. In addition, linear FAE biodegradate faster than APEs. Besides they have better detergency properties than LAS over different types of dirt and over most types of fabrics, and are especially effective to eliminate grease from synthetic fibres. They also perform well at low temperature, that is why they have become one of the major and most common ingredients in household detergent formulations. Amine, amide and fatty acid esters are also broadly employed in personal care products. For example, coconut dieathanolamide has good foaming properties, stabilizing the anionic surfactant foam. Finally, sugar based nonionic surfactants, alkylpolyglucosides (APGs) have an extremely fast biodegradability, low toxicity and a high dermatologic tolerance. Besides, they can be elaborated from natural raw materials. The structure and the nomenclature of the main nonionic surfactant families are shown in **Fig. 1.4**.

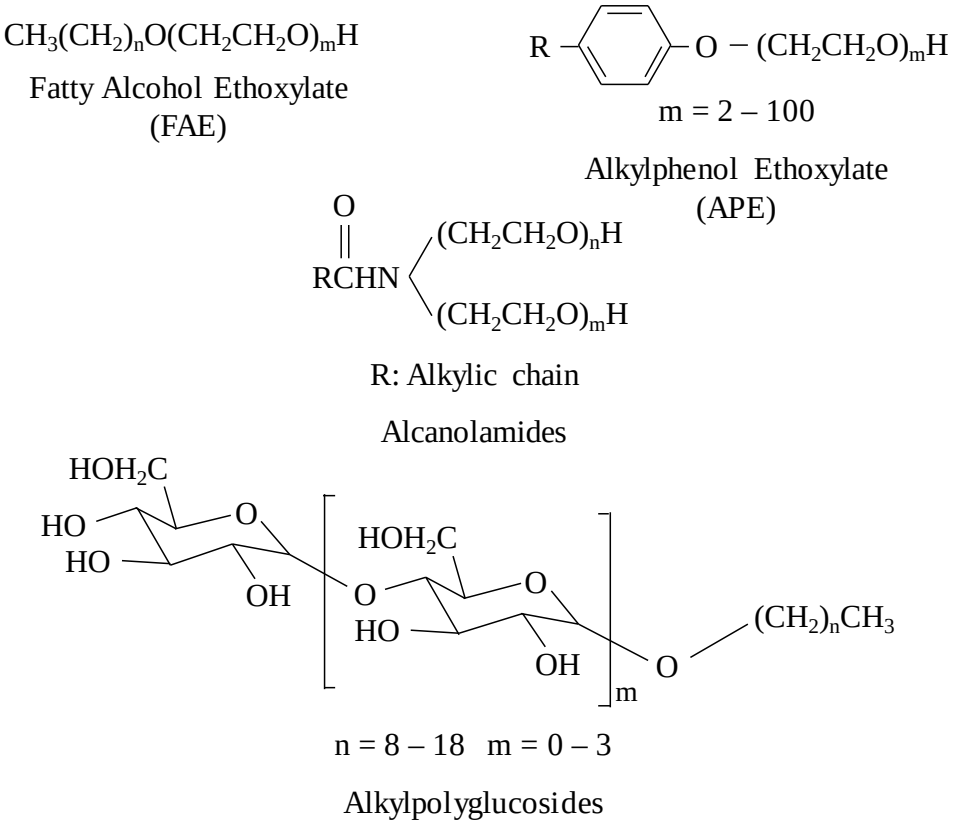


Fig. 1.4. Structure and nomenclature of the main nonionic surfactant families.

1.2.2.4. Amphoterics

Finally, molecules with acidic and basic characteristics are amphoterics or double ions. They are compounds that carry both a positive and a negative charge simultaneously. Some of these compounds have weak acidic or basic groups and they can act as anionics or cationics depending on the pH. They are usually used in conjuction with other surfactants (anionics or nonionics) to boost desired properties such as foam or detergency. Since the optimal activity of amphoterics takes place around neutral pH, they are particularly appreciated in personal care products (shower gels, foam baths, shampoos, etc.) for their mildness and skin compatibility, being less irritating than cationic and anionic surfactants. Formulation of these products is not easy due to the possible precipitation of the surfactant when the pH is near to their isoelectric point. They can be used, with NaOH, in alkaline cleaners for greasy surfaces, and as acidic cleaners with HCl for rusted surfaces, as a result of their stability and functionality over wide pH ranges. A big number of amphoteric surfactants are extensively known natural products, as lecithin. An additional family of amphoteric surfactant that show a quaternary ammonium group are the alkyllbetaines (**Fig. 1.5**)

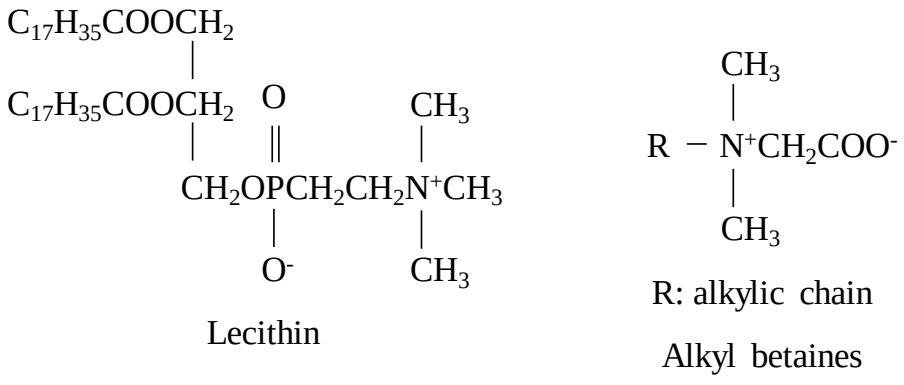


Fig. 1.5. Structure and nomenclature of the main amphoteric surfactant families.

1.2.3. Fatty alcohol ethoxylates

These kind of surfactants, produced from the reaction of fatty alcohols with EO, are obtained as complex mixtures of oligomers with the structure:

$$CH_3(CH_2)_{n-1}(OCH_2CH_2)_mOH$$

that can be shortened as C*n*E*m*, where *n* adopts values between 8 and 18 and where *m* ranges between 0 and 30, and average EO being often represented by $\overline{m}$.

1.2.3.1. Properties and applications

The most used nonionic surfactants nowadays in household products are FAEs, being also used in the formulation of cosmetics, dispersing agents in herbicides, etc. The ether groups provide them with enough polarity to guarantee their water solubility. Even though the EO chain is not so polar as an ionized group, the combination of 5 to 10 EO units can be enough to reach a remarkable hydrophilic character. FAE are excellent moisturizing agents, compatible with both anionic and cationic surfactants, and their detergency is not reduced by the presence of Ca^{+2} or Mg^{+2}. They tend to be liquids or waxes with a low melting point, therefore, they are not usually employed in the formulation of powder detergents. Another drawback is their tendency to precipitate at high temperatures or at high ionic strengths, due to the decreased solvation of the EO chains compared to the ionic groups. Besides, at high temperatures, the statistical weight of the polar conformations of the EO chain is reduced, so that the polar zone loses much of its hydrophilic character. In these conditions the surfactant splits from the aqueous medium forming a different phase (cloud point). Linear FAE are roughly characterized, by both the range of *n* (also known as “hydrophobic cut”), and the average number of EO; however, for both industrial quality control and environmental analysis, information of the oligomer distribution is often required. Characterization of FAE is important because their physico-chemical properties,

are strongly influenced by the lengths and distributions of their hydrophobic and hydrophilic chains [12-18].

1.2.3.2. Analysis methods

The determination and full characterization of FAE is not an easy task. Due to their low volatility and the low thermal stability of the EO chain, FAEs with m > 4 cannot be determined by gas chromatography (GC) [12, 19-21]. The lack of a suitable detector has been the main drawback for the analysis of FAE by high HPLC [12, 22]. This problem has been partially solved with the use of detectors such as the refractive index detector (RID) [23-26] and mainly by the evaporative light scattering detector (ELSD) [24, 27- 31]; however, the limits of detection (LODs) for the RID detector are high, and the unfeasibility to use gradient elution hinder the rapid elution of the more hydrophobic oligomers. As for the use of ELSD, its sensitivity is compromised for volatile compounds such as non-ethoxylated alcohols and FAE oligomers with a low ethoxylation grade ($m < 3$) [32, 33]. Lowest LODs can be attained with precolumn derivatization with chromogenic and fluorogenic agents [13, 34-51].

In the determination of FAE using mass spectrometry (MS), formation of aducts with positive ions, using both electrospray ionization (ESI) or Atmospheric pressure chemical ionization (APCI), is problematic due to the decrease of sensitivity as m decreases, which is especially noteworthy for $m < 4$. Finally, non-ethoxylated alcohols ($m = 0$) cannot be detected by MS [12, 20, 22, 51-56]. To overcome this drawback, derivatization procedures on which a chromophore group or a permanent charge is added to the FAE oligomers have been developed [36, 38, 41, 46, 47, 51, 54-56]. However, FAE derivatization is not an easy task, because an anhydrous medium is frequently needed [43, 51, 54, 57], so the excess of derivatization reagent and byproducts of the reaction may increase the risk of

interferences [39, 40, 48, 49, 54]. On the other hand, and due to the risk of losing the lower ethoxymers by volatilization, water content in samples must be carefully reduced, what prolongs the time of analysis [54]. Nevertheless, derivatization with cyclic anhydrides tolerates the presence of small amounts of water in the samples [48-51].

1.2.4. Alkyl ether sulfates

Alkyl ether sulfates are an anionic surfactant class broadly used in cleaning products and personal hygiene [58, 59]. They are obtained by esterification of FAE using sulfur trioxide or chlorosulfuric acid. The molecular structure of AES is formed by a hydrocarbon chain linked to an EO chain with a sulfate group at the end [60-62]. These compounds are rarely pure, being generally mixtures, due to the raw material where they come from and to the wide range of EO numbers of the oligomers. They are obtained as complex mixtures of oligomers with the next structure:

$$CH_3(CH_2)_{n\text{-}1}(OCH_2CH_2)_mOSO_3^-$$

that can be abbreviated as C*n*E*m*S, where n adopts values between 12 and 16 and where m ranges between 0 and 3, and the average EO is represented by $\overline{m}$.

1.2.4.1. Properties and applications

Alkyl ether sulfate aqueous solutions show a special behaviour, because their viscosity increases at the beginning by the addition of electrolytes as sodium chloride at low concentrations, but diminishes with further additions. The highest viscosity, as for the salt concentration to add, depends on the ether sulfate structure. In comparison to ASs, the corresponding AESs are more water soluble and show better resistance to the presence of Ca^{+2} and Mg^{+2}. The addition of an EO chain to the AS tends to increase the foaming power of the surfactant, especially in hard

water, since it allows an intensification of the interfacial adsorption. Moisturizing and emulsifying properties are also enhanced. Thus AES are often used in bar soap formulations, in different cosmetic and pharmaceutical products and in the treatment of textiles [63, 64].

1.2.4.2. *Analysis methods*

The main features of these surfactants (viscosity, detergency, foam formation and skin compatibility), as their environmental impact, depend on the distribution of their hydrocarbon and EO chains [12-18, 62, 65]. Hence, it is important to develop analytical methods for their characterization and determination. However, due to the complexity of the samples, the lack of chromophores and the wide range of polarity of the oligomers, the detailed analysis of AES is not an easy task. Besides, the absence of commercial standards difficults their accurate determination.

Classical methods for the AES determination include the methylene blue test or the two-phase mixed indicator titration method [66] which are used to determine the total amount of anionic surfactants. Determination of ASs and AESs has been achieved by ion pair chromatography with indirect UV detection [67, 68], ionic chromatography with conductometric detection [69, 70]. CE with indirect UV detection has also been used for the analysis of AES [71, 72]. The use of GC for direct analysis is limited to AS and AES due to required hydrolysis and derivatization reactions in order to increase their volatility [73, 74]. Transesterification with cyclic aromatic anhydrides has been also reported for HPLC-UV analysis of AES [75, 76]. Liquid chromatography (LC) with tandem MS is lately the preferred technique for AES determination and several methods applied to different environmental matrices have been reported [77-80].

1.2.5. Linear alkylbenzene sulfonates

Linear alkylbenzene sulfonates are the most widely used anionic surfactants in the formulation of laundry and industrial detergents. Commercial LAS consist of a mixture of homologues mainly containing the C10-C13 homologues. Each homologue comprises from four to six positional isomers which differ in the attachment point of the *p*-sulfonate phenyl group to the linear alkyl chain, which can be found at any position except in the terminal carbon atoms. LAS is produced by sulphonation of linear alkylbenzene (LAB) using oleum, sulfuric acid, or gaseous sulfur trioxide. On an industrial scale, sulfonation with SO_3 is the most common process. LAB, the precursor of LAS, is manufactured in large scale industrial processes by alkylating benzene with linear mono-olefins or alkyl halides such as chloro-paraffins by using HF or $AlCl_3$ as the alkylation catalyst [81].

1.2.5.1. Properties and applications

Linear alkylbenzene sulfonates are nowadays one of the most frequently used anionic surfactants because of its relatively low production cost, good cleaning performance, the fact that it can be easily dried to a stable powder, and also because of its biodegradable environmental friendliness, making it a very cost-effective surfactant. The distribution of isomers significantly determines the physical properties of LAS; although symmetric isomers are more water soluble compared to external ones, alkaline-earth salts of external isomers are more soluble. Optimum general properties of LAS are reached for carbon chain lengths ranging between 9 and 15. The C11-C12 range favors wetting and foaming, whereas the C13-C14 range is more beneficial for detergency. Due to its very high detersive action, LAS has a low compatibility with skin and is scarcely used in cosmetics. LAS is also used as foaming agent in the manufacture of plaster boards,

as an emulsifier in phytosanitary liquids and emulsion polymerization and as dispersing agent for dyes in paper and textile industries [10].

1.2.5.2. Analysis methods

Classical spectrophotometric methods have been commonly used for analysis of LAS, mostly in aqueous matrices [82-85]. Sensitivity improves when an ion pair between the surfactant and a cationic dye is formed. Determination of LAS by GC is not possible directly because the LAS oligomers are not volatile enough, thus derivatization procedures should be applied to obtain their sulfonyl chloride or methyl ester derivatives [86-88]. The presence of a chromophore group (the phenyl group) allows the relatively easy detection of LAS by HPLC-UV or fluorescence detection [89-92]. HPLC-MS is also a commonly used technique, especially for the environmental analysis of LAS and other surfactants, because it ensures the correct assignment of the chromatographic peaks [93-95]. In the absence of additives, and using isocratic or gradient elution with hydroorganic mobile phases, reverse phase liquid chromatography (RP-LC) with a C4 or a C8 column give rise to wide bands with poor repeatability [76]; however, retention of LAS becomes reproducible and efficiency improves largely by adding either sodium perchlorate or tetraalkylammonium salts to the mobile phase [89, 90, 96, 97]. Another technique for the determination of LAS is CE. The advantages with regard to HPLC are the use of small sample volumes, low consumption of solvent, a reduced time of analysis and the possibility to separate the positional isomers. [98, 99].

1.2.6. Soaps

Carboxylate salts or soaps can be directly produced by the alkaline hydrolysis (or saponification) of animal and vegetable glycerides or can result from the neutralization of fatty acids obtained by the acidification of carboxylates.

Commercial fatty acids coming from natural sources are generally not pure and involve mixtures of different fatty acids with various chain lengths and unsaturation degrees.

1.2.6.1. Properties and applications

Free fatty acids are not used as surfactants, due to their low solubility in water. However water-soluble fatty acids salts show good water affinity and are widely used, especially the alkaline ones and those resulting from the neutralization with short-chain amines (ethanol amine, diethanol amine, triethanol amine). Potassium and alkanolamine soaps are more fluid and also more soluble than sodium soaps. The extremely low solubility of alkaline-earth and heavy metal fatty acid salts makes this class of surfactants unappropriate for use in hard water. The main applications of fatty acid carboxylates are the fabrication of soap bars for hand washing of fabrics, but they are also extensively used in liquid laundry detergents in combination with other surfactants. Water-soluble soaps are mainly used in skin cleansers, shaving products and deodorant sticks [10].

1.2.6.2. Analysis methods

The traditional analysis technique for the carboxylate salts analysis is GC, previous esterification in order to increase the volatility, enhance detection sensitivity and improve chromatographic resolution. The most widely derivatization route is the direct transesterification of the fatty acids into methyl esters using methanol. However, the derivatization procedures can cause a variety of inconveniences limiting their applications. The use of HPLC has been also broadly reported for soap analysis. Fatty acids are typically separated according to the increasing alkyl chain length on RP columns with MeOH/water or ACN/water mobile phases. Retention increases if the pH is lowered. Analysis is performed

either at low pH without an ion-pairing agent or at a high pH (7-8) with ion-pairing. Low wavelength UV, RID, and evaporative detectors are adequate. The ESI-MS sensitivity in the ion-negative mode is also enhanced by buffering the mobile phase to pH 7 (e.g., with ammonium acetate), or by post column addition of an ammonium solution to ionize the carboxylate terminal group and to form adducts with ammonium in the gas phase [100].

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CHAPTER 2.

Liquid chromatography

2.1. Liquid Chromatography

Liquid chromatography is a physical separation method in which the analytes are separated according to their distribution between two phases: the stationary phase which remains fix, and the mobile phase, which flows in a defined direction. A chromatographic system (**Fig. 2.1**) is composed at least of a pumping module, a device to introduce the sample or injector, a column, a detector and an appropriate data acquisition and control system.

Depending on the elution mode, pumps employed in LC can be of two kinds: isocratic or gradient pumps. In the isocratic mode, the mobile phase composition remains constant throughout separation, whereas in gradient elution the mobile phase composition is modified during the separation process. The gradient pump modules generate mixtures of variable composition. Depending on the design of the pump module, mixtures are produced at low or high pressure, being different the characteristics, advantages and limitations of each type.

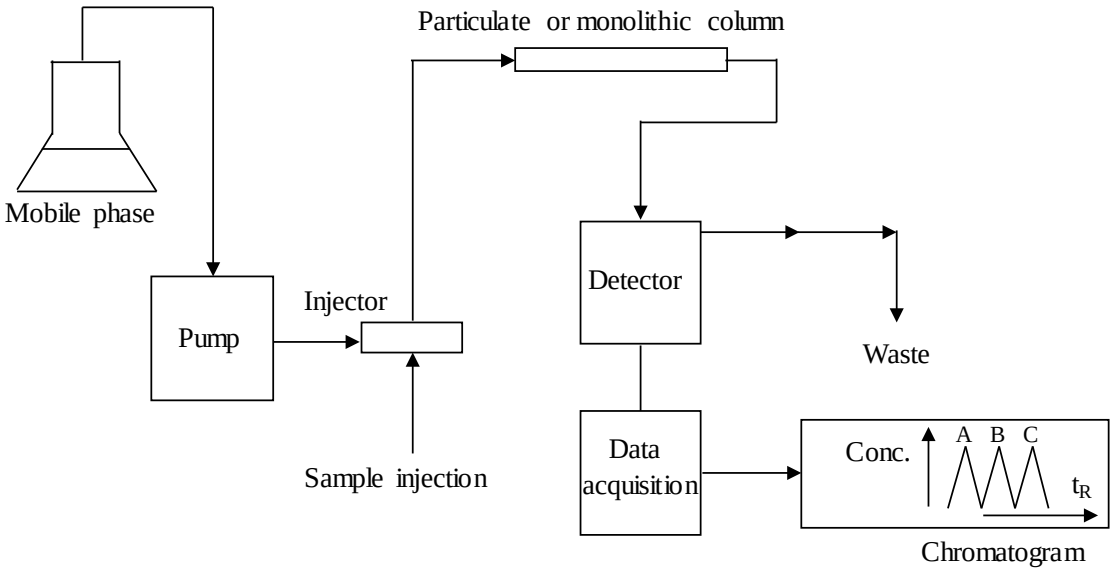

Fig. 2.1. Flow chart of the essential components of a liquid chromatograph.

Either in LC as in other analytical techniques, the sample is introduced in the flow of the mobile phase through an injection loop using a 6-port-2 position valve that can be manual or automatic. The loop is inserted between the pump module and the column.

The type of column to be used depends on the desired retention mechanism. The more common LC modes are:

i) Partition chromatography: a column with a bounded liquid phase. Depending on the relative polarities of the mobile phase and the stationary phase, two modalities can be distinguished: reverse-phase, in which the stationary phase is nonpolar and the mobile phase is polar, or normal-phase, where the stationary phase is polar and the mobile phase is nonpolar. A special chromatographic mode in NP is hydrophilic interaction liquid chromatography (HILIC), in which the mobile phase is less polar than the stationary phase, but keeps miscibility with water.

ii) Ion chromatography: the stationary phase is a cationic or an anionic exchanger consisting either of a strong acid, strong base, weak acid or a weak base. A cationic stationary phase is used to separate anions whereas an anionic stationary phase is used to separate cations. The mobile phase contains anions or cations, and the elution of the analytes can be accomplished by running mobile phases with high concentration of the competitive ions through the column.

iii) Size-exclusion chromatography: the porous of a microporous solid or a gel is used as stationary phase. In this mode molecules are sorted by size. Small molecules penetrate deep into the pores eluting later than larger molecules that cannot enter the pores.

On the other hand, it is important to degas the solvents to prevent the formation of air bubbles in the instrument, which can disturb the separation and cause detection noise. For that, liquid chromatographs usually include an on-line degasser, inserted before the passage of the mobile phase to the pump.

Miniaturization, an emerging trend in analytical chemistry along the last third of the last century, has been also introduced in the different chromatographic modes. Among the advantages of miniaturization, it is worth noting the reduction of the time analysis, the volume of the generated wastes and the amount of sample that is required. At the same time, improvement in the sensitivity, reduction of the LODs and higher efficiencies is obtained. The higher instrumental requirements, as the use of micro-pumps and micro/nano-nebulizers can be indicated as the main drawbacks. Among the developments in chromatography, multidimensional liquid chromatography (MDLC) is a high performance separation technique that is getting more and more popular over the years for the separation of complex samples including biomedical, pharmaceutical, food and industrial samples such as synthetic polymers or surfactants [1,2]. In MDLC the sample is subjected to more than one separation mechanism. Each mechanism is considered an independent separation dimension. The two dimensional version of MDLC, 2D-HPLC, has gained popularity as a column-based separation method with much higher resolution, selectivity and peak capacity than a single-column separation method.

2.1.1. Detectors

Today, the most employed detection techniques in LC are UV-vis spectrophotometry and MS. As alternative detectors, refractive index (RID) and evaporative detectors such as ELSD and charged aerosol detector (CAD), are also used. For specific applications, amperometric and fluorimetric detectors are used. Conductometric detection is commonly used in ion chromatography.

2.1.1.1. UV-Vis detector

In UV-vis spectrophotometry, the signal is proportional to the molar concentration of the solute, whose molar absorptivity depends on the nature of the

absorbent group or groups. When the LOD is not low enough, preconcentration techniques can be used. Alternatively, the sensitivity can be enhanced by derivatization. Spectrophotometric detectors follow two types of design: variable wavelength and diode array detector (DAD). In the former, a fixed wavelength is selected for the measurement, while the later detectors are able to scan the whole UV-vis spectrum several times per second. In these detectors, the monochromator is placed after the radiation of all frequencies that reaches the sample. Once the beam goes through the sample, the transmitted radiation is dispersed so that each photodiode measures the corresponding intensity within a small range of wavelengths.

2.1.1.2. Evaporative light scattering detection

The evaporative light scattering detector is a quasi-universal detector mainly used in LC that responds to all compounds that are less volatile than the mobile phase and is independent of the compound's optical properties. The operation principle of ELSD consists mainly of three successive processes (as shown in **Fig. 2.2**): nebulization of the chromatographic effluent, evaporation of the mobile phase and detection of the non-volatile residual particles, by means of the measurement of the scattered light [3]. In the first step, the chromatographic eluent passes through the heated nebuliser and is mixed with the incoming nebuliser gas stream, usually nitrogen. The mixed gas and eluent stream form an aerosol containing a cloud of dispersed droplets that passes into the evaporation section. In the evaporation tube, the solvent is evaporated and the size of the aerosol droplets is reduced. In this step the evaporation temperature should be selected in accordance to the mobile phase and analyte volatilities and to the mobile phase flow rate. The nebuliser temperature and the flow rate of the nebulizing gas can be used to optimize signal response in addition to the evaporator

temperature. Lastly the aerosol, after the evaporation process, enters the optical cell and passes through a light beam. When only the mobile phase is being evaporated, only its vapor passes through the light path and the amount of light scattered to the photomultiplier is small and gives a constant baseline response. When a non-volatile solute is present a particle cloud passes through the light path, causing light to be scattered. This scattered light enters the optical aperture of the detection system and generates a signal response from the photodiode in real time. The quantity of light detected is dependent on the solute mass rate and solute particle size detection.

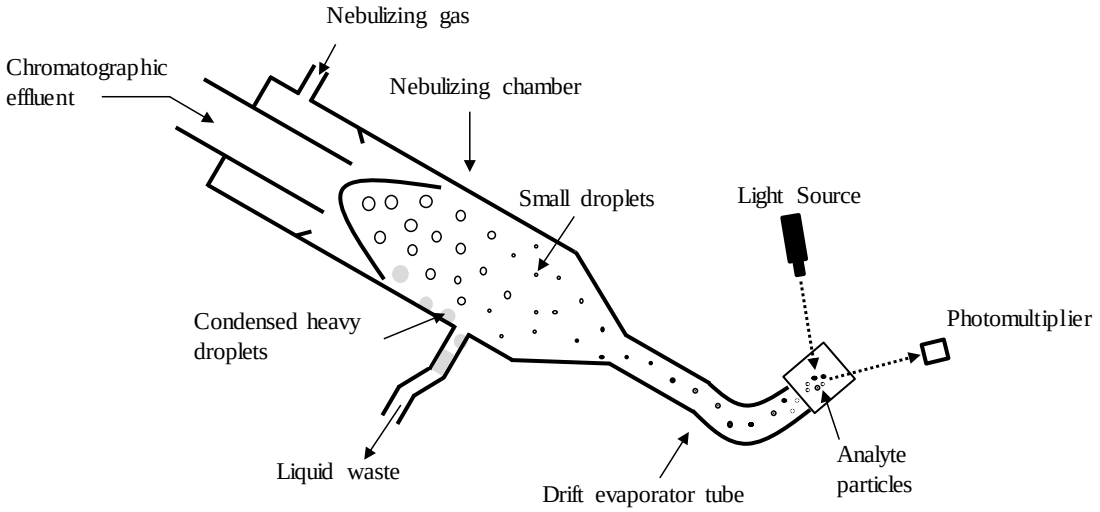

Fig. 2.2. Scheme of the main steps of ELSD operation.

This detector has a number of advantages over other detectors. In many applications the need for spectrophotometric derivatization can be avoided with the use of an ELSD. Other special characteristics are its compatibility with gradient elution and insensitivity to temperature variations (unlike RID), better detectability than RID for most molecular classes and its lower cost and easy operation (in comparison to mass spectrometers). On the other hand, this detector presents also some limitations. The main drawback is the restriction on the mobile phase volatility. Non-volatile modifiers, several ion pairing reagents and a restricted list

of acids, bases and buffers cannot be used with ELSD. If it is used with other detectors it should be the last in the line because it is a destructive detector. Another drawback, especially in the frame of routine work is that in most applications ELSD presents a non-linear response, thus exponential and polynomial regressions have to be used in order to achieve a good correlation between peak areas and the analyte mass [4-6]. A solution would be to use a CAD, whose response is more linear as that of the ELSD along a wider calibration range. A CAD works essentially as an ELSD but the final measurement is not made on the scattered light, but on the amount of electrical current the aerosol is capable of carrying under a certain bias voltage.

2.1.2. Measurement of chromatographic parameters

To carry out a chromatographic separation, the analyst has to stablish if it is possible to appropriately separate the analyte from the rest of components of the sample, and if the amount of analyte present in the samples is enough to be detected and/or quantified. The time that elapses from the injection until the analyte detection is its retention time or t_R. Besides, the capacity factor or relative retention (k) expresses the overall retention in units of the dead time, t_0, or the retention time of a non-retained compound:

$$k = \frac{t_{R,i} - t_0}{t_0} \tag{2.1}$$

where $t_{R,i}$ is the retention time of analyte i. The optimal range of k values is between 1 and 5, even though values between 0.2 and 10 are acceptable. Values lower than 0.2 indicate low retention, excessive preference of the solute for the mobile phase. On the contrary, k values higher than 20 indicate too high retention, produced by an excessive preference of the solute by the stationary phase. This involves very

long analysis times and generally wide and short peaks, difficult to be detected and measured accurately, thus with higher LODs and lower accuracy than expected.

The capacity of a chromatographic system to distinguish between two solutes is expressed using the selectivity factor $\alpha_{i,j}$, that can be calculated as the relation between the relative retention of both solutes:

$$\alpha_{i,j} = \frac{k_j}{k_i} \tag{2.2}$$

being *i* and *j* two neighbouring peaks and *i* the less retained.

The separation degree between two solutes is measured by the resolution, *R*, which is calculated as:

$$R = \frac{t_{R,i} - t_{R,j}}{0{,}5(w_i + w_j)} \tag{2.3}$$

being w_i and w_j the width of the base of the peaks of compounds *i* and *j*.

For its part, efficiency describes the band broadening degree in relation to the retention volume. A high efficiency is achieved when all the peaks remain narrow despite requiring a high volume of mobile phase for elution. The global efficiency is described by the number of theoretical plates (*N*), and the efficiency per length unit by the equivalent height to a theoretical plate (*H*), or by its reverse (*1/H*). For a given solute, *N* can be calculated from the expressions:

$$N = 16 \cdot \left(\frac{t_R}{w}\right)^2 \quad \text{and} \quad N = 5{,}54 \cdot \left(\frac{t_R}{w_{1/2}}\right)^2 \tag{2.4}$$

where t_R is the retention time of the solute and *w* and $w_{1/2}$ are the peak width at its base, and its width at medium height, respectively. Additionally, *H* is related with *N* through the expression:

$$N = \frac{L}{H} \tag{2.5}$$

where *L* is the column length.

The Van Deemter equation describes the contributions to *H*, this is, it indicates how the different building and working factors of the column affect over the efficiency. In the case of microparticulated columns in GC, HPLC and CEC, the next simplified expression can be used:

$$H = A + \frac{B}{\bar{u}} + C \cdot \bar{u} \qquad (2.6)$$

where $\bar{u}$ is the average linear velocity of the mobile phase.

The *A* term of the van Deemter equation is known as the eddy-diffusion parameter, which is related to the different length of the followed paths and the different velocities of the solutes on their movement through the chromatographic bed. (**Fig. 2.3, part A**). The contribution to the band broadening is due to the fact that molecules move at different velocities depending on the width of the followed route. Furthermore, the mobile phase moving through the centre of the channels travels faster than the one moving close to the walls. This contribution to *H* is just a function of the filling geometry, this is, not depending on $\bar{u}$.

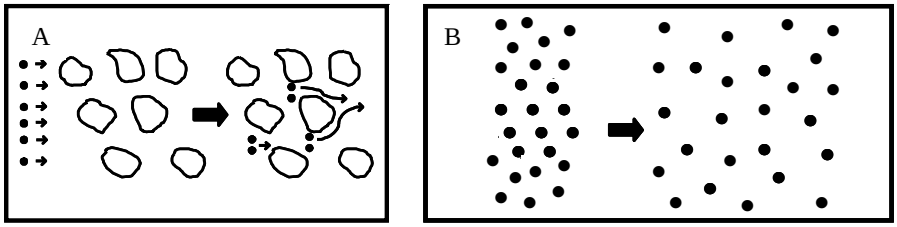

Fig. 2.3. A) Eddy diffusion B) longitudinal molecular diffusion.

The *B* term represents the longitudinal molecular diffusion (in the axial direction), which is related to the diffusion of the solutes at a molecular level (**Fig. 2.3, part B**). This diffusion is proportional to the solutes diffusibility and the dwelling time within the column. The longer the solutes stay in, the bigger the diffusion is, thus the *B* term becomes important at low flows. This time dependence is reflected on the invers proportionality to the contribution on $\bar{u}$.

The *C* term corresponds to the combined contribution of the mass transfer of the solute rates between the mobile phase and the stationary phase. This can be calculated in two terms, C_M and C_S, depending on the diffusion coeffcients within each phase. This term is proportional to $\bar{u}$ because the movement of the mobile phase strives in terms of velocity with the mass transfer of the solute between both phases, thus the importance of the *C* term increases with the velocity of the mobile phase as the equilibration time between both phases is more seriously delayed. The delay in the mass transfer after each "stage" of advance of the mobile phase origins a broadening in the zone occupied by the solute.

The terms *A* and *C* of the van Deemter equation indicate that the column efficiency can be improved using smaller particles of the stationary phase, or more uniform packing. The shape of the van Deemter plot gives information about the quality of the packaging of the chromatographic column. The lower the contributions of the *A* and *C* terms the higher the number of theoretical plates can be achieved at a certain mobile phase flow. In **Fig. 2.4** a typical representation of this equation is shown.

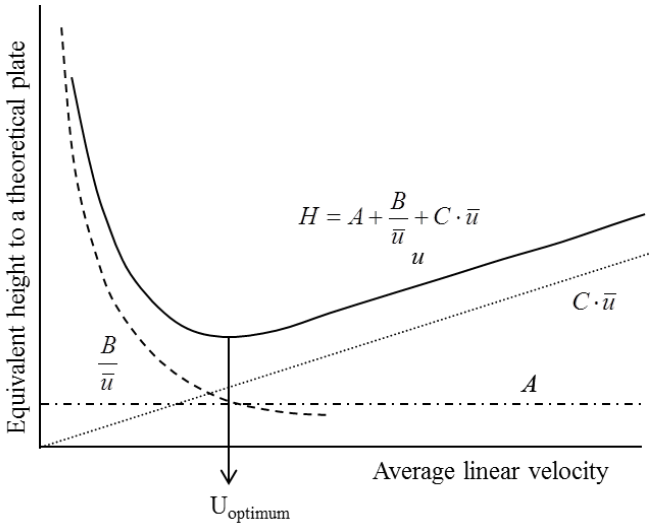

Fig. 2.4. *H* versus $\bar{u}$ (van Deemter plot).

2.2. Two-dimensional chromatography

One-dimensional chromatography is a well-known technique used to the analysis of a widespread range of samples in several fields. However, such separation methods often do not provide sufficient resolving power for the separation of target components in many real-world samples. As previously introduced, in multidimensional chromatograpy a much higher resolving power can be achieved thanks to the use of multiple separation stages. The most commonly used MDLC technique is 2D-HPLC, in which complex samples are separated using two different columns. By positioning these columns in the proper order, and by selecting the mobile phase composition of the two separations, it is possible to selectively take elements from the first column and transfer them to a second columns for additional separation.

The first 2D-HPLC separation was described by Erni and Frei [7]. They separated glycosides on a gel permeation column as first dimension, and RP conditions by means of a C18 column as second dimension. As shown in **Fig. 2.5** the instrumental setup consisted on an eight-port loop valve (valve 2) combined with the action of two pumps.

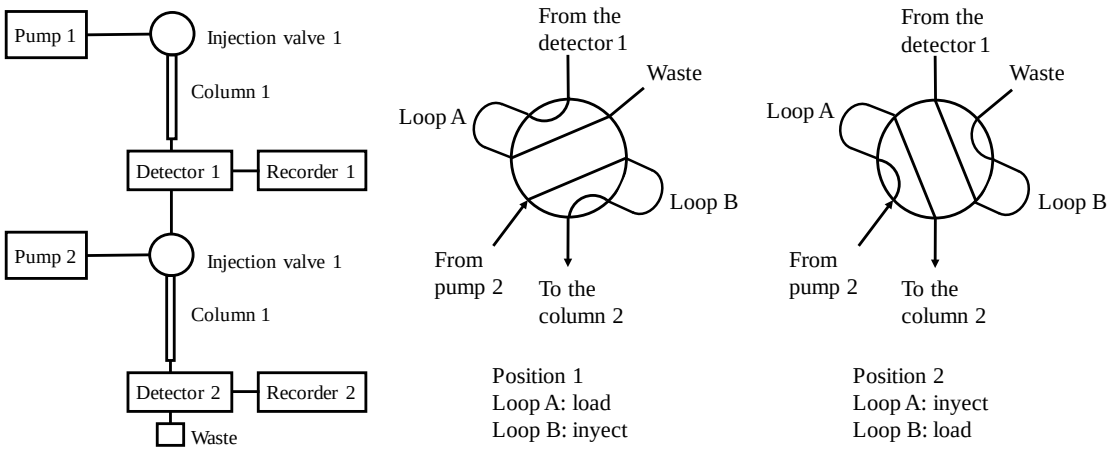

Fig. 2.5. Scheme of the online 2D-HPLC described by Erni and Frei .

