FACULTAT DE QUÍMICA

Widening the possibilities of liquid chromatography through the use of secondary equilibria with additives and hydrophilic interaction chromatography

Memoria para alcanzar el Grado de Doctor en Química dentro del Programa de Doctorado en Química (RD 1999/2011) presentada por:

Ester Peris García

Directores:
Dra. María Celia García Álvarez-Coque
Dra. María José Ruiz Ángel
Dr. Samuel Carda Broch

Valencia, Julio 2019

Dña. MARÍA CELIA GARCÍA ÁLVAREZ COQUE, Catedrática de Universidad, Dña. MARÍA JOSÉ RUIZ ÁNGEL, Profesora Titular, adscritas al Departamento de Química Analítica de la Universidad de Valencia, y SAMUEL CARDA BROCH, Profesor Titular, adscrito al Departamento de Química Física y Analítica de la Universidad Jaume I,

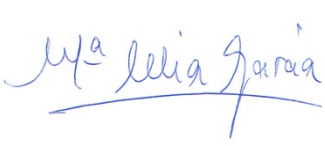
CERTIFICAN

Que la presente Memoria, "Widening the possibilities of liquid chromatography through the use of secondary equilibria with additives and hydrophilic interaction chromatography", constituye la Tesis Doctoral de

Dña. ESTER PERIS GARCÍA

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

Y para que conste a los efectos oportunos, firmamos la presente en Burjassot, a dos de julio de dos mil diecinueve.



María Celia García Álvarez-Coque	María José Ruiz Ángel	Samuel Carda Broch

Quien me conoce, sabe que soy risueña, estudiosa, así como luchadora y aventurera. Y tal vez, es de esto de lo que se ha tratado estos últimos años de mi vida. Una fascinante aventura que empezó ya años atrás, cuando durante la licenciatura un profesor de la UJI, llamado Samuel Carda, me habló por primera vez en su despacho de la posibilidad de hacer la Tesis Doctoral. Y aunque por aquel entonces, Samuel ya había hecho que la Química Analítica hiciera mella en mí, haciendo de ella mi predilección, yo veía demasiado lejana esa vía. Sin embargo, como buen mentor, durante el verano del cuarto año de licenciatura me ofreció empezar a ir a su laboratorio de investigación para una primera toma de contacto. Y, ¿cómo me iba yo a negar a semejante aventura? "Investigación y Química Analítica", palabras que eran música celestial para mis oídos.

Allí, no sólo aprendí a manejar a nuestros queridos cromatógrafos y cacharrear con ellos (tal y como el propio Samuel me decía), sino que aprendí que hay profesores que enseñan Química, y otros, que además transmiten pasión por lo que hacen.Y por ello, Samuel, no puedo dejar de olvidar que eres, sin duda, mi precursor y mi mentor, pero sobre todo, la persona que se fijó y confió en mí desde los inicios.

Y como no podría ser de otra forma, y seguida por tu recomendación, me embarqué en mi siguiente aventura, venir a hacer el "Máster de Técnicas Experimentales en Química" a la Universidad de Valencia.

Recuerdo ese primer día como si fuera ahora. Cuando abrí la puerta del despacho de la Profesora María Celia García para hablar del trabajo de final de Máster: emocionada, nerviosa y con miedo, todo a la vez. Nada más lejos de la realidad, porque lo que encontré fue a una profesora cercana, atenta, con mucha paciencia, con un conocimiento inconmensurable, y sobre todo, apasionada de la Química, tanto, que creo que ha hecho de su hobby, su profesión. Pero si algo te caracteriza, Celia, es la perfección, contigo he aprendido el valor de esa palabra, si cabe, a un nivel superior.

Tras viajes de ida y vuelta, todos los días Vila-real-Valencia, que hay quien ya los consideraba una aventura diaria, acabé presentando mi TFG y finalizando mi Máster. Sin embargo, durante ese año, yo ya había empezado a pensar en mi próxima aventura, y después de varias reuniones con Celia, comenzamos a solicitar el tan ansiado contrato pre-doctoral para la realización de mi Tesis Doctoral.

Durante la espera, y dado mi carácter luchador, yo no cesaba en mi empeño por mejorar mi curriculum. Y por si no tuviera bastante con publicar artículos, empecé a trabajar en Repol, una empresa de plásticos situada en Almazora. Así que mis viajes Vila-real-Valencia tuvieron que dejar hueco a Almazora. Y aunque parecía una aventura imposible con un horario de 8 horas y jornada partida, yo sacaba tiempo para venir al laboratorio a seguir con mi investigación.

Pero al fin, salió la resolución de concesión, y vi mi nombre en el listado de concedidas. No me lo podía creer, no sabía si gritar, llorar o reír. Lo cierto era, que al fin, iba a poderme dedicar al completo a la investigación.

De modo, que empecé el contrato pre-doctoral y mi aventura por excelencia bajo la dirección de la Profesora María José Ruiz, conjuntamente, por supuesto, con Celia y Samuel. María José ha sido mi guía durante todos estos años, bajo su supervisión y directrices he ampliado mi conocimiento analítico y he aprendido a sacar adelante los diferentes trabajos, a reconducir un experimental, y sobre todo, a perder el miedo en desmontar el equipo cuando surge un problema. A ti, María José, te debo, en gran medida el éxito de mis trabajos.

Durante estos años, no puedo dejar de mencionar a los otros dos profesores integrantes de nuestro grupo de investigación, Juan José Baeza y José Ramón Torres, ambos expertos en hacer magia con un ordenador. He tenido la suerte de colaborar en varios trabajos junto a ti Juanjo, y de ellos he aprendido que si juntas la Química (ya de por si fascinante) con las Matemáticas y un ordenador, el resultado es deslumbrante.

Pero si de aventuras se trata, las vividas con Tamara, José Antonio, Nikita, Joan y Noemí son incontables. Con ellos he tenido la suerte de compartir laboratorio, investigación, docencia, congresos, charlas divulgativas, en definitiva, nuestra vocación, la Química. Sin embargo, como vosotros mismos sabéis, nuestras aventuras no se han limitado al laboratorio, porque junto a vosotros ha habido risas, comidas y cenas de celebración, visitas turísticas, buñuelos falleros, viajes tortuosos, tazas compartidas y un sinfín de historias que me harían falta páginas para enumerarlas. Y es que con todos vosotros venir al laboratorio a trabajar cada día, me hacía olvidar muchas veces que de trabajo se trataba.

No obstante, no todo han sido aventuras en el Departamento de Química Analítica de la Universidad de Valencia. Deberíais de haber visto las caras de mi alrededor cuando dije que me iba de estancia, nada más y nada menos, que a Estados Unidos. In this case, I would like to express my gratitude to Prof. Anderson for giving me the opportunity of working in his research group at the Iowa State University. It has contributed to improve the quality of my thesis and my knowledge, making me grow up professionally and personally. In fact, this experience brings me the opportunity to meet Maria, Idaira, Ariana, Jakub, Fahad and Israel (The cool people of Ames). Because I never imagined finding so far from home, people that will make me feel just that. Be like in my own home!

En conjunto, estos profesores y compañeros, formamos el grupo de investigación FUSCHROM, aunque para mí es prácticamente la familia Fuschrom, por todo el tiempo y el afecto compartido. Aunque, si de familia se trata, son innumerables las aventuras, el apoyo y el cariño que he recibido a lo largo de estos años, tanto de la familia Peris como de la García, sin olvidar a la familia Villanueva Negre.

Y contra todo pronóstico, yo tengo dos familias más, mis queridas Desmelenás y mis alocados Etilikos, porque dicen que los amigos son la familia que se elige, y yo doy fe de ello. Especialmente, Gema, Montse, Ana, Lidia y Pedro, para los que las locuras junto a mí en Ludiente no tienen fin. Por no hablar de Paloma, María Elena, Esther y Sandra, ejemplos de que la distancia no es sinónimo, ni mucho menos, de olvido. Con todos vosotros se han escrito las aventuras más divertidas durante estos años, y habéis hecho que jamás me falte una sonrisa o un simple abrazo. Por ello, con vosotros la palabra amistad cobra sentido.

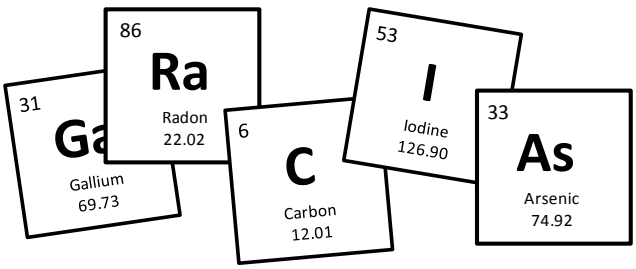
Y en toda aventura o historia que se precie siempre hay un personaje chistoso. Aquí jugaría un papel importantísimo Víctor. Y es que tú, tete, siempre has estado a mi lado, haciendo de cualquier cosa un chiste hasta en los peores momentos, apoyándome y caminando siempre junto a mi lado. Y por si no fuera suficiente, tú y Carmen me habéis dado uno de los mejores regalos de mi vida, la oportunidad de ser tía. Tía de dos increíbles sobrinos, mis dos soletes, Víctor y Jordi. Con vosotros no sólo disfruto jugando, paseando, haciendo los deberes, o simplemente mirando lo tranquilos que estáis cuando dormís. Sino que vosotros me habéis enseñado lo bonita que es la inocencia, lo inteligentes que sois los niños, que la docencia adopta otro carácter cuando sois vosotros los que me pedís que

hagamos "experimentos" y que una sonrisa vuestra sirve para curar todos los males. De hecho, vosotros conseguís iluminar hasta mi noche más oscura.

Pero todas estas aventuras no hubieran sido posibles sin Hipólito y Rosa. Porque vosotros papás, sois los dos grandes pilares en los que se fundamenta mi vida. Desde bien pequeña, me habéis brindado un cariño, una ayuda, una dedicación, una paciencia, una educación y una comprensión que no conoce límites, pero sobre todo un apoyo incondicional en cada una de mis decisiones y aventuras. Por ello, vosotros sois mi mejor ejemplo. Y es que a vosotros os debo todo lo que soy.

Y entre todas estas aventuras, surgió un 28 de julio de 2018, probablemente la más bonita en mi vida hasta el momento. Y ésta fue junto a ti, Noel. Contigo las palabras sobran, porque eres capaz de entenderme con tan sólo una mirada. Capaz de calmarme a la par que ilusionarme. Capaz de adorar a mi amada Chuni como si fuera tuya. Capaz de quererme tanto por mis virtudes como por mis defectos. Capaz de hacerme sentir la chica más afortunada y amada con tan sólo un beso. Describir lo que siento por ti con un simple "Te Quiero", se queda corto. Pero es que a veces ocurre que dos personas se juntan, y de repente surge la Química.

Y es de Química, de mi querida Química, de lo que se ha tratado todo este tiempo. Como ya veis, muchas aventuras con increíbles personajes que convierten en tarea difícil narrar todo lo vivido y reconocer todo lo aportado a esta Tesis Doctoral a todos ellos en tan pocas líneas. ¿En una palabra?



ABBREVIATIONS

A: Left peak half-width

B: Right peak half-width

BMIM: 1-Butyl-3-methylimidazolium

Brij-L4 / C12E4: Polyoxyethylene(4)lauryl ether

Brij-35 / Brij-L23 / C12E23: Polyoxyethylene(23)lauryl ether

C1: Methylsiloxane

C12E10: Polyoxyethylene(10)lauryl ether

C13E10: Polyoxyethylene(10)tridecyl ether

C18: Octadecylsiloxane

C8: Octylsiloxane

CD: Cyclodextrin

CE: Capillary electrophoresis

CMC: Critical micellar concentration

CTAB: Cetyltrimethylammonium bromide

D: Apparent octanol-water partition coefficient

EMIM: 1-Ethyl- 3-methylimidazolium

ESI: Electrospray ionization

GC: Gas chromatography

HCl: Hydrochloric acid

HILIC: Hydrophilic interaction liquid chromatographic

HMIM: 1-Hexyl-3-methylimidazolium

HPLC: High performance liquid chromatography

HSLC: High submicellar liquid chromatography

ICH: International Conference of Harmonization

IGEPAL CO-630: Polyoxyethylene(9)nonylphenyl ether

IIC: Ion-interaction chromatography

IL: Ionic liquid

IP: Ion pair

IPC: Ion pair chromatography

k: Retention factor

LOD: Limit of detection

LOQ: Limit of quantification

LSS: Linear solvent strength

M: Molar concentration (mol/L)

MLC: Micellar liquid chromatography

MS: Mass spectrometry

MW: Molecular weight

NaOH: Sodium hydroxide

NPLC: Normal phase liquid chromatography

OVAT: One-variable-at-a-time

pK_a: Acid dissociation constant

$P_{O/W}$: Octanol-water partition coefficient

QSAR: Quantitative structure-activity relationship

R^2: Determination coefficient

RP: Reversed-phase

RPLC: Reversed-phase liquid chromatography

RSD: Relative standard deviation

S: Elution strength parameter

SDS: Sodium dodecyl sulphate

Span 20: Sorbitanmonolaurate

TCAs: Tricyclic antidepressants

t_R: Retention time

Triton-X 100: Polyethylene glycol

Triton-X 114: Polyethylene glycol tert-octylphenyl ether

UV: Ultraviolet

w: Peak width

WHO: World Health Organization

RESUMEN

La cromatografía líquida en fase inversa (RPLC), cuyo mecanismo de separación se basa principalmente en la polaridad de los solutos, es el modo de cromatografía líquida más habitual en los laboratorios analíticos, debido a su amplia aplicabilidad, fiabilidad, robustez y sensibilidad. Sin embargo, los compuestos ionizados, que son altamente polares, muestran poca o ninguna retención con las fases móviles convencionales formadas exclusivamente por agua y disolvente orgánico. Además, existen graves problemas (largos tiempos de retención, y picos anchos y asimétricos) en el análisis de los compuestos catiónicos básicos, debido a su interacción con los grupos silanol residuales aniónicos presentes en las fases estacionarias de base sílice (que son las más frecuentes). Estos problemas dificultan una aplicabilidad universal de este modo cromatográfico. Una posible solución es el uso de aditivos de diferente naturaleza para inducir equilibrios secundarios.

Con el fin de incrementar el intervalo de polaridades de los compuestos analizados en RPLC y mejorar los perfiles de pico, durante el transcurso de la Tesis Doctoral, se han realizado diversas investigaciones en las que se han utilizado diversos surfactantes y líquidos iónicos como aditivos de la fase móvil, así como una modalidad cromatográfica (la cromatografía líquida de interacción hidrofílica, HILIC), que permite la separación de compuestos de elevada polaridad.

Los estudios realizados han implicado un gran esfuerzo experimental, diseñado para explorar y extraer información sobre el comportamiento cromatográfico de compuestos de diferente naturaleza (sulfonamidas, β-bloqueantes, antidepresivos tricíclicos, flavonas, diuréticos y nucleósidos). Se ha estudiado el uso de surfactantes de diferente carácter como modificadores de la fase móvil: aniónico (dodecilsulfato de sodio, SDS) y neutro (polioxetilen(23)lauriléter (conocido como Brij-35), polioxetilen(10) lauriléter,

polioxetilen(10)trideciléter, Brij-L4, Triton X-100, Triton X-114, IGEPAL CO-360 y Span 20), además de los líquidos iónicos cloruro de 1-hexil-3-metilimidazolio (HMIM·Cl) y tetrafluoro-borato de 1-hexil-3-metilimidazolio (HMIM·BF_4). Por último, se ha examinado el efecto de cuatro tampones de distinta naturaleza (acetato, formiato, citrato y fosfato), en presencia y ausencia de un líquido iónico, sobre el comportamiento cromatográfico.

A lo largo de todo el trabajo, se han inspeccionado una gran diversidad de condiciones experimentales, utilizando fases móviles acuo-orgánicas con acetonitrilo, fases móviles micelares puras (conteniendo tan sólo un surfactante) e híbridas (conteniendo un surfactante y disolvente orgánico), fases móviles micelares mixtas (conteniendo dos surfactantes), y fases móviles que contienen un líquido iónico, en algunos casos en ambos modos isocrático y de gradiente. El trabajo ha supuesto el uso de una gran variedad de columnas: C18 y C8 en RPLC, y de diverso tipo (sílice, neutra, catiónica, aniónica y zwiteriónica, incluyendo tres columnas de reciente comercialización) en HILIC.

Debe destacarse el gran esfuerzo bibliográfico realizado sobre los diferentes temas investigados. Ello ha implicado la búsqueda, lectura y organización de una gran cantidad de información valiosa, que se refleja adecuadamente en cada apartado de la Tesis Doctoral.

Los capítulos de la Memoria del trabajo realizado incluyen estudios fundamentales sobre el comportamiento cromatográfíco observado en RPLC con fases móviles de surfactantes y líquidos iónicos, y en HILIC con fases móviles de agua-acetonitrilo, así como el desarrollo de métodos para el análisis de preparados farmacéuticos y fluidos fisiológicos, acompañados de una extensa validación. A continuación se detallan ambos tipos de estudios.

1. Estudios fundamentales

Los estudios fundamentales tienen el propósito de comparar el comportamiento cromatográfico de diferentes grupos de analitos de diversa polaridad, carácter ácido-base y carga, examinando la retención y la forma de los picos obtenidos en los cromatogramas. En algunos estudios, se han ajustado modelos matemáticos que permiten predecir la retención de los solutos. Por otro lado, se han trazado gráficos que describen la variación de la anchura y asimetría de los picos cromatográficos (los denominados gráficos de semianchura, que revelan la cinética de interacción de los solutos con la fase estacionaria), y la correlación entre los tiempos de retención en distintas condiciones (que indican diferencias en la selectividad alcanzada con distintas columnas o condiciones experimentales). Estos gráficos permiten analizar la calidad de las condiciones experimentales ensayadas, en términos de tiempo de análisis, forma de los picos y resolución. En los estudios realizados, se muestran los cromatogramas de mezclas de los analitos correspondientes a las condiciones óptimas de resolución.

Los estudios fundamentales englobados dentro de la Tesis Doctoral, que se describen a continuación, pertenecen a tres campos de interés:

1.1. Cromatografía líquida micelar (CLM)

A pesar de la variedad de columnas disponibles en el mercado, su química todavía es limitada. Sin embargo, mediante la adición de reactivos a la fase móvil, el intervalo de selectividades de las columnas comerciales utilizadas en RPLC puede incrementarse, mejorando su rendimiento en el análisis de una mayor variedad de compuestos químicos. Se realizaron varios estudios que profundizaron en el conocimiento de los factores que producen el

ensanchamiento y distorsión de las señales cromatográficas en RPLC, y se desarrollaron estrategias para mejorar el perfil de los picos cromatográficos y su resolución. Se amplió, asimismo, el intervalo de polaridades de los compuestos analizados.

Los aditivos investigados fueron el surfactante SDS y varios surfactantes no iónicos, añadidos a la fase móvil por encima de su concentración micelar crítica. En estas condiciones, los surfactantes se adsorben sobre la fase estacionaria modificando su naturaleza y su exceso permanece en la fase móvil influyendo en su fuerza eluyente. El interés se centró principalmente en el uso del surfactante no iónico Brij-35. Este surfactante es considerado en CLM como una alternativa al surfactante aniónico SDS, ampliamente utilizado en este modo cromatográfico. Sin embargo, a pesar de la relevancia del Brij-35 en las relaciones cuantitativas estructura-actividad (QSAR), su uso es muy limitado. A lo largo de la Tesis Doctoral, se ha realizado un estudio detallado del campo de aplicación del Brij-35 en cromatografía líquida y de las condiciones más adecuadas para realizar los análisis en los modos isocrático y de gradiente. Se examinó también la posibilidad de utilizar otros surfactantes no iónicos y las ventajas del uso de fases móviles mixtas de Brij-35 y SDS.

En primer lugar, se estudió la posibilidad de utilizar fases móviles puramente micelares del surfactante no iónico Brij-35 (es decir, fases constituidas por agua y jabón), en RPLC. La metodología desarrollada puede considerarse como un método de análisis “verde”, que permite minimizar la contaminación y los residuos, aumentando así la sostenibilidad.

La presencia de Brij-35 en la fase móvil ofrece una selectividad particular, que afecta a la retención global de los analitos. Se comprobó que las fases móviles puramente micelares de Brij-35 son adecuadas para la separación cromatográfica de compuestos de polaridad alta o intermedia, o compuestos

polares que interactúan con el grupo terminal hidroxilo de la cadena etoxilada del surfactante no iónico por formación de enlaces de hidrógeno. Este último comportamiento se observó con varios flavonoides y sulfonamidas, que contienen grupos hidroxilo y amino, respectivamente. Por contra, se demostró que la polaridad de los β-bloqueantes ensayados y la formación de enlaces de hidrógeno internos entre los grupos hidroxilo y amino adyacentes, en estos compuestos, no permiten una interacción suficientemente intensa con la fase estacionaria modificada con Brij-35, por lo que no resultan suficientemente retenidos. Sin embargo, aunque fue posible eluir los flavonoides y las sulfonamidas con fases móviles puramente micelares de Brij-35, el uso de fases móviles híbridas conteniendo una baja concentración de Brij-35 y acetonitrilo mejoró significativamente el rendimiento cromatográfico (los picos fueron más estrechos y los tiempos de análisis más bajos).

Los picos cromatográficos obtenidos para flavonoides y sulfonamidas con Brij-35, en elución isocrática, presentaron una elevada simetría, revelando los gráficos de semianchura diferentes cinéticas de interacción con la fase estacionaria modificada por el surfactante. Para los flavonoides, la cinética de interacción depende de la posición de los sustituyentes hidroxilo en los anillos de benceno de estos compuestos. Por otro lado, utilizando elución en gradiente, la anchura de los picos se redujo significativamente cuando se utilizó una concentración fija de Brij-35 y una concentración creciente de acetonitrilo, o una concentración creciente de ambos Brij-35 y acetonitrilo. La anchura de pico también se redujo a alta temperatura (50 ^{o}C), lo que permitió mejorar la resolución de los cromatogramas.

Se comprobó que el uso de gradientes de acetonitrilo, utilizando una concentración fija de Brij-35, es especialmente interesante para el análisis de fármacos en fluidos fisiológicos, permitiendo la inyección directa de la muestra

en el cromatógrafo. Se comprobó que puede ser conveniente iniciar el análisis utilizando un medio puramente micelar de Brij-35, y una vez que las proteínas de la muestra han sido eluidas, aplicar un gradiente de acetonitrilo para incrementar la fuerza eluyente.

Dadas las ventajas que puede ofrecer el uso de surfactantes no iónicos como modificadores de la fase móvil en cromatografía líquida y la escasez de referencias sobre otros surfactantes no iónicos en CLM, distintos del Brij-35, se consideró interesante realizar un amplio estudio sobre las posibilidades de surfactantes no iónicos alternativos, en este modo cromatográfico. Dichos surfactantes deberían poseer prestaciones similares a las del Brij-35, e incluso, mejorar significativamente su comportamiento cromatográfico.

El estudio mostró que el polioxietileno(10)tridecil éter (C13E10) y el polioxietileno(10)lauril éter (C12E10) pueden ser utilizados en CLM, debido a su alta solubilidad en agua y nula absorción UV en el intervalo de detección habitual. La solubilidad de otros surfactantes no iónicos fue demasiado baja (Brij L4, TritonX-114 o Span 20), o su absorción UV demasiado elevada (Triton X 100 e IGEPAL CO-630). Se examinaron las propiedades cromatográficas de las fases móviles de C12E10 y C13E10, comparándolas con las obtenidas con el Brij-35. El estudio mostró que la fuerza eluyente de las fases móviles de los tres surfactantes es lo suficientemente intensa para eluir sulfonamidas, β-bloqueantes y antidepresivos tricíclicos (TCAs), sin la necesidad de añadir un disolvente orgánico. También es posible analizar mezclas de flavonoides, pero para estos compuestos fue necesario añadir una cantidad relativamente pequeña de acetonitrilo a las fases móviles micelares, con el fin de disminuir los tiempos de retención a valores prácticos.

Los β-bloqueantes y los TCAs (compuestos básicos que muestran carga positiva al pH de la fase móvil) se retuvieron principalmente de acuerdo a su

polaridad. Esto se evidenció por la alta correlación entre el coeficiente de reparto octanol-agua (log $P_{o/w}$) y los valores logarítmicos de las constantes de asociación (K_{AS} y K_{AM}), que miden las interacciones de los solutos con la fase estacionaria y la fase móvil, respectivamente. También se comprobó la mayor intensidad de las interacciones de las sulfonamidas analizadas con las fases estacionaria y móvil, que se traduce en tiempos de retención mayores en comparación a los compuestos básicos, lo que puede ser debido a la interacción del grupo sulfonamida con la cadena etoxilada de los surfactantes no iónicos.

Por otro lado, los picos cromatográficos obtenidos con fases móviles de C12E10 y C13E10 fueron más estrechos y simétricos, en comparación con los obtenidos con Brij-35. En el caso de los flavonoides, para los que se necesitó la adición de una pequeña cantidad de acetonitrilo para ser eluidos en tiempos prácticos, los picos fueron más anchos y asimétricos. El estudio indicó que el comportamiento cromatográfico de los compuestos analizados en presencia de C12E10 y C13E10 es similar y depende de la naturaleza del soluto. Sin embargo, entre los tres surfactantes no iónicos (Brij-35, C12E10 y C13E10), sólo el C13E10 aparentemente no se adsorbe irreversiblemente sobre la fase estacionaria, siendo esta característica muy interesante para la conservación de las prestaciones de las columnas.

Una ventaja común de los tres surfactantes es que su adsorción sobre la fase estacionaria incrementa su polaridad, con la consiguiente disminución de los tiempos de retención, para la mayoría de los solutos, evitando en muchos casos la necesidad de un disolvente orgánico y haciendo así la técnica más sostenible medioambientalmente. Sin embargo, a pesar del incremento de polaridad de la fase estacionaria neutra modificada con los surfactantes no iónicos ensayados, que permite analizar compuestos de polaridad baja o intermedia, para aquellos compuestos polares que no pueden establecer interacciones específicas con el

Brij-35 la retención es muy baja, o incluso nula. Éste es el caso de la mayoría de β-bloqueantes. Además, con Brij-35, las eficacias son más pobres que con SDS.

Por otro lado, los tiempos de retención de los compuestos básicos, analizados con las fases móviles micelares del surfactante aniónico SDS, son demasiado elevados para ser prácticos, lo que obliga a la adición de una elevada cantidad de disolvente orgánico. La alta retención obtenida es debida a la interacción electrostática de los compuestos protonados (catiónicos) con el surfactante aniónico adsorbido sobre la fase estacionaria. Se consideró así que el uso de una fase móvil mixta que contuviera simultáneamente SDS y Brij-35 podría resolver todas estas limitaciones. Puesto que la fase móvil se prepara con agua, en ausencia de disolvente orgánico, se trata de un método "verde", que permitiría el análisis de compuestos básicos de diversa polaridad.

En este estudio se comprobó que, efectivamente, para los compuestos básicos (como los β-bloqueantes y TCAs), la capacidad de retención de la fase estacionaria modificada con la fase móvil mixta es mayor en comparación con una fase estacionaria modificada exclusivamente con Brij-35, y significativamente inferior a la conseguida con una fase estacionaria modificada exclusivamente con SDS. Se verificó que las interacciones entre los solutos básicos y las micelas de Brij-35 y la fase estacionaria modificada (medida por las constantes de asociación K_{AS} y K_{AM}) son diferentes a las interacciones establecidas con el sistema mixto Brij-35/SDS, y por consiguiente, también se observaron diferencias en la selectividad. Por otro lado, los picos fueron prácticamente simétricos, similares a los obtenidos con fases móviles que contienen tan sólo el surfactante SDS (en este caso, en presencia de una pequeña cantidad de disolvente orgánico). Esto indica que el efecto del SDS en la fase móvil mixta sobre los grupos silanol de la columna se

conserva, lo que dificulta el acceso de los compuestos básicos a los grupos silanol de la fase estacionaria, dando lugar a una cinética de interacción con los solutos suficientemente rápida.

El estudio mostró que la retención de compuestos básicos polares y moderadamente polares (como es el caso del grupo de los β-bloqueantes analizados), demasiado baja cuando se utilizan fases móviles que contienen sólo Brij-35, se puede modular a valores prácticos mediante la adición de SDS a la fase móvil que contiene Brij-35. El sistema micelar mixto dio lugar a una buena resolución y tiempos de análisis adecuados. Sin embargo, para los TCAs, es preferible el uso de fases móviles que contienen únicamente Brij-35.

1.2. Líquidos iónicos como aditivos de la fase móvil en cromatografía líquida

En la última década, el interés por el uso de líquidos iónicos como aditivos en RPLC se ha incrementado. La mayoría de las investigaciones que utilizan líquidos iónicos se refieren a la separación de compuestos básicos, intentando eliminar el problema de los silanoles residuales de las columnas convencionales de base sílice y mejorando así el rendimiento cromatográfico. Las interacciones de los líquidos iónicos con los analitos pueden ser más complejas que las observadas con los surfactantes, ya que tanto el catión como el anión en el líquido iónico pueden adsorberse sobre la fase estacionaria. Por otro lado, los tampones añadidos a la fase móvil también pueden afectar al rendimiento cromatográfico. De ahí que se investigara el efecto de tampones de diverso tipo añadidos a la fase móvil, en presencia y ausencia de un líquido iónico, para lo que se seleccionó al HMIM·Cl. La elección de este aditivo para realizar el estudio se debe a que la adsorción del catión ($HMIM^+$) en las columnas C18 es muy significativa, mientras que el anión cloruro no posee apenas afinidad por esta fase estacionaria.

Se evaluó la variación en la retención y la forma de los picos de seis β-bloqueantes, eluidos con fases móviles que contenían cuatro sistemas tampón de distinta naturaleza (ácido acético/acetato, ácido cítrico/dihidrógenocitrato, ácido fórmico/formiato y ácido fosfórico/dihidrógenofosfato), a concentraciones crecientes a pH 3, en presencia y ausencia del líquido iónico. Los sistemas tampón polipróticos se estudiaron también a pH 7. Se comprobó que la retención se incrementa mediante la adición de una mayor concentración de todos los sistemas tampón ensayados, tanto en ausencia como en presencia de HMIM·Cl, lo que indica la importancia de controlar la concentración de tampón en los procedimientos analíticos cromatográficos.

En ausencia de líquido iónico, la(s) especie(s) aniónica(s) de los tampones se adsorben directamente sobre las cadenas alquílicas de la fase estacionaria. La especie aniónica del tampón adsorbido sobre la fase estacionaria atrae a los compuestos básicos catiónicos, originando una mayor retención al aumentar la concentración del tampón. La tendencia observada en la retención indicó un inicio de saturación para la concentración de tampón más elevada. Mientras tanto, en presencia del líquido iónico, los aniones de los tampones deben ser atraídos a la fase estacionaria por el catión del líquido iónico. Este comportamiento se evidencia por la tendencia lineal observada para la retención de los compuestos catiónicos: al aumentar la concentración del anión del tampón crece la intensidad de la interacción establecida con la fase estacionaria (y por consiguiente, con los solutos básicos catiónicos), debido a la atracción electrostática hacia la fase estacionaria recubierta por $HMIM^+$.

Los cambios en la retención al añadir el ion $HMIM^+$ fueron significativamente mayores, en comparación a los observados al incrementar la concentración de los tampones. Sin embargo, se observó una elevada correlación entre los factores de retención en presencia y ausencia del líquido

iónico, y para concentraciones variables de los tampones. Este comportamiento, así como el mantenimiento del orden de elución de la mezcla de β-bloqueantes en presencia de los distintos sistemas tampón ensayados, sugiere una selectividad similar, lo que se puede explicar principalmente por la similitud de la naturaleza de las interacciones (de tipo electrostático) que se establecen con $HMIM^+$ y/o los aniones de los tampones adsorbidos sobre la fase estacionaria.

Tal como se ha comentado, en ausencia de aditivo, se obtuvieron picos asimétricos debido a la interacción de los compuestos básicos catiónicos con los grupos silanoles libres, especialmente a pH 7. La obtención de picos estrechos y prácticamente simétricos, en presencia de HMIM·Cl a cualquier concentración de tampón, indica una eficaz protección de la columna por parte del líquido iónico, que recubre la superficie de la fase estacionaria C18. En ausencia del líquido iónico, el acceso a los grupos silanol se obstaculiza más eficazmente al aumentar la concentración del tampón, pero el efecto es menor que el observado con $HMIM^+$. De hecho, en presencia de este aditivo, no hay un cambio aparente en la anchura y simetría de los picos, al variar la concentración de los tampones.

Por último, debe indicarse que el ion citrato parece poseer una mayor afinidad por la fase estacionaria, debido a los mayores tiempos de retención y mejores simetrías de pico. En el caso del tampón fosfato, la retención fue algo menor que para los tampones de acetato y formiato, cuyo efecto sobre la retención fue similar. Aunque a pH 3 las especies predominantes, en la fase móvil, para los sistemas de acetato y formiato no son las formas básicas (aniónicas), el aumento en la retención de los β-bloqueantes al incrementarse la concentración de estos tampones sugiere una desprotonación inducida de los sistemas monopróticos, debido a la adsorción de los aniones sobre la fase estacionaria.

1.3. Cromatografía líquida de interacción hidrofílica (HILIC).

La cromatografía líquida de interacción hidrofílica permite el análisis cromatográfico de compuestos altamente polares. Inicialmente, se utilizaron columnas de fase normal para este modo cromatográfico. Sin embargo, en los últimos años, se han desarrollado nuevas fases estacionarias para obtener un rendimiento adecuado para una variedad de compuestos. HILIC es un campo de rápido crecimiento a lo largo de las últimas dos décadas. El tipo de interacciones que se establecen en este modo cromatográfico es similar a las obtenidas con columnas modificadas con surfactantes y líquidos iónicos. Por ello, se consideró interesante estudiar las características de los picos cromatográficos en este modo cromatográfico, desde una perspectiva similar a la utilizada en los apartados anteriores.

En la investigación realizada, se compararon las propiedades cromatográficas de siete columnas HILIC de distinta naturaleza, disponibles comercialmente, en términos de retención, selectividad, forma de pico y resolución, utilizando como compuestos de prueba un grupo de nucleósidos (adenosina, citidina, guanosina, xantosina, timidina, y uridina) y uracilo. Con este propósito, se hizo uso del mismo protocolo utilizado para las columnas de RPLC modificadas con aditivos. El comportamiento observado reveló información sobre las interacciones establecidas entre los solutos y las fases estacionarias.

Para realizar los estudios, se verificó el acondicionamiento y la regeneración de las columnas, realizando ciclos de cinco inyecciones consecutivas de citidina cada 60 min, hasta conseguir valores estables de tiempo de retención. Para comprobar la reproducibilidad de las medidas, se inyectó también citidina al principio y al final de cada jornada. Se necesitó un tiempo de equilibrado inicial de al menos 12 horas a un flujo de 1 mL/min para el acondicionamiento,

mientras que una vez que la columna había sido acondicionada, sólo fueron necesarios 30 min para cambiar a una fase móvil con mayor contenido de agua.

Para comprender el comportamiento de las columnas HILIC, se tuvo en cuenta el estado de ionización de los nucleósidos. Por un lado, adenosina, citidina, guanosina y xantosina se hallan parcialmente protonadas a pH 3, y por otro, timidina, uracilo y uridina existen principalmente en forma catiónica a pH ácido. Las columnas zwitteriónica, amino, HILIC-N y HILIC-B mostraron una mayor retención para todos los compuestos estudiados, lo que revela un carácter más hidrófilo para estas fases estacionarias, donde la capa acuosa adsorbida debe ser más gruesa. Además, el estudio parece indicar que el efecto de los silanoles sobre los compuestos catiónicos se encuentra enmascarado por la capa acuosa adsorbida en las columnas HILIC. Así, aunque las columnas de sílice y diol poseen un carácter neutro sin capacidad de intercambio iónico, la ionización de los silanoles puede proporcionar silanoles aniónicos libres que incrementarían la retención de las bases protonadas (catiónicas). Sin embargo, no se observó dicho comportamiento, ya que los nucleósidos analizados mostraron tiempos de retención bajos cuando se eluyeron con ambas columnas.

Se observaron comportamientos inesperados en la retención ofrecida por las columnas neutra (HILIC-N) y catiónica (la HILIC-A), comercializadas recientemente por ACE. Así, la retención con la columna HILIC-N fue significativamente mayor a lo esperado para los nucleósidos, lo que indica una elevada polaridad para esta columna, a pesar de su capacidad de intercambio iónico presumiblemente baja. Mientras tanto, a pesar de la alta capacidad de intercambio catiónico, reivindicada por los fabricantes, la columna HILIC-A dio lugar a los factores de retención más bajos que otras columnas. Por otro lado, el orden de elución de la mezcla de nucleósidos, especialmente para

adenosina y citidina, dependió significativamente de la naturaleza de la columna.

Las alta correlación entre los factores de retención para las columnas de sílice, diol y HILIC-A, por un lado, y las HILIC-B, amino y HILIC-N, por otro, indican una selectividad similar. En cambio, la columna zwitteriónica mostró una selectividad particular, pudiéndose explicar parcialmente por la cantidad significativamente distinta de agua adsorbida sobre las columnas.

En términos generales, los picos cromatográficos fueron simétricos para todas las columnas, incluso al eluir solutos catiónicos. Este comportamiento también indica que la actividad de los silanoles residuales es enmascarada, presumiblemente, por la capa acuosa adsorbida. Sin embargo, los picos fueron algo más anchos para las columnas de sílice y HILIC-A, y algo más asimétricos para las amino (picos con colas), seguidos de las columnas de sílice, zwitteriónica y HILIC-N (picos con desviaciones frontales). Los mejores resultados en términos de simetría se obtuvieron para las columnas diol, HILIC-A y HILIC-B.

Finalmente, la falta de dispersión de los gráficos de semianchura para todas las columnas HILIC (excepto la zwitteriónica) indicó una cinética de interacción similar para el grupo de nucleósidos en las condiciones estudiadas. La resolución sólo fue satisfactoria con la columna zwitterionica.

También se realizó una investigación detallada, con las siete columnas HILIC, sobre los modelos matemáticos más adecuados para modelizar su comportamiento de retención. Además, se comparó el comportamiento con el observado para dos columnas de RPLC en el análisis de compuestos polares (sulfonamidas y diuréticos). Se estudió la exactitud de siete modelos de retención de diversa complejidad, incluyendo modelos que describen mecanismos de retención mixtos (reparto en la capa rica en agua adsorbida

sobre las columnas, junto con interacciones polares y electrostáticas). En general, los datos de HILIC proporcionaron mejores ajustes en comparación con los datos de RPLC, probablemente debido a la menor variación de la retención observada para las columnas de HILIC. La calidad del ajuste aumentó con el número de parámetros del modelo. Así, los modelos de dos y tres parámetros muestran un mejor comportamiento, con errores promedio del 1.0% y 0.7%, respectivamente.

Además del estudio de los modelos de retención, se realizó una investigación sobre la fuerza eluyente. En primer lugar, se observó una fuerte correlación entre los valores del parámetro *S* (que cuantifica la fuerza de elución de la fase móvil en cada soluto) y la ordenada en el origen del modelo LSS (que cuantifica el nivel de retención del soluto), encontrándose las mejores correlaciones para sílice, diol, HILIC-A en HILIC, y Zorbax y Chromolith en RPLC, lo que indica una menor dispersión en el comportamiento del conjunto de analitos. Sin embargo, la conclusión más destacada es que la correlación es muy similar para todas las columnas. De hecho, el comportamiento similar en la capacidad de elución de las columnas HILIC y RPLC es sorprendente, considerando los diferentes mecanismos de retención.

2. Aplicaciones

Además de los trabajos fundamentales descritos, la Tesis Doctoral incluye diversos apartados en los que se comprueba la viabilidad de las modalidades cromatográficas estudiadas para su aplicación al desarrollo de métodos de análisis. Concretamente, se han realizado tres aplicaciones, una de ellas al análisis de fluidos fisiológicos, y las otras dos dirigidas al control de preparados farmacéuticos.

En primer lugar, se desarrolló un método analítico, utilizando gradientes de acetonitrilo y una concentración fija de Brij-35 o de SDS, para analizar sulfonamidas en muestras de orina y leche. El objetivo era diseñar un procedimiento que permitiera la inyección directa de muestras fisiológicas. El comportamiento observado, en este estudio, para las sulfonamidas eluidas con las fases móviles de Brij-35 puede explicarse debido a una combinación de las interacciones establecidas con una fase estacionaria modificada de mayor polaridad, y la formación de puentes de hidrógeno entre los grupos amino de las sulfonamidas y el extremo de la cadena de polioxietileno del surfactante no iónico adsorbido. La mayor polaridad de la fase estacionaria modificada se verificó comparando el comportamiento de retención de la serie homóloga de alquilbencenos en ausencia y presencia de Brij-35.

Los métodos desarrollados para leche y orina que utilizan gradientes de acetonitrilo, en presencia de un surfactante, e inyección directa de las muestras, son muy prácticos, debido a su simplicidad, bajo tiempo de re-equilibrado y la eliminación de cualquier tratamiento previo a la etapa cromatográfica, exceptuando el filtrado y dilución de la muestra. La implementación de gradientes de acetonitrilo en el modo micelar, utilizando SDS, permitió la separación de las sulfonamidas en tiempos de análisis prácticos.

En presencia de Brij-35, el tiempo de análisis disminuyó considerablemente en comparación a la elución isocrática, pero la resolución fue insuficiente. En cambio, el uso de elución en gradiente con acetonitrilo, en presencia de Brij-35, puede ser útil para analizar mezclas simples de sulfonamidas. El análisis de muestras de leche fortificada utilizando gradientes de acetonitrilo, en presencia de SDS o Brij-35, no reveló ninguna interferencia de los componentes endógenos de la matriz. Para facilitar la solubilización de las proteínas, lo que

incrementó la protección de la columna, se añadió una etapa isocrática de 2 min previamente a la aplicación del gradiente.

Por su parte, el desarrollo de métodos para el control de fármacos en preparados farmacéuticos, se realizó en el modo isocrático con fases móviles que contenían aditivos. El primer método se aplicó a la determinación de β-bloqueantes, utilizando una fase móvil preparada con una mezcla de los surfactantes aniónico SDS y no iónico Brij-35, en ausencia de disolvente orgánico. El segundo método se aplicó a la determinación de antidepresivos tricíclicos. En este caso, la fase móvil se preparó con una mezcla de acetonitrilo-agua en presencia del líquido iónico HMIM·Cl.

En ambos casos, se realizó una validación exhaustiva. Ésta se llevó a cabo siguiendo las recomendaciones de la guía de la Conferencia Internacional de Harmonización (ICH), en la que se recomienda evaluar diversos factores, como son los parámetros de calibración (linealidad), la precisión y exactitud intra- e inter-día, los límites de detección (LODs) y cuantificación (LOQs), la robustez y la recuperación de los analitos. La linealidad se evaluó mediante la obtención de rectas de calibrado y la medida de sus pendientes y ordenadas en el origen, durante tres días no consecutivos y a lo largo de tres semanas distintas. Por su parte, la exactitud (error relativo) y precisión (desviación estándar relativa) intra- e inter-día se investigaron durante tres días no consecutivos, a tres concentraciones distintas de los analitos, dentro del intervalo lineal de las rectas de calibrado, realizando seis réplicas para cada analito. Los LODs y LOQs se determinaron como las concentraciones de los analitos que proporcionaban relaciones señal/ruido 3:1 y 10:1, respectivamente. Por su parte, la robustez se examinó utilizando el método OVAT (*one-variable-at-a-time*), centrándose en los factores flujo, pH, y concentración de disolvente orgánico y de los

aditivos. En cualquier caso, se tuvieron en cuenta las desviaciones estándar relativas (RSD) de los tiempos de retención y de las áreas de los picos.

Por último, para medir la recuperación de los analitos en las muestras, se pesó una cantidad apropiada de cada muestra (varias cápsulas homogeneizadas de los preparados farmacéuticos) y se prepararon disoluciones de aproximadamente 25 µg/mL de cada fármaco por quintuplicado, realizándose finalmente inyecciones duplicadas de las mismas. De acuerdo a la guía ICH, el intervalo aceptable de recuperación se debe encontrar entre el 80 y 120%, como se comprobó para todos los preparados farmacéuticos analizados.

El método desarrollado con las fases móviles mixtas de SDS y Brij-35 permitió la elución de los β-bloqueantes atenolol, celiprolol, metoprolol, oxprenolol y propranolol, en tiempos de análisis relativamente cortos, con resultados de similar calidad a un procedimiento que utilizaba fases móviles de SDS 0.15 M y 15% (*v*/*v*) de 1-propanol. El método que utiliza fases móviles mixtas de SDS y Brij-35 evita, sin embargo, la necesidad de añadir un disolvente orgánico y permite la obtención de tiempos de análisis más bajos. Además, se obtuvieron picos simétricos y una buena resolución cuando se analizaron mezclas de los β-bloqueantes.

Una de las ventajas del método propuesto con SDS y Brij-35 es que la preparación de la muestra es muy sencilla y sólo requiere su solubilización y filtración antes de la inyección. La validación del método de acuerdo a las directrices de la ICH ofreció buenos resultados para los medicamentos analizados. Todas las rectas de calibrado cumplieron con los requisitos de linealidad, con coeficientes de determinación $R^2 > 0.9990$. Además, las pendientes y las ordenadas en el origen de las rectas de calibrado se mantuvieron estables durante todo el proceso de validación, lo que indicó la gran estabilidad de la columna a lo largo de los estudios realizados, así como

una buena capacidad predictora de las concentraciones de los analitos. Se obtuvo una buena exactitud y precisión intra- e inter-días, generalmente por debajo del 2%, y los LODs y LOQs fueron inferiores a 0.14 μg/mL y 0.26 μg/mL, respectivamente. Para finalizar, los ensayos de robustez, considerando el flujo, el pH y las concentraciones de Brij-35 y SDS en la fase móvil, arrojaron valores de RSD para los tiempos de retención, generalmente en el intervalo entre el 1% y 2%, observándose una mayor variabilidad para Brij-35 y para las áreas de pico para todos los factores estudiados.