The effluent of the first column fills alternatively one or another of the two loops connected to valve 2. While one loop is being filled, the other one is eluted by the second pump through the second column. A requirement of this setup, that limits their possible applications, is the fact that the eluate from the first column has to have a less eluent force than the mobile phase used in the second column. Otherwise, dilution effects are produced when the content of the loops is introduced in the second column.

In 2D-HPLC two main approaches can be considered: either heart-cutting or comprehensive [8]. In the heart-cutting mode of operation, one or several discrete zones are collected from the first-dimension column and reinjected into the second-dimension separation system. The resulting data are one or more individual one-dimensional datasets and are useful for resolving fused peaks from specific region(s) of the first-dimension separation system. In fully comprehensive separation the entire effluent from the first column, divided in small fractions, is sequentially introduced in a second column with a different selectivity. The resulting chromatograms are put into the rows of a matrix that is usually represented as a contour map or topological map, with the time of the corresponding separations over the two cartesian axes [9].

Commercial surfactants are not usually used as a single compound, but as a mixture of compounds with a similar structure that have distributions of chain lengths but with a charged group of constant size, for example sulfate, phosphate or sulfonate. These surfactants constitute complex mixtures susceptible to be analysed by 2D-HPLC. Many applications of 2D-HPLC to the analysis of surfactants are focused on the separation of FAE, due to the dual distributions (alkyl and ethylene oxide) and the lack of full resolution in one-dimensional separation system. Separation of underivatized FAE by 2D-HPLC was first described by Murphy *et al.* [9] using the setup shown in **Fig. 2.6.**

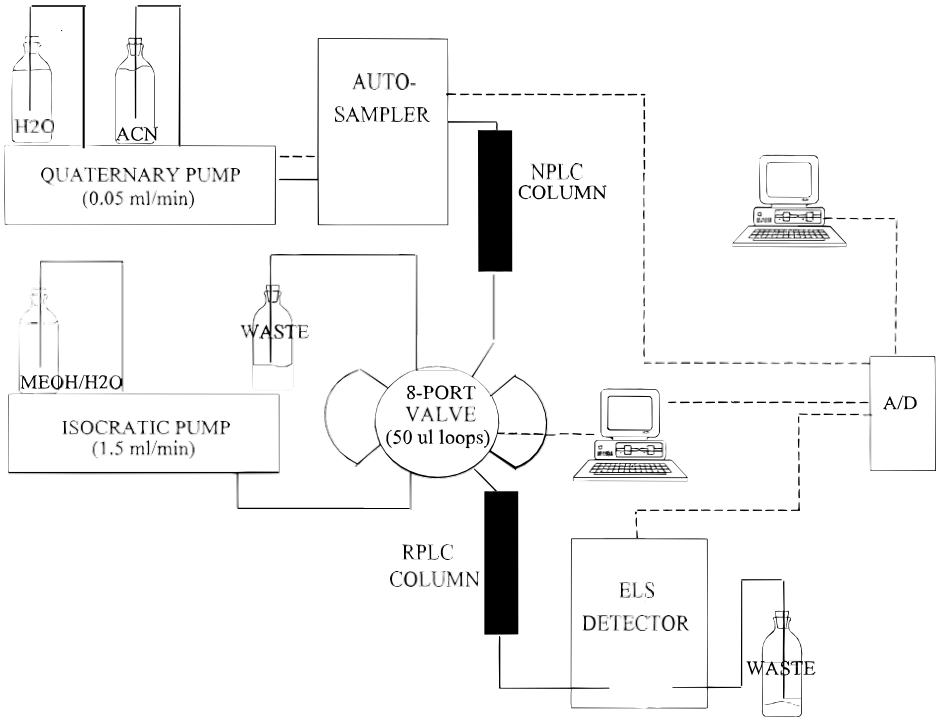

Fig. 2.6. Experimental setup used by Murphy [9].

In this setup a quaternary pump drives the mobile phase to the first column where a separation in normal phase is produced. In this column an ACN/water gradient partially separates the FAE oligomers according to the EO chain. Thanks to the 8-port valve, the eluate is alternatively stored in one of both 50 µL loop. While one loop is being filled, the other one is inserted in the flow path coming from the isocratic pump, bringing the content of the loop to the reverse phase

column. In this column a 95/5 MeOH/water isocratic mobile phase separates the oligomers according to the length of the hydrocarbon chain. The use of miscible phases (ACN/water and MeOH/water) is necessary to avoid mixing problems at the beginning of the second column.

Separation of FAE by 2D-HPLC was also explored by Trathnigg et al. [10-12]. The first approach proposed by these authors combined LC under critical conditions (LCCC) in the first dimension, and liquid exclusion-adsorption chromatography (LEAC) in the second dimension. In the first dimension, the separation was consistent with the hydrocarbon series, and in the second dimension the oligomers within each series were separated according to degree of ethoxylation. Detection of underivatized FAEs was achieved using a RID and a density detector. LCCC separations are not common. For instance, the separation of FAE can be achieved using a C18 column and with high concentrations of MeOH, such as 95-97 % and a 5-3 % of water. The for separation in LEAC, it is carried out in a C18 column with a 10 nm pore size, using ACN/water phases that are miscible with the eluate of the first dimension.

A comprehensive 2D-HPLC approach for the separation of a FAE mixture was tackled by Raust *et al.* [13] based on the separation on a C18 column according to the hydrocarbon chain with a MeOH/water gradient, whereas an isocratic flow of isopropanol/water on a polar Chromolith Si column gave the separation according to the oligomer chain length. Detection was accomplished using ELSD and the method allowed to separate mixtures of FAE with a hydrophobic chain length from 10 to 18 and a 6-7 EO average value and nonylphenol ethoxylates with an average of EO chain length of 10 as can be observed in **Fig. 2.7.**

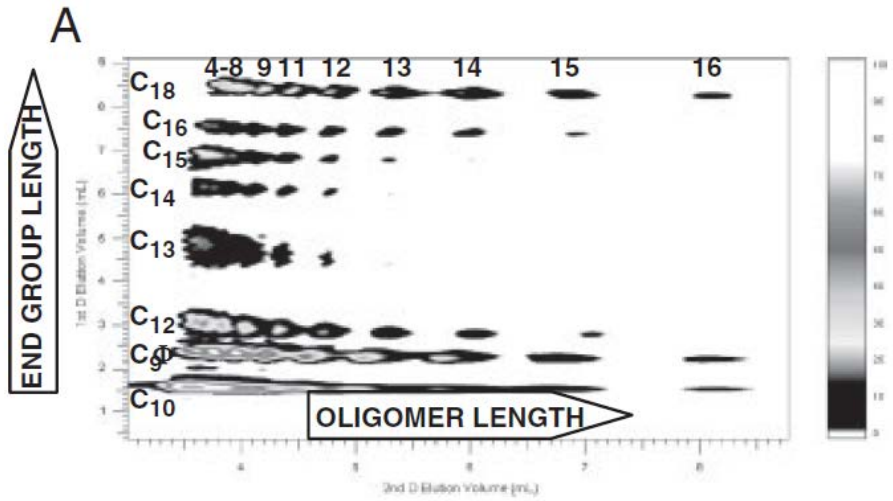

Fig. 2.7. Two-dimensional plot of the FAE model blend (20 mg/mL in methanol–water 80/20); end-group separation (first dimension) along the Y-axis and oligomer separation (second dimension) along the X-axis [13].

Different heart-cutting 2D-HPLC methods have been developed for the separation of FAE. Okada et al [14] presented the procedure to separate heart-cuts of the eluate corresponding to the series n = 12, 14, 16 and 18, separated on a styrene-divinylbenzene copolymer gel. These cuts were sequentially introduced in the second dimension to separate the ethylene oxide oligomers or ethoximers within each series. For the separation along the second dimension, a strong cation exchanger (SCX) column was used. The homologues were separated depending on the degree of complexation with the K^+ cation, using a KCl methanolic mobile phase. The separating system was constituted by three pumps and three columns, two of them were separating columns and the third one was used for preconcentration. The focusing of fractions was also employed by Thrathnigg *et al.* using the separation mechanisms previously described [10], but incorporating an intermediate retention step of the solutes between both dimensions consisting on a full absorbtion/desorption (FAD) technique [12]; the sections of interest in the first dimension were trapped in the FAD column and reinjected in the second column to separate the analites according to the number of EO units. This same

bidimensional system was used for the separation of fatty acid polyglycol ethers [12]. In all the described methods the instrumental setup used at least two or more pumps to achieve the bidimensional separation. Micó et al. [15] used a column selection valve and a 6-port 2-position injection valve to implement bidimensional LC in a system driven by a single pump for the separation of FAE. For the separation in the first dimension, according to the hydrocarbon series of FAE, a propyl-diol column and an ACN/aqueous ammonium acetate gradient were used. Selected segments of the first dimension separation were transferred to the second column, where the successive oligomers within the isolated series were resolved using a C8 column and an ACN/water gradient.

Separation of complex mixtures of different kind of surfactants has been also accomplished using 2D-HPLC. Haefliger [16] described a setup for 2D chromatography capable of separating a complex mixture of cationic, amphoteric, non-ionic and anionic surfactants. A diol column was set as first dimension on which separation by surfactant families was achieved. Elution on this column was performed with ACN/water mobile phases in the presence of 0.1% trifluoroacetic acid. Proportions of ACN/water, near to the proportion 60:40, were optimized in order to achieve the maximum retention of the oligomers on the diol column. Separation on the second dimension was done on RP columns, being a C4 and C2 the recommended stationary phases. Elution over these columns was performed with ACN/water gradients, adjusting in each case the elution strength to the nature of the surfactant family to be separated. In all cases ELSD was used.

Fig. 2.8 shows the experimental setup, which was based on a 10-port 2-position valve. One of the disadvantages of this system is the necessity of three pumps. The first pump is used to elute the analytes along the diol column. A column with 1 mm of diameter is used to minimize the dispersion effect that can involve pumping eluate portions with a high eluent strength into the second

dimension. The second pump is used to reduce the eluent strength, and focus the solutes at the head of the second column. Finally, the third pump is used to implement the gradient along the second dimension. The 10-port valve allows the connection of the C4 and C2 alternatively in one or another of the next positions:

(a) inserted between the exit of the first dimension and the waste, in order to transfer an eluate cut from one dimension to another.

(b) Inserted between the quaternary pump and the detector, in order to examine the chromatogram in the second dimension.

Complex surfactant mixtures were analysed with this system, allowing fast separation but with low efficiency. Its main advantage was the separation in a single bidimensional chromatogram of a mixture of at least 6 surfactants with very different properties.

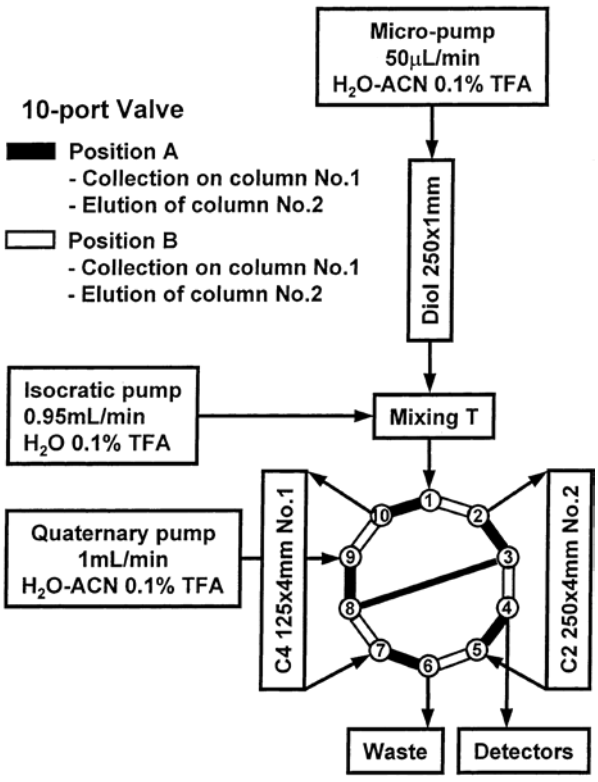

Fig. 2.8. Experimental setup for two-dimensional HPLC analyses described by Haefliger [16].

The separation of a mixture of fatty alcohol derivatives including anionic (AES and AS), non-ionic (APGs and FAE) and amphoteric (cocamidopropyl betaines) surfactants has been also accomplished by comprehensive 2D-HPLC coupled with mass spectrometry [17]. In this work several columns were first evaluated in order to select the best conditions for the separation of the surfactant mixtures according to their characteristics. For the first dimension a ZIC-HILIC column was selected to separate the polyethoxylated surfactants according to their EO number. For this purpose, ACN with an NH_4AcO buffer was used. A 10-port 2-position valve was used as the interface to transfer the first dimension eluate to the second dimension column, which consisted in a Reprosphere 100 C8-Aqua column. In this dimension MeOH with a NH_4AcO buffer allowed the separation by the alkyl chain length. Two different LCxLC-QTOF MS systems were assayed with similar conditions to demonstrate that a transfer of the method was possible. However, small differences in delay volume and extra-column volume between the systems led to changes concerning peak shape, sensitivity and analysis time.

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CHAPTER 3.

Capillary electrochromatography and monolithic columns

3.1. Capillary electrochromatography

Capillary electrochromatography is a chromatographic technique that combines the high efficiency of capillary zone electrophoresis and the high selectivity of HPLC. In CEC, separation is accomplished in capillary columns which are totally or partially filled with a stationary phase. In presence of high electric fields, the electroosmotic flow (EOF) acts as the pumping system of the mobile phase. This flow is generated in the capillary due to the presence of charges on the surface of the column packing. The EOF gives rise to a planar flow profile inside the capillary, different from parabolic profile obtained in hydrodynamic flows, which makes possible to achieve higher efficiencies than in systems driven by pressure.

In CEC the separation mechanism is double [1]. On one hand, there is a chromatographic mechanism, because there is a distribution of the solutes between a mobile and a stationary phase. On the other hand, ionic solutes are also separated with an electrophoretic mechanism, based on the differences of their electrophoretic mobilities. Thus the nature of the packing determines the EOF and affects the separation selectivity.

An instrument for CEC (see scheme in **Fig. 3.1**) is comprised by a high voltage power source, a solvent and/or sample delivery system to the vials in the inlet and outlet of the column, a capillary column with a stationary phase on which the EOF is generated and where takes place the electrochromatographic separation, an isothermal compartment for the capillary column and a detection system capable to detect the concentration profiles of the analites in the background electrolyte (BGE).

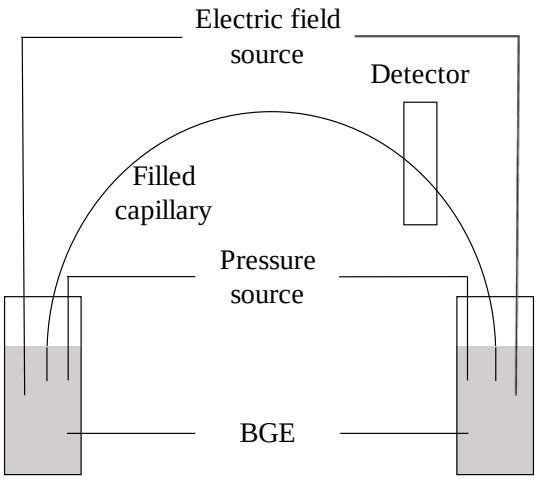


Fig 3.1. Scheme of a CEC instrument

The same instrumentation used for CE can be used for CEC, but in the later a pressurization system of the inlet and outlet vials is necessary. Pressurization is needed to avoid the formation of bubbles that can disrupt the current. Bubbles can be originated because of several reasons, either local differences in the EOF flow [2], in the electric field, loss of trapped gas in the porous of the stationary phase, gas produced electrochemically [3] or by heating [4, 5], or in the case of packed columns, because of cavitation at the frits that retain the packing [6]. Pressurization is applied to the inlet and outlet vials to assure a reproducible flow. To pressurize the vials an inert gas, usually N_2, at approximately 10 bars is used. In commercially available CE instruments, these parameters are automatically controlled, thus giving significant improvements in the reproducibility and security of the separations.

Detection is done in the column itself, using as detection cell a small section made by removing the protective polymeric layer, adjacent to the bed/packing. This step is not necessary for capillaries with transparent coatings. Spectrophotometric UV-vis detection is the most employed in CEC [7-10] being also possible to work in the indirect detection mode. Other widely used detection techniques are laser-induced fluorescence [11-13] and MS [14-16].

3.2. Monolithic columns and its application to CEC

The word “monolith” comes from greek meaning a single stone, which in chromatographic terms is equivalent to a separation over a “continuous bed”. The porous structure of monoliths allows to work in HPLC with high flows obtaining fast separations, without a significant increase in the back-pressure, as happens with particulated columns. In CEC, monolithic columns are also alternatives to packed columns, with some interesting advantages. Given their continuous structure, it is not necessary to use frits at the ends of the monolithic bed, since they are covalently attached to the wall of the capillary. Besides, they can be prepared *in situ*, making the manufacturing of monolithic beds relatively easy compared to the particle packing techniques.

Monolithic columns can be classified into two main categories, silica based and polymeric. Silica columns are prepared using the sol-gel technique [17]. The structure of a silica monolith is composed by interconnected frameworks which create a particular pore distribution. In **Fig. 3.2** the cross-section of a silica monolith (**Fig. 3.2A**) and a zoom of the surface (**Fig. 3.2B**), obtained by scanning electron microscopy (SEM) are shown. These images show a bimodal porous structure composed by macroporous from 1 to 3 μm (pore size related to the framework size) and mesoporous from 10 to 20 nm[*].

[*]The IUPAC divide the porous into three categories according to their size: Macroporous, with diameters over 50 nm; mesoporous, with diameters comprised between 50 and 2 nm; and microporous with a size lower than 2 nm.

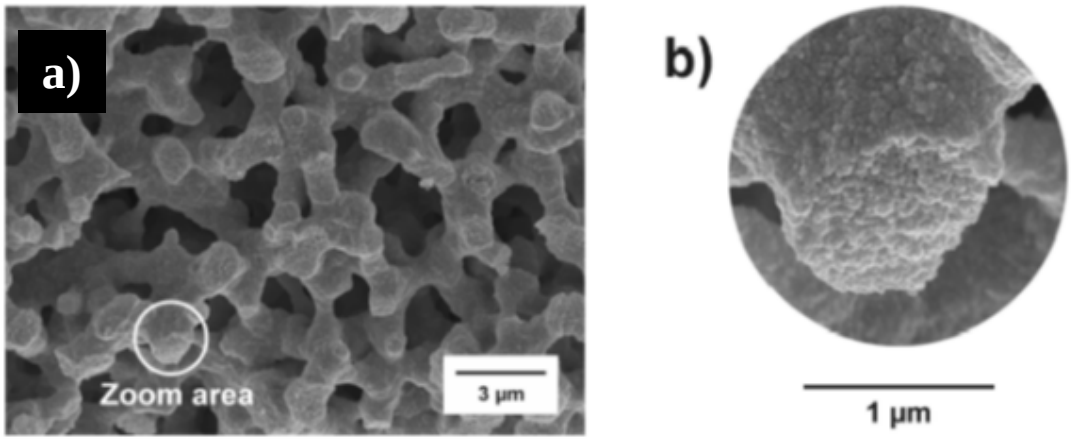


Fig. 3.2. Microphotographs showing the morphology of silica monolithic columns. (A) Cross-section. (B) Zoom of the surface.

On the other hand, preparation of polymeric monolithic columns is easily accomplished by filling capillary columns with a polymerization mixture comprised by monomers, a cross-linker, a porogenic mixture of solvents and a radicalary initiator. The resultant hydrophobicity of the monolith can be controlled selecting the nature of the monomer [18, 19]. The presence of monomers derived from acrylic and sulfonic acids or quaternary ammonium salts in the polymerization mixture [20] guarantees the EOF. Polymerization starts thermally, chemically or by UV radiation. The latter option also allows the formation of monoliths in a specific area by the use of transparent capillaries, passing the radiation through a mask in a pseudo-lithographic process. Once the polymerization is completed, the seals are removed and the capillary is connected to a pump to eliminate the porogens and other soluble compounds that can remain within the monolith.

To avoid any displacement of the monolith along the column, it is necessary to attach the polymer to the inner wall of the capillary. To do so, before introducing the polymerization mixture into the capillary, the inner walls of the capillary are

silanized. For this purpose, 3-(trimethoxysilyl)propyl methacrylate (silane binding) is used in most cases.

To synthesize monoliths different types of polymers have been used, distinguishing mainly between derivatives of acrylamide, polystyrene and esters of methacrylate or acrylate. The first described monolithic columns were prepared with acrylamide and methacrylamide [21-23]. These polymers were synthesized by the polymerization of acrylamide, methacrylamide or derivatives thereof in the presence of methylenebisacrylamide or piperazine diacrylamide as cross-linking agents. Monolithic columns based on polystyrene [24, 25] are obtained by polymerization of styrene and derivatives with divinylbenzene as cross-linking agent. Monoliths based on methacrylate esters [26-28] are prepared by polymerization of butylmethacrylate or other methacrylate ester derivatives, using ethyleneglycol dimethacrylate as cross-linking agent.

3.2.1. Monolithic columns based on methacylate and acrylate esters

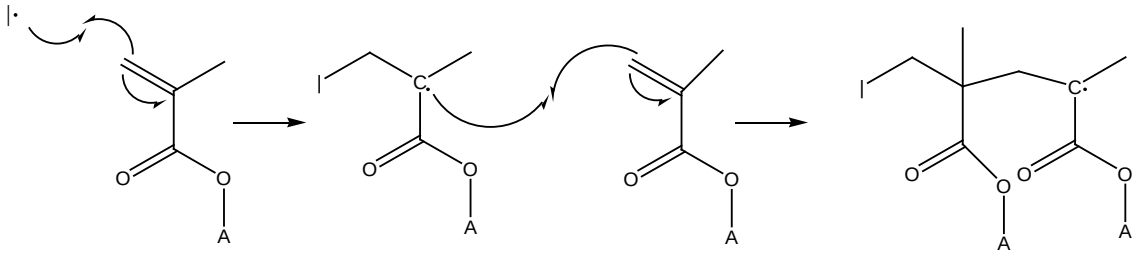
Monolithic columns based on polymethacrylate are more common and better characterized, being extensively developed by Svec et al. [27, 28], who have described either HPLC and CEC applications. Methacrylate and acrylate polymers have mechanical and chemical characteristics that make them highly appropriate as stationary phases. They are stable in a wide pH range (2-12), unlike silica based stationary phases, which degrade easily over pH 9. Their synthesis is fast and easy, and it is possible to start from monomers of very diverse polarity.

Polymerization of methacrylate and/or acrylate monoliths is carried out by a radical reaction, generally initiated by an elevated temperature, UV irradiation, or by chemical agents at room temperature. For the thermal initiation, azobisisobutyronitrile (AIBN) [27-29], benzoyl peroxide [30] or other peroxides [31] are usually added to the monomer mixture.

The formation mechanism of the macroporous monolith is based on a radical reaction, where the monolith is prepared *in situ* through a chain polymerization. The set of reactions could be described as follows:

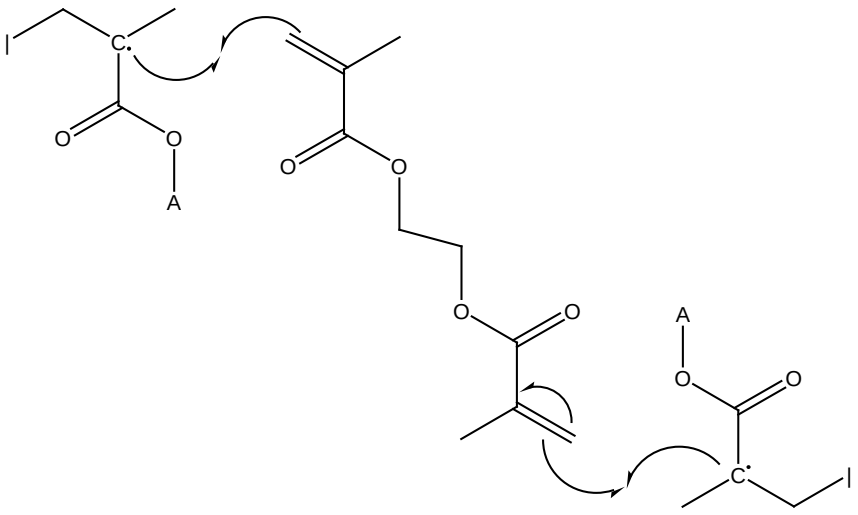
First, the radical initiator, which is capable of generating free radicals from weak bonds, is decomposed and polymerization is initiated.

The monomer reacts with the formed radical, with subsequent growth of the polymer chain. In the case of methacrylate esters, this step can be generally represented by the following scheme:



where A is an end group that depends on the selected monomer.

Sequentially, the reaction takes place between the monomer and the cross-linker. Following the example of methacrylate polymers, and using EDMA as cross-linker, we have:



As the polymer chains grow, the solubility in the medium decreases and the generated nuclei are separated from the remaining mixture. The monomers are thermodynamically better solvents of the polymer than the porogens, thus the nuclei are preferably solvated with the remaining monomers in the polymerization mixture. As the monomer concentration inside the nuclei is higher than in the solution, polymerization preferentially continues in the nuclei for kinetic reasons [32], resulting in an increase in size and formation of microbeads. These continue to increase in size and become interconnected, creating the final morphology of the monolith. The process finally gives rise to a two-phase system: a continuous white monolithic solid and an inert porogenic liquid that fills the pores of the structure. The volume occupied by the porogens corresponds therefore to the volume of macropores of the monolithic bed.

The resulting morphology of the macroporous monoliths constitutes a complex system, with a structure constituted by a series of interconnected microbeads, partially aggregated in larger clusters, that form the polymer body. Irregular gaps between the microglobule groups are the macropores. The organization of the globules and their aggregates depends both on the composition of the polymerization mixture and on the reaction conditions used in the preparation of the monolith.

Svec *et al.* [27, 28] have shown that the chromatographic properties (efficacy, selectivity, permeability, etc.) of these materials can be altered by varying the composition of the polymerization mixture (monomers, cross-linker, porogenic solvent and/or initiator), as well as the type of initiation used, which is an interesting way, not only for the development and optimization of chromatographic separations, but also for applications of environmental, biochemical and industrial interest.

3.2.2 Monolith characterization

Monolithic materials can be characterized by studying both their morphological and electrochromatographic properties. There are numerous techniques that provide information about the influence of various factors on the morphological properties of monolithic materials.

For the study of the morphological properties of monolithic materials, there are numerous methods and analytical tools. Among them it is worth to mention SEM [33], mercury intrusion porosimetry [34], nitrogen adsorption/desorption, evaluated by the Brunauer-Emmet-Teller equation [35] and chromatographic permeability. The monolithic columns used in this thesis have been physically characterized by SEM.

Using SEM, images of the structure of a material can be obtained. A beam of electrons is focused on the surface of the material. This beam scans the surface of the material, producing mainly the emission of secondary electrons of low energy and backscattered electrons of greater energy, both being collected by means of suitable detection systems [36]. The information obtained varies according to the characteristics of the detector used. The secondary electrons are formed in a thin surface layer, in the order of 5 to 10 nm in thickness. Part of the signal consists of electrons that emerge from the sample with energy lower than 50 eV. On the other hand, being low energy electrons, they can easily vary their initial path, and information can be obtained from areas that are not in sight of the detector. This particularity is fundamental to give the signal the possibility of providing a three-dimensional information of the topography of the sample, being perhaps the most well-known feature of this technique.

On the other hand, the main utility of the backscattered electron signal, which consists of the electrons that emerge from the sample with an energy greater than 50 eV, lies in the fact that its emission depends strongly on the atomic number

of the elements of the sample. For this reason, two zones with different chemical composition will be revealed with different intensity, although there are no topography differences between them.

To apply SEM the sample to be analyzed must be dry. Otherwise, the low pressure in the microscope would cause the evaporation of the volatile components that would be violently fired, altering the structure of the sample. In addition, the surface must be conductive, which is achieved by coating it with a film of conductive material. For this purpose, high vacuum cathodic spray techniques are used. On the other hand, the reduced thermal stability of the polymers limits the voltage that can be applied to obtain the images.

The electrochromatographic behaviour of monolithic columns can be evaluated from the same chromatographic parameters used to characterize chromatographic separations in conventional liquid chromatography. These parameters have been already explained in section 2.2.1.

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SECTION II.

REVISION ON DERIVATIZATION REACTIONS OF HYDROXYL GROUPS

CHAPTER 4.

Derivatization of hydroxyl functional groups for liquid chromatography and capillary electroseparation

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Review

Derivatization of hydroxyl functional groups for liquid chromatography and capillary electroseparation

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ABSTRACT

The derivatization reactions commonly used to enhance the analytical signal in the HPLC and CE determination of compounds with hydroxyl functional groups are revised. Focus is placed on the determination of compounds having aliphatic alcohols and phenols while lacking other reactive functional groups. The derivatization with acyl chlorides, organic anhydrides, isocyanates and a variety of other approaches, including oxidation of primary and secondary alcohols, sulfonation, esterification with carboxylic acids, and the use of azides, sulfonyl chlorides and other reagents having miscellaneous leaving groups, is covered. Reactions mainly addressed to introduce a chromophore or a fluorophore in the analyte molecule, or to introduce a charge to enhance sensitivity in MS detection, or to enable CE separation are included. Applications related to the industrial quality control of raw materials and manufactured products, and to the evaluation of their environmental impact are emphasized. The problem of the different response factors of the derivatives when complex mixtures of oligomers are derivatized, as occurs with non-ionic surfactants (mainly fatty alcohol ethoxylates) and soluble synthetic polymers, is discussed. Other applications related to the biochemical, biomedical, pharmaceutical, nutritional and toxicological fields are also reviewed. The reactions, the criteria to be

applied to select the reagent, and the characteristics of the derivatives in relation to separation and detection, are discussed.

Keywords: *Acyl chlorides / Derivatization / Fatty alcohol ethoxylates / Hydroxyl functional groups/ Isocyanates / Organic anhydrides*

4.1. Introduction

Analyte derivatization is commonly used in HPLC and CE to enhance the detector response. Ideally, derivatization reactions should proceed rapidly and quantitatively under soft conditions, giving rise to derivatives exhibiting a large molar or mass response in the selected detector. Derivatization can be carried out either in the pre-column or post-column mode. Excellent discussions about the advantages and limitations of each approach can be found elsewhere [1]. Derivatization procedures mostly designed to be used in the pre-column mode, which is much simpler and less demanding than the post-column approach, are collected in this review.

Most frequently, derivatization is performed to add a chromophore or a fluorophore to the analyte molecule. The introduction of a charge has also become a fairly common resource to enhance the response at a mass spectrometer, being also useful to provide non-zero mobility in CE. The introduction of a charge is also a way of procuring an enhanced response for volatile analytes when a light-scattering or charged-aerosol evaporative detector is used. Several commercial autosamplers also provide an increased capability for mixing, heating and extracting, which makes pre-column derivatization much attractive. On the other hand, there are many compounds of industrial, environmental or biomedical interest with hydroxyl functional groups while lacking a chromophore or a charge, thus giving very low sensitivities in relation to UV-vis, fluorescence and APCI-MS and ESI-MS detection. Further, in many cases, mainly in the environmental and biomedical fields, the sensitivity given by the analyte at the selected detector is not enough to reach the very low limits of detection which are required. In these cases, sensitivity enhancement by derivatization can provide good solutions. Derivatization relies on the presence of a reactive group in the analyte molecule, including amino, hydroxyl (either aliphatic alcohol or phenol), ketone, aldehyde,

carboxylate, thiol and other groups. Excellent reviews covering the derivatization of these functional groups have been published along the last decade [2-6]. A wide variety of fluorogenic derivatization methods, mainly related to biomedical applications, were revised by Yamaguchi and Ishida [2]. In the review by Toyo'oka [3], the focus was on chiral drug resolution upon diastereomer formation with chiral derivatization reagents. In the reviews by Gao *et al.* [4], Santa [5] and Iwasaki *et al.* [6], the main topic was sensitivity enhancement for atmospheric pressure mass spectrometry detection, including APCI-MS and ESI-MS. The increase of chromatographic retention and efficiency upon derivatization was also discussed.

In this work, an overview with emphasis on the derivatization of analytes having a hydroxyl group, including mainly aliphatic alcohols but also phenols, while lacking other reactive groups, is given. Chromogenic and fluorogenic reactions and those addressed to enhance detection in APCI-MS and ESI-MS, and to enable separation in CE by introducing a charge in the analyte molecule, are covered. Industrial and environmental applications are mainly discussed; however, methods used to quantify analytes of biomedical interest are also included, since successful approaches in this field could be useful to develop industrial and environmental applications, and vice versa. Hydroxyl functional group derivatization has been frequently applied to the determination of fatty alcohol ethoxylates (FAE), since these non-ionic surfactants lack both a chromophore and a charge. Also, the low volatility of the oligomers with more than four ethylene oxide (EO) units prevents the use of GC. Derivatization of FAE and other related non-ionic surfactants, and soluble polymers as polyethyleneglycol (PEG), has been mainly applied to quality control of industrial raw materials and manufactured products, as well as to the evaluation of its impact on the aquatic environment. Thus, influents and effluents of sewage treatment plants, sewage sludge, and river

and sea water, have been also analyzed. On the other hand, toxic compounds, hormones, nutrients, drugs and other biologically active compounds have been analyzed in a wide variety of matrices, including urine, plasma, tissues, cosmetics and food.

The reagents have been classified by groups, including acyl chlorides (Section 4.3), organic anhydrides (Section 4.4), isocyanates (Section 4.5) and miscellaneous (Section 4.6), involving oxidation reactions for primary and secondary alcohols, sulfonation, esterification with carboxylic acids, and the use of azides, sulfonyl chlorides and other reagents having different leaving groups. The reagents used to quantify some analytes, followed by the separation and detection of the derivatives by HPLC or CE, are critically compared, and trends are indicated.

4.2. The problem of the response factors in compound classes constituted by complex mixtures of oligomers

In the characterization and determination of compound classes constituted by mixtures of oligomers (homologues or isomers), as occurs with surfactants, the variation of the response factors of the oligomers with the number or position of structural repetitive units or groups in the molecule is an important issue. This point will be discussed first, since it is common to all these compound classes, independently from the derivatization reaction to be used. The response factor of an oligomer is established as the sensitivity ratio with respect to a standard compound which is used as reference. The response factors could depend on both, the variations in the derivatization yield and differences of detection sensitivity. However, since the reactions commonly used in analysis are thoroughly quantitative, usually the response factors are mainly dependent on the differences of detection sensitivity among the oligomers and the reference compound. For

instance, in UV detection, the response factors will depend on the variations produced by the analyte residue on the electron density of the chromophore, and in MS they could depend on the variations of volatility, charge, or readiness to form adducts. In the case of ethoxylated surfactants, the response factor of the derivatizated oligomers of a given hydrocarbon series (same number of carbon atoms in the alkyl chain) varies mainly with the number of EO units of the oligomer, and in a lesser amount also with the length of the alkyl chain. However, these variations are strongly dependent on both the derivatizing reagent used and the detection technique.

When the derivatives show a narrow range of response factors, all the individual oligomers and the whole compound class can be quantified using a single compound as reference. For this purpose, a single oligomer, or even a mixture of oligomers with a known composition, can be used. When response factors are not needed, the evaluation of the oligomer distribution and the quantitation of the compound class is greatly simplified. Otherwise, when the response factors of the derivatives vary largely, either multi-oligomer calibration, or the application of previously tabulated response factors, is required. This will make unbiased quantification of the compound class to be long and tedious, as well as prone to accumulate systematic errors. In addition, with the exception of the light oligomers of the most common classes of compounds, heavy oligomers in a sufficiently purified form to be used as calibration standards will be expensive, and likely those oligomers belonging to less common compound classes (as alkyl ether sulfates, AES) will not be commercially available. In these cases, a solution could be the use of mixtures of known composition as standards, but in this case the oligomer distribution of both sample and standard should satisfactorily match to reduce the calibration systematic error as much as possible.

4.3. Acyl chlorides

Acyl chlorides are very reactive carboxylic acid derivatives frequently used to derivatize hydroxyl functional groups, and also to prepare acid anhydrides, esters and amides. Since the chloride group is an excellent leaving group, acyl chlorides react even with weak nucleophiles, such as aliphatic alcohols, in the presence of a base to give esters (**Fig. 4.1**). As shown in **Table 4.1**, acyl chlorides have been used to introduce a chromophore or fluorophore in a wide variety of compounds having an aliphatic alcohol or a phenol group.