Por último, se evaluó la viabilidad del uso de fases móviles que contienen los líquidos iónicos de 1-hexil-3-metilimidazolio asociado a los iones cloruro (HMIM·Cl) y tetrafluoroborato (HMIM·BF_4), para el análisis de compuestos básicos de carácter hidrofóbico (antidepresivos tricíclicos, TCAs) en preparados farmacéuticos. Estos fármacos se asocian fuertemente a las cadenas alquílicas de las fases estacionarias convencionales, por lo que los tiempos de análisis son muy elevados cuando se utilizan métodos convencionales en RPLC. Además, los TCAs interactúan con los silanoles ionizados residuales presentes en las fases estacionarias convencionales de base sílice, lo que se traduce en una mayor retención, y picos anchos y con colas.

Por ello, se pensó que la adición de un líquido iónico podría mejorar el rendimiento cromatográfico de los TCAs. Efectivamente, ambos líquidos iónicos ensayados (HMIM·Cl y HMIM·BF_4) originaron picos estrechos y simétricos para los compuestos básicos, cuando se añadieron a las fases móviles hidro-orgánicas. Sin embargo, se observó un efecto de sobrecarga con la columna C18, en presencia de los aditivos, lo que se interpretó por la acumulación de los TCAs hidrofóbicos en la cabeza de la columna. El efecto fue más acusado con HMIM·BF_4, debido a la alta afinidad del anión (tetrafluoroborato) hacia la fase estacionaria, que atrae con mayor intensidad a

los analitos catiónicos hacia su superficie, mediante interacciones electrostáticas.

El efecto de sobrecarga se minimizó utilizando una columna C8, en presencia del líquido iónico HMIM·Cl en concentración 10 mM. En estas condiciones, se obtuvieron tiempos de análisis razonables y se mejoró el perfil de los picos cromatográficos, utilizando un contenido de acetonitrilo relativamente bajo en la fase móvil (30% *v*/*v*), a pH 3.

La validación del método, realizada siguiendo la guía ICH mostró buenos resultados para todos los analitos. Todas las rectas de calibrado cumplieron con los requisitos de linealidad, con coeficientes de determinación $R^2 > 0.9964$, manteniéndose estables las pendientes y las ordenadas en el origen durante todo el proceso de validación. Además, las precisión intra- e inter-día estuvo por lo general por debajo del 1.0%. Por su parte, la exactitud intra-día se encontró en el intervalo entre el –2.1% y el +2.4%, y la precisión inter-día entre el –3.0% y el +2.3%. Los LODs y los LOQs fueron, por lo general, inferiores a 0.25 μg/mL y 0.09 μg/mL, respectivamente para los distintos analitos.

Por último, los valores de RSD en los estudios de robustez sobre la retención oscilaron entre el 0.4% y 1.6% para el flujo, la concentración de acetonitrilo y el pH, pero el intervalo de variación fue mayor para la concentración de HMIM·Cl (en el intervalo entre el 2.8% y 4.8%). Respecto a las áreas de los picos, la variabilidad fue significativamente menor para la concentración de HMIM·Cl (0.4–0.9%), y similar para los otros parámetros analizados. Debe indicarse que el método desarrollado no permite el cribado de los TCAs, aunque arrojó excelentes resultados para el análisis de preparados farmacéuticos que contienen sólo uno de estos fármacos. Para realizar el análisis, la muestra sólo debe disolverse y filtrarse previamente a su análisis.

INDEX

OBJECTIVES AND DEVELOPMENT OF THE RESEARCH 1

CHAPTER 1: Introduction 19

1.1. Reversed Phase Liquid Chromatography 21

1.2. Secondary equilibria in RPLC 24

1.3. Hydrophilic Interaction Liquid Chromatography 30

1.4. Half-widths plots, a simple useful tool to study peak profiles 34

1.5. Validation in Liquid Chromatography 36

1.6. References 38

PART 1: MICELLAR LIQUID CHROMATOGRAPHY WITH NON-IONIC SURFACTANTS 49

CHAPTER 2: Fundamentals of Micellar Liquid Chromatogrpahy 51

2.1. Abstract 53

2.2. Introduction 54

2.3. The chromatographic system 55

2.3.1. The mobile phase 55

2.3.2. The stationary phase 57

2.4. Partitioning behaviour 59

2.5. Correlation between retention and polarity.....61

2.6. Effect of pH on retention 62

2.7. Modelling of retention 64

2.7.1. Pure micellar mobile phases 64

2.7.2. Hybrid micellar mobile phases 66

2.7.3. Simultaneous effect of surfactant, organic solvent, and pH 67

2.8. Peak shape 68
2.9. Separation performance 69
2.10. Analytical applications 72
2.11. Further reading 77

CHAPTER 3: Isocratic and gradient elution in Micellar Liquid Chromatography with Brij-35 81
3.1. Abstract 83
3.2. Introduction 84
3.3. Experimental 87
3.3.1. Reagents, column and apparatus 87
3.3.2. Procedures 88
3.4. Results and discussion 89
3.4.1. An overview of the capability of Brij-35 as modifier in MLC 89
3.4.2. Retention behaviour in isocratic elution 91
3.4.3. Peak profiles in isocratic elution 95
3.4.4. Gradient elution.....98
3.5. Conclusions 105
3.6. References 106

CHAPTER 4: Effect of sodium dodecyl sulphate and Brij-35 on the analysis of sulphonamides in physiological samples using direct injection and acetonitrile gradients 113
4.1. Abstract 115
4.2. Introduction 116
4.3. Experimental 118
4.3.1. Reagents and columns 118
4.3.2. Apparatus and software 121

4.3.3. Experimental designs 122
4.3.4. Analysis of physiological samples 124
4.4. Results and discussion 124
4.4.1. Isocratic separation of sulphonamides 124
4.4.2. Interactions with the unmodified and modified stationary phases 126
4.4.2.1. Electrostatic attraction and formation of hydrogen bonds 127
4.4.2.2. Stationary phase polarity 128
4.4.2.3. Interactions of sulphonamides 130
4.4.3. Retention behaviour in gradient elution 133
4.4.4. Column re-equilibration 135
4.4.5. Analysis of physiological samples 137
4.5. Conclusions 139
4.6. References 142

CHAPTER 5: Search of non-ionic surfactants suitable for Micellar Liquid Chromatography 149
5.1. Abstract 151
5.2. Introduction 152
5.3. Experimental 154
5.3.1. Reagents 154
5.3.2. Apparatus and columns 160
5.4. Results and discussion 161
5.4.1. Preliminary studies 161
5.4.2. Retention capability of stationary phases modified with the non-ionic surfactants 163

5.4.3. Association of solutes to the modified stationary phase and micelles in the mobile phase 170
5.4.4. Peak profiles 176
5.4.5. Surfactant desorption from the stationary phase 182
5.5. Conclusions 184
5.6. References 187

CHAPTER 6: Reversed-Phase Liquid Chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A green method for the analysis of basic compounds 193
6.1. Abstract 195
6.2. Introduction 196
6.3. Experimental 199
6.3.1. Reagents 199
6.3.2. Apparatus and column 200
6.3.3. Experimental design.....200
6.4. Results and discussion 201
6.4.1. Retention capability of the mixed Brij-35/SDS micellar systems 201
6.4.2. Solute-stationary phase and solute-mobile phase interactions 207
6.4.3. Peak profiles in the mixed Brij-35/SDS micellar systems 210
6.4.4. Selectivity and resolution.....214
6.5. Conclusions 217
6.6. References 219

CHAPTER 7: Analysis of basic drugs by Liquid Chromatography with environmentally friendly mobile phases in pharmaceutical formulations 227

7.1. Abstract 229

7.2. Introduction 230

7.3. Experimental 233

7.3.1. Reagents 233

7.3.2. Apparatus and chromatographic conditions 234

7.3.3. Procedure 235

7.4. Results and discussion 235

7.4.1. Chromatographic behaviour of β-blockers in hybrid and mixed MLC 235

7.4.2. Peak shape 238

7.4.3. Method validation 242

7.4.4. Analysis of pharmaceutical formulations 247

7.5. Conclusions 250

7.6. References 253

PART 2: IONIC LIQUIDS AS MOBILE PHASE ADDITIVES IN LIQUID CHROMATOGRAPHY 257

CHAPTER 8: Effect of buffer nature and concentration on the chromatographic performance of basic compounds in the absence and presence of 1-hexyl-3-methylimidazolium chloride 259

8.1. Abstract 261

8.2. Introduction 262

8.3. Experimental 265
8.3.1. Reagents 265
8.3.2. Apparatus and columns 266
8.4. Results and discussion 267
8.4.1. pK_a and pH variation of the selected buffers with solvent composition.....267
8.4.2. Dominant acid-base species at the pH of the assayed mobile phases.....269
8.4.3. Effect of ionic liquid on retention 271
8.4.4. Effect of buffer on retention.....274
8.4.5. Effect of ionic liquid and buffer on peak shape 278
8.4.6. Selectivity and resolution.....289
8.5. Conclusions 295
8.6. References 297

CHAPTER 9: Suitability of 1-hexyl-3-methyl imidazolium ionic liquids for the analysis of pharmaceutical formulations containing tricyclic antidepressants 305
9.1. Abstract 307
9.2. Introduction 308
9.3. Experimental 310
9.3.1. Reagents 310
9.3.2. Apparatus and chromatographic conditions 311
9.3.3. Procedure 312
9.4. Results and discussion 312
9.4.1. Experimental design 312
9.4.2. Effect of ionic liquids on retention and peak profile of TCAs 315
9.4.3. Selection of stationary phase and mobile phase additive 316

9.4.4. Method validation 319
9.4.5. Analysis of pharmaceuticals formulations 329
9.5. Conclusions 335
9.6. References 336

PART 3: HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY 343

CHAPTER 10: Protocol to compare column performance applied to Hydrophilic Interaction Liquid Chromatography 345
10.1. Abstract 347
10.2. Introduction 348
10.3. Experimental 350
10.3.1. Reagents 350
10.3.2. Apparatus and columns 353
10.4. Results and discussion 355
10.4.1. Retention capability of HILIC columns 355
10.4.2. Selectivity 361
10.4.3. Column performance based on peak shape 363
10.4.4. Resolution 371
10.5. Conclusions 374
10.6. References 375

CHAPTER 11: Study on the retention modelling using Hydrophilic Interaction Liquid Chromatography columns 385
11.1. Abstract 387
11.2. Introduction 388
11.3. Theory 390

11.4. Experimental 392
11.4.1. Reagents 392
11.4.2. Apparatus and columns 397
11.4.3. Column conditioning and regeneration 398
11.5. Results and discussion 400
11.5.1. Retention behaviour in HILIC and RPLC columns 400
11.5.2. Performance of retention models applied to HILIC columns 404
11.5.3. Correlation between the parameters of the LSS model.....415
11.5.4. Variation of the elution strength with the modifier concentration.....420
11.6. Conclusions 427
11.7. References 429

SUMMARY AND CONCLUSIONS 435
C.1. Micellar Liquid Chromatography with non-ionic surfactants.....438
C.1.1. Isocratic and gradient elution in Micellar Liquid Chromatography with Brij-35 438
C.1.2. Effect of sodium dodecyl sulphate and Brij-35 on the analysis of sulphonamides in physiological samples using direct injection and acetonitrile gradients 440
C.1.3. Search of non-ionic surfactants suitable for Micellar Liquid Chromatography 442
C.1.4. Reversed-Phase Liquid Chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A green method for the analysis of basic compounds 444
C.1.5. Analysis of basic drugs by Liquid Chromatography with environmentally friendly mobile phases in pharmaceutical formulations 445

C.2. Ionic liquids as mobile phase additives in Liquid Chromatography 447

C.2.1. Effect of the buffer nature and concentration on the chromatographic performance of basic compounds in the absence and presence of 1-hexyl-3-methylimidazolium chloride.....447

C.2.2. Suitability of 1-hexyl-3-methyl imidazolium ionic liquids for the analysis of pharmaceutical formulations containing tricyclic antidepressants 450

C.3. Hydrophilic Interaction Liquid Chromatography 452

C.3.1. Protocol to compare column performance applied to Hydrophilic Interaction Liquid Chromatography 452

C.3.2. Retention modelling with Hydrophilic Interaction Liquid Chromatography columns 455

CONTRIBUTION OF THE PhD WORK TO PUBLISHED ARTICLES 457

OBJECTIVES AND DEVELOPMENT OF THE RESEARCH

Reversed phase liquid chromatography (RPLC) is the most usual LC mode in analytical laboratories, due to its wide applicability, reliability, robustness and sensitivity. Unfortunately, the loss of efficiency (especially for basic compounds, whose broad and asymmetrical peaks are mainly explained by the interaction with anionic residual silanol groups present in the column packing), and the inadequate retention for diverse compounds (too short or excessive), hinder a more universal applicability of this chromatographic mode. Since the development of the technique, much effort has been devoted to solve these problems. However, it is necessary to still progress on the knowledge of the chromatographic separation mechanisms, considering its thermodynamics and kinetics aspects, and improve the analytical methodologies, especially in the field of pharmaceutical and food analysis.

The PhD. work collected in this Project gathers some proposals of two types to improve the HPLC performance. The objectives are next briefly summarised:

Objective 1. *Use of additives of different nature to induce secondary equilibria.*

In spite of the variety of columns available in the market, their chemistry is still limited. However, by adding additives to the mobile phase, the range of selectivities of the comercial columns in RPLC can increase, improving their performance. In this work, several studies are presented which have deepened on the knowledge of the factors that produce the broading and distortion of the chromatographic signals, as well as on the development of strategies that improve the profile of the chromatographic peaks and the resolution. The investigated additives were the surfactants sodium dodecyl sulphate (SDS) and several from the non-ionic Brij-35 family (added to the mobile phase above their critical micellar concentration, in the so-called micellar liquid

chromatography or MLC), as well as 1-hexyl-3-methyl imidazolium ionic liquids associated to chloride and tetrafluoroborate.

Throughout the development of this PhD. work, our purpose was:

Objective 1.1: Minimise the undesirable effects of the residual silanols in reversed-phase HPLC columns. With this aim, the cited additives were adsorbed onto the stationary phase, forming a protective layer that masked the access of basic solutes to the residual silanol groups, giving rise to an improvement in the peak shape (width and asymmetry).

Objective 1.2: Progress in the application of MLC. We were particularly interested in the development of gradient elution using Brij-35, exploring new non-ionic surfactants for MLC, and checking the viability and possible advantages of the use of mixtures of the anionic SDS and non-ionic Brij-35.

Objective 1.3: Further investigate the use of ionic liquids (ILs) as mobile phase additives in liquid chromatography, and the effect on the chromatographic behaviour of the use of different buffers to fix the pH, in the absence and presence of ILs.

Objective 1.4: Develop new methodologies for the analysis of physiological fluids and pharmaceuticals, using surfactants and ILs as additives of the mobile phase.

Objective 2. *Studies on hydrophilic interaction liquid chromatography*.

In recent years, diverse hydrophilic interaction liquid chromatography (HILIC) columns have been manufactured for the determination of high polarity compounds. HILIC is a fast growing field with a number of authors interested, along the last two decades, on the investigation of the mechanisms of retention in HILIC columns of different nature and their comparison. The

research group has developed diverse methodologies to investigate the column performance for RPLC. It has been considered of interest applying the developed tools to HILIC columns of different nature. Our purpose was:

Objective 2.1: Study and compare the retention, peak shape, selectivity and resolution in the analysis of polar compounds using commercially available HILIC columns of different nature.

Objective 2.2: Explore the possibility of modelling the retention behaviour in HILIC, using equations of diverse complexity.

To improve the understanding of the contents of the PhD. Project, the publications developed in this period have been ordered, divided in three parts:

Part 1. *Micellar Liquid Chromatography with non-ionic surfactants.*

Part 2. *Ionic liquids as mobile phase additives in Liquid Chromatography.*

Part 3. *Hydrophilic Interaction Liquid Chromatography.*

The work has implied a large experimental effort, designed to explore and extract information on the chromatographic behaviour of compounds of different nature (sulphonamides, β-blockers, tricyclic antidepressants, flavonoids, diuretics and nucleosides). A great diversity of experimental conditions has been assayed, using aqueous-organic mobile phases with acetonitrile, pure and hybrid micellar mobile phases, mixed micellar mobile phases, and mobile phases containing ILs. The work has involved a large variety of columns for RPLC and HILIC.

The large effort in several literature surveys on the different topics investigated in this work should be also highlighted. This has implied the search, reading and organization of a large amount of valuable information that is properly reflected in each part of the PhD. Project.

Supervisors and research laboratories

The research work leading to the PhD. degree in Chemistry was started in September 2014, once the Master degree on "Experimental Techniques in Chemistry" offered by the Departments of Analytical Chemistry and Inorganic Chemistry at the University of Valencia was finished. The experimental work in this Project was developed in the Department of Analytical Chemistry (University of Valencia), under the supervision of Profs. María Celia García Álvarez-Coque, María José Ruiz Ángel and Samuel Carda Broch. Acknowledge should be also given to the valuable collaboration of Prof. Juan José Baeza Baeza in some fundamental studies.

The PhD. period included a three-months stay (September to December 2018**)**, under the supervision of Prof. Jared L. Anderson in the Department of Chemistry at the Iowa State University in Ames (USA), working in the field of argentation chromatography. The aim of the research was to develop a gas chromatography stationary phase, based on the coordination of silver ions to ionic liquids for the analysis of unsaturated compounds. The column should be thermally stable at temperatures above 150 °C and sufficiently high efficiency.

Publications

The publications included in this PhD. Project are the following (the journal impact factor, IF, and ranking in the category of Analytical Chemistry and Multidisciplinary Chemistry are given):

1. Ester Peris García, Casandra Ortiz Bolsico, Juan José Baeza Baeza, María Celia García Álvarez-Coque
   Isocratic and gradient elution in micellar liquid chromatography with Brij-35.
   Journal of Separation Science 38 (2015) 2059–2067 (Chapter 3).
   IF (2015): 2.741 (Analytical Chemistry: 21/75)

2. María José Ruiz Ángel, Ester Peris García, María Celia García Álvarez-Coque
   Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A method for the analysis of basic compounds.
   Green Chemistry 17 (2015) 3561–3570 (Chapter 6).
   IF (2015): 8.506 (Multidisciplinary Chemistry: 16/163)

3. Ester Peris García, María Teresa Úbeda Torres, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Effect of sodium dodecyl sulphate and Brij-35 on the analysis of sulphonamides in physiological samples using direct injection and acetonitrile gradients.
   Analytical Methods 8 (2016) 3941–3952 (Chapter 4).
   IF (2016): 1.900 (Analytical Chemistry: 43/76)

4. Ester Peris García, María José Ruiz Ángel, Samuel Carda Broch, María Celia García Álvarez-Coque
   Analysis of basic drugs by liquid chromatography with environmentally friendly mobile phases in pharmaceutical formulations.
   Microchemical Journal 134 (2017) 202–210 (Chapter 7).
   IF (2017): 2.746 (Analytical Chemistry: 24/80)

5. Sonia Calabuig Hernández, Ester Peris García, María Celia García Álvarez-Coque, María José Ruiz Ángel
   Suitability of 1-hexyl-3-methylimidazolium ionic liquids for the analysis of pharmaceutical formulations containing tricyclic antidepressants.
   Journal of Chromatography A 1559 (2018) 118–127 (Chapter 9).
   IF (2018): 3.858 (Analytical Chemistry: 15/84)

6. Ester Peris García, Jorge Rodríguez Martínez, Juan José Baeza Baeza, María Celia García Álvarez-Coque, María José Ruiz Ángel
   Search of new non-ionic surfactants for micellar liquid chromatography.
   Analytical and Bioanalytical Chemistry 410 (2018) 5043–5057
   (Chapter 5).
   IF (2018): 3.286 (Analytical Chemistry: 18/84)

7. María Celia García Álvarez-Coque, María José Ruiz Ángel, Ester Peris García
   Micellar liquid chromatography
   Encyclopedia of Analytical Science (edited by P.J. Worsfold, A. Townshend, C. Poole, M. Miró), Elsevier, Waltham, MA, 3rd ed., Vol. 6, 2019, pp. 133–142 (chapter written by invitation) (Chapter 2). The chapter was also published on line in Reference Module in Chemistry,

Molecular Sciences and Chemical Engineering Series (edited by J. Reedijk), Elsevier, Waltham, MA, 2018.

8. Raquel Burgos Gil, Ester Peris García, María José Ruiz Ángel, Juan José Baeza Baeza, María Celia García Álvarez-Coque
   Protocol to compare column performance applied to hydrophilic interaction liquid chromatography.
   Microchemical Journal 149 (2019) 103973 (Chapter 10).
   IF (2018): 3.206 (Analytical Chemistry: 20/84)

9. Ester Peris García, Samuel Carda Broch, María Celia García Álvarez-Coque, María José Ruiz Ángel
   Effect of buffer nature and concentration on the chromatographic performance of basic compounds in the absence and presence of 1-hexyl-3-methylimidazolium chloride
   Journal of Chromatography A 1602 (2019) 397–408. (Chapter 8).
   IF (2018): 3.858 (Analytical Chemistry: 15/84)

10. Ester Peris García, María José Ruiz Ángel, Juan José Baeza Baeza, María Celia García Álvarez-Coque
    Study on the retention modelling using hydrophilic interaction liquid chromatography columns
    Article in preparation (2019) (Chapter 11).

The work not included in this PhD. Project is:

11. Ester Peris García, N. Pankajkumar-Patel, Samuel Carda Broch, María José Ruiz Ángel, María Celia García Álvarez-Coque,
    Oil-in-water microemulsion liquid chromatography
    Separation & Purification Reviews (in press, 2019).
    IF (2018): 4.714 (Analytical Chemistry: 12/84)

12. N. Pankajkumar-Patel, Ester Peris García, María José Ruiz Ángel, Samuel Carda Broch, María Celia García Álvarez-Coque,
    Modulation of retention and selectivity in oil-in-water microemulsion liquid chromatography: A review
    Journal of Chromatography A 1592 (2019) 91–100 (selected to be published in the special issue "Editor´s Choice XII").
    IF (2018): 3.858 (Analytical Chemistry: 15/84)

Congress communications

The developed research has been also presented in 13 scientific conferences in the period June 2013 to August 2019 (11 communications in international conferences and 9 communications in national conferences, 18 posters and 2 oral communications). The PhD. candidate has attended to 7 of these conferences: Analytica'2015 (Valencia), SECyTA'2016 (Sevilla), Workshop/November 2016 (Valencia), Euroanalysis'2017 (Stockholm), SEQA'2017 (Valencia), ISSS'2018 (Jasná, Slovakia), and Young Researchers in Chemistry Workshop'2019 (Burjassot):

39th International Symposium on High Performance Liquid Phase Separation and Related Techniques (HPLC'13)
Amsterdam (Netherlands), June 2013 (international)

1. Juan Peris Vicente, Inmaculada Casas Breva, Samuel Carda Broch, Mónica Villarreal Traver, David Fabregat Safont, Noelia Fuentes Navarro, Ester Peris García, Josep Esteve Romero
   Hospital monitorization in blood of a highly active antiretroviral therapy regime
   (Poster BIOM 25-MO)

30th International Symposium on Chromatography (ISC'2014)
Salzburg (Austria), September 2014 (international)

2. María Celia García Álvarez-Coque, Ester Peris García, Casandra Ortiz Bolsico, Juan José Baeza Baeza, María José Ruiz Ángel
   Reversed-phase liquid chromatography with water and detergent
   (Poster P073-FF-MO)

14th Conference on Instrumental Analysis (JAI'2014)
Barcelona (Spain), October 2014 (national)

3. Juan José Baeza Baeza, Ester Peris García, Casandra Ortiz Bolsico, María Celia García Álvarez-Coque
   Reversed-phase liquid chromatography with Brij-35: A chromatographic mode with water and soap
   (Poster CTQ-P03)

6th International Conference and Exhibition on Analytical and Bioanalytical Techniques (Analytica'2015)
Valencia (Spain), September 2015 (international)

4. María José Ruiz Ángel, María Teresa Úbeda Torres, Ester Peris García, María Celia García Álvarez-Coque
   Comparison of the performance of amines and ionic liquids as additives in RPLC for the analysis of basic compounds
   (Oral communication)

5. Ester Peris García, María Teresa Úbeda Torres, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Comparison of gradients of organic solvent in micellar liquid chromatography using the surfactants sodium dodecyl sulphate and Brij-35 (Poster AA12)

6. María José Ruiz Ángel, Ester Peris García, María Celia García Álvarez-Coque
   Validation of green chromatographic method for the analysis of β-blockers that uses a surfactant aqueous solution as mobile phase
   (Poster AA21)

XVI Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECyTA'2016)
Sevilla (Spain), November 2016 (national)

7. Ester Peris García, Samuel Carda Broch, María Celia García Álvarez-Coque, María José Ruiz Ángel
   Analysis of basic drugs in pharmaceutical formulations using liquid chromatography with water and detergent
   (Poster P-CPA-6)

8. Ester Peris García, Sonia Calabuig Hernández, Samuel Carda Broch, María Celia García Álvarez-Coque, María José Ruiz Ángel
   Amines versus ionic liquids as silanol blockers in reversed-phase liquid chromatography
   (Poster P-FCH-3)

First Workshop on Separation Strategies in Chromatography
Burjassot, Valencia (Spain), November 2016 (national)

9. Ester Peris García, María José Ruiz Ángel, Samuel Carda Broch, María Celia García Álvarez-Coque
   Green RPLC with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate
   (Oral communication)

XIX Europe's Analytical Chemistry Meeting (Euroanalysis'2017)
Stockholm (Sweden), August-September 2017 (international**)**

10. Ester Peris García, Sonia Calabuig Hernández, María Celia García Álvarez-Coque, María José Ruiz Ángel
    Use of eluents containing ionic liquids to improve the HPLC analysis of tricyclic antidepresants
    (Poster 121 Thu-PHA)

11. Ester Peris García, Jorge Rodríguez Martínez, Juan José Baeza Baeza, María Celia García Álvarez-Coque, María José Ruiz Ángel
    RPLC using phases containing non-ionic surfactants
    (Poster 255 Thu-SAM)

XXI Meeting of the Spanish Society of Analytical Chemistry (SEQA'2017)
Valencia (Spain), September 2017 (national)

12. María José Ruiz Ángel, Samuel Carda Broch, Ester Peris García, María Celia García Álvarez-Coque
    Extent of the influence of phosphate buffer and ionic liquids on the reduction of the silanol effect in a C18 stationary phase
    (Poster PO-TS-02)

13. Samuel Carda Broch, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
    Microemulsion liquid chromatography: A useful technique for drug analysis
    (Poster PO-TS-03)

24th International Symposium on Separation Sciences (ISSS'2018)
Jasná (Slovakia), June 2018 (international)

14. Ester Peris García, María José Ruiz Ángel, Samuel Carda Broch, María Celia García Álvarez-Coque
    Effect of buffers and ionic liquids on the reduction of silanol activity in RPLC
    (Poster P059)

15. Ester Peris García, N. Pankajkumar-Patel, Samuel Carda Broch, María José Ruiz Ángel, María Celia García Álvarez-Coque
    Microemulsion liquid chromatography: Main features and applications
    (Poster P108)

32nd International Symposium on Chromatography (ISC'2018)
Cannes-Mandelieu (France), September 2018 (international)

16. María Celia García Álvarez-Coque, Ester Peris García, Raquel Burgos Gil, Ana Ribera Castelló, Juan José Baeza Baeza, María José Ruiz Ángel
    Evaluation of several hydrophilic interaction liquid chromatography stationary phases for analysis of nucleosides: Modeling the retention behaviour
    (Poster PS-07-06)

48th International Symposium on High Perfomance Liquid Phase Separations and Related Techniques (HPLC'2019)
Milan (Italy), June 2019 (international)

17. María Celia García Álvarez-Coque, Ester Peris García, Raquel Burgos Gil, Juan José Baeza Baeza, María José Ruiz Ángel
    Comparison of the performance of HILIC columns
    (Poster P 396)

1st Workshop "Young Researchers in Chemistry"
Burjassot, June 2019 (national)

18. Ester Peris García, Nikita Pankajkumar-Patel, María José Ruiz Ángel, María Celia García Álvarez-Coque
    Modulation of retention and selectivity in oil-in-water microemulsion liquid chromatography
    (Poster P1)
19. Nikita Pankajkumar-Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
    Analysis of β-blockers in microemulsion liquid chromatography
    (Poster P2)

XXII Reunión de la Sociedad Española de Química Analítica (SEQA'2019) Valladolid, July 2019 (national)

20. Nikita Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
    Microemulsion liquid chromatography versus high submicellar liquid chromatography for the analysis of β-blockers

    (Poster TSE-P14)

The research along the PhD. Project was funded by three national Research Projects:

1. Project CTQ2013-42558-P: *"Modulation of selectivity and efficiency in HPLC using multi-column strategies to enhance the resolution of complex samples"*, funded by Ministry of Economy and Competitiveness, January 2014–December 2016. Main researchers: María Celia García Álvarez-Coque and José Ramón Torres Lapasió.
2. Project PROMETEO/2016/128: *"Multi-column strategies to enhance the performance in the separation of complex samples by liquid chromatography"*, funded by Generalitat Valenciana (Direcció General d'Universitat, Investigació i Ciència), January 2016–December 2019. Main researcher: María Celia García Álvarez-Coque.
3. Project CTQ2016-75644-P: *"Design of methodologies to optimize the separation quality in liquid chromatography"*, funded by Ministry of Economy, Industry and Competitiveness, January 2017–December 2019. Main researchers: María Celia García Álvarez-Coque and José Ramón Torres Lapasió.

Along the PhD period, three grants were obtained:

1. UV-AYCOLINV-193063, funded by University of Valencia (September 2014–January 2015).
2. UV-INV-PREDOC16F1-384313, funded by University of Valencia (July 2016–June 2020).
3. VALi+d Ref. ACIF/2016/319, funded by Generalitat Valenciana (this grant was obtained at the same time as UV-INV-PREDDOC16F1 grant, but was dropped to enjoy the grant offered by the University of Valencia).

Before enjoying the PhD. grant funded by the University of Valencia, the following professional activities were carried out:

1. REPOL (industry dedicated to the manufacture of technical plastics), contract as analyst in the quality control laboratory, Almassora, Castellón (April 2015–February 2016).
2. SOIVRE (Servicio Oficial de Inspección, Vigilancia y Regulación de Exportaciones, *Official Service of Inspection, Surveillance and Regulation of Exports*), laboratory of the territorial direction of foreign trade in Valencia, Ministry of Industry, Trade and Tourism. Grant for specialization in analytical control (Disposición 2939 del BOE num. 73, March 25, 2016) (March 2016-June 2016). The grant was dropped to enjoy the PhD grant.

CHAPTER 1

INTRODUCTION

This section has been written to introduce some relevant topics that are treated throughout the PhD. work, and from which it is convenient to explain some basic concepts:

1.1. Reversed Phase Liquid Chromatography

Reversed Phase Liquid Chromatography (RPLC) is a mode of High-Performance Liquid Chromatography (HPLC) that employs a nonpolar stationary phase and a polar mobile phase constituted by water and at least a water-miscible organic solvent, which performs as a modifier [1–3]. RPLC has become the most popular HPLC mode, representing the vast majority of all HPLC separations. In fact, its applications cover a wide variety of fields, such as environmental control, food, clinical, pharmaceutical and industrial analysis, drug and chemical manufacturing (at both analytical quality control and preparative scales), biomedical studies, and measurement of physico-chemical properties.

In this technique, retention is fundamentally determined by the distribution of solutes between mobile phase and stationary phase, as they progress along the chromatographic column. For this reason, the choice of the stationary phase in RPLC plays a fundamental role. It should be mechanically stable and have the desired non-polar, moderately polar or polarisable properties to interact with the analytes. Among the diversity of types of stationary phases in RPLC, silica is the most ideal support in view of its properties, being the C_{18} column the most widely used, followed by C_8 phases. Two reasons of this preference are the relatively high organic content for analyte interaction, and the better stability at both extremes of the working pH range, compared to shorter alkyl chain phases [4,5].

However, although bonded silica stationary phases have many advantages, they have important limitations associated to free silanols and pH. On the one hand, unreacted accessible silanols can interact with polar groups of solutes, as hydroxyl and amino groups, via specific hydrogen bonding or dipole-dipole interactions. Moreover, silanols are weakly acidic, being ionised within the working pH range of typical RPLC columns, in different extent. This gives rise to a negatively charge allowing weak cation-exchange interactions, which increase the retention of protonated basic compounds [6–8], which are positively charged, showing broad tailing peaks for these compounds [8,9], due the slow sorption-desorption kinetics of the cationic solutes on free silanols.

On the other hand, the pH range where most chemically modified silicas are stable is relatively narrow, ranging from pH 2 to 8, although this range and the degradation rates in acidic and basic media depend on the type of silica, bonded reagent, bonding method and bonding density [1].

Nonetheless, throughout the years, other RPLC packing materials, providing different retention and selectivity, have been introduced. In general, an alkyl chain and one or two functional groups interacting with the solutes through two or three different mechanisms can be found in these materials. The functional group can be a polarisable aryl group, a permanent dipole as cyano or amide, or a hydrogen donating or accepting group as diol or amine. The most popular types are alkyl-phenyl, alkyl-fluoro-phenyl and alkyl-cyano phases. These phases offer different selectivity due to the possibility of induced dipole or π-π interactions, while permanent dipole-dipole interactions are significant for the retention of polar solutes on cyano phases [10].

Once a suitable column has been selected, the other important choice is the composition of mobile phase, which has considerable influence on the

separation performance. The use of binary mixtures of water and an organic solvent in the mobile phase is common, but ternary or quaternary mixtures of water, and two or three organic solvents, can be an option to control the elution strength and selectivity. The selection of pH and the mobile phase composition will determine the degree of interaction between solute and stationary phase.

With regard to organic solvents, only a few of the wide range of water-miscible organic solvents that can be used as modifiers are usual in RPLC: acetonitrile, methanol, ethanol (greener), tetrahydrofuran, and isopropanol [11,12]. Among them, acetonitrile followed by methanol is by far the most frequently used. The reasons that make acetonitrile the most popular choice as modifier are its lower viscosity that reduces the pump back-pressure, lower cut-off for UV detection, sufficiently large elution strength, reduced reactivity, and ability to dissolve a wide range of compounds.

Moreover, the separation can be enhanced by addition of other kind of modifiers, such as buffers, neutral salts, weak acids, surfactants, and ion-pairing reagents (as ammonium salts) to the aqueous-organic mobile phases [13]. Some of them can interact electrostatically with ionised silanols, acting as silanol blockers or masking agents. This is the case of anionic surfactants, which associate with the bonded alkyl chains, masking effectively the silanols by restricting the accessibility [8]. Silanol blockers may have, however, some undesirable effects, such as the difficulty of removal from the stationary phase, chemical reaction with some functional groups, and the generation of additional background noise in evaporative light-scattering and mass spectrometric detection.

RPLC is the technique of choice for the separation of complex mixtures of analytes within a wide range of polarities, where the retention is mainly related to solute hydrophobicity. In this technique, separations can be carried out in

either the isocratic or gradient elution modes. In isocratic elution, the mobile phase composition is held constant during the separation, while in gradient elution, it is varied.

When the chromatogram contains only the single peak of an analyte, or peaks of two or more analytes within a small or moderate polarity range, the isocratic mode can be used with success, achieving good resolution. This, together with its inherent simplicity, lower cost and higher robustness, explains that many RPLC separations are carried out in this chromatographic mode [14]. However, with more complex mixtures containing solutes with different polarities, this isocratic mode is not recommended due to the high retention times obtained for the most retained compounds on the one hand, and the poor resolution or even elution close to the dead time of the less retained compounds. This has been called the “general elution problem of RPLC”, due to its impossibility to improve both extremes of the chromatogram at the same time by using isocratic elution [3].

The usual solution to the general elution problem is the application of gradient elution with programmed changes of organic modifier [15–17]. By implementing gradient elution, adequate resolution and acceptably short times during a single analytical run is possible by modifying the elution strength of the mobile phase through the separation process.

1.2. Secondary chemical equilibria in RPLC

In spite of the extensive use of RPLC, it has still some limitations associated to the shorter or even absence of retention of ionised compounds, inorganic anions and metal anions, which are polar. In fact, this has been a big challenge in environmental, clinical, and food chemistry throughout the

development of RPLC. However, as commented above, the addition of reagents (additives) to the mobile phase enable the separation of these compounds, using conventional instrumentation, silica-based materials, and hydro-organic mixtures, thanks to a variety of secondary equilibria which have given rise to several chromatographic modes.

These secondary reactions on the support or within the mobile phase involves dissociation-protonation of ionisable compounds by tuning the pH, ion-exchange processes through adsorption of an ionic lipophilic reagent on the stationary phase to attract analytes with an opposite charge or suppress the silanol activity, formation of analyte-reagent ion pairs in the mobile phase, or metal complexation, among others [13]. These equilibria can be expressed as:

$$A + X \leftrightarrows AX \qquad (1.1)$$

where A is the analyte or the silanol group on the support, and X is H^+, a lipophilic ion, a ligand, or other added species. The observed retention factor (k) is a weighted average:

$$k = k_A \delta_A + k_{AX} \delta_{AX} = \frac{k_A + k_{AX} K[X]}{1 + K[X]} \qquad (1.2)$$

where δ_A and δ_{AX} are the molar fractions of A and AX, [X] is the molar concentration of X in the mobile phase, and K the formation constant (for an acid-base reaction, $\log K = pK_a$, where K_a is the dissociation constant).

The term Ion Pair Chromatography (IPC) has been coined to refer to the combination of the analyte and lipophilic ions of opposite charge to form an ion pair in the mobile phase, able to partition into the nonpolar bulk-liquid stationary phase [18]. This is a dynamic ion-exchange mechanism, which considers that the lipophilic ion is dynamically distributed between the mobile

phase and stationary phase, where it is adsorbed (immobilised), behaving as an ion exchanger for oppositely charged analytes.

Although in IPC the chromatographic performance, in terms of selectivity and peak shape, is improved in some cases by these secondary equilibria, understanding the mechanisms under these conditions can be far more complex, owing to the coexistence of more than one equilibrium inside the column.

In this context, a first step in a chromatographic protocol is to control the pH, and consequently, the acid-base secondary equilibria, since small variations of pH in the mobile phase at values close to pK_a results in significant changes in retention and selectivity [19,20]. For this reason, the addition of an appropriate buffer is needed to achieve reproducible retention for ionisable compounds. Common buffer acid-base systems are phosphoric, citric, tris(hidroxymethyl) aminomethane, phthalic, acetic, formic, and ammonium. Column temperature should also be controlled, since it affects strongly the ionisation degree of analytes and buffer.

Both, cell calibration and test solution measurements, must be adequately done to assure accuracy [21]. One procedure to calibrate a pH-meter makes use of the ${}^{s}_{w}\mathrm{pH}$ scale, where reference aqueous buffers are used, followed by measurement of pH in the aqueous-organic mixture. This procedure is easier, since it avoids the problem of preparing reference buffers in mobile phases at different composition.

The major advantage of the dynamic coating in IPC is the possibility of controlling the column ion-exchange capacity by varying the mobile phase composition, promoting secondary equilibria. A quite distinct alternative is the equilibration of the stationary phase with a highly lipophilic ion, which is then removed from the mobile phase in the actual separation step. This coating is

strongly bound and persists for long periods of subsequent use. The method is known as permanent coating ion interaction chromatography (IIC).

In principle, any salt containing a lipophilic ion can be used as IIC reagent. The longer the alkyl chain the more hydrophobic the reagent, and the stronger the adsorption on the stationary phase bonded chains. The same column can be converted into an anion exchanger or a cation exchanger by the addition of an appropriate additive. The most common reagents are salts of alkylammonium or tetraalkylammonium for anions, and alkyl sulphates or alkylsulphonates for cations. Other newer reagents are ionic liquids and chaetropic ions [22].

By modifying the reagent concentration, the retention of the analytes can be modulated. The adsorbed layer of lipophilic ion can be easily removed by washing the column with an organic solvent. In order to maintain a reproducible ion-exchange capacity, both IIC reagent and organic solvent, should be kept constant in the mobile phase at specified concentrations. Other consideration is the requirement of the equilibration time to get a constant counterion coating, especially in gradient elution.

Surfactant coatings are an easy and inexpensive way of converting silica-based RPLC packings into ion exchangers. Its attractiveness arises from their different ion-exchange capacities and selectivities, by just altering the coating conditions. When surfactant concentration is above the critical micellar concentration (CMC), micelles (dynamic aggregates of surfactant monomers) are formed in the mobile phase, producing a new phase (pseudophase), which gives rise to additional interactions with the analyte. This chromatographic mode is called Micellar Liquid Chromatography (MLC) [23,24].

Due to the possibility of interactions between the analyte and both, stationary phase and micelles, in MLC analytes are separated on the basis of their differential partitioning between the bulk aqueous phase and the

micellar aggregates or the surfactant-coated stationary phase. Therefore, a secondary equilibrium is added to the mobile phase, which can be altered for ionisable compounds by tuning the pH [25].

An organic solvent can be added to the micelar mobile phase in order to decrease retention times of apolar compounds and improve the peak shape. This particular MLC is called “hybrid MLC”. Furthermore, the addition of a high percentage of the organic solvent to the mobile phases produce the disruption of the micelles creating the so-called “submicellar RPLC”, with the free monomers of surfactant in the mobile phase, but not forming micelles.

Surfactants used in MLC are from diverse nature, ionic, nonionic and zwiterionic. We will not extend further here, since the information on the MLC mode will be expanded through the next chapters.

As commented in Section 1.1, one of the limitations of the silica stationary phases in RPLC is the ionisation of residual silanols, due to the long retention times, peak tailing, poor efficiency, and strong dependence of retention on sample size obtained for basic compounds. This problem can be reduced masking the electrostatic interaction with IIC reagents, such as amines, ionic liquids (ILs), alkyl sulphates, alkylsulphonates, perfluorinated carboxylates, and other anions.

Amines show a high silanol suppression effect, especially for compounds with bulky substituents. Alkylsulphonates present a disadvantage because of the difficult column regeneration, while perfluorinated carboxylates are preferable for evaporative light scattering and mass spectrometry detection, and suitable for preparative chromatography thanks to their volatility. Ionic liquids, which are molten salts with melting points equal or lower than 100 °C, present an interesting feature as suppressors of silanophilic activity, due to their dual character (both cation and anion are absorbed on the stationary phase). The

large variety of possible combinations of cations and anions (and consequently, new liquid reagents) offer a wide range of different additives with different properties to be use as IIC reagents [26]. The masking capability is larger for more lipophilic cations (e.g., 1-hexyl-3-methylimidazolium and 1-butyl-3-methylimidazolium) [27,28].

The physico-chemical properties of ILs strongly depend on both the nature and size of the cation and anion. Both species may contribute to the chromatographic behaviour. Cation and anion create a bilayer, positively or negatively charged depending on the relative strength of the adsorption of cation and anion, respectively. This allows specific electrostatic interactions (attraction or repulsion) with analytes. Hydrogen bonding is also thought to exist between the oxygen or halide atoms on the anion, and the hydrogen atoms on the imidazolium or pyridinium ring of the cation in ILs [29,30]. Specific solute-salt interactions can also take place in the mobile phase, where ILs can act as ion-pairing reagents.

All these interactions, in addition to those existing in conventional organic solvents, increase the complexity of the chromatographic system (and consequently, the mechanisms of retention), with regard to other common additives. The retention mechanisms are, thus, mixed and involve hydrophobic partitioning, ion-exchange and ion-pairing. The extension of these interactions depends on the selected IL, and its concentration. It is known that ions can adsorb on hydrophobic stationary phases in amounts depending on the Hofsmeister series:

For cations:

$Al^{3+} > Ca^{2+} > Mg^{2+} > H^{+} > Na^{+} > K^{+} > Rb^{+} > Cs^{+} > NH_4^{+} > N(CH_3)_4^{+} > NH(CH_3CH_2)_3^{+}$

and for anions:

$PF_6^- > SCN^- > ClO_4^- \sim BF_4^- > NO_3^- > I^- > Br^- > Cl^- > F^- > H_2PO_4^- > SO_4^{2-} >$ citrate($^{3-}$).

This ranking was first proposed in 1888 and was related to a special physico-chemical property of the ions called “lyotropy”, a notion close to “hydrophobicity”. This property is correlated with the degree of hydration.

All this information about ILs will be expanded in Part II of this PhD. work, where ILs are used as silanol masking reagents.

1.3. Hydrophilic Interaction Liquid Chromatography

The analysis of very polar compounds is also limited in RPLC, due to poor retention. However, Hydrophilic Interaction Liquid Chromatography (HILIC), where the mobile phase is constituted by an eluent rich in an organic solvent (typically acetonitrile over 70% *v*/*v*) that also contains and appreciable amount of water (at least 2.5% *v*/*v*), and the stationary phase is polar [31], excels in the separation of small and very polar compounds. This chromatographic mode provides an alternative selectivity to RPLC, good retention for hydrophilic compounds [32,33], together with low operational back pressure owing to the low viscosity of the organic-rich mobile phases used.

Other well-known advantages of HILIC are good peak shape and high loadability for basic compounds, compared to RPLC (where the use of additives in IPC is required to obtain retention) [33], the flatter van Deemter curves at high mobile phase velocity resulting from the larger solute diffusivity in the organic-rich mobile phases [34] that make feasible the use of long columns [35], and finally, the possibility of direct injection into HILIC columns of some biological samples in bioanalytical applications, without the need for evaporation and reconstitution after protein precipitation with acetonitrile.

Nevertheless, there are some significant drawbacks associated with the difficulty in column selection for method development: the large column diversity, sample solubility, poorer method robustness, and lack of guidance in the development of new HILIC analysis, which difficult the applicability of new methodologies.

The selection of the column in HILIC has the greatest effect on the selectivity, and consequently, it is probably one of the most important factors to get success in the analysis of a sample. Luckily, nowadays there are many references available where different HILIC stationary phases have been used, as reported in some recent reviews [36–42], and the number of reports is continuously growing.

The HILIC method robustness is frequently discussed, but many of these issues can be solved by adequate equilibration of HILIC columns prior to use. The time taken for equilibration is longer with regard to RPLC. It is usually recommended to equilibrate a brand new column with 60–80 column volumes, while for second and subsequent runs only 20 column volumes are needed [43]. The main reason of this higher equilibration time is the need of forming the stable hydration layer around the stationary phase particles, which is mandatory to get reproducible HILIC chromatographic methods. Also, if the HILIC method includes a gradient, then appropriate equilibration between injections is also required.

On the other hand, choosing the correct sample diluent in HILIC is often perceived to be difficult due to analyte solubility concerns and diluent mismatch with the HILIC eluent. Poorly optimised diluents can significantly reduce the chromatographic performance and peak shape. For this reason, the optimisation of the sample diluent is recommended, exploring acetonitrile to buffer ratios between 20% and 80%, in 20% increments [43]. Furthermore, if

possible, higher concentrations of analytes and smaller injection volumes are preferred because this can minimise disruption of the absorbed water layer.

The effect of various experimental variables including the nature of the stationary phase, buffer pH, buffer concentration, organic solvent and its concentration, temperature, and some properties of solutes (such as their apparent log $P_{o/w}$ (log D values) on the selectivity cannot be overlooked.

Many new stationary phases and columns have become commercially available in the last decade [44]. The separation materials used in HILIC are quite diverse and can be classified by the type (neutral, charged, and zwitterionic) and by the functional groups on the structure. The stationary phases are made mostly of bare silica or silica modified with amino, amide, cyano, diol, zwitterionic, cyclodextrin (CD), saccharide, or other polar functional groups [36,37,45]. Materials such as zirconia [46], titania [47], and hydrophilic polymer [48], have been also used as supports for HILIC applications. Aminopropyl-bonded silica is particularly suitable for the HILIC separations of acidic compounds, diol closely resembles naked silica in overall polarity presenting high polarity and hydrogen bonding capability, cyanopropyl silica has almost disappear due to low retention of polar compounds in aqueous organic phases, amide bonded phases are less sensitive to pH than amino phases since amide group does not possess basic properties, and CD-based silica are used for chiral separations.

Zwitterionic sulfoalkylbetaine stationary phases have been employed in HILIC, especially for the separations of inorganic salts, small organic ionic compounds, and proteins. These columns exhibit unique characteristics due to the presence of a very low net negative surface charge on the bonded layer (attributed to a larger distance of the sulphonic groups from the silica gel surface with respect to the ammonium group) that is scarcely affected by pH

[49]. As a consequence, ion-exchange interactions of the zwitterionic stationary phase are weaker than those of ion exchangers and bonded amino phases. In addition, electrostatic interactions were also reported to be weaker with regard to bare silica, amide, and other bonded phases, as residual silanols are efficiently shielded by self-association of oppositely charged functional groups [50].

Finally, new stationary phases have been recently employed with some macromolecules, such as CD sandcyclofructans. These macromolecules contain multiple polar groups, which can provide good hydrophilicity [39]. The last advances are focused on the preparation of IL-based stationary phases. Owing to their properties (in particular their high hydrophilicity) ILs appear to be particularly suitable for HILIC.

Regarding to the mobile phase, mixtures of acetonitrile and water, or buffer solutions, are preferentially used as mobile phases. Protic solvents, such as methanol, are normally avoided due to their ability to disturb the aqueous layer surrounding the stationary phase. As in RPLC, the pH is an important parameter for ionisable compounds. Using a pH value above or below the pK_a of an ionisable species changes the analyte ionisation state, which in turn affects its hydrophilicity. The pH will also affect the polarity of the stationary phase surface, which additionally influences the retention mechanisms. For this reason, the addition of a buffer is needed. A variety of buffer salts can be used in HILIC. It is essential the buffer be soluble in the high organic solvent content, which discards inorganic buffers, such as phosphates. Among the possible buffers in HPLC, ammonium formate is the most widely used.

In HILIC, retention mechanisms are still debated [38,51]. The most accepted model is based on the partitioning mechanism between the bulk eluent and a water-enriched layer immobilised on the stationary phase [36,37]. The

thickness of the water layer depends on many factors, including the composition of the stationary phase, and the mobile phase temperature and ionic strength [52]. However, there are some studies [45,53–55] that have evidenced the functional groups on the surface contribute to selectivity in HILIC, suggesting therefore the hypothesis of a multimodal retention process in HILIC, made of partitioning of solutes between the surface water layer and the bulk mobile phase, adsorption via processes such as hydrogen bonding, dipole/dipole interactions, and electrostatic forces between ionised silanols on silica materials (or charged ligands on other types of columns) and ionised solutes.

In the last few years, HILIC has become one of the preferred analytical techniques for the separation of hydrophilic compounds, which results in a wide variety of applications in different research fields, such as pharmaceutical analysis [56], agricultural and food chemistry [57], medicinal chemistry and bioanalysis [58], proteomics and metabolomics [59], and glycomics [60].

1.4. Half-width plots, a simple useful tool to study peak profiles

Peak profiles in chromatography are characterised by their height, position, width and asymmetry, the latter depending on the values of the left (*A*) and right (*B*) peak half-widths. In this context, it is interesting to build plots of the half-widths versus the retention time, which have been called half-width plots. These graphics are easily obtained and facilitate the prediction of chromatograms in different situations, assist in method development, and characterise different columns and experimental conditions [61].

The plots follow an almost linear trend, which can be represented according to the following equations:

$$A = m_A t_R + A_0 \quad (1.3)$$

$$B = m_B t_R + B_0 \quad (1.4)$$

where m_A and m_B are the slopes of the linear correlations, and the intercepts A_0 and B_0 indicate the extra-column contributions to the peak broadening: the sum and ratio of the intercepts ((A_0+ B_0) and B_0/A_0) explain, at least partially, the peak width and asymmetry for scarcely retained compounds, respectively. The sum of slopes (m_A +m_B) indicates the peak broadening rate inside the column, whereas their ratio (m_B/m_A) is the asymmetry of peaks eluting at a time where the extra-column contribution is non-significant. The slope for the right half-width is usually larger, indicating the tailing character of most chromatographic peaks. Nevertheless, in some cases, the lines converge, changing the peaks from tailing to fronting above a certain retention time. The coincidence of slopes ($m_A = m_B$) indicates that the peaks of compounds eluting at different retention times will be symmetrical.

This kind of plots can be useful to reveal differentiated mass transfer kinetics of solutes in different situations. With fast mass transfer kinetics, or similar kinetics for all eluted compounds or experimental conditions, the points in the plots will follow the same trend of variation, with a nice correlation. In contrast, particular slow kinetics for the interaction between a given solute and the stationary phase will make the behaviour depart from the common trend [62–64]. This will give rise to some scattering, especially for the right half-width. In addition, if the mobile phase contains an additive that modifies the stationary phase nature, when the additive concentration is increased, the interaction rate for some compounds may change, which will yield again some scattering. Half-width plots are also highly convenient to observe changes in column performance with the experimental factors, or compare the behaviour

of different columns, with regard to the direct comparison of chromatograms. This interesting tool can be used in both, isocratic and gradient elution [65].

During the last years, our research group has used these plots with optimisation purposes in a variety of situations: with sets of compounds of different nature, different columns and experimental conditions (different organic solvents, additives, pH, temperature and flow rate), with great success [66,67]. Half-width plots offer interesting graphical information about column performance [62,63,68–70]. This tool has been extensively used along this PhD. work.

1.5. Validation in Liquid Chromatography

Analysts are becoming more and more conscious that a method validation is needed during the method development in order to achieve reliable results, to guarantee its quality. Method validation is an applied approach to verify that a method is suitable and rugged enough to function as a quality control tool in different locations and times [71,72]. It has received considerable attention in the literature from industrial committees and regulatory agencies, and today is required by most regulations and quality standards that impact laboratories.

However, in liquid chromatography, method validation is not an easy task due to the variety of existing fields (biological fluids, pharmaceuticals, impurities, microbials, food, and botanicals, among others) and the variety of applied detection techniques. The validation process is complex and time-consuming. Therefore, with the aim of making this process easier, a validation plan is first developed. This should include owners, responsibilities and deliverables. The first step is to define the scope of the method. This includes the compounds and concentration ranges, sample matrix, specific equipment to be used, and location where the method should be performed. Once the target

analysis is known, the performance parameters, performance tests and acceptance criteria should be defined. Test protocols are then developed with all experimental details, and these are executed according to the protocols. Tests results are compared with acceptance criteria. Finally, routine method procedures are developed to verify constant system performance at the time of analysis. Tests may include system suitability testing and the analysis of quality control samples. All experimental conditions and validation results must be documented in a validation report.

Despite the validation exercise may appear tedious, costly and time-consuming, it eventually turns out to pay for itself, eliminating annoying repetitions and leading to better time management on the long term. The internationally renowned organisations have been offering guidelines on method validation, along the years, on a tentative exhaustive basis [73–85]. It should be noted that the validation parameters being evaluated can be different among guidelines.

In general, in liquid chromatography, linearity, range of application (which is closely related to linearity), precision, accuracy and limits of detection and quantification, are the most common parameters being evaluated [71,72]. Specificity and selectivity seem to have medium to low interest to the authors, but some include in their studies the matrix effect, which is usually related to selectivity. Evaluation of recovery is not too high because sometimes it is used as indicative of accuracy. This is sometimes also termed trueness. Sample stability is also an important parameter that is not sufficiently evaluated. It is also surprising that robustness is rarely included in the validation process, given the importance of the variability of the results with a change in any experimental parameter in many developed methods. Robustness is really a relevant validation parameter that authors should take more into consideration.

Finally, the least studied parameters have been ruggedness, system suitability, and uncertainty.

Unfortunately, the validation criteria are not always applied with the same rigor. Some authors fully validate their methods, but on the contrary, others evaluate only some parameters, such us linearity, accuracy, precision, LOQ and LOD, and without following any specific criteria, or just following what other authors did. Some authors call these studies "optimisation", instead of validation. Nowadays, most authors tend to meticulously follow guidelines to validate their methods. For this reason, when validating a method, it is compulsory to indicate the followed guideline, since the validation criteria should be clearly established for each parameter.

In this PhD. work, the International Conference of Harmonisation (ICH) guideline has been followed to assure the quality of the results by the evaluation of linearity, intra- and inter-day accuracy and precision, limits of detection and quantification, robustness and accuracy.

1.6. References

[1] J.J. Pesek, M.T. Matyska, Reversed-phase chromatography: Description and applications (edited by J. Cazes), in *Encyclopedia of Chromatography*, Taylor and Francis, New York, 2006, pp. 719–722.

[2] A. Soliven, S. Kayillo, R.A. Shalliker, Reversed phase liquid chromatography (edited by J. Reedijk), in *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, Elsevier, Amsterdam, 2013.

[3] M.C. García Álvarez-Coque, J.J. Baeza Baeza, G. Ramis Ramos, Reversed phase liquid chromatography, in *Analytical Separation Science*

Series (edited by J.L. Anderson, A. Stalcup, A. Berthod, V. Pino), Vol. 1 *Liquid Chromatography*, Wiley-VCH, New York, 2015, pp. 159–197.

[4] M.J.J. Hetem, J.W. de Haan, H.A. Claessens, L.J.M. van de Ven, C.A. Cramers, J.N Kinkel, Influence of alkyl chain length on the stability of *n*-alkyl-modifiedreversed phases: 1. Chromatographic and physical analysis, *Anal. Chem.* 62 (1990) 2288–2296.

[5] H.A. Claessens, M.A van Straten, Review on the chemical and thermal stability of stationary phases for reversed-phase liquid chromatography, *J. Chromatogr. A* 1060 (2004) 23–41.

[6] J. Nawrocki, The silanol group and its role in liquid chromatography, *J. Chromatogr. A* 779 (1997) 29–71.

[7] D.H. Marchand, P.W. Carr, D.V. McCalley, U.D. Neue, J.W. Dolan, L.R. Snyder, Contributions to reversed-phase column selectivity: II. Cation exchange, *J. Chromatogr. A* 1218 (2011) 7110–7129.

[8] M.J. Ruiz Ángel, S. Pous Torres, S. Carda Broch, M.C. García Álvarez-Coque, Performance of different C18 columns in reversed-phase liquid chromatography with hydro aqueous-organic and micellar-organic mobile phases, *J. Chromatogr. A* 1344 (2014) 76–82.

[9] J.J. Baeza Baeza, M.J. Ruiz Angel, S. Carda Broch, M.C. García Álvarez-Coque, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

[10] K. Croes, A. Steffens, D.H. Marchand, L.R. Snyder, Relevance of π-π and dipole-dipole interactions for retention on cyano and phenyl columns in reversed-phase liquid chromatography, *J. Chromatogr. A* 1098 (2005) 123–130.

[11] V.J. Barwick, Strategies for solvent selection, *Trends Anal. Chem.*16 (1997) 293–309.

[12] G. Ramis Ramos, M.C. García Álvarez-Coque, Solvent selection in liquid chromatography, in *Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, P.J. Schoenmakers, D. Lloyd), Elsevier, Amsterdam, 2013, pp. 223–249.

[13] M.C. García Álvarez-Coque, J.A. Navarro Huerta, J.R. Torres Lapasió, Secondary chemical equilibria in reversed-phase liquid chromatography, in *Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, M.L., Riekkola), Elsevier, Amsterdam, 2nd ed., 2017, pp. 125–146.

[14] J.W. Dolan, LC Method Scaling, Part I: Isocratic separations, *LC GC North America*, 32 (2014) 98, 100, 102.

[15] P. Jandera, Can the theory of gradient liquid chromatography be useful in solving practical problems?, *J. Chromatogr. A* 1126 (2006) 195–218.

[16] J.E. Haky, D.A. Teifer, Gradient elution, in *Encyclopedia of Chromatography*, Taylor and Francis, New York, 2006, pp. 393–396.

[17] J.W. Dolan, L.R. Snyder, Gradient elution chromatography, en *Encyclopedia of Analytical Chemistry*, Wiley, New York, 2012.

[18] T. Cecchi, Ion pairing chromatography, *Crit. Rev. Anal. Chem.* 38 (2008) 161–213.

[19] U.C. Neue, C.H. Phoebe, K. Tran, Y.F. Cheng, Z. Lu Dependence of reversed-phase retention of ionisable analytes on pH, concentration of organic solvent and silanol activity, *J Chromatogr. A* 925 (2001) 49–67.

[20] M. Rosés, E. Bosch, Influence of mobile phase acid-base equilibria on the chromatographic behaviour of protolytic compounds, *J. Chromatogr. A* 982 (2002) 1–30.

[21] M. Rosés, Determination of the pH of binary mobile phases for reversed-phase liquid chromatography, *J. Chromatogr. A* 1037 (2004) 283–298.

[22] A. Bartha, G. Vigh, Z. Varga Puchony, Basis of the rational selection of the hydrophobicity and concentration of the ion-pairing reagent in reversed-phase ion-pair high-performance liquid chromatography, *J. Chromatogr. A* 499 (1990) 423–434.

[23] A. Berthod, M.C. García Álvarez-Coque, *Micellar Liquid Chromatography*, New York, Marcel Dekker, 2000.

[24] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, New insights and recent developments in micellar liquid chromatography, *Sep. Purif. Rev.* 38 (2009) 45–96.

[25] M.J. Ruiz Ángel, S. Carda Broch, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Retention mechanisms in micellar liquid chromatography, *J. Chromatogr. A* 1216 (2009) 1798–1814.

[26] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, A. Berthod, S. Carda Broch, On the use of ionic liquids as mobile phase additives in high-performance liquid chromatography, *Anal. Chim. Acta* 883 (2015) 1–21.

[27] Y. Wang, M. Tian, W. Bi, K.H. Row, Application of ionic liquids in high performance reversed-phase chromatography, *Int. J. Mol. Sci.* 10 (2009) 2591–2610.

[28] J.J. Fernández Navarro, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, The role of the dual nature of ionic liquids in the reversed-phase liquid chromatographic separation of basic drugs, *J Chromatogr A* 1218 (2011) 398–407.

[29] D. Han, M. Tian, D.W. Park, D.K. Choi, K.H. Row, Application of ionic liquids as mobile phase additives and surface-bonded stationary phase in liquid chromatography, *Korean J. Chem. Eng.* 26 (2009) 1353–1358.

[30] D. Han, K.H. Row, Recent applications of ionic liquids in separation technology, *Molecules* 15 (2010) 2405–2426.

[31] A. Cavazzini, M. Catani, A. Felinger, Hydrophilic liquid interaction, in *Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, M.L., Riekkola), Elsevier, Amsterdam, 2nd ed., 2017, pp. 147–169.

[32] D.V. McCalley, Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography, *J. Chromatogr. A* 1217 (2010) 3408–3417.

[33] D.V. McCalley, The challenges of the analysis of basic compounds by high performance liquid chromatography: Some possible approaches for improved separations, *J. Chromatogr A* 1217 (2010) 858–880.

[34] D.V. McCalley, Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds?, *J. Chromatogr. A* 1171 (2007) 46–55.

[35] D.V. McCalley, Evaluation of the properties of a superficially porous silica stationary phase in hydrophilic interaction chromatography, *J. Chromatogr. A* 1193 (2008) 85–91.

[36] P. Hemström, K. Irgum, Hydrophilic interaction chromatography, *J. Sep. Sci.* 29 (2006) 1784–821.

[37] P. Jandera, Stationary phases for hydrophilic interaction chromatography, their characterization and implementation into multidimensional chromatography concepts, *J. Sep. Sci.* 31 (2008) 1421–37.

[38] Y. Guo, Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC), *Analyst* 140 (2015) 6452–6466.

[39] L. Qiao, X. Shi, G. Xu, Recent advances in development and characterization of stationary phases for hydrophilic interaction chromatography, *TrAC Trends Anal. Chem.* 81 (2016) 23–33.

[40] E.M. Borges, Silica, hybrid silica, hydride silica and non-silica stationary phases for liquid chromatography, *J. Chromatogr. Sci.* 53 (2015) 580–97.

[41] X. Shi, L. Qiao, G. Xu, Recent development of ionic liquid stationary phases for liquid chromatography, *J. Chromatogr. A* 1420 (2015) 1–15.

[42] D. García Gómez, E. Rodríguez Gonzalo, R. Carabias Martínez, Stationary phases for separation of nucleosides and nucleotides by hydrophilic interaction liquid chromatography, *TrAC Trends Anal. Chem.* 47 (2013) 111–28.

[43] Advanced Chromatography Technologies (ACE). *ACE HILIC Methods Development Guide. A Step by Step Approach to Developing Reproducible HILIC Methods.*

[44] Y. Guo, S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography, *J. Chromatogr. A* 1218 (2011) 5920–5938.

[45] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, Chromatographic characterization of hydrophilic interaction liquid chromatography stationary phases: Hydrophilicity, charge effects, structural selectivity, and separation efficiency, *J. Chromatogr. A* 1218 (2011) 5903–19.

[46] J. Randon, S. Huguet, C. Demesmay, A. Berthod, Zirconia based monoliths used in hydrophilic-interaction chromatography for original selectivity of xanthines, *J. Chromatogr. A* 1217 (2010) 1496–500.

[47] T. Zhou, C.A. Lucy, Hydrophilic interaction chromatography of nucleotides and their pathway intermediates on titania, *J. Chromatogr. A* 1187 (2008) 87–93.

[48] M. Guerrouache, A. Pantazak, M.C. Millot, B. Carbonnier, Zwitterionic polymeric monoliths for HILIC/RP mixed mode for CEC separation applications, *J. Sep. Sci.* 33 (2010) 787–792.

[49] R.I. Chirita, C. West, S. Zubrzyckia, A.L. Finaru, C. Elfakir, Investigations on the chromatographic behaviour of zwitterionic stationary phases used in hydrophilic interaction chromatography, *J. Chromatogr. A* 1218 (2011) 5939–5963.

[50] Y. Guo, S. Gaiki, Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography, *J. Chromatogr. A* 1074 (2005) 71–80.

[51] G. Greco, T. Letzel, Main interactions and influences of the chromatographic parameters in HILIC separations, *J. Chromatogr. Sci.* 51 (2013) 684–93.

[52] J.C. Heaton, J.J. Russell, T. Underwood, R. Boughtflower, D.V. McCalley, Comparison of peak shape in hydrophilic interaction chromatography using acidic salt buffers and simple acid solutions, *J. Chromatogr. A* 1347 (2014) 39–48.

[53] B. Dejaegher, D. Mangelings, Y. Vander Heyden, Method development for HILIC assays, *J. Sep. Sci.* 31 (2008) 1438–1448.

[54] Z. Hao, B. Xiao, N. Weng, Impact of column temperature and mobile phase components on selectivity of hydrophilic interaction chromatography (HILIC), *J. Sep. Sci.* 31 (2008) 1449–1464.

[55] G.W. Jin, Z.M. Guo, F.F. Zhang, X.Y. Xue, Y. Jin, X.M. Liang, Study on the retention equation in hydrophilic interaction liquid chromatography, *Talanta* 76 (2008) 522–527.

[56] B. Dejaegher, Y.V. Heyden, HILIC methods in pharmaceutical analysis, *J. Sep. Sci.* 33 (2010) 698–715.

[57] A.L.N. van Nuijs, I. Tarcomnicu, A. Covaci, Application of hydrophilic interaction chromatography for the analysis of polar contaminants in food and environmental samples, *J. Chromatogr. A* 1218 (2011) 5964–5974.

[58] W. Jian, R.W. Edom, Y. Xu, N. Weng, Recent advances in application of hydrophilic interaction chromatography for quantitative bioanalysis, *J. Sep. Sci.* 33 (2010) 681–697.

[59] K. Spagou, H. Tsoukali, N. Raikos, H. Gika, I.D. Wilson, G. Theodoridis, Hydrophilic interaction chromatography coupled to MS for metabonomic/metabolomic studies, *J. Sep. Sci.* 33 (2010) 716–727.

[60] L.R. Ruhaa, C. Huhn, W.J. Waterreus, A.R. de Boer, C. Neusüss, C.H. Hokke, Hydrophilic interaction chromatography-based high-throughput sample preparation method for *N*-glycan analysis from total human plasma glycoproteins, *Anal. Chem.* 80 (2008) 6119–6126.

[61] J.J. Baeza Baeza, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, S. Carda Broch, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

[62] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[63] J.J. Fernández Navarro, J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Silanol suppressing potency of alkyl-imidazolium ionic liquids on C18 stationary phases, *J. Chromatogr. A* 1232 (2012) 166–175.

[64] S. Pous Torres, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Comparison of the performance of Chromolith Performance RP-18e, 1.8-μm Zorbax Eclipse XDB-C18 and XTerra MS C18, based on modelling approaches, *Anal. Bioanal. Chem.* 405 (2013) 2219–2231.

[65] J.J. Baeza Baeza, C. Ortiz Bolsico, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Approaches to model the retention and peak profile in linear gradient reversed-phase liquid chromatography, *J. Chromatogr. A* 1284 (2013) 28–35.

[66] J.J. Fernández Navarro, J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, 1-Hexyl-3-methyl imidazolium tetrafluoro-borate: An efficient column enhancer for the separation of basic drugs by reversed-phase liquid chromatography, *J. Chromatogr. A* 1258 (2012) 168–174.

[67] S. Pous Torres, J.R. Torres Lapasió, M.J. Ruiz-Ángel, M.C. García Álvarez-Coque, Interpretive optimisation of organic solvent content and flow-rate in the separation of β-blockers with a Chromolith RP-18e column, *J. Sep. Sci.* 32 (2009) 2793–2803.

[68] J.J. Baeza Baeza, S. Pous Torres, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Approaches to characterise chromatographic column performance based on global parameters accounting for peak broadening and skewness, *J. Chromatogr. A* 1217 (2010) 2147–2157.

[69] J.J. Fernández Navarro, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, The role of the dual nature of ionic liquids in the reversed-phase liquid chromatographic separation of basic drugs, *J. Chromatogr. A* 1218 (2011) 398–407.

[70] J.J. Baeza Baeza, Y. Dávila, J.J. Fernández Navarro, M.C. García Álvarez-Coque, Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC, *Anal. Bioanal. Chem.* 404 (2012) 2973–2984.

[71] M. Rambla Alegre, J. Esteve Romero, S. Carda Broch, Is it really necessary to validate an analytical method or not? That is the question, *J. Chromatogr. A* 1232 (2012) 101–109.

[72] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, S. Carda Broch, Are analysts doing the method validation in liquid chromatography?, *J. Chromatogr. A* 1353 (2014) 2–9.

[73] Association of Official Analytical Chemists (AOAC), Official Methods of Analysis, Vol. 1, 15th ed., Arlington, VA (USA), 1990.

[74] ISO 5725, Application of the Statistics-Accuracy (Trueness and Precision) of the Results and Methods of Measurement: Parts 1 to 6, International Organization for Standardization (ISO), Geneva (Switzerland), 1994.

[75] IUPAC, Pure Appl. Chem. 62 (1995) 149.

[76] H. Holcombe (Ed.), EURACHEM Guide: The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics, LGC, Teddington (United Kingdom), 1998.
Also available at http://www.eurachem.org/guides/pdf/valid.pdf

[77] FAO, Validation of Analytical Methods for Food Control, Report of a Joint FAO/IAEA Expert Consultation, December 1997, in: FAO Food and Nutrition Paper No. 68, Rome (Italy), 1998.

[78] International Union of Pure Applied Chemistry, Harmonized Guidelines for In-House Validation of Methods of Analysis (Technical Report), IUPAC, Budapest (Hungary), 1999.
http://old.iupac.org/divisions/V/501/draftoct19.pdf

[79] Guidance for Industry: Bioanalytical Method Validation, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Rockville, MD (USA), 2001.
http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf

[80] European Commission, Off. J. Eur. Commun. L 221 (2002) 8.

[81] ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, Q2(R1), Geneva (Switzerland), 2005. http://www.ich.org/LOB/media/MEDIA417.pdf

[82] ISO International Vocabulary of Basic and General Terms in Metrology (VIM), 3rd ed., Geneva (Switzerland), 2006.

[83] United States Pharmacopoeia (USP 29-NF 24), Section 1225, Validation of Compendia: 1. Methods, United States Pharmacopeia Convention Inc., Rockville, MD (USA), 2006.

[84] AOAC International, Statistics Manual of the AOAC, Gaithersburg, MD (USA), 1975.

[85] W.D. Pocklington, Guidelines for the Development of Standard Methods by Collaborative Trial, Laboratory of the Government Chemist, Middlesex (United Kingdom), 1990.

Part 1

MICELLAR LIQUID CHROMATOGRAPHY WITH NON-IONIC SURFACTANTS

CHAPTER 2

FUNDAMENTALS OF
MICELLAR LIQUID CHROMATOGRAPHY

2.1. Abstract

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatographic mode with a solution containing a surfactant above the critical micellar concentration, as mobile phase. Most procedures use the anionic surfactant sodium dodecyl sulphate. However, pure micellar solutions are in general useless as mobile phases, except for the analysis of highly polar compounds. A small amount of an organic solvent (usually a short/medium chain alcohol) is added to improve the elution strength and peak shape. The interaction of solutes with the surfactant monomers adsorbed on the stationary phase, in combination with the solubilisation capability of micelles, has profound implications on retention, selectivity and efficiency. This is the result of the ability of micelles to compartmentalise and organise solutes at the molecular level and the interaction of surfactant monomers adsorbed on the bonded phase with the analytes. The most relevant features of MLC are the elution of compounds (neutral and ionic) in a wide range of polarities using isocratic elution, and the feasibility of direct injection of physiological fluids after simple filtration. Nowadays, MLC seems to be an alternative to conventional RPLC with hydro-organic mobile phases, and with increasing interest in “green” Chemistry.

2.2. Introduction

In the late 1970s, ionic surfactants were added for the first time to polar aqueous-organic mobile phases in reversed-phase liquid chromatography (RPLC) to form ion pairs. The concentration of surfactant in the mobile phases was kept below the critical micellar concentration (CMC) to avoid micelle formation. In 1980, Armstrong reported the possibility of using solutions containing micelles for separation purposes. This gave rise to a new RPLC mode, which has been called micellar liquid chromatography (MLC).

Some qualities of MLC were outlined in the first descriptions: the relatively low cost, low toxicity and ease of operation. Other qualities were revealed as the technique was developed: the possibility of analysing solutes presenting a wide range of polarities within a single isocratic run, the direct injection of physiological fluids, the high reproducibility of retention times, and their accurate prediction through modelling. The latter feature facilitates the optimisation of the separation conditions. The existence of micelles in the mobile phase and the adsorption of surfactant monomers on the stationary phase produce notable changes in the chromatographic behaviour (retention, elution strength, peak shape, selectivity, and resolution).

The weak elution strength and poor efficiencies of pure micellar solutions make them generally useless as mobile phases, except for analysing highly polar compounds. An organic solvent is required to decrease the analysis times to acceptable values and enhance the efficiencies. However, the concentration of the organic solvent is appreciably smaller with regard to conventional aqueous-organic RPLC. The stabilisation of the organic solvent by the micellar medium also decreases the risk of evaporation.

2.3. The chromatographic system

2.3.1. The mobile phase

The mobile phase in MLC consists of an aqueous solution of surfactant forming micelles, or a ternary mixture of water, surfactant and organic solvent (called hybrid micellar mobile phase). In the latter case, the concentration of organic solvent is maintained low enough to permit the formation of micelles; the micellisation process is, however, altered.

Surfactants of different types (non-ionic, anionic, cationic, and zwitterionic) have been assayed, but the anionic surfactant sodium dodecyl sulphate (SDS, CMC = 8.2×10^{-3} M at 25 ^{o}C) is used in most reported procedures. Other common surfactants are the cationic cetyltrimethylammonium bromide (CTAB, CMC = 9×10^{-4} M) and the non-ionic Brij-35 (polyoxyethylene (23) dodecyl ether, CMC = 1×10^{-4} M). Common solvents in RPLC (methanol, ethanol, 1-propanol and acetonitrile) are suitable for MLC. The less polar 1-butanol and 1-pentanol can also be used, since their miscibility with water increases in the presence of micelles. 1-Propanol is the most frequent organic solvent, although acetonitrile may have similar elution strength and usually improves the peak shape. The stronger 1-butanol and 1-pentanol permit the elution of apolar solutes at sufficiently short retention times.

The extreme concentrations of surfactant and organic solvent are imposed by practical limitations in the chromatographic system. The lower concentration of surfactant must be well above the CMC. Its solubility and the viscosity of the resulting mobile phase determine the upper concentration. The concentration of organic solvent is conditioned by the maximal value that ensures the integrity of micelles. For SDS, micelles are disrupted at concentrations (*v*/*v*) above 30–40% methanol, 30% ethanol, 22% 1-propanol, 10% 1-butanol,

7% 1-pentanol, and 30% acetonitrile. Also, CTAB micelles do not exist in solution above 20% methanol. More recently, high contents of organic solvents in the mobile phase (which disrupt the micelles) have been demonstrated to produce significant modifications in the elution strength, selectivity, and peak shape, enhancing the resolution. This RPLC mode, which shows good performance in the analysis of cationic polar and apolar solutes, has been called high submicellar liquid chromatography (HSLC).

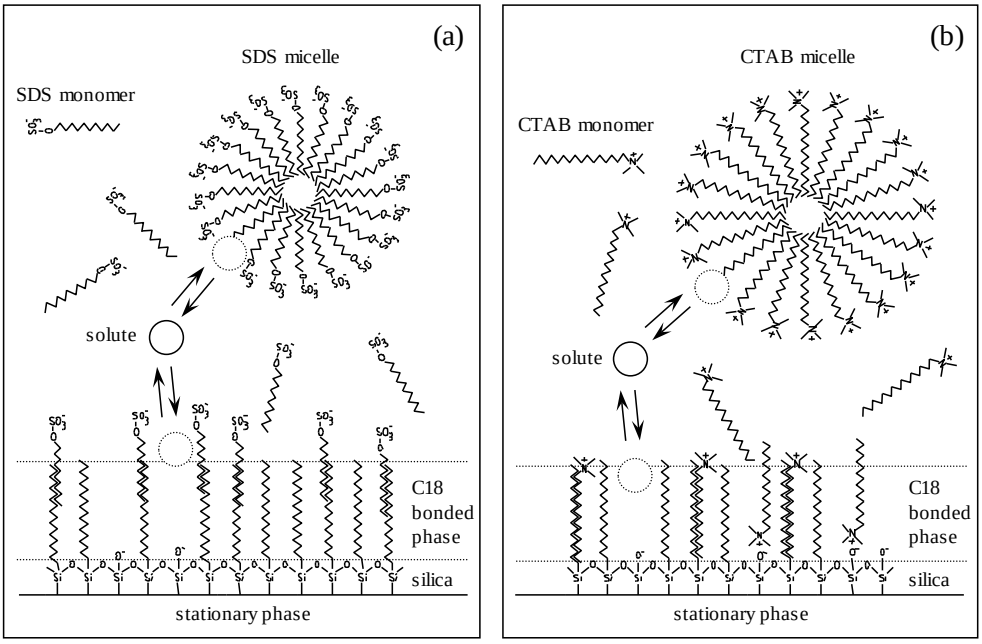


Figure 2.1. Solute interactions with stationary phase and micelle in pure micellar liquid chromatography (MLC) systems containing the surfactants: (a) sodium dodecyl sulphate (SDS), and (b) cetyltrimethylammonium bromide (CTAB).

2.3.2. The stationary phase

Separations in MLC are usually carried out in conventional octadecylsiloxane (C18) columns. A significant number of surfactant molecules may be adsorbed on these stationary phases, giving rise to a structure similar to an open micelle (Figure 2.1). Consequently, column properties change radically, although the subjacent stationary phase (the bonded moiety) still plays a role in the interaction with solutes. Octylsiloxane (C8), cyanopropylsiloxane, and ultrawide pore and monolithic C18 columns are useful for some specific applications.

At increasing surfactant concentration, the amount of adsorbed surfactant on the stationary phase increases rapidly, reaching often a plateau close to the CMC. However, for some surfactants and stationary phases, the adsorption increases beyond the CMC. Nuclear magnetic resonance studies have indicated that on the densely grafted stationary phase, the hydrophobic chain of anionic surfactants is inserted in the bonded organic layer with the functional group protruding towards the mobile phase (Figure 2.1a). Similar behaviour is found with non-ionic surfactants. Cationic surfactants, instead, may either associate with the alkyl-bonded layer like anionic surfactants, or their cationic head be attracted to the residual free silanols buried inside the bonded layer (Figure 2.1b). This explains why the SDS-modified alkyl-phases (with negative charge) are more polar than the CTAB-modified phases (with positive charge). The behaviour of surfactant-modified stationary phases is partially explained considering that surfactant molecules coat the interior walls of the pores in the column support without completely filling them.

Ionic compounds added to the micellar system for pH or ionic strength adjustment increase usually surfactant adsorption. Organic solvents, in contrast, decrease the amount of adsorbed surfactant. Alcohol and surfactant molecules

compete for adsorption sites. The alkyl chains of 1-propanol and longer alcohols form on the stationary phase a monolayer similar to that of adsorbed surfactant molecules, with the hydroxyl group oriented towards the aqueous phase. For ionic surfactants, the desorbing ability depends on the organic solvent polarity (methanol < ethanol < propanol < butanol < pentanol). Also, the amount of adsorbed surfactant molecules decreases with the organic solvent concentration.

Some care is needed to preserve the column performance for long time periods of intensive MLC use, which can be similar or even longer compared to conventional RPLC. Some recommendations are next given to preserve the column. First, since most micellar solutions are able to dissolve minute amounts of silica, the mobile phase should be saturated in silica by inserting a short precolumn before the injection valve. Second, the micellar solution should never stay motionless in the chromatographic system to avoid the formation of surfactant crystals that could clog the system or ruin the column. When the system is not being used, the flow rate can be reduced to a minimal value (e.g., 0.1 mL/min). Finally, before stopping the flow, the column should be cleaned with water to later allow the passage of pure methanol or a 75:25 methanol/propanol mixture for a few minutes. Operating in this way, the layer of SDS monomers can be completely removed from the stationary phase, but CTAB and Brij-35 cannot be removed so easily. With convenient experimental caution, hundreds of injections can be made without modification of the chromatographic system or pressure build-up.

The existence of a plateau of adsorbed ionic surfactant above the CMC permits rapid analyses using gradients of surfactant. Beyond the CMC, changes in total surfactant concentration only modify the concentration of micelles in the mobile phase; the stationary phase is not affected. Also, with hybrid

micellar eluents, gradients of organic solvent can be used without apparent modification of the surfactant layer. This means that the initial conditions with either gradient of surfactant or organic solvent can be recovered without re-equilibration time. However, the particular elution strength behaviour of MLC makes gradient elution usually unnecessary.

2.4. Partitioning behaviour

MLC is a good example of the use of secondary equilibriums in liquid chromatography. Solutes are partitioned between the mobile phase and the stationary phase, which are modified by the presence of micelles and surfactant monomers. In order to understand the partitioning behaviour in MLC, it should be considered that the attraction of solutes to the surface of the surfactant-modified stationary phase is stronger than the attraction to micelles in the mobile phase. However, since the amount of adsorbed surfactant on the stationary phase remains constant, an increase in the concentration of surfactant in the mobile phase (and consequently, an increase in the number of micelles) results in decreased retention.

In the mobile phase, depending on polarity and steric factors, solutes can remain outside the micelle, associate to the polar head of the surfactant, form a part of the outer palisade layer, or penetrate into the micelle core. Organic solvents added to the micellar solution experience similar interactions: acetonitrile and methanol associate weakly with micelles, while 1-butanol and 1-pentanol occupy the inter-monomer spaces of the micelle palisade.

In both mobile phase and stationary phase, neutral solutes interact hydrophobically with surfactants. Meanwhile, charged solutes experience both

hydrophobic and electrostatic interactions with ionic surfactants. In this case, two distinct situations are possible:

- Charges on solute and surfactant are of the same sign. Electrostatic repulsion from the surfactant-modified stationary phase yields low retention (the solute may be even eluted with the void volume), in the absence of sufficiently strong hydrophobic interaction. However, a particular behaviour has been observed with methylsiloxane (C1)- and cyanopropylsiloxane-bonded phases, where the amount of adsorbed surfactant is small: the retention increases at increasing micelle concentration, since the repulsion of solutes from micelles is now dominant (anti-binding behaviour). The electrical double layer surrounding the micelles can be narrowed in a solution containing higher concentration of ions, which facilitates the approximation of the solute to the micelle assembly to establish hydrophobic interactions. By increasing the ionic strength, anti-binding solutes can thus adopt non-binding or even binding behaviour.
- Charges on solute and surfactant are opposite. Electrostatic and hydrophobic interactions with the stationary phase are usually sufficiently large to offset the increase in micelle attraction, and the retention of the charged solutes will be strong.

Water-insoluble apolar solutes exhibit large affinity for the micelles and the surfactant-coated stationary phase and, consequently, can be directly transported between them. This direct transfer mechanism has also been observed with cationic solutes eluted with mobile phases of the anionic SDS. By addition of organic solvent to the mobile phase, the solubility of apolar solutes increases and the direct transfer loses importance with regard to the usual two-step mechanism (micelle-bulk solvent and bulk solvent-stationary phase) (Figure 2.1).

2.5. Correlation between retention and polarity

MLC is useful for measuring the polarity of compounds. The regular linear increase in log k with the number of carbon atoms, n_C, in a homologous series, observed in aqueous-organic RPLC is not usually valid with micellar mobile phases; k versus n_C is linear instead. This behaviour has been explained by the location in the micelle of different members in the homologous series. The more hydrophobic homologues tend to be located in more apolar microenvironments, experiencing smaller change in polarity upon transfer from the micelle to the stationary phase.

Good correlation has also been found between retention in MLC and the logarithm of octanol-water partition coefficient, log $P_{o/w}$, for series of neutral compounds of diverse polarities. The type of general relationship (log k *versus* log $P_{o/w}$ or k *versus* log $P_{o/w}$) depends on the selected set of compounds and the characteristics of both the mobile phase and the stationary phase. The linearity of both correlations is improved by addition of alcohols. The organic solvent apparently provides an environment closer to the octanol-water mixture than pure micellar systems. Correlation of retention with log $P_{o/w}$ is poorer for ionisable compounds, due to the extra ionic interactions. The measured $P_{o/w}$ values depend strongly on the solute ionisation degree, and consequently, non-linear relationships of log k or k *versus* log $P_{o/w}$ are obtained, unless $P_{o/w}$ values of the molecular and ionic forms are available or a correction term is added to consider the ionisation degree.

Retention can also be estimated from solute log $P_{o/w}$ values. The most suitable organic solvent to be used as modifier of the mobile phase should be chosen according to the polarity of the eluted compound. For SDS, a low propanol content (~1%, *v*/*v*) is useful to separate compounds with log $P_{o/w} < -1$, such as amino acids. A larger amount of propanol (~5–7%) is

needed for compounds in the range $-1 < \log P_{o/w} < 2$, such as diuretics and sulphonamides. Other alcohols (< 10% butanol or < 7% pentanol) are required for apolar compounds with $\log P_{o/w} > 3$, such as steroids. This rule of thumb is, however, not always valid: propanol is too weak for cationic solutes, such as phenethylamines ($0 < \log P_{o/w} < 1.7$) or β-blockers ($1 < \log P_{o/w} < 3$). For these compounds, the additional electrostatic attraction to the anionic surfactant molecules adsorbed on the stationary phase requires a stronger solvent.

Micelles are recognised as simple biomembrane models. Biomembranes and micelles have amphiphilic properties and are anisotropic, providing hydrophobic and electrostatic sites of interaction. Particularly, Brij-35 has drawn attention because RPLC with solutions containing micelles of this surfactant seem to emulate *in vitro* the partitioning process in biomembranes better than conventional RPLC, which is useful for quantitative retention-activity relationships studies.

2.6. Effect of pH on retention

Chromatographic retention of ionisable compounds depends on the pH of the mobile phase. Organic solvent and surfactant shift the protonation equilibriums, due to the modification of the polarity of bulk solvent and the association of solutes with micelles and surfactant monomers adsorbed on the stationary phase. Since the intrinsic retention of both acid-base species is different, a sudden change in retention time will happen at pH values close to the logarithm of the apparent protonation constant in the mobile phase medium, K'_H . The full protonation process covers several pH units and is sigmoidal. It resembles conventional acid-base titration curves and is shifted more or less depending on the concentrations of organic solvent and surfactant. However, the whole change in retention is not observed when the working pH of the

column is narrow, as happens with conventional alkyl-bonded stationary phases (pH 3–7). In this case, retention *versus* pH plots may show different patterns (Figure 2.2).

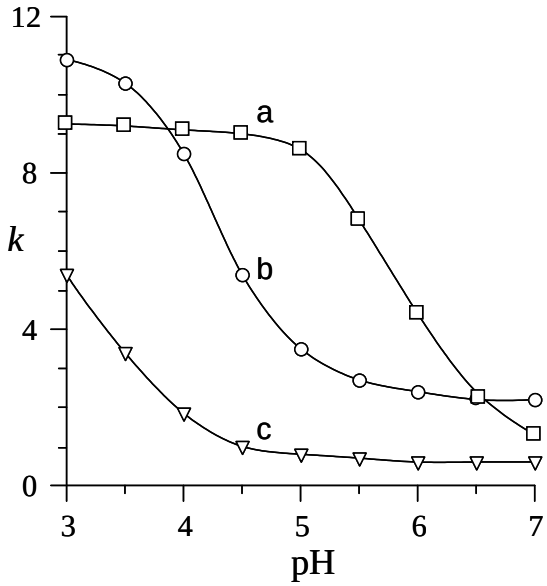


Figure 2.2. Retention *versus* pH plots for: (a) xipamide, (b) ethacrynic acid, and (c) tyrosine. Chromatographic system: ODS-2/0.10 M SDS/4% (*v*/*v*) 1-propanol.

Usually, K'_H diminishes as the organic solvent concentration increases. For ionic surfactants, electrostatic interactions of solute with surfactant are responsible for the shift direction in K'_H at increasing surfactant concentration. These interactions also explain the direction in the trend of the *k*–pH dependence. For weak acids having neutral acidic and anionic basic species (or weak bases having cationic acidic and neutral basic species), K'_H increases with surfactant concentration for anionic surfactants, since the acidic species is stabilised by interaction with micelles and adsorbed monomers on the

stationary phase. Meanwhile, retention decreases with pH, due to the weaker association of the basic species with the surfactant-modified stationary phase (Figure 2.2). The shift in K'_H to higher pH benefits the observation of the maximal retention (the retention of the acidic species) within the operable limits of silica-based columns. When a cationic surfactant is used instead, both the shift in K'_H and the *k versus* pH dependence are opposite to those observed with anionic surfactants, since cationic surfactant molecules stabilise the basic species.

The pH of the micellar mobile phase is usually buffered using the phosphoric or citric acid-base systems. Potassium ion cannot be used with SDS as potassium dodecyl sulphate precipitates from aqueous solutions. Only one peak is observed for each solute in the chromatograms because acid-base equilibriums are much faster than solute-micelle or solute-stationary phase dynamics.