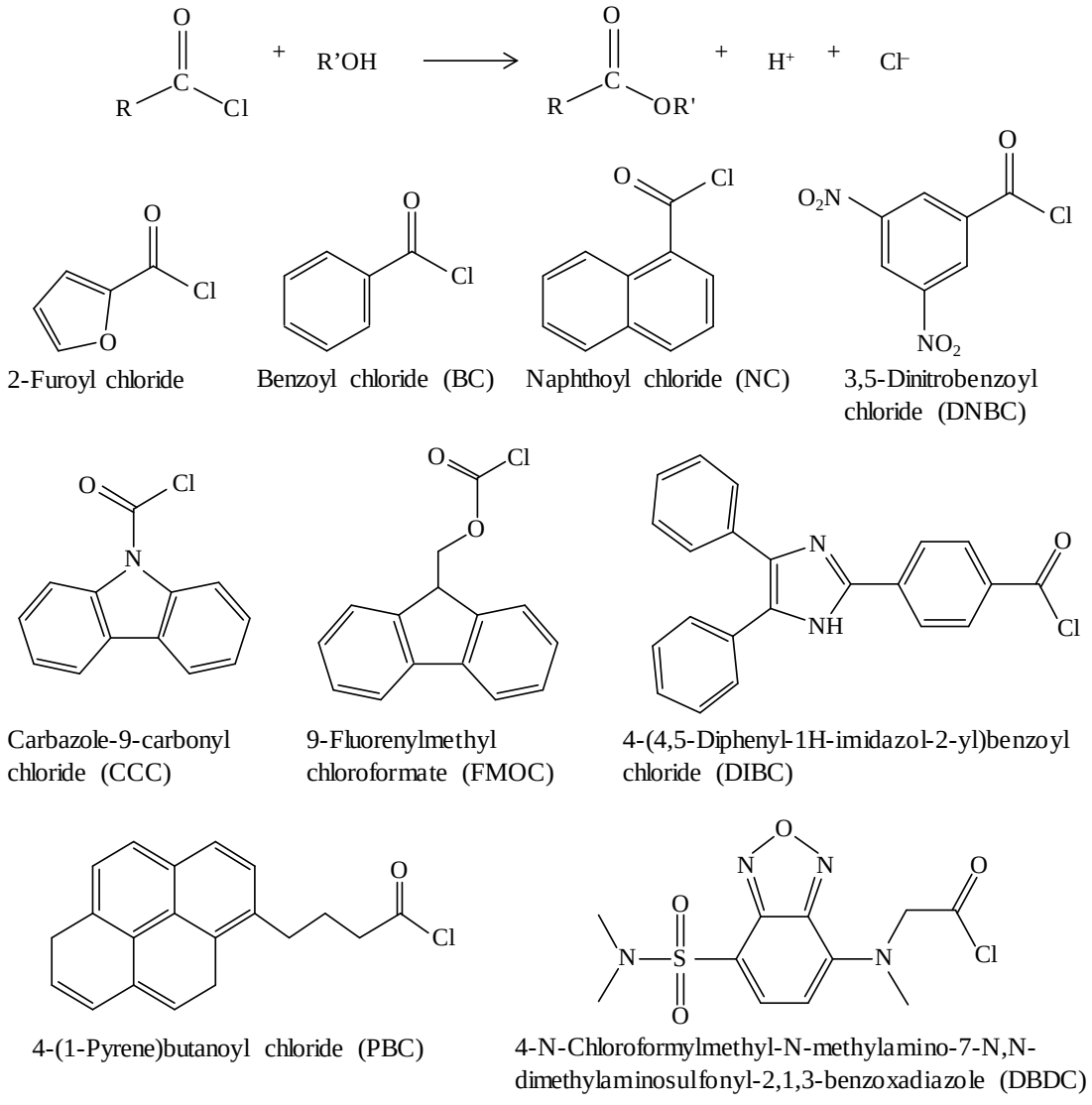

Fig. 4.1. Derivatization reaction of hydroxyl functional groups with acyl chlorides and chemical structures of the derivatizing reagents discussed in Section 4.3.

Table 4.1. Derivatization of hydroxyl functional groups with acyl chlorides, and working conditions for the separation and detection of the derivatives[a]

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation [b]	Samples	LOD	Ref.
2-Furoyl chloride	FAE, AES	ACN, pyridine	30 min, 60 ºC	HPLC-UV (254)	Raw materials	0.4 mmol	[7]
	Anionic surfactants	ACN, pyridine	30 min, 60 ºC	HPLC-UV (249)	Raw materials	5-7 μg L^{-1} [c]	[8]
Benzoyl chloride (BC)	Ethylene glycol	Water, NaOH	5 min, r.t.	HPLC-UV (237)	Serum	10-20 μg L^{-1} [c]	[18]
	FAE, PEG	ACN, pyridine	30 min, 80 ºC	HPLC-UV (228) HPLC-FL (ex228, em295)	Sewage sludge	-	[9]
	Propylene glycol	Pentane, NaOH	30 min, r.t.	HPLC-UV (237)	Plasma and tissues	1 mg L^{-1}	[18]
	Total glycerides	Hexane, NaOH	30 min, r.t.	HPLC-UV (230)	Plasma	2 μM	[20]
	Free glycerol	Hexane, NaOH	30 min, r.t.	HPLC-UV (230)	Plasma	0.2 μM	[20]
	Glycerol	Hexane, NaOH	4 h, 40 ºC	HPLC-UV (231)	Plasma and tissues	70 pmol	[21]
	Myo-inositol	Hexane, NaOH	5 h, 40 ºC	HPLC-UV (231)	Plasma and tissues	17.5 pmol	[21]
	Free choline	NaH_2PO_4, NaOH	5 min, r.t.	HPLC-UV (228)	Cell culture medium	10 μM	[22]
Naphthoyl chloride (NC)	FAE	-	-	HPLC-FL (ex300, em385)	Sewage sludge	5 μg L^{-1}	[13]
	PEG	-	-	HPLC-FL (ex228, em368)	Sewage sludge	10 μg L^{-1}	[13]
	FAE	ACN, 1-methylimidazole	30 min, 60 ºC	HPLC-FL (ex300, em385)	Sewage sludge	20-30 mg L^{-1}	[14]
	FAE	ACN, pyridine	20 min, r.t.	HPLC-FL (ex220, em383) HPLC-MS/MS	Enviromental water samples	0.10 mg L^{-1}, 0.09 μg L^{-1}	[15]
	FAE	ACN, 1-methylimidazole	30 min, 60 ºC	HPLC-MS	Waste water and sludge	0.3-1.3 ng	[17]
	Digoxin and metabolites	ACN, 4-dimethylaminopyridine	1 h, 50 ºC	HPLC-FL (ex217, em340)	Serum	-	[23]
	Oleandrin	ACN, 4-dimethylaminopyridine	2 h, 80 ºC	HPLC-FL (ex220, em345)	Gastrointestinal content	0.05 mg L^{-1}	[24]
	Steroideal alkaloids	ACN, thionyl chloride	45 min, 88 ºC	HPLC-UV (224)	*Fritillaria* bulbs	0.05 mg L^{-1}	[25]

[a] Acronyms: alkylether sulfates (AES), dichloromethane (DCM), fatty alcohol ethoxylates (FAE), polyehyleneglycol (PEG)

[b] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

[c] Limit of quantitation (LOQ)

Table 4.1. (Continued)

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation [b]	Samples	LOD	Ref.
3,5-Dinitrobenzoyl chloride (DNBC)	FAE	THF, pyridine	30 min, 65 ºC	HPLC-UV (254)	Raw materials	-	[12]
	FAE	Benzene, Mg	30 min, 80 ºC	HPLC-UV (254)	Crude oil	-	[10]
	PEG	DCM, Mg	30 min, 40-80 ºC	HPLC-UV (276)	Raw materials and textile finish	0.14 ng	[28]
	sn-1,2-Diacylglycerols	Pyridine	10 min, 60 ºC	HPLC-UV (254)	Human tissues	-	[29]
Carbazole-9-carbonyl chloride (CCC)	FAE	ACN, 1-methylimidazole	30 min, 65 ºC	HPLC-FL (ex228, em318)	Raw materials	-	[30]
9-Fluorenylmethyl chloroformate (FMOC)	Methanol, ethanol, propanol, butanol	ACN, Na_2HPO_4 pH 12.5	14 min, r.t.	HPLC-FL (ex259, em311)	Air	4-70 pmol	[31]
	FAE	ACN, pyridine	1 h, r.t.	HPLC-FL (ex260, em310)	Raw materials	-	[30]
	Erythromycin	ACN, phosphate buffer pH 7.5	1 h, 45 ºC	HPLC-FL (ex260, em305)	Animal origin food	25-50 mg kg^{-1}	[32]
	Oleandomycin	ACN, phosphate buffer pH 7.5	1 h, 45 ºC	HPLC-FL (ex260, em305)	Animal origin food	50-100 mg kg^{-1}	[32]
4-(4,5-Diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIBC)	Phenol	ACN, triethylamine	30 min, 60 ºC	HPLC-FL (ex340, em450)	Urine	0.2 pmol	[35]
	Cresols	ACN, triethylamine	30 min, 60 ºC	HPLC-FL (ex340, em450)	Urine	0.2-0.4 pmol	[35]
	Xylenols	ACN, triethylamine	30 min, 60 ºC	HPLC-FL (ex340, em450)	Urine	0.3-1.6 pmol	[35]
	Bisphenol A	ACN, triethylamine	20 min, r.t.	HPLC-FL(ex350, em475)	Rat brain	0.3 µg L^{-1}	[36]
	1- and 2-Naphthol	ACN, triethylamine	20 min, 40 ºC	HPLC-FL (ex350, em480)	Plasma	16-14 fmol	[37]
4-(1-Pyrene)butanoyl chloride (PBC)	Bisphenols	ACN, K_2CO_3	30 min	HPLC-FL (ex345, em475)	Water contained on a baby bottle	3-5 fmol	[33]
	Tyrosine	ACN, K_2CO_3	1 h, 60 ºC	HPLC-FL (ex345, em475)	Human urine	4.5 fmol	[34]
	Tyramine	ACN, K_2CO_3	1 h, 60 ºC	HPLC-FL (ex345, em475)	Human urine	2.6 fmol	[34]
4-N-Chloroformylmethyl-N-methylamino-7-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBDC)	Anandamide	ACN	2 h, 60 ºC	HPLC-FL (ex450, em560)	Rat Brain	10 fmol	[38]

[a] Acronyms: alkylether sulfates (AES), dichloromethane (DCM), fatty alcohol ethoxylates (FAE), polyehyleneglycol (PEG)

[b] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

[c] Limit of quantitation (LOQ)

Bachus and Stain [7] used 2-furoyl chloride to derivatize FAE. The procedure was also applied to the anionic surfactant class of AES, previous hydrolysis of the ester bond to yield the corresponding FAE and free inorganic sulfate. In comparison to other derivatizing reagents for FAE and AES, an advantage of 2-furoyl chloride was the small variation of the UV response factors of the derivatized oligomers. Using a C18 column and an ACN/water gradient, the 2-furoyl chloride derivatives of FAE oligomers were eluted in the order of increasing number of carbon atoms of the hydrocarbon series, with fairly good resolution between the successive oligomers within the series. The 2-furoyl chloride method was applied to the industrial surfactants Brij 30 and Brij 56. Morvan *et al.* [8] also used 2-furoyl chloride to derivatize FAE and, previous hydrolysis of the ester bond, also to AES and other anionic surfactant classes including alkyl sulfosuccinates, alkyl sulfoacetates and alkyl phosphates. Correction of the injected volume variations was achieved by using biphenyl as internal standard. To correct the hydrolysis, extraction and derivatization yields, nonyl sulfate was also used as internal standard. The two internal standards, which eluted at the beginning of the chromatogram, did not interfere.

Zanette *et al.* [9] studied benzoyl chloride (BC), 4-nitrobenzoyl chloride, 1-naphthoyl chloride (NC), 2-naphthoyl chloride and 1-naphthyl isocyanate (NIC, see Section 4.5) to evaluate the biodegradation of FAE and PEG in aqueous environments. The molar absorptivities were 2.3 and 2.0 times larger for the naphthyl derivatives (NIC gives carbamates instead of esters, see Section 4.5), than for the phenyl esters obtained with BC and 4-nitrobenzoyl chloride, respectively, also enabling fluorimetric detection. Further, the molar absorptivities of the derivatives obtained with NIC were a *ca.* 10% larger than those of the esters obtained with NC and 2-naphthoyl chloride. On the other hand, the fluorescence quantum yield of the NIC derivatives was *ca.* 1.7 times that obtained using NC and

2-naphthoyl chloride. The fluorescence quantum yields of the NIC and NC derivatives were independent of the EO number of the FAE oligomers, with the exception of the derivatives of the non-ethoxylated alcohols which exhibited slightly lower sensitivities.

Separation of the NC derivatives of FAE oligomers was studied using a C18 stationary phase. With an ACN/water gradient, the oligomers within the hydrocarbon series were resolved, whereas a MeOH/water gradient led to the isolation of the consecutive FAE series without separation of the oligomers differing in the number of EO units within the series [9]. This behavior is common to the FAE derivatives obtained with several derivatization reagents, including acyl chlorides and organic anhydrides [10-12]. The advantage obtained with a MeOH/water gradient is the possibility of quantifying the surfactant, including the relative percentages of the hydrocarbon series, without overlapping of peaks of oligomers belonging to different series. Further, using a MeOH/water gradient only a single peak per hydrocarbon series should be integrated; however, as commented in Section 4.2, a systematic error arises if the response factors of the derivatives vary with the EO number of the oligomer. Derivatization with NC and NIC followed by HPLC-UV and HPLC-FL was also applied to the determination of PEG [9]. The fluorescence quantum yield was very low for $n < 3$, and increased at higher polymerization numbers, at least up to $n = 600$. The fate of FAE and PEG oligomers during activated sludge sewage treatment was investigated using derivatization with NC followed by HPLC-FL [13]. This method was used to monitor FAE in sludge samples from several European countries [14]. Zgola-Grześkowiak and Grześkowiak [15] also used NC to derivatize FAE followed by either HPLC-FL or HPLC-MS. Derivatization with NC followed by HPLC-FL has been also used to determine polypropylene glycol homologues (PPG) in environmental samples [16]. Cassani *et al.* [17] have compared three approaches

to detect FAE in waste water treatment plant influents and effluents and sludge using RP-HPLC-MS, i.e. in the ion-positive mode as Na^+ adducts of underivatized FAE, in the ion-negative mode previous sulfation to convert FAE into AES, and as NC derivatives.

Derivatization with acyl chlorides has been also applied to the determination of light glycols, glycerol and other compounds of toxicological, pharmaceutical and biomedical interest. Ethylene glycol is a toxic liquid, widely used as antifreeze, having the risk of being accidentally consumed due to its sweet taste. Thus, the rapid detection of ethylene glycol in serum is important in defining appropriate clinical management. Ethylene glycol in serum was determined by derivatization with BC and HPLC-UV [18]. 1,3-Propanediol was used as internal standard, and no interference by other common glycols and alcohols was observed. Propylene glycol (1,2-propanediol), which is used as an excipient for medications as well as in personal care products, was determined in plasma after derivatization with BC followed by HPLC-UV [19]. In this method, ethylene glycol was used as internal standard. BC has been also used to derivatize glycerol in serum [20]. Glycerol was not quantitatively derivatized; however, *ca.* 100% recoveries were achieved using 1,2,4-butanetriol as internal standard. This method was extended to the determination of total lipids (previous hydrolysis to fatty acids and glycerol), *myo*-inositol and other polyhydroxy compounds in plasma and tissues [21]. Phloroglucinol was used as internal standard. The determination of free choline in bacterial culture media was accomplished using BC derivatization followed by RP-HPLC [22].

As indicated, in comparison to BC, naphthoyl chloride (NC) has the advantages of a higher molar absorptivity of the derivatives and the possibility of using fluorimetric detection, which is of particular interest in the determination of compounds present in very low concentrations in biological matrices. Thus, NC

followed by HPLC-FL has been used to determine phytosteroids and steroidal glycosides. Steroidal glycosides, including digoxin (a cardiac stimulant) and its metabolites in serum [23], and oleandrin (a toxic steroidal glycoside found in *Nerium oleander L.*) in gastrointestinal contents of cattle [24], have been determined by HPLC-FL previous derivatization with NC. Veticine and other alkaloids of bulbs of *Fritillaria* were determined by derivatization with NC followed by either HPLC-UV or HPLC-MS [25].

Nozawa and Ohnuma [12] introduced 3,5-dinitrobenzoyl chloride (DNBC) to derivatize FAE previous to HPLC-UV. Derivatization with DNBC has been also used for the determination of FAE in products employed to enhance mineral oil recovery from reservoir rocks [26]. An advantage of DNBC is a similar response factor for the derivatized FAE oligomers, independently from the alkyl chain length and number of EO units [10]. DNBC derivatives of FAE oligomers were also separated using 2D-HPLC [27]. DNBC has been used to derivatize PEG [28] and 1,2-diacylglycerols [29]. The DNBC derivatives of PEG oligomers up to 0.4 kDa (*ca.* 9 EO units) were separated using RP-HPLC-UV on a C8 column, whereas derivatives up to 2 kDa (*ca.* 45 EO units) were separated using NP-HPLC-UV on an amino-propyl-silica column. DNBC was also used to analyze PEG in textile finishers [28]. Derivatization with DNBC followed by NP-HPLC-UV was used to determine 1,2-diacylglycerols in tissues (muscle) [29] using a silica column and a cyclohexane/diethyl ether/ethanol mobile phase.

Meissner and Engelhardt [30] described the use of carbazole-9-carbonyl chloride (CCC) and fluorenylmethyloxycarbonyl chloride (FMOC) for the fluorogenic derivatization of FAE, followed by RP-HPLC-FL. The sensitivity obtained with CCC was twice that obtained with FMOC or NC, but the CCC excess led to a broad and strongly tailing reagent peak which hindered trace determination of FAE. This problem was overcome by removing the reagent excess by SPE.

Huang et al. [31] reported the use of FMOC to derivatize low-molecular-weight aliphatic alcohols, and applied the method to the determination of methanol in laboratory air and ethanol content in beer. Derivatization with FMOC followed by RP-HPLC-FL was used in the determination of erythromycin and oleandomycin (antibiotics against a wide range of Gram-positive bacteria) in food of animal origin including meat, liver, kidney, raw milk and egg [32]. The determination of these two antibiotics demonstrated that FMOC was useful not only as a labeling reagent for amino groups, but also for hydroxyl groups.

Yoshida *et al.* [33] developed a novel fluorimetric method for the determination of bisphenols based on derivatization with 4-(1-pyrene)butanoyl chloride (PBC). The reagent labeled the two hydroxyl groups of bisphenols forming an intramolecular excimer (excited dimer). The migration of bisphenol A, a component of polycarbonate and other plastic materials also exhibiting hormone-like properties, from baby bottles to water was evaluated. Also, PBC was used for the determination of tyrosine and related compounds in urine by HPLC-FL [34]. The LC-MS analysis of the derivatization reaction products confirmed that PBC labeled both the amino and the hydroxyl functional groups of tyrosine, as also occurs with FMOC [32].

Nakashima *et al.* [35] analyzed phenol, cresols and xylenols in human urine using derivatization with 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIBC) followed by HPLC-FL. Derivatization with DIBC was also used to determine bisphenol A in microdialysis samples of rat brain with a column switching HPLC setup consisting of dual C_{18} semi-microcolumns [36]. Ohyama *et al.* [37] used DIBC to label 1-naphthol and 2-naphthol, followed by HPLC-FL. These substances, which are metabolites of naphthalene, were analyzed in plasma. The derivatives were isocratically separated with ACN/water on a C18 column. Finally, Arai *et al.* [38] used 4-N-chloroformylmethyl-N-methylamino-7-N,N-

dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBDC) to determine anandamide, an endogenous cannabinoid neurotransmitter, in rat brain. The determination was performed using coupled-column RP-HPLC-FL.

4.4. Organic anhydrides

Organic anhydrides contain two acyl groups bonded to the same oxygen atom. As shown in **Fig. 4.2**, organic anhydrides react with alcohols to afford an ester. Usually a basic compound as pyridine or imidazol is added to speed up esterification. Symmetric anhydrides should be used for analytical purposes, since asymmetric anhydrides give rise to two different esters per analyte. Hemiesters with an ionizable carboxylate group are obtained by using cyclic anhydrides, whereas non-cyclic anhydrides yield uncharged derivatives. Then, the correct choice for analysis is usually a symmetric cyclic anhydride. However, symmetric aliphatic anhydrides, as propionic anhydride can be conveniently used for HPLC-MS, with the advantage of the increased volatility of the uncharged derivatives. For UV detection, an aromatic anhydride should be preferred; however, maleic anhydride which provides two carbonyls conjugated through a double bond in the middle has been also used. In comparison to the use of aromatic anhydrides, maleic anhydride yields derivatives with lower molar absorptivities. Benzoic anhydride, which is symmetric but not cyclic, is of interest for both HPLC-MS and HPLC-UV [39]. Further, benzoic anhydride, which gives derivatives lacking a ionizable carboxylate group, could be of interest to derivatize highly polar analytes which are weakly retained on reversed phase stationary phases. However, the solubility of the benzoic anhydride derivatives in water rich mobile phases will be lower than that of the corresponding phthalic anhydride derivatives. On the other hand, cyclic anhydrides are frequently used to introduce a ionizable carboxylic acid group in the analyte molecule, as required to gain sensitivity in HPLC-ESI-MS [40], and to

enable electrophoretic mobility for CZE separation [41]. To increase retention of the resulting hemiesters on reversed phase stationary phases by protonation of the carboxylate group, as well as to suppress ionization of the silanol groups of the stationary phase, a small concentration of acetic acid or any other weak acid, is usually added to the mobile phase.

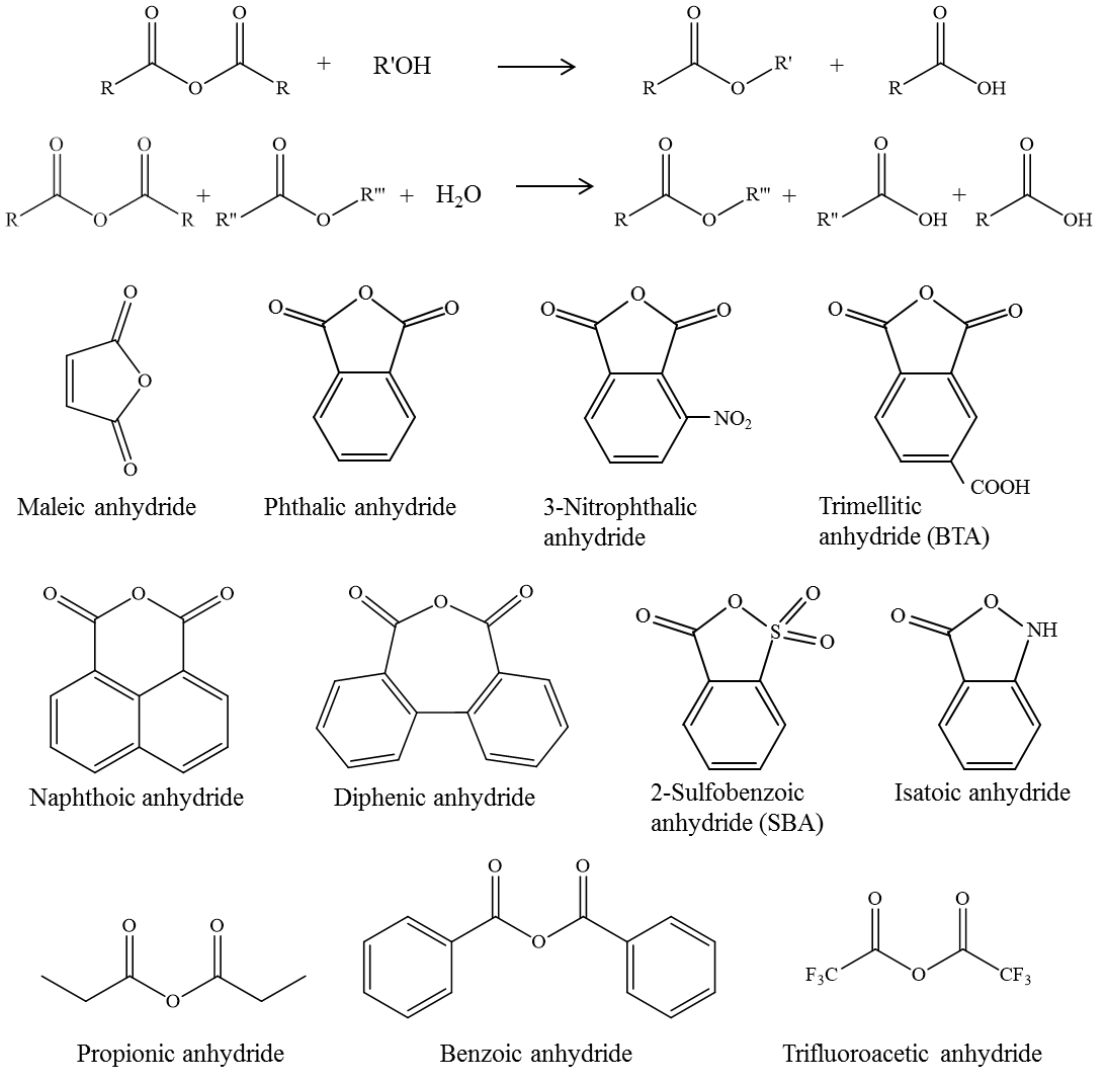

Fig. 4.2. Derivatization reaction of hydroxyl functional groups with organic anhydrides and chemical structures of the derivatizing reagents discussed in Section 4.4.

Symmetric anhydrides react also with esters to yield a new ester and a carboxylic acid (two esters per analyte and two carboxylic acids are obtained with an asymmetric anhydride). Thus, transesterification with symmetric anhydrides is

useful for the chromogenic or fluorogenic derivatization of esters, but may interfere in the determination of alcohols. In the frequent case where a sample contains both alcohols and esters, these two functional groups will be jointly derivatized by the anhydride. Even if all the water is carefully removed from the sample, esterification of the alcohols will provide enough water to promote partial or total transesterification of the esters. In the case of esters containing the same alcohol residue than the free alcohols present in the sample, the derivatives coming from the alcohols and the corresponding esters will be indistinguishable. This is a frequent case in many fields, including surfactants [42], essential oils [43] and cosmetics [44]. If the sample contains sufficient water, or more hydroxyl functional groups than the corresponding ester groups, the peak areas will be proportional to the sum of both functional groups. Therefore in these cases, the separation of the alcohols and the corresponding esters previous to derivatization with an anhydride is necessary to separately quantifying the analytes. When the esters have a charge, as occurs with AES, separation can be carried out by ion-exchange on SPE cartridges [42]. The joint quantification of alcohols and the corresponding esters is not a problem exclusive to derivatization with organic anhydrides. Other derivatization reagents, as acyl chlorides, also yield derivatives with the alcohol residue of the esters if water is present in the sample.

Table 4.2. Derivatization of hydroxyl functional groups with organic anhydrides, and working conditions for the separation and detection of the derivatives[a]

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation [b]	Samples	LOD	Ref.
Maleic anhydride	FAE	Direct melting	90 min, 120 ºC	CZE-UV (214)	Raw materials	-	[58]
	FAE	1,4-Dioxane, urea	15 min, 80 ºC	HPLC-UV (200)	Raw materials, cleaners, river and sea waters	1.3 μM	[48,59]
Phthalic anhydride	FAE	ACN	8 h, 100 ºC	CZE-UV (200)	Laundry cleaner and shower gel	-	[57]
	FAE	Direct melting	90 min, 120 ºC	CZE-UV (214)	Raw materials	-	[58]
	FAE	Pyridine	1 h, 85 ºC	HPLC-ESI-MS	Sewage influent and effluent	0.02 μg L^{-1}	[40]
	FAE	1,4-Dioxane, urea	1 h, 110 ºC	HPLC-UV (200, 230)	River and sea water	0.1-0.2 μM	[42,60]
	1-Octanol, 1-decanol and 2-octanol	Pyridine, imidazole	30 min, 100 ºC	HPLC-UV (230)	Standards	-	[55]
	PEG	Pyridine, imidazole	1 h, 100 ºC	CGE-UV (275, 280)	Raw materials	-	[41]
	PEG	Pyridine, imidazole	45 min, 85 ºC	CZE-UV (205)	Raw materials	-	[56]
	PEG	Pyridine, imidazole	16 h, 95 ºC	CZE-UV (220)	Raw materials	-	[46]
	Glycerin-based polyols	ACN, 1,4-diazabycyclo[2,2,2] octane, imidazole	30 min, 100 ºC	CZE-UV (220)	Raw materials	-	[61]

[a] Acronyms: adenosin diphosphate (ADP), adenosin monophosphate (AMP), adenosin triphosphate (ATP), decision limit (CCα), fatty alcohol ethoxylates (FAE), polyehyleneglycol (PEG), polypropyleneglycol (PPG)

[b] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

Table 4.2. (Continued)

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation [b]	Samples	LOD	Ref.
Diphenic anhydride	FAE	1,4-Dioxane, urea	90 min, 105 °C	HPLC-UV (220)	Wastewater and sea water	0.07-0.1 μM	[48,62]
	Linear aliphatic alcohols ($18 \leq NC \leq 30$), terpene alcohols and 4-methylsterols	THF, urea	120 min, 60 °C	HPLC-UV (200) HPLC-MS	Vegetable oils	-	[63]
1,2,4-Benzenetricarboxylic anhydride (BTA)	PEG	THF	5 h, 100 °C	CZE (210)	Raw materials	-	[45]
	PEG, PPG	THF	16 h, 95 °C	CZE (220)	Raw materials	-	[46]
2-Sulfobenzoic anhydride (SBA)	FAE	ACN, 4-dimethylaminopyridine	1 h, r.t.; 2 h, 80 °C	HPLC-ESI-MS	Raw materials	-	[49]
Propionic anhydride	Cytokinins, adenosin, AMP, ADP and ATP	ACN, N-methylimidazole	30 min, 37 °C	HPLC-ESI-MS	*Arabidopsis thaliana*	0.17-110 fmol	[39]
Benzoic anhydride	Adenosin, AMP, ADP and ATP	ACN, N-methylimidazole	30 min, 37 °C	HPLC-ESI-MS	*Arabidopsis thaliana*	35-14000 fmol	[39]
Trifluoroacetic acid anhydride	Endectocides	ACN, 1-methylimidazole, triethylamine	30 min, 70 °C	HPLC-FL (ex365, em470)	Milk	0.1 $\mu g\ kg^{-1}$	[65]
	Endectocides	ACN, 1-methylimidazole, triethylamine	50 min, 65 °C	HPLC-FL (ex364, em470)	Milk	0.1-50.6 $\mu g\ kg^{-1}$ ($CC\alpha$)	[66]

[a] Acronyms: adenosin diphosphate (ADP), adenosin monophosphate (AMP), adenosin triphosphate (ATP), decision limit ($CC\alpha$), fatty alcohol ethoxylates (FAE), polyehyleneglycol (PEG), polypropyleneglycol (PPG)

[b] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

Concerning to the methods summarized in Table 4.2, and as could be expected, two derivatives per analyte were obtained by using the asymmetric 1,2,4-benzenetricarboxylic anhydride (BTA or trimellitic anhydride) [45]. However, BTA was used to derivatize PEG followed by CZE. Oudhoff et al. [46] also used BTA for the determination of the monomer-number distribution in PEG and PPG. Another asymmetric cyclic anhydride, 3-nitrophtalic anhydride, was used to determine farnesol and geranylgeraniol in rat liver and testis [47]. In this case, owing to the electron-withdrawing inductive effect of the nitro group, esterification was produced mainly at the 2-position (next to the nitro group). However, Micó-Tormos et al. [48] also tried 3-nitrophthalic anhydride to esterify 1-dodecanol, but two peaks showing a ca. 30:70 area ratio were obtained. Exceptions to the symmetry rule are 2-sulfobenzoic anhydride (SBA), isatoic anhydride and N-methyl isatoic anhydride. These reagents are asymmetric, but the ester can be formed exclusively through the carbonyl side, giving rise to a single derivative per analyte. Thus, Zu et al. [49] developed an HPLC-MS procedure for the characterization of commercial FAE with precolumn derivatization with SBA. Also, in the determination of citokinins, a class of substances that promote cell division in plants, isatoic anhydride reacted with the hydroxyl groups of ribose to yield fluorescent anthraniloyl derivatives [50]. Isatoic and N-methyl isatoic anhydrides have been also used to derivatize saccharides [51] and peptides [52], respectively.

Phthalic anhydride, classically used to determine hydroxyl compounds by titration after derivatization [53,54], has been also extensively applied to precolumn derivatization for HPLC and CZE analysis. Primary and secondary aliphatic alcohols were determined by HPLC-UV after derivatization with phthalic anhydride [55], and to evaluate the oligomer distributions of FAE and PEG by capillary gel electrophoresis (CGE) [41] and CZE [46,56,57]. FAE were also

derivatized with maleic and phthalic anhydrides, and the derivatives were separated by CZE [58]. The method included a previous rinsing of the capillary with a quaternary diammonium salt to provide effective EOF control. The reaction rates and derivatization yields of FAE with maleic anhydride increased largely by adding grinded urea to the reaction mixture [59]. The procedure was tolerant to the presence of relatively large amounts of water. Grinded urea also showed a moderate positive influence on the reaction rate for the derivatization of FAE with phthalic anhydride [60]. Phthalic anhydride derivatization was also used to determine the degree of polymerization and polydispersity of glycerin-based polyols by CZE [61]. Sparham *et al.* [40] derivatized FAE in sewage influent and effluent samples with phthalic anhydride, followed by HPLC-ESI-MS. The derivatization of FAE with maleic, phthalic and diphenic anhydrides followed by HPLC-UV were compared by Micó *et al.* [48]. Diphenate derivatives provided significantly higher sensitivity than that achieved with phthalates, and much higher when compared to maleates. The procedure was applied to raw materials, cleaning products, sewage effluents, and river and sea water. Naphthoic anhydride was also tried, but the FAE derivatives, which were rapidly formed, could not be dissolved in water, nor in any of the hydroorganic mixtures and hydrophobic solvents tried. This was attributed to the presence of both the highly hydrophobic naphthyl group and the hydrophilic carboxylate group of the hemiesters in the molecular structure of the derivatives. Diphenic anhydride was also used to characterize FAE using a novel single-pumped 2D-HPLC system [62]. Lerma-García *et al.* [63] used diphenic anhydride to derivatize the alcoholic fraction of vegetable oils. Separation and identification of the derivatives by both HPLC-UV and HPLC-MS, followed by linear discriminant analysis (LDA) of the chromatographic data allowed the classification of vegetable oils according to their botanical origin.

The derivatives of compound classes containing an EO chain, as FAE and AES, also show the properties of the parent underivatized oligomers. These include an increased polarity at decreasing temperatures, a slightly higher polarity of the oligomers having an odd number of EO units with respect to those having an even EO number, and an enhanced polarity of the first oligomers of a hydrocarbon series, i.e. those with less than three EO units, with respect to the oligomers with at least three EO units [60,64]. The last effect is due to coiling of the EO chain by formation of intramolecular bonds between the EO units, which increases the hydrophobicity of the oligomers with three or more EO units. Coiling of the EO chain in the hydroorganic mobile phases is not possible with one or two EO units, which enhances the polarity and hydrophilicity of the light oligomers of the hydrocarbon series. For this reason, when a hydrocarbon series of derivatized FAE or AES is eluted in RP-HPLC, the peaks of the two or three first members of the series overlap with those of other oligomers of the same series. The relative retention of the peaks of the lighter oligomers with respect to the heavier oligomers of the same series varies with the column temperature, and also with the nature of the derivatizing agent. Almost perfectly resolved series, well ordered according to the EO number, were obtained in RP-HPLC by using diphenic anhydride derivatives and low column temperatures [62].

Propionic anhydride and benzoic anhydride have been used to esterify the hydroxyl group of cytokinins and other biochemically important compounds such as AMP, ADP and ATP, in *Arabidopsis thaliana* [39]. Derivatization reduced the polarity range of the compounds, making them more hydrophobic, which allowed their separation in a single chromatogram, also improving the ESI-MS response. A rather different approach was developed by Berendsen *et al.* [65] to derivatize endectocides, which are important antihelmitic drugs, including avermectins and milbemycins. These compounds bear a tetrahydrofuran ring with two hydroxyl

substituents which were derivatized in two steps. Trifluoroacetic anhydride and 1-methylimidazole were used first to esterify the hydroxyl functional groups. In a second step, a strong base as triethylamine was used to cleave the ester bonds and form fluorescent derivatives bearing a phenyl group. This procedure, followed by RP-HPLC-FL, was applied to the determination of endectocides in milk [65,66].

4.5. Alkyl and aryl isocyanates

Isocyanates (R–N=C=O, where R can be either an alkyl or aryl residue) are derivatives of isocyanic acid. Isocyanates are extremely reactive towards a variety of nucleophiles including alcohols, amines and water. Their reactions typically involve an attack on the carbon atom of the isocyanate group by the nucleophile to form urethanes or “carbamates” (**Fig. 4.3**). As shown in **Table 4.3**, analytical methods making use of both alkyl and aryl isocyanates to derivatize hydroxyl functional groups have been described.