2.7. Modelling of retention

2.7.1. Pure micellar mobile phases

The retention behaviour is the final result of the competition of the equilibriums existing in the separation system. In MLC, solutes partition between three environments: water, micelle and stationary phase (Figure 2.1). If the solute interacts with the micelle, retention is explained by:

$$\frac{V_e - V_0}{V_S} = \frac{k}{\Phi} = \frac{P_{WS}}{1 + \upsilon(P_{WM} - 1)[M]} \qquad (2.1)$$

where V_e represents the total volume of mobile phase needed to elute the solute from the column, V_S is the volume of active surface of the stationary phase, V_0 is the column void volume, $\Phi = V_S/V_0$ is the phase ratio, υ is the partial

specific volume of surfactant monomers in the micelle, P_{WS} and P_{WM} are the partition coefficients between water and stationary phase, and between water and micelle, respectively, and [M] is the concentration of micellised surfactant (total concentration of surfactant minus CMC).

When micelles are not present in the mobile phase, Eq. (2.1) is reduced to the partition equation of aqueous-organic RPLC:

$$V_e = V_0 + V_S P_{WS} \tag{2.2}$$

Since in MLC the stationary phase is modified by surfactant adsorption, P_{WS} in conventional RPLC and MLC will be different for the same column.

A more practical model can be expressed by considering the interactions of solute inside the column, in terms of association equilibriums of solute in bulk solvent with surfactant monomers in the stationary phase and micelles:

$$k = \Phi \frac{[AS]}{[A]+[AM]} = \frac{\Phi P_{WS}[S]}{1 + K_{AM}[M]} \tag{2.3}$$

which results in the following linear model:

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}[M] = c_0 + c_1[M] \tag{2.4}$$

where K_{AS} is used instead of ΦP_{WS} [S], and K_{AM} is the association constant of the solute with a surfactant monomer in the micelle (to be referred to the whole micelle, it should be multiplied by the aggregation number). MLC provides a convenient method of estimating K_{AM}, since solute concentration need not be known, the impurities in the sample are separated, and the chromatographic process makes the simultaneous determination of the constants for several solutes possible.

The three equations (Eqs. (2.2), (2.3) and (2.4)) have been checked to be valid for apolar, uncharged polar, and ionic solutes, chromatographed with anionic, cationic, and non-ionic surfactants, in C8, C18 and cyanopropylsiloxane-bonded columns.

2.7.2. Hybrid micellar mobile phases

The addition of organic solvent to the micellar mobile phase shifts the partition equilibriums of the solute in bulk solvent with both the stationary phase and the micelle (Figure 2.1). The retention is described by:

$$\frac{1}{k} = \frac{1 + K_{AM}\,\dfrac{1+K_{MD}\,\varphi}{1+K_{AD}\,\varphi}[\mathrm{M}]}{K_{AS}\,\dfrac{1+K_{SD}\,\varphi}{1+K_{AD}\,\varphi}} \qquad (2.5)$$

φ being the volumetric fraction of organic solvent. The constants K_{AD}, K_{SD} and K_{MD} measure the relative variation in the concentration of solute in bulk water, stationary phase, and micelle, respectively, due to the reduction in bulk solvent polarity and the modification of the stationary phase and micelle produced by the organic solvent. The K_{SD} term is only needed for highly apolar solutes, which are strongly associated to the stationary phase. In other cases, $K_{SD} = 0$ and Eq. (2.5) can be simplified to:

$$\frac{1}{k} = c_0 + c_1\,\varphi + c_2\,[\mathrm{M}] + c_{12}\,\varphi[\mathrm{M}] \qquad (2.6)$$

The analogous effects of organic solvent on both microenvironments (stationary phase and micelle) are evident in the parallel variations of solute-stationary phase and solute-micelle partition coefficients, as the concentration of organic solvent changes.

2.7.3. Simultaneous effect of surfactant, organic solvent, and pH

Very often, chromatographic separations are carried out in a buffered medium. In this case, Eqs. (2.4)–(2.6) are appropriate to describe the retention of compounds exhibiting acid-base behaviour. For these compounds, retention is a weighted mean of the retention of the basic and acidic species. At varying pH:

$$k = k_A \delta_A + k_{HA} \delta_{HA} = k_A \frac{1}{1 + K_H' h} + k_{HA} \frac{K_H' h}{1 + K_H' h} = \frac{k_A + k_{HA} K_H' h}{1 + K_H' h} \tag{2.7}$$

where k_A and k_{HA} are the retention factors of the basic and acidic species, respectively, and h is the concentration of hydrogen ion. In a pure micellar mobile phase:

$$\frac{1}{k} = \frac{1 + \frac{K_{AM} + K_{HAM} K h}{1 + K h} [M]}{\frac{K_{AS} + K_{HAS} K h}{1 + K h}} \tag{2.8}$$

When the simultaneous effect of the three factors (surfactant, organic solvent, and pH) is considered, the retention is given by:

$$\frac{1}{k} = \frac{\left(1 + K_{AM} \frac{1 + K_{MD} \varphi}{1 + K_{AD} \varphi} [M]\right) + \left(1 + K_{HAM} \frac{1 + K_{HMD} \varphi}{1 + K_{HAD} \varphi} [M]\right) K h}{\left(K_{AS} \frac{1}{1 + K_{AD} \varphi}\right) + \left(K_{HAS} \frac{1}{1 + K_{HAD} \varphi}\right) K h} \tag{2.9}$$

K_{AS}, K_{AM}, K_{AD}, and K_{MD} are constants associated to the basic species, and K_{HAS}, K_{HAM}, K_{HAD}, and K_{HMD} are constants associated to the acidic species; K is the protonation constant in the aqueous-organic mixture.

2.8. Peak shape

The thick surfactant film formed on the surface of the organic bonded layer is partially responsible for the loss of efficiency (i.e., broad and tailing peaks) observed when pure micellar mobile phases are used, with regard to aqueous-organic mixtures, which is especially true for apolar compounds. Peak shape deteriorates at larger surfactant concentration. This is a major drawback that hindered the development of MLC. The addition of a small amount of organic solvent was proposed to remediate this problem. Hybrid micellar mobile phases containing surfactant and organic solvent often yield similar or even improved efficiency relative to aqueous-organic mixtures, but the efficiency for highly apolar compounds is clearly inferior. Also, peak broadening in the presence of Brij-35 is larger.

The reason for the favourable behaviour produced by the addition of organic solvent is the reduction of the viscosity and thickness of adsorbed surfactant on the stationary phase. This enhances the rate of solute mass-transfer between bulk solvent and stationary phase. Higher temperature may also yield a significant improvement in the efficiency. In an interesting example involving a set of flavonoids, peak shape with Brij-35 mobile phases at 25 °C was significantly poorer with regard to acetonitrile-water mixtures. At increasing temperature, the efficiency of Brij-35 increased, approaching at 80 °C the values already reached with acetonitrile at 25 °C.

The anionic surfactant SDS, used in combination with an organic solvent to reduce the surfactant layer on the stationary phase, has proved to suppress efficiently the silanol effect on basic compounds giving rise to symmetrical peaks (Figure 2.3, and Figures 2.4a and b). SDS reduces the penetration depth of the cationic protonated solutes into the bonded phase, and the kinetics of the electrostatic association between the anionic head group of the surfactant and cationic solutes seems to be easier than ion-exchange processes involving the silanols on the silica surface. Unfortunately, retention of the cationic solutes is significantly increased due to their attraction to the adsorbed anionic surfactant, being required the addition of high amounts of organic solvent to the mobile phase that often reach the HSLC conditions.

2.9. Separation performance

In MLC, the retention behaviour (elution strength and selectivity) can be quite different from conventional RPLC, even after the addition of an organic solvent. The range of interactions provided by MLC is by far superior to that in aqueous-organic systems. This feature confers micellar mobile phases the ability of making cationic, anionic, uncharged polar, and apolar solutes compatible within the same chromatographic run. Furthermore, perhaps, the main strength of MLC lies in the capability of performing the isocratic separation of mixtures of compounds exhibiting a relatively wide range of polarities (Figure 2.4). In the micellar mode, the retention of the least retained compounds is larger than for conventional RPLC (which is interesting with regard to the direct injection of samples), and all compounds appear more evenly distributed in the chromatograms. This effect is comparable to the use of gradients of organic solvent in conventional RPLC, but with a clear saving in organic solvent.

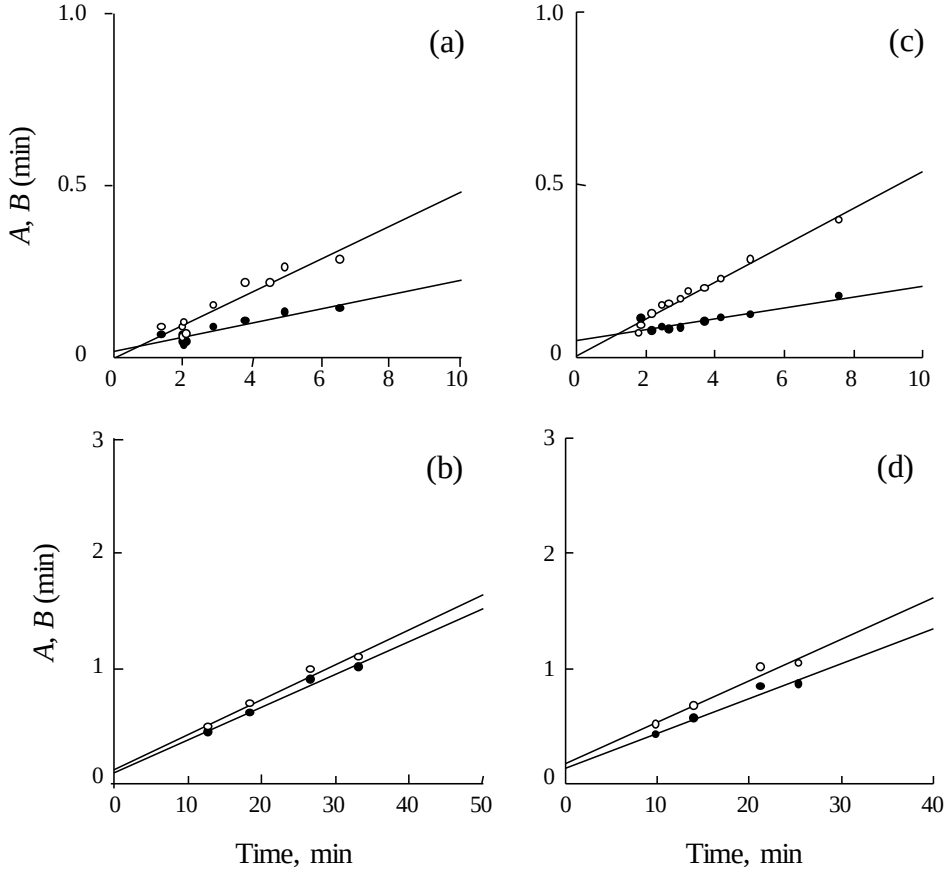


Figure 2.3. Plots of the half-widths of chromatographic peaks at 10% peak height *versus* their retention time for a set of β-blockers, using Inertsil (a,b), and XTerra (c,d) columns, and acetonitrile/water (a,c) or SDS/acetonitrile (b,d) mobile phases. Half-widths: left, A (●), and right, B (○).

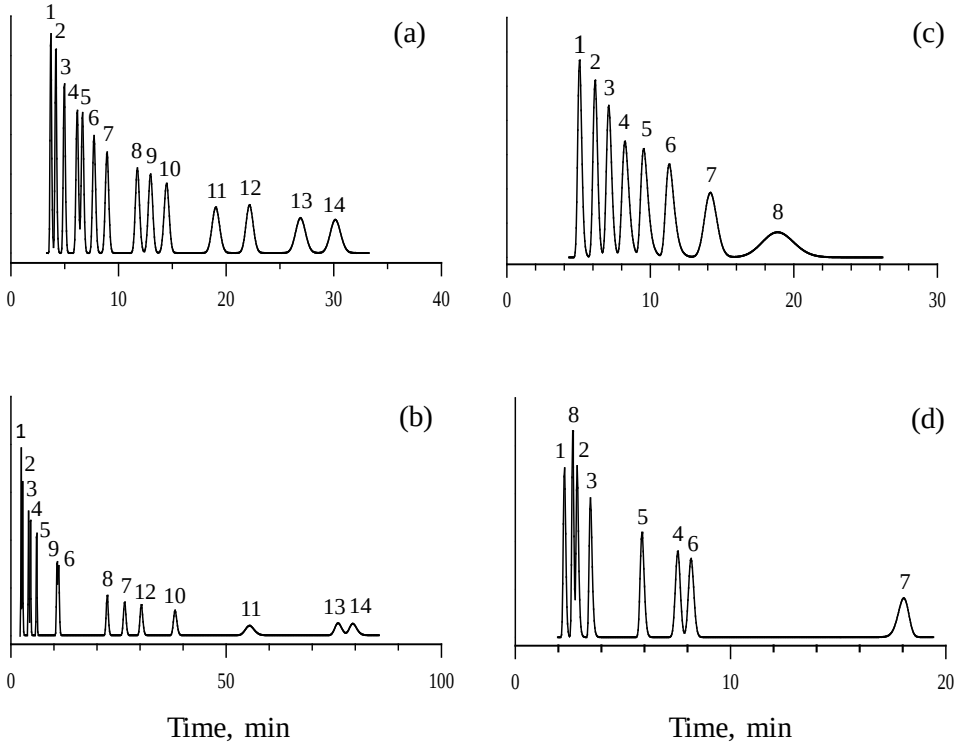


Figure 2.4. Chromatograms of mixtures of 14 β-blockers (a,b) and 8 steroids (c,d). Chromatographic systems: (a) ODS-2/0.10 M SDS/15% (*v*/*v*) 1-propanol, (b) XTerra MS/15% (*v*/*v*) acetonitrile, (c) ODS-2/0.12 M SDS/6% (*v*/*v*) 1-pentanol, and (d) ODS-2/52% (*v*/*v*) acetonitrile. Compounds in (a,b): (1) atenolol, (2) sotalol, (3) carteolol, (4) nadolol, (5) pindolol, (6) acebutolol, (7) celiprolol, (8) esmolol, (9) timolol, (10) bisoprolol, (11) labetalol, (12) oxprenolol, (13) propranolol, and (14) alprenolol. Compounds in (c,d): (1) dehydrotestosterone, (2) testosterone, (3) methyltestosterone, (4) medroxy-progesterone acetate, (5) dydrogesterone, (6) progesterone, (7) testosterone propionate, and (8) nandrolone.

Resolution and selectivity in MLC can be optimised by wise selection of the nature and concentration of both the surfactant and the organic solvent and, occasionally, the pH. For polar compounds, the elution strength of the surfactant may be larger or similar to the organic solvent; the opposite situation is found for apolar compounds, which need a small amount of butanol or pentanol in the micellar mobile phase to achieve sufficiently low retention times (Figure 2.4). Eqs. (2.4)–(2.9) are useful for predicting the retention. Optimisations based on these models can be carried out using economical experimental designs.

With Brij-35, moderately polar compounds can be separated in appropriate retention times from C18 columns, without the need of adding an organic solvent. Highly polar compounds that interact with the hydroxyl end group in Brij-35 are retained by formation of hydrogen bonds. As shown in Chapter 6, cationic basic compounds, which are highly retained on the stationary phases modified with the anionic SDS (requiring a strong organic solvent or high amounts of acetonitrile or propanol), can be analysed in proper analysis times using a mobile phase containing only the two surfactants SDS and Brij-35. The biodegradable character of SDS and Brij-35 gives rise to a promising “green” liquid chromatography mode.

2.10. Analytical applications

Most applications in MLC make use of hybrid micellar mobile phases. The reported procedures usually correspond to the assay of drugs in formulations and physiological fluids, but interesting procedures have been also reported for the separation of bioactive compounds in cosmetics, food, water, and environmental samples, among other types of samples. Some examples of MLC procedures are given in Table 2.1 and Figure 2.5.

Table 2.1. Representative examples of analytical use of micellar chromatographic systems.

Analytes	Samples	Chromatographic system
Alkaloids, tricyclic antidepressants, antihistamines, benzodiazepines, β-blockers, catecholamines, diuretics, opiates, parabens, phenethylamines, steroids and sulfonamides	Medicinal plants and pharmaceuticals	Phenyl / SDS C18 / SDS / 1-propanol, acetonitrile, butanol, or pentanol / pH 3 C18 / Brij-35 / pH 3 C18 / CTAB / pentanol
Sugars	Infant formula and syrups	Amino / SDS / ethanol / pH 6.7
Preservatives, sunscreen products	Cosmetics	C_{18} / Brij–35 / 1-propanol / pH 3 C_8 / SDS / 2-propanol / trimethylamine / pH 3
Antibacterials, anticancer drugs, anticonvulsants, antidepressants, β-blockers, opiates, phenols, radioligands and steroids	Biological samples (urine, plasma, serum, milk)	C_{18} / SDS / 1-propanol / triethylamine / pH 4 C_{18} / SDS / butanol or pentanol pH 3 or 7 C_{18} / CTAB / acetonitrile pH 5
Folylpolyglutamate hydrolase activity	Mouse kidney	C_{18} / SDS
Recombinant human growth hormone	*Escherichia coli*	C_4 / SDS / 1-propanol / pH 6.4
Antibacterials	Honey, milk, egg	C_{18} / SDS / 2-propanol / pH 3 or 3.5
Antioxidants	Oils, olive extracts	C_{18} / SDS / 1-propanol / pH 3 C_{18} / SDS / methanol / pH 7

Table 2.1 (continued).

Analytes	Samples	Chromatographic system
Amino acids and biogenic amines	Wines	C_{18} / SDS / 1-propanol / pH 3
Curcumin	Spice samples	C_{18} / SDS / 1-propanol / pH 7
Melamine	Dietetic supplements	C_{18} / SDS / pH 3
Proteins	Mixtures of proteins and beef heart	C8 / Neodol 91–6, pH 7
Tetracyclines	Animal feeds	C_{18} / SDS / 1-butanol
Maleic hydrazide	Tobacco	C_{18} / CTAB / pH 7
Aromatic amines	Waste waters	C_{18} / SDS / 1-pentanol / pH 7
Carbamates	Water and commercial pesticide	C_{18} / Brij-35
Fungicides	Pond water	Cyano / CTAB / methanol / pH 7

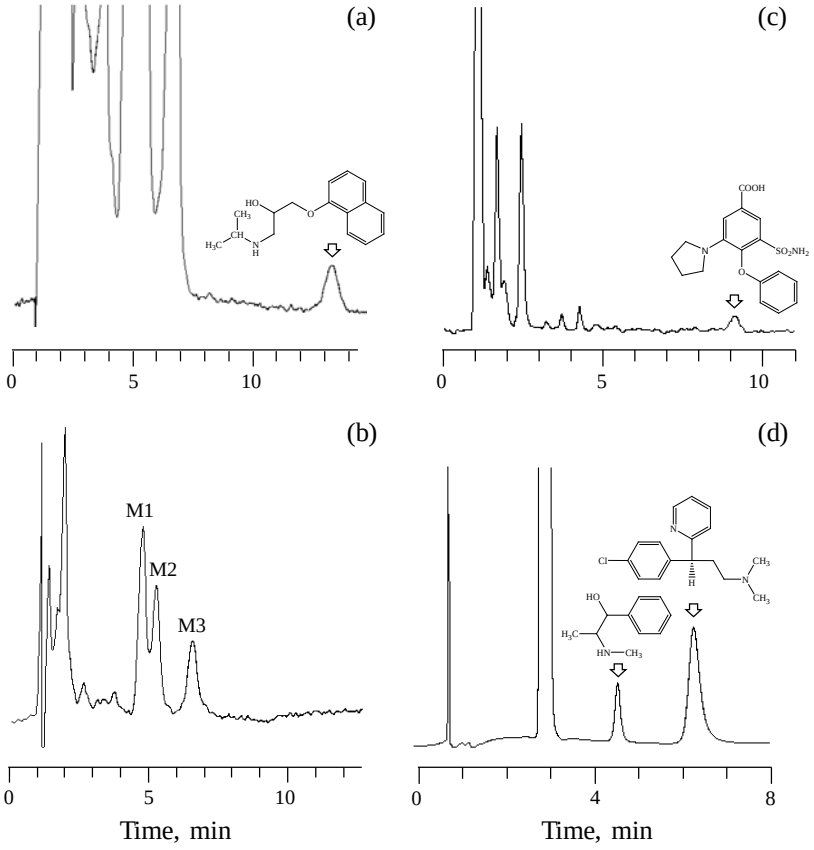


Figure 2.5. Analysis of several drugs in a urine sample (a to c) and in a cough-cold formulation (d): (a) Propranolol (2 h after a 10 mg dose intake), determined by direct injection of the urine without dilution using 0.1 M SDS/15% (*v*/*v*) 1-propanol/1% triethylamine mobile phase and fluorimetric detection; (b) propranolol metabolites (M1–M3) determined after a 1:25 dilution of the previous sample; (c) piretanide (2 h after a 6 mg dose intake), determined after a 1:25 dilution of a urine sample using 0.055 M SDS/8% (*v*/*v*) 1-propanol and fluorimetric detection; (d) pseudo-ephedrine (preceding peak) and dexchlorpheniramine using 0.15 M SDS/6% (*v*/*v*) 1-pentanol and spectrophotometric detection.

Micellar solutions can be used to dissolve samples or extract analytes. Apolar and polar compounds, derivatisation reagents, and products can be cosolubilised. These media can induce favourable shifts in the equilibrium constants and expedite reactions through micellar catalysis. Also, they are useful to minimise the photochemical degradation of drugs under the influence of ultraviolet (UV) radiation. Micelles improve the analytical performance of various spectroscopic (UV-visible spectrophotometry, fluorimetry, phosphorimetry, and atomic absorption), and electrochemical (amperometry) detection methods, by increasing the sensitivity, modifying the selectivity, and overcoming some problems associated with the use of aqueous-organic mixtures. An interesting example is the use of MLC with non-ionic surfactant to separate amino acids or cholesterol derivatives with postcolumn immobilised enzyme reactors. Micellar phases are gentler for the enzyme activity than aqueous-organic mixtures or aqueous buffers.

One of the main appealing factors of MLC is the possibility of determining drugs in physiological fluids in a few minutes, without the need of previous separation of the proteins present in the samples. Micelles bind to proteins releasing bound drugs, whereas the proteins rather than precipitating on the column are solubilised and swept away, eluting with or shortly after the solvent front. However, although hundreds of repetitive serial injections are possible, the sample should be diluted when possible before performing the direct injection to keep the column operative for a longer time.

The anionic SDS is the most common surfactant in these analyses, but the non-ionic Brij-35 can also be employed. Cationic surfactants are not compatible because they cause the precipitation of proteins. MLC and HSLC can be combined to analyse compounds in a wide range of polarities with screening purposes, in physiological fluids. The sample can be directly injected into the

column in the presence of micelles and a very small concentration of organic solvent, or even, without organic solvent. Once the proteins are eluted with the solvent front, the concentration of organic solvent may be rapidly increased to elute the most retained compounds under submicellar conditions.

2.11. Further reading

[1] D.W. Armstrong, F. Nome, Partitioning behavior of solutes eluted with micellar mobile phases in liquid chromatography, *Anal. Chem.* 53 (1981) 1662–1666.

[2] J.J. Baeza Baeza, Y. Dávila, J.J. Fernández Navarro, M.C. García Álvarez-Coque, Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC, *Anal. Bioanal. Chem.* 404 (2012) 2973–2984.

[3] A. Berthod, M.C. García Álvarez-Coque, *Micellar liquid chromatography*, Marcel Dekker, New York, 2000.

[4] R.D. Caballero, M.J. Ruiz Ángel, E. Simó Alfonso, M.C. García Álvarez-Coque, Micellar liquid chromatography: Suitable technique for screening analysis, *J. Chromatogr. A* 947 (2002) 31–45.

[5] L. Escuder Gilabert, J.J. Martínez Pla, S. Sagrado, R.M. Villanueva Camañas, M.J. Medina Hernández, Biopartitioning micellar separation methods: Modelling drug absorption, *J. Chromatogr. B* 797 (2003) 21–35.

[6] J. Esteve Romero, S. Carda Broch, M. Gil Agustí, M.E. Capella Peiró, D. Bose, Micellar liquid chromatography for the determination of drug materials in pharmaceutical preparations and biological samples, *Trends Anal. Chem.* 24 (2005) 75–91.

[7] J. Esteve Romero, J. Albiol Chiva, J. Peris Vicente, A review on the development of analytical methods to determine monitorable drugs in serum and urine by micellar liquid chromatography using direct injection, *Anal. Chim. Acta* 926 (2016) 1–16.

[8] M.C. García Álvarez-Coque, J.R. Torres Lapasió, Quantitation of hydrophobicity in micellar liquid chromatography, *Trends Anal. Chem.* 18 (1999) 533– 543.

[9] M.C. García Álvarez-Coque, J.R. Torres Lapasió, J.J. Baeza-Baeza, Modeling of retention behaviour of solutes in micellar liquid chromatography, *J. Chromatogr. A* 780 (1997) 129–148.

[10] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, S. Carda Broch, Micellar Liquid Chromatography: Fundamentals, in *Analytical Separation Science* (edited by J.L. Anderson, A. Berthod, V. Pino, A.M. Stalcup), Vol. 2, Wiley-VCH, New York, 2015, pp. 371–406.

[11] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, S. Carda Broch, Micellar liquid chromatography: Method development and applications, in *Analytical Separation Science* (edited by J.L. Anderson, A. Berthod, V. Pino, A.M. Stalcup), Vol. 2, Wiley-VCH, New York, 2015, pp. 407–460.

[12] S. López Grío, M.C. García Álvarez-Coque, W.L. Hinze, F.H. Quina, A. Berthod, Effect of a variety of organic additives on retention and efficiency in micellar liquid chromatography, *Anal. Chem.* 72 (2000) 4826–4835.

[13] R. Nakao, M. Schou, C. Halldin, Direct plasma metabolite analysis of positron emission tomography radioligands by micellar liquid chromatography with radiometric detection, *Anal. Chem.* 84 (2012) 3222–3230.

[14] M.J. Ruiz Ángel, S. Carda Broch, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Retention mechanisms in micellar liquid chromatography, *J. Chromatogr. A* 1216 (2009) 1798–1814.

[15] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[16] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Chromatographic efficiency in micellar liquid chromatography: Should it be a topic of concern? *Sep. Purif. Rev.* 42 (2013) 1–27.

[17] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, High submicellar liquid chromatography, *Sep. Purif. Rev.* 43 (2014) 124–154.

[18] M.J. Ruiz Ángel, E. Peris García, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A method for the analysis of basic compounds, *Green Chem.* 17 (2015) 3561–3570.

[19] J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, M.H. Abraham, Micellar versus hydro-organic reversed-phase liquid chromatography: A solvation parameter-based perspective, *J. Chromatogr. A* 1182 (2008)176–196.

CHAPTER 3

ISOCRATIC AND GRADIENT ELUTION IN MICELLAR LIQUID CHROMATOGRAPHY WITH BRIJ-35

3.1. Abstract

Polyoxyethylene(23)lauryl ether (known as Brij-35) is a non-ionic surfactant, which has been considered as an alternative to the extensively used in micellar liquid chromatography anionic surfactant sodium lauryl (dodecyl) sulphate, for the analysis of drugs and other types of compounds. Brij-35 is the most suitable non-ionic surfactant for micellar liquid chromatography, owing to its commercial availability, low cost, low toxicity, high cloud temperature and low background absorbance. However, it has had minor use. In this work, we gather and discuss some results obtained in our laboratory with several β-blockers, sulphonamides and flavonoids, concerning the use of Brij-35 as mobile phase modifier in the isocratic and gradient modes. The chromatographic performance for purely micellar eluents (with only surfactant) and hybrid eluents (with surfactant and acetonitrile) is compared. Brij-35 increases the polarity of the alkyl-bonded stationary phase and its polyoxyethylene chain containing a hydroxyl end group allows hydrogen-bond interactions, especially for phenolic compounds. This offers the possibility of using aqueous solutions of Brij-35 as mobile phases with sufficiently short retention times. The use of gradients of acetonitrile to keep the concentration of Brij-35 constant is another interesting strategy that yields a significant reduction in the peak widths, which guarantee high resolution.

3.2. Introduction

Solutions containing surfactants, above the critical micelle concentration (CMC), have been used with separation purposes in hundreds of analytical reports during the last three decades, in the so-called micellar liquid chromatography (MLC) [1,2]. In MLC, the stationary phase is modified by adsorption of surfactant monomers, and those in excess are associated in the mobile phase to form micelles. The presence of organised entities of surfactant in both phases allows the establishment of new and strong interactions, and the ability to modulate the chromatographic behaviour of solutes, expanding the possibilities of separation. The retention behaviour (elution strength and selectivity) in MLC can be very different from that observed with aqueous-organic mixtures in reversed-phase liquid chromatography (RPLC), in the absence of additives [3–6].

Some of the attractive features of MLC were detailed in the first descriptions of the technique: the reduction of toxicity and wastes due to the surfactant biodegradability and the smaller amount of organic solvent in the mobile phases, the possibility of direct injection of physiological fluids [7], the accurate prediction of the retention behaviour that greatly facilitates the optimisation of separation conditions [8], and the capability of analysing solutes in a wide range of polarities with isocratic elution in sufficiently short analysis times, performing a single injection of the sample [9]. In some cases, gradient elution may help to reduce the analysis times. However, following the enthusiastic studies carried out by Dorsey, Khaledi and co-workers on the development of gradient elution in MLC [10–13], only a small number of MLC procedures have been described that apply this elution mode. Recently, a report describing in detail the implementation of gradient elution has been published to encourage analysts to implement gradients of organic solvent in MLC, which

is rather simple and allows rapid analytical procedures without pre-treatment or the need of re-equilibration [14].

Although surfactants of various types (with non-ionic or ionic end groups) may be used for the preparation of mobile phases in MLC, most reports involve the anionic surfactant sodium dodecyl (lauryl) sulphate (SDS, $C_{12}H_{25}SO_4^-\ Na^+$), followed by the cationic cetyltrimethylammonium bromide ($C_{16}H_{33}N(CH_3)_3^+\ Br^-$) [1,2]. SDS has some favourable characteristics that explain its success: it is commercially available at high purity, it dissolves effectively proteins in biological matrices, and there are extensive studies on the dynamics of micelle formation, much more numerous than for other surfactants. These features, along with a habit of use, have relegated exploring the possibilities offered by other surfactants in MLC, such as non-ionic surfactants.

Borgerding and Hinze were the first to study the feasibility, advantages and limitations of the non-ionic surfactant polyoxyethylene(23)lauryl ether ($C_{12}H_{25}(OC_2H_4)_{23}OH$, also known as Brij-35) in MLC [15]. For this purpose, the authors used a test mixture composed of benzyl alcohol, acetophenone, methyl benzoate, benzaldehyde, benzene, and dimethyl terephthalate, which were eluted with purely mobile phases of Brij-35 (aqueous solutions of the surfactant) or hybrid mobile phases of Brij-35 and ethanol. These researchers studied later the retention mechanism of the homologous series of alkylbenzenes in MLC with Brij-35 [3], and the modification of the stationary phase by this surfactant [16]. Cline-Love and Fett found that Brij-35 was optimal for analysing drugs in urine samples by direct injection [17]. However, despite the promising results of these early studies, the interest in the analytical use of Brij-35 declined. Some years later, two interesting reports with Brij-35 were published on the behaviour of 15 benzene and naphthalene derivatives

[18], and the analysis of Al(III) and Fe(II) in serum samples as complexes with desferrioxamine [19]. More recent reports describe the determination of carbamate pesticides [20], and mixtures of various esters of benzoic and *p*-hydroxybenzoic acid in cosmetics and food samples [21]. A particularly interesting application using Brij-35 and a cyano column is the determination of cholesterol and its metabolites in food samples, using an enzymatic method in the detection step [22]. In all these reports, isocratic elution was used.

In our laboratory, we have developed a method for the analysis of apolar tricyclic antidepressants, using purely micellar mobile phases of Brij-35 [23]. A study of the effect of temperature on the isocratic elution of flavonoids with purely micellar mobile phases of Brij-35 was also carried out, where a significant enhancement in the efficiency was observed by increasing the column temperature up to 80 °C, approaching the performance achieved in aqueous-organic RPLC [24]. Mobile phases containing mixed micelles of Brij-35 and SDS have also been suggested to improve the separation of organic compounds in pharmaceutical preparations [25,26], coumarins [27], and alkaloids [28]. Brij-35 has also attracted attention in the field of quantitative structure-activity relationships (QSAR): the ability of a Brij-35 MLC system to mimic biopartitioning processes has proven very useful in describing the biological behaviour of diverse drugs [29–33].

In this work, an overview of the performance of Brij-35 in MLC with isocratic and gradient elution of three families of compounds with different polarities and structures is presented, which can guide analysts to develop future applications. The type of compounds that can be analysed with this surfactant and the required experimental working conditions are discussed.

3.3. Experimental

3.3.1. Reagents, column and apparatus

The following compounds were analysed (ChemAxon predicted values of octanol-water partition constants taken from http://www.drugbank.ca/ are given, which correlate with the order of retention): three β-blockers (pindolol, oxprenolol, and propranolol, log $P_{o/w}$ = 1.69, 2.17 and 2.58, respectively), six sulphonamides (sulphamethazine, sulphamerazine, sulphamethoxazole, sulphisoxazole, sulphadimethoxine, and sulphaquinoxaline, log $P_{o/w}$ = 0.52, 0.65, 0.73, 0.79, 1.26, and 1.55), and three flavonoids (fisetin, quercetin, and chrysin, log $P_{o/w}$ = 1.81, 2.16, and 3.01), all from Sigma (St. Louis, MO, USA). All compounds were dissolved in a small amount of acetonitrile and diluted with water. The concentration of the injected solutions was 10 µg/mL for β-blockers and sulphonamides, and 50 µg/mL for flavonoids.

Mobile phases containing Brij-35 (99% purity, mean molecular mass ≈ 1200 g/mol) from Fluka (Steinheim, Germany) or/and acetonitrile from Scharlau (Sentmenat, Barcelona, Spain) were prepared, buffered at pH 3 with 0.01 M anhydrous sodium dihydrogen phosphate from Fluka. Nanopure water, obtained with a Thermo Scientific Barnstead purification system (Dubuque, IA, USA), was used in all experiments. The solutions of the probe compounds and mobile phases were filtered through 0.45 µm Nylon membranes from Micron Separations (Westboro, MA, USA). A Zorbax Eclipse XDB-C18 column (125 mm × 4.6 mm i.d., 5 µm particle size) from Agilent (Waldbronn, Germany) was used.

The liquid chromatograph was from Agilent, equipped with the following modules: a quaternary pump (HP 1200) operated at 1 mL/min, an autosampler (HP 1100) provided with 2 mL vials, a temperature controller (HP 1100), and a

variable wavelength UV-visible detector (HP 1100), set at 254 nm. The system was governed with an OpenLAB CDS LC ChemStation (Agilent B.04.03). The half-widths of chromatographic peaks were measured with the MICHROM software [34].

3.3.2. Procedures

In isocratic elution, 0.01 to 0.05 M aqueous solutions of Brij-35 without organic solvent were first assayed as mobile phases (the molarities were calculated based on the surfactant mean molecular weight; 0.05 M is close to the solubility of Brij-35 in water). Other measurements were carried out with seven hybrid mobile phases prepared with 0.01 M Brij-35 and acetonitrile in the range 0 to 50% (*v*/*v*).

Four types of linear gradients were built: (i) gradients of Brij-35 in the absence of organic solvent, (ii) gradients of organic solvent keeping constant the concentration of Brij-35, (iii) simultaneous gradients of Brij-35 and acetonitrile, where the concentration of both components was increased, and (iv) simultaneous gradients of Brij-35 and acetonitrile, where acetonitrile was increased and Brij-35 decreased. Solvent A was always 0.01 M Brij-35, and solvent B was 0.05 M Brij-35 for gradient (i), 0.01 M Brij-35 and 50% acetonitrile for gradient (ii), 0.05 M Brij-35 and 50% acetonitrile for gradient (iii), and 50% acetonitrile for gradient (iv). Chromatograms for gradients of Brij-35 in the absence of organic solvent were obtained at 25 °C and 50 °C.

In all cases (isocratic and gradient elution), duplicate runs were carried out. The dead time in the presence of surfactant was measured from the first baseline disturbance, being $t_0 = 1.10 \pm 0.04$ min. The dwell time was 0.89 min.

3.4. Results and discussion

3.4.1. An overview of the capability of Brij-35 as modifier in MLC

Both Brij-35 and SDS, have an alkyl chain of 12 carbon atoms, but differ in the terminal group. When Brij-35 is added to a mobile phase in RPLC, the surfactant alkyl chain is associated with the bonded alkyl chain on the stationary phase, modifying its properties. The polar hydrophilic head of the Brij-35 molecule (the polyoxyethylene chain with the hydroxyl end group) is oriented away from the surface of the stationary phase. This increases the polarity of the stationary phase, which remains neutral. The hydroxyl end group in the polyoxyethylene glycol chain of Brij-35 can also interact with polar or moderately polar solutes by formation of hydrogen bonds with hydroxyl (and amino) groups, which would increase the retention. Brij-35 also forms large micelles in the mobile phase [35], which contain an apolar dodecyl core and a relatively polar outer region formed by the surfactant polyoxyethylene glycol chains, which provide hydrophobic and hydrophilic interaction sites, similar to those experienced by the solutes on the modified stationary phase.

Previous studies in our laboratory, together with reports published by other authors, have suggested that purely micellar mobile phases of Brij-35 may be suitable for the determination of compounds of intermediate or relatively low polarity, or for polar (even highly polar compounds) that interact with the hydroxyl end group in the surfactant by formation of hydrogen bonds, such as imipramine and nortriptyline [23], flavonoids [24], diazepam, and oxazepam [30], and arbutin and hydroquinone [36].

To study the capability of Brij-35 as modifier in MLC, three sets of probe compounds were selected, covering a relatively wide range of polarities and structures: three β-blockers, six sulphonamides, and three flavonoids (see

Section 3.3.1). The β-blockers (with a polarity range similar to the assayed flavonoids) showed the shortest retention: with aqueous 0.01 M Brij-35 at pH 3, pindolol eluted close to the dead time and the retention times for oxprenolol and propranolol were 2.7 and 11.8 min, respectively. The polarity of the β-blockers, and the formation of internal hydrogen bonds between the adjacent hydroxyl and amino groups in these compounds, do not allow a sufficiently strong interaction with the stationary phase modified with Brij-35. However, the most apolar β-blockers, such as oxprenolol and propranolol, may be analysed using a purely micellar mobile phase of Brij-35 at low concentration (the retention times of these β-blockers significantly decreased with 0.02 M Brij-35).

As shown below, flavonoids, and to a lesser extent, sulphonamides (which are more polar), show larger retention than β-blockers in MLC with Brij-35, which can be explained by the formation of hydrogen bonds between the amino or hydroxyl groups in sulphonamides and flavonoids with the hydroxyl end group of Brij-35. In previous work [24], the interaction of flavonoids with the stationary phase modified by Brij-35, and consequently the retention behaviour, was explained by the number and position of the hydroxyl groups in the aromatic rings of flavonoids.

Since the retention of the assayed sulphonamides and flavonoids was not excessively high, it was possible in both cases to elute the compounds with purely micellar mobile phases of Brij-35 at high concentration. However, hybrid mobile phases containing a low concentration of Brij-35 and acetonitrile, improved significantly the performance (narrower peaks and shorter analysis times). Mobile phases with added surfactant increase the viscosity; therefore, acetonitrile was found more convenient than methanol as modifier. Note that acetonitrile was found also superior to ethanol and 1-

propanol to enhance the peak profiles in MLC with SDS [37]. Next, the chromatographic behaviour of the sets of sulphonamides and flavonoids studied in this work is described in detail, using isocratic and gradient elution.

3.4.2. Retention behaviour in isocratic elution

In Figures 3.1 and 3.2, the retention (expressed as the logarithm of the retention factor, ln k) is depicted against the concentration of acetonitrile, φ, for some of the probe compounds studied in this work, eluted with mobile phases containing acetonitrile, in the absence (Figures 3.1a and 3.2a) and presence of 0.01 M Brij-35 (Figures 3.1b and 3.2b). In both cases, the lines in the plots can be described by the classical polynomial:

$$\ln k = c_0 + c_1 \varphi + c_2 \varphi^2 \qquad (3.1)$$

where c_0, c_1 and c_2 are fitting parameters. It should be noted that the concave trend in aqueous-organic mobile phases (Figures 3.1a and 3.2a) is significantly changed when Brij-35 is added to the mobile phase, resulting in sigmoidal (for sulphonamides, Figure 3.1b), or convex (for flavonoids, Figure 3.2b) plots.

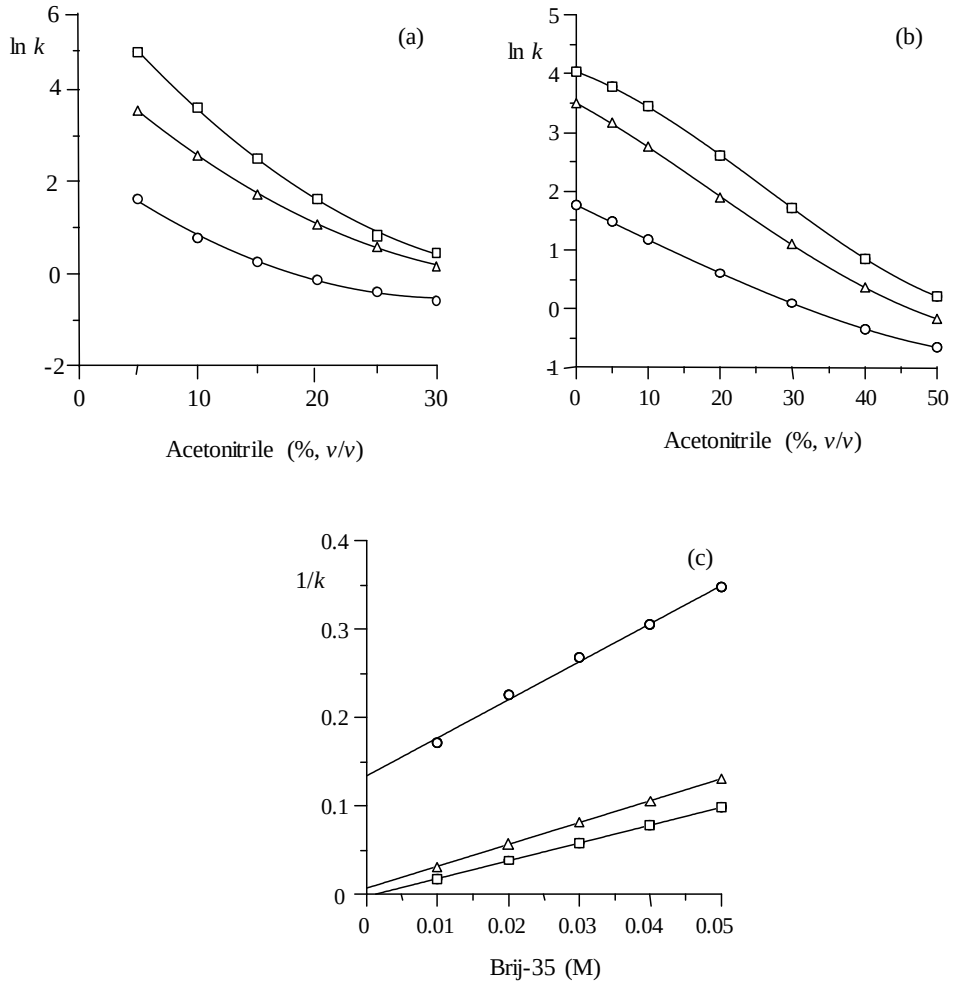


Figure 3.1. Retention plots describing the isocratic elution of the probe compounds: (a) aqueous-organic mixtures in the absence of Brij-35, (b) micellar mobile phases containing 0.01 M Brij-35 and acetonitrile, and (c) purely micellar mobile phases of Brij-35. Compounds: sulphamerazine (○), sulphisoxazole (Δ), sulphaquinoxaline (□).

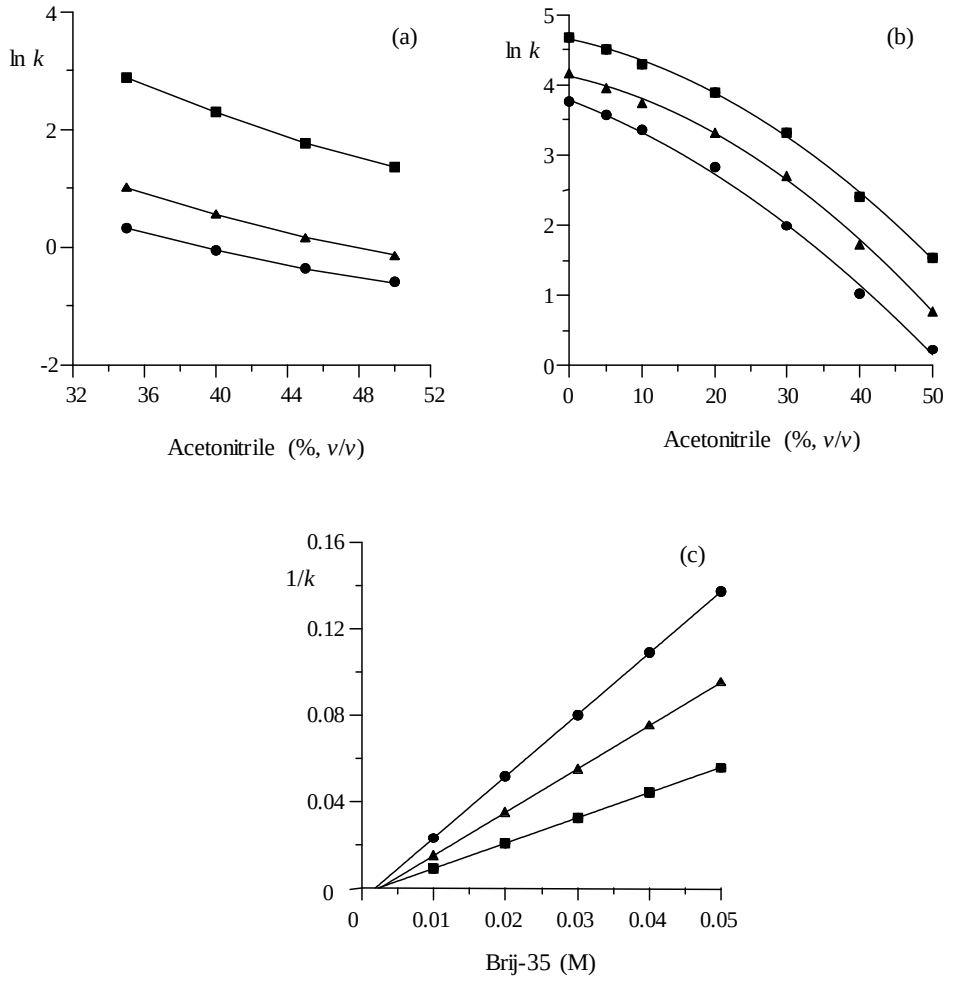


Figure 3.2. Retention plots describing the isocratic elution of the probe compounds: (a) aqueous-organic mixtures in the absence of Brij-35, (b) micellar mobile phases containing 0.01 M Brij-35 and acetonitrile, and (c) purely micellar mobile phases of Brij-35. Compounds: fisetin (●), quercetin (▲), and chrysin (■).

For both sets of compounds, with the first additions of acetonitrile, the elution strength in the presence of Brij-35 was relatively low (i.e., the slope of the curves was small), which could be explained at least partially by the stabilisation of the organic solvent by the micelles in the mobile phase. With further additions of organic solvent, the elution strength was similar to that observed with aqueous-organic mixtures (at least for sulphonamides), due to the excess of organic solvent and micelle disruption. On the other hand, when comparing the chromatographic behaviours achieved with purely micellar mobile phases of 0.01 M Brij-35 and aqueous-organic mixtures, it is observed that the surfactant may yield shorter or longer retention times, depending on the interaction with solutes.

Meanwhile, in MLC, the retention at varying concentration of surfactant ([M]) is better described by the reversed retention factor, which yields a simple linear representation. Following the Cline-Love approach [8,38]:

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}[M] \qquad (3.2)$$

Eq. (3.2) provides information about the strength of the interactions between the solute and the stationary phase or mobile phase; K_{AS} and K_{AM} are the solute-stationary phase and solute-micelle association constants, respectively. Figures 3.1c and 3.2c correspond to the behaviour observed in purely micellar mobile phases of Brij-35. It should be observed that the intercept of the straight-lines ($1/K_{AS}$ in Eq. (3.2)) for sulphaquinoxaline and sulphisoxazole (Figure 3.1c, also for sulphadimethoxine, which is not shown), and for the three flavonoids (Figure 3.2c) is close to zero. This indicates a strong interaction of these compounds with the surfactant monomers associated to the stationary phase (i.e., a high value for K_{AS}). The interaction of the less retained

sulphonamides (sulphamerazine, sulphamethazine and sulphamethoxazole, the two latter not shown) is much weaker. Sulphamerazine and sulphamethazine are more polar, and contain both a pyrimidin-2-yl ring.

Fitting the *k* versus [M] data (and considering the mean molar mass of Brij-35) by non-linear regression (which improves the fittings for the most retained compounds), the following association constants were obtained for sulphamethazine, sulphamerazine, sulphamethoxazole, sulphisoxazole and sulphadimethoxine: K_{AS} = 6.97 ± 0.07, 7.81 ± 0.27, 80.4 ± 1.6, 216 ± 18, and 1165 ± 202, and K_{AM} = 25.7 ± 0.7, 35.7 ± 2.8, 200.2 ± 5.2, 553 ± 54, and 2544 ± 455, respectively (whose magnitude agrees with the elution order). The evaluation of the association constants for sulphaquinoxaline and the three flavonoids was, however, not possible due to the negative intercept in the 1/*k* versus [M] plot, which must be interpreted by the direct transfer between the micelles and the modified stationary phase to which these compounds should be strongly associated [3].

3.4.3. Peak profiles in isocratic elution

The information obtained from chromatograms for different types of compounds has shown that the profile of chromatographic peaks can be described by simple models, based on the left (*A*) and right (*B*) peak half-widths, or the widths for symmetrical peaks [39]. The plots representing the peak half-widths versus the retention time, which have been called half-width plots, give direct information about the changes in the peak width and asymmetry of solutes eluted in a chromatographic column. The models are parabolic:

$$A = a_0 + a_1 t_R + a_2 t_R^2 \quad (3.3)$$

$$B = b_0 + b_1 t_R + b_2 t_R^2 \quad (3.4)$$

although, as will be seen, in sufficiently short ranges they may be approximated to straight-lines.

Figure 3.3 depicts the half-width plots for sulphoquinoxaline and chrysin in isocratic elution, when either the concentrations of Brij-35 or acetonitrile are varied. The half-widths were measured at 10% peak height to estimate better the asymmetry. Similar plots at smaller retention times were obtained for the other sulphonamides and flavonoids. In all cases, the plots show clear trends. For all compounds, the slope of the line that represents the right half-width (*B*) is only slightly larger, indicating that for the studied compounds the peaks were virtually symmetrical for all conditions in the experimental designs using Brij-35. The plots obtained for mobile phases with different concentrations of Brij-35 are convex for sulphonamides (Figure 3.3a) and nearly straight-lines for flavonoids (Figure 3.3c). In contrast, the change in the acetonitrile contents linearises the behaviour for sulphonamides (Figure 3.3b), and yields concave lines for flavonoids (Figure 3.3d). The different shape of the plots for sulphonamides and flavonoids indicate the different kinetic behaviour of both types of compounds with the chromatographic column modified with Brij-35. For flavonoids, the width decrease is larger for the first additions of surfactant and organic solvent (note that the retention is reduced at increasing concentration of both modifiers).

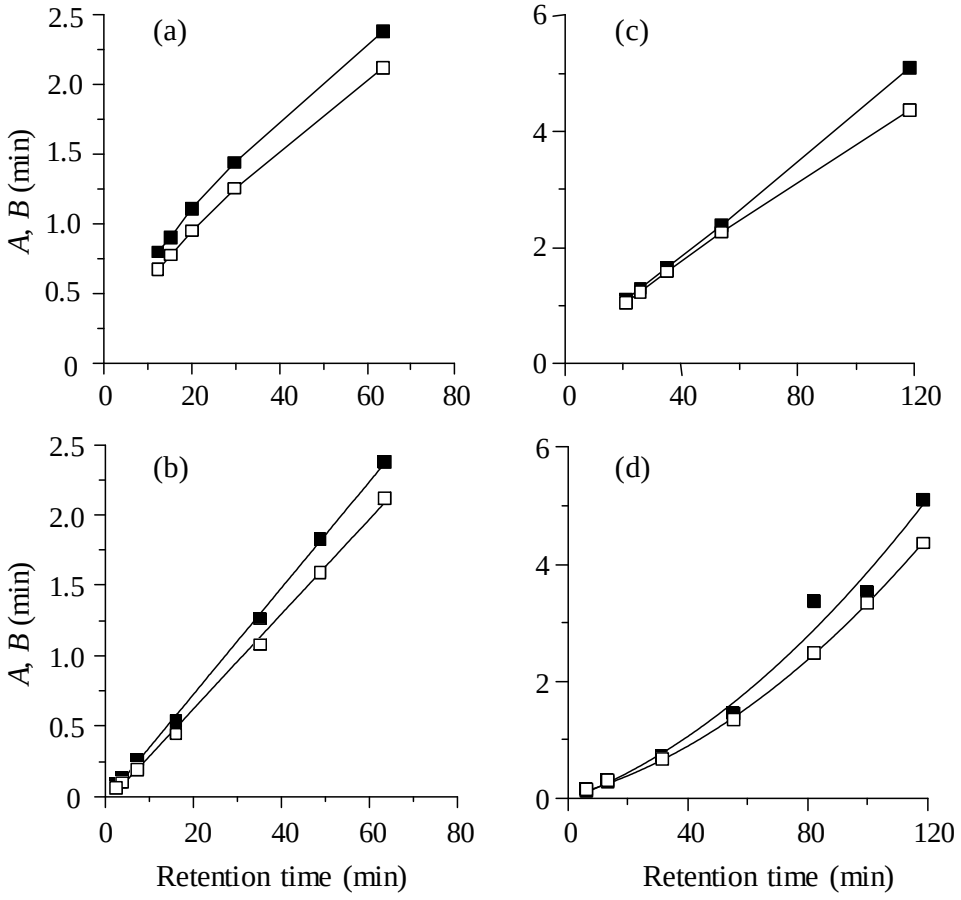


Figure 3.3. Peak half-width plots corresponding to the isocratic elution of sulphaquinoxaline (a,b), and chrysin (c,d), using different concentrations of Brij-35 without organic solvent (a,c), and different concentrations of acetonitrile in the presence of 0.01 M Brij-35 (b,d). The points correspond to: (a,c) 0.01 (highest retention), 0.02, 0.03, 0.04, and 0.05 M (lowest retention) Brij-35, and (b,d) 0 (highest retention), 5, 10, 20, 30, 40 and 50% (*v*/*v*) (lowest retention) acetonitrile. Left half-width (*A*, □), and right half-width (*B*, ■).

3.4.4. Gradient elution

Figures 3.4 and 3.5 show some chromatograms obtained at different conditions using gradient elution with Brij-35 and acetonitrile, after baseline correction. The reduction in the peak width for sulphonamides and flavonoids in gradient elution, with regard to isocratic elution, is noteworthy especially for acetonitrile gradients, either keeping constant or decreasing the concentration of Brij-35 (Figures 3.4c,e and 3.5c,e). Also, when comparing the chromatograms obtained with gradients where the concentration of Brij-35 was increased, the peak width was smaller when the acetonitrile content was simultaneously increased (compare Figures 3.4a,d, and 3.5a,d). At higher temperature the peaks were narrower, which should yield better resolution (compare Figures 3.3a,b and 3.5a,b, corresponding to chromatograms for sulphonamides and flavonoids at 25 ^{o}C and 50 ^{o}C).

According to Jandera and Churácek [40], the peak width by applying gradient elution approximately agrees with the width of a peak in isocratic elution, if the solute were eluted using the gradient composition when its peak maximum reaches the column outlet. Therefore, Eqs. (3.3) and (3.4) are also valid to describe the peak profiles in gradient elution, if the instantaneous retention time at the column outlet were known.

In Figure 3.6, the widths obtained at different times are compared in isocratic and gradient elution, for the two sets of probe compounds.

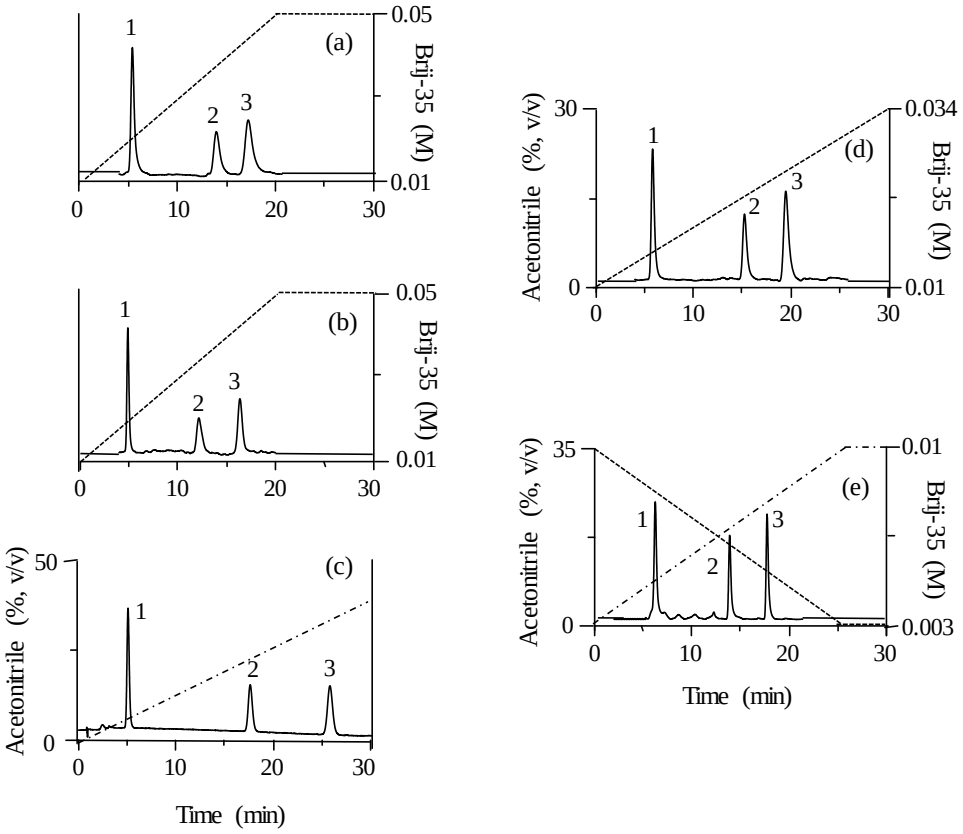


Figure 3.4. Chromatograms obtained for the sulphonamides, applying different types of gradients (concentration range in the gradient, gradient time, and temperature are given). Gradients of Brij-35: (a) 0.01–0.05 M, t_G = 20 min, 25 ºC, and (b) 0.01–0.05 M, t_G = 20 min, 50 ºC. Gradients of acetonitrile or acetonitrile and Brij-35 (25 ºC): (c) 0–40% acetonitrile, t_G = 40 min at fixed Brij-35 (0.01 M), (d) simultaneous increase in acetonitrile (0–30%) and Brij-35 (0.01–0.034 M), t_G = 30 min, and (e) acetonitrile increase (0–35%) and Brij-35 decrease (0.01–0.003 M), t_G = 25 min. The gradient programs correspond to: acetonitrile (-.-) and Brij-35 (---). Compounds: (1) sulphamerazine, (2) sulphisoxazole and (3) sulphaquinoxaline.

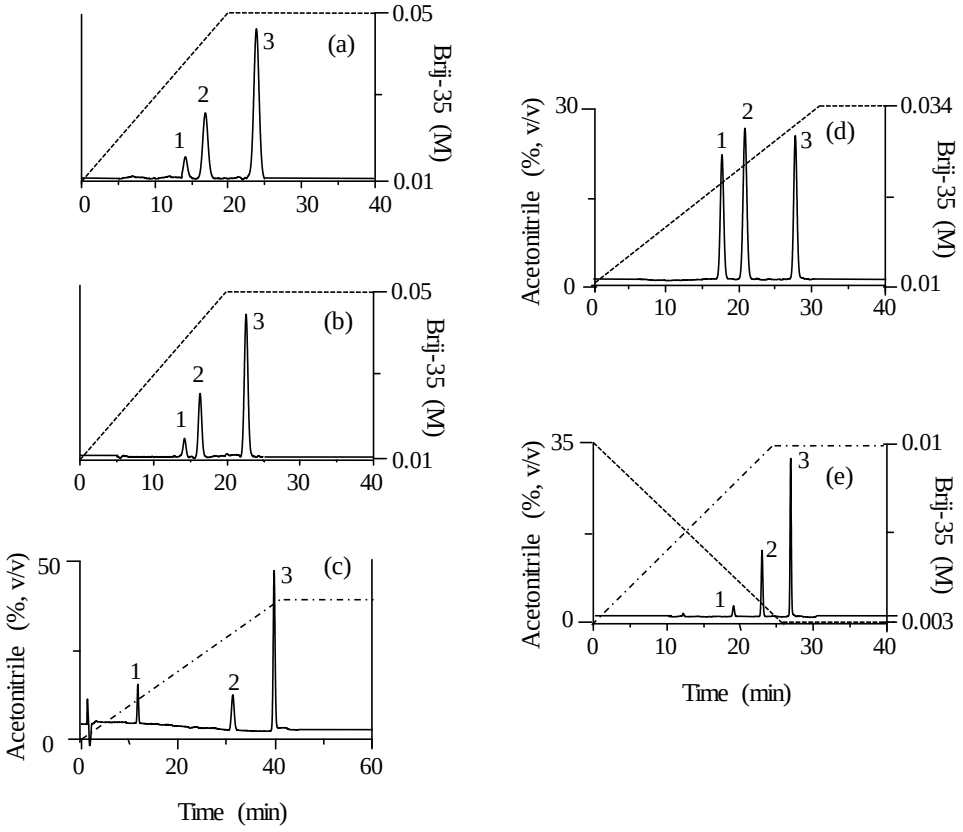


Figure 3.5. Chromatograms obtained for the flavonoids, applying different types of gradients. See Figure 3.4 for details on the gradients. Compounds: (1) fisetin, (2) quercetin, and (3) chrysin.

The plots in Figures 3.6a and d describe the isocratic elution for the six sulphonamides and three flavonoids, respectively, with a mobile phase containing only Brij-35. The lines represent a kind of simplified chromatograms. As observed, the behaviour is perfectly linear for the sulphonamides, while the data for the flavonoids exhibit a significant dispersion. According to a previous study carried out in isocratic elution, this should be explained by differences in the kinetics of interaction of each flavonoid with the stationary phase modified by Brij-35, which depends on the position of the hydroxyl substituents on the benzene rings of these compounds [24]. The almost perfect alignment of the data for the sulphonamides in Figure 3.6a suggests that the interaction rate of the six compounds with the modified stationary phase is similar.

On the other hand, the intercept of the lines in Figure 3.6 is significantly different from zero (especially for flavonoids), indicating that the peaks have a significant width close to the dead time. With an aqueous-organic mobile phase, peaks eluting close to the dead time are much narrower (see lower dashed line in Figure 3.7d, which virtually goes through the origin). The significant width close to the dead time is also observed with SDS in MLC, but it is more remarkable with Brij-35.

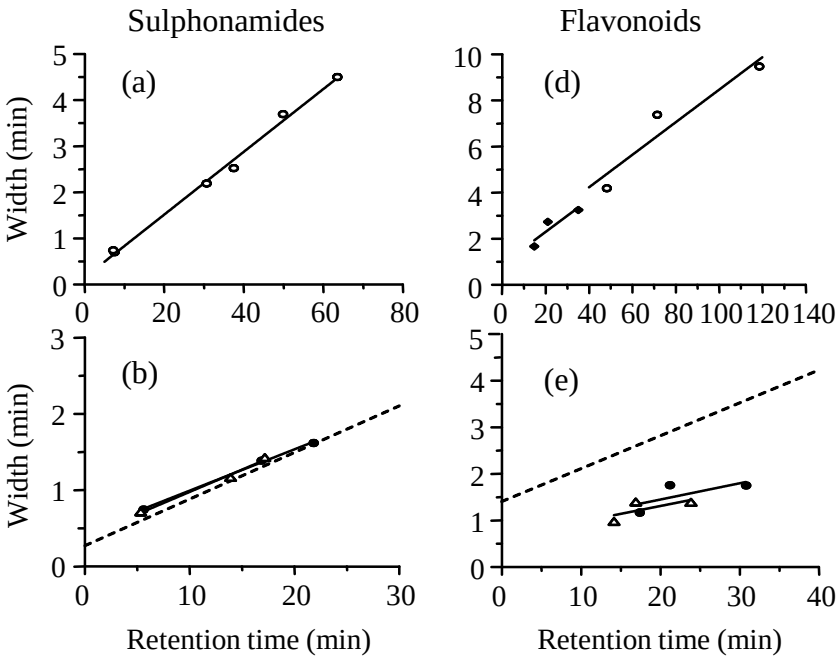


Figure 3.6. Peak width plots corresponding to different gradient programs (the gradient slope, *m*, is given) for: (a,d) isocratic elution (*m* = 0) with 0.01 M (○), and 0.03 M (♦) Brij-35; (b,e) gradient of Brij-35 with *m* = 0.001 (●) and 0.002 (Δ); and (c,f) gradient of acetonitrile with *m* = 0.5 (●), 1.0 (Δ), 1.5 (◊), and 2.0 (+) at fixed Brij-35 (0.01 M). In (b), (c), (e) and (f), the dashed line represents the behaviour in isocratic elution with 0.01 M Brij-35. Compounds: (a) sulphamerazine, sulphametazine, sulphisoxazole, sulphamethoxazole, sulphadimethoxine, and sulphaquinoxaline, (b,c) sulphamerazine, sulphisoxazole, and sulphaquinoxaline, and (d, e and f) fisetin, quercetin and chrysin.

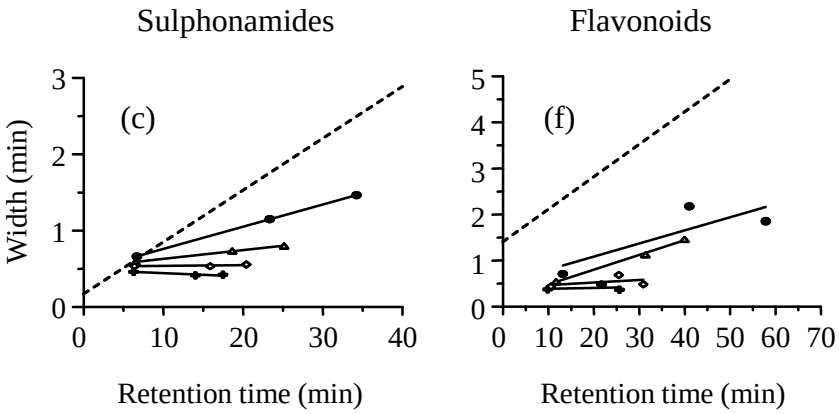


Figure 3.6 (continued).

To evaluate the effect of different gradients, the behaviour obtained with the isocratic mobile phase of 0.01 M Brij-35 (Figures 3.6a and d) has been also depicted as a dashed straight-line in the plots in Figures 3.6b, c, e and f, and Figure 3.7 (in these figures, only the data for sulphamerazine, sulphisoxazole and sulphaquinoxaline are shown, together with those for the three flavonoids). When the sulphonamides were eluted using a gradient of Brij-35 (Figure 3.6b), the peak widths were similar (or slightly larger) than those obtained in isocratic elution with 0.01 M Brij-35, while for flavonoids (Figure 3.6e) the peak widths decreased considerably.

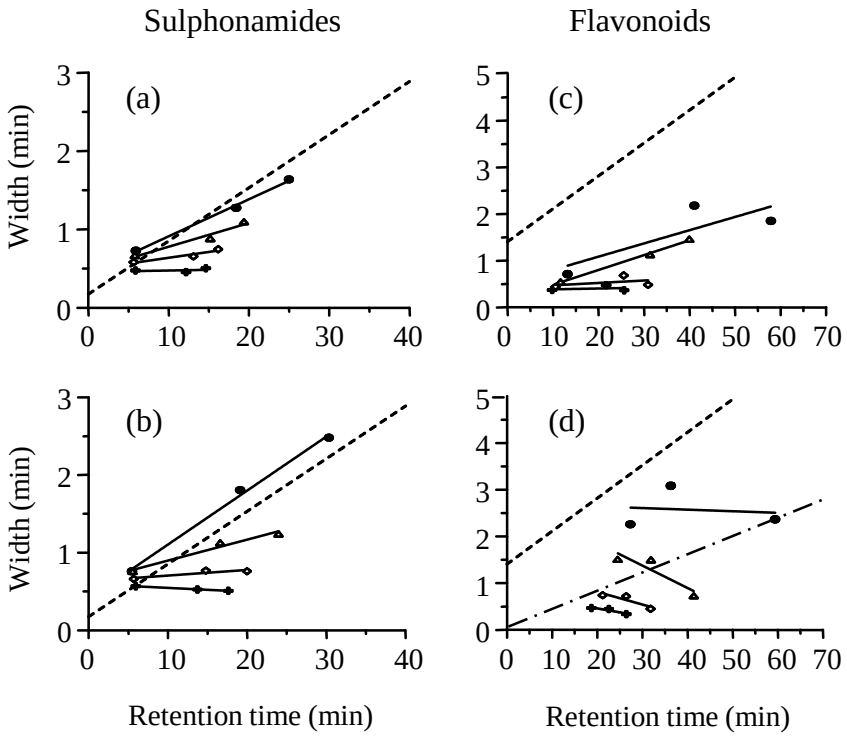


Figure 3.7. Peak width plots for: (a,c) gradients where Brij-35 and acetonitrile are simultaneously increased, and (b,d) gradients where acetonitrile is increased and Brij-35 decreased. Gradient slopes for acetonitrile (a to d): m = 0.5 (●), 1.0 (Δ), 1.5 (◊), and 2.0 (+). Gradient slopes for Brij-35: (a,c) $m = 4\times10^{-4}$ (●), 8×10^{-4} (Δ), 1.2×10^{-3} (◊), and 1.6×10^{-3} (+), and (b,d) $m = -1\times10^{-4}$ (●), -2×10^{-4} (Δ), -3×10^{-4} (◊), and -4×10^{-4} (+). The dashed line represents the behaviour in isocratic elution with 0.01 M Brij-35. In (d), the behaviour in isocratic elution with an aqueous-organic mobile phase of 28% acetonitrile is also represented as (–.–). Compounds: (a,b) sulphamerazine, sulphisoxazole and sulphaquinoxaline, and (c,d) fisetin, quercetin and chrysin.

On the other hand, by applying a gradient of acetonitrile (Figures 3.6c and f), keeping constant the concentration of surfactant (0.01 M), the peak widths decreased significantly, and to a greater extent as the gradient slope increased. Similar behaviour was obtained for the simultaneous gradients of Brij-35 and acetonitrile (Figure 3.7). Again, sulphonamides yielded a perfect linear behaviour, while significant data dispersion was observed for flavonoids. Note, finally, the large effect of the gradient slope on the peak profile of flavonoids with gradients decreasing Brij-35 and increasing acetonitrile (Figure 3.7d).

3.5. Conclusions

This work had the global aim of exploring the capability of Brij-35 as modifier in MLC using both isocratic and gradient elution, with acetonitrile as organic solvent. To observe the behaviour of a chromatographic system using mobile phases of Brij-35, in terms of retention and peak profiles, several plots were drawn for two families of compounds showing significant retention, and different polarity range and structure. Two interesting strategies are envisioned to be used in MLC with Brij-35: (i) the work with eluents containing exclusively Brij-35 in isocratic and gradient elution, which represents a kind of "green chromatography" (chromatography with water and detergent), which obviously is only interesting for compounds and mobile phases showing sufficiently short retention times, and (ii) the work with gradients of acetonitrile at fixed concentration of Brij-35. It should be noted that using SDS instead of Brij-35, only highly polar compounds can be eluted with purely micellar mobile phases at practical times [41]. The range of compounds that can be eluted with purely micellar mobile phases is increased with Brij-35. In these conditions, the peak widths can be significantly reduced at high temperature, enhancing the chromatographic performance [24].

With regard to the second strategy (the use of gradients of acetonitrile, fixing the concentration of Brij-35), the most outstanding feature is the significant reduction in the peak width with increasing concentration of acetonitrile. The high efficiencies will guarantee high resolution, while the presence of Brij-35 offers particular selectivity and also affects the global retention. This strategy is especially interesting for the analysis of drugs in physiological fluids by direct injection of the sample. In this case, a purely micellar medium of Brij-35 can be used initially, and once the proteins are eluted from the sample, a gradient of acetonitrile would be applied to increase the elution strength, achieving short retention times for all analytes [14,42,43]. This work has the aim to encourage other authors to develop new applications with the non-ionic surfactant. The ability to model the observed behaviours can greatly facilitate the development of these methodologies.

3.6. References

[1] A. Berthod, M.C. García Álvarez-Coque, *Micellar Liquid Chromatography*, Marcel Dekker, New York, 2000.

[2] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, New insights and recent developments in micellar liquid chromatography, *Sep. Purif. Rev.* 38 (2009) 45–96.

[3] M.F. Borgerding, F.H. Quina, W.L. Hinze, J. Bowermaster, H.M. McNair, Investigation of the retention mechanism in nonionic micellar liquid chromatography using an alkylbenzene homologous series, *Anal. Chem.* 60 (1988) 2520−2527.

[4] B.K. Lavine, S. Hendayana, J. Tetreault, Selectivity in micellar reversed-phase liquid chromatography: C-18 and C-8 alkyl bonded phases, *J. Anal. Chem.* 66 (1994) 3458–3465.

[5] R.D. Caballero, S. Carda Broch, M.C. García Álvarez-Coque, Hydro-organic and micellar-organic reversed-phase liquid chromatographic procedures for the evaluation of sulphonamides in pharmaceuticals, *Anal. Lett.* 34 (2001) 1189–1203.

[6] M.J. Ruiz Ángel, S. Carda Broch, J.R. Torres Lapasió, E.F. Simó Alfonso, M.C. García Álvarez-Coque, Micellar-organic versus aqueous-organic mobile phases for the screening of β-blockers, *Anal. Chim. Acta* 454 (2002) 109–123.

[7] M.C. García Álvarez-Coque, S. Carda Broch, Direct injection of physiological fluids in micellar liquid chromatography, *J. Chromatogr. B* 736 (1999) 1–18.

[8] M.C. García Álvarez-Coque, J.R. Torres Lapasió, J.J. Baeza Baeza, Modelling of retention behaviour of solutes in micellar liquid chromatography, *J. Chromatogr. A* 780 (1997) 129–148.

[9] M.J. Ruiz Ángel, R.D. Caballero, E. Simó Alfonso, M.C. García Álvarez-Coque, Micellar liquid chromatography: Suitable technique for screening analysis, *J. Chromatogr. A* 947 (2002) 31–45.

[10] J.S. Landy, J.G. Dorsey, Rapid gradient capabilities of micellar liquid chromatography, *J. Chromatogr. Sci.* 22 (1984) 68–70.

[11] J.G. Dorsey, M.G. Khaledi, J.S. Landy, J.L. Lin, Gradient elution micellar liquid chromatography, *J. Chromatogr. A* 316 (1984) 183–191.

[12] L.S. Madamba-Tan, J.K. Strasters, M.G. Khaledi, Gradient elution in micellar liquid chromatography: I. Micelle concentration gradient, *J. Chromatogr. A* 683 (1994) 321–334.

[13] L.S. Madamba, J.K. Strasters, M.G. Khaledi, Gradient elution in micellar liquid chromatography: II. Organic modifier gradients, *J. Chromatogr. A* 683 (1994) 335–345.

[14] J. Rodenas Montano, C. Ortiz Bolsico, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Implementation of gradients of organic solvent in micellar liquid chromatography using DryLab®: Separation of basic compounds in urine samples, *J. Chromatogr. A* 1344 (2014) 31–41.

[15] M.F. Borgerding, W.L. Hinze, Characterization and evaluation of the use of nonionic polyoxyethylene(23)dodecanol micellar mobile phases in reversed-phase high-performance liquid chromatography, *Anal. Chem.* 57 (1985) 2183–2190.

[16] M.F. Borgerding, W.L. Hinze, L.D. Stafford, G.W. Fulp Jr., W.C. Hamlin Jr., Investigations of stationary phase modification by the mobile phase surfactant in micellar liquid chromatography, *Anal. Chem.* 61 (1989) 1353–1358.

[17] L.J. Cline Love, J.J. Fett, Optimization of selectivity in micellar chromatographic procedures for the determination of drugs in urine by direct injection, *J. Pharm. Biomed. Anal.* 9 (1991) 323–333.

[18] M.L. Marina, O. Jiménez, M.A. García, S. Vera, Study of the separation selectivity of a group of benzene and naphthalene derivatives in micellar liquid chromatography, *Microchem. J.* 53 (1996) 215−224.

[19] P. Menéndez Fraga, A. Blanco González, A. Sanz Medel, J.B. Cannata Andia, Micellar versus reversed phase liquid chromatography for the determination of desferrioxamine and its chelates with aluminium and iron in uremic serum, *Talanta* 45 (1997) 25−33.

[20] M. Gil Agustí, L. Álvarez Rodríguez, L. Monferrer Pons, D. Bose, A. Durgbanshi, J. Esteve Romero, Chromatographic determination of carbaryl and other carbamates in formulations and water using Brij-35, *Anal. Lett.* 35 (2002) 1721−1734.

[21] N. Memon, M. Iqbal Bhanger, M.Y. Khuhawer, Determination of preservatives in cosmetics and food samples by micellar liquid chromatography, *J. Sep. Sci.* 28 (2005) 635−638.

[22] A. Berthod, S. Tomer, J.G. Dorsey, Polyoxyethylene alkyl ether nonionic surfactants: Physicochemical properties and use for cholesterol determination in food, *Talanta* 55 (2001) 69−83.

[23] J.J. Fernández Navarro, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography without organic solvent for determination of tricyclic antidepressants, *J. Sep. Sci.* 35 (2012) 1303–1309.

[24] J.J. Baeza Baeza, Y. Dávila, J.J. Fernández Navarro, M.C. García Álvarez-Coque, Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC, *Anal. Bioanal. Chem.* 404 (2012) 2973–2984.

[25] T.A Biemer, Simultaneous analysis of acetaminophen, pseudoephedrine hydrochloride and chlorpheniramine maleate in a cold tablet using an isocratic, mixed micellar high-performance liquid chromatographic mobile phase, *J. Chromatogr. A* 410 (1987) 206−210.

[26] M.R. Hadjmohammadi, P. Ebrahimi, Optimization of the separation of anticonvulsant agents in mixed micellar liquid chromatography by experimental design and regression models, *Anal. Chim. Acta* 516 (2004) 141−148.

[27] P. Ebrahimi, M.R. Hadjmohammadi, Simultaneous optimization of resolution and analysis time in mixed micellar liquid chromatography of coumarins by use of a utility function, *Anal. Bioanal. Chem.* 384 (2006) 851−858.

[28] J. Sun, J. Mao, X. Liu, Y. Wang, Y. Sun, Z. He, Separation and mechanism elucidation for six structure-like Matrine-type alkaloids by micellar liquid chromatography, *J. Sep. Sci.* 32 (2009) 2043−2050.

[29] L. Escuder Gilabert, S. Sagrado, R.M. Villanueva Camañas, M.J. Medina Hernández, Quantitative retention-structure and retention-activity relationship studies of local anesthetics by micellar liquid chromatography, *Anal. Chem.* 70 (1998) 28−34.

[30] M. Molero Monfort, Y. Martín, S. Sagrado, R.M. Villanueva Camañas, M.J. Medina Hernández, Micellar liquid chromatography for prediction of drug transport, *J. Chromatogr. A* 870 (2000) 1−11.

[31] S. Wang, G. Yang, H. Zhang, H. Liu, Z. Li, QRAR models for cardiovascular system drugs using biopartitioning micellar chromatography, *J. Chromatogr. B* 846 (2007) 329–333.

[32] Y. Chen, L.P. Wu, C. Chen, L.M. Ye, Development of predictive quantitative retention-activity relationship models of alkaloids by mixed micellar liquid chromatography, *Biomed. Chromatogr.* 24 (2010) 195–201.

[33] C.R. Yin, L.Y. Ma, J.G. Huang, L. Xu, Z.G. Shi, Fast profiling ecotoxicity and skin permeability of benzophenone ultraviolet filters using biopartitioning micellar chromatography based on penetrable silica spheres, *Anal. Chim. Acta* 804 (2013) 321–327.

[34] J.R. Torres-Lapasió, *MICHROM Software*, Marcel Dekker, New York, 2000.

[35] G. Tóth, A. Madarász, Structure of Brij-35 nonionic surfactant in water: A reverse Monte Carlo study, *Langmuir* 22 (2006) 590–597.

[36] W. Thogchai, B. Liawruangrath, Micellar liquid chromatographic determination of arbutin and hydroquinone in medicinal plant extracts and commercial cosmetic products, *Int. J. Cosmetic Sci.* 35 (2013) 257–263.

[37] M.J. Ruiz Ángel, J.R. Torres Lapasió, S. Carda Broch, M.C. García Álvarez-Coque, Performance of short-chain alcohols versus acetonitrile in the surfactant-mediated reversed-phase liquid chromatographic separation of β-blockers, *J. Chromatogr. A* 1217 (2010) 7090–7099.

[38] M. Arunyanart, L.J. Cline-Love, Model for micellar effects on liquid chromatography capacity factors and for determination of micelle-solute equilibrium constants, *Anal. Chem.* 56 (1984) 1557–1561.

[39] J.J. Baeza Baeza, M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

[40] P. Jandera, J. Churácek, Gradient elution in liquid chromatography: II. Retention characteristics (retention volume, band width, resolution, plate number) in solvent-programmed chromatography-theoretical considerations, *J. Chromatogr. A* 91 (1974) 223–235.

[41] S. Carda Broch, J.S. Esteve Romero, M.C. García Álvarez-Coque, Liquid chromatographic determination of some thiazide diuretics in pharmaceuticals with a sodium dodecyl sulfate mobile phase, *Analyst* 123 (1998) 301–306.

[42] R. Nakao, C. Halldin, Mixed anionic and non-ionic micellar liquid chromatography for high-speed radiometabolite analysis of positron emission tomography radioligands, *J. Chromatogr. A* 1281 (2013) 54–59.

[43] R. Nakao, M. Schou, C. Halldin, Direct plasma metabolite analysis of positron emission tomography radioligands by micellar liquid chromatography with radiometric detection, *Anal. Chem.* 84 (2012) 3222–3230.

CHAPTER 4

EFFECT OF SODIUM DODECYL SULPHATE AND BRIJ-35 ON THE ANALYSIS OF SULPHONAMIDES IN PHYSIOLOGICAL SAMPLES USING DIRECT INJECTION AND ACETONITRILE GRADIENTS

4.1. Abstract

Micellar liquid chromatography (MLC) is a reversed-phase (RP) mode, which often does not require gradient elution, since the peaks of mixtures of compounds within a large range of polarities appear more evenly distributed in the chromatograms of isocratic elution, giving rise to a “gradient effect”. However, the use of a gradient of organic solvent may still be convenient to shorten the total analysis time. This work compares the separation of 15 sulphonamides using conventional hydro-organic RPLC and MLC with the surfactants sodium dodecyl sulphate (SDS) and Brij-35, and both isocratic and gradient elution with acetonitrile. The observed behaviour is rationalised attending to the interactions in the different environments formed inside the chromatographic column, in the absence and presence of the two surfactants. The retention of alkylbenzenes is used to evaluate the relative polarity of the stationary phase under hydro-organic and micellar conditions. The most favourable gradients in MLC were used to analyse the sulphonamides in milk and urine samples, directly injected into the chromatograph. The addition of an initial step of a micellar mobile phase containing only SDS or Brij-35 facilitated the direct injection of the sample, which was followed by a gradient of acetonitrile to elute the most retained compounds.

4.2. Introduction

In reversed-phase liquid chromatography (RPLC), the addition of a surfactant to the hydro-organic mixtures modifies the alkyl-bonded stationary phase by adsorption of surfactant monomers. Above the critical micelle concentration (CMC), the monomers also aggregate in the mobile phase to form small clusters or micelles able to interact with the analytes [1–3]. The effect of the surfactant on the elution strength is larger for hydrophobic compounds, which are more easily desorbed from the stationary phase transported by the micelles into the mobile phase [4]. In contrast, polar compounds are more strongly retained with regard to classical hydro-organic RPLC. This means that more compounds per time unit are eluted in MLC, using isocratic conditions. As a result, the peaks appear more evenly distributed in the chromatograms [5], the effect being equivalent to the implementation of a gradient of organic solvent in hydro-organic RPLC. This behaviour, known as the "gradient effect", is the main reason that explains that almost all reported MLC procedures have been developed under isocratic conditions. However, gradient elution can be useful in MLC to expedite some analyses or improve the separation capability, as will be shown in this work.

Only few studies concerning gradient elution have been reported in MLC [6–14]. Most reports apply gradients of organic solvent (acetonitrile, butanol or pentanol), and keep the concentration of the surfactant (always the anionic sodium dodecyl sulphate, SDS) constant. Only Bryant and Altria used simultaneous gradients of SDS and organic solvent (pentanol) [10]. The separation capability of gradient elution in the presence of surfactant has been recently enhanced using submicellar RPLC at a high concentration of organic solvent [12,14]. Under these conditions, the concentration of organic solvent reached during the gradient is so high that micelles breakdown [15].

However, the combination of micellar and submicellar conditions allows the direct injection of physiological samples into the column in the presence of micelles and a very small concentration of organic solvent, or even, without organic solvent. Once the proteins are eluted with the solvent front, the concentration of organic solvent may be rapidly increased to elute the most retained compounds under submicellar conditions [12,14].

The use of the non-ionic surfactant polyoxyethylene(23)lauryl ether, commercially known as Brij-35, has been explored in MLC by a few authors, as an alternative to SDS with satisfactory results in the analysis of diverse compounds [16–20]. Brij-35 has been also reported as the ideal surfactant in quantitative structure-activity relationship (QSAR) studies, due to its capability to mimic biopartitioning processes [21]. More recently, its relevance in the analysis of basic compounds and flavonoids has been demonstrated [22–24]. The higher polarity of the stationary phase modified with Brij-35 decreases significantly the retention times in isocratic elution. Therefore, usually only a low concentration of organic solvent (or no organic solvent) is needed to obtain sufficiently small retention times. However, the use of gradient elution in MLC with Brij-35 has been scarcely investigated [25].

In this work, we explore the separation capability of MLC with SDS and Brij-35 under gradient conditions, for the screening of sulphonamides. The elution of a group of 15 sulphonamides was studied using a fixed amount of surfactant and a linear acetonitrile gradient. The procedure was applied to the analysis of milk and urine samples, which were directly injected into the chromatograph after only filtration and dilution. For comparison purposes, acetonitrile gradients without surfactant were also run.

4.3. Experimental

4.3.1. Reagents and columns

The following sulphonamides were analysed (see Table 4.1): (1) sulphaguanidine, (2) sulphanilamide, (3) sulphacetamide, (4) sulphadiazine, (5) sulphathiazole, (6) sulphapyridine, (7) sulphamerazine, (8) sulphamethazine, (9) sulphamethizole, (10) sulphamonomethoxine, (11) sulphachloropyridazine, (12) sulphamethoxazole, (13) sulphisoxazole, (14) sulphadimethoxine, and (15) sulphaquinoxaline, all from Sigma (St. Louis, MO, USA). Also, four alkylbenzenes (ethylbenzene, propylbenzene, butylbenzene and amylbenzene) from Sigma were used to evaluate the relative polarity of the stationary phase under hydro-organic and micellar conditions. All compounds were dissolved in a small amount of acetonitrile (Scharlau, Sentmenat, Barcelona, Spain), with the aid of an ultrasonic bath (Elmasonic, Singen, Germany), and diluted with water. The solutions were stable at 4 ºC during at least two months. The concentration of the injected solutions was *ca.* 20 mg/L for isocratic elution, and 6 mg/L for gradient elution. Urine samples were collected from healthy human volunteers who consented to the use of their urine in this study, whereas milk was purchased from a local supermarket.

The mobile phases contained sodium dodecyl sulphate from Merck (99% purity, Darmstadt, Germany) or Brij-35 (Sigma), and acetonitrile. The pH was buffered at 3.0 with 0.01 M anhydrous NaH_2PO_4 (Fluka, Steinheim, Germany) and HCl (Scharlau). All experiments were performed with nanopure water, obtained with a Barnstead ultrapure water purification system from Thermo Scientific (Dubuque, IA, USA). The drug solutions and mobile phases were filtered through 0.45 µm Nylon membranes from Micron Separations (Westboro, MA, USA).

Table 4.1. Structures, dissociation constants (pK_a) and octanol-water partition coefficients (log $P_{o/w}$) of the studied sulphonamides.

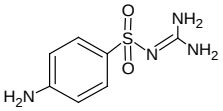
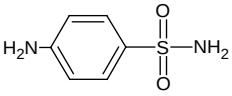
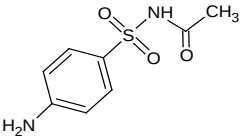
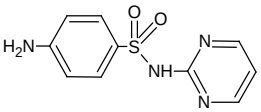
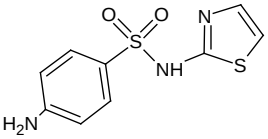
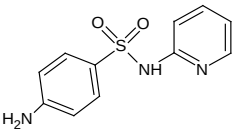
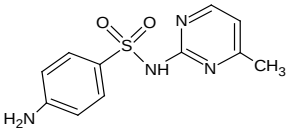
Compound	Structure	pK_a	log $P_{o/w}$
Sulphaguanidine		2.8, 12.1[a]	–1.07[a]
Sulphanilamide		2.4, 10.4[a]	–0.77[a]
Sulphacetamide		1.8, 6.1[a]	–0.19[a]
Sulphadiazine		2.0, 6.4[a]	–0.06[a]
Sulphathiazole		2.1, 7.1[a]	–0.04[a]
Sulphapyridine		2.4, 8.2[b]	0.03[a]
Sulphamerazine		2.2, 7.0[a]	0.11[a]

Table 4.1 (continued).