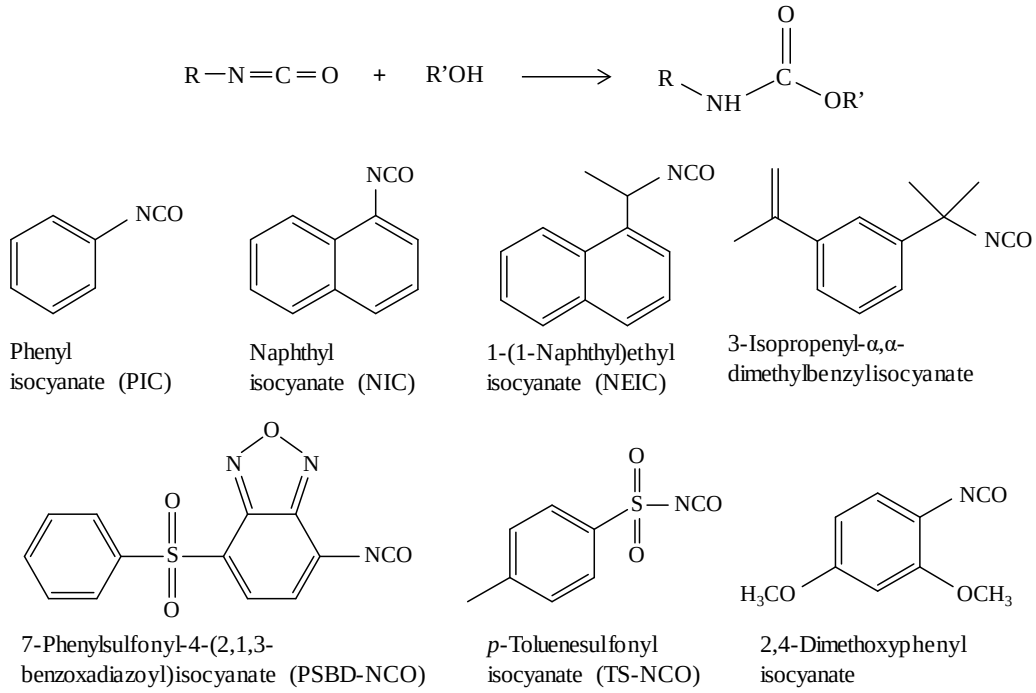

Fig. 4.3. Derivatization reaction of hydroxyl functional groups with isocyanates and chemical structures of the derivatizing reagents discussed in Section 4.5.

Table 4.3. Derivatization of hydroxyl functional groups with isocyanates, and working conditions for the separation and detection of the derivatives[a]

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation [b]	Samples	LOD	Ref.
Phenyl isocyanate (PIC)	FAE	DCM	45 min, 55 ºC	HPLC-UV (235)	Wastewater	3 µg L^{-1}	[67]
	FAE	ACN	60 s, microwave	HPLC-UV (235)	Standard solutions	-	[68]
	Aliphatic *n*-alcohols	ACN	60 s, microwave	HPLC-UV (235)	Standard solutions	-	[68]
	PEG	ACN	2 h, 50 ºC	HPLC-UV (235)	River water	0.002-0.019 µg mL^{-1}	[70]
	Irganox 1076	ACN	12-15 h, 50 ºC	HPLC-UV (230)	Plastic polymers	0.4-3 ng	[71]
	1,2-*sn*- and 2,3-*sn*-Diacylglycerols	DCM, pyridine	24 h, 55 ºC	HPLC-UV (254)	Lipase hydrolyzates	-	[72]
Naphtyl isocyanate (NIC)	FAE	ACN	30 min, r.t.	HPLC-UV (222)	Raw materials	0.001%	[73]
	PEG	ACN	2 h, 60 ºC	HPLC-FL (ex232, em358)	Raw materials	-	[75]
	PPG	ACN	20 min, 35 ºC	HPLC-FL (ex225, em362)	River water	0.001-0.010 µg mL^{-1}	[70,76]
	2-Ethoxyethanol	Acetone	3 h, r.t.	HPLC-UV (230)	Cosmetic formulations	0.6-7.6 µg mL^{-1}	[77]
	Choline	ACN, MgO	15 min, r.t.	HPLC-FL (ex220, em350)	Human plasma	0.5 µmol L^{-1}	[78]
1-(1-Naphthyl)ethyl isocyanate (NEIC)	Diacyl-*sn*-glycerols	Toluene, 4-pyrrolidine	Overnight, 50 ºC	HPLC-UV (280)	Maize oil	-	[79]
	Eliprodil	DCM	40 min, 70 ºC	HPLC-FL (ex275, em336)	Plasma and urine	0.15-50 ng mL^{-1}	[80]
3-Isopropenyl-α,α-dimethylbenzylisocyanate	2-Propanol,	DCM, trifluoroacetic acid	5 h, r.t.	HPLC-UV (201)	Standard solutions	-	[81]
	1-Naphthol, 2-isopropoxyphenol	DCM, triethylamine	5 h, r.t.	HPLC-UV (201)	Standard solutions	-	[81]
2,4-Dimethoxyphenylisocyanate	2-Propanol	DCM, trifluoroacetic acid	5 h, r.t.	HPLC-UV (201)	Standard solutions	-	[81]
	1-Naphthol, 2-isopropoxyphenol	DCM, triethylamine	5 h, r.t.	HPLC-UV (201)	Standard solutions	-	[81]
7-Phenylsulfonyl-4-(2,1,3-benzoxadiazoyl) isocyanate (PSBD-NCO)	Aliphatic *n*-alcohols	ACN	4 h, 60 ºC	HPLC-FL (ex368, em490)	Standard solutions	5.6-10.7 fmol [b]	[82]
p-Toluenesulfonyl isocyanate (TS-NCO)	3α-Hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel	ACN	2 min, r.t.	HPLC-ESI-MS	Plasma	20 pg mL^{-1}	[83]
	Diethylene glycol and polypropylene glycol	ACN	10 min, r.t.	HPLC-UV (227)	Drugs	0.019-0.022 µg mL^{-1}	[84]

[a] Acronyms: dichloromethane (DCM), fatty alcohol ethoxylates (FAE), polyehyleneglycol (PEG), polypropyleneglycol (PPG)

[a] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

[b] Limit of quantitation (LOQ)

Derivatization with phenyl isocyanate (PIC) followed by RP-HPLC-UV was used to determine FAE in effluents of sewage treatment plants [67]. Chavez et al. [68] investigated the use of microwave irradiation to speed up the off-line and on-line derivatization of both FAE and light aliphatic alcohols up to 12 carbon atoms with either PIC or BC. Pre-column off-line derivatizations were completed in 2 min. Also, using a flow-reactor located in a 450 W microwave oven, derivatization was completed in 45 s. The derivatives were separated on C8 and C18 stationary phases with MeOH/water [68]. The use of microwave irradiation to derivatize FAE with PIC was also studied by Arias et al. [69]. Irradiation at 600 W for 30 s was used, and the derivatives were separated by non-aqueous capillary electrophoresis (NACE). In biodegradation studies, PEG was isolated from river water matrices, derivatized with PIC, and the derivatives were separated on a C18 column with a MeOH/water mobile phase [70]. Baillet et al. [71] also used derivatization with PIC and HPLC-UV for the determination of phenolic antioxidants in synthetic polymers. Although the analytes absorb in the UV, derivatization improved the limits of detection in a factor of 5-10 times. Derivatization with either PIC or 4-nitrophenyl isocyanate (NPIC) was used for the chiral analysis of 1,2-sn- and 2,3-sn-diacylglycerol enantiomers [72]; using HPLC-UV with a chiral stationary phase and n-hexane/ethanol as mobile phase, the stereopreferences of triacylglycerol lipases were investigated.

Derivatization with NIC followed by RP-HPLC-UV was used for the determination of trace amounts of *n*-octanol, *n*-decanol and *n*-dodecanol in industrial alkyl sulfates [73]. Phenyl and 2-anthryl isocyanates were also tried for derivatization, but NIC, which showed the best balance between reaction rate and molar absorptivity of the derivatives, was preferred. NIC was also applied to the derivatization of PEG, and the derivatives were separated by RP-HPLC-UV [74]. The NIC derivatives of PEG 600, PEG 1000 and PEG 3000 were separated by

Rissler *et al*. [75] using gradient elution with ACN-THF-water mobile phases on bare silica columns. This unusual pseudo-reversed-phase separation yielded better efficiencies than the common alkyl-bonded stationary phases. Biodegradation of PPG was monitored by RP-HPLC-FL and RP-HPLC-MS after derivatization with NIC [70,76]. 2-Ethoxyethanol, which is potentially hazardous to humans, was analyzed in cosmetics by derivatization with NIC followed by RP-HPLC-UV [77]. NIC was also used to determine free choline in plasma. Dry magnesium oxide was added to the reaction mixture to remove most of the water. Since the choline molecule has a permanent positive charge on the quaternary ammonium group, a cationic derivative, 2-(1-naphthylurethane)-ethyltrimethylammonium, was obtained. This derivative was separated by HPLC on an ion-exchange cationic column. The mobile phase contained 10 mM tetramethyl ammonium hydroxide, 20 mM glycolic acid and 15 % water in ACN [78].

The use of chiral isocyanates as derivatizating agents has been also investigated. Laakso and Christie [79] used (*R*)- or (*S*)- forms of 1-(1-naphthyl)ethyl isocyanate (NEIC) to derivatize diacyl-*sn*-glycerols. Separation of the diastereomeric diacylglycerol derivatives was carried out by NP-HPLC-UV on a silica gel column using a 2-propanol/hexane mobile phase. (*S*)-(+)-NEIC was also used to derivatize (*S*)-(+)- and (R)-(-)-eliprodil, a neuroprotector drug [80]. The analytes were first preconcentrated using on-line clean-up of the plasma and urine samples, then the diastereoisomeric derivatives were formed and separated by HPLC-FL with column switching. As indicated in Section 1, more about chiral derivatization reagents can be found in the review by Toyo'oka [3].

3-Isopropenyl-α,α-dimethylbenzylisocyanate and 2,4-dimethoxy phenylisocyanate were synthetized by Vandenabeele-Trambouze *et al*. [81], in an attempt to find universal reagents to derivatize nucleophilic pollutants including alcohols, phenols, amines, thiols and oximes; however, the derivatization yield of

alcohols, phenols and thiols decreased largely as the analyte concentration decreased. Uchiyama *et al*. [82] designed fluorogenic reagents with the benzofuran skeleton in order to derivatize linear *n*-alcohols previous to RP-HPLC-FL. Among them, 7-phenylsulfonyl-4-(2,1,3-benzoxadiazoyl) isocyanate (PSBD-NCO) gave the highest quantum yield, and thus it was recommended to determine biologically important alcohols, including steroids and prostaglandins.

p-Toluenesulfonyl isocyanate (TS-NCO) was successfully used by Zuo *et al*. [83] to derivatize hydroxyl functional groups. An advantage of this reagent is the enhanced reactivity towards nucleophiles, which is due to the polar sulfonyl group next to the isocyanate group. The reagent was used to derivatize two pharmacologically active 3-hydroxy metabolites of tibolone, a synthetic steroid used in the hormonal replacement therapy for postmenopause. Separation was achieved on a C18 stationary phase, using gradient elution with MeOH/ aqueous ammonium acetate. TS-NCO was also used by Zhou *et al*. [84] to derivatize diethylene glycol and propylene glycol in drugs, prior to HPLC-UV.

4.6. Miscellaneous derivatization reactions

In this Section, alternatives to the use of acyl chlorides, organic anhydrides and isocyanates to derivatize hydroxyl functional groups for analysis are presented. The reagents and the reaction schemes are given in **Fig. 4.4**, and the related analytical methods are summarized in **Table 4.4**.

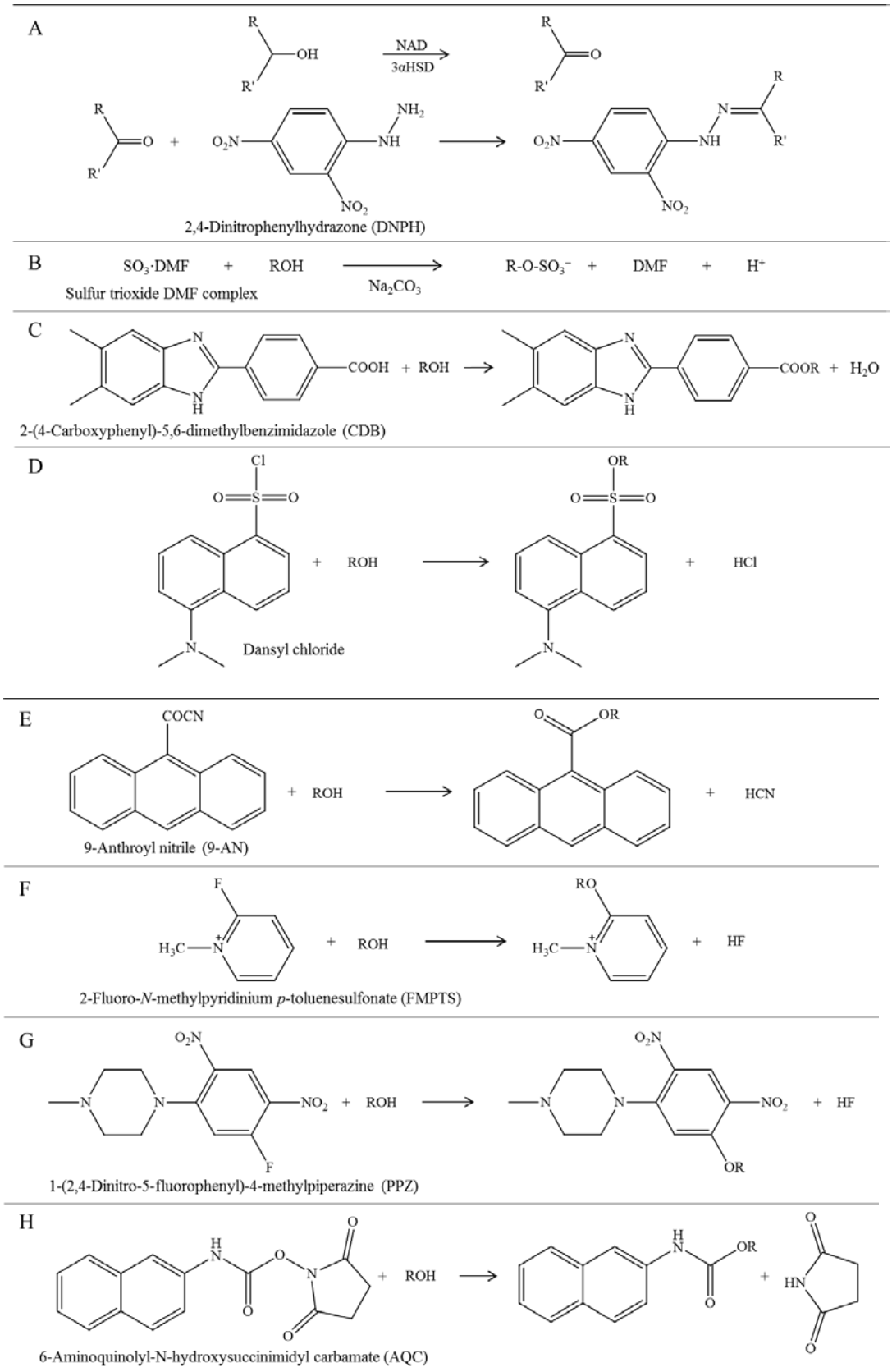

Fig. 4.4. Derrivatization of hydroxyl functional groups with the miscellaneous reactions discussed in Section 4.6.

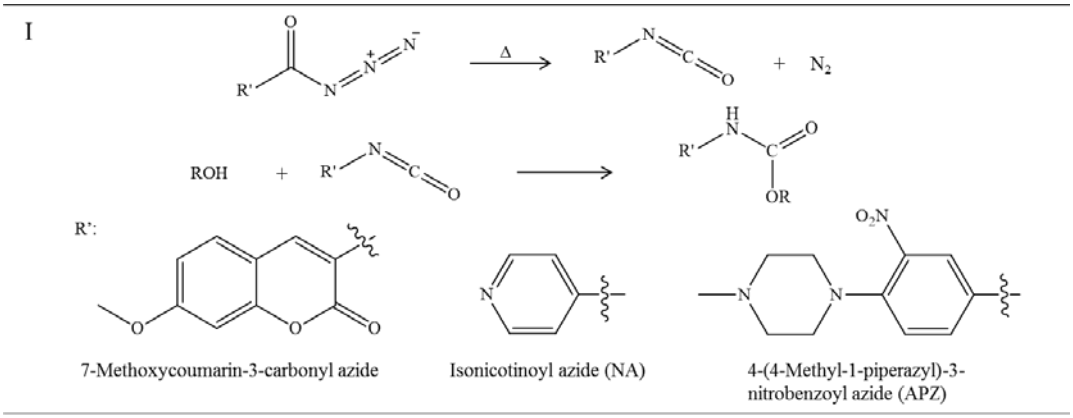

Fig. 4.4. (Continued)

4.6.1. Oxidation

Beneito *et al.* [85] oxidized primary aliphatic alcohols and FAE dissolved in acetone with a chromic oxide solution in a water/sulfuric acid mixture. The resulting carboxylic and ethoxy-carboxylic acids were isolated by extraction in ethyl acetate, and the extracts were directly infused on the ESI source of a MS. The method was useful to quantify cetyl and stearyl alcohols in cosmetics, and a variety of fatty alcohols and phytol in sea water. However, the sum of the primary alcohols and the corresponding carboxylic acids present in the samples were quantified by this method. On the other hand, pyridinium dichromate and pyridinium chlorochromate, which are soluble in a variety of organic solvents, can be used to oxidize primary and secondary alcohols to aldehydes and ketones, respectively. To enhance RP-HPLC retention, Doehl *et al.* [86] have oxidized prostaglandins to the corresponding oxo prostaglandins with pyridinium dichromate.

Une *et al.* [87] developed a method for the determination of bile alcohols which makes use of an enzymatic reaction to convert first a secondary alcohol to a ketone, which is next derivatized using a common reaction for ketones. Namely, the 3α-hydroxy group of the analytes was oxidized with NAD and 3α-hydroxysteroid dehydrogenase, with subsequent derivatization of the resulting keto groups with 2,4-dinitrophenylhydrazine (DNPH, **Fig. 4.4A**). Ketones react with the hydrazide group of DNPH to yield a hydrazone linkage. The method

allowed the separation of bile alcohol stereoisomers on a phenyl column using a MeOH/water gradient.

4.6.2. Sulfonation

Cassani *et al*. [88,89] used the sulfur trioxide dimethylformamide complex (SO_3-DMF) to convert FAE into AES (**Fig. 4.4B**). The reaction is an esterification where anhydrous SO_3 instead of sulfuric acid is used. The resulting AES were separated on a C18 stationary phase with a MeOH/water gradient, followed by HPLC-MS. The advantage of converting non-ionic FAE to anionic AES is that MS detection in the ion-negative mode can be used. With the ion-negative mode, all the AES oligomers gave almost constant response factors; on the contrary, FAE oligomers should be detected in the ion-positive mode as adducts of Na^+ or other cations present in the mobile phase. The molar response of these adducts varies largely with the number of EO units of the oligomer, being almost zero for the non-ethoxylated alcohols, increasing rapidly with the length of the alkyl chain at least up to 8 EO units. The SO_3-DMF-pyridine complex has been also used to determine sterols at the attomol level [90].

Table 4.4. Derivatization of hydroxyl functional groups with miscellaneous reagents, and working conditions for the separation and detection of the derivatives[a]

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation	Sample	LOD	Ref.
Sulfur trioxide dimethyl formamide complex	FAE	DCM in N_2 atmosphere	3 h, r.t.	HPLC-ESI-MS, HPLC-APCI-MS	Raw materials	-	[88,89]
Picolinic acid and 2-methyl-6-nitrobenzoic anhydride	7α-Hydroxy-4-cholesten-3-one	THF, triethylamine, 4-dimethylaminopyridine	30 min, r.t.	HPLC-ESI-MS/MS	Human serum,	0.1 ng mL^{-1}	[93]
	Aldosterone	THF, triethylamine, 4-dimethylaminopyridine	30 min, r.t.	HPLC-ESI-MS/MS	Human serum,	5 pg mL^{-1}	[94]
	Testosterone	THF, triethylamine, 4-dimethylaminopyridine	30 min, r.t.	HPLC-ESI-MS/MS	Human serum, prostate tissue	5 pg mL^{-1}, 0.17 pg mg^{-1}	[95]
	5α-Dihydrotestosterone	THF, triethylamine, 4-dimethylaminopyridine	30 min, r.t.	HPLC-ESI-MS/MS	Human serum, prostate tissue	6 pg mL^{-1}, 0.33 pg mg^{-1}	[95]
2,4-Dinitrophenylhydrazone (DNPH)	Bile alcohols	Incubation with NAD and 3α-hydroxysteroid dehydrogenase in $Na_4P_2O_7$; followed by DNPH in MeOH	20 h, 25 ºC; 1 h, 60 ºC	HPLC-UV (364)	Human bile	20 ng	[87]
6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)	*n*-Alcohols	ACN, borate at pH 7.5	2 min, 25 ºC	HPLC-FL (ex290, em365)	Alcoholic beverages	100 pmol	[112]
	Secondary alcohols	ACN, borate at pH 7.5	2 min, 25 ºC	HPLC-FL (ex290, em365)	Alcoholic beverages	250 pmol	[112]
2-(4-Carboxyphenyl)-5,6-dimethylbenzimidazole (CDB)	Bisphenol A	ACN, IDC, 4-piperidinopyridine	1 h, 40 ºC	HPLC-FL (ex336, em440)	Rat serum	0.1 pg mL^{-1}	[91]
9-Anthroyl nitrile (AN)	Triamcinolone	ACN, quinoclidine, triethylamine	30 min, 22 ºC	HPLC-FL (ex460, em360)	Human plasma	1 ng mL^{-1}	[105]
2-Fluoro-*N*-methylpyridinium *p*-toluensulfonate	FAE	ACN, triethylamine	2 h, r.t.	HPLC-ESI-MS	Water, waste water, river sediments	0.7-10 ng L^{-1} 0.3-78 ng g^{-1}	[106,107]

[a] Acronyms: dichloromethane (DCM), 2,4-dinitrophenylhydrazine (DNPH), fatty alcohol ethoxylates (FAE), 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC), nicotinamide adenine dinucleotide (NAD)

[b] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

Table 4.4. (Continued)

Reagent	Analyte	Reaction medium	Reaction time and temperature	Separation	Sample	LOD	Ref.
Dansyl chloride	Ethynylestradiol	$NaHCO_3$/NaOH pH 10.5	3 min, 60 ºC	HPLC-ESI-MS/MS	*Rhesus* monkey plasma	0.2 fg mL^{-1}	[96]
	Estrone, estradiol, ethynylestradiol	MeOH (on-line reaction)	4 min, 40 ºC	HPLC-MS/MS	Wastewater	0.4-0.7 ng L^{-1}	[97]
	Testosterone, cholesterol, vitamin A, vitamin D3, 12-OH dodecanoic acid, 3-OH palmitic acid, 6β-OH testosterone, deoxycholic acid, hydrocortisone	DCM, 4-(dimethylamino)-pyridine, *N*,*N*-diidopropylethylamine	1 h, 65 ºC	HPLC-MS/MS	Standards	2.9-109 pg	[104]
7-Methoxycoumarin-3-carbonyl azide (MCCA)	7α-Hydroxycholesterol (7-HC)	Ethyl acetate	40 min, 140 ºC	HPLC-FL (ex338, em411)	Dog plasma	4 pg	[115]
	Brevetoxins	-	-	MEK-LIF (ex354, em410) MEKC-UV	Fish	4 pg g^{-1} 1 ng g^{-1}	[114]
Isonicotinoyl azide	5α-Androstane-3α,17β-diol	Benzene	30 min, 80 ºC	HPLC-ESI-MS/MS	Rat brain	0.1 ng g^{-1}	[116,117]
	Androsterone	Benzene	30 min, 80 ºC	HPLC-ESI-MS/MS	Rat brain		[118]
	7α-Hydroxy-4-cholesten-3-one	Benzene	30 min, 80 ºC	HPLC-ESI-MS/MS	Rat brain		[119]
4-(4-Methyl-1-piperazyl)-3-nitrobenzoyl azide (APZ)	Dehydroepiandrosterone, pregnolone, 5α-dihydrosterone	Benzene	30 min, 80 ºC	HPLC-ESI-MS/MS	Human serum	1.0-1.4 fmol	[97]
1-(2,4-Dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ)	Estrone, estradiol	Acetone, $NaHCO_3$	30 min, 60 ºC	HPLC-ESI-MS/MS	Human serum	2.6 fmol	[97]

[a] Acronyms: dichloromethane (DCM), 2,4-dinitrophenylhydrazine (DNPH), fatty alcohol ethoxylates (FAE), 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC), nicotinamide adenine dinucleotide (NAD)

[b] Between parenthesis, in nm, absorption wavelength, or excitation and emission wavelengths

4.6.3. Esterification with carboxylic acids

Bisphenol A was determined in rat serum by RP-HPLC-FL after derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB, **Fig. 4.4C**) [91]. Yamashita *et al.* [92] used a mixture of 2-methyl-6-nitrobenzoic anhydride and picolinic acid to introduce pyridine-carboxylate moieties into hydroxysteroids to enhance sensitivity in RP-HPLC-ESI-MS/MS. It should be noted that derivatives of picolinic acid were formed, while the anhydride worked as activator and dehydrating agent. Probably esters of the anhydride were not formed due to steric hindrance with the ortho methyl and nitro groups. The method was applied to the quantification of aldosterone, testosterone, 7α-hydroxy-4-cholesten-3-one and dihydrotestosterone in human serum [93-95].

4.6.4. Sulfonyl chlorides

Analogously to acyl chlorides, aryl-sulfonyl chlorides have a chloride leaving group, and react with hydroxyls to yield aryl-sulfonates of the alcohol residue. 5-(Dimethylamino)naphthalene-1-sulfonyl chloride, popularly known as dansyl chloride, a typical reagent for the derivatization of amino groups, has been also used in the derivatization of estrogenic hormones (**Fig. 4.4D**). These compounds have both a phenol group and one or two aliphatic hydroxyl groups; however, the former is derivatized with dansyl chloride. The reaction was used to enhance the sensitivity in the RP-HPLC-ESI-MS/MS determination of ethinylestradiol, an estrogen used in contraceptive preparations [96]. This estrogen was determined in plasma. Dansylation was also used to determine estrogenic hormones in river and waste water using HPLC-MS/MS [97-102]. Xu and Spink [103] have used dansyl chloride and other sulfonyl chlorides, including 1,2-dimethylimidazole-4-sulfonyl chloride (DMISC), pyridine-3-sulfonyl (PS) chloride, and 4-(1H-pyrazol-1-yl)benzenesulfonyl (PBS) chloride, to enhance the sensitivity in the ESI-MS detection of estrogens. Fragmentation of the derivatives in the gas phase to give specific MS transitions also improved detection reliability. Unlike phenols, derivatization of aliphatic hydroxyl functional groups with

sulfonyl chlorides has not been extensively reported. Thus, Tang and Guengerich [104] reported a RP-HPLC-MS method to determine testosterone, vitamin A, hydrocortisone and 12-hydroxydodecanoic acid after derivatization of the aliphatic hydroxyl groups with dansyl chloride.

4.6.5. Miscellaneous leaving groups

In acyl nitriles, like 9-anthroyl nitrile (9-AN, **Fig. 4.4E**), the leaving group is hydrogen cyanide. Główka *et al.* [105] determined triamcinolone, a synthetic corticosteroid used to reduce the body's immune responses in the presence of endogenous corticosteroids. In pharmacokinetic studies, the analyte was labeled with 9-AN, followed by RP-HPLC-FL.

Another common leaving group is fluoride. Dunphy *et al.* [106] developed an HPLC-ESI-MS method for the determination of FAE in water and wastewater samples. The terminal hydroxyl group of FAE was derivatized using 2-fluoro-*N*-methylpyridinium *p*-toluenesulfonate (FMPTS, **Fig. 4.4F**), which provided a permanent positive charge. This made possible the ESI-MS detection of the analytes in the ion-positive mode without relying on the formation of adducts. Dyer *et al.* [107] applied this method to the study of the FAE oligomer distributions in river sediments. Using solvent extraction followed by derivatization with FMPTS and HPLC-MS, an average LOD of 6 ng g^{-1} for individual oligomers was reached. Morin *et al.* [108] used FMPTS to derivatize the phytosterol fraction of rape seed oil. The derivatized phytosterols bear a positive charge and possess UV absorbance due to the pyridinium group, which allowed separation and detection by CZE-UV. A non-aqueous buffer containing trimethyl-β-cyclodextrins, which improved selectivity and resolution, was used as background electrolyte. FMPTS has been also used to determine testosterone and dihydrotestosterone [109] and the hypnotic agent propofol [110]. Differently from FMPTS, 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ, **Fig. 4.4G**) does not introduce a charge into the analyte molecule, but it is a strong fluorofor [111].

As shown in **Fig. 4.4H**, using an N-hydroxysuccinimidyl carbamate, the leaving group is succinimide, and a carbamate of the alcohol residue is formed.

Similarly, N-hydroxysuccinimidyl esters yield the corresponding esters of the alcohol residue. A method for the determination of *n*-alcohols and secondary alcohols in alcoholic beverages after fluorimetric derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was described by Motte *et al.* [112] (**Fig. 4.4H**). Full resolution of the derivatized alcohol mixture was achieved by HPLC-FL using a C18 column and gradient elution with ACN/sodium acetate. Similarly, N-methyl-nicotinic acid N-hydroxysuccinimide ester (C1-NANHS) has been used to enhance the MS detection of Vitamin E components [113].

4.6.6. Azides

Azides, which react via the formation of an isocyanate intermediate, have been used for the derivatization of hydroxyl functional groups (**Fig. 4.4I**). Thus, brevetoxins, which are cyclic polyether neurotoxins, have been determined in fish using 7-methoxycoumarin-3-carbonyl azide (MCCA) [114]. Separation was performed by MEKC using a sodium borate/SDS running buffer, followed by UV and LIF detection. MCCA was also used by Saisho *et al.* [115] to determine 7α-hydroxycholesterol in dog plasma. Separation was performed on an HPLC phenyl column with ACN/water. Higashi *et al.* [116] developed a method for the determination of 5α-androstane-3α,17β-diol, a neurosteroid, in rat brain. The derivatives, obtained with isonicotinoyl azide (NA, **Fig. 4I**), were analyzed using RP-HPLC with ion-positive ESI-MS/MS detection. The method was extended to other neurosteroids [117-119]. Nishio *et al.* [111] used 4-(4-methyl-1-piperazyl)-3-nitrobenzoyl azide (APZ, **Fig. 4.4I**) and PPZ (**Fig. 4.4G**) for the HPLC-ESI-MS determination of estrogens and 5-ene-steroids/5α-reduced steroids bearing hydroxyl functional groups in serum. Derivatization with these reagents provided a 500-2000 fold increase of sensitivity compared to the intact analytes.

4.7. Conclusions

A variety of reagents and reaction schemes addressed to introduce a chromophore, a fluorophore, a ionizable group or a permanent charge in analytes having an aliphatic alcohol or a phenol group while lacking other reactive groups, have been described. The introduction of a charge is of interest both to enhance MS sensitivity, to enable CE separation, as well as to decrease volatility, thus to enhance the sensitivity of volatile analytes in evaporative detectors. Derivatization reagents with the same reactive group may also differ in reactivity, which depends on the presence of activating groups, as well as in the extension of the chromogenic or fluorogenic conjugated system. However, an increase of reactivity is frequently offset by the reduced stability of the reagent, and reagents having either high reactivity and a large conjugate system are usually expensive. Reagents containing a single aromatic ring are usually adequate for industrial quality control, but large conjugated systems, which assure a large molar absorptivity or a high fluorescence quantum yield, are frequently required for both environmental studies and biochemical applications. Reagents containing condensed rings usually provide large fluorescent quantum yields. However, it should be noted that condensed ring systems tend to be carcinogenic. Further, reagents with large hydrophobic regions will led to derivatives with increased retention in the common RP-HPLC stationary phases, but they can be also incompatible with the common hydro-organic mobile phases if excessively hydrophobic. For this reason, large conjugated systems are frequently complemented with polar groups strategically located along the reagent molecule. Among the anhydrides, symmetric cyclic aromatic structures have the advantages of giving rise to a single derivative per analyte, providing both a chromophore and a ionizable carboxylate group. However, asymmetric reagents as SBA and isatoic anhydride, and non-cyclic symmetric anhydrides, as those used to enhance volatility for MS detection or GC separation, can be also useful.

Several authors have compared acyl chlorides and isocyanates. A few studies have also compared different organic anhydrides for the derivatization of FAE, as well as a few other reagents for the derivatization of other analytes. Concerning to the stability of the derivatives, it should be noted that esters are slowly hydrolyzed in aqueous and hydroorganic media. Therefore, the derivatives formed by reaction with acyl chlorides and organic anhydrides should be analyzed immediately or stored in a freezer. Otherwise, since hydrolysis proceeds at different rates for analytes having different alcohol residues (for instance, the esters of non-ethoxylated alcohols hydrolyze much slowly than those of ethoxylated oligomers with same alkyl chain), biased conclusions will be obtained.

Concerning to the application fields, derivatization of hydroxyl functional groups has been mainly used in industrial quality control and evaluation of the environmental impact of non-ionic aliphatic surfactants (mainly FAE). In a few cases, it has been also applied to the determination of anionic surfactants which are esters of inorganic acids as sulfuric or phosphoric acids. Other analytes of interest in the industrial and environmental fields which have been also derivatized with the reactions here reported include soluble polymers (as PEG), and small polar molecules (as glycols and others). Among the analytes with a remarkable biological activity, phytosterols, hormones, di- and mono-glycerides, and many others have been derivatized. These include simple molecules as choline, or very complex as endectocides. The matrices include all types of cell cultures, biological fluids, tissues, food and others. The different reagents and sample preparation procedures used in the biochemical and related fields can be of interest in other fields also requiring high sensitivities, as it is the usual case in environmental studies, and vice versa.

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SECTION III.

CHROMATOGRAPHIC METHODS FOR SURFACTANT ANALYSIS

CHAPTER 5.

Single-pump heart-cutting two-dimensional liquid chromatography applied to the determination of fatty alcohol ethoxylates

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Single-pump heart-cutting two-dimensional liquid chromatography applied to the determination of fatty alcohol ethoxylates

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ABSTRACT

A setup for heart-cutting bi-dimensional liquid chromatography (LC–LC), constructed with a chromatograph provided with a single pump, an auxiliary 6-port 2-position valve ($V_{6/2}$) and a column selector valve (V_{CS}), is described. The possible ways of connecting the two valves for LC–LC, namely with $V_{6/2}$ first followed by V_{CS} and vice versa, are compared. The possibility of using the setups for preconcentration followed by the backwards transfer of the preconcentrated solutes to the detector or to a second column is also shown. The $V_{6/2}$-first configuration for LC–LC was applied to the characterization of industrial fatty alcohol ethoxylates (FAEs) using UV–vis detection. For this purpose, the phthalates of the FAE oligomers were first obtained. The hydrocarbon series were separated along the 1st dimension by MeOH/water gradient elution on a C8 column at 60 ºC. Selected segments of the eluate were transferred to the 2nd dimension, where the EO oligomers of the isolated series were resolved by gradient elution with a complementary ACN/water mobile phase on a C8 column at 25 ºC. In addition, an average response factor of the hydrocarbon series of FAEs was proposed. To apply the factors, the average EO number of the series is first established by the chromatographic profile of one of the series along the 2nd dimension. Then, the factors are used to correct the peak areas of the isolated series

which are obtained along the 1st dimension chromatogram, thus allowing the fast and accurate determination of the series in industrial FAEs. The method is particularly useful to characterize FAEs having large average EO numbers or constituted by mixtures of even and odd series.

Keywords: *Average response factor of an ethoxylated series; Complementary mobile phases; Fatty alcohol ethoxylates; Heart-cutting bi-dimensional liquid chromatography.*

5.1. Introduction

Fatty alcohol ethoxylates (FAEs) are widely used non-ionic surfactants [1–3]. Industrial and household cleaners, and many other commercial products, contain large concentrations of them. Obtained by condensation of fatty alcohols with ethylene oxide (EO), industrial FAEs are mixtures of oligomers with the structure:

$$CH_3(CH_2)_{n-1}(OCH_2CH_2)_mOH$$

where *n* indicates the length of the hydrocarbon chain and *m* is the number of EO units of the polar chain. The oligomer distributions of a FAE mixture are usually described by the hydrophobic cut or range of n values of the oligomers which are present at significant concentrations, and by the average number of EO units, *m*. FAEs with $10 \leq n \leq 18$ and $\overline{m}$ values ranging from 3 to 8 are usually found in commercial cleaners; however, some specialty surfactants have much larger EO chains. To characterize FAEs in raw materials and manufactured products, both the proportion of the hydrocarbon series in the mixture and the EO distribution of the series should be established [3–5].