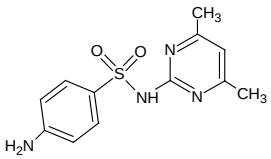
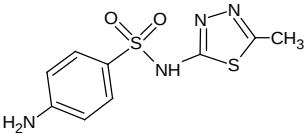
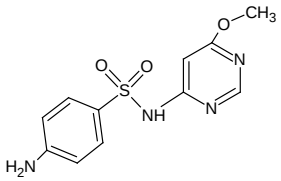
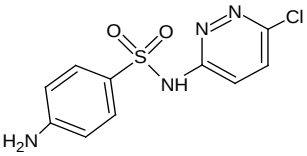
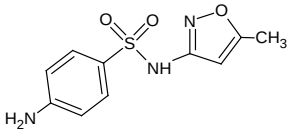
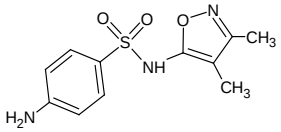
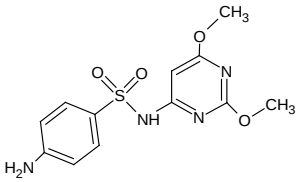
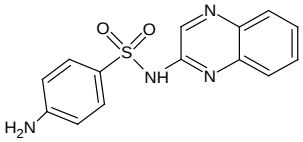
Compound	Structure	pK_a	log $P_{o/w}$
Sulphamethazine		2.4, 7.4[a]	0.27[a]
Sulphamethizole		2.0, 5.4[a]	0.47[a]
Sulphamonomethoxine		NA	0.74[c]
Sulphachloropyridazine		1.9, 5.1[a]	0.71[a]
Sulphamethoxazole		1.7, 5.6[a]	0.85[a]
Sulphisoxazole		1.8, 5.0[b]	0.81[a]

Table 4.1 (continued).

Compound	Structure	pK_a	log $P_{o/w}$
Sulphadimethoxine		1.8, 6.2[a]	1.66[a]
Sulphaquinoxaline		5.5[a]	1.45[a]

[a] From Ref. [26]. [b] From Ref. [27]. [c] From http://www.chemicalize.org/.
NA: Not available.

Two analytical Kromasil C18 columns (150 mm × 4.6 mm i.d., 5 µm particle size) from Scharlau and Teknokroma (Barcelona, Spain) were used, connected to a similar 30 mm guard column. Brij-35 was observed to show an irreversible adsorption. Therefore, it needed a dedicated column. The flow rate was fixed at 1 mL/min. Duplicate injections were made using a volume of 20 µL.

4.3.2. Apparatus and software

An Agilent (Waldbronn, Germany) instrument was used, which was equipped with the following modules: a quaternary pump (HP 1200), an autosampler (HP 1100) with 2 mL vials, and a UV-visible detector (HP 1100)

set at 254 nm. Temperature was controlled at 25 ºC with a thermostated column compartment (HP 1100). The maximal operating pump pressure was 400 bar. The system was controlled with an OpenLAB CDS LC ChemStation (Agilent B.04.03). The chromatographic peaks were processed with the MICHROM software to obtain the peak parameters (retention time and peak half-widths) [28].

The dwell time (t_D) was determined from the midpoint of the signal obtained with a 0–100% linear gradient, formed by mixing water and 0.1% acetone. For this purpose, the column was removed from the system and the injector connected directly to the detector. The mean value from several runs was $t_D = 0.32$ min. The dead time (t_0) obtained from the peak of uracil was $t_0 = 1.39$ min for hydro-organic RPLC, 1.52 min for MLC with SDS, and 1.46 min for MLC with Brij-35.

4.3.3. Experimental designs

Five isocratic MLC mobile phases containing 0.05–0.075 M SDS and 2–5% (*v*/*v*) acetonitrile, and other five containing 0.01–0.02 M Brij-35 and 5–8% acetonitrile, were assayed to model the retention behaviour. The molar concentration of Brij-35 was calculated based on the surfactant mean molecular weight. All mobile phases contained 0.01 M NaH_2PO_4 at pH 3.0. For comparison purposes, the sulphonamides were also analysed with three hydro-organic mobile phases in the 15–20% acetonitrile range, without surfactant. Minimal and maximal concentrations of the surfactants and acetonitrile in the mobile phase were selected to get enough retention for the most polar sulphonamides and not excessive retention for the less polar.

MLC analysis of sulphonamides was also carried out using gradients of organic solvent and a fixed concentration of the surfactants. Solvent A

contained 0.05 M SDS or 0.01 M Brij-35, and solvent B, 50% acetonitrile and 0.05 M SDS or 0.01 M Brij-35. Both solvents also contained 0.01 M NaH_2PO_4. Runs were also made in the absence of surfactant, where solvent A was an aqueous solution of 0.01 M NaH_2PO_4 and solvent B contained 100% acetonitrile. The assayed gradients are indicated in Table 4.2.

Table 4.2. Assayed experimental conditions in gradient elution.[a]

Reservoir A	Reservoir B	A_i	B_i	A_f	B_f	t_G
0.01 M sodium dihydrogen phosphate	100% acetonitrile	100	0	80	20	40
				70	30	20
				70	30	30
				60	40	30
0.05 M SDS 0.01 M sodium dihydrogen phosphate	0.05 M SDS 50% acetonitrile 0.01 M sodium dihydrogen phosphate	100	0	80	20	20
				60	40	20
				80	20	30
				60	40	30
0.01 M Brij-35 0.01 M sodium dihydrogen phosphate	0.01 M Brij-35 50% acetonitrile 0.01 M sodium dihydrogen phosphate	100	0	60	40	30
				60	40	40
				40	60	30
				80	20	30

[a] A_i and B_i are the initial percentages (%) in solutions A and B, and A_f and B_f are the final percentages (%); t_G is the gradient time (min).

4.3.4. Analysis of physiological samples

Aliquots of 1 mL milk or urine samples were fortified with the 15 sulphonamides, and diluted to 25 mL with solutions containing 0.05 M SDS or 0.01 M Brij-35, and 0.01 M NaH_2PO_4 at pH 3.0. The 1:25 diluted samples were filtered directly into the autosampler vials to be analysed. The concentration of sulphonamides in the injected solution was 6 mg/L. The injection of undiluted physiological samples allows reaching lower limits of detection. However, dilution of the samples, if possible, protects the column for a longer time.

4.4. Results and discussion

4.4.1. Isocratic separation of sulphonamides

The experimental conditions yielding maximal resolution in the isocratic analysis of sulphonamides were first obtained, assisted by a new version of the MICHROM optimisation software [28], which considers both conventional RPLC and MLC modes. For this purpose, experimental designs of three and five mobile phases of different compositions were used for the hydro-organic and MLC modes, respectively (see Section 4.3.3). Optimal resolution in the hydro-organic mode was reached at 15.2% (*v*/*v*) acetonitrile. This concentration was decreased in the micellar mode to 3.5% and 5%, in the presence of SDS and Brij-35, respectively. The corresponding chromatograms are shown in Figure 4.1.

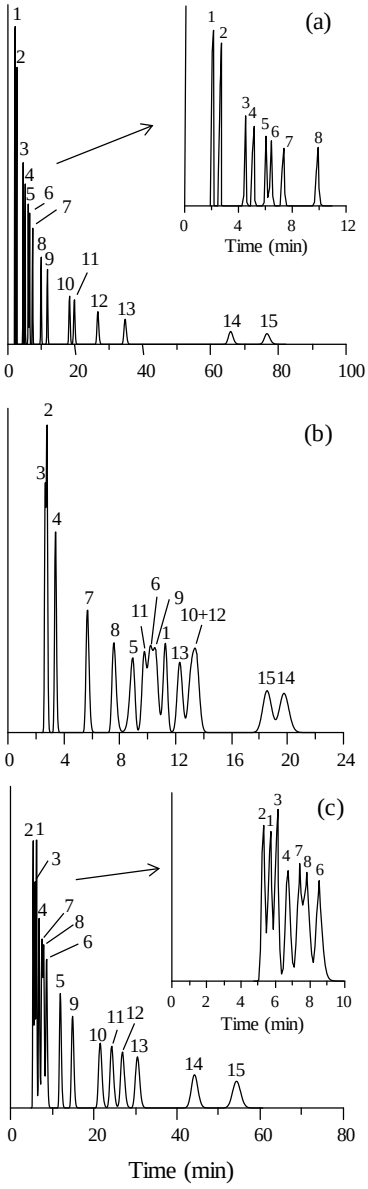


Figure 4.1. Chromatograms of an aqueous mixture of the group of sulphonamides eluted isocratically: (a) 15.2% (v/v) acetonitrile, (b) 0.065 M SDS/3.5% acetonitrile, and (c) 0.012 M Brij-35/5% acetonitrile. For peak identity, see Section 4.3.1.

The best resolution in the hydro-organic mode was associated to a large analysis time of approximately 80 min, with only a small overlapping between sulphathiazole (peak 5) and sulphapyridine (peak 6) (Figure 4.1a). The analysis time decreased with a larger concentration of acetonitrile, but with significant overlapping for the less retained compounds. In the presence of SDS (Figure 4.1b), the analysis time was significantly shorter, with peaks more evenly distributed in the chromatogram. However, the resolution was poor. In the presence of Brij-35 (Figure 4.1c), the elution pattern was somehow similar to that obtained in the hydro-organic mode. The retention was longer compared to the mobile phases with SDS, but significant overlapping was observed for the sulphonamides eluted below 10 min.

Consequently, the selectivity and elution strength of sulphonamides were different for the hydro-organic mode and micellar mode with the two surfactants, especially in the presence of SDS. However, the performance was not satisfactory in either mode. It was evident that the separation of the mixture of 15 sulphonamides required the use of gradient elution to allow the resolution of the early eluting compounds and the reduction of the analysis time.

4.4.2. Interactions with the unmodified and modified stationary phases

Before addressing the implementation of gradients, it is interesting to rationalise the interactions that take place in the different environments formed inside the C18 column, using RPLC in the absence and presence of the two surfactants. To understand the retention behaviour of sulphonamides, we will discuss first the behaviour of protonated basic compounds (which besides hydrophobic interactions, experience electrostatic attraction to the stationary phase), and a homologous series (which experiences only hydrophobic interactions).

4.4.2.1. Electrostatic attraction and formation of hydrogen bonds

SDS and Brij-35 are significantly adsorbed on an alkyl-bonded stationary phase, creating a bilayer [1]. The C12 chain in SDS associates to the C18 chain bonded to the stationary phase, with the sulphate group oriented to the mobile phase [29]. This yields a negatively charged stationary phase. Brij-35 is also adsorbed by association of its C12 chain to the C18 column, with its long hydrophilic polar end oriented away from the stationary phase surface. This increases the stationary phase polarity, which remains neutral.

The chromatographic behaviour in RPLC is governed by the differences in polarity among the stationary phase, mobile phase and analytes, although other types of interactions may be dominant [30]. The behaviour of protonated basic compounds, which are positively charged, has been extensively studied in MLC and is useful to understand the behaviour of some sulphonamides. The cationic species of basic compounds are electrostatically attracted to the SDS modified stationary phases. This increases the retention and forces the addition of a relatively high concentration of organic solvent to the mobile phase to elute moderately polar and apolar compounds at convenient times. The elution of the cationic species may require a strong eluent, such as butanol or pentanol. With hydro-organic mobile phases, the attraction of cationic basic compounds to the anionic silanols also increases the retention, although in a smaller extent.

With micellar mobile phases containing the neutral Brij-35, the polar interaction of the basic compounds with the modified stationary phase is the main effect, although additional ion-exchange interactions with free silanols on the packing may also happen if the stationary phase is not sufficiently covered with the surfactant. Besides this, the polar group of Brij-35 may establish a strong interaction with some solutes, such as flavonoids, by the formation of hydrogen bonds [23].

In MLC with Brij-35, in the absence of particular interactions, the retention is often weaker with respect to MLC with SDS and the hydro-organic mode. Therefore, a smaller concentration of organic solvent is required. The elution of some basic compounds may be carried out even without the need of organic solvent (i.e., using a mobile phase containing only Brij-35). The separation of tricyclic antidepressants is a typical example of this behaviour [22]. These compounds have a strong basic character and octanol-water partition coefficients in the range log $P_{o/w}$ = 3.9–5.3. Their elution with SDS requires thus a strong solvent, such as pentanol, due to their low polarity and the attraction of the cationic protonated species to the anionic stationary phase covered by SDS. In contrast, with pure micellar mobile phases of Brij-35 (without an organic solvent), the analysis time is below 30 min. Note that more polar basic compounds, such as β-blockers with log $P_{o/w} < 2$ are not retained with Brij-35 [25]. All these behaviours can be explained, at least partially, by the lower polarity of the stationary phase modified with Brij-35. However, with the cationic compounds, the main difference in retention between the SDS and Brij-35 modified stationary phases is explained by the electrostatic interaction with the adsorbed monomers of SDS, which does not exist with Brij-35.

4.4.2.2. Stationary phase polarity

The comparison of the retention behaviour of the homologous series of neutral alkylbenzenes, which only experience hydrophobic interactions, is useful to evaluate the relative polarity of the modified stationary phases. For this purpose, ethylbenzene, propylbenzene, butylbenzene and amylbenzene were eluted under isocratic conditions with hydro-organic and hybrid micellar mobile phases containing SDS or Brij-35, and acetonitrile (Fig. 4.2).

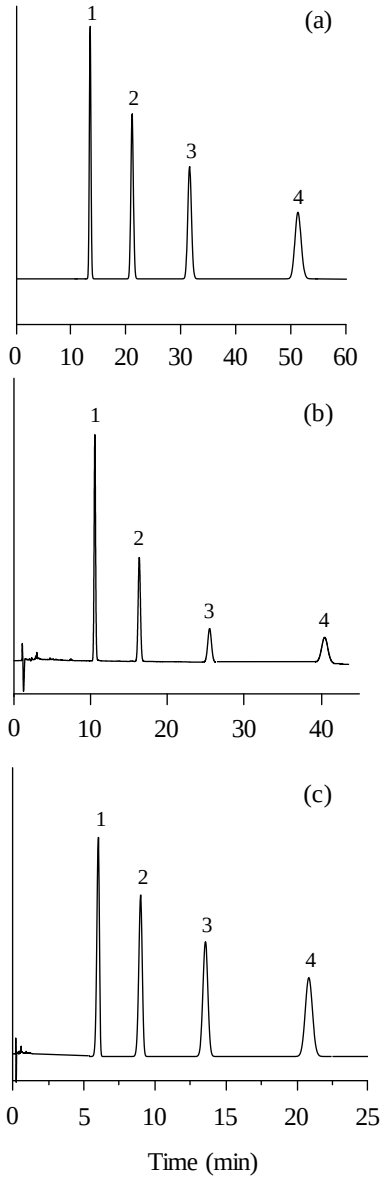


Figure 4.2. Chromatograms obtained for the mixture of alkylbenzenes eluted isocratically: (a) 60% acetonitrile, (b) 0.01 M SDS/60% acetonitrile, and (c) 0.01 M Brij-35/60% acetonitrile. Peak identity: (1) ethylbenzene, (2) propylbenzene, (3) butylbenzene, and (4) amylbenzene.

The low polarity of alkylbenzenes forced the use of a high concentration of acetonitrile in the mobile phases (60%) to obtain sufficiently short retention times. Under these conditions, micelles are not formed in the presence of surfactant (submicellar conditions). Also, with increasing concentration of organic solvent in the mobile phase, the surfactant monomers are gradually desorbed from the stationary phase. However, as demonstrated in previous work, the stationary phase is still covered by the surfactant at a relatively high acetonitrile concentration [31,32].

In the hydro-organic mode (Figure 4.2a), the strong hydrophobic interaction between alkylbenzenes and the alkyl-bonded stationary phase yielded long analysis times, close to 50 min. In the presence of SDS and Brij-35 (Figures 4.2b and c), the analysis times decreased significantly, especially for the non-ionic surfactant. This behaviour reveals the higher polarity of the stationary phase covered by this surfactant.

4.4.2.3. Interactions of sulphonamides

The studied sulphonamides (except sulphaguanidine, sulphanilamide and sulphacetamide) have both an amine group of aniline and a heterocyclic base, which is specific for each sulphonamide (Table 4.1). A third functional group with acid-base behaviour is the sulphonamide group, whose proton can be lost relatively easily. Therefore, sulphonamides may be described by three pK values (pK_{a1}, pK_{a2} and pK_{a3}), corresponding to the diprotonated, monoprotonated and neutral forms, respectively [26,27].

The acidity strength of the double protonated form, which is a strong acid with very low pK_{a1} (< 2), is usually not reported. The pK_{a2} values are in the 1.5–3 range, indicating that the corresponding base is rather weak. Finally, the pK_{a3} values are in the 5–8 range, indicating that the sulphonamide group is

weakly acidic. Sulphaguanidine and sulphanilamide are unusual compared to the other sulphonamides, because of the much lower acidity of the sulphonamide group (pK_{a3} = 12.1 and 10.4, respectively).

Although the above pK_a values were obtained in aqueous solution, at the pH of the assayed mobile phases (pH = 3.0) the neutral species should be dominant, except for sulphaguanidine whose pK_{a2} is somewhat larger (2.8). From the correlation of the retention times in the hydro-organic mode (Figure 4.1a) with the log $P_{o/w}$ values (Table 4.1), it can be inferred that the elution order follows the polarity of sulphonamides. The elution order in MLC with Brij-35 (Figure 4.1c) is similar, with some changes in the least retained compounds (peaks 1 and 2, and 5 to 8). However, the retention capability of the column is weaker, and therefore, a smaller concentration of organic solvent is needed to elute optimally the mixture of sulphonamides (5% against 15.2% for the hydro-organic mode). The observed behaviour with Brij-35 may be explained due to a combination of a more polar modified stationary phase and the interaction of the amine groups in sulphonamides with the polyoxyethylene tail of the non-ionic surfactant.

Such specific interaction does not happen with SDS, giving rise to a significantly weaker retention (note that the concentration of acetonitrile was only 3.5% for the optimal conditions). The elution order in MLC with SDS (Figure 4.1b) also changes significantly with respect to hydro-organic RPLC and MLC with Brij-35. The most significant change is the late elution of sulphaguanidine (peak 1, which appears close to the dead time in hydro-organic RPLC and MLC with Brij-35). As commented, the sulphonamide group of sulphaguanidine is weaker (pK_{a2} = 2.8 in aqueous solution) with respect to the other assayed compounds (pK_{a2} = 1.7–2.4), and the cationic species may be dominant at the working pH.

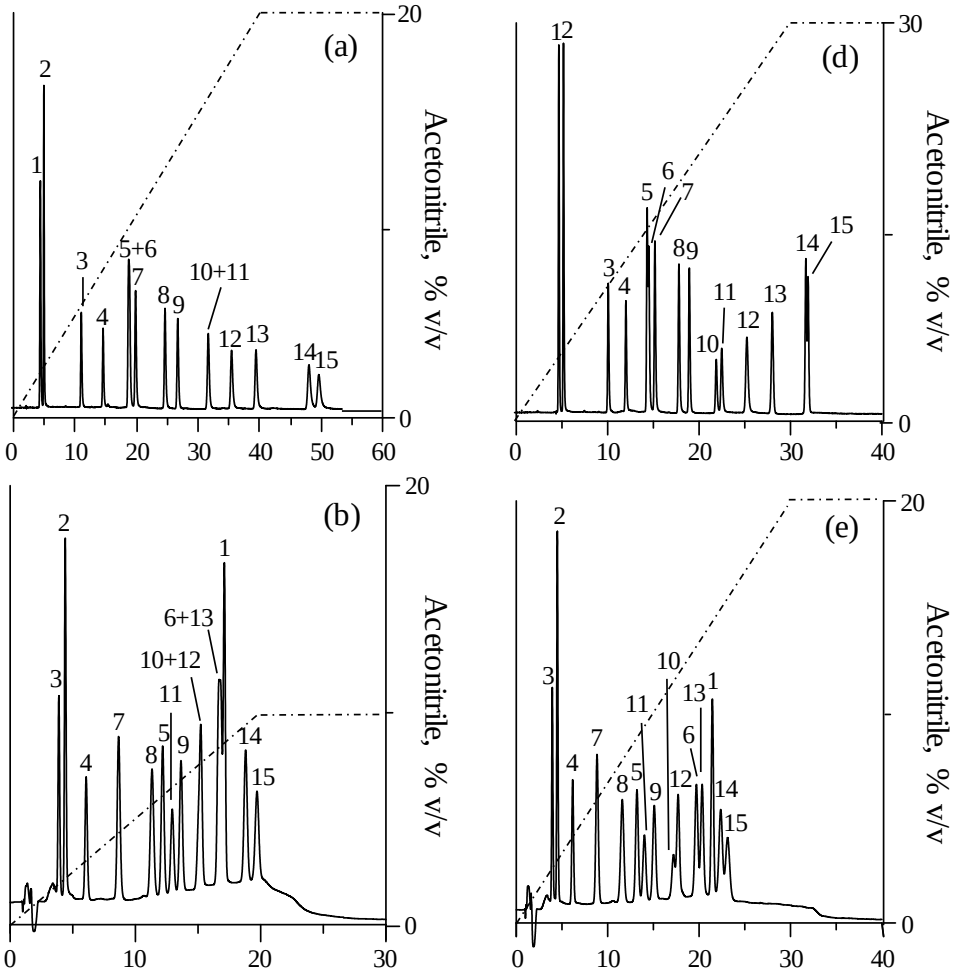


Figure 4.3. Chromatograms obtained using gradient elution for an aqueous mixture of the 15 sulphonamides injected in the hydro-organic mode (a and d), and the micellar mode with SDS (b and e), and Brij-35 (c and f). In the micellar mode, the concentrations of SDS and Brij-35 were fixed at 0.05 and 0.01 M, respectively. The gradient profiles are depicted on the chromatograms. For peak identity, see Section 4.3.1.

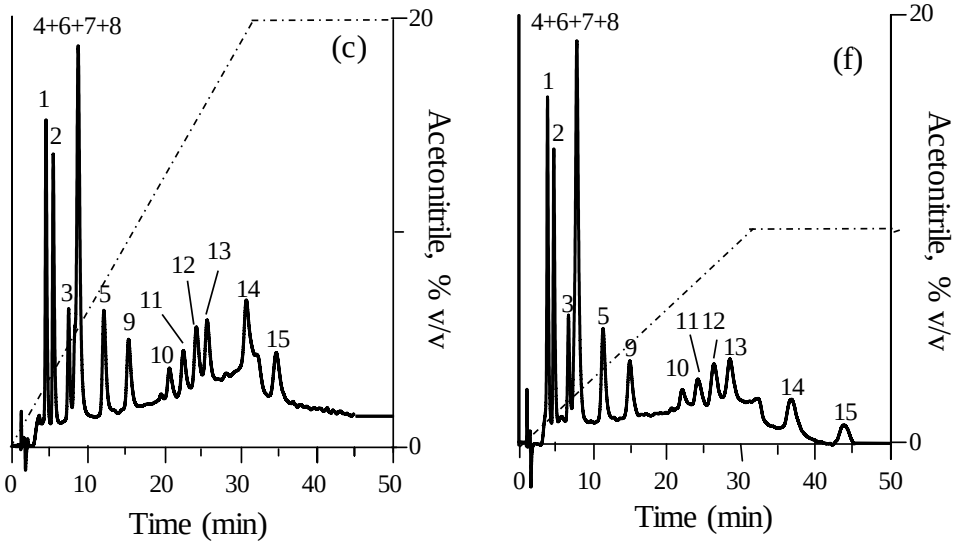


Figure 4.3 (continued).

The stronger retention of sulphaguanidine can be thus explained by the attraction of the cationic species to the negatively charged stationary phase modified with SDS. Other changes in the elution order of the analysed sulphonamides could be also explained by differences in the formation degree of the cationic species.

4.4.3. Retention behaviour in gradient elution

The use of acetonitrile gradients in hydro-organic RPLC and in the micellar modes with a constant concentration of SDS and Brij-35 was next explored. Linear gradients were applied in all cases, which were built by mixing gradually solvents A and B as indicated in Table 4.2. Figure 4.3 shows some of the chromatograms obtained under different gradient conditions in the

hydro-organic mode (Figures 4.3a and d), and the micellar modes in the presence of SDS (Figures 4.3b and e) and Brij-35 (Figures 4.3c and f). The implementation of gradient elution allowed the reduction of the analysis time (compare with the chromatograms in Figure 4.1).

The gradients were designed based on the optimal concentrations of the organic solvent and surfactant in the isocratic mode. In the hydro-organic mode, using isocratic elution, 15.2% acetonitrile was needed to elute the sulphonamides with optimal resolution (Figure 4.1a). Therefore, the gradients were designed to increase the concentration of acetonitrile linearly from 0 to 20%, 30% and 40% over different time periods (t_G) between 20 and 40 min, in order to assure the elution of the most retained sulphonamides at more convenient analysis times. As observed in Figures 4.3a and d, using gradient elution, the peaks were narrower with respect to those obtained in the isocratic mode, and the resolution of the early eluting sulphonamides was improved. However, three critical peak clusters were still observed, formed by: (i) sulphathiazole (peak 5), sulphapyridine (peak 6) and sulphamerazine (peak 7), (ii) sulphamonomethoxine (peak 10) and sulphachloropyridazine (peak 11), and (iii) sulphadimethoxine (peak 14) and sulphaquinoxaline (peak 15). Note that the latter compounds could be baseline resolved with isocratic elution.

In the micellar mode, an optimal isocratic chromatogram was obtained with 0.065 M SDS and a low concentration of acetonitrile (3.5%). However, several sulphonamides co-eluted. In gradient elution, a fixed concentration of SDS of 0.05 M (close to the optimal composition in isocratic elution) was selected, together with a linear increase from 0 to 10% or 20% acetonitrile, and gradient times of 20 min or 30 min, to guarantee enough resolution. Under these conditions, the analysis time was similar to that obtained in the isocratic mode (compare Figures 4.3b and e with Figure 4.1b). However, the chromatograms

show still overlapping of the peaks of sulphamonomethoxine (peak 10) and sulphamethoxazole (peak 12) on the one hand, and sulphapyridine (peak 6) and sulphisoxazole (peak 13), on the other.

The elution of sulphonamides using acetonitrile gradients in the presence of 0.01 M Brij-35 was less favourable, since it yielded a baseline with an extremely positive slope (data not shown), which could not be completely corrected (Figures 4.3c and f). The resolution was also poorer. In this case, gradient elution was implemented by increasing the acetonitrile content from 0 to 10%, 20% or 30% with gradient times of 30 min or 40 min. The elution pattern was significantly different from that found with SDS. Gradient elution in the presence of Brij-35 decreased considerably the analysis time with respect to the isocratic mode. However, the resolution did not improve sufficiently for the least retained sulphonamides, remaining a strong overlapping between sulphadiazine (peak 4), sulphapyridine (peak 6), sulphamerazine (peak 7), and sulphamethazine (peak 8).

4.4.4. Column re-equilibration

After each gradient run, the column should be regenerated to avoid variability in the retention times among consecutive injections, which may affect especially early eluting compounds. Columns are re-equilibrated by flushing several volumes of the initial eluent composition before the next run. If the time required for regenerating the column is long, the increase in the total time will be significant.

In MLC, the amount of free surfactant in the mobile phase is approximately constant. Any change in the total surfactant concentration will result only in a change in the micelle concentration. Adsorption isotherms with SDS have shown that, under these conditions, no or little changes occur in the stationary

phase [33]. Therefore, using a gradient of surfactant above the CMC, no change would be observed in the adsorbed amount of surfactant when returning to the initial conditions. The only required re-equilibration time will be related to the amount of eluent needed to flush the mixer and the injector, together with other pre-column volumes [6,34]. In contrast, when a gradient of organic solvent is run, this may desorb significant amounts of surfactant monomers from the stationary phase [35]. Also, the surfactant CMC will change with the concentration of organic solvent [36]. However, different studies have shown that the re-equilibration times for gradients of organic solvent in the presence of SDS are short, [7,12,14] which has been attributed to the limited organic solvent content along the gradient. The re-equilibration time in the hydro-organic mode is also short, due to the consistent solvation of the stationary phase by the organic solvent [37].

In previous work, the implementation of acetonitrile gradients in the hydro-organic mode, and 1-propanol gradients in the micellar mode with SDS, needed re-equilibration times below 2 min [14], significantly smaller than previous values reported two decades before [7]. However, no data have been reported for gradients of organic solvent in the presence of Brij-35. Therefore, a re-equilibration study was carried out with this surfactant. The results were compared with those obtained in the hydro-organic mode and in the presence of SDS with the same column. Three sulphonamides with intermediate elution (sulphacetamide, sulphamethazine and sulphathiazole) were used for this study. The elution conditions were selected to obtain retention times of approximately 10 min for the hydro-organic mode (0–30% acetonitrile with t_G = 30 min), micellar mode with 0.05 M SDS (0–10% acetonitrile with t_G = 20 min), and micellar mode with 01 M Brij-35 (0–20% acetonitrile with t_G = 30 min).

An automatic injector was used with an injection routine that started just at the time the gradient top value was reached, which took approximately 2 min. Considering the dwell time (0.32 min), the re-equilibration time was below 2 min for the hydro-organic mode and micellar mode in the presence of SDS, which agrees with recent values reported in the literature [12,14]. In the presence of Brij-35, the re-equilibration time was longer (around 3 min), which means that the gradual increase in the concentration of organic solvent during the gradient affects more significantly the adsorbed layer of the non-ionic surfactant on the stationary phase. Desorption of surfactant along the acetonitrile gradient is also the reason for the steep baseline in the chromatograms. However, the re-equilibration time is sufficiently short to be practical.

4.4.5. Analysis of physiological samples

Milk and urine are composed of a matrix containing proteins and endogenous compounds. In hydro-organic RPLC, prior treatment to eliminate or reduce the amount of proteins is needed to avoid damage of the chromatographic column. In MLC, the micelles in the mobile phase are able to solubilise the proteins, which are swept away from the column as a broad band at the solvent front [38]. The presence of endogenous compounds can be, however, problematic, since these may overlap the peaks of analytes.

With micellar mobile phases, the stationary phase is also protected by a layer of surfactant monomers, even in the presence of organic solvents. However, to increase the protection against the proteins in the physiological samples, we decided to add a 2 min isocratic step at the beginning of the gradients, consisting of an eluent containing exclusively 0.05 M SDS or 0.01 M Brij-35 and a buffer. In both cases, after this step, the acetonitrile

content was increased from 0 to 20% over 30 min. For the urine samples, blanks from different males and females of different ages, diets and weights were also injected to check the elution of endogenous compounds.

Figures 4.4 and 4.5 show the chromatograms of milk and urine blanks and fortified samples with the whole set of sulphonamides (15) and 7 sulphonamides, eluted with acetonitrile in the gradient mode in the presence of SDS and Brij-35, respectively. With milk samples, no interference from endogenous components in the matrix sample was observed with both surfactants (see Figures 4.4a and b for SDS, and Figures 4.5a and b for Brij-35), but with urine samples, sulphanilamide (peak 2) and sulphacetamide (peak 3) overlapped with the peaks of two endogenous compounds in the presence of SDS (Figures 4.4c and d), and sulphanilamide (peak 2) and sulphadiazine (peak 4) overlapped with the front band of proteins and a prominent peak of an endogenous compound, respectively, in the presence of Brij-35 (Figures 4.5c and d). The situation was the same for urine samples from other individuals.

The addition of an isocratic step with a purely micellar solution (without an organic solvent) slightly modified the retention times and decreased the resolution, giving rise to overlapping between sulphamonomethoxine (peak 10) and sulphamethoxazole (peak 12), and between sulphadimethoxine (peak 14) and sulphaquinoxaline (peak 15), in the presence of SDS (see Figures 4.4b and d). With Brij-35, a smaller number of sulphonamides could be resolved under the studied conditions (Figures 4.5b and d).

4.5. Conclusions

The isocratic elution of the mixture of 15 sulphonamides did not allow their screening in either the hydro-organic or micellar modes with SDS or Brij-35, using acetonitrile as organic solvent. In the hydro-organic mode, only the most polar sulphonamides were eluted at practical analysis times, whereas the most hydrophobic required times above 60 min. Previous work with other chromatographic columns in the hydro-organic mode also yielded long analysis times or low resolution for the same group of sulphonamides [39]. Although in the micellar mode the screening of sulphonamides was neither possible, an interesting feature is the small concentration of the organic solvent (< 5% acetonitrile) required with both SDS and Brij-35. This allows the implementation of particular procedures for the determination of different sulphonamides, which may be classified inside the field of "green chromatography" [3].

In contrast to this situation, the implementation of acetonitrile gradients in the micellar mode using SDS allowed the screening of sulphonamides at practical analysis times. However, using Brij-35, the results did not improve with respect to the isocratic elution. Although the peaks appeared more evenly distributed in the chromatogram, these were broad and strongly overlapped for different sulphonamides. Nevertheless, the use of gradient elution with acetonitrile in the presence of Brij-35 may be an option to analyse mixtures containing a reduced number of sulphonamides.

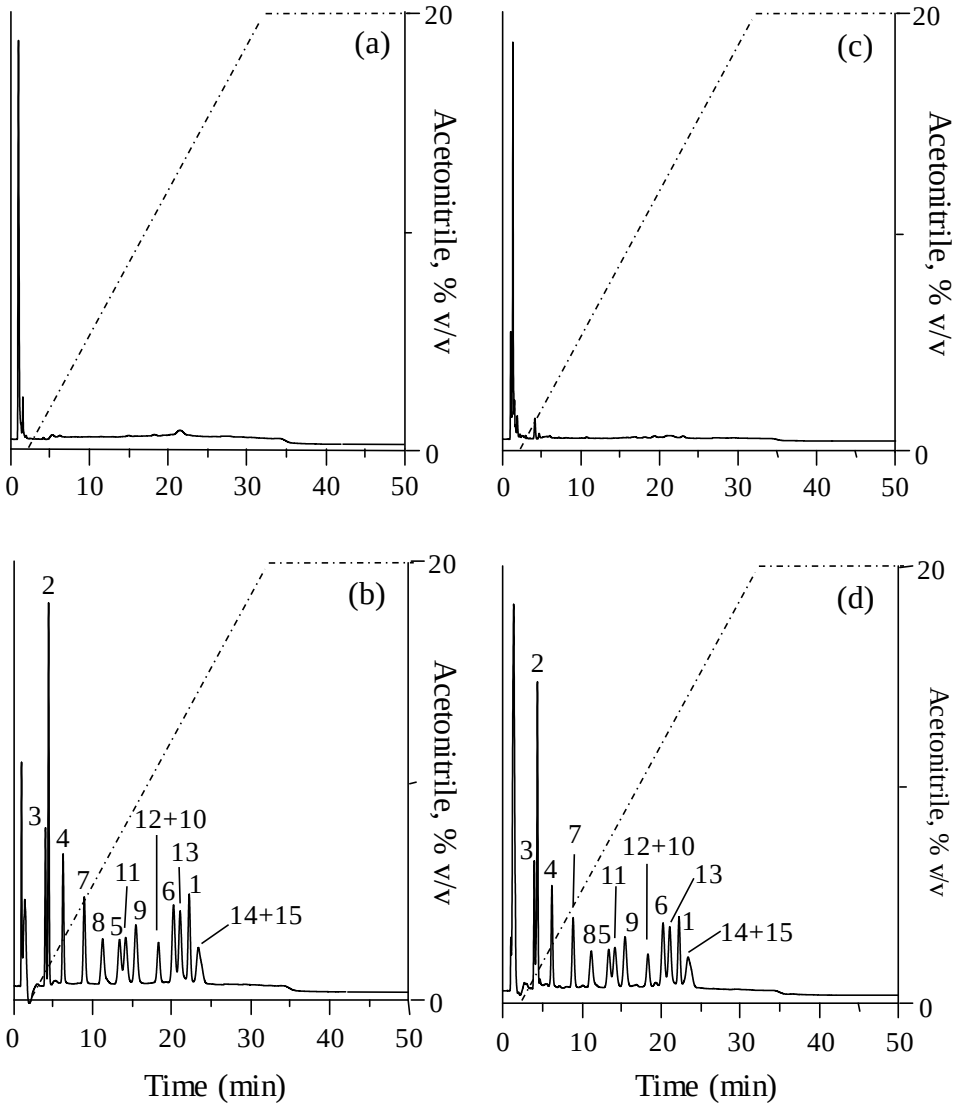


Figure 4.4. Chromatograms obtained using gradient elution in the hybrid-micellar mode with 0.05 M SDS: milk (a) and urine (c) blanks, and milk (b) and urine (d) samples fortified with 6 mg/L of the 15 sulphonamides. See Figure 4.3 for more details.

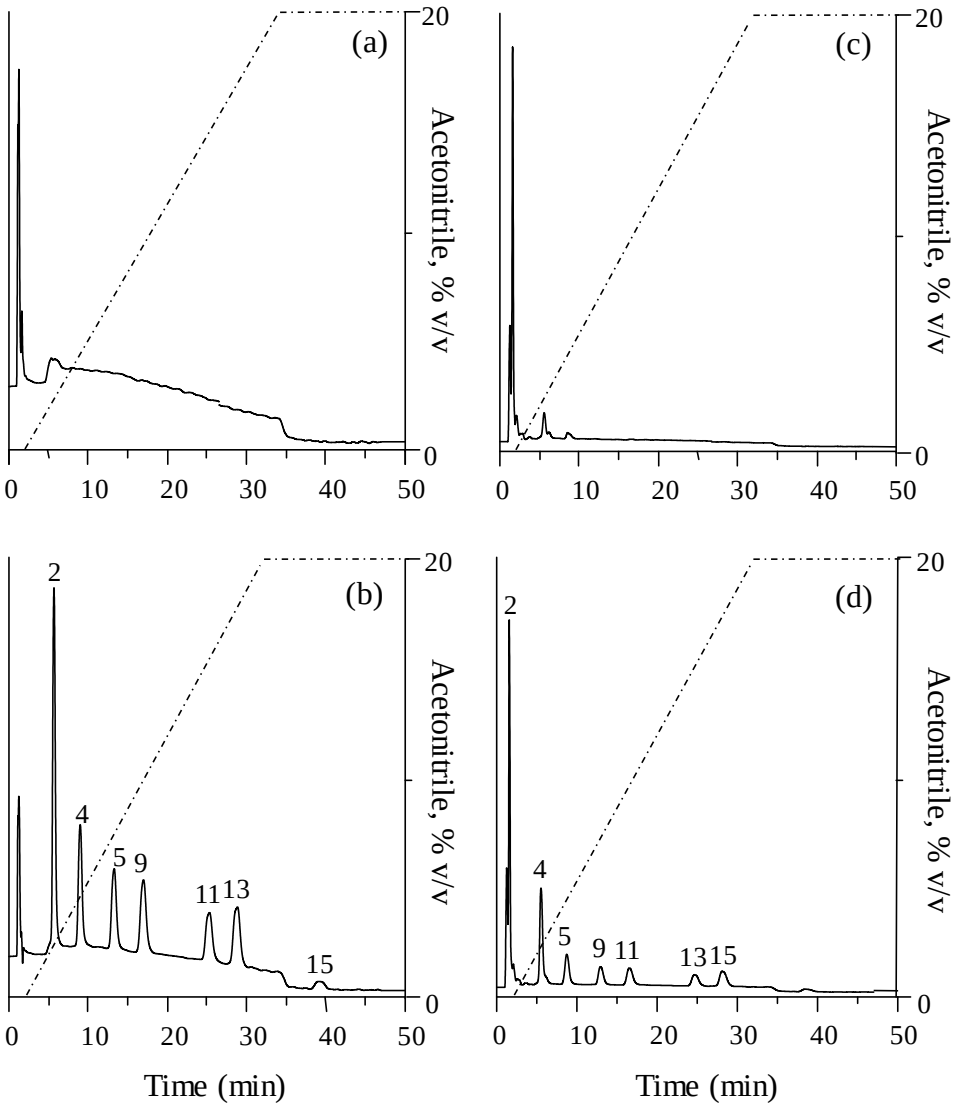


Figure 4.5. Chromatograms obtained using gradient elution in the hybrid-micellar mode with 0.01 M Brij-35: milk (a) and urine (c) blanks, and milk (b) and urine (d) samples fortified with 6 mg/L of seven sulphonamides. See Figure 4.3 for more details.

The micellar mode has the advantage of making the direct injection of the physiological samples (milk and urine) possible, without the need of prior treatment to avoid column damage. The sample should preferably be eluted initially with a pure micellar solution containing only SDS or Brij-35, above the CMC, to facilitate the solubilisation of the proteins. Once the proteins have been eluted (which happens in the first few minutes of the chromatographic analysis), the elution strength can be increased using a positive linear gradient of organic solvent to obtain convenient analysis times.

This work shows promising results to implement a useful screening method for sulphonamides in urine and milk samples, using direct injection. The optimisation of the resolution was made by trial-and-error in a few runs. In future work, it is intended to develop an interpretive methodology to optimise gradients of organic solvent in MLC, in order to improve the resolution. The estimated limits of detection for sulphonamides with the optimised gradient in the presence of SDS were approximately 0.01–0.04 mg/L, which are similar to those usually reported for other RPLC methods. However, still a full validation of the method is needed.

4.6. References

[1] A. Berthod, M.C. García Álvarez-Coque, *Micellar Liquid Chromatography* (edited by J. Cazes), Marcel Dekker, New York, 2000.

[2] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, New insights and recent developments in micellar liquid chromatography, *Sep. Purif. Rev.* 38 (2009) 45–96.

[3] R.N. El Shaheny, M.H. El Magharabey, F.F. Belal, Micellar liquid chromatography from green analysis perspective, *Open Chem.* 13 (2015) 877–892.

[4] M.C. García Álvarez-Coque, J.R. Torres Lapasió, Quantitation of hydrophobicity in micellar liquid chromatography, *Trends Anal. Chem.* 18 (1999) 533–543.

[5] M.J. Ruiz Ángel, R.D. Caballero, E. Simó Alfonso, M.C. García Álvarez-Coque, Micellar liquid chromatography: Suitable technique for screening analysis, *J. Chromatogr. A* 947 (2002) 31–45.

[6] L.S. Madamba Tan, J.K. Strasters, M.G. Khaledi, Gradient elution in micellar liquid chromatography: I. Micelle concentration gradient, *J. Chromatogr. A* 683 (1994) 321–334.

[7] L.S. Madamba Tan, J.K. Strasters, M.G. Khaledi, Gradient elution in micellar liquid chromatography: II. Organic modifier gradients, *J. Chromatogr. A* 683 (1994) 335–345.

[8] E.K. Paleologos, M.G. Kontominas, On-line solid-phase extraction with surfactant accelerated on-column derivatization and micellar liquid chromatographic separation as a tool for the determination of biogenic amines in various food substrates, *Anal. Chem.* 76 (2004) 1289–1294.

[9] A.R. Ghorbani, F. Momenbeik, J.H. Khorasani, M.K. Amini, Simultaneous micellar liquid chromatographic analysis of seven water-soluble vitamins: Optimization using super-modified simplex, *Anal. Bioanal. Chem.* 379 (2004) 439–444.

[10] S.M. Bryant, K.D. Altria, An initial assessment of the use of gradient elution in microemulsion and micellar liquid chromatography, *J. Sep. Sci.* 27 (2004) 1498–1502.

[11] J. Cao, H. Qu, Y. Cheng, Micellar and aqueous-organic liquid chromatography using sub-2 μm packings for fast separation of natural phenolic compounds, *J. Sep. Sci.* 33 (2010) 1946–1953.

[12] R. Nakao, M. Schou, C. Halldin, Direct plasma metabolite analysis of positron emission tomography radioligands by micellar liquid chromatography with radiometric detection, *Anal. Chem.* 84 (2012) 3222–3230.

[13] R. Nakao, M. Schou, C. Halldin, Rapid metabolite analysis of positron emission tomography radioligands by direct plasma injection combining micellar cleanup with high submicellar liquid chromatography with radiometric detection, *J. Chromatogr. A* 1266 (2012) 76–83.

[14] J. Rodenas Montano, C. Ortiz Bolsico, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Implementation of gradients of organic solvent in micellar liquid chromatography using Drylab®: Separation of basic compounds in urine samples, *J. Cromatogr. A* 1344 (2014) 31–41.

[15] M.J. Ruiz Angel, S. Carda Broch, M.C. García Alvarez-Coque, High submicellar liquid chromatography, *Separ. Purif. Rev.* 43 (2014) 124–154.

[16] L.J. Cline-Love, J.J. Fett, Optimization of selectivity in micellar chromatographic procedures for the determination of drugs in urine by direct injection, *J. Pharm. Biomed. Anal.* 9 (1991) 323–333.

[17] A. Berthod, S. Tomer, J.G. Dorsey, Polyoxyethylene alkyl ether nonionic surfactants: Physicochemical properties and use for cholesterol determination in food, *Talanta* 55 (2001) 69–83.

[18] N. Memon, M.I. Bhanger, M.Y. Khuhawer, Determination of preservatives in cosmetics and food samples by micellar liquid chromatography, *J. Sep. Sci.* 28 (2005) 635–638.

[19] W. Thogchai, B. Liawruangrath, Micellar liquid chromatographic determination of arbutin and hydroquinone in medicinal plant extracts and commercial cosmetic products, *Int. J. Cosmetic Sci.* 35 (2013) 257–263.

[20] Y.M. Dong, N. Li, Q. An, N.W. Lu, A novel nonionic micellar liquid chromatographic method for simultaneous determination of pseudoephedrine, paracetamol, and chlorpheniramine in cold compound preparations, *J. Liq. Chromatogr. Rel. Technol.* 38 (2015) 251–258.

[21] L. Escuder Gilabert, S. Sagrado, R.M. Villanueva Camañas, M.J. Medina Hernández, Quantitative retention-structure and retention-activity relationship studies of local anesthetics by micellar liquid chromatography, *Anal. Chem.* 70 (1998) 28−34.

[22] J.J. Fernández Navarro, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography without organic solvent for determination of tricyclic antidepressants, *J. Sep. Sci.* 35 (2012) 1303–1309.

[23] J.J. Baeza Baeza, Y. Dávila, J.J. Fernández Navarro, M.C. García Álvarez-Coque, Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC, *Anal. Bioanal. Chem.* 404 (2012) 2973–2984.

[24] M.J. Ruiz Ángel, E. Peris García, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A method for the analysis of basic compounds, *Green Chem.* 17 (2015) 3561–3570.

[25] E. Peris García, C. Ortiz Bolsico, J.J. Baeza Baeza, M.C. García Álvarez-Coque, Isocratic and gradient elution in micellar liquid chromatography with Brij-35, *J. Sep. Sci.* 38 (2015) 2059–2067.

[26] S. Carda Broch, A. Berthod, Countercurrent chromatography for the measurement of the hydrophobicity of sulfonamide amphoteric compounds, *Chromatographia* 59 (2004) 79–87.

[27] A. Białk-Bielinska, S. Stolte, M. Matzke, A. Fabianska, J. Maszkowska, M. Kołodziejska, B. Liberek, P. Stepnowski, J. Kumirska, Hydrolysis of sulphonamides in aqueous solutions, *J. Hazard. Mater.* 221–222 (2012) 264–274.

[28] J.R. Torres Lapasió, *MICHROM Software*, Marcel Dekker, New York, 2000.

[29] B.L. Lavine, W.T. Cooper, Y. He, S. Hendayana, J.H. Han, J. Tetreault, Solid-state C-13 NMR-studies of ionic surfactants adsorbed on C18 and C8 silicas: Implications for micellar liquid chromatography, *J. Colloid Interface Sci.* 165 (1994) 497–504.

[30] M.C. García Álvarez-Coque, J.J. Baeza Baeza, G. Ramis Ramos, Reversed phase liquid chromatography in *Analytical Separation Science Series* (edited by J.L. Anderson, A. Stalcup, A. Berthod, V. Pino), Vol. 1, Wiley, New York, 2015, pp. 159–198.

[31] M.J. Ruiz Ángel, J.R. Torres Lapasió, M.C. García Álvarez-Coque, S. Carda Broch, Retention mechanisms for basic drugs in the submicellar and micellar reversed-phase liquid chromatographic modes, *Anal. Chem.* 80 (2008) 9705–9713.

[32] C. Ortiz Bolsico, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Adsorption of the anionic surfactant sodium dodecyl sulfate on a C18 column under micellar and high submicellar conditions in reversed-phase liquid chromatography, *J. Sep. Sci.* 38 (2015) 550–555.

[33] A. Berthod, I. Girard, C. Gonnet, Micellar liquid chromatography: Adsorption isotherms of two ionic surfactants on five stationary phases, *Anal. Chem.* 58 (1986) 1356–1358.

[34] T.J. McCormick, J.P Foley, C.M. Riley, D.K. Lloyd, The effect of stationary phase pore size on retention in micellar liquid chromatography, *Anal. Chem.* 72 (2000) 294–301.

[35] M.F. Borgerding, W.L. Hinze, L.D. Stafford, G.W. Fulp Jr., W.C. Hamlin Jr., Investigations of stationary phase modification by the mobile phase surfactant in micellar liquid chromatography, *Anal. Chem.* 61 (1989) 1353–1358.

[36] S. López Grío, J.J. Baeza Baeza, M.C. García Álvarez-Coque, Influence of the addition of modifiers on solute-micelle interaction in hybrid micellar liquid chromatography, *Chromatographia* 48 (1998) 655–663.

[37] L.A. Cole, J.G. Dorsey, Reduction of reequilibration time following gradient elution reversed phase liquid chromatography, *Anal. Chem.* 62 (1990) 16–21.

[38] M.C. García Álvarez-Coque, S. Carda Broch, Direct injection of physiological fluids in micellar liquid chromatography, *J. Chromatogr. B* 736 (1999) 1–18.

[39] C. Ortiz Bolsico, J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Comparison of two serially-coupled column systems and optimization software in isocratic liquid chromatography for resolving complex mixtures, *J. Chromatogr. A* 1281 (2013) 94–105.

CHAPTER 5

SEARCH OF NON-IONIC SURFACTANTS SUITABLE FOR MICELLAR LIQUID CHROMATOGRAPHY

5.1. Abstract

Most reports in reversed-phase liquid chromatography (RPLC) with micellar mobile phases make use of the anionic sodium dodecyl sulphate. This surfactant masks efficiently the silanol groups that are the origin of the poor efficiencies and tailing peaks observed for basic compounds in conventional RPLC. However, it has the handicap of yielding excessive retention, which forces the addition of an organic solvent to reduce the retention times to practical values. Other surfactants, such as the non-ionic polyoxyethylene(23)lauryl ether (Brij-35), are rarely used. Brij-35 allows the separation of a large range of analytes in adequate retention times, without the need of adding an organic solvent to the mobile phase. However, this non-ionic surfactant shows irreversible adsorption on chromatographic columns and peak shape is poorer. Therefore, the search of non-ionic surfactants with similar properties to Brij-35, but showing reversible adsorption and better peak shape, can be of great interest. In this work, the adequacy of several non-ionic surfactants as modifiers in RPLC has been explored, being polyoxyethylene(10)tridecyl ether particularly attractive. The separation of different types of compounds was checked: sulphonamides (acidic), β-blockers and tricyclic antidepressants (basic with diverse polarity), and flavonoids (with and without hydroxyl groups on the aromatic rings). The chromatographic behaviours were examined in terms of retention and peak shape. The results were compared with those obtained with Brij-35.

5.2. Introduction

In reversed-phase liquid chromatography (RPLC), the addition of a surfactant above its critical micelle concentration (CMC) to aqueous solutions or hydro-organic mixtures gives rise to a particular chromatographic mode, known as micellar liquid chromatography (MLC). After three decades from the birth of the technique, MLC appears to be a solid alternative to conventional RPLC with hydro-organic mobile phases [1–3]. This mode is characterised by the existence of clusters of surfactant monomers or micelles in the mobile phase, and the adsorption of surfactant monomers on the stationary phase, which is significantly modified. Both the ability of micelles to organise solutes at the molecular level, and the association of surfactant monomers to the bonded phase, modulate the retention times and efficiencies, and gives rise to unique selectivity.

Among the surfactants usually added to the mobile phase in MLC, the anionic sodium dodecyl sulphate (SDS) is, by far, the most popular [1–3]. The main reasons are its commercial availability at high purity and relatively low cost. It also efficiently dissolves proteins in physiological matrices (urine, plasma, serum, etc.), allowing the direct injection of the samples in the chromatograph without any other treatment than filtration [4], which is not possible with cationic surfactants. The non-ionic surfactant polyoxyethylene(23)lauryl ether (C12E23), commercially known as Brij-35, has been used as an alternative to SDS in the analysis of compounds of different nature, with diverse results [5–8]. Brij-35 is adsorbed on the alkyl-bonded stationary phase, with the hydrophilic polar end of the molecule oriented away from the surface. The polarity of the stationary phase, which remains neutral, is thus increased. This reduces significantly the retention time of basic compounds, which are strongly retained in a stationary phase modified

with the anionic SDS [9,10]. In contrast to SDS, mobile phases with Brij-35 require only a small concentration of organic solvent (if needed) to elute solutes at practical retention times. However, there are still two problems to be solved: broad and asymmetrical peaks are usually obtained with the non-ionic surfactant, and polar compounds are not retained unless specific interactions are established with its polyoxyethylene chain. The search of alternative non-ionic surfactants with similar properties to Brij-35, but able to significantly enhance its performance, can be thus of great interest in the field of MLC. However, the use of non-ionic surfactants different from Brij-35 has been almost anecdotic [5,11–13].

This work explores the suitability of seven non-ionic surfactants as mobile phase additives in MLC. The surfactants belong to the Brij, Triton, Igepal and Span series, and were selected due to their commercial availability, low cost and low toxicity. The separation of different types of compounds was checked for those surfactants soluble in water and with low UV absorption: sulphonamides (acidic), β-blockers and tricyclic antidepressants (TCAs) (basic with diverse polarity), and flavonoids (with and without hydroxyl groups on the aromatic rings). These drugs are usually prescribed for bacterial infections (sulphonamides), to remedy neurological, neuropsychiatric and cardiovascular disorders (β-blockers), and in the treatment of anxiety and depressive disorders (TCAs). Flavonoids are extensively used due to their antioxidant, anti-inflammatory and antimicrobial properties. The chromatographic behaviour using a stationary phase modified by the non-ionic surfactants was studied in terms of retention, peak shape, and adsorption capability. The results were compared with those obtained with the traditional Brij-35.

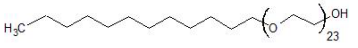
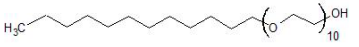
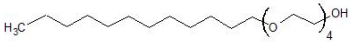
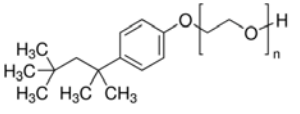
5.3. Experimental

5.3.1. Reagents

The non-ionic surfactants assayed in this work were: Brij-35, polyoxyethylene(10)lauryl ether (C12E10), polyoxyethylene(10)tridecyl ether (C13E10), Brij-L4, Triton-X100, Triton-X114, IGEPAL CO-630, and Span 20, all purchased from Sigma (St. Louis, MO, USA). Their structures, Sigma product number, linear formula, and average molecular weight are indicated in Table 5.1. It should be noted that non-ionic surfactants are usually a mixture of ethers. Thus, for example, the synthesis of polyoxyethylene non-ionic surfactants involves ethylene oxide polymerisation, where the final C*n*E*m* product is actually a mixture of molecules with *n* carbons in the alkyl chain if the starting alcohol was pure, and a variable number of ethylene oxide units (*m*).

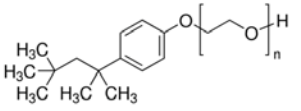
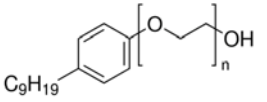
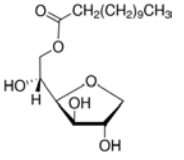
As commented below, among the assayed non-ionic surfactants, only mobile phases containing C12E10, C13E10, and Brij-35 could be prepared. For tricyclic antidepressants, the anionic surfactant SDS from Merck (Darmstadt, Germany) was also assayed. In all cases, the pH of the mobile phase was buffered at 3.0 with 0.01 M sodium dihydrogen phosphate (Fluka, Buchs, Switzerland) and hydrochloric acid (Scharlab, Barcelona, Spain). When the mobile phase contained organic solvent (acetonitrile, methanol, or pentanol), the pH was adjusted before this was added.

Table 5.1. Linear formula, structure and average molecular weight of the non-ionic surfactants examined in this work.

Surfactant	Linear formula	Sigma product number	Structure	MW (g/mol)
Polyoxyethylene(23) lauryl ether (Brij-35, Brij-L23 or C12E23)[a]	$C_{12}H_{25}(OCH_2CH_2)_{23}OH$	P1254		1198
Polyoxyethylene(10) lauryl ether (C12E10)[a]	$C_{12}H_{25}(OCH_2CH_2)_{10}OH$	P9769		627
Polyoxyethylene(10) tridecyl ether (C13E10)[a]	$C_{13}H_{27}(OCH_2CH_2)_{10}OH$	P2393		640
Polyoxyethylene(4) lauryl ether (Brij-L4 or C12E4)[b]	$C_{12}H_{25}(OCH_2CH_2)_4OH$	235989		362
Polyethylene glycol tert-octylphenyl ether(Triton-X100)[a]	$C_{14}H_{21}O(OCH_2CH_2)_nH$ (n = 9-10)	X-100		625

Solubility assayed in this work: [a] Soluble in water. [b] Soluble in 20–35% acetonitrile-water mixtures. [c] Soluble in >10% acetonitrile. [d] Insoluble in water and acetonitrile-water mixture.

Table 5.1 (continued).

Surfactant	Linear formula	Sigma product number	Structure	MW (g/mol)
Polyethylene glycol tert-octylphenyl ether (Triton-X114)[c]	$C_{14}H_{21}O(OCH_2CH_2)_nH$ (n = 7-8)	X-114		537
Polyoxyethylene(9) nonylphenyl ether (IGEPAL CO-630)[a]	$C_{15}H_{24}(OCH_2CH_2)_nOH$ (n = 9-10)	542334		617
Sorbitanmonolaurate (Span 20)[d]	$C_{18}H_{34}O_6$	S6635		346

Solubility assayed in this work: [a] Soluble in water. [b] Soluble in 20–35% acetonitrile-water mixtures. [c] Soluble in >10% acetonitrile. [d] Insoluble in water and acetonitrile-water mixture.

The test compounds were four sulphonamides (sulphadimethoxine, sulphaguanidine, sulphathiazole and sulphamethizole), three β-blockers (oxprenolol, alprenolol and propranolol), three TCAs (doxepin, amitriptyline and imipramine), and three flavonoids (flavone, 3-hydroxyflavone and 5-hydroxyflavone), all from Sigma. Table 5.2 shows the compound structures, together with their acid-base dissociation constants (pK_a), and octanol-water partition coefficients ($\log P_{o/w}$).

Stock solutions containing 100 µg/mL of each compound were prepared by dissolving the solids in a few milliliters of acetonitrile (Scharlab), with the aid of an Elmas 15h ultrasonic bath from Elmasonic (Singen, Germany), and dilution with water. These solutions remained stable during at least two months at 4 ºC, and were again diluted before injection into the chromatograph with aqueous solutions of the surfactants (10 mM C12E10, C13E10, Brij-35, or SDS), up to a final concentration of 20 µg/mL. The diluted solutions of flavonoids were daily prepared and protected from light to avoid degradation. All solutions (test compounds and mobile phases) were filtered through 0.45 µm Nylon membranes (Micron Separations, Westboro, MA, USA), and degassed in an ultrasonic bath. Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

Table 5.2. Structures, dissociation constants (pK_a) in water, and octanol-water partition coefficients (log $P_{o/w}$) of the probe compounds.

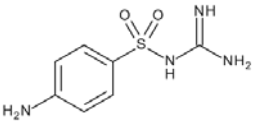
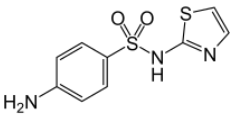
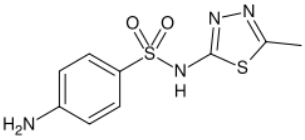
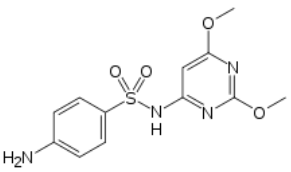
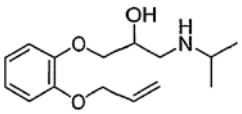
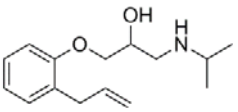
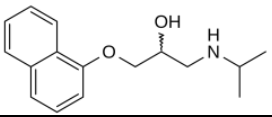
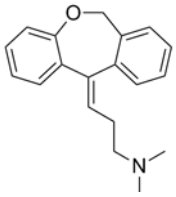
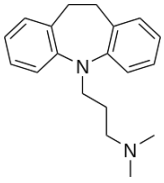
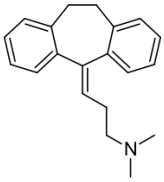
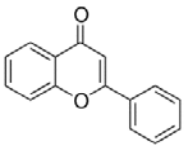
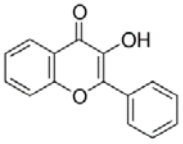
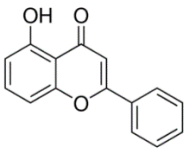
Compound	Structure	pK_a	log $P_{o/w}$
Sulphaguanidine		2.5, 11.2[a]	–1.07[a]
Sulphathiazole		2.1, 7.1[a]	–0.04[a]
Sulphamethizole		2.2, 5.3[a]	0.47[a]
Sulphadimethoxine		3.4, 5.8[a]	1.66[a]
Oxprenolol		9.1[b]	2.30[b]
Alprenolol		9.3[b]	3.15[b]
Propranolol		9.3[b]	3.41[b]

Table 5.2 (continued).

Compound	Structure	pK_a	log $P_{o/w}$
Doxepin		9.0[c]	3.88[c]
Imipramine		9.5[c]	4.41[c]
Amitriptyline		9.4[c]	4.64[c]
Flavone		ND	3.56[d]
3-Hydroxyflavone		9.12[c]	3.76[d]
5-Hydroxyflavone		ND	3.10[d]

[a] From Ref. [14]. [b] From Ref. [15]. [c] From Ref. [16]. [d] From Ref. [17].

5.3.2. Apparatus and columns

An Agilent chromatograph, equipped with a quaternary pump (Series 1200), an autosampler (Series 1100), a thermostated column compartment (Series 1100), a UV-visible wavelength detector (Series 1260), and an HPChemStation (Agilent, B.04.03) for data acquisition, was used. Sulphonamides, TCAs and flavone were monitored at 254 nm, β-blockers at 225 nm, and 3-hydroxyflavone and 5-hydroxyflavone at 350 and 274 nm, respectively. The flow rate was 1 mL/min. Duplicate injections of 20 µL were carried out.

A UV-visible double beam spectrophotometer (Model V-650) from Jasco (Tokyo, Japan) was used to obtain the spectra of non-ionic surfactants. Mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA), whereas chromatographic peaks were integrated assisted by the software MICHROM [18].

A particular Zorbax Eclipse XDB C18 column (Agilent, Waldbronn, Germany) was used to assay the chromatographic behaviour in the presence of each non-ionic surfactant (C12E10, C13E10, and Brij-35). The characteristics of the analytical columns were the following: 150 mm × 4.6 mm i.d., 5 µm particle size, 180 m^2/g surface area, 80 Å pore size, and 10% carbon load. A Zorbax Eclipse XDB C8 column (150 mm × 4.6 mm i.d., 5 µm particle size) was used for TCAs in the presence of SDS-pentanol. All columns were connected before injection to a similar 30 mm guard column.

5.4. Results and discussion

5.4.1. Preliminary studies

Pure aqueous micellar mobile phases require complete surfactant solubilisation in water. With the exception of Tritons, it was not possible to find information about the solubility of the non-ionic surfactants studied in this work, either in water or in acetonitrile-water mixtures. For this reason, a preliminary solubility study in both media was carried out at room temperature. Solutions containing 5 mM surfactant were initially prepared in water. In case of complete dissolution, the concentration was progressively increased up to 50 mM. For water-insoluble surfactants, acetonitrile was gradually added until complete dissolution. The obtained results are indicated in Table 5.1. As observed, C12E10, C13E10, Triton X-100, and IGEPAL CO-630 were completely solubilised in water at the assayed concentration range (5–50 mM). Brij L4 and Triton X-114 were soluble in acetonitrile-water mixtures, and Span 20 was insoluble in both water and acetonitrile.

In view of these results, C12E10, C13E10, Triton X-100, and IGEPAL CO-630 were considered initial candidates for MLC. However, it was still necessary to confirm possible absorption in the UV-visible region. Figure 5.1 depicts the spectra of the four surfactants. Triton X-100 and IGEPAL CO-630 showed two absorption maxima at approximately 225 and 275 nm. Meanwhile, C12E10 and C13E10 combined complete solubility in water with low absorption in the UV-visible region. For this reason, only these two surfactants were selected for the chromatographic study, together with Brij-35.

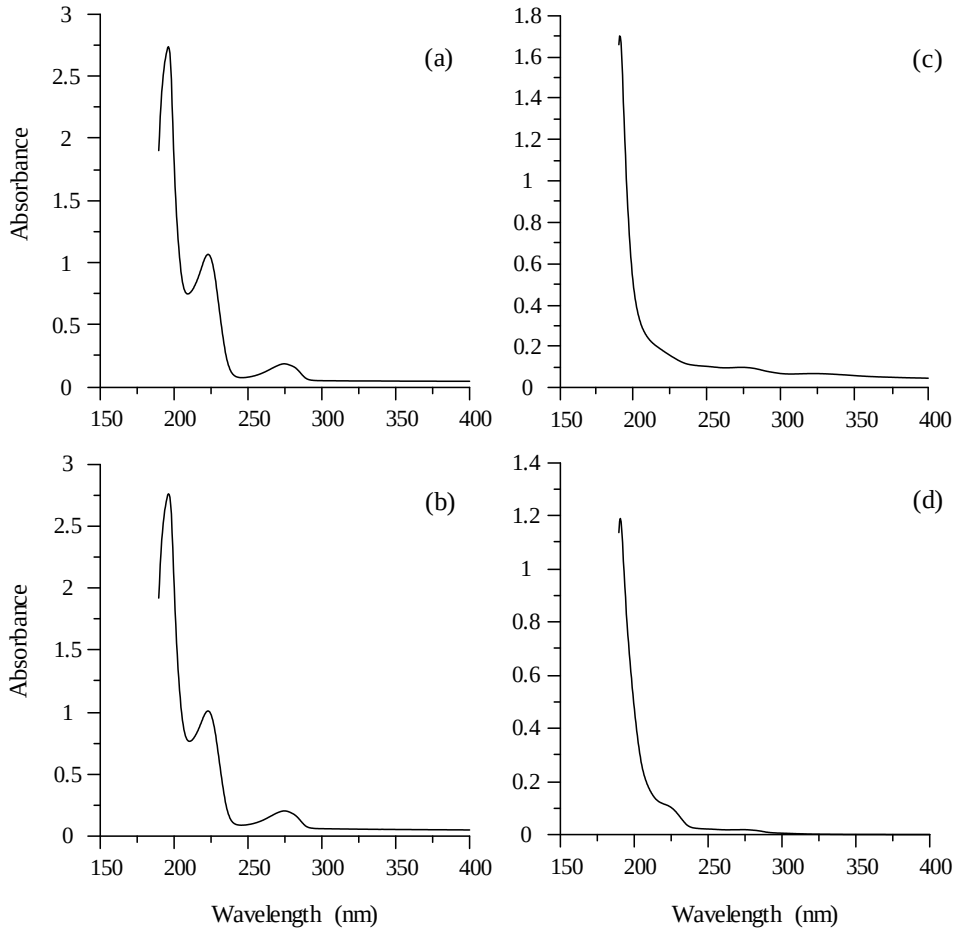


Figure 5.1. UV-visible absorption spectra obtained for four selected non-ionic surfactants: (a) Triton X-100, (b) IGEPAL CO-630, (c) C12E10, and (d) C13E10. Surfactant concentration was 10 mM.

5.4.2. Retention capability of stationary phases modified with the non-ionic surfactants

A C18 stationary phase modified with adsorbed non-ionic surfactant is more polar than the original bonded phase. This reduces the retention times of the analysed compounds if no specific interactions are established with the adsorbed surfactant. In the mobile phase, micelles formed by an apolar core and a relatively polar surface are formed, which are able to interact with solutes. Thus, the micellised surfactant in the mobile phase also changes the elution strength and relative retention (selectivity).

Owing to solubility or absorptivity problems, only C12E10, C13E10 and Brij-35 (among the assayed non-ionic surfactants) were considered for the chromatographic study. Figure 5.2 shows the changes in retention for the sulphonamides, β-blockers, and TCAs studied in this work, and Figures 5.3a to c describe the retention behaviour for the three flavonoids. The relative error in the measurement of retention times is in the 0.1–0.4% range.

Pure mobile phases at four different concentrations of the surfactants were assayed: C12E10 and C13E10 (20, 25, 30 and 35 mM), and Brij-35 (20, 30, 40 and 50 mM). Hybrid mobile phases containing a fixed concentration of the surfactants, and 5, 10, 15 and 20% (*v*/*v*) acetonitrile were also investigated for flavonoids. The CMC (mM) for Brij-35 (C12E23), C12E10 and C13E10 in aqueous medium is 0.06, 0.09 and 0.026 (estimated), respectively [1,5]. Therefore, the concentrations selected to prepare the mobile phases guaranteed micelle formation. Mobile phases containing 25% and 45% acetonitrile in the absence of surfactant were also assayed.

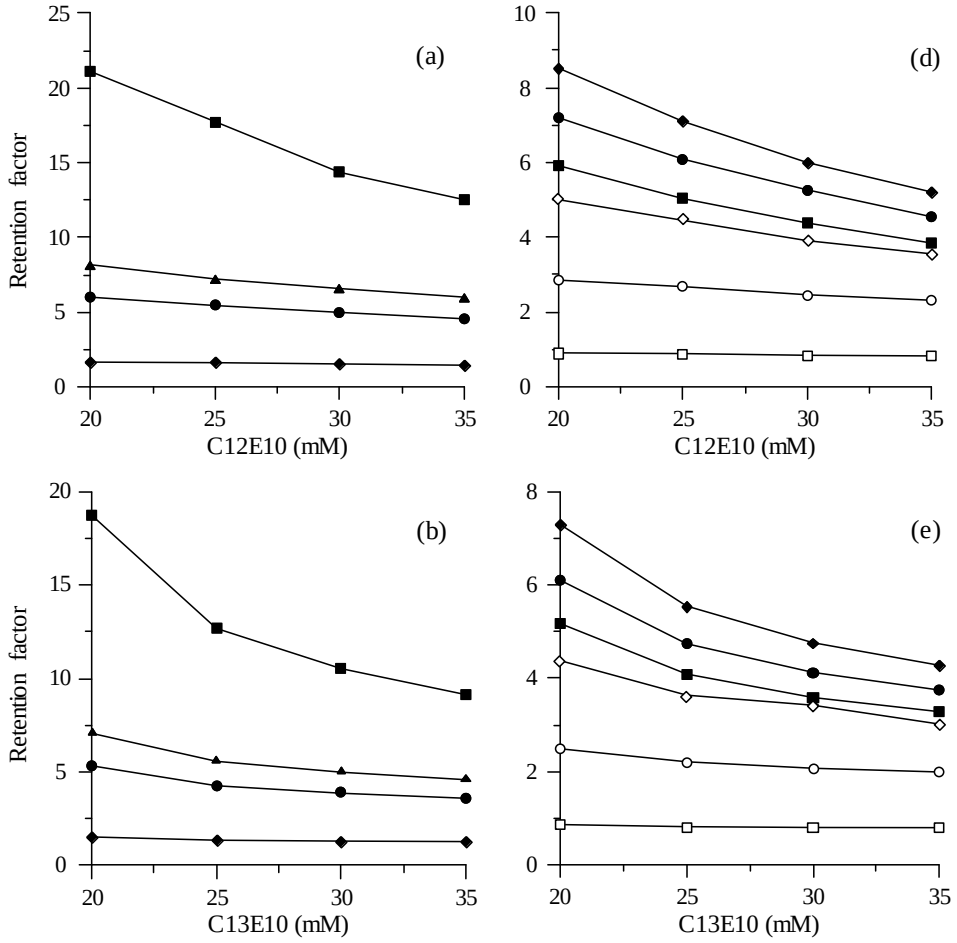


Figure 5.2. Effect of the addition of increasing concentrations of surfactant on the retention of sulphonamides, β-blockers and TCAs. Compound identities: (a,b,c) sulphadimethoxine (■), sulphamethizole (▲), sulphathiazole (●), and sulphaguanidine (◆); (d,e,f) the TCAs amitryptilyline (◆), imipramine (●), doxepin (■), and the β-blockers propranolol (◊), alprenolol (○), and oxprenolol (□).

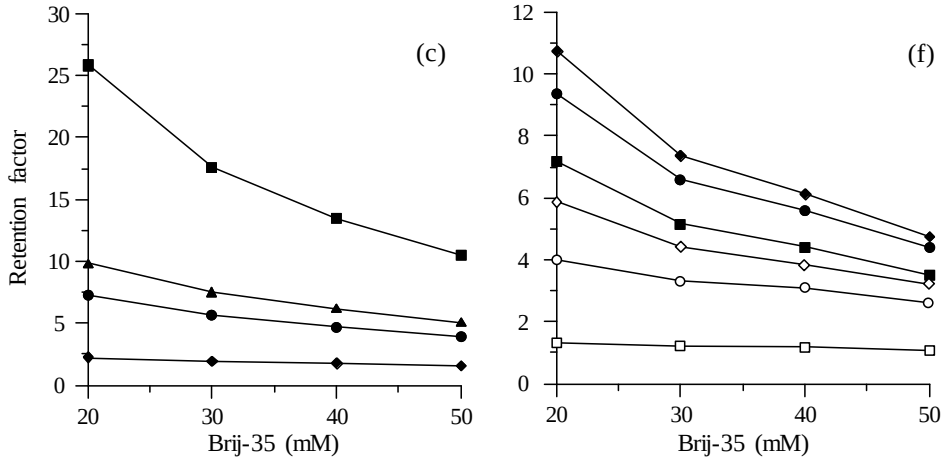


Figure 5.2 (continued).

Dead times, obtained from the first perturbation of the baseline in the chromatograms, were (min): 1.29 ± 0.13 and 1.17 ± 0.04 for 25% and 45% acetonitrile, respectively, 0.994 ± 0.009 for C12E10, 0.981 ± 0.019 for C13E10, and 0.92 ± 0.03 for Brij-35. The dead times for C12E10, C13E10, and Brij-35 correspond to the mean values obtained at variable concentrations of the surfactants.

The mobile phase pH was fixed at 3.0. β-Blockers and TCAs are basic compounds with pK_a = 9–10. Therefore, at the usual working pH range of mobile phases, the analysed species are positively charged. Meanwhile, for sulphonamides, which are acidic, neutral species should be dominant.

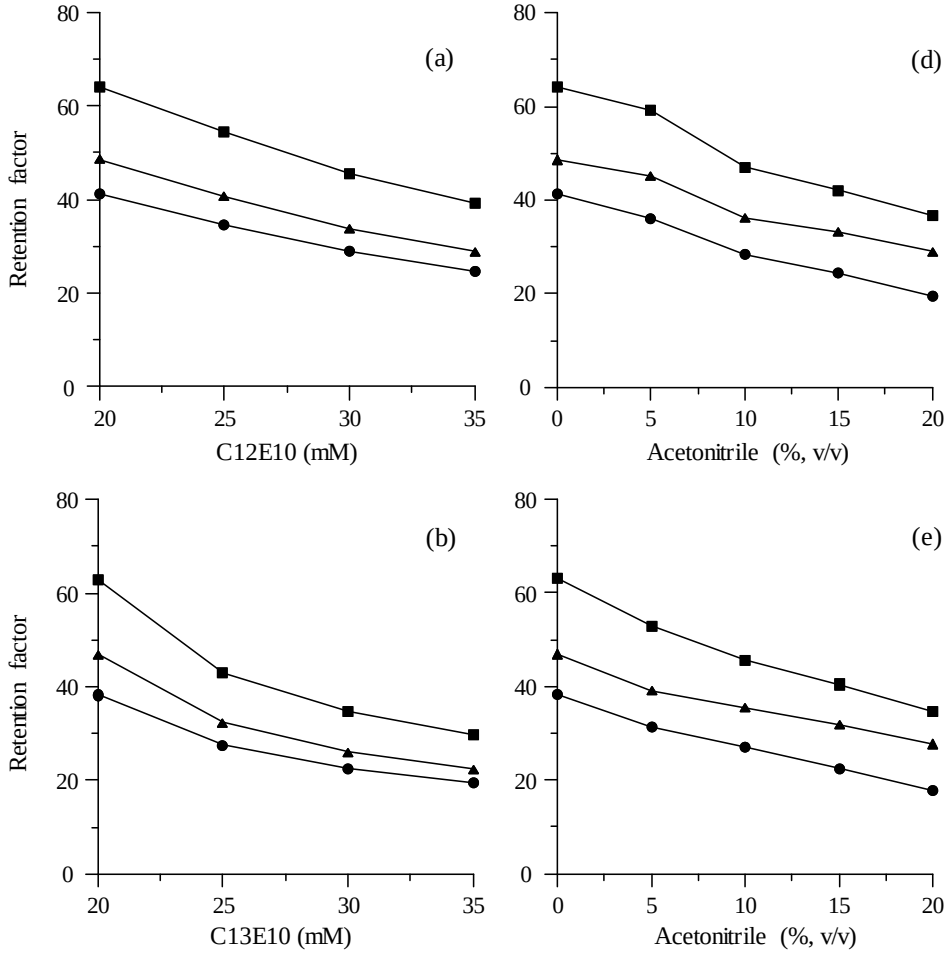


Figure 5.3. Effect of the addition of increasing concentrations of the non-ionic surfactants (a,b,c) and acetonitrile (d,e,f) on the retention of flavonoids. In (d,e,f), mobile phases contained a fixed concentration of the non-ionic surfactants (20 mM): (d) C12E10, (e) C13E10, and (f) Brij-35. Compound identities: 5-hydroxyflavone (■), 3-hydroxyflavone (▲), and flavone (●).

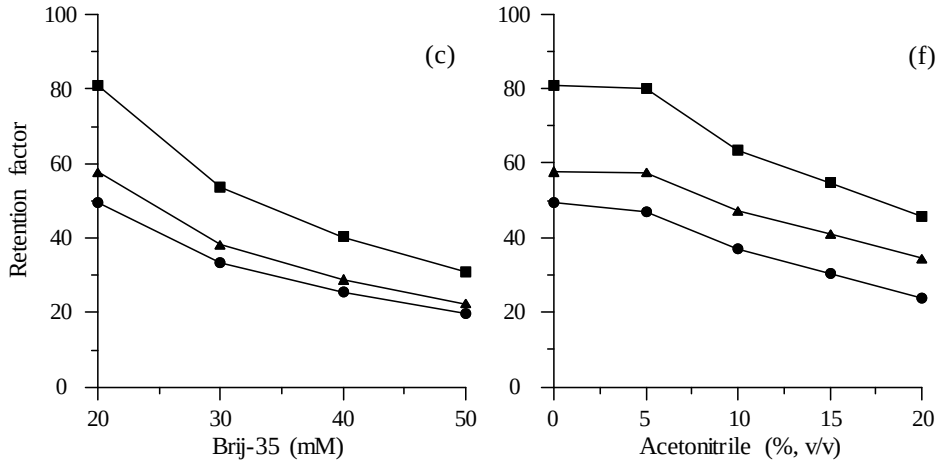


Figure 5.3 (continued).

Minimal and maximal concentrations of the surfactants in the mobile phase were selected to achieve enough retention for the most polar compounds (sulphonamides) and not excessive retention for the most retained ones (flavonoids). Retention times (min) using pure micellar mobile phases of C12E10, C13E10, and Brij-35 were in the ranges 2.4–9.1, 2.2–7.9, and 2.4–10.0, respectively, for sulphonamides (except sulphadimethoxine); 13.5–21.9, 9.7–19.4, and 10.6–24.7 for sulphadimethoxine; 1.8–6.0, 1.7–5.3 and 1.9–6.3 for β-blockers; 4.8–9.5, 4.2–8.1 and 4.1–10.8 for TCAs; and 25.3–64.8, 20.1–62.9 and 18.9–74.8 for flavonoids. As observed, the retention is higher for flavonoids in the presence of the non-ionic surfactants. All these values should be compared with those obtained with hydro-organic mobile phases. Thus, for sulphonamides, β-blockers and TCAs, eluted with 25% acetonitrile, the retention times were in the ranges 1.7–10.4 min, 4.8–8.9 min, and 18.1–47.3

min, respectively. The range was 9.3–23.8 min for flavonoids eluted with 45% acetonitrile.

As observed in Figures 5.2 and 5.3, successive additions of the non-ionic surfactants to the mobile phase gradually reduced the retention owing to the increased micelle concentration. The retention factors were similar for C12E10 and C13E10, and somewhat shorter than those obtained with Bri-35. The elution strength of the three non-ionic surfactants was strong enough to elute the sulphonamides, β-blockers and TCAs, avoiding the use of organic solvent.

Owing to the long retention times for flavonoids, the addition of a relatively small amount of acetonitrile to the non-ionic micellar mobile phases was needed to decrease the retention times to practical values. Figures 5.3d to f show the influence of acetonitrile (5–20%) on the modulation of the retention of flavonoids at a fixed concentration of the three non-ionic surfactants (20 mM).

Upon addition of the organic solvent, the expected trend of decreased retention times was observed. Figures 5.4a and b show chromatograms of the three assayed flavonoids with mobile phases containing 20 mM C13E10 and acetonitrile at two concentrations (5% and 20%). The worst performance of methanol (20%) used as modifier is also shown in Figure 5.4c. As observed, the elution strength of acetonitrile is significantly stronger than that obtained with methanol. Acetonitrile also gives rise to better efficiencies. The CMC values of C13E10 are changed in complicated manner when an organic solvent is added in a wide range. The micelles of C13E10 are possibly disrupted in solution with large amount of acetonitrile (20%) and methanol (20%), changing drastically the separation mechanism.

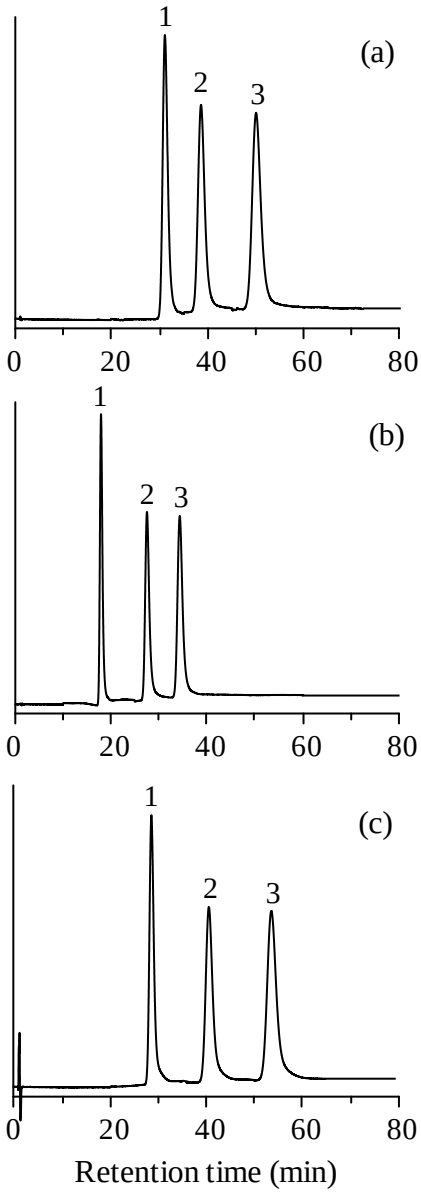


Figure 5.4. Chromatograms of mixtures of the three flavonoids eluted with mobile phases containing 20 mM C13E10 and: (a) 5% acetonitrile, (b) 20% acetonitrile, and (c) 20% methanol. Compound identity: (1) flavone, (2) 3-hydroxyflavone, and (3) 5-hydroxyflavone.

5.4.3. Association of solutes to the modified stationary phase and micelles in the mobile phase

A three-phase model (stationary phase, water and micelle) was proposed succesfully in the 80's to explain the observed retention behaviour in MLC. This model describes the changes in solute retention at increasing concentrations of surfactant, in the presence and absence of organic solvent [19–21]. The approach is valid for both ionic and non-ionic surfactants, and considers the following chemical equilibria proposed by Arunyanart and Cline-Love [20]:

$$A + S \leftrightarrows AS \tag{5.1}$$

$$A + M \leftrightarrows AM \tag{5.2}$$

where A is the solute in bulk water that associates with the stationary phase binding sites (S), and with the surfactant monomers in the micelles dissolved in the mobile phase (M). The displacement of the equilibria in Eqs. (5.1) and (5.2) is quantified by the association constants K_{WS} and K_{AM}, respectively. These constants can be obtained considering the linear dependence between the reversed retention factor ($1/k$), and the molar concentration of surfactant monomers in the mobile phase, [M]:

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}[M] \tag{5.3}$$

The equilibrium constant K_{AS} accounts for the product $\phi[S]K_{WS}$, where ϕ is the phase ratio and [S] the concentration of active sites on the stationary phase. This constant measures the strength of the interaction between solute and stationary phase, and is obtained by extrapolation of the linear $1/k$ versus [M]

plot. Meanwhile, K_{AM} describes the solute-micelle interaction and should be estimated from the slope of the regressed straight-line.

The values of K_{AS} and K_{AM} estimated from Eq. (5.3) are given in Table 5.3 for the studied solutes, eluted with mobile phases that contain only the non-ionic surfactants. Obviously, these constants should be positive. The high uncertainties associated to the estimated values for sulphadimethoxine, amitriptyline, and flavonoids for mobile phases containing C12E10 are explained by the strong interaction of these compounds with the modified stationary phase, and the fact that K_{AS} is obtained from an extrapolated value (see Eq. (5.3)). Note that the high uncertainty in K_{AS} also affects the estimation of K_{AM}.

Since the solute-stationary phase interactions were even stronger for C13E10 and Brij-35 (giving rise to extremely long retention times), the estimation of the association constants of sulphadimethoxine and flavonoids with the stationary phase modified with these surfactants was not possible. The strength of these interactions can be explained by the low polarity of these compounds and the possibility of hydrogen bonding with the monomers of the non-ionic surfactants. For C12E10, although the association constants show certain correlation with the flavonoids polarity, the retention is affected significantly by the number and position of hydroxyl groups in the aromatic rings, as observed in previous work [22].

Table 5.3. K_{AS} and K_{AM} association constants for pure mobile phases of non-ionic surfactants.

	C12E10		C13E10		Brij-35	
Compound	K_{AS}	K_{AM}	K_{AS}	K_{AM}	K_{AS}	K_{AM}
Sulphaguanidine	1.95 ± 0.06	8.64 ± 1.2	2.03 ± 0.29	18.8 ± 5.8	3.01 ± 0.12	17.0 ± 1.2
Sulphathiazole	10.3 ± 0.16	35.4 ± 0.8	13.0 ± 4.3	76.2 ± 28	17.2 ± 1.2	67.6 ± 5.2
Sulphamethizole	15.4 ± 0.47	44.8 ±1.8	20.5 ± 7.9	101 ± 41	26.3 ± 2.1	83.0 ± 6.9
Sulphadimethoxine	322 ± 329	705 ± 720	–	–	–	–
Oxprenolol	1.05 ± 0.04	8.80 ± 1.3	0.98 ± 0.06	7.25 ± 2.2	1.50 ± 0.08	7.64 ± 1.4
Alprenolol	4.16 ± 0.13	23.0 ± 1.3	3.67 ± 0.38	25.3 ± 4.5	5.91 ± 0.6	24.7 ± 3.7
Propranolol	11.5 ± 0.65	63.9 ± 4.1	9.64 ± 2.0	62.6 ± 15	12.0 ± 1.6	54.1 ± 7.9

Table 5.3 (continued).

	C12E10		C13E10		Brij-35	
Compound	K_{AS}	K_{AM}	K_{AS}	K_{AM}	K_{AS}	K_{AM}
Doxepin	20.5 ± 0.88	122 ± 5.5	19.5 ± 8.7	145 ± 67	21.8 ± 6.2	103 ± 30
Imipramine	33.2 ± 5.2	179 ± 28	29.2 ± 17	199 ± 120	34.1 ± 12	133 ± 49
Amitriptyline	58.5 ± 6.4	291 ± 32	73.5 ± 74	473 ± 607	55.6 ± 30	210 ± 114
Flavone	625 ± 502	694 ± 558	–	–	–	–
3-Hydroxyflavone	714 ± 526	676 ± 498	–	–	–	–
5-Hydroxyflavone	526 ± 209	353 ± 141	–	–	–	–

In general, it can be observed that K_{AM} is significantly larger than K_{AS} (except for flavonoids), indicating stronger interaction with micelles. As commented, the association of solutes with non-ionic surfactants should obey both the differential polarity between the solutes and modified stationary phase, and the possibility of establishing additional interactions (mainly, hydrogen bonds of solutes with the hydroxyl group in the ethoxylated chain of the surfactant monomers). These specific interactions are not possible when solutes lack of groups able to form hydrogen bonds, or these groups are not easily accessible to the surfactant.

The solutes included in our study correspond to different categories. On the one hand, the basic compounds (β-blockers and TCAs), which are cationic at the mobile phase pH, were mainly retained according to their polarity, as shown in Figures 5.5a and b for C13E10. In the plots, the logarithmic values of the association constants (log K_{AS} and log K_{AM}, Table 5.3) are plotted *versus* the log $P_{o/w}$ values given in Table 5.2. As observed, the correlation was satisfactory (similar correlations were obtained for C12E10 and Brij-35).

Figures 5.5c and d also indicate that the association constants (and consequently, the retention) for sulphonamides (except sulphadimethoxine) also obey solute polarity. However, the interactions with both stationary and mobile phases were stronger with regard to the basic compounds, according to their polarity (compare with Figures 5.5a and b). This can be explained by the interaction of the sulphonamide group with the ethoxylated chain of the non-ionic surfactants. The even higher strength of the interactions of sulphadimethoxine with both stationary and mobile phases should be justified by the presence of the methoxyl groups.

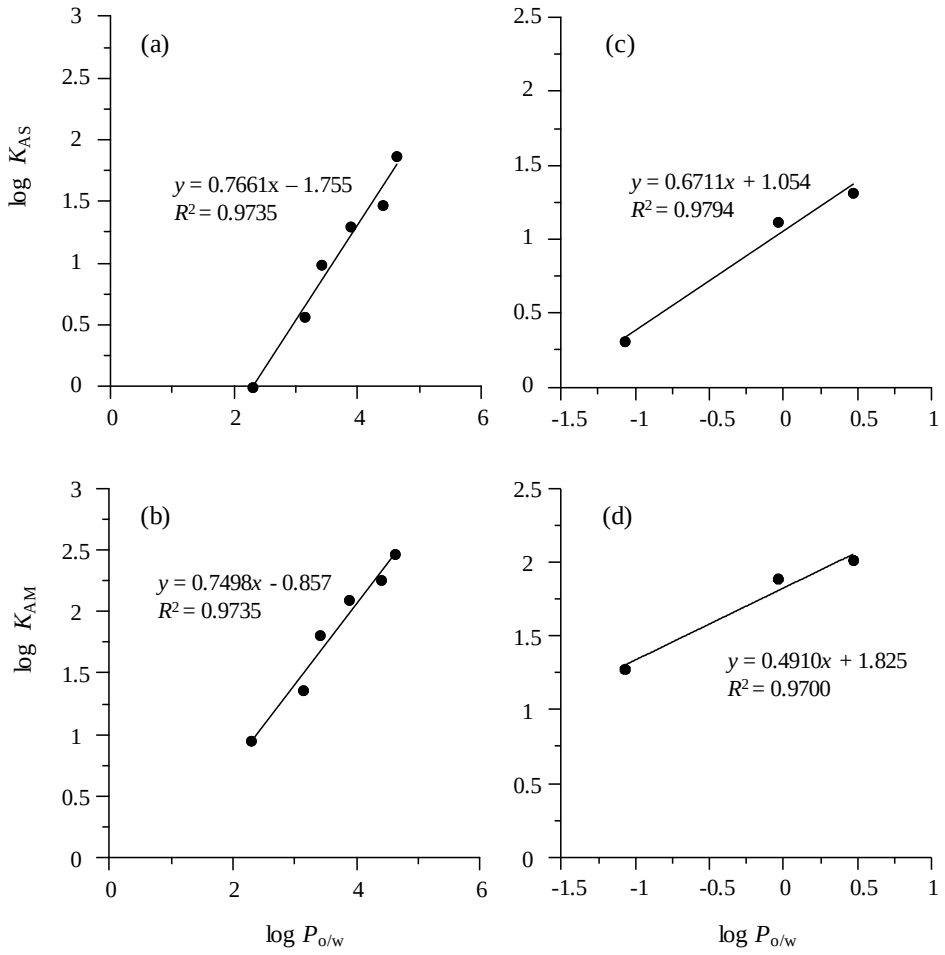


Figure 5.5. Correlations between the octanol-water partition coefficients and solute-stationary phase (a,c), or solute-mobile phase (b,d) association constants for β-blockers and TCAs (a,b), and sulphonamides (c,d), using C13E10.