A variety of methods for the quality control of FAEs in raw materials, and for the characterization and determination of FAEs in samples also containing other surfactant classes, including manufactured products [6–17] and environmental waters and sediments [2, 18–25] have been described. Whether underivatized or derivatized with a variety of chromogenic and fluorogenic reagents, FAE oligomers with short EO chains are well separated using RP-HPLC with ACN/water mobile phases on C8 columns [11–15]. Separation is primarily achieved by series, in the order of increasing values of *n*, with fairly good resolution of the EO oligomers within the series. However, using mono-dimensional HPLC, mixtures containing large EO chains are not well resolved, the

last eluting oligomers of a series overlapping with the first eluting oligomers of the following series [14]. Further, mixtures containing short EO chains cannot be resolved either if series with both even and odd values of *n* are present. This badly limits the use of HPLC for the characterization of FAEs. The problem of FAE characterization in these difficult cases can be tackled by two-dimensional HPLC (2D-HPLC).

Two different 2D-HPLC approaches have been applied to the characterization of mixtures of surfactants, namely heart-cutting 2D-HPLC (LC–LC), in which selected segments of the 1st dimension eluate are transferred to the 2nd dimension column, and comprehensive 2D-HPLC (LC × LC) in which small segments of the 1st column eluate are continuously transferred. The LC–LC approach has been used to isolate cationic, amphoteric, non-ionic and anionic surfactant classes [26]. Complex mixtures of surfactant classes have been analyzed by LC × LC [27] and by comprehensive two-dimensional GC (GC × GC) of the silylated derivatives [28,29]. LC–LC and LC × LC have been also used to characterize industrial FAEs. Thus, using LC–LC, Okada [30] separated first the hydrocarbon series on a styrene–divinylbenzene copolymer gel, followed by the separation of the EO oligomers on a strong cation-exchanger in the presence of potassium ions. Trathnigg *et al.* [31,32] separated the series by RP-HPLC on C18, followed by exclusion/adsorption chromatography to resolve the EO oligomers within the series. Using LC × LC, FAE oligomers have been separated first according to the EO distribution on a silica column, followed by separation of the alkyl chains on a C18 column [33]. Raust *et al.* [34] used RP-HPLC followed by adsorption HPLC to separate FAE oligomers according to the alkyl chain length and the EO distribution, respectively. Elsner *et al.* [27] used HILIC to separate first the FAE oligomers by the EO number, followed by separation of the alkyl chains by RP-HPLC on a C8 column.

As shown in a previous work [35], heart-cutting LC–LC can be implemented in a simple way by complementing a single pump chromatograph with both an auxiliary 6-port 2-position valve ($V_{6/2}$) and a column selector valve (V_{CS}). In the present work, it is shown that these two valves can be combined in two non-equivalent ways, namely $V_{6/2}$ first followed by V_{CS} and vice versa, and that each one of these two combinations can be used either for LC–LC or for solute preconcentration followed to backwards transfer to the detector. The $V_{6/2}$ first configuration can be also used to backwards transfer the preconcentrated solutes to a second column for further separation. Thus, depending on which valve is located first, and which ports are used, four non-equivalent ways of connecting these two valves are possible. In our previous work [35], the V_{CS}-first configuration for LC–LC was introduced and applied to the characterization of FAEs. Instead of this, in the present work we have used the $V_{6/2}$-first configuration for LC–LC. The main advantage of this approach is the possibility of selecting different stationary phases to resolve the solute groups with rather different properties that can be found in the eluate segments of the 1st dimension. Other advantages and limitations of the four configurations for either LC–LC or solute preconcentration are also discussed.

Also, in our previous work [35], separation of the FAE oligomers was carried out by first isolating the series on a propyl-diol column. Selected segments of the eluate were transferred to a C8 column, on which the EO oligomers within the series were resolved. Both columns were eluted with ACN/water gradients, using room temperature for the 1st dimension and a lower temperature for the 2nd column. Thus, orthogonality was mainly achieved by using different stationary phases to implement the 1st and 2nd dimensions. However, owing to the limited inter-series resolution along the 1st dimension, FAE mixtures containing series with both even and odd values of *n* were not resolved. In the present work, we have

largely improved the selectivity of the LC–LC separation of the FAE series along the 1st dimension by using a MeOH/water gradient on a C8 column. According to literature [20,33], the use of a MeOH/water gradient on a C18 column is excellent to achieve the separation of the FAE series without distinguishing the EO oligomers; however, we found that isolation of the series was better achieved with C8 which required lower MeOH concentrations than C18. Also, according to reported data [11,36], the polarity of the EO chain is enhanced at low temperatures, also increasing the polarity differences between the successive EO oligomers. Then, a superior focusing of the series into narrower bands was achieved by using a MeOH/water gradient at high column temperatures. On the other hand, to resolve the oligomers within the series (2nd dimension separation), the use of an ACN/water gradient on a C8 column was maintained. As indicated, this system is usually recommended for this purpose [11–15]. Also, the $V_{6/2}$-first configuration offers the possibility of automatically changing the column used for the 2nd dimension separation, which is useful when segments containing solutes with rather different properties are transferred. However, since FAE oligomers with the same EO number but belonging to different series have rather similar properties, stationary phases different from C8 were not required to chromatograph the consecutive series. Excellent separations of the successive oligomers were achieved by simply adjusting the initial and final ACN gradient concentrations according to the polarity of the n-series. Thus, orthogonality between the two dimensions was achieved by changing both the mobile phase and the column temperature. Mixtures of FAEs containing consecutive even and odd series were very well resolved, independently from the length of the EO chain.

The isolation of the FAE series along the 1st dimension, without distinguishing the oligomers according to the EO number, is of interest for the quick total determination of FAEs, also providing information about the proportion

of the series in the mixture. However, owing to the different UV–vis response factors of the oligomers, the peak areas obtained along the 1st dimension chromatogram for the isolated series cannot be directly related to the series concentrations. In this work, this problem was sorted out by introducing an average response factor of the series. This factor, which is a function of both the response factors of the oligomers and their EO distribution within the series, made possible the quick FAE quantification along the 1st dimension, with correction of the systematic error associated to the use of a given single oligomer as calibration standard. Another application of the proposed series response factors is the quick unbiased evaluation of the proportions of the series by using the 1st dimension chromatogram. To calculate the average response factor of a series, the EO chain distribution of the series, as measured by $\overline{m}$, should be known. However, in a given FAE raw material, all the series have the same EO distribution. This is so because fatty alcohols are condensed with ethylene oxide giving rise to closely similar values of $\overline{m}$, almost independently from the length of the alkyl chain. Cleaner formulations contain one or at most two FAE raw materials, which could have the same or different values of $\overline{m}$; therefore, in raw materials and in most samples, all the series along the 1st dimension chromatogram can be accurately determined after having established the $\overline{m}$ values by chromatographing one or two series along the 2nd dimension. Further, it is shown that if $\overline{m}$ is large, the average response factors of the series depend only slightly on $\overline{m}$. Then, for FAE separations where mono-dimensional LC is not enough to separate the series and LC–LC is needed, accuracy in the determination of $\overline{m}$ is not required. This method was applied to the characterization and determination of FAE mixtures containing both even and odd series, also including a specialty FAE with very long EO chains.

5.2. Experimental

5.2.1. Instrumentation and reagents

An 1100 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany), provided with a quaternary pump, a thermostated column compartment, an auxiliary 6-port 2-position valve (part G1316#055), a column selector valve (part G1159A) and a UV–vis diode array detector, was used. A second thermostated column compartment for HPLC (STH 585, Dionex Softron GmbH, Germering, Germany) was used for the 2nd dimension column. Two Ascentis Express fused-core C8 columns (Sigma–Aldrich, Steinheim, Germany) were used for the 1st (5 µm, 90 Å, 10 cm × 4.6 mm) and 2nd (2.7 µm, 90 Å, 15 cm × 4.6 mm) dimensions. The following analytical grade reagents were used: acetic acid, methanol (MeOH), acetonitrile (ACN), 1,4-dioxane (Scharlab, Barcelona, Spain) and urea (Fluka, Buchs, Switzerland). Phthalic anhydride (Sigma–Aldrich, Steinheim, Germany) was used for FAE derivatization. The following industrial FAE mixtures were used: Dehydol LT-7 (n = 12, 14, 16 and 18, $\overline{m}$ = 7), Lutensol AO7 (n = 13and 15, $\overline{m}$ = 7) (BASF, Germany) and Brij 35 (n = 12, 14 and 16, $\overline{m}$ = 23, Sigma–Aldrich). Also Dehydol LS-2 DEO, Dehydol LS-3DEO-N (n = 12, 14, 16 and 18, $\overline{m}$ = 2 and $\overline{m}$ = 3, respectively) and Lutensol AO3 (n = 13 and 15, and $\overline{m}$ = 3) were purposely synthetized for this work and kindly donated by BASF (Barcelona, Spain). Decaethylene glycol mono-dodecylether (DEGDE, nominally $C_{12}E_{10}OH$, Sigma–Aldrich) was also used. It should be noted that DEGDE is not $C_{12}E_{10}OH$, in fact contains large concentrations of the n = 12–18 even series, all them showing the typical EO distributions with $\overline{m}$ = 10.

5.2.2. Instrumental setup for single-pump LC–LC

Two possible ways of connecting a 6-port 2-position valve ($V_{6/2}$) and a column selector valve (V_{CS}) to implement LC–LC, namely with$V_{6/2}$ first followed by V_{CS} and vice versa, are shown in **Fig. 5.1**. For the two configurations, the operations to be performed to implement either LC–LC or solute preconcentration, as well as the necessary position of the valves, are summarized in **Table 5.1**. As further discussed in Section 5.3.2, the two configurations are not equivalent. In this work, only the $V_{6/2}$-first configuration (**Fig. 5.1A**) for LC–LC was used, whereas the reversed V_{CS}-first configuration (**Fig. 5.1B**) was used in the previous work [35]. To implement LC–LC using the$V_{6/2}$-first configuration, the flow was fed to port 1 of $V_{6/2}$, which was used to select either the mainpass through the 1st dimension (position I, continuous lines in **Fig. 5.1**) or a bypass to V_{CS} (position II, dashed lines in **Fig. 5.1**). Valve V_{CS} was used to direct the flow towards a 2nd dimension column (C2, inserted between the paired ports 1-1’ in **Fig. 5.1**) or to bypass the 2nd dimension through the shorted ports 3-3’. Five out of the six paired ports of V_{CS} remained available for use with different 2nd dimension columns if necessary. With $V_{6/2}$ in mainpass (position I) and V_{CS} in bypass (3-3’) it was possible to optimize the 1st dimension separation, and to elute in a single dimension those parts of the chromatogram not requiring further separation. With $V_{6/2}$ in mainpass (position I) and V_{CS} in the 1-1’ channel, selected segments of the eluate of the 1st column were transferred into the 2nd column. With both valves in bypass ($V_{6/2}$ in II and V_{CS} in 3-3’), the system was flushed with the new mobile phase required to either start gradient elution on the 2nd column or to resume elution on the 1st column with the previously used mobile phase. Finally, with $V_{6/2}$ in bypass (position II) and V_{CS} in 1-1’, gradient elution along the 2nd dimension was carried out. Independent temperature control of the two columns was also implemented. Automatic control of the times to operate both the injection valve

and the pump module (to control the flow rate), and to switch both $V_{6/2}$ and V_{CS}, was programmed using the ChemStation LC software (v.10.02, Agilent). A drawback of this software was the low maximal number of rows of program, which was limited to a sequence of 22 commands. To overcome this problem, instead of a single method, a sequence of methods, the first one to start elution along the 1st dimension, and the following ones to start elution along the 2nd dimension after each transfer of eluate to C2, followed by resuming elution of C1, was programmed. The sample was injected only once, with the first method, and all the other methods that followed were programmed with false or "zero volume" injections.

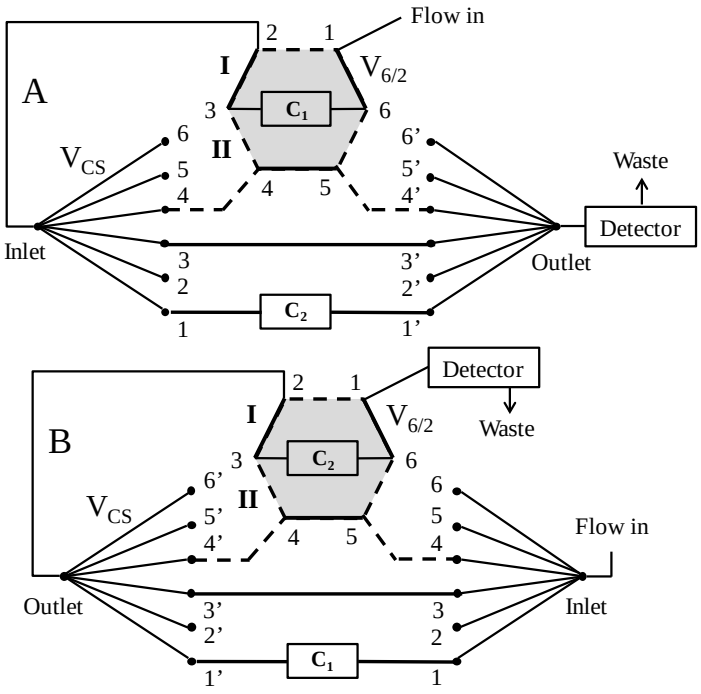

Fig. 5.1. The $V_{6/2}$ first (A) and V_{CS} first (B) ways of connecting a 6-port 2-positionvalve ($V_{6/2}$) with a column selector valve (V_{CS}) to carry out LC–LC (continuous lines). Positions of $V_{6/2}$: continuous thick lines, I; dashed lines, II. The dashed lines between ports 4 and 4' of V_{CS} and ports 4 and 5 of $V_{6/2}$ can be optionally used for solute preconcentration on either C1(A) or C2(B), followed by flow inversion; other details in Table 5.1.

Table 5.1. Operations to perform for either LC-LC or solute preconcentration, and required position of the valves for the $V_{6/2}$-first and V_{CS}-first configurations[a].

Purpose	Operations	$V_{6/2}$-first $V_{6/2}$; V_{CS}	V_{CS}-first V_{CS}; $V_{6/2}$
LC-LC	Condition or elution of C_1	I; 3-3’	1-1’; II
	Transfer of C_1 to C_2	I; 1-1’	1-1’; I
	System flush	II; 3-3’	3-3’; II
	Condition or elution of C_2	II; 1-1’	3-3’ or 4-4’; I
Preconcentration optionally followed by mono-dimensional LC	Condition or preconcentration in C_1	II; 4-4’	-
	Backwards elution of C_1 to detector	I; 3-3’ or 4-4’	-
	Backwards transfer of C_1 to C_2	I; 1-1’	-
	System flush	II; 3-3’	
	Condition or elution of C_2	II; 1-1’	-
Preconcentration (not followed by LC)	Condition or preconcentration in C_2	-	3-3’; I
	Backwards elution of C_2 to detector	-	4-4’; II
	System flush	-	3-3’; II

[a] Valve positions according to Fig. 5.1: continuous and dashed lines (positions I and II in Fig. 5.1, respectively) for $V_{6/2}$, and connected paired ports for V_{CS}; in addition to 1-1’, other columns can be inserted in the other channels of V_{CS}, but preserving one for shorting and optionally another one for nesting (e.g. 3-3’ and 4-4’, respectively, in this table and in Fig. 5.1).

5.2.3. Derivatization and elution conditions

Derivatization was performed according to the procedure described elsewhere [14]; briefly, ca. 40 mg of the samples (industrial FAEs and their mixtures), 1 g phthalic anhydride and 0.5 g grinded urea were directly weighed in screw-cap tubes, and 4 mL 1,4-dioxan was added. The tubes were shaken and introduced in a silicone-oil thermostatic bath at 105◦C for 90 min. After cooling, the residue was dissolved with a 2:1 MeOH/water mixture containing 0.1 M NH_3. The volume was completed up to 10 mL with this mixture. The solutions were injected immediately or stored at -20 ºC. The gradient along the 1st dimension was achieved by mixing 50:50MeOH/water (A) with 100% MeOH (B). Gradient elution along the 2nd dimension was performed by mixing 50:50 ACN/water (C) with 100% ACN (D). Phases A–D also contained 0.1% acetic acid. Except during

eluate transfer or valve operation, the flow rate was1 mL/min. Detection was performed at 230 ± 10 nm (360 ± 40 nm as reference). Before injection of the 20 µL aliquots, all solutions were passed through a 0.45 µm pore-size nylon filter.

5.3. Results and discussion

5.3.1. System selection

According to literature [11–15], a C8 column and an ACN/water gradient performs very well in separating the oligomers within the hydrocarbon series. Thus, this system, using a core-shell type C8 column to achieve an enhanced efficiency, was selected to perform the 2nd dimension separations. In addition, FAE oligomers with the same EO number but belonging to different hydrocarbon series have rather similar properties, only differing slightly on the hydrophobicity of the hydrocarbon chain. This made unnecessary to select a different stationary phase, such as C4 or C18, to chromatograph the different series after their isolation along the 1st dimension. Further, as shown below, all the series from n = 12 to 18 were well resolved using ACN/water gradients with similar concentration ranges. After fixing the 2nd dimension system, the stationary and mobile phases for the 1st dimension separations were selected. Orthogonality between the two dimensions is most frequently achieved by selecting different stationary phases. With this aim, we tried several alkyl columns, including C1, C4 and C18, as well as propyl-amide, propyl-cyan and propyl-penta-fluoro-phenyl. Using ACN/water gradients, none of these systems was able of separating the series without also separating the oligomers within them. Instead of this, a single band for each series was achieved using MeOH/water gradients. This also agreed with reported data [11,36]. Focusing of the series into bands without distinguishing the oligomers within them was better achieved with alkyl columns. Among them, C18 gave excessive retention of the n = 16 and 18 series. On the other hand, the differences

between the C1, C4 and C8 columns were small. However, as shown below, further focusing of the series into narrow bands along the1st dimension was achieved by using high column temperatures. Then, since the pH and thermal stability of bonded alkyl chains shorter than C8 are low [37,38], C8 was selected. The blindness of MeOH/water mobile phases to the presence of the EO chain is a rarity, then, the successful substitution of MeOH/water by any other mobile phase in combination with another stationary phase to resolve the series without also resolving the EO oligomers is unlikely. In fact, we believe that the relative success achieved in our previous work [35] with an alkyl-diol stationary phase to resolve at least mixtures of even FAE series can be explained by the similarity of the diol group to MeOH. Both are basic ligands, in which retention by acceptance of protons predominates. Thus, the lack of acidic protons along the EO chain could explain its unusual chromatographic behaviour.

5.3.2. Comparison of the $V_{6/2}$-first and V_{CS}-first configurations for either LC–LC or solute preconcentration

The $V_{6/2}$-first and V_{CS}-first configurations for either LC–LC or for solute preconcentration are illustrated in **Fig. 5.1**. To implement LC–LC, the following operations should be possible: (i) to alternatively bypass the 1st or the 2nd column, thus to independently conditioning or eluting each dimension; (ii) to serially connect the two columns for eluate transfer; and (iii) to simultaneously bypass the two columns for system flushing. As shown in **Table 5.1**, these operations can be performed using either the $V_{6/2}$-first and V_{CS}-first configurations. However, $V_{6/2}$ provides only two alternative pathways, one of which being required for bypassing, whereas V_{CS} provides up to five alternative channels plus the bypass. In LC–LC, the 1st dimension is normally used to separate the sample into families or groups of compounds, groups which will be next separated along the 2nd

dimension. Since the properties of the solutes may largely differ from a group to another, then, in a large number of applications, different columns will be required to carry out the 2nd dimension separation. The reverse situation, in which a sample is separated using different columns to give rise to solute groups which can be treated with the same 2nd dimension column, is much less common. Then, in relation to the V_{CS}-first configuration of the previous work [35], the $V_{6/2}$-first configuration used in this work is potentially applicable to a much larger number of real cases. However, in this work we have always used the same C8 column along the 2nd dimension because the eluate segments of the 1st dimension contained FAE hydrocarbon series with rather similar properties. For all the series, the oligomers were very well resolved using a C8 column and ACN/water gradients, other stationary phases being not required. On the other hand, both the $V_{6/2}$-first and V_{CS}-first configurations also provide the possibility of inverting the flow in the column inserted in the mainpass of $V_{6/2}$, which is useful for solute preconcentration. To implement this, the ports of one of the channels of V_{CS} (4 and 4' in **Fig. 5.1**, **parts A and B**) should be connected with ports 4 and 5 of $V_{6/2}$. In this way, $V_{6/2}$ is "nested" in V_{CS} (dashed line connections in **Fig. 5.1**). The necessary valve positions required to invert the flow in the column inserted in $V_{6/2}$ are also indicated in **Table 5.1**. Solute preconcentration at one end of this column should be first achieved by using a weak mobile phase. High sensitivities can be obtained by subsequently inverting the flow and using a strong mobile phase. However, as shown in **Table 5.1**, there is again an important difference between the two configurations. Thus, using $V_{6/2}$-first, the inverted flow through C1 can be driven to the detector either through C2 or bypassing C2. Instead of this, using V_{CS}-first, the inverted flow through C2 can be driven only directly to the detector. Thus, the $V_{6/2}$-first configuration is again the most useful; however, solute preconcentration was not performed in this work. Finally, instead of a valve of

each type, either two $V_{6/2}$ or two V_{CS} valves of the same type can be also combined to implement LC–LC, but solute preconcentration can be achieved only by combining a valve of each type.

5.3.3. Optimization of the 1st dimension separation

Optimization was performed with a mixture of Dehydol LT7 and Lutensol AO7 (ca. 20 mg each). This mixture contains FAE series with even and odd values of *n*, all them with wide EO distributions ($\overline{m}$ = 7). Elution of this mixture using ACN/water gradients on a C8 column (2.7 µm, 15 cm) led to the separation of the consecutive hydrocarbon series with excellent resolution of the successive EO oligomers; however, the series overlap largely (chromatograms not shown). The oligomers of Brij 35, which contains only even series but with significant concentrations of EO oligomers with very large values of *m*, could not be resolved either. As reported [20,33], a much better inter-series resolution, at the cost of lack of resolution between the EO oligomers, can be achieved by using MeOH/water gradients. As shown in **Fig. 5.2**, traces A and B, with a MeOH/water gradient, the Dehydol LT7 and Lutensol AO7 mixture was resolved into isolated peaks, one single peak per each series, with little dispersion of the EO oligomers within the series. Further, at increasing temperatures, the inner dispersion of the series was reduced more than the average retention times of the series, which resulted in an improvement of the inter-series resolution. This is shown in **Fig. 5.2**, trace B, where superior resolution between consecutive series up to *n* = 18 with a short total analysis time (less than 20 min) was achieved at 60 ºC. Further, in these conditions, portions of flat baseline between the peaks allowed easy eluate segment transfer from the 1st to the 2nd dimensions without cross-contamination between consecutive series. Also, the capability of the method to also isolate the very long EO chain series of Brij 35 is shown in **Fig. 5.2**, **part C**. As deduced by comparing

traces B and C of **Fig. 5.2**, the width of the single peaks of the series increased only slightly by increasing $\overline{m}$ from 7 to 23.

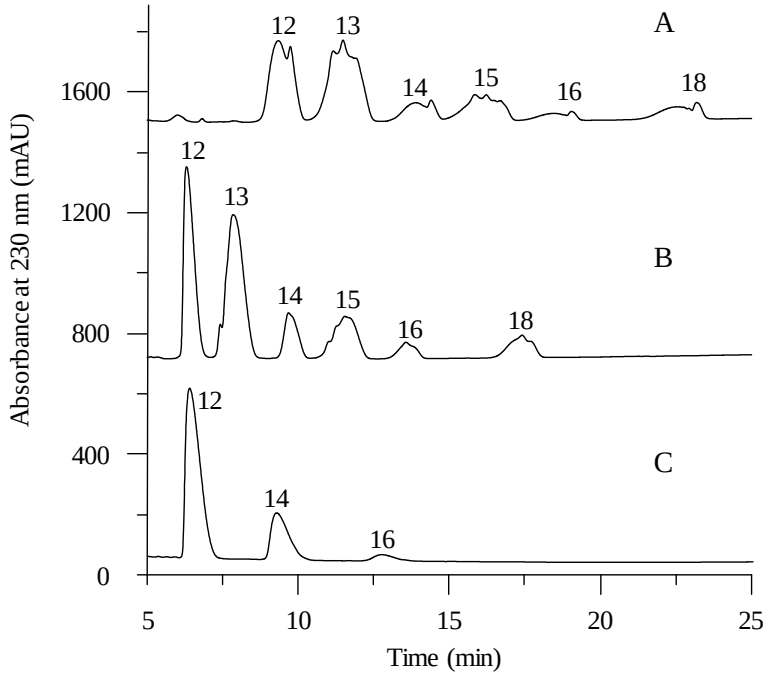

Fig. 5.2. Chromatograms of a derivatized mixture of Dehydol LT-7 and Lutensol AO7 obtained along the 1st dimension at 25ºC (A) and 60 ºC (B), and chromatogram of Brij 35 at 60 ºC (C). A gradient elution from 75 to 90% MeOH in 25 min on a C8 column (5 µm, 10 cm) was used. The number of carbon atoms of the series (*n*) is indicated at the peaks.

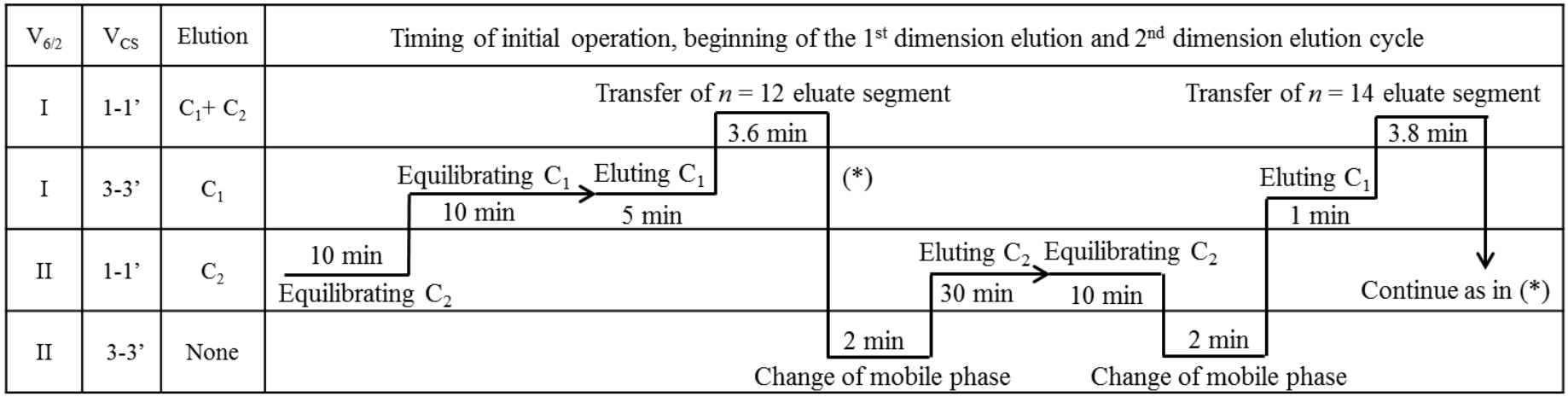

Fig. 5.3. Diagram of times. $V_{6/2}$ can be set at the mainpass or bypass positions (in Fig. 1, positions I and II, respectively); 1-1' and 3-3' are the paired ports of V_{CS} between which the 2nd dimension column and a bypass, respectively, are inserted. With $V_{6/2}$ at II and V_{CS} at 3-3', the whole system (except the columns) is flushed with a new mobile phase; C_1 and C_2 are equilibrated with the adequate mobile phase before starting the gradients, and C_2 is re-equilibrated with the mobile phase to be used in the following cycle after finishing the gradient. The flow rate was 1 mL min^{-1}, but was reduced to 0.5 mL min^{-1} during transfer.

5.3.4. Transfer of eluate segments to the 2nd dimension

A scheme of the system operation for LC–LC is shown in **Fig. 5.3**. The two columns should be eluted in series during transfer of eluate segments from the 1st to the 2nd dimension. Thus, to avoid overpressure during transfer, the particle size and length of the 1st dimension column were limited to 5 μm and 10 cm, whereas 2.7 μm and 15 cm were used for the 2nd dimension column, respectively. An advantage of this approach was short analysis times along the 1st dimension separations. Also, the flow rate, which was 1 mL min^{-1}during elution along either the 1st or 2nd dimensions, was reduced to 0.5 mL min^{-1} during transfer. A high column temperature in the 1st dimension also helped in reducing backpressure during eluate transfer. To avoid leaking, the flow was momentarily reduced down to zero before the switching of any valve, and restored immediately after. As further commented below, the 1stdimension chromatograms are of much interest in industrial quality control for the quick evaluation of both the FAE contents and the proportions of the series.

5.3.5. Optimization of the 2nd dimension separation

After optimization of the 1st dimension separation, selected segments of the eluate of C1, each one comprising a given series, were transferred to C2. Optimization of the separation along the 2nd dimension was also performed with a C8 column, which was inserted in one of the channels of V_{CS} (**Fig. 5.1A**). After transfer of an eluate segment, the shorted 3-3’ channel of V_{CS} was used to flush the system with ACN/water, thus displacing the previously used MeOH/water mixture. Separation of the oligomers was achieved by eluting the 2nd dimension column with an ACN/water gradient. For this purpose, the ACN concentration was initially increased from 50 to 95%. Satisfactory separations of the oligomers of all the series were achieved; however, to save analysis time, the starting and ending

concentrations of the ACN gradients, and the gradient times, were optimized according to the length of the hydrocarbon chain of each series. The optimized values are indicated in **Figs. 5.4** and **5.5**, where chromatograms of the resolved peaks of selected series of Dehydol LT-7, Lutensol AO7 and Brij 35 are shown. Using the optimized conditions, the separation of the most hydrophilic oligomers of all series (e.g. $m \approx 20$ for Dehydol LT-7 and Lutensol AO7 which have $\overline{m} = 7$) began a few minutes after starting the ACN gradient, the most hydrophobic oligomer ($m = 2$) eluting a few minutes before ending the gradient time. This reduced the elution time along the 2nd dimension for all series to a minimum. As expected [11,14], the peaks of the oligomers followed the decreasing order of m, except for the derivatives of the $m = 0$ and 1 oligomers, which are more hydrophilic than the $m = 2$ and 3 oligomers, thus overlapping with the peaks of other oligomers of the corresponding series. Thanks to the separation by series along the 1st dimension, it was possible to observe oligomers with $m > 20$ in mixtures containing both even and odd series, without any sign of cross-contamination between consecutive series. As also shown in **Fig. 5.4**, traces B and D, the 2nd dimension chromatograms of the Lutensol AO7 series were more complex than those of Dehydol LT7 series, the oligomers showing double peaks. This was attributed to the presence of both branched isomers and impurities, likely corresponding to FAEs of mineral origin. Finally, **Fig. 5.5** shows the 2nd dimension chromatograms of the $n = 12$, 14 and 16 series of Brij 35. As observed, the series were resolved up to $m > 40$ without inter-series cross-contamination.

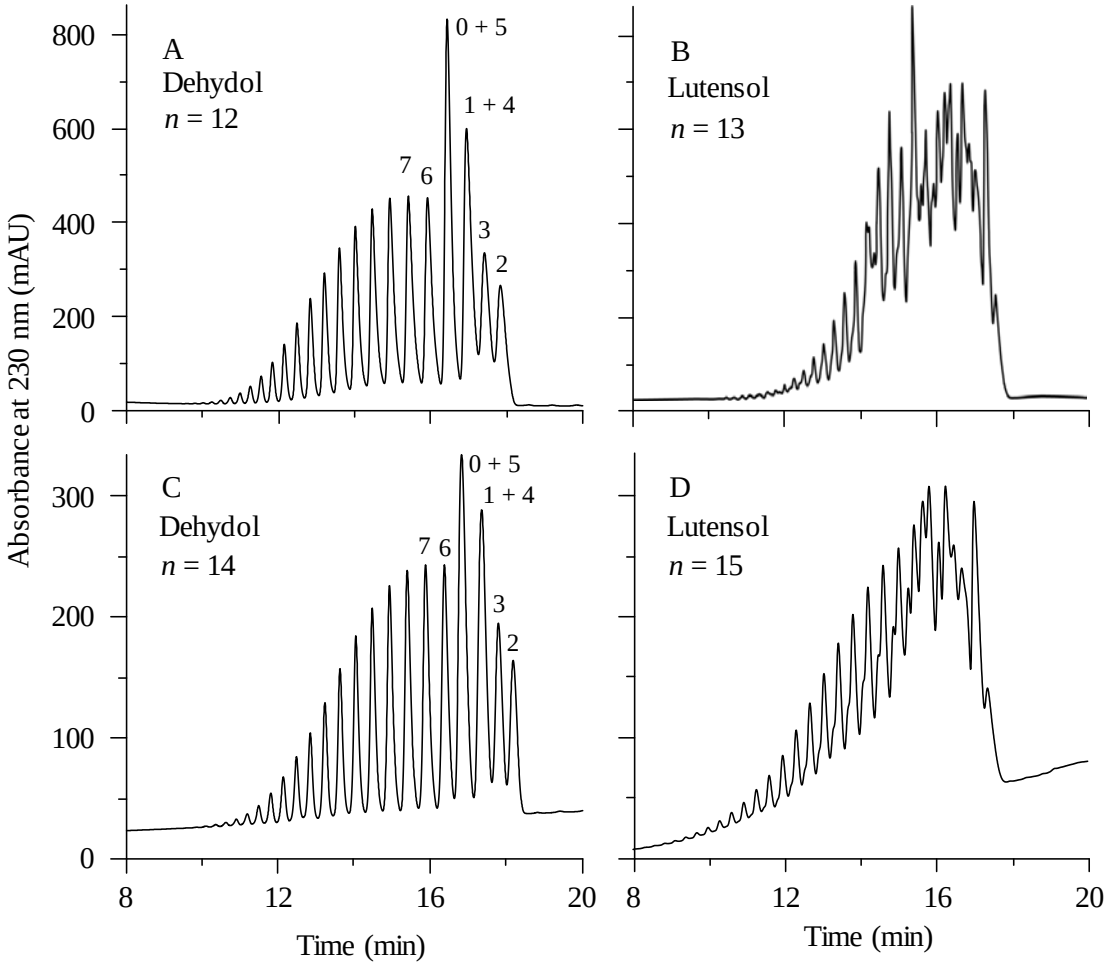

Fig. 5.4. Chromatograms obtained by gradient elution along the 2nd dimension of segments of the 1st dimension eluate for a mixture of Dehydol LT-7 and Lutensol AO7 (Fig. 5.2, trace B). The time is counted from the beginning of the ACN gradient; ACN increased within the following ranges: 60–85%, 63–88%, 66–91% and 69–94%, for the n = 12, 13, 14 and 15 series, respectively. Gradient time was 23 min. On the peaks of the Dehydol LT-7 series, the EO numbers of some representative oligomers are indicated.

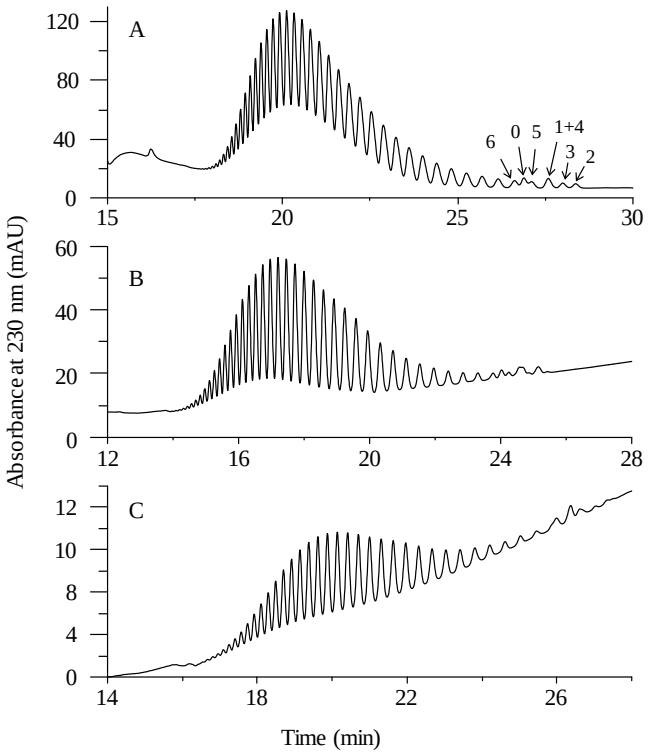

Fig. 5.5. Chromatograms along the 2nd dimension for the n = 12 (A), n = 14 (B) and n = 16 (C) series of Brij 35. Elution of the n = 16 series, 72–97% ACN in 23 min; other conditions as in Fig. 5.4.

5.3.6. Evaluation of the series distribution using the 1st dimension chromatogram

After optimization of the LC–LC separation, the possible use of the 1st dimension chromatograms to determine FAEs and to establish the proportion of the hydrocarbon series in industrial samples was studied. In comparison to the integration of the peaks of all the oligomers along the 2nd dimension chromatograms of the successive series, to integrate a single peak per series along the 1st dimension saves time and reagents, and is much simpler. However, the areas of the peaks of the series cannot be related to the series concentration without taking into account the different UV–vis response factors of the EO oligomers with respect to the compound used as calibration standard. Lauryl alcohol, $C_{12}OH$, has been frequently used as standard, the response factors of the oligomer being

calculated with respect to it [6]. The response factors of the oligomers usually differ from one. Thus, the response factors of the phthalates increase slightly with *n* and decrease with *m* at least up to *m* = 4 [14]. Since the response factors of the oligomers depend on *m*, to establish the molar concentration of a series by using the peak areas of the series along the 1st dimension having a single compound as reference standard is not straightforward. However, as next deduced, this is possible if the peak areas of the series are divided by an average response factor of the series. To establish the average factors of the series, a condition is that, for a series with *n* carbon atoms in the alkyl chain and an average EO number *m*, the peak area of the series along the 1st dimension should equal the sum of the peak areas of the oligomers of the same series along the 2nd dimension:

$$A_{n,\bar{m}} = \sum_{m} A_{n,m} \tag{5.1}$$

where the sum is extended to all the significant peaks of the oligomers of the series along the 2nd dimension chromatogram. We have checked Eq. (5.1) using the experiments given in **Figs. 5.2, 5.4 and 5.5** (and other chromatograms), having found a perfect agreement between the peak areas, with relative standard deviations of the differences ranging from 0.6% to 2.9% for the *n* = 12 and *n* = 14 series, respectively. The concentration of a given series with *n* carbon atoms in the alkyl chain, C_n, should equal the peak area of the series along the 1st dimension, $A_{n,\bar{m}}$, divided by the sensitivity (slope of the calibration curve) for the derivative of the reference compound ($C_{12}OR$), $s_{12,0}$, and by the proposed average response factor of the series, $f_{n,\bar{m}}$:

$$C_n = \sum C_{n,m} = \frac{A_{n,\bar{m}}}{s_{12,0}\ f_{n,\bar{m}}} \tag{5.2}$$

where $C_{n,m}$ is the concentration of the derivative of the *m*-oligomer of the *n*-series, C_nE_mOR, also including that of the non-ethoxylated alcohol (*m* = 0), and where the sum is extended to all the EO oligomers which constitutes the *n*-series. In Eq. (5.2), the bar over the subscript $\bar{m}$ indicates that the area corresponds to the peak of a series along the 1st dimension chromatogram, and that the response factor is an averaged value for the whole series. The total FAE concentration is the sum of the concentrations of all the series of the mixture:

$$C_{FAE} = \sum C_n \tag{5.3}$$

By substituting Eq. (5.2) in Eq. (5.3), the molar fraction of an *n*-series can be obtained as:

$$\frac{C_n}{C_{FAE}} = \frac{A_{n,\bar{m}} / f_{n,\bar{m}}}{\sum \left(A_{n,\bar{m}} / f_{n,\bar{m}} \right)} \tag{5.4}$$

The average response factors of the series, $f_{n,\bar{m}}$, can be established as next explained. For the derivative of a given oligomer, C_nE_mOR, we have:

$$C_{n,m} = \frac{A_{n,m}}{s_{12,0}\ f_{n,m}} \tag{5.5}$$

where $A_{n,m}$ and $f_{n,m}$ are the peak area of the oligomer derivative along the 2nd dimension and the response factor of the oligomer derivative with respect to that of the reference compound, respectively. Reorganizing in Eq. (5.2), and substituting the value of $C_{n,m}$ given by Eq. (5.5), we have:

$$f_{n,\bar{m}} = \frac{A_{n,\bar{m}}}{\sum \frac{A_{n,m}}{f_{n,m}}} \tag{5.6}$$

As deduced from Eq. (5.6), the proposed average response factors of the series depend on both the response factors of the EO oligomers and the distribution of their peak areas within the series. Therefore, the average response factors also depend on the concentration distributions of the EO oligomers. The EO distributions of the n = 12 and 14 series of surfactants having $\overline{m}$ = 3, 7, 10 and 23, calculated as molar fractions of the oligomers, are plotted in **Fig. 5.6**. To obtain the molar fractions, the peak areas of the oligomers along the 2nd dimension chromatograms were first corrected by dividing by their respective response factors [14]. Then, the corrected peak areas were divided by the sum of all the corrected peak areas of the EO oligomers of the series. As observed, by comparing the traces for the n = 12 and 14 series, the EO molar distributions are approximately the same for all the series which constitute an industrial FAE. Therefore, the average response factors of the series can be tabulated as functions of n and $\overline{m}$ for all industrial FAEs.

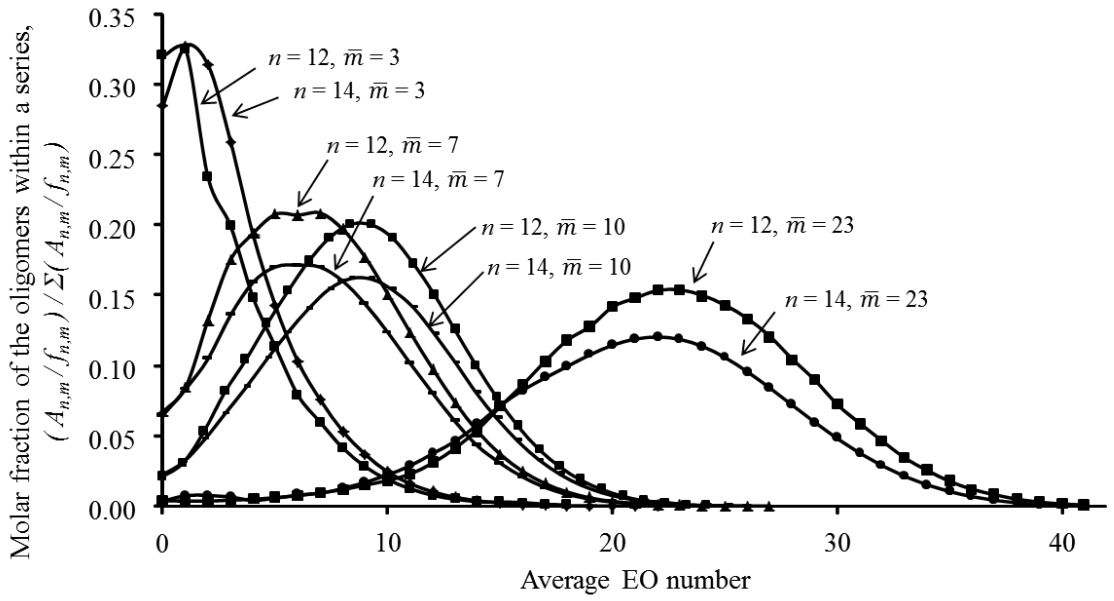

Fig. 5.6. Distribution of the EO oligomers of the n = 12 and 14 series of industrial FAEs: Dehydol LS-3 DEO-N ($\overline{m}$ = 3), Dehydol LT-7 ($\overline{m}$ = 7), DEGDE ($\overline{m}$ = 10) and Brij 35 ($\overline{m}$ = 23). On the vertical axis the molar fractions of the EO oligomers within the series are plotted.

To calculate the average factors of the series, $f_{n,\bar{m}}$, the oligomer distributions of **Fig. 5.6** and the UV–vis response factors of the oligomers, $f_{n,m}$, which were taken from a previous work [14], were used. A plot of $f_{n,\bar{m}}$ as a function of *m* at increasing values of *n* is given in **Fig. 5.7**, and for convenience, tabulated values of $f_{n,\bar{m}}$, including the experimental and a few extrapolated data, are given in **Table 5.2**. To use this table, the value of *m* should be known. If not known, it can be obtained by chromatographing one of the series along the 2nd dimension. However, for industrial products containing mixtures of raw materials with different values of $\bar{m}$, it would be necessary to chromatograph more than one series.

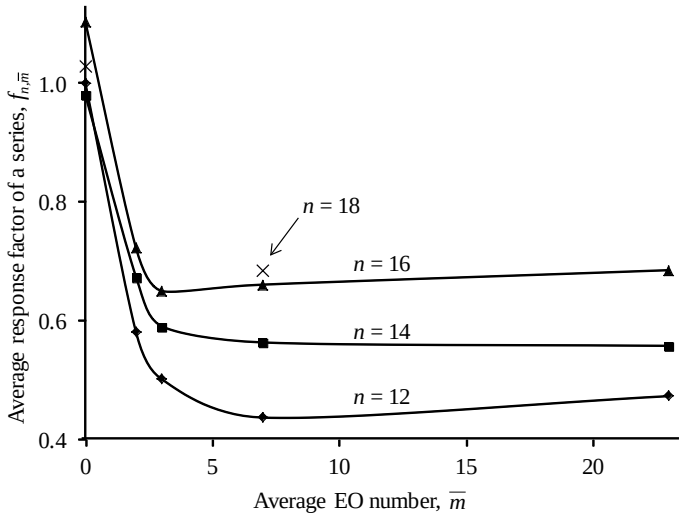

Fig. 5.7. Dependence of the average response factor of the hydrocarbon series of industrial FAEs with both *n* and $\bar{m}$. Values of $f_{n,\bar{m}}$ calculated according to Eq. (5.6) using the EO distributions of **Fig. 5.6** and others.

Table 5.2. Average response factors of the series for industrial FAEs

$\bar{m}$	*n*			
	12	14	16	18
0	1.00	0.98	1.10	1.03
2	0.58	0.67	0.72	0.76*
3	0.50	0.59	0.65	0.66*
7	0.44	0.56	0.66	0.68
10	0.44	0.56	0.69	0.72*
23	0.47	0.56	0.69	0.72*

*Extrapolated values

In **Table 5.3**, the molar fractions of the series of the industrial FAEs, obtained by the proposed quick method, based on measuring the series peak areas along the 1st dimension chromatogram followed by application of Eq. (5.4), are compared to those obtained by using the uncorrected peak areas of the series. As observed, if the peak areas are not corrected, the n = 12 series is underestimated in all cases, the other series being overestimated. The accuracy in establishing the relative series concentrations according to this procedure depends on both the accuracy of the response factors of the oligomers and that of the EO distribution. As shown, this later is established by chromatographing a series along the 2nd dimension, which can be done either within the same run in which the peak areas of the other series along the 1st dimension are established, or with a separate run. On the other hand, the UV–vis response factors of the phthalates of the oligomers used in this work were established in a previous work using a large number of standards [14]. The response factors should be established again if either a different derivatization reaction or another detection technique is used. Another question of practical interest is the influence of the accuracy of both the m values and the global response factors of the series on the accuracy of the determinations, including both the series distribution and the FAE concentration. As deduced from **Fig. 5.7**, $\bar{m}$ has little influence on the average response factor of the series, except

when $\overline{m}$ < 5. Then, for $\overline{m}$ > 5, inaccurate values of $\overline{m}$ can be used without diminishing the accuracy of the determinations. This is due to the negligible weight of the $\overline{m}$ < 3 oligomers on the global response factors of the series when $\overline{m}$ is large. This is important, because the main interest of using the peak areas of the series along the 1st dimension is in the analysis of mixtures having large values of *m*. When *m* = 3 or lower, an ACN/water gradient over one-dimension is enough to separate both the series and the oligomers within them with negligible overlapping of consecutive series [6,39]. Further, this can be extended to mixtures having up to *m* = 5 if series with only even or odd values of *n* are present. Then, accurate values of m are needed only when both even and odd series with m values ranging from 3 to 5 are present in the mixture.

Table 5.3. Comparison of the molar fractions of the series (in percentages) obtained by using Eq. (5.4) and by dividing the uncorrected peak areas of the series by the sum of the peak areas along the 1st dimension chromatograms

Industrial FAE	$\overline{m}$	*n* = 12		*n* = 14		*n* = 16		*n* = 18	
		Eq. (5.4)	Uncorr.	Eq. (5.4)	Uncorr.	Eq. (5.4)	Uncorr.	Eq. (5.4)	Uncorr.
Dehydol LS-2	2	76.3	73.5	22.6	25.2	1.1	1.3	-	-
Dehydol LS-3	3	77.1	74.0	21.8	24.6	1.1	1.4	-	-
Dehydol LT-7	7	64.6	56.1	20.3	23.8	7.7	10.1	7.4	10.0
Brij 35	23	75.4	71.8	21.7	24.2	2.9	4.0	-	-

5.4. Conclusions

Two ways of combining a 6-port 2-position valve and a column selector valve to implement a single-pump LC–LC system have been described and compared. Both the $V_{6/2}$-first and V_{CS}-first configurations have the capability of using different mobile and stationary phases to elute the 1st and 2nd dimensions, and also offer the possibility of automatically selecting different columns along

the dimension where V_{CS} is located. Both configurations also provide the ability of inverting the flow in the mainpass of $V_{6/2}$, which can be useful for the on-line preconcentration of solutes in the sample; however, further separation of the preconcentrated solutes on a 2nd column is only possible with the $V_{6/2}$-first configuration. The V_{CS}-first configuration was used in a previous work [35]. In this work, the $V_{6/2}$-first configuration was applied to the LC–LC separation of industrial FAEs according to the hydrocarbon series and the EO oligomers along the 1st and 2nd dimensions, respectively. For this purpose, two C8 columns and two MeOH/water and ACN/water complementary mobile phases at two different temperatures were used. Mixtures of FAEs containing both even and odd hydrocarbon series with long EO chains were well resolved along the 1stdimension, and the EO oligomers within the series were well separated along the 2nd dimension. This method is also of interest at preparative scale, to obtain purified fractions of the EO oligomers, particularly those having large values of *m*. In addition, the use of the 1st dimension chromatogram to accurately quantify the proportion of the series in industrial FAEs has been demonstrated. For this purpose, the use of an average response factor of the series has been proposed. The average response factors depend on both the length of the alkyl chain of the series, *n*, and the average EO number, $\overline{m}$. However, all FAE raw materials with a given nominal value of the average EO number, contain series with closely similar values of that number; further, samples containing just one FAE or as much as two raw materials are the most common. Thus, if not previously known, $\overline{m}$ can be established by chromatographing only one or two of the FAE series along the 2nd dimension. Although not common in industrial analysis, full resolution of all the series along the second dimension could be necessary with complex samples. Further, the average response factors depend only slightly on $\overline{m}$ when $\overline{m} > 5$. Thus, accurate values of m are required only when $\overline{m} < 5$, then, for samples which will

probably not require LC–LC separation. The proposed method is quick and simple, and can be implemented in any industrial laboratory provided with an ordinary HPLC system with minimal investment in new equipment. Further, the time required to operate a heart-cutting two-dimensional system can be largely reduced by using the currently available two-pumped 2D systems [40,41]. With them, the idle times for column conditioning and system flushing (ca. 15 min each time a mobile phase is replaced) will be avoided. In addition, quick elution at high pressures along one or the two dimensions may be also provided.

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CHAPTER 6.

Determination of the four major surfactant classes in cleaning products by reversed-phase liquid chromatography using serially connected UV and evaporative light-scattering detection.

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Determination of the four major surfactant classes in cleaning products by reversed-phase liquid chromatography using serially connected UV and evaporative light-scattering detection

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ABSTRACT

A method for the simultaneous determination of the most frequently used surfactant families- linear alkyl benzenesulphonates (LAS), alkyl ether sulphates (AES), fatty alcohol ethoxylates (FAE) and oleins (soaps, fatty acid salts) – in cleaning products, has been developed. The common reversed phase octyl (C8), pentafluorophenyl and biphenyl columns were not capable of separating the anionic LAS and AES classes; however, since only LAS absorbs in the UV, these two classes were independently quantified using a C8 column and serially connected UV and ELSD detection. The best compromise to resolve the four surfactant classes and the oligomers within the classes was achieved with a C8 column and an ACN/water gradient. To enhance retention of the anionic surfactants, ammonium acetate, as an ion-pairing agent compatible with ELSD detection, was used. Also, to shift the olein peaks with respect to that of the FAE oligomers, acetic acid was used. In the optimized method, modulation of the mobile phase, using ammonium acetate during elution of LAS and AES, and acetic acid after elution of LAS and AES, was provided. Quantitation of the overlapped LAS and AES classes was achieved by using the UV detector to quantitate LAS and the ELSD to determine AES by difference. Accuracy in the determination of AES was achieved by using a quadratic model, and by correcting the predicted

AES concentration according to the LAS concentration previously established using the UV chromatogram. Another approach also leading to accurate predictions of the AES concentration was to increase the AES concentrations in the samples by adding a standard solution. In the samples reinforced with AES, correction of the predicted AES concentration was not required. FAE and olein were quantified using also quadratic calibration.

Keywords: *Evaporative light scattering detection, fatty acids, fatty alcohol ethoxylates, alkyl ether sulphates, linear alkyl benzenesulphonates, olein, cleaning products.*

6.1. Introduction

Industrial and household cleaners mainly contain four major classes of surfactants, namely linear alkylbenzene sulfonates (LAS), alkyl ether sulfates (AES), fatty alcohol ethoxylates (FAE) and oleins or soaps, which are mixtures of salts of fatty acids [1, 2]. LAS and AES are anionic at all pHs and FAE are non-ionic, whereas the olein components are ionized at pH values over pH = pKa ≈ 5. LAS is obtained as mixtures mainly containing the C10-C13 homologues. Each homologue comprises from four to six isomers which differ in the attachment point of the p-sulfonate phenyl group to the linear alkyl chain, starting from the second carbon atom. LAS is most frequently analyzed by HPLC-UV using C8 columns and ACN/water in the presence of an ion-pairing agent such as sodium perchlorate [3-7] or a tetraalkylammonium salt [4, 8, 9, 10].

Both AES and FAE contain series of oligomers that differ both in the length of the hydrocarbon chain (the hydrophobic cut), and in the number of the condensed ethylene oxide (EO) units of the hydrophilic moiety. AES are the sulfuric acid esters of FAE, and then AES and FAE only differ in the nature of the terminal group of the hydrophilic tail, a sulfate and a hydroxyl, respectively. The separation of underivatized AES and FAE oligomers is most conveniently carried out using HPLC on C8 columns with an ACN/water gradient; however, an ion-pairing agent should be added to enhance retention of the oligomers of both LAS and AES [11]. Finally, the palm olein components (fatty acid salts) are also well resolved using the same chromatographic mode [12, 13].

LAS are normally detected using UV [14], although fluorescence can be also used [15]. The olein components can be also detected using UV at a low wavelength, but their molar absorptivities are low. Instead of this, UV detection of AES and FAE requires previous derivatization. This can be achieved with anhydrides [16-23] and other reagents [17, 18, 24]; however, upon hydrolysis of

the sulfate ester bond, AES gives rise to the same derivatives as FAE. For this reason, if these two classes should be independently characterized and quantified, separation of them before derivatization is mandatory. This can be achieved by ion-exchange on SPE cartridges [7, 8]. Instead of derivatization followed by UV, mass spectrometry [24-28], evaporative-light-scattering (ELSD) [18, 29] or charged-aerosol detection (CAD) [30, 32] of the underivatized AES and FAE oligomers can be used. However, it should be noted that the non-ethoxylated FAE oligomers (the fatty alcohols) are too volatile to be detected in an evaporative detector, and that the mono-ethoxylated FAE oligomers, that are also fairly volatile, are underestimated. Underestimation of these oligomers results in a small systematic error, unless FAE standards having the same EO distribution as that in the samples would be used for calibration. This is not a problem in quality control of manufactured products, since the EO distribution of the samples is known, but requires correction according to the observed EO distribution when an unknown sample is analyzed [33].

At the present time, HPLC-MS has become the preferred technique for the analysis of surfactants in industrial and environmental samples [15, 34, 35] because of its specificity, high capacity for identification of homologues and ethoxymers and capability of determining different surfactants at the same time [36, 37]. Another powerful tool which is gaining more importance in the analysis of cleaning product samples, due to the elevated amount of components of different nature that these samples contain, is two-dimensional HPLC (2D-HPLC) [33. 38, 39]. However, the development of HPLC-MS and 2D-HPLC methods, requires expensive instrumentation, which is not affordable for a big range of small industries and quality control laboratories. Instead of this, traditional and cheaper detection methods, such as UV-vis and ELSD, can still be used for quality control

of raw materials and industrial samples, where high sensitivity is not required as occurs for environmental samples.

Therefore, in this work, a cheap, practical and simple method capable of determining the four major surfactant classes mainly used in all types of cleaning products (LAS, AES, FAE and oleins) all in a single chromatographic run using UV-ELSD detection, has been developed. The best separation was achieved with a single C8 column, an ACN/water gradient, and with modulation of the concentrations of ammonium acetate (NH_4AcO, used as an ELSD compatible ion-pairing agent [40]) and acetic acid (HAcO) in the mobile phase. Using RP-HPLC, and in the presence of an ion-pairing agent, anionic surfactants elute first, followed by FAE and oleins. We have not found any RP system capable of separating the LAS and AES classes; however, in this work, we present an alternative method to HPLC-MS [37] for the independent evaluation of these two classes. First, using the UV chromatogram the LAS concentration was established, followed by the subtraction of this concentration from the sum of the LAS and AES concentrations obtained using ELSD, to finally obtain the AES concentration. The difficulties derived from the non-linear nature of the ELSD signal were overcome by using two different strategies which consisted in performing a quadratic calibration and the addition of an excess of AES to the samples. On the other hand, the olein peaks also overlap with the peaks of a few FAE oligomers; however, the successive FAE oligomers were well resolved, following a highly regular distribution pattern that made possible the accurate prediction of the areas of the overlapped peaks. Using this procedure, the high cost in instrumentation or the required time to make successive extractions of the different surfactant classes, using SPE protocols [7,8] and further derivatization can be avoided.

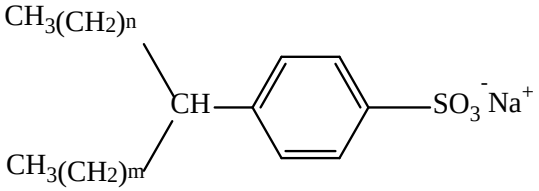
6.2. Experimental

6.2.1. Reagents, samples and standard solutions

The following analytical grade reagents were used: acetic acid (HAcO), methanol (MeOH), acetonitrile (ACN) (Scharlab, Barcelona, Spain) and ammonium acetate (NH_4AcO, Riedel de Haën, Seeltze, Germany). The industrial surfactants Dehydol LT-7 (fatty alcohol ethoxylates, FAE, with n = 12, 14, 16 and 18 carbon atoms in the hydrophobic tail, and an average EO number of 7, Cognis, Monheim, Germany), Lutensol AO7 (FAE with n = 13 and 15 and an average EO number of 7, BASF, Germany), alkyl ether sulfates (AES, sodium salts, with $12 \leq n \leq 18$ and average EO number of 3, Limsa, Barcelona, Spain), lineal alkylbenzenesulfonates (LAS, mixture of the $10 \leq n \leq 13$ homologues, Fluka, Steinheim, Germany) were used. Palm olein and other components of cleaning products were kindly supplied by Químicas Oro (San Antonio de Benagéber, Valencia, Spain). In **Table 6.1**, the structure and specifications of the used standards can be observed. Stock standard solutions of 10 g L^{-1} of LAS and AES, and 5 g L^{-1} of FAE, were prepared in water. A stock standard solution of 5 g L^{-1} of palm olein was prepared by dissolving the proper amount with a NaOH solution in methanol, followed by dilution with water. Dilutions of the stock standard solutions were made with water. Samples of liquid detergents and dishwashers, supplied by Químicas Oro, were prepared in ca. 1 kg batches according to the full formulations of 6 different commercial products including laundry cleaners, handwashers and dishwashers. These formulations are constituted, by one or more surfactants and other components including water, boric acid, sodium chloride, triethanolamine, ethylendiaminetetraacetic sodium salt, alkyl phosphonate sodium salts, colorants, fragrances, opacifiers and preservatives. Portions of these samples were weighed and diluted with water. Deionized water (Barnstead deionizer, Sybron, Boston, MA) was used in all cases. Identification of the olein components

was made by injecting 100 mg L^{-1} standard solutions of the following fatty acids: palmitic (C16:0), stearic (C18:0) (Sigma-Aldrich, St. Louis, USA), myristic (14:0), myristoleic (14:1) and oleic (C18:0) acids (Fluka, Steinheim, Germany). These solutions were prepared in a 85:15 propanol-methanol mixture containing 40 mM NH_3.

Table 6.1. Structure and main features of the main surfactant classes studied in this work.

Name	Structure	n	m
Linear alkyl benzenesulfonate (**LAS**)		n+m = 7-10	
Alkyl ether sulfate (**AES**)	$CH_3(CH_2)_nO(CH_2CH_2O)_mOSO_3^-Na^+$	$11 \leq n \leq 17$	$\overline{m}$ = 3
Olein/Soap	$CH_3(CH_2)_nCOO^-Na^+$	$13 \leq n \leq 17$	
Fatty Alcohol Ethoxylate (**FAE**)	$CH_3(CH_2)_nO(CH_2CH_2O)_mH$	Lutensol AO7 n = 12,14 Dehydol LT-7 n= 11, 13, 15, 17	$\overline{m}$ = 7

6.2.2. Instruments and HPLC separation

A 1100 Series HPLC chromatograph (Agilent Technologies, Waldbronn, Germany), provided with a quaternary pump, including a thermostated column compartment and a UV-Vis diode array detector connected in tandem with an ELSD (385-ELSD, Agilent Technologies), was used. For the optimized procedure, an Ascentis Express fused-core C8 column (Sigma-Aldrich, Steinheim, Germany, 2.7 μm, 90 Å, 15 cm x 4.6 mm) was used. Other tested columns were an Ascentis Express fused-core C8 column (Sigma-Aldrich, 5 μm, 90 Å, 10 cm x 4.6 mm), a Kinetex pentafluorophenyl column (F5, Phenomenex, 2.6 μm, 100 Å, 10 cm x 4.6 mm) and a Kinetex biphenyl column (BP, Phenomenex, 2.6 μm, 100 Å, 10 cm x

4.6 mm). The solutions were passed through a 0.45 μm pore-size nylon filter (Albet, Barcelona, Spain) and 20 μL was injected. The flow rate was 1 mL min^{-1}. UV detection was set at 225 ± 10 nm using 360 ± 60 nm as reference. For ELSD, the nebulizer and evaporator temperatures were set at 40 ºC, and the gas flow was set at 1.6 SLM (standard liter per min). In the optimized procedure, separations were performed at 15 ºC using an ACN-water gradient achieved by mixing four solutions by pairs (A-B and C-D) as follows. Thus, solution A contained 10 mM NH_4AcO (pH = 6.7) in water and solution B was a 90:10 ACN-water mixture also containing 10 mM NH_4AcO. Solution C was a 17.5 mM (0.1%) HAcO aqueous solution (pH = 3.2), whereas solution D also contained 17.5 mM (0.1%) HAcO but in a 90:10 ACN/water mixture. Lower and higher NH_4AcO and HAcO concentrations were used during optimization of the chromatographic separation. Gradient elution was started by mixing A and B. For the optimized procedure, the percentage of B was linearly increased from 50 to 65% in 12 min. After t = 12 min, solutions A and B were substituted by solutions C and D, respectively, but maintaining 65% of D at the time of replacing the solutions. Then, D was increased from 65 to 100 % in 28 more min (the total ACN gradient time was 40 min). Substitution of the solutions was easily implemented by programming the quaternary pump.

6.3. Results and discussion

6.3.1 Selection of the stationary and mobile phases

In order to resolve the four surfactant classes in a single run, the following RP columns were tried: C8, F5 and BP. A mixture of the four surfactant classes (500 μg mL^{-1} of each) was injected. Concerning the mobile phase, both ACN-water and MeOH-water were tested. The F5 and BP columns always showed closely similar chromatograms. That obtained with the F5 column with ACN/water is shown in **Fig. 6.1A**. As observed, in the absence of ionic additives in the mobile

phase, the anionic surfactant classes, LAS and AES, were not sufficiently retained [11]. After a few minutes, the successive hydrocarbon series of the FAE oligomers, and the peaks of the components of olein (mainly palmitic and oleic acids), were eluted. Also in the absence of an ion-pairing agent, the C8 column gave the same elution order as the two other columns, but with a low-efficient separation of the bands of the four LAS homologues that overlapped with the bands of the unresolved AES oligomers (**Fig. 6.1C**). Instead of this, the peaks of the EO oligomers within the FAE hydrocarbon series were well resolved by the C8 column. According to the literature, retention of the LAS oligomers on a C8 column increases and becomes reproducible in the presence of ion-pairing agents as $NaClO_4$ [3-6], a tetraalkylammonium salt [9] or using NH_4AcO [10, 40]; NH_4AcO which is compatible with ELSD detection, was used in this work. Also, an ion-pairing agent has been recommended to increase retention of the AES oligomers [11]. As shown in **Fig. 6.1B** for the F5 column, retention of both LAS and AES, increased in the presence of NH_4AcO, and the LAS homologues and AES oligomers were also resolved within the respective classes, although these two anionic surfactant classes, LAS and AES, largely coeluted. On the other hand, the presence of NH_4AcO did not modify the elution of the FAE and olein components. In the presence of NH_4AcO, both the BP and C8 columns showed chromatograms similar to that obtained with the F5 column (**Fig. 6.1B**), with the only remarkable difference that the C8 column also resolved the EO oligomers within the FAE series. With respect to the absence of ionic additives, both LAS and AES also showed a slightly higher retention and high-efficiency reproducible peaks by adding HAcO to the mobile phase (chromatograms not shown), which is assumed to be mainly due to the modification of the stationary phase [40].

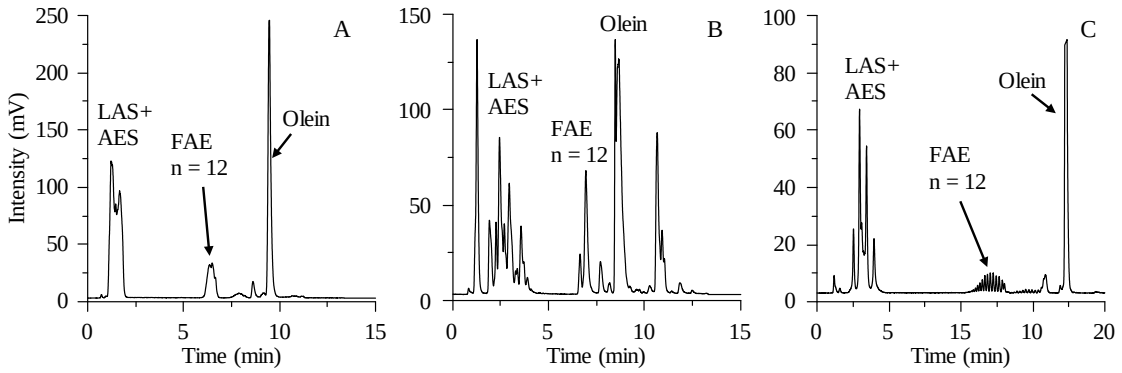


Fig. 6.1. Chromatograms of a mixture of the four surfactant classes (500 μg mL^{-1} of each class, Dehydol was used as FAE); from 40 % to 90% ACN in 20 min. F5 column, in the absence (A) and presence of 20 mM NH_4AcO (B); C8 column in the absence of ionic additives (C). Flow rate: 1 mL min^{-1}. Column temperature: 25 ºC. ELSD detection.

In any case, retention of the FAE oligomers was not significantly modified by the presence of NH_4AcO and HAcO; however, retention of the olein components increased by increasing the HAcO concentration of the mobile phase, which should be attributed to protonation of the carboxylate groups of these analytes. Substitution of ACN by MeOH gave rise to similar chromatograms, with the only remarkable difference that the EO oligomers of the FAE series were not resolved.

In order to distinguish the oligomers of the coeluting LAS and AES series, both the UV-Vis and ELSD detectors were serially connected. In this way, UV-Vis was used to monitor LAS, whereas the ELSD, that detected all the surfactant classes, was used to quantify AES by difference, as well as to quantify the FAE oligomers and the olein components. On the other hand, the conditions to achieve the best separation of the four classes were explored using pairs of serially connected columns. For this purpose, the C8 columns and the combinations F5-C8 and BP-C8, as well as the reverse combinations, were tried. In all cases, ACN/water gradients in the presence of 20 mM NH_4AcO, and HAcO concentrations ranging from 0 up to 35mM (0.2%), were tried. The best resolution

between the successive oligomers of all classes was obtained with the C8 columns. Thus, the 15-cm long C8 column was selected. In **Fig. 6.2**, parts A-C, the effect of increasing the NH_4AcO concentration using this column is shown. As observed, the retention of LAS and AES increased, but that of the FAE oligomers was only slightly modified. Then, to preserve a segment of flat baseline between the last peak of the AES class and the first one of FAE, 10 mM NH_4AcO was selected. The chromatogram obtained with both additives, namely 10 mM NH_4AcO and 17.5 mM (0.1%) HAcO (pH = 4.3), is shown in **Fig. 6.2D**. The effect of increasing the HAcO concentration using 3.5, 17.5 and 87.5 mM (0.02, 0.1 and 0.5%, respectively), while keeping constant the NH_4AcO concentration at 10 mM, was investigated. LAS and AES were more retained at increasing HAcO concentrations, thus HAcO further reduced the flat baseline region before elution of the first eluting oligomer of the n = 12 series of FAE. The peaks of the FAE oligomers were not shifted when the HAcO concentration was increased, but the olein components eluted at progressively longer retention times.

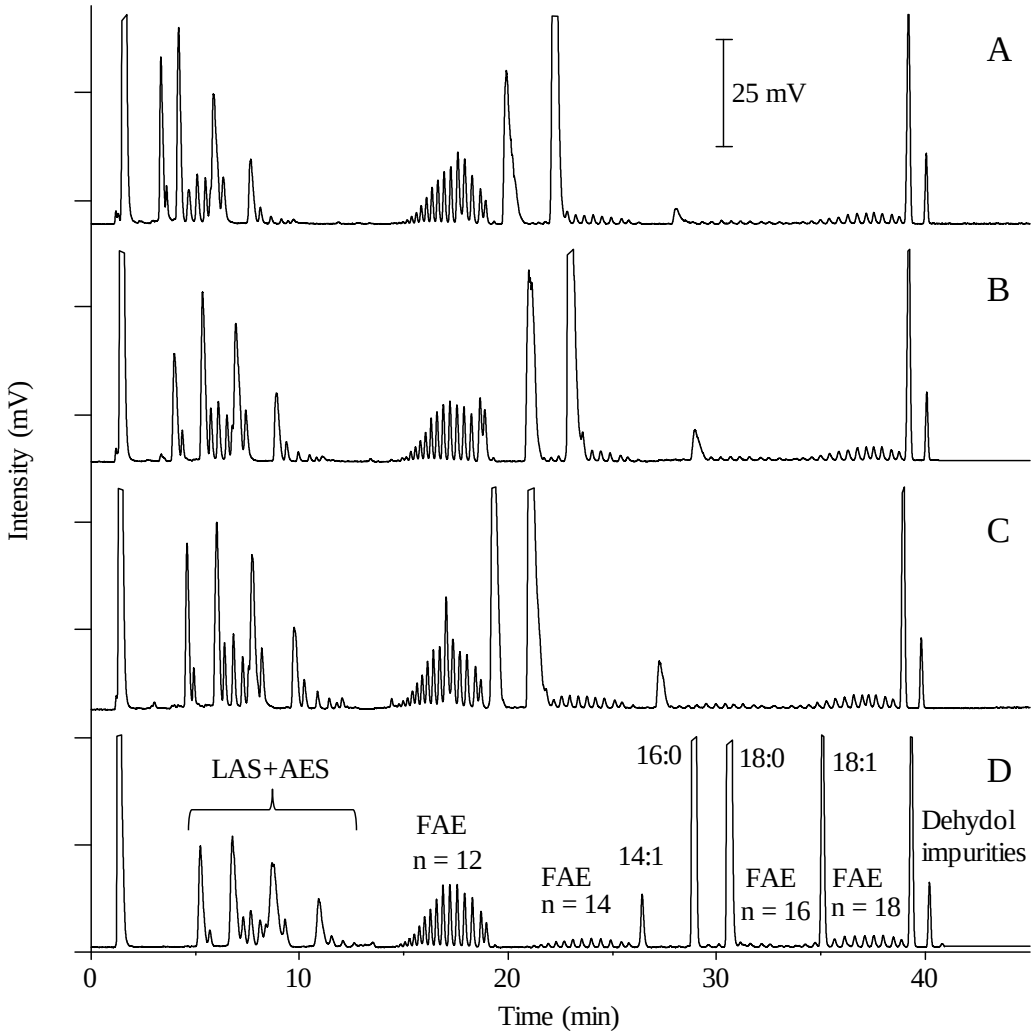


Fig. 6.2. Chromatograms of a mixture of the four surfactant classes (500 µg mL^{-1} of each class, Dehydol was used as FAE): C8 column (Ascentis Express, 2.7 µm, 150x4.6mm), from 45% to 90% ACN in 40 min in the presence of÷ 5 (A), 10 (B) and 20 mM NH_4AcO (C); both 10 mM NH_4AcO and 17.5 mM HAcO (D). Flow rate: 1 mL min^{-1}. Column temperature: 25 ºC. ELSD detection Identification of components: n=12 to n=18 are the successive FAE hydrocarbon series, showing the resolved oligomers according to their EO numbers; (14:1) myristoleic acid, (16:0) palmitic acid, (18:0) stearic acid and (18:1) oleic acid, the two last peaks are Dehydol impurities.

Using the optimized conditions of **Fig. 6.2D**, mobile phase modulation was implemented in order to enhance retention of LAS and AES and independently shifting the peaks of the olein components according to the pH of the mobile phase. For this purpose, a 10 mM NH_4AcO was selected to provide enough retention of

the LAS and AES peaks by ion-pairing, while preserving a 2-min long flat baseline region after elution of the anionic surfactants and before elution of the n = 12 FAE series. By introducing the HAcO after t = 12 min, modifications of the pH of the mobile phase, which are required to modify retention of the olein components, can be performed without altering the elution of the LAS and AES classes. To preserve the retention time region before t = 12 min unaltered was also essential to avoid changes in the slopes of the calibration curves of the LAS and AES classes during the quantitation studies. Then, elution of the FAE oligomers and olein components was made in the absence of NH_4AcO but in the presence of variable amounts of HAcO. For this purpose, at t = 12 min phases A and B, each containing 10 mM NH_4AcO, were substituted by phases C and D, which could contain variable HAcO concentrations. This allowed the modification of the retention of the olein components with respect to that of the FAE oligomers without disturbing retention of the already eluted LAS and AES oligomers. The ACN gradient, was not altered by the substitution of the phases, since phases A and B contained the same ACN concentrations as phases C and D, respectively, and they were mixed also in the same proportions. Namely a 65% of B was substituted by a 65% of D when t = 12 min. A chromatogram obtained in these optimized conditions, with a C8 column, and by substituting the constant 10 mM NH_4AcO concentrations by a constant 17.5 mM (0.1%) HAcO concentration at t = 12 min, just before elution of the n = 12 FAE series, is shown in Fig. 3, part B. The effect of the HAcO during the second part of the chromatogram was to delay the peaks of the olein components with respect to that of the FAE oligomers. Thus, using 17.5 mM (0.1%) HAcO, the first peak of the olein components, corresponding to the 14:1 oligomer (myristoleic acid), appeared at the end of elution of the n = 14 FAE series, whereas the main peaks of palm olein, that of the 16:0 and 18:1 components (palmitic and oleic acids) overlapped with the peaks of the oligomers of the n = 16 FAE series.

Overlapping of the FAE and olein peaks is not a problem, since the areas of the overlapped FAE peaks can be accurately predicted by interpolation, using the areas of the neighbouring peaks within the same hydrocarbon series and along the EO series. Further, the interpolated areas can be subtracted from the total peak area to obtain the corrected area of the corresponding olein component. If necessary, retention of the olein components can be increased without altering the retention times of the other surfactant classes by increasing the HAcO concentration in mobile phase components C and D.

In **Fig. 6.3**, the effect of temperature on the optimized procedure is also shown. As observed, elution of LAS and AES was not modified by varying the column temperature from 15 to 35 ºC, respectively. However, the successive EO oligomers within the FAE series were better resolved at a low temperature; thus, 15 ºC was selected to perform the quantitation studies. In **Figs. 6.2** and **6.3**, the last two peaks of the traces were exclusively present in the injections of the Dehydol standards and in mixtures containing Dehydol, then, they were attributed to impurities of this raw material. They could correspond to esters because their retention times did not vary by modifying the HAcO concentrations, indicating that they were not fatty acids.

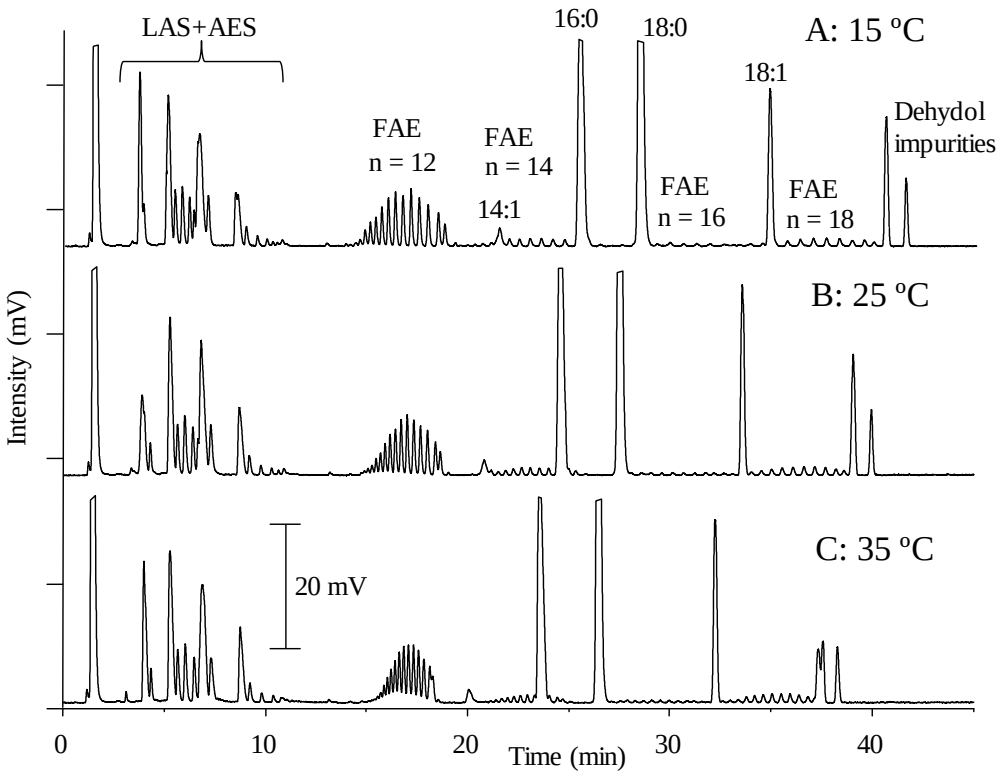


Fig. 6.3. Chromatograms of a mixture of the four surfactant classes: C8 column, from 45% to 90% ACN in 40 min in the presence of 10 mM NH_4AcO before t = 12 min and 17.5 mM HAcO afterwards. Chromatograms were obtained at 15 ºC (A), 25 ºC (B) and 35 ºC (C). Other details as in Fig. 6.2.

6.3.2. Quantitation of the LAS and AES classes.

Calibration curves of the surfactant classes were next constructed. Since anionic and non-ionic surfactants did not overlap each other, then binary mixtures of the LAS and AES classes on one side, and FAE (both Dehydol and Lutensol) and olein solutions on the other side, were prepared for calibration. Series of standard mixtures containing different amounts of LAS and AES were prepared as follows. The sum of the LAS and AES concentrations, x_1, can be defined as:

$$x_1 = C_{LAS} + C_{AES} \qquad (6.1)$$

C_{LAS} and C_{AES} being the total LAS and AES concentrations in mg L^{-1}, respectively. The percentage of LAS in the mixtures, x_2, can be defined as:

$$x_2 = 100\% \cdot C_{LAS} / x_1 = 100\% \cdot C_{LAS} / (C_{LAS} + C_{AES}) \quad (6.2)$$

Accordingly, seven series of six standards each were prepared for calibration. The series contained the following six values of the sum of concentrations, x_1 = 100, 300, 500, 800, 1000 and 1200 mg L^{-1}. The series differed from each other in the LAS percentage as follows: x_2 = 0, 10, 25, 50, 75, 90 and 100%. Unfortunately, the chromatograms did not show any region with wholly isolated peaks of either, LAS nor AES, also showing enough intensity. Then, as indicated in **Fig. 6.4**, calibration was tried by integrating the peaks along four different retention time zones, as well as by using the sum of all the four time zones (integration along all the peaks of the two classes from line a to line b of **Fig. 6.4**). Only region 3 of **Fig. 6.4** corresponded to a partially isolated peak of AES, the other three zones containing extensively overlapped peaks of both LAS and AES. The small peaks that appeared after elution of the last LAS homologue also correspond to isolated AES oligomers, but they were too small in relation to the total area of the surfactant class, then, this final elution region was not used for calibration. Calibration to establish the total concentrations of the two classes was tried using the calibration approaches next explained, by using both the independent areas of the zones 1 to 4, and the sum of them (from the time a to time b lines of **Fig. 6.4**).

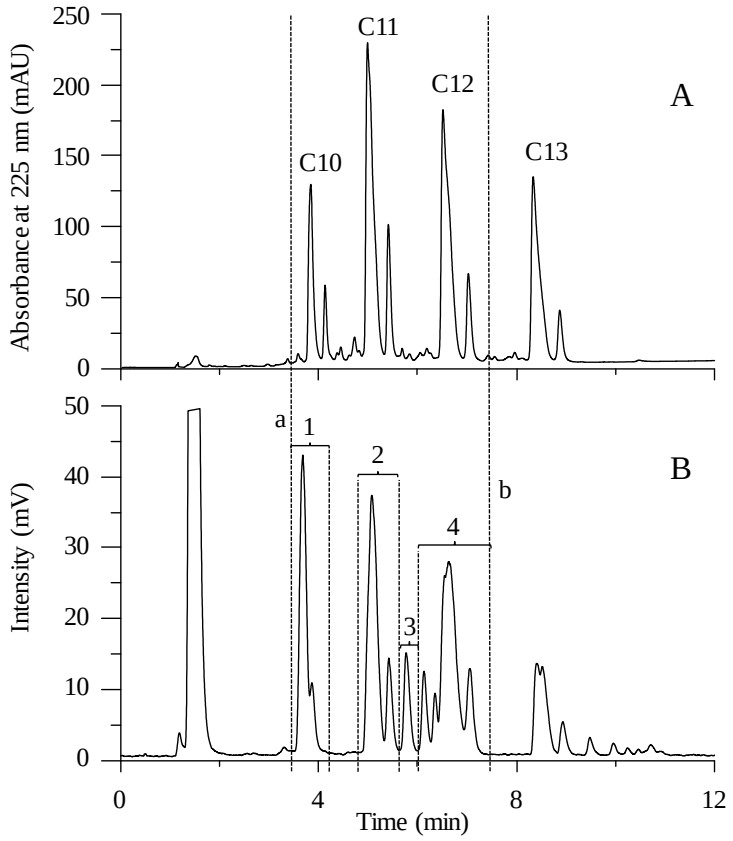


Fig. 6.4. Chromatograms of a mixture of LAS and AES obtained as indicated in Fig. 3A, Detection: UV (A) and ELSD (B). Sections 1 to 4 indicate the time intervals used for calibration of LAS and AES; Total area in the time interval from a to b was eventually used in the optimized calibration procedures. In part A, the labels on the groups of two peaks indicate the LAS homologues.

This latter approach is the simplest, and provided the smallest systematic errors with all the calibration approaches that were tried, thus, it was eventually adopted. As expected, the UV chromatogram gave rise to a linear calibration for LAS, whereas convex curves were obtained in all cases for ELSD. It has been reported [41, 42] that the ELSD convex curves fit well to a potential equation:

$$y_1 = a\, x_1^b \qquad (6.3)$$

where y_1 is the integrated peak area, and a and b are the fitted model parameters; however, as shown in **Fig. 6.5**, another excellent fit was obtained using the quadratic equation:

$$y_1 = a_1 + b_1 x_1 + c_1 x_1^2 \quad (6.4)$$

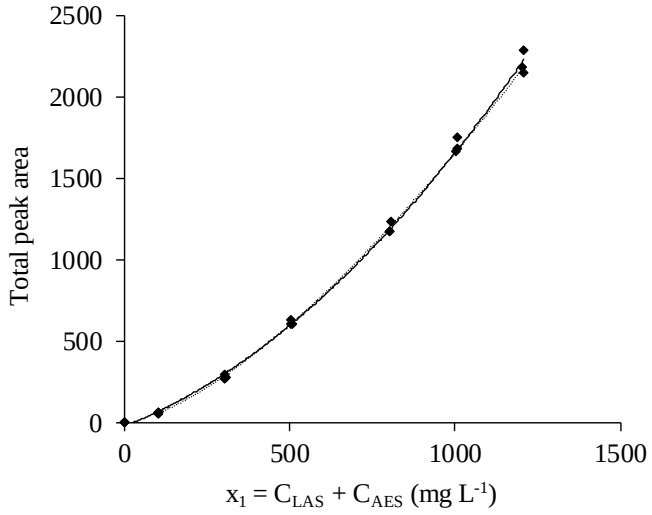


Fig. 6.5. ELSD calibration curves of LAS+AES mixtures according to the mixtures of standards (see text for details). Continuous line: quadratic fitting according to Eq. (6.4); dashed line: potential fitting according to Eq. (6.3); Total peak area corresponds to the total area obtained for the sum of the LAS and AES peaks (from line a to line b of Fig. 6.4).

An advantage of the quadratic model is that the zero point can be included, which is not possible using the exponential equation. The quadratic model was selected. Then, at the sight of the excellent fittings, it seemed possible to use ELSD calibration according to Eq. (6.4) to estimate the sum of the LAS and AES classes, x_1, and then to obtain the AES concentration, C_{AES}, after subtraction of the LAS concentration, C_{LAS}, that can be obtained from the UV chromatogram. We have:

$$y_{UV} = b_{UV} C_{LAS} \quad (6.5)$$

where y_{UV} is the area of the LAS peaks on the UV chromatogram. Then, we have:

$$C_{AES} = x_1 - C_{LAS} \quad (6.6)$$

where x_1 and C_{LAS} are obtained from Eqs. (6.4) and (6.5), respectively. As shown in **Table 6.2**, the C_{AES} values calculated using Eqs. (6.4), (6.5) and (6.6), were biased because the fitted parameters, b_1 and c_1 (the 1st and 2nd degree ELSD sensitivities), also depended on the percentage of LAS in the mixture, x_2. The dependence of the peak areas respecting x_2 was observed for the 4 integration zones of **Fig. 6.4**, as well as for the sum of them. For the sum of the four integration zones, the dependence of the peak areas regarding x_2 is depictured in **Fig. 6.6**.

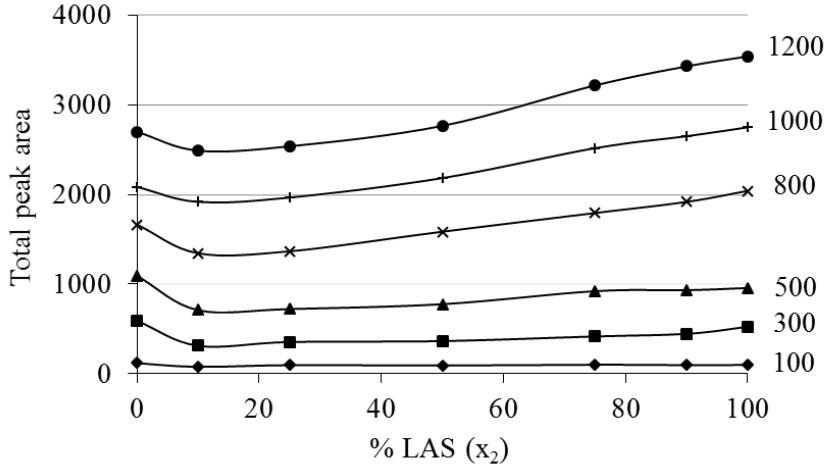


Fig. 6.6. Total peak area of the mixtures of LAS and AES obtained using ELSD detection plotted against the LAS percentage, x2, at increasing values of the sum of the concentrations, $x_1 = C_{LAS}+C_{LES}$. The numbers at the right of the lines are total concentrations, x_1, in mg L^{-1}. Chromatographic conditions as in Fig. 6.3A.

In this figure, the total peak area, y_1, is plotted against x_2 at increasing values of the sum of concentrations of the classes, x_1. As observed in **Fig. 6.6**, the sensitivity depended very little on x_2 along an extensive region, roughly within the limits $0.1 \leq x_2 \leq 0.5$, and particularly at low x_1 values, increasing significantly outside this region. Thus, direct application of the quadratic model of Eq. (6.4) produces systematic errors if the influence of the LAS/AES ratio on the sensitivity is not taken into account. This can be observed on **Table 6.2** (results according to footnote *a* of the table), where the quadratic model (Eq. 6.4) was applied to the analysis of 6 commercial cleaning products without any further correction. As

observed, C_{AES} was obtained with large positive systematic errors in all cases. Thus, to avoid bias, two strategies based on the modification of either the calibration model or the sample composition was tried. A possibility is to use multiple quadratic regression:

$$y_1 = a_1 + b_1 x_1 + c_1 x_1^2 + b_2 x_2 = a_1 + b_1 x_1 + c_1 x_1^2 + b_2 C_{LAS} / x_1 \quad (6.7)$$

The main drawback of this and other similar approaches is that they result in complex models with many fitting parameters, being also of a 3rd or higher degree in x_1. A more simple solution is to predict x_1 using the quadratic model of Eq. (6.4), followed by a correction of the predicted value of x_1. For this correction, the already known value of C_{LAS} can be used. We have used the following simple linear model:

$$C_{AES} = a_2 + b_2 y_1 + c_2 C_{LAS} \quad (6.8)$$

The coefficients of Eq. (6.8) were obtained by linear fitting using the calibration data. These were $a_2 = –0.036$, $b_2 = –1.124$, and $c_2 = –0.906$. Eq. (6.8) was used to correct the C_{AES} values obtained by using Eqs. (6.4) to (6.6). As also shown in **Table 6.2**, this calibration approach, that is, to correct the C_{AES} value obtained from Eq. (6.4) according to Eq. (6.8) led to a significant reduction of the systematic errors. As observed in **Table 6.2** (results according to footnote *b* of the table), these did not surpassed 7.7% for the prediction of C_{AES} in any of the six real samples.

However, another approach based on a modification of the sample composition was also tried. As observed in **Fig. 6.6**, within the region $0.1 \leq x_2 \leq 0.5$, the sensitivity depended only slightly on x_2. Thus, another solution tried to avoid bias was to use only standards and samples within the limits $0.1 \leq x_2 \leq 0.5$, making sure that the samples were also confined within these limits. Usually, detergents contain higher amounts of LAS than AES ($C_{LAS} > C_{AES}$), then, virtually all samples can be made to contain a LAS percentage within the $0.1 \leq x_2 \leq 0.5$

limits by adding a large fixed amount of AES to them. Accordingly, a fixed amount of AES was added to the 6 real samples before injection. This was made by adding an aliquot of the 10 g L^{-1} AES stock solution to all the samples, thus to increase the AES concentration of the injected solutions in an additional amount equal to 2000 mg L^{-1}. Then, the simple quadratic model, Eq. (6.4), was obtained by fitting using only the calibration mixtures with $0.1 \leq x_2 \leq 0.5$. The values of C_{AES} of the samples were obtained by subtracting 2000 mg L^{-1} from the predicted C_{AES}. This subtraction did not increase significantly the systematic error of the predicted C_{AES} values, since the amount of standard added to the samples was always accurately known. As also shown in **Table 6.2** (results according to footnote *c* of the table), the systematic errors for the AES spiked samples were small, even smaller than those obtained by correcting the predictions according to Eq. (6.8).

The limits of detection (LOD) and quantization (LOQ) of the method, were obtained using the ratios $3s_b/b$ and $10\ s_b/b$ respectively. Where, s_b is the standard deviation of the background and b the curve calibration slope. The S_b was estimated by measuring 10 standard of each surfactant in a concentration close to their respectively LOD. Calibration slope, b, was obtained from the linear fitting for the standards located within the low concentration range. LOD and LOQ were 2 and 6 mg L^{-1} for LAS and 20 and 60 mg L^{-1} for AES, respectively.

Table 6.2: Determination of LAS and AES in 6 real cleaning products.

Sample	Calibration method	LAS+AES (%)		LAS (%)		AES (%)	
		Declared	Found (Rel. Error %)	Declared	Found (Rel. Error %)	Declared	Found (Rel. Error %)
1	a	5.2	6.1 (18.0)	0	0 (not applies)	5.2	6.1 (18.0)
	b		6.1 (18.0)				5.5 (5.6)
	c		5.3 (3.3)				5.5 (6.1)
2	a	5.5	6.0 (10.2)	1.6	1.6 (3.7)	3.9	4.7 (19.9)
	b		6.0 (10.2)		1.6 (3.7)		3.6 (-7.7)
	c		5.5 (0.3)		1.6 (2.3)		3.9 (0.2)
3	a	8.0	9.4 (17.8)	2.0	2.2 (9.8)	6.0	7.6 (27.8)
	b		9.4 (17.8)		2.2 (9.8)		6.0 (0.1)
	c		7.9 (-0.5)		2.1 (3.1)		5.5 (-7.7)
4	a	10.9	12.9 (18.5)	4.7	4.9 (4.5)	6.2	9.7 (57.2)
	b		12.9 (18.5)		4.9 (4.5)		6.1 (-0.9)
	c		10.9 (-0.2)		4.7 (-0.9)		6.2 (-0.3)
5	a	8.7	10.9 (25.4)	5.3	5.6 (5.9)	3.4	8.1 (136.8)
	b		10.9 (25.4)		5.6 (5.9)		3.5 (4.3)
	c		8.7 (0.2)		5.3(0.4)		3.5 (1.7)
6	a	9.3	10.3 (10.6)	0.6	0.6 (-2.5)	8.7	11.7 (34.3)
	b		10.3 (10.6)		0.6 (-2.5)		8.6 (-0.9)
	c		9.2 (-1.4)		0.6 (-8.0)		8.2 (-5.6)

[a]: Mass percentage of the class predicted according to Eq. (6.4).
[b]: Mass percentage of the LAS and AES classes predicted according to Eq. (6.4) with correction of C_{AES} according to Eq. (6.8).
[c]: Mass percentage of the LAS and AES classes predicted according to Eq. (6.4) after increasing C_{AES} in a further 2000 mg L^{-1} in the injected sample solution.

6.3.3. Quantitation of FAE and olein

To determine the FAE and the olein classes a blank independent series of six standard solutions of Dehydol, Lutensol and palm olein containing from 50 to 2000 mg L^{-1} were injected. In all cases, the quadratic expression of Eq. (6.4) was used for calibration. The total concentration of the FAE and olein classes in the samples was established by measuring the sum of all the oligomers. The areas of the FAE peaks overlapping with olein peaks were established as the average area of the peaks of the two neighboring FAE oligomers. The interpolated areas were added to the total area of the FAE peaks and subtracted from the area of the corresponding olein peak. The corrections slightly increased the positive errors of FAE and decreased that of oleins. As observed in **Table 6.3**, satisfactory accuracy was obtained in all cases.

The LODs and LOQs for FAEs and olein were obtained as described previously for LAS and AES in section 6.3.2. LOD and LOQ were, 50 and 150 mg L^{-1} for FAEs and 10 and 30 mg L^{-1} for olein, respectively.

Table 6.3: Determination of FAE and olein in 6 real cleaning products.

Sample	FAE (%)		Olein (%)	
	Declared	Found[a] (Rel. Error %)	Declared	Found[a] (Rel. Error %)
1	4.3	4.5 (4.3)	3.1	3.2 (3.0)
2	0.0	0 (n. c)	4.6	4.5 (-2.2)
3	5.4	5.6 (4.2)	2.5	2.5 (-1.3)
4	4.1	4.1 (-0.6)	3.0	3.0 (-1.4)
5	5.6	6.1 (8.2)	3.4	3.4 (0.6)
6	3.4	9.0 (8.7)	4.4	4.4 (-0.1)

[a] Mass percentage of the class predicted according to Eq. (6.4).

6.4. Conclusions

An affordable HPLC procedure capable of determining, using a single chromatographic run, the four major surfactant classes present in household cleaning products, has been developed. Anionic surfactants, including LAS and AES, elute earlier than non-ionic surfactants (fatty alcohol ethoxylates) and fatty acid salts (soaps); however, a reversed-phase HPLC system capable of separating the anionic LAS and AES classes was not found; then, the strategy followed was to use serially connected UV and ELSD detection. Only LAS provides peaks by using UV, then the LAS concentration can be obtained from the UV record and the resulting value can be subtracted from the sum of the LAS and AES concentrations predicted from the ELSD chromatogram, thus to obtain the AES concentration; however, the ELSD calibration is non-linear also depending slightly on the LAS/AES ratio. For this reason, quadratic calibration gives rise to a systematic error. Two solutions were successfully tried in this work. First, to linearly correct the predicted AES concentrations making use of the LAS concentrations obtained from the UV chromatogram. Second, to raise the AES concentrations of the samples, thus to perform the determinations at low LAS/AES concentration ratios, within the region where the sensitivity depends very little from this factor. This later was achieved by adding a known amount of AES to the samples before analysis. These two approaches maybe of interest in any other cases in which coeluting species are calibrated using UV and ELSD. The procedure has been applied to the quantification of the four most frequently found surfactant classes in a range of common cleaning products with success.

Acknowledgments

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6.5. References

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SECTION IV.

MONOLITHIC STATIONARY PHASES

CHAPTER 7.

Preparation and characterization of octadecyl acrylate monoliths for capillary electrochromatography by photochemical, thermal and chemical initiation

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Research Article

Preparation and characterization of octadecyl acrylate monoliths for capillary electrochromatography by photochemical, thermal, and chemical initiation†

ABSTRACT

Octadecyl acrylate based monolithic stationary phases for capillary electrochromatography using different initiating systems (UV irradiation, thermal and chemical initiation) in the presence of lauroyl peroxide as initiator were synthesized. For each initiation mode, the influence of porogenic solvent composition on both morphological and electrochromatographic properties of the resulting monoliths was investigated. Under optimal conditions, excellent efficiencies for photo- and chemical polymerized monoliths (minimum plate heights of 6.9-10.7 µm and 6.5-12.6 µm, respectively) were achieved. Thermal initiated columns gave lower efficiency values, permeabilities and longer analysis times compared to these initiating systems. The produced monolithic stationary phases were evaluated in terms of reproducibility, giving RSD values below 9.2, 10.6 and 9.8 % for UV-, thermal- and chemical-initiated columns, respectively.

Keywords: *Acrylate ester-based monolithic columns/ Capillary electrochromatography / Initiating systems/ Lauroyl peroxide*

7.1. Introduction

Capillary electrochromatography (CEC) is a separation technique defined as a hybrid of CE and HPLC [1], which combines the use of an electroosmotically driven mobile phase (EOF) and the typical separation mechanism of a stationary phase. Among CEC supports, polymeric stationary phases based on acrylate- and methacrylate-based monoliths, introduced in the early 1990s by Svec *et al.* [1, 2], are the most popular materials for CEC applications. The advantages of these stationary phases are its easy preparation, facile functionalization and outstanding chemical stability over a wide pH range [2-4]. These monoliths are usually prepared *via* a free-radical polymerization of a mixture containing one or more functional monomers, including a cross-linker, a porogenic solvent and an initiator. Heat [5-13] and UV irradiation [10, 14-20] are the most common ways of initiating polymerization. Photopolymerization provides several advantages over thermal initiation such as fast preparation, higher bed uniformity and easy selection of polymerization regions by using masks, which is particularly important in relation to the manufacturing of microfluidic chips [21]. However, UV initiation requires the use of transparent capillaries, which are not compatible with some commercially available instruments (*i.e.* conventional and capillary LC systems), being thermal initiation commonly adopted. Additionally, thermal and UV initiation need a water bath or a UV lamp to initiate the polymerization reaction, respectively. Apart from heat and UV light, the polymerization of acrylate or methacrylate monoliths can also be initiated by redox initiators [22-24]. Usually, the redox initiation system for these monoliths contains a peroxide oxidant and an aromatic amine reductant that compose the redox initiator-couple.

On the other hand, most of literature concerning acrylate-based monoliths is focused on the use of short alkyl chain monomers, such as butyl acrylate [9, 14, 16] and hexyl acrylate [15, 25-27] as bulk monomer. However, few studies related

to the employ of other longer alkyl chain non-polar monomers such as lauryl acrylate (LA) [9, 24, 28], stearyl acrylate [5-7] or octadecyl acrylate [29-32] can be found.

Furthermore, the search of an adequate initiator compatible with the initiating systems described in this paper for the synthesis of long alkyl chain monolithic columns is not a trivial task. In this way, lauroyl peroxide (LPO), as other diacyl peroxides, constitutes a source of free radicals when decomposed by thermolysis, UV irradiation or activated by several promoters such as tertiary amines [33-35]. In fact, this compound has been employed as thermal- [10, 36, 37] and photo-initiator [10, 38] for the preparation of methacrylate/acrylate monoliths and also its combination with N,N,N',N'-tetramethylethylenediamine (TEMED) has demonstrated to be a feasible choice as chemical initiating system for the preparation of LA-based monoliths [24]. Thus, LPO could constitute a good candidate to perform a comparative study between these radical polymerization modes to obtain long alkyl chain acrylate-based monolithic columns. To our knowledge, this investigation has not yet been described in the literature.

In this work, the preparation of ODA-based monolithic columns for CEC by photochemical, thermal and chemical initiation is described. The influence of porogenic solvent composition on morphological and CEC properties was evaluated for each initiation system. SEM images were used to characterize the morphology of monoliths, whereas CEC performance of different columns was evaluated by measuring the retention factor and efficiency of a test mixture of neutral solutes. Additionally, a comparison in terms of efficiency, permeability and reproducibility of columns polymerized under each initiating mode was performed.

7.2. Experimental

7.2.1 Chemicals and materials

ODA, 1,3-butanediol diacrylate (BDDA), [2-(methacryloyloxy)ethyl] trimethyl ammonium chloride (75% in water, META), 1,4-butanediol, LPO and 3-(trimethoxysilyl)propyl methacrylate were purchased from Aldrich (Milwaukee, USA); 1-propanol, ACN and methanol were obtained from Scharlau (Barcelona, Spain); TEMED and Tris were provided by Fluka (Buchs SG, Switzerland). Thiourea and the following polycyclic aromatic hydrocarbons (PAHs) standards: naphthalene, fluorene, phenanthrene, anthracene, pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, perilene, benzo[e]pyrene, benzo[a]pyrene, dibenz[a,h]anthracene and benzo[g,h,i]perilene (Riedel de Haën, Seelze, Germany) were used. Deionized water was obtained by using a Barnstead deionizer (Sybron, Boston, MA). Uncoated fused-silica capillaries of 375 μm od × 100 μm id with either polyimide or UV-transparent coating (Polymicro Technologies, Phoenix, AZ, USA) were used.

7.2.2 Instrumentation

CEC experiments were performed on a HP^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array UV detector and connected to an external nitrogen pressure source. Data acquisition was performed with ChemStation Software (Rev.A.10.01, Agilent). Prior to use, all mobile phases for CEC were degassed with a D-78224 ultrasonic bath (Elma, Germany). To photoinitiate polymerization, the capillaries were placed into an UV crosslinker (Model CL1000) from UVP (Upland, CA, USA) equipped with 5 UV lamps of 8 W emitting a wavelength of 254 nm. SEM images were taken with a scanning electron microscope (S-4100, Hitachi, Ibaraki, Japan) provided with a field emission gun, a back secondary electron detector and an EMIP 3.0 image data

acquisition system (Rontec, Normanton, UK). For SEM analysis, the capillary ends were cut off and the capillary tube was fixed to the sample holder, through the use of double-sided carbon type. Then, they were sputtered with a thin Au/Pd layer. The photomicrographs of the monoliths materials were taken at 10 kV under several magnifications (900 or 9000 ×), according to the best visualization of the morphology of the stationary phase.

7.2.3 Preparation of polymeric monolithic columns

To ensure covalent attachment of monolithic beds to the inner capillary wall, a previous surface modification of this wall was performed with 3-(trimethoxysilyl)propyl methacrylate [3]. Monoliths were prepared from polymerization mixtures obtained by weighing amounts of ODA (functional monomer), BDDA (crosslinker), META a (a positively charged monomer to generate EOF), and as porogenic solvents, 1,4-butanediol and 1-propanol, and LPO as initiator. The details of compositions of the monolith solutions prepared are given in **Tables 7.1-3**. After mixing these compounds, polymerization mixtures were sonicated for 10 min and purged with nitrogen for 10 min.

For each initiating system, one end of a 33.5 cm long modified capillary was immersed in the final polymerization mixture and filled up to a length of either 8.5 or 25 cm. Thermal polymerization was performed at 70ºC for 20 h, whereas UV initiation was carried out at room temperature at 0.9 J/cm^2 (maximum irradiation energy supplied by the crosslinker oven) for 10 min. For chemical polymerization, a proper amount of a 40 wt% TEMED solution in methanol was added to reach a 1.5 wt% TEMED in the polymerization mixture, and the polymerization reaction proceeded at room temperature for 24 h.

After polymerization and using an HPLC pump, the resulting columns were flushed first for 30 min with methanol, thus to remove the pore-forming solvents and any possible unreacted components, and then with mobile phase. When it was

required, a detection window was made adjacent to the monolithic material by burning the polyimide coating.

Table 7.1. CEC properties of ODA-based monoliths prepared by photo-polymerization

1,4-Butanediol/ 1-propanol (wt/wt)	u (mm s^{-1})[a]	k_{pyrene}[a]	H_{min} naphthalene (µm)	H_{min} anthracene (µm)	H_{min} benzo(k)fluorantene (µm)
25:75	3.40	NM[b]	NM[b]	NM[b]	41.8
20:80	3.15	2.18	15.7	17.8	15.2
17:83	3.02	2.86	10.7	8.5	6.9
10:90	2.90	2.94	20.6	14.4	9.4
6:94	1.94	3.84	23.4	19.7	17.3

[a]Flow rate and retention measured at 25 KV. Mobile phase, 80:20 (v:v) ACN:water (5 mM Tris buffer pH = 8.0).
[b]Not measured

Table 7.2. CEC properties of ODA-based monoliths prepared by thermal initiation with LPO

1,4-Butanediol/ 1-propanol (wt/wt)	u (mm s^{-1})[a]	k_{pyrene}[a]	H_{min} naphthalene (µm)	H_{min} anthracene (µm)	H_{min} benzo(k)fluorantene (µm)
25:75	2.57	1.92	NM[b]	NM[b]	53.0
20:80	1.96	2.72	21.5	24.3	22.9
17:83	1.68	3.74	18.4	18.4	17.9
14:86	1.42	4.21	31.5	31.2	28.7

[a]Measured in the same conditions as Table 7.1.
[b]Not measured.

Table 7.3. CEC properties of ODA-based monoliths prepared by chemical initiation with LPO-TEMED

1,4-Butanediol/ 1-propanol (wt/wt)	u (mm s^{-1})[a]	k_{pyrene}[a]	H_{min} naphthalene (μm)	H_{min} anthracene (μm)	H_{min} benzo(k)fluorantene (μm)
17:83	2.14	2.70	23.3	21.2	14.3
10:90	2.20	2.95	17.5	15.4	13.2
6:94	2.10	3.17	12.6	9.0	6.5
3:97	1.94	3.91	31.5	25.2	16.7

[a]Measured in the same conditions as Table 7.1.

7.2.4 CEC procedures

Each monolithic column was placed in the CEC instrument and equilibrated with mobile phase by applying a stepwise increase in voltage from 5 kV up to 25 kV, until a stable current and baseline was observed at each voltage. Separations were performed at 25 ºC and at several voltages. In all cases, nitrogen was used to pressurize at 10 bar (1 MPa) both vials. To prepare mobile phases, an aqueous 100 mM Tris buffer was adjusted to pH 8.0 with 1 M HCl, diluted and mixed with ACN at several volume ratios. A total Tris buffer content of 5 mM was used in the resulting mobile phases. Test mixtures containing PAHs (six or thirteen) and thiourea as EOF marker (100 μg mL^{-1} of each compound) were prepared in mobile phase and used to evaluate the CEC performance of columns. The sample solution was injected electrokinetically at 5 kV for 3 s. Detection was performed at 214 and 254 nm.

Chromatographic permeability of columns (K) was determined by using Darcy's law:

$$K = \frac{\eta \cdot L \cdot u}{\Delta P} \tag{7.1}$$

where η is the mobile phase viscosity, L the column length, u the average mobile phase velocity and ΔP the pressure drop across the column. K was measured in the CEC instrument by injecting thiourea in 8.5 cm long columns and applying a pressure of 10 bar to move it from the outlet end to the detection window. The u-value was obtained as the ratio between monolithic bed length and retention time of thiourea. The viscosity of the 80:20% (v/v) ACN:H_2O was taken as $5.3 \cdot 10^{-4}$ Pa·s [39].

7.3. Results and discussion

7.3.1 Preparation and characterization of photo-, thermal- and chemical-initiated ODA-based monolithic columns

The conditions to prepare photopolymerized ODA-based monoliths were adapted from a previous work, where LA-based monoliths were synthetized using LPO as initiator [24]. The selected composition contained 40 wt% of monomers (69.8 wt% ODA, 29.9 wt% BDDA and 0.3 wt% META) and 60 wt% of porogens (17 wt% 1,4-butanediol, 83 wt% 1-propanol). When a PAH test mixture was injected in this monolith, a satisfactory separation of all the analytes was achieved (**Fig. 7.1**).

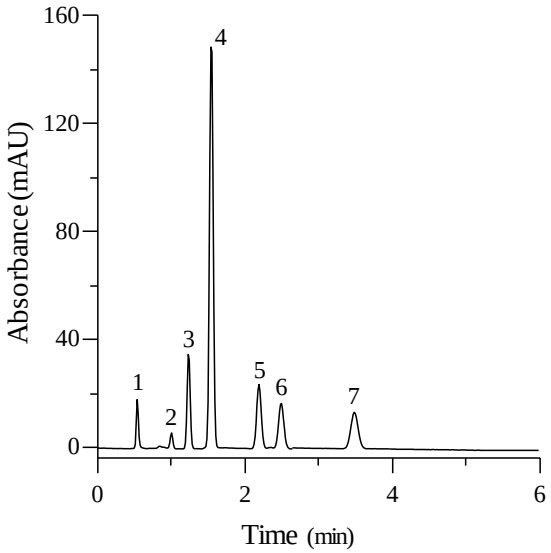

Fig. 7.1. CEC separation of a PAH test mixture in ODA-based monoliths of optimized composition prepared by UV irradiation. Details of composition of the polymerization mixtures are given in the text. CEC conditions: mobile phase, 80:20% (v/v) ACN: 5 mM Tris (pH = 8.0); 8.5 cm monolithic bed length; UV detection at 254 nm; applied voltage, 25 kV; injection, 5 kV for 3 s. Peak identification: (1) thiourea, (2) naphthalene, (3) fluorene, (4) anthracene, (5) pyrene, (6) benz[a]anthracene and (7) benzo[b]fluoranthene.

In order to check the possibility of achieving shorter analysis time while keeping satisfactory resolution between analytes, the influence of porogenic solvent composition on the monolith morphology and CEC performance was next investigated (**Table 7.1**). For this purpose, morphology was evaluated by SEM images, and the mixture of PAHs was used to measure the retention and efficiency values (by giving the minimum plate heights, H_{min} obtained from van Deemter plots).

When the ratio of 1,4-butanediol/1-propanol was reduced (from 25:75 to 6:94), a decrease in *u*-values and an increase in the retention properties (*k*-values) of columns were produced. This behaviour was consistent with SEM pictures of these monoliths (**Figs. 7.2A** and **7.2B**), where a decrease in the size of globules and voids was observed with decreasing 1,4-butanediol content. Similar effect of

the composition of porogens on the monolith morphology has been also reported for monoliths prepared by UV-initiated polymerization [10, 38].

Regarding to the efficiency, the 17:83 (wt/wt) 1,4-butanediol/1-propanol ratio provided the best H_{min} values (6.9-10.7 μm) with low mass transfer contributions (*C*-term) (6.3-11.0 ms), giving naphthalene the highest deviating mass transfer behavior. **Fig. 7.3A** shows the van Deemter plot obtained for the optimal photopolymerized ODA-based monolith for naphthalene, anthracene and benzo[k]fluorantene. **Fig. 7.1** shows the separation of PAH solutes obtained under the best polymerization conditions for UV-initiated ODA monoliths.

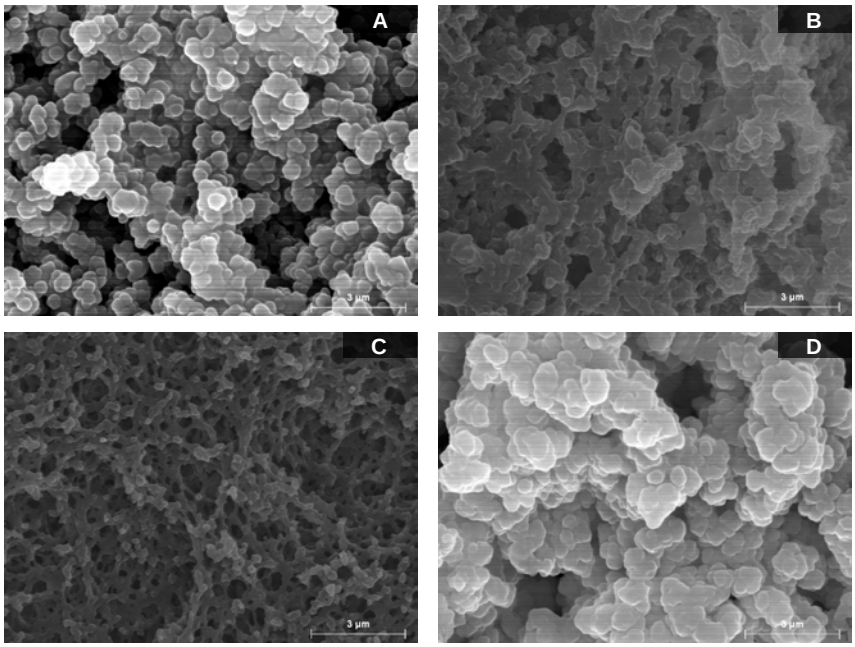

Fig. 7.2. SEM photographs of ODA-based monoliths polymerized under different initiating systems: Photopolymerized(UV-initiated) columns prepared at several ratios 1,4-butanediol/1-propanol: (A) 17:83 and (B) 6:94 (wt/wt), (C) thermally initiated monolith prepared with 17:83 (wt/wt) 1,4-butanediol/1-propanol, and (D) chemically initiated monolith prepared with 6:94 (wt/wt) 1,4-butanediol/1-propanol. Other details of polymerization conditions are given in the text.

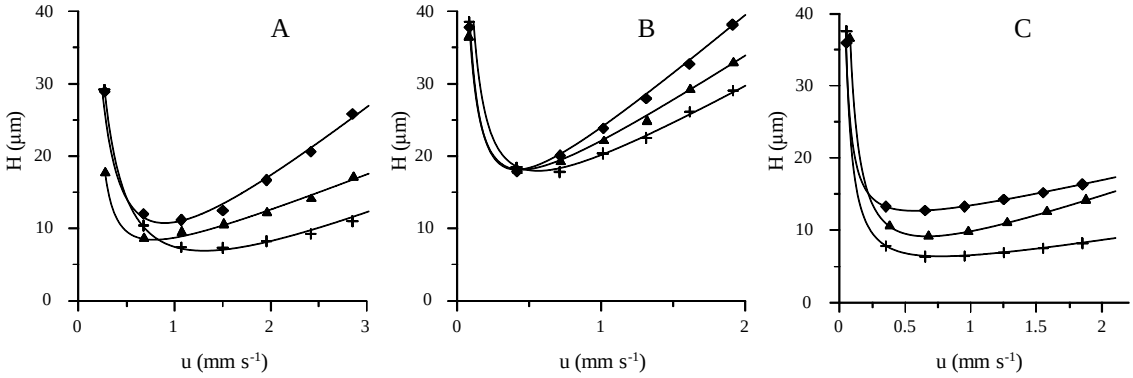

Fig. 7.3. Van Deemter plots of ODA-based monolithic columns of optimized composition prepared by (A) UV irradiation, (B) thermal and (C) chemical initiation. Compounds: (♦) naphthalene, (▲) anthracene, (+) benzo[k]fluoranthene. CEC conditions: mobile phase, 80:20% v/v ACN: 5mM Tris (pH = 8.0); UV detection at 254 nm; injection, 5 kV for 3 s.

A similar optimization study of the porogenic solvent composition was performed with thermally and chemically polymerized monoliths. Thus, a series of thermal ODA-based monolithic columns were first prepared by modifying the percentage of 1,4-butanediol in the porogenic solvent, at fixed ratios of monomers/porogens (40:60 wt%) and ODA/BDDA (70:30 wt%) (**Table 7.2**). Monoliths prepared at ≤ 10:90 (wt/wt) 1,4-butanediol/1-propanol exhibited poor permeabilities, whereas ratios above 25:75 (wt/wt) led to poor separation performances. As shown in **Table 7.2**, when the 1,4-butanediol/1-propanol ratio was decreased, a decrease in *u*- and an increase in *k*-values were observed. This behavior was consistent with the SEM pictures of these monoliths (see **Fig. 7.S1**) and with that previously described in several reports for thermal polymerization [3, 4, 8, 10, 37]. An example of porous structure of these monoliths (prepared at 17:83 (wt/wt) 1,4-butanediol/1-propanol) is given in **Fig. 7.2C**.

The column efficiency was also evaluated on thermally polymerized monoliths. As shown in **Table 7.2**, the limits of the studied range of 1,4-butanediol (14:86 and 25:75 wt/wt) gave the highest H_{min} values, which could explained as follows. The low efficiency achieved for the lower limit of 1,4-butanediol range

could be explained by the peak broadening resulting from the double-layer overlap [4, 8]. On the other hand, the upper limit could be justified taking into account the large globule size found at this 1,4-butanediol content (see **Fig. 7.S1**). **Fig. 7.3B** shows the van Deemter plot for the column prepared with 17:83 (wt/wt) 1,4-butanediol/1-propanol using the PAH analytes. Using these column, the solutes provided the lowest H_{min} from thermally initiated ODA-based monoliths, with *C*-term values comprised between 11.4 and 17.3 ms. **Fig. 7.4** shows the electrochromatogram of the PAH test mixture obtained under the best polymerization conditions for thermal ODA-based monoliths.

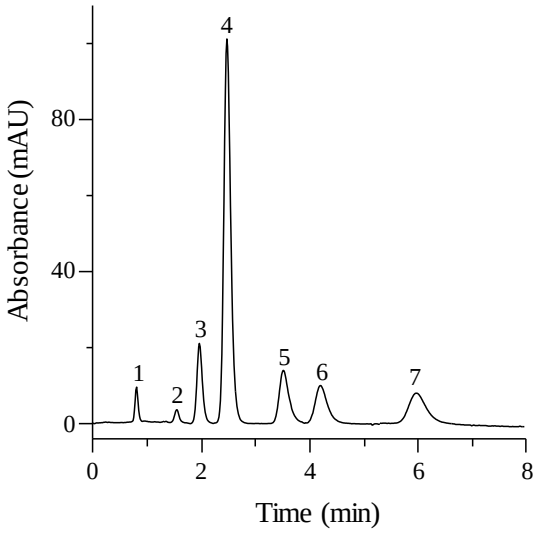

Fig. 7.4. CEC separation of a PAH test mixture in ODA-based monoliths of optimized composition prepared by thermal initiation. Details of composition of the polymerization mixtures are given in the text. Other conditions as in Fig. 7.1.

Next, the preparation of ODA monolithic columns by chemical polymerization was carried out. The influence of 1,4-butanediol/1-propanol on CEC properties is shown in **Table 7.3**. Thus, the 1,4-butanediol content was varied between 3 and 17 wt% in the porogenic solvent. Columns made with < 3 wt% 1,4-butanediol showed a reduced permeability with certain resistance to flow, giving long analysis times and low efficiencies. On the other hand, contents above 17 wt% 1,4-butanediol exhibited poor CEC performance. As observed in **Table 7.3**,

minor variations in *u*-values, with a slight increase in *k*-values were observed. This behavior was consistent with the SEM pictures, where small changes in morphology along the studied 1,4-butanediol range were observed (**Fig. 7.S2**). This trend was in agreement with that previously reported for methacrylate and acrylate monoliths prepared by chemical initiation mode [22-24]. A representative example of porous structure of these monoliths is given in **Fig. 7.2D**. Since the effect of porogenic solvent composition on porous properties of chemical column is less pronounced in chemical ODA monoliths than in UV- and thermal-initiated ODA columns, this initiation mode is more suitable for a fine control of the pore size of ODA-based monoliths.

Regarding to the efficiency, chemical initiated monoliths prepared with 6 wt% 1,4-butanediol in the porogenic solvent provided the best H_{min} values ranged between 6.5 and 12.6 μm, with very low *C*-term values ranged between 3.0 and 8.4 ms (see van Deemter curves given in **Fig. 7.3C**). Under these optimum polymerization conditions, the PAHs were separated within 5 min (**Fig. 7.5**).

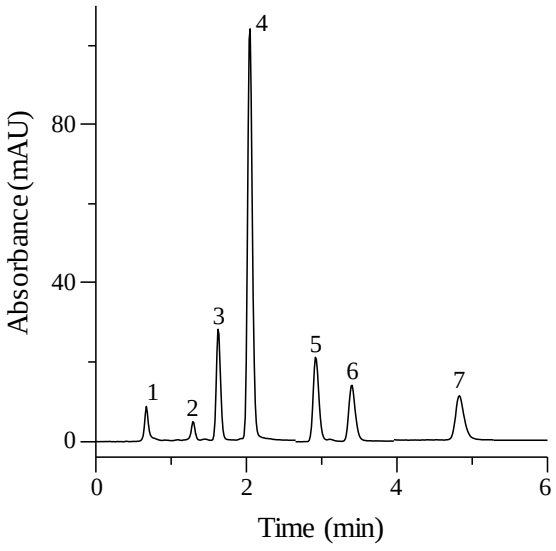

Fig. 7.5. CEC separation of a PAH test mixture in ODA-based monoliths of optimized composition prepared by chemical initiation. Details of composition of the polymerization mixtures are given in the text. Other details as in Fig. 7.1.

7.3.2 Comparison of photo-, thermal- and chemical-initiated ODA based monolithic columns

Although different experimental conditions (polymerization temperature, 1,4-butanediol/1-propanol ratio, etc.) have been employed for each initiation mode, several findings related to the morphology and CEC performance for each polymerization process could be derived and critically discussed. The features of columns prepared under similar 1,4-butanediol range, are shown in **Tables 7.1-3**.

At a given 1,4-butanediol/1-propanol ratio (*i.e.* 17:83 wt/wt), photoinitiated columns gave lower retention than the corresponding thermal columns, whereas the chemical monoliths showed similar *k*-values than those obtained for UV initiation. These results were consistent with SEM pictures of these monoliths (**Figs. 7.2A, 7.2C** and **7.2D**), showing that thermally polymerized monoliths had smaller globule sizes compared with the photo- and chemical-initiated ones.

The different retention characteristics showed for each polymerization process could be attributed to the differences in preparation conditions. The differences in 1,4-butanediol content and polymerization temperature will affect the solvating power and viscosity of the porogenic solvents, and consequently the morphological properties of the resulting polymers [40]. Furthermore, the temperature causes variations in the polymerization kinetics [41, 42]. Thus, the low polymerization temperature used in photopolymerization (room temperature in this work) in relation to that selected for thermal initiation (70ºC) favors a reduction in the decomposition rate of the initiator, the number of growing radicals and the overall polymerization rate [41, 43]. The formation of lower number of chains but with longer chain lengths is preferentially produced compared with polymerization at higher temperatures, where short-chain polymers are generated, giving as a result, monoliths with larger pore and globule size [40, 41, 43]. Furthermore, a change in solvency of porogens could result from an increase in the

polymerization temperature. Then, an increase in the solubility of the monomer could happen, thereby resulting in late phase separation, giving differences in morphology between thermal and UV initiation modes. Also, differences between the nucleation mechanisms observed for the chemical mode compared to UV- and thermal-initiated polymerizations should be considered. As we described in **Tables 7.1-3**, in general, better efficiencies were achieved for photo- and chemical polymerized columns than for those thermally initiated. The optimum thermal polymerized column showed higher H_{min} values for PAHs (17-9-18.4 μm) and slightly worse *C*-term values (11.4-17.3 ms) than those obtained for photo- and chemical polymerized monoliths. A comparison in terms of efficiency with reported monolithic columns synthetized with ODA was also performed. Thus, our efficiencies were slightly higher than those obtained for neutral ODA columns thermally initiated with AIBN [29], where the average H_{min} values for alkyl benzene compounds was *ca*. 6 μm.

Figs. 7.1, 7.4 and **7.5** shows the CEC separation of a test mixture of PAHs in ODA monoliths polymerized by UV irradiation, thermally and chemically respectively under its respective optimal conditions. As observed, the photo- and chemical initiated columns showed similar efficiencies, although the former one provided shorter analysis time. The monoliths thermally initiated gave the lowest efficiencies and the longest analysis times.

The significant differences shown above in monolithic structure between the different initiated monoliths gave rise to changes in permeability. Thus, the permeability measured for the optimal photoinitiated monolith was $7.8 \cdot 10^{-13}$ m^2, the chemically initiated monolith was $5.1 \cdot 10^{-13}$ m^2 whereas thermally polymerized column was $8.5 \cdot 10^{-15}$ m^2. These permeability differences could deteriorate the quality of separation or even hindering the preparation of long monolith columns. When a test mixture containing thiourea and 13 PAHs was injected in ODA

columns of bed length of 25 cm polymerized by UV irradiation, thermally and chemically under its respective optimal polymerization conditions, excellent separations of all analytes were achieved for photo- and chemical polymerized monoliths within 20-25 min (**Fig. 7.6**). However, the monolith thermally initiated gave lower efficiencies, with some peak coelutions in a longer analysis time.

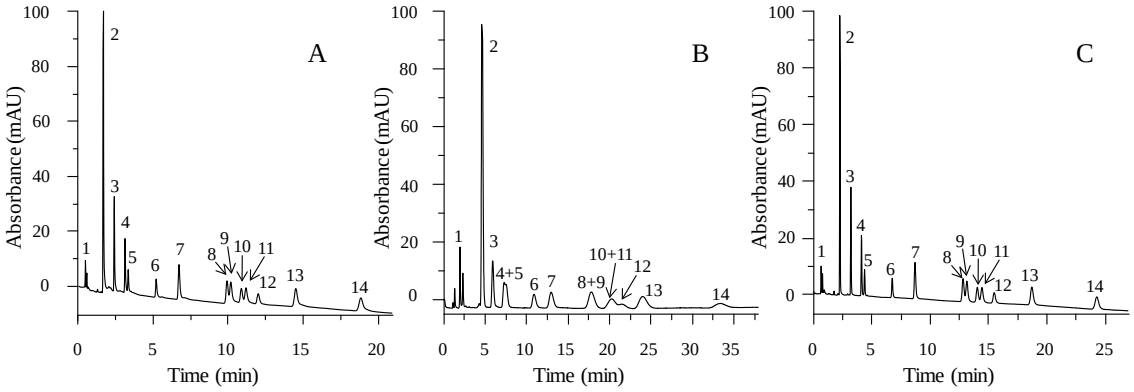

Fig. 7.6. CEC separation of a test mixture containing thiourea and 13 PAHs in ODA-based monoliths of optimized composition prepared by (A) UV irradiation, (B) thermal and (C) chemical initiation. Details of composition of the polymerization mixtures are given in the text. CEC conditions: mobile phase, 70:30% (v/v) ACN: 5 mM Tris (pH = 8.0); 25 cm monolithic bed length; UV detection at 254 nm; applied voltage, 25 kV; injection, 5 kV for 3 s. Peak identification: (1) thiourea, (2) naphthalene, (3) fluorene, (4) phenanthrene, (5) anthracene, (6) pyrene, (7) benz[a]anthracene, (8) benzo[b]fluoranthene, (9) benzo[k]fluoranthene (10) benzo[e]pyrene, (11) perilene, (12) benzo[a]pyrene, (13) dibenz[a,h]anthracene and (14) benzo[g,h,i]perylene.

The reproducibility of monolithic columns prepared using the three initiating systems was also evaluated. For this purpose, polymerization mixtures that provided the optimum separation performance in each polymerization process were selected. Several column parameters (EOF time, retention factor of pyrene and Hmin of naphthalene) were determined to both test column-to-column and batch-to-batch reproducibilities. Three separated batches of three columns for each polymerization process (photochemically, thermally or chemically initiated), yielding a total of eighteen columns, were prepared and examined. The results are summarized in Table 7.4. The column-to-column reproducibility obtained for UV-initiated ODA-based monoliths was satisfactory with

RSD values for the three parameters comprised between 4.1 and 6.2%. These values were very similar to those obtained for thermal (4.5-6.7%) and chemical (3.8-5.4%) polymerized columns. The batch-to-batch reproducibilities were also examined in both columns, and quite acceptable RSD values for photo-polymerized (9.2%), thermal (10.6%) and chemical (9.8%) monoliths were achieved, which confirmed the good reproducibility of both column fabrication processes.

Table 7.4. Reproducibility of CEC properties of ODA-based monoliths prepared by UV, thermally, and chemically initiated polimerization [a)]

Parameter	UV initiation		Thermal initiation		Chemical initiation	
	Column-to-column (n = 3)	Batch-to-batch (n = 3)	Column-to-column (n = 3)	Batch-to-batch (n = 3)	Column-to-column (n = 3)	Batch-to-batch (n = 3)
	Mean; RSD%	Mean; RSD%	Mean; RSD%	Mean; RSD%	Mean; RSD%	Mean; RSD%
u (mm s^{-1})	2.97; 4.1	3.12; 5.4	1.74; 4.5	1.97; 6.2	2.07; 3.8	2.17; 5.8
k_{pyrene}	2.83; 5.7	3.21; 7.2	3.59; 5.9	3.72; 7.8	3.15; 4.8	3.32; 8.4
H_{min} (μm)[b]	10.9; 6.2	11.6; 9.2	18.7; 6.7	22.3; 10.6	12.8; 5.4	13.7; 9.8

[a]Polymerization mixtures prepared from 17:83 (wt/wt) 1,4-butanediol/1-propanol for UV- and thermal initiation, and 6:94 (wt/wt) for chemical initiation; other conditions were as in Table 8.1.

[b]Values obtained for naphthalene.

7.4. Conclusions

ODA-based monolithic columns for CEC have been prepared using either UV irradiation, thermal and chemical initiation. Photo- and thermal-polymerized columns were initiated with LPO, whereas the couple LPO-TEMED was used for chemical polymerization. The effect of composition of porogenic solvent on the porous and chromatographic properties for each initiating system has been studied. Differences in retention properties and morphologies observed for each initiation mode could be explained taking into account several factors such as polymerization temperature, 1,4-butanediol/1-propanol content, nucleation mechanism, among others. Additionally, under chemical initiation system, a fine control of the pore size in monoliths over the 1,4-butanediol/1-propanol ratio could be more easily accomplished with respect to the other initiating systems. Under optimized conditions, a comparison in terms of efficiency, permeability and reproducibility, for the three initiation modes was performed. Columns synthetized under UV- and chemical-initiation showed higher permeabilities and better efficiencies than those prepared by thermal. Besides, UV initiation provides a fast preparation of monoliths, whereas the chemical initiation does not require additional equipment (water bath or UV lamp) for its synthesis. These good features make both initiation modes excellent candidates to be used in the preparation of long monolithic columns and miniaturized devices. Additionally, the three initiating systems provided a satisfactory column-to-column and batch-to-batch reproducibilities in their electrochromatographic behavior.

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Supplementary Material

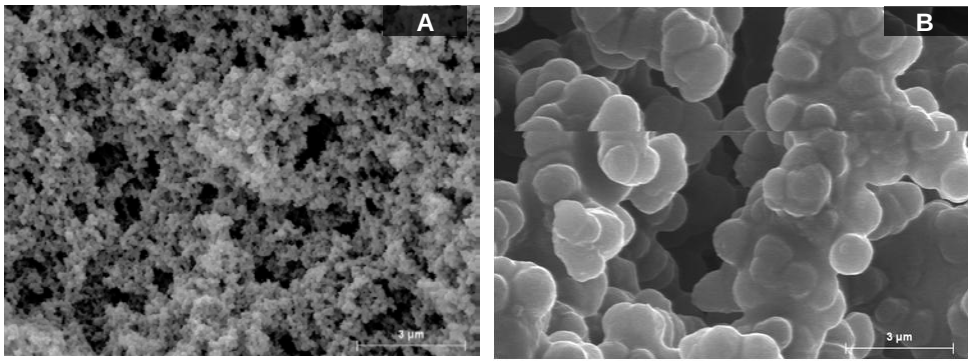

Fig. 7.S1. SEM photographs of ODA-based monoliths thermally polymerized at different ratios 1,4-butanediol/1-propanol: (A) 25:75 and (B) 14:86 (wt/wt). Other details of polymerization conditions are given in the text.

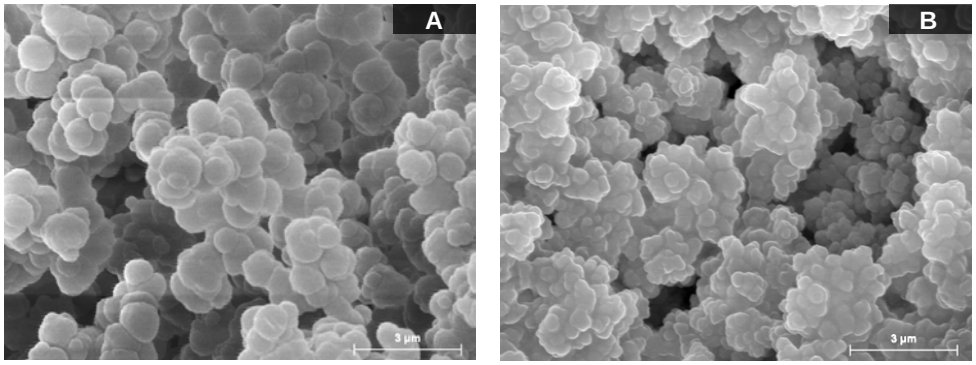

Fig. 7.S2. SEM photographs of ODA-based monoliths chemically polymerized at different ratios 1,4-butanediol/1-propanol: (A) 17:83 and (B) 3:97 (wt/wt). Other details of polymerization conditions are given in the text.

SECTION V.

SUMMARY AND CONCLUSIONS

This PhD thesis reports the development of advanced chromatographic methods for the determination of different families of surfactants present in raw industrial material and household cleaning products. The proposed methods try to cover the demand of the industries to have reliable quality control methods to properly check raw materials and their products to secure that they are produced according to the laws that regulate their manufacture. In this sense, the analysed samples are complex mixtures of surfactants and other components, representing difficult analytical challenges. Another part covered by this thesis was the development of ODA monolithic columns for capillary electrophoresis comparing different initiation methods for their polymerization.

In this section of the PhD Thesis and, as required by the aforementioned regulations of the University of Valencia, a summary of the results and the most relevant conclusions is presented.

Part I: Revision on derivatization reactions of hydroxyl groups

I. Derivatization of hydroxyl functional groups for liquid chromatography and capillary electroseparation

Derivatization reactions are frequently used to introduce a chromophore or a fluorophore to an analyte in order to achieve detection or enhance the detection signal in UV, fluorescence or MS. In this revision, we focused on the derivatization of molecules bearing hydroxyl functional groups for their analysis by liquid chromatography and capillary electrochromatography.

The derivatization methods have been classified by the reactive group of the derivatizing agent, including acyl chlorides, organic anhydrides, isocyanates and other miscellaneous derivatization reactions. The reactions and methods have

been conveniently sorted in a series of tables for a global overview of the main features of each method.

Most of the reviewed reactions are used to introduce a chromophore or a fluorophore in analytes having an aliphatic alcohol group, in order to enable their detection or to enhance sensitivity. Other reactions are addressed to introduce an ionisable group or a permanent charge to perform CE separations, enhance MS sensitivity or decrease volatility, thus to enhance the sensitivity of volatile analytes in evaporative detectors.

Concerning to the applications, a high amount of the reviewed reactions involve the derivatization of analytes with hydroxyl functional groups which are of interest in industrial quality control and evaluation of the environmental impact of non-ionic aliphatic surfactants (mainly FAE), and other compounds of industrial interest as ethylene glycol and their condensed polymers such as PEG, glycerine based polyols or light aliphatic alcohols. The derivatization of other remarkable substances in the biological field such as hormones, mono- and di- glycerides or endocrine disruptors has been also covered. The studied matrices in all these methods comprise from typical environmental samples as fresh water, wastewater, sediments and sludges to biological matrices as cell cultures, biological fluids or tissues, also including several industrial samples as raw materials, food and beverages.

Part II: Chromatographic methods for surfactant analysis

II.1. Single-pump heart-cutting two-dimensional liquid chromatography applied to the determination of fatty alcohol ethoxylates

In this work, the application of a heart-cutting bi-dimensional liquid chromatography system driven by a single pump and using an auxiliary 6-port 2-

position valve ($V_{6/2}$) and a column selector valve (V_{CS}) for the determination of fatty alcohol ethoxylates in raw materials has been developed.

First, the selection of the stationary and mobile phases for both the first and the second dimensions were made according to the properties of the FAE oligomers. The separation of the FAE oligomers within the hydrocarbon series is well performed with C8 columns and ACN/water mixtures, thus these conditions were chosen as second dimension. For the first dimension separation, different alkyl columns were tried. The use of ACN/water gradients on a C8 column did not permit the separation of the series without also separating the oligomers within them. On the contrary, single peaks for each series were achieved using MeOH/water gradients. An improvement of the inter-series resolution and a short analysis time was achieved at 60 ºC. As aforementioned, in the second dimension separation with ACN/water gradients on a C8 column at 25 ºC were used for the oligomer separation, optimizing the starting and ending concentrations of the ACN gradients according to the length of the hydrocarbon chain of each series. Mixtures of FAE containing both even and odd hydrocarbon series with long EO chains were well resolved along the 1st dimension, and the EO oligomers within the series were well separated along the second dimension.

The optimized LC-LC separation was used for the determination of FAE and the proportion of the hydrocarbon series in industrial samples. For this purpose, an average response factor of the series has been proposed. These factors take into account the different UV-vis response factor of the EO oligomers and have been tabulated for each series as functions of *n* and $\overline{m}$. The average response factors are used to correct the peak areas of the isolated series along the 1st dimension chromatogram. It has also been demonstrated that the average response factors depend slightly on $\overline{m}$ when $\overline{m}$>5. This allows the use of inaccurate values of $\overline{m}$ without diminishing the accuracy of the determinations.

In conclusion, a heart-cutting bi-dimensional liquid chromatography setup, constructed with a chromatograph provided with a single pump, an auxiliary 6-port 2-position valve and a column selector valve can be used for the characterization of raw FAE samples. In the first dimension FAE are separated according to their hydrocarbon chain, whereas in the second dimension each FAE series can be separated according to the length of the EO chain. Orthogonality in the separation is achieved by using complementary mobile phases in the different dimensions. An average response factor of the hydrocarbon series for the correction of the peak areas was also proposed.

II.2. Determination of the four major surfactant classes in cleaning products by reversed-phase liquid chromatography using serially connected UV and evaporative light-scattering detection.

A method for the simultaneous determination of the most frequently used surfactant families- LAS, AES, FAE and oleins (soaps, fatty acid salts) in cleaning products using a single chromatographic injection, has been developed. The common reversed phases octyl (C8), pentafluorophenyl and biphenyl were not capable of separating the anionic LAS and AES classes; however, since only LAS absorbs in the UV, these two classes were independently quantified using a C8 column and serially connected UV and ELSD detectors.

Different RP columns (C8, F5 and BP) as well as combinations of serially connected columns were tried. Concerning to the mobile phase, both ACN/water and MeOH/water gradients were tried. The best results to resolve the four surfactant classes and the oligomers within the classes was achieved with a C8 column and an ACN/water gradient. To enhance retention of the anionic surfactants, ammonium acetate, as an ion-pairing agent compatible with ELSD detection, was used. The presence of the ionic additive did not modify the elution

of the FAE and olein components. The presence of acetic acid in the mobile phase was also studied and was observed that it mainly affected the retention of the olein peaks with respect to the FAE oligomers. Modulation of the mobile phase was implemented using ammonium acetate during elution of LAS and AES, and acetic acid after elution of LAS and AES, in order to enhance retention of LAS and AES and independently shifting the peaks of the olein components according to the pH of the mobile phase. Finally, the effect of the temperature was also investigated, being significant in the resolution of the EO oligomers, but mantaining practically invariable the separation of the rest of surfactants. The optimized chromatographic method consisted in the separation on a single C8 column with a gradient starting from 40 % until a 90 % ACN in 40 min in the presence of 10 mM NH_4AcO before t = 12 min and using 17.5 mM HAcO afterwards at 15 ºC.

Quantitation of the overlapped LAS and AES classes was achieved by using the UV detector to quantitate LAS and the ELSD to determine AES by substraction of the LAS concentration to the total LAS and AES concentration. However, sensitivity was influenced by the LAS/AES ratio. Two strategies were tried to overcome this difficulty. The first approach was to use a simple linear calibration model to calculate the AES concentration, which considers both the LAS concentration and the ELSD signal as the sum of LAS and AES. Also, it was observed for LAS and AES mixtures, that in the region of $0.1 \leq \%\ LAS \leq 0.5$ the sensitivity depended only slightly on the percentage of LAS. To avoid bias, the alternative solution was to use only standard and samples within these concentration limits. To do so, samples were spiked with a large fixed amount of AES. The values of the AES concentration of the samples were obtained by subtracting the known amount from the predicted AES concentration. The LODs and LOQs were 2 and 6 mg L^{-1} for LAS and 20 and 60 mg L^{-1} for AES respectively.

Determination of the FAE and olein can be easily done with their individual calibration curves. The areas of the FAE peaks overlapping with olein peaks were established as the average area of the two neighboring FAE oligomers. The corrections slightly increased the positive errors of FAE and decreased that of oleins. The LODs and LOQs were 50 an 150 mg L^{-1} for FAE and 10 and 30 mg L^{-1} for oleins, respectively.

In conclusion, a chromatographic method for the simultaneous determination of the four major surfactant classes in cleaners by RP-HPLC using a single run was developed. The separation was achieved by mobile phase modulation, consisting on a change of the mobile phase additives, i.e. ammonium acetate in a first elution stage and acetic acid afterwards. Also, the lack of a proper method to resolve LAS and AES oligomers, and therefore their proper quantitation, was overcomed by the use of serially connected UV and ELSD detectors. Finally, the problem of the variation of sensitivity of the ELSD response with the LAS/AES ratio was tackled by increasing the AES concentration of the samples.

Part III: Monolithic stationary phases

III.1. Preparation and characterization of octadecyl acrylate monoliths for capillary electrochromatography by photochemical, thermal and chemical initiation

In this work, monolithic stationary phases based on octadecyl acrylate for CEC using different initiating systems (UV irradiation, thermal and chemical initiation) in the presence of lauroyl peroxide as initiator were synthesized and their chromatographic performance was compared. The influence of porogenic solvent composition (1,4-butanediol/1-propanol ratio) on the morphological and

electrochromatographic properties was evaluated for each initiation system. The CEC performance of the different columns was evaluated by measuring the retention factor and efficiency of a test mixture of PAHs, whereas SEM images were used to evaluate the morphological characterization of the monoliths.

The comparison of the three initiation modes in terms of efficiency, permeability and reproducibility under optimized conditions was performed. It was observed that the photochemically and chemically polymerized monoliths gave better efficiencies (minimum plate heights of 6.9-10.7 and 6.5-12.6 μm, respectively) and higher permeabilities than the termally initiated columns, which also gave longer analysis times, compared to the other two inititation systems. Column-to column and batch-to-batch reproducibilities were also evaluated under optimal conditions giving RSD values below 9.2, 10.6 and 9.8 % for UV, thermally, and chemically initiated columns respectively. Further, UV initiation provides a fast way of monolith preparation, whereas chemical initiation does not require additional equipment (water bath or UV lamp).

In conclusion, the preparation of octadecyl acrylate monolithic stationary phases using different initiating systems (UV irradiation, thermal, and chemical initiation) and the study of their CEC properties and morphology, revealed that UV and chemical initiation gave monolithic columns with the best performances, making both initiation modes excellent candidates to be used in the preparation of long monolithic columns and miniaturized devices.

Other developments

This PhD thesis should also include three other chapters whose experimental parts are today finished, but the writing is still being done. Because of time requirments to present the thesis, these three parts have not been included in this report but have been recorded here to be considered as genuine part of the thesis. These three parts are next shortly comented:

a) Comparative study of different chromatographic methods for the determination of biocides commonly used in cleaning products and cosmetics.

b) Revision of the UNE-EN 14669 standard which establishes the standard method of determination of anionic surface agents and soaps in detergents and cleaning products, determining the associated errors in the application of the same.

c) Study of the dependence of the ELSD response on analyte concentration. A dependence of the analyte concentration with the peak efficiency in the ELSD response has been determined to obtain linear calibrations, what makes possible to simplify the calculations carried out for the joint determination of LAS and AES described in Chapter 6.