5.4.4. Peak profiles

Half-width plots give a global overview of the changes that occur in the width and asymmetry of chromatographic peaks, for a given column and mobile phase. They are graphical representations of the left (*A*) and right (*B*) half-widths versus the retention time. The half-widths were measured at 10% peak height to monitor the asymmetry (measured as *B*/*A*), without interference of the baseline noise of chromatograms. The validity of these plots was demonstrated in previous work for micellar and hydro-organic RPLC with different families of compounds and types of columns [23,24]. The plots will be described here by the following equations:

$$A = m_A\, t_R + A_0 \quad (5.4)$$

$$B = m_B\, t_R + B_0 \quad (5.5)$$

where m_A and m_B are the slopes of the linear correlations for the left and right half-widths, respectively, and A_0 and B_0 include the extra-column contribution to the peak broadening. Eqs. (5.4) and (5.5) describe the peak shape behaviour for peaks of compounds eluted at different retention times. These parameters are also useful to characterise chromatographic columns.

The sum of slopes ($m_A + m_B$) describes the peak broadening rate inside the column, whereas the ratio (m_B/m_A) is a measurement of the asymmetry of peaks eluted at a time where the extra-column contribution is non-significant. Global plots were built considering all mobile phases prepared for each surfactant at varying concentration for groups of compounds, in order to study the effect on the peak shape.

Figures 5.6a and b depict the half-width plots for mobile phases containing C13E10 and Brij-35. They describe the peak profile of both β-blockers and TCAs studied in this work. Similar plots to those for C13E10 were obtained for C12E10. The slope for the right half-widths was always appreciably larger than for the left half-width, indicating that the peaks were tailing. Compared to Brij-35, peaks were narrower and more symmetrical with C12E10 and C13E10.

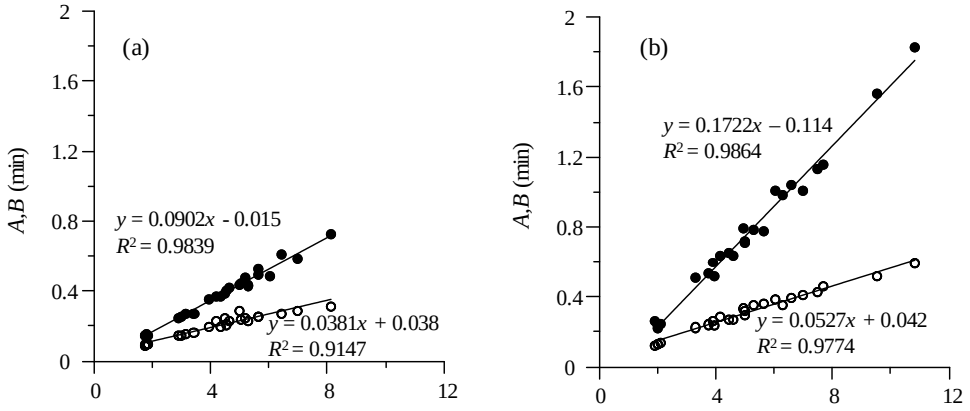


Figure 5.6. Half-width plots obtained for β-blockers and TCAs, eluted with pure mobile phases of C13E10 (a), and Brij-35 (b). Surfactants were used at different concentrations. Left (*A*, ○) and right (*B*, ●) half-widths.

Figures 5.7a to d show half-widths plots for the group of flavonoids eluted with mobile phases of C13E10 and Brij-35, in the absence (Figures 5.7a and c) and presence of acetonitrile (Figures 5.7b and d). It can be observed that, in the presence of the organic solvent, peaks were broader and more asymmetrical, especially for Brij-35.

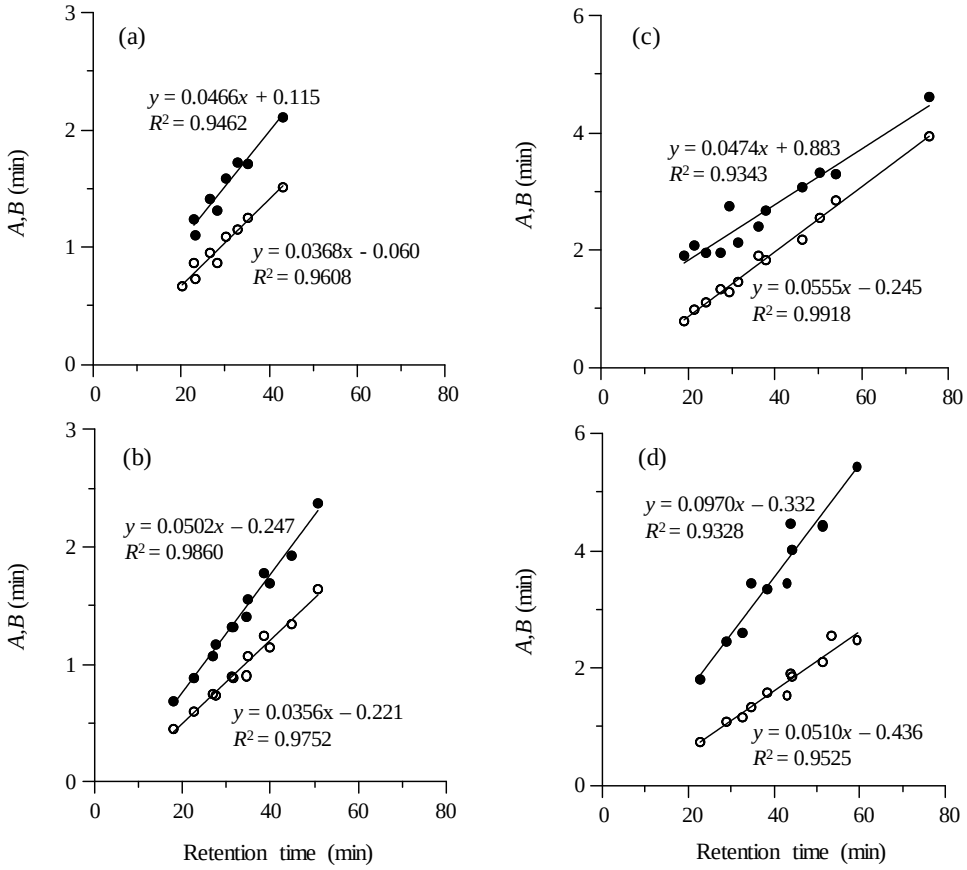


Figure 5.7. Half-width plots obtained for flavonoids eluted with pure mobile phases of C13E10 (a), and Brij-35 (c), and hybrid mobile phases of C13E10 and acetonitrile (b), and Brij-35 and acetonitrile (d). Surfactants and acetonitrile were used at different concentrations. Left (*A*, ○) and right (*B*, ●) half-widths.

The good alignement of the data in the plots in Figure 5.6 and 5.7 shows that peak broadening follows a similar pattern for the basic compounds, on the one hand, and for flavonoids on the other. The reason of making different representations for the peak half-widths of basic compounds and flavonoids is not only the appreciably larger retention for flavonoids, but also their particular kinetics due to their strong interaction with the polar groups of the non-ionic surfactants, through hydrogen bonding. This hinders the desorption of flavonoids from the modified stationary phase, as demonstrated in previous work for Brij-35 [22]. The sum of slopes of the linear segments for the left (m_A) and right (m_B) half-widths, and its ratio, are given in Table 5.4 for the three surfactants. As observed, the asymmetry measured as m_B/m_A was significantly above unity (tailing peaks), except for pure micellar mobile phases of Brij-35, which yielded fronting peaks. In general, better performance (regarding peak width and asymmetry) was observed for mobile phases of C12E10 and C13E10, compared to Brij-35.

The combination of the data in Figures 5.2, 5.6a and 5.6b, on the one hand, and Figures 5.3 and 5.7a to d, on the other, gives information on the peak shape for each compound at different mobile phase compositions.

The chromatograms depicted in Figure 5.8 illustrate the obtained performance in the separation of TCAs, with mobile phases of the non-ionic surfactants C12E10 (Figure 5.8a), C13E10 (Figure 5.8b), and Brij-35 (Figure 5.8c). As commented, the retention of basic compounds with mobile phases of the anionic surfactant SDS is much higher, compared to that obtained with the non-ionic surfactants.

Table 5.4. Half-width plots parameters for mobile phases containing a non-ionic surfactant.[a]

		$m_A + m_B$	m_B/m_A
β-Blockers and TCAs			
Pure micellar	C12E10	0.129 ± 0.0034	2.909 ± 0.18
	C13E10	0.128 ± 0.0034	2.368 ± 0.16
	Brij-35	0.225 ± 0.0045	3.245 ± 0.13
Flavonoids			
Pure micellar	C12E10	0.068 ± 0.0051	1.061 ± 0.16
	C13E10	0.084 ± 0.0050	1.266 ± 0.15
	Brij-35	0.103 ± 0.0043	0.839 ± 0.076
Hybrid with acetonitrile	C12E10	0.100 ± 0.0020	1.564 ± 0.071
	C13E10	0.086 ± 0.0026	1.389 ± 0.087
	Brij-35	0.148 ± 0.0100	1.902 ± 0.23

[a] m_A and m_B are the slopes of the left and right half-width plots, respectively.

The chromatogram in Figure 5.8d was obtained with a mobile phase containing SDS and a C8 column instead of the C18 column used for the non-ionic surfactants. Even with the C8 column (giving rise to smaller retention), a strong modifier (pentanol) was needed to get sufficiently short times. The best peak performance and shortest analysis time were obtained with C13E10 (Figure 5.8b). Previous work has shown that when eluted with mobile phases of SDS, sulphonamides [25], β-blockers [9], and flavonoids [26] also require the addition of organic solvent to provide sufficiently short retention times.

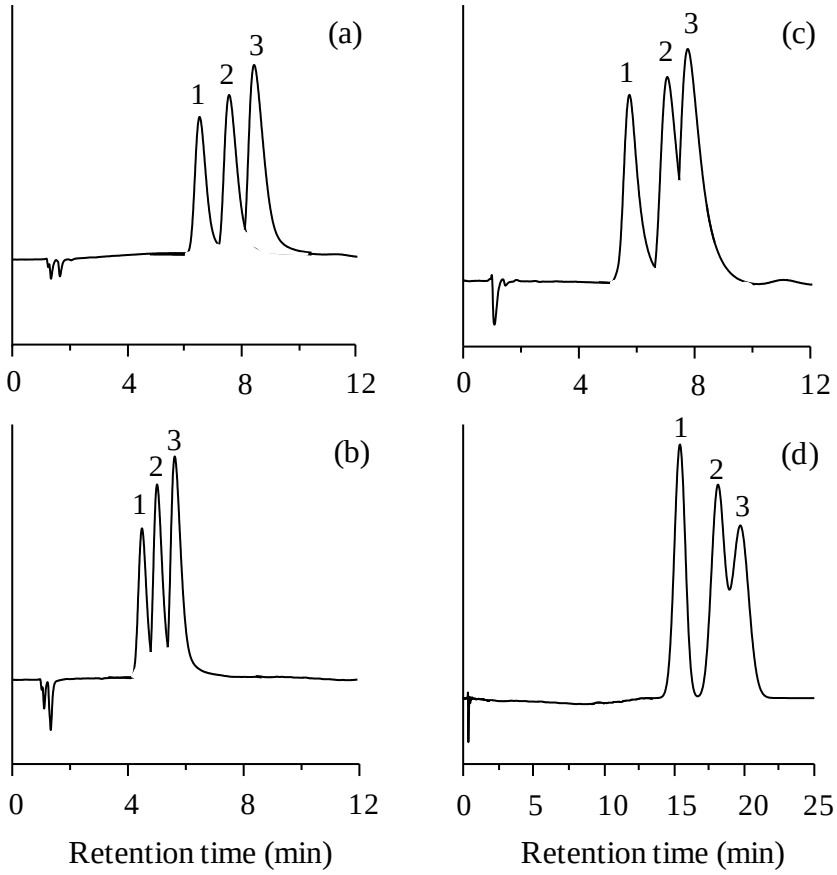


Figure 5.8. Chromatograms of mixtures of three TCAs eluted with micellar mobile phases containing: (a) 30 mM C12E10, (b) 30 mM C13E10, (c) 30 mM Brij-35, and (d) 0.12 M SDS–2% 1-pentanol. Column: (a to c) Zorbax Eclipse XDB C18, and (d) Zorbax Eclipse XDB C8. Compound identity: (1) doxepin, (2) imipramine, and (3) amitriptyline.

5.4.5. Surfactant desorption from the stationary phase

In MLC, there is some concern about surfactant desorption from the stationary phase, in order to recover the original column conditions before changing the nature of the mobile phase. After three decades of MLC experience, it is widely demonstrated that SDS can be almost or completely removed from C18 stationary phases [27–30]. The information about the adsorption of Brij-35 is still insufficient, although in a recent report we checked some irreversible adsorption on a C18 column [9]. To our knowledge, no information about the reversible/irreversible adsorption of C12E10 and C13E10 on C18 columns is available in the literature. For this reason, a study was performed consisting on the comparison of retention times for the whole set of compounds eluted with 45% acetonitrile at pH 3, before and after flushing the column with pure or hybrid mobile phases (i.e., without and with acetonitrile). In all experiments, column regeneration was made with methanol. A dedicated column was used for each surfactant.

Table 5.5 shows the initial retention times obtained with an acetonitrile-water mixture before starting the chromatographic study with the non-ionic surfactants, and the times obtained after using mobile phases with C12E10 and C13E10, and cleaning the column.

Table 5.5. Retention times obtained with 45% acetonitrile-water before and after the elution with the non-ionic surfactants C12E10 and C13E10.

Compound	t_R (min)[a]	t_R (min)[b]	t_R (min)[c]
Sulphaguanidine	1.48	1.52	1.53
Sulphathiazole	1.63	1.60	1.62
Sulphamethizole	1.85	1.81	1.84
Sulphadimethoxine	2.85	2.72	2.81
Oxprenolol	1.64	1.56	1.57
Alprenolol	1.98	1.85	1.88
Propranolol	1.94	1.82	1.84
Doxepin	2.30	2.09	2.14
Imipramine	2.90	2.58	2.66
Amitriptyline	3.19	2.82	2.91
Flavone	9.50	8.86	9.54
3-Hydroxyflavone	15.00	13.93	14.77
5-Hydroxyflavone	23.87	20.95	22.92

[a] Before elution with the micellar mobile phases.

[b] After elution with micellar mobile phases containing C12E10.

[c] After elution with micellar mobile phases containing C13E10.

Figure 5.9 depicts representative chromatograms for compounds belonging to each group studied in this work. No significant differences were observed for the retention times obtained with the column exposed to C13E10 (the observed changes can be partially due to small differences in the concentration of acetonitrile in the prepared mobile phases, in the absence and presence of non-ionic surfactants). In contrast, after eluting the test compounds with C12E10, the retention times decreased significantly, especially for the group of TCAs and flavonoids, which could be attributed to incomplete desorption of C12E10 after cleaning the stationary phase.

5.5. Conclusions

Most reported analytical applications in MLC make use of the anionic surfactant SDS, being few the references on MLC with non-ionic surfactants, with the exception of Brij-35 (C12E23). This work examines the potential of two non-ionic polyoxyethylene alkyl ethers with a shorter ethoxylated chain (C12E10 and C13E10), as surfactants in MLC for separating diverse types of compounds (sulphonamides, β-blockers, TCAs, and flavonoids). The chromatographic behaviour with these surfactants was compared with Brij-35. Other non-ionic surfactants (Brij-L4, Triton-X100, Triton-X114, Span 20, and IGEPAL CO-630) were checked to be less feasible or unfeasible for MLC, due to their low solubility in water or high absorptivity above 200 nm.

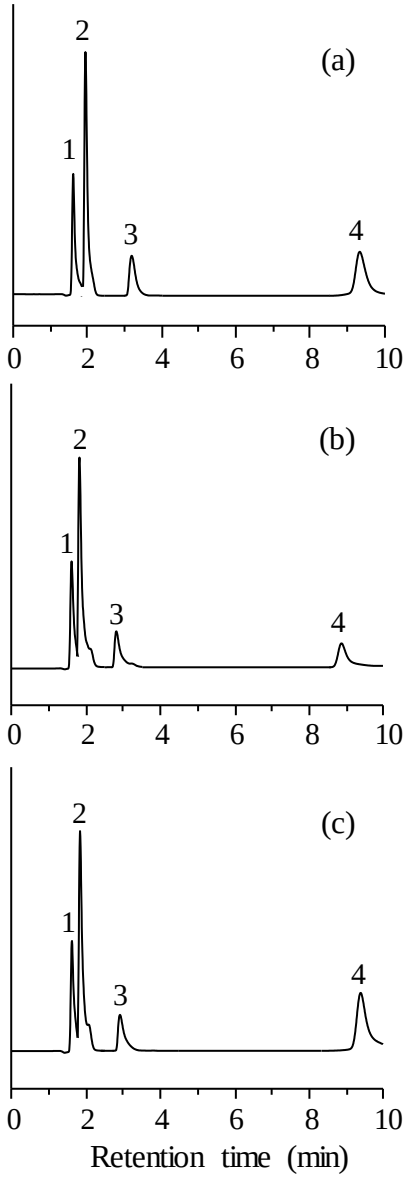


Figure 5.9. Chromatograms of mixtures of four selected compounds eluted with a hydro-organic mobile phase containing 45% acetonitrile, before (a) and after flushing the column with C12E10 (b), and C13E10 (c). Compound identity: (1) sulphathiazole, (2) propranolol, (3) amitriptyline, and (4) flavone.

The results showed that the chromatographic behaviour of the selected compounds in the non-ionic micellar media of C12E10 and C13E10 was similar and dependent on solute nature. In the presence of C12E10 and C13E10, the polarity was increased, and consequently, the retention times of sulphonamides, β-blockers, and TCAs decreased. The retention is similar to that achieved with hydro-organic mobile phases with a relatively high amount of organic solvent. In contrast, the retention of flavonoids is high in the presence of the non-ionic surfactants, due to hydrogen bonding of these compounds with the ethoxylated chain in these surfactants. Chromatographic peaks were narrower and more symmetrical in the presence of C12E10 and C13E10, with regard to Brij-35 with a longer polyoxyethylene chain (C12E23). The addition of an organic solvent, such as acetonitrile, to the non-ionic micellar mobile phases decreased the retention times of the highly retained flavonoids, but the peak shape was deteriorated.

As commented, the adsorption of the three non-ionic surfactants (C12E10, C13E10, and Brij-35) on the stationary phase confers higher polarity that decreases the retention times, for most solutes. This avoids in many cases the addition of organic solvent to the micellar mobile phase, which increases the greenness of the RPLC separation of basic compounds with respect to classical RPLC or MLC with hybrid mobile phases of SDS and organic solvent. Another important advantage is the biodegradable character of the reagents used in the mobile phase: SDS is a fatty alcohol sulphate that is aerobically degraded [31], and polyoxyethylene lauryl and tridecyl ethers are derivatives of fatty alcohol ethoxylates, developed as eco-friendly alternatives to alkyl phenol ethoxylates [32]. However, it should be reminded that organic modifiers may be useful to modulate the selectivity and significantly improve the efficiency. Pure micellar mobile phases can thus result in significant limitations in this regard.

Finally, among the three surfactants, only C13E10 is seemingly non-irreversibly adsorbed on the stationary phase. However, further studies are still necessary to appraise the usefulness of this surfactant. Although the obtained results do not solve the problem of poor efficiency when non-ionic surfactants are used in MLC, this work shows that the reduction in the number of ethylene oxide units from 23 (Brij-35) to 10 (C12E10 and C13E10) improves the chromatographic performance. It should be noted, however, that a further decrease seems not possible, due to solubility problems, as shown for Brij L4 (C12E4).

5.6. References

[1] A. Berthod, M.C. García Álvarez-Coque, *Micellar Liquid Chromatography*, Marcel Dekker, New York, 2000.

[2] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, New insights and recent developments in micellar liquid chromatography, *Sep. Purif. Rev.* 38 (2009) 45–96.

[3] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, S. Carda Broch, Micellar liquid chromatography: Method development and applications in *Analytical Separation Science Series* (edited by J. Anderson, A. Berthod, A. Stalcup, V. Pino Estévez), Vol. 2, Wiley-VCH, New York, 2015, pp. 407–460.

[4] J. Esteve Romero, S. Carda Broch, M. Gil Agustí, M.E. Capella Peiró, D. Bose, Micellar liquid chromatography for the determination of drug materials in pharmaceutical preparations and biological samples, *Trends Anal. Chem.* 24 (2005) 75–91.

[5] A. Berthod, S. Tomer, J.G. Dorsey, Polyoxyethylene alkyl ether nonionic surfactants: Physicochemical properties and use for cholesterol determination in food, *Talanta* 55 (2001) 69–83.

[6] N. Memon, M.I. Bhanger, M.Y. Khuhawer, Determination of preservatives in cosmetics and food samples by micellar liquid chromatography, *J. Sep. Sci.* 28 (2005) 635–638.

[7] W. Thogchai, B. Liawruangrath, Micellar liquid chromatographic determination of arbutin and hydroquinone in medicinal plant extracts and commercial cosmetic products, *Int. J. Cosmet. Sci.* 35 (2013) 257–263.

[8] Y.M. Dong, N. Li, Q. An , N.W. Lu, A novel nonionic micellar liquid chromatographic method for simultaneous determination of pseudoephedrine, paracetamol, and chlorpheniramine in cold compound preparations, *J. Liq. Chromatogr. Relat. Technol.* 38 (2015) 251–258.

[9] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Comparison of the performance of non-ionic and anionic surfactants as mobile phase additives in the RPLC analysis of basic drugs, *J. Sep. Sci.* 34 (2011) 623–630.

[10] J.J. Fernández Navarro, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography without organic solvent for determination of tricyclic antidepressants, *J. Sep. Sci.* 35 (2012) 1303–1309.

[11] R. Nakao, C. Haldin, Mixed anionic and non-ionic micellar liquid chromatography for high-speed radiometabolite analysis of positron emission tomography radioligands, *J. Chromatogr. A* 1281 (2013) 54–59.

[12] E.K. Paleologos, S.D. Chytiri, I.N. Savvaidis, M.G. Kontominas, Determination of biogenic amines as their benzoyl derivatives after cloud point extraction with micellar liquid chromatographic separation, *J. Chromatogr. A* 1010 (2003) 217–224.

[13] R. Halko, M. Hutta, Study of high-performance liquid chromatographic separation of selected herbicides by hydro-methanolic and micellar liquid chromatography using Genapol X–080 non-ionic surfactant as mobile phase constituent, *Anal. Chim. Acta* 466 (2002) 325–333.

[14] S. Carda Broch, A. Berthod, Countercurrent chromatography for the measurement of the hydrophobicity of sulfonamide amphoteric compounds, *Chromatographia* 59 (2004) 79–87.

[15] S. Carda Broch, A. Berthod, pH dependence of the hydrophobicity of β-blocker amine compounds measured by counter-current chromatography, *J. Chromatogr. A* 995 (2003) 55–66

[16] C.J. Drayton (Ed.), *Comprehensive Medicine Chemistry*, Vol. 6, Pergamon Press, Oxford, 1990.

[17] *ACD/ChemSketch software for Academic and Personal Use* (http://www.acdlabs.com/resources/freeware/chemsketch/)

[18] J.R. Torres Lapasio, *MICHROM Software*. Marcel Dekker, New York, 2000.

[19] D.W. Armstrong, F. Nome, Partitioning behavior of solutes eluted with micellar mobile phases in liquid chromatography, *Anal. Chem.* 53 (1981) 1662–1666.

[20] M. Arunyanart, L.J. Cline-Love, Model for micellar effects on liquid chromatography capacity factors and for determination of micelle-solute equilibrium constants, *Anal. Chem.* 56 (1984) 1557–1561.

[21] M.C. García Álvarez-Coque, J.R. Torres Lapasió, J.J. Baeza Baeza, Modelling of retention behaviour of solutes in micellar liquid chromatography, *J. Chromatogr. A* 780 (1997) 129–148.

[22]. J.J. Baeza Baeza, Y. Dávila, J.J. Fernández Navarro, M.C. García Álvarez-Coque, Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC, *Anal. Bioanal. Chem.* 404 (2012) 2973–2984.

[23] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[24] J.J. Baeza Baeza, M.J. Ruiz Ángel, M.C. García Álvarez Coque, S. Carda Broch, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

[25] R.D. Caballero, S. Carda Broch, M.C. García Álvarez-Coque, Hydro-organic and micellar-organic reversed-phase liquid chromatographic procedures for the evaluation of sulphonamides in pharmaceuticals, *Anal. Lett.* 34 (2001) 1189–1203.

[26] M.R. Hadjmohammadi, S. Saman, S.J. Nazari, Separation optimization of quercetin, hesperetin and chrysin in honey by micellar liquid chromatography and experimental design, *J. Sep. Sci.* 33 (2010) 3144–3151.

[27] A. Berthod, I. Girard, C. Gonnet, Micellar liquid chromatography: adsorption isotherms of two ionic surfactants on five stationary phases, *Anal. Chem.* 58 (1986) 1356–1358.

[28] A. Berthod, I. Girard, C. Gonnet, Stationary phase in micellar liquid chromatography: Surfactant adsorption and interaction with ionic solutes, *ACS Symp. Ser.* 342 (1987) 130–141.

[29] D.E. Keller, R.G. Carbonell, P.K. Kilpatrick, Adsorption equilibria and desorption rates of charged ethoxylated surfactants on octadecyl silica: Role of electrostatics, *J. Colloid Interface Sci.* 155 (1993) 124–136.

[30] C. Ortiz Bolsico, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Adsorption of the anionic surfactant sodium dodecyl sulfate on a C18 column under micellar and high submicellar conditions in reversed-phase liquid chromatography, *J. Sep. Sci.* 38 (2015) 550–555.

[31] O.R.T. Thomas, G.F. White, Metabolic pathway for the biodegradation of sodium dodecyl sulfate by Pseudomonas sp. C12B, *Biotechnol. Appl. Biochem.* 11 (1989) 318–327.

[32] M.T. Scott, M.N. Jones, The biodegradation of surfactants in the environment, *Biochim. Biophys. Acta* 1508 (2000) 235–251.

CHAPTER 6

REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH MIXED MICELLAR MOBILE PHASES OF BRIJ-35 AND SODIUM DODECYL SULPHATE: A GREEN METHOD FOR THE ANALYSIS OF BASIC COMPOUNDS

6.1. Abstract

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatographic (RPLC) mode, which uses a surfactant as a modifier, with significant changes in retention and selectivity with regard to the classical RPLC mode that employs mixtures of water and organic solvent. The anionic sodium dodecyl sulphate (SDS) is the most usual surfactant in MLC, but it also requires the addition of an organic solvent to decrease the retention times and increase the efficiency. In particular, positively charged basic compounds are strongly retained by the stationary phase modified by adsorption of SDS monomers and require the addition of a strong solvent, such as propanol or pentanol. The non-ionic surfactant Brij-35 is much less common in MLC, but has the interesting feature of increasing the stationary phase polarity which remains neutral. This decreases the retention significantly and can eliminate the need of an organic solvent, giving rise to successful "green" RPLC procedures. However, the retention of polar compounds may be too short if these do not exhibit specific interactions with the non-ionic surfactant. In this work, MLC with mixtures of Brij-35 and SDS without organic solvent are investigated for the analysis of basic compounds. The research has been carried out with tricyclic antidepressants (TCAs) and β-blockers, which are compounds of pharmaceutical interest with different polarities. The chromatographic performance in the mixed micellar system is examined in terms of retention behaviour and peak profiles, and compared with the performance achieved with MLC systems containing a single surfactant. In the mixed micellar system, the analysis of β-blockers of diverse polarity is carried out with good resolution and adequate analysis time. For TCAs, mobile phases with only Brij-35 are preferable.

6.2. Introduction

The idea of adding a surfactant to the mobile phase in reversed-phase liquid chromatography (RPLC) is a practice that has been explored over the three last decades, with significantly different results in the analysis of compounds of diverse nature with respect to those obtained in classical RPLC that employs mixtures of water and organic solvent [1–3]. Surfactant monomers are adsorbed on the alkyl-bonded chains of the stationary phase (usually C8 or C18) through hydrophobic interactions, modifying its nature. This creates a neutral or charged double layer (depending on the nature of the adsorbed surfactant), which interacts with solutes. For stationary phases modified with a charged surfactant, a dynamic ion-exchanger is yielded. Moreover, above the critical micelle concentration, surfactant monomers in the mobile phase aggregate to form small clusters or micelles that also interact with solutes. The formation of micelles has given rise to the most accepted name for this chromatographic mode: micellar liquid chromatography (MLC). However, the main changes in the observed chromatographic performance are due to the adsorption of surfactant monomers on the stationary phase.

An attractive feature of MLC is the significant reduction in the amount of organic solvent with respect to the classical RPLC. Another fascinating feature is the capability of micelles of some surfactants to solubilise proteins that has been effectively exploited for the direct injection of untreated biological fluids onto RPLC columns, avoiding previous extraction steps with organic solvents [4,5]. For this reason, MLC is considered a “green” RPLC mode [6].

Although several surfactants of diverse natures can be used in MLC, the anionic sodium dodecyl sulphate (SDS) has been selected in most reports [1,2]. The frequent use of SDS has somehow relegated the research on the potential of other surfactants as modifiers, such as non-ionic surfactants. One of such

surfactants is polyoxyethylene(23)lauryl ether ($(C_2H_4O)_{23}C_{12}H_{25}OH$), commercially known as Brij-35, which has been explored by a few authors as an alternative to SDS with satisfactory results [7–17]. Brij-35 has been also reported as an ideal modifier in quantitative structure-activity relationship (QSAR) studies in RPLC, due to its capability to mimic biopartitioning processes [18,19].

When RPLC columns are used with mixtures of water and organic solvent, solute retention is mainly based on the hydrophobic interaction with the alkyl-bonded layer of the stationary phase, together with the solving power of the organic solvent in the mobile phase. When cationic compounds are analysed, additional ion-exchange interactions with residual anionic silanols on the silica packing are established. These interactions are also characterised by slow kinetics, which results in broad and skewed peaks [20,21]. Mobile phases containing SDS minimise the interaction of cationic solutes with the residual silanols: the long hydrophobic chain of SDS monomers covers the stationary phase with the sulphate group oriented outside, resulting in a negatively charged stationary phase [22]. This enhances remarkably the efficiency and peak symmetry of basic compounds, such as tricyclic antidepressants (TCAs) and β-blockers.

However, due to the attraction of the cationic basic compounds to the anionic SDS modified stationary phase their retention increases significantly. This forces the addition of a relatively high amount of acetonitrile or propanol to elute most β-blockers [23,24], and pentanol is required to elute TCAs [25]. If Brij-35 is used instead of SDS, its monomers are adsorbed on the stationary phase with the hydrophilic polar end of the molecule oriented away from the surface. This increases the polarity of the stationary phase without providing a net charge, which allows compounds of low or intermediate polarity be eluted

without the addition of organic solvent [26,27]. However, polar compounds as most β-blockers, which do not establish specific interactions with Brij-35, are not retained.

In this work, it is shown that a solution for the described limitations of mobile phases containing a single surfactant (Brij-35 or SDS), in the RPLC analysis of β-blockers, is the use of mobile phases that include both surfactants, so that the favourable characteristics of each surfactant are combined. These mixed systems have been investigated in the last few decades outside the field of chromatography [28]. Thus, it is known that when an anionic surfactant (such as SDS) and a non-ionic surfactant (such as Brij-35) are mixed in aqueous solution, their tails establish hydrophobic interactions, and their head groups ion-dipole and hydrophilic interactions, giving rise to the formation of mixed micelles. Systems containing mixed surfactants have been scarcely used in MLC [29–32], the combination of Brij-35 and SDS being the most common. The mixed systems may result in improvements in the chromatographic performance with respect to the use of mobile phases containing a single surfactant.

The capability of mobile phases containing exclusively Brij-35 or the combination of Brij-35 and SDS to elute basic compounds, specifically TCAs and β-blockers, is studied here. The results are analysed in terms of retention, peak profiles, selectivity and resolution. Since there is no organic solvent in the mobile phase, the greenness of the method is increased with respect to classical RPLC or MLC with hybrid mobile phases of SDS and organic solvent. Another important advantage is the biodegradable character of the reagents used in the mobile phase: SDS is a fatty alcohol sulphate that is aerobically degraded [33], and Brij-35 is a derivative of fatty alcohol ethoxylate, developed as an

eco-friendly alternative to alkyl phenol ethoxylates [34]. It is shown how their combined use gives rise to a successful "green" RPLC separation of β-blockers.

6.3. Experimental

6.3.1. Reagents

The probe compounds were seven TCAs (doxepin, amitriptyline, clomipramine, imipramine, maprotiline, nortriptyline, and trimipramine) and six β-blockers (alprenolol, atenolol, celiprolol, metoprolol, oxprenolol, and propranolol), all from Sigma (St. Louis, MO, USA). All these compounds are basic (pK_a = 9–10), which means that at the working pH of the mobile phase (~3) they are positively charged. Most experiments were carried out with the seven TCAs and the two most hydropobic β-blockers (propranolol and alprenolol), all of them sufficiently retained with Brij-35. As will be commented below, atenolol, celiprolol, metoprolol and oxprenolol were eluted close to the dead time with Brij-35.

Stock solutions of 100 μg/mL of the drugs were prepared in a small amount of ethanol with the aid of an Elmas 15h ultrasonic bath from Elmasonic (Singen, Germany), and diluted with water. These solutions were stable for at least two months at 4 ^{o}C and were diluted before injection with an aqueous solution of 0.02 M Brij-35 (Fluka, Buchs, Switzerland) up to a final concentration of 20 μg/mL. Uracil (Acros Organics, Geel, Belgium) was used as dead time marker.

Mobile phases containing Brij-35 or a mixture of Brij-35 and SDS (99% purity, Merck, Darmstad, Germany) were prepared at different concentrations, buffered at pH ~3.0 with 0.01 M sodium dihydrogen phosphate (Panreac, Barcelona, Spain) and HCl, to reduce the amount of free silanols in

the column. The solutions of the probe compounds and mobile phases were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA). Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

6.3.2. Apparatus and column

An Agilent chromatograph (Waldbronn, Germany), equipped with a quaternary pump (Series 1260), an autosampler (Series 1200), a thermostated column compartment (Series 1100) set at 25 ^{o}C, a diode array detector, and an HPChemStation (Agilent, B.02.01) for data acquisition, were used. TCAs and β-blockers were monitored at 254 and 225 nm, respectively.

The chromatographic column was a Zorbax Eclipse C18 (Agilent) with the following characteristics: 150 mm × 4.6 mm i.d., 5 μm particle size, 10% carbon load, 180 m^2/g surface area, and 80 Å pore size, which was connected to a similar 30 mm pre-column for protection. The flow rate was 1 mL/min. Duplicate injections were made using an injection volume of 20 μL. The mobile phases were recycled between runs and also during the analysis (as long as a small number of injections was made) to reduce the consumption of reagents. This increases the sustainability of the procedure. The chromatographic system was periodically rinsed with water and methanol (around 20 mL) to remove the surfactant from the stationary phase.

6.3.3. Experimental design

Based on previous experience [24–26], two mobile phases containing either 0.02 M Brij-35 or 0.15 M SDS were selected as references. SDS was added to the 0.02 M Brij-35 solution at the following concentrations: 0.02, 0.04, 0.08,

0.12, and 0.15 M. Similarly, Brij-35 was added to the 0.15 M SDS solution at the concentrations: 0.01, 0.02, 0.03, 0.04, and 0.05 M (the latter concentration being close to the solubility of Brij-35 in water). The minimal and maximal concentrations of the surfactants in the mobile phase were selected to achieve enough retention for the most polar compounds, and not excessive retention for the most apolar.

6.4. Results and discussion

6.4.1. Retention capability of the mixed Brij-35/SDS micellar systems

The modified stationary phase coated by polyoxyethylene chains of Brij-35 is significantly more polar than the original C18 bonded phase. This reduces the retention times of the analysed compounds, if no specific interactions with the adsorbed surfactant are established, such as hydrogen-bonding between the hydroxyl groups in the surfactant and phenolic compounds [27]. The micellised surfactant in the mobile phase also changes the elution strength and selectivity (relative retention). Micelles formed by Brij-35 contain a dodecyl apolar core (similarly to SDS) and a relatively polar surface formed by oxyethylene chains, which interact with the solutes in the mobile phase.

Surfactant monomers of SDS and Brij-35 compete for adsorption sites on the stationary phase. The long hydrophobic chain of SDS monomers is inserted into the alkyl-bonded layer (similarly to Brij-35), with the sulphate group oriented outside (Figure 6.1). Therefore, in the mixed system, the modified stationary phase will have a negative charge, although with smaller density than in a system exclusively modified with SDS. Different studies have also demonstrated that Brij-35 and SDS form mixed micelles in the mobile phase,

with a common core involving their hydrophobic chains [35]. Therefore, mixed micellar systems should provide different chromatographic behaviour with respect to the single surfactant systems.

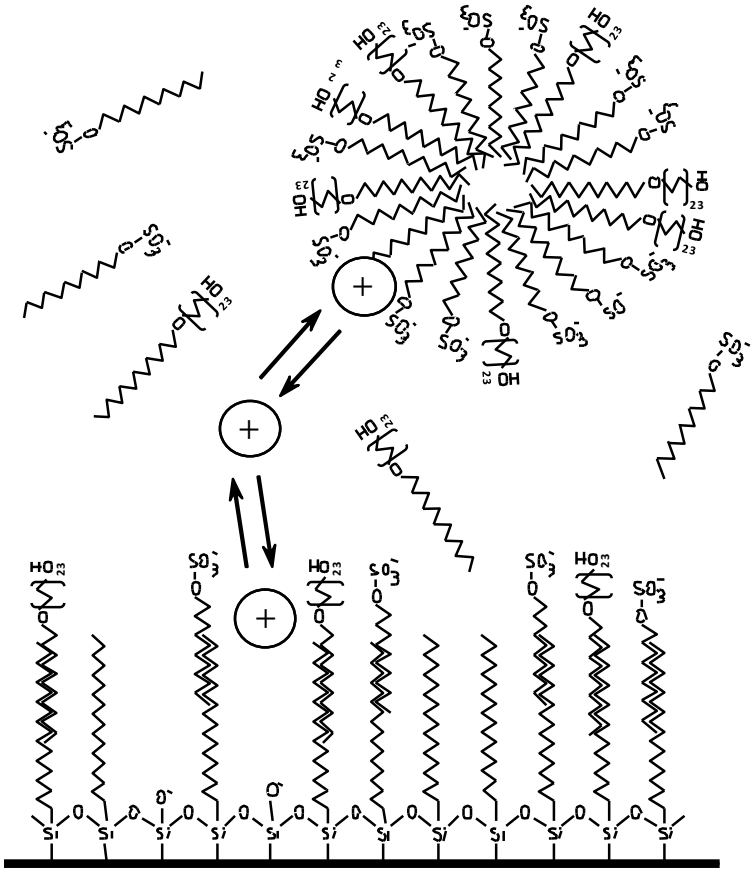


Figure 6.1. Simplified scheme of the environment of cationic solutes in a C18 stationary phase, in the presence of both Brij-35 and SDS.

Figure 6.2a shows the changes in retention for the whole set of TCAs and the two most apolar β-blockers eluted with a mobile phase containing 0.02 M Brij-35 and increasing concentrations of SDS in the 0.02–0.15 M range. As observed, the trends are similar for TCAs and β-blockers. It can be observed that the retention factors increased dramatically with the first addition of SDS. This is mainly due to the strong electrostatic attraction of the basic compounds (positively charged) to the anionic SDS monomers adsorbed on the stationary phase. Further addition of SDS reduces the retention, due to the increase in micelle concentration which attracts the cationic solutes towards the mobile phase.

Figure 6.2b depicts the changes in retention by adding increasing concentration of Brij-35 into a 0.15 M SDS mobile phase. The retention of TCAs and β-blockers in the absence of Brij-35 was excessively large (often above 80 min) and could not be measured. However, the addition of a small amount of Brij-35 (0.01 M) decreased the retention factors to practical analysis times. Successive additions of the non-ionic surfactant gradually reduced the retention, although in a smaller extent than the addition of SDS to a mobile phase containing a fixed amount of Brij-35.

When TCAs and β-blockers are eluted with SDS mobile phases, the addition of a relatively high amount of organic solvent (such as acetonitrile, propanol, butanol or pentanol) (Figure 6.3a, and Figures 6.4b and c), or the use of a column with a shorter alkyl-bonded chain (e.g., a C8 column) is required to decrease the retention times to practical values [23–26]. Using a C18 column, the retention times of propranolol and alprenolol (not shown) were above 120 and 30 min with SDS mobile phases in the presence of 10 and 45% acetonitrile, respectively.

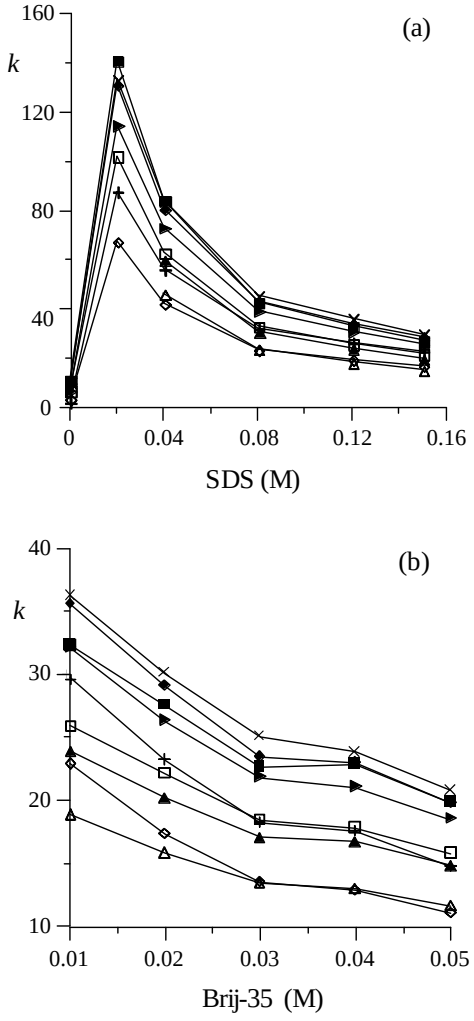


Figure 6.2. Effect of the addition of increasing concentrations of surfactant on the retention of TCAs and β-blockers in a mobile phase containing a fixed concentration of a second surfactant: (a) 0.02 M Brij-35 and increasing concentrations of SDS, and (b) 0.15 M SDS and increasing concentrations of Brij-35. Compound identity: alprenolol (+), propranolol (◊), amitriptyline (□), clomipramine (■), doxepin (△), imipramine (▲), maprotiline (×), nortriptyline (◆), and trimipramine (►).

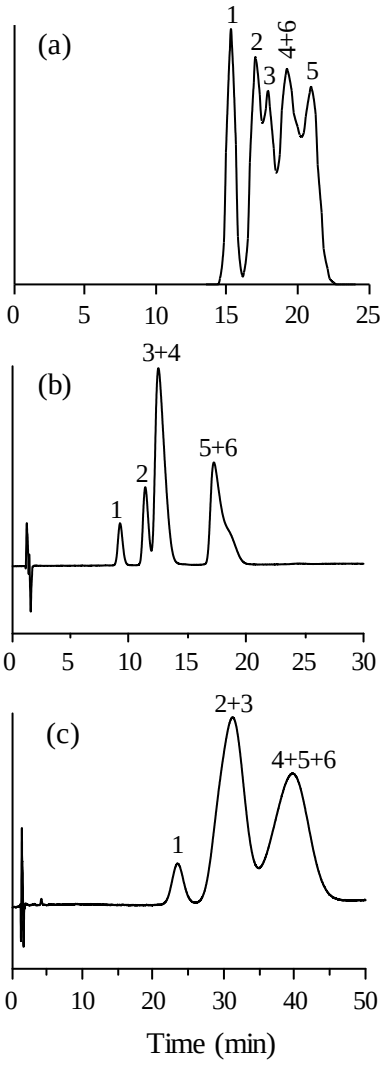


Figure 6.3. Chromatograms for a mixture of TCAs eluted with: (a) 0.10 M SDS and 3.4% v/v pentanol (Eclipse XDB C8 column), (b) 0.02 M Brij-35 (Zorbax Eclipse C18), and (c) mixed micellar system composed of 0.02 M Brij-35 and 0.15 M SDS (Zorbax Eclipse C18). Compound identity: (1) doxepin, (2) imipramine, (3) amitriptyline, (4) trimipramine, (5) nortriptyline, and (6) clomipramine.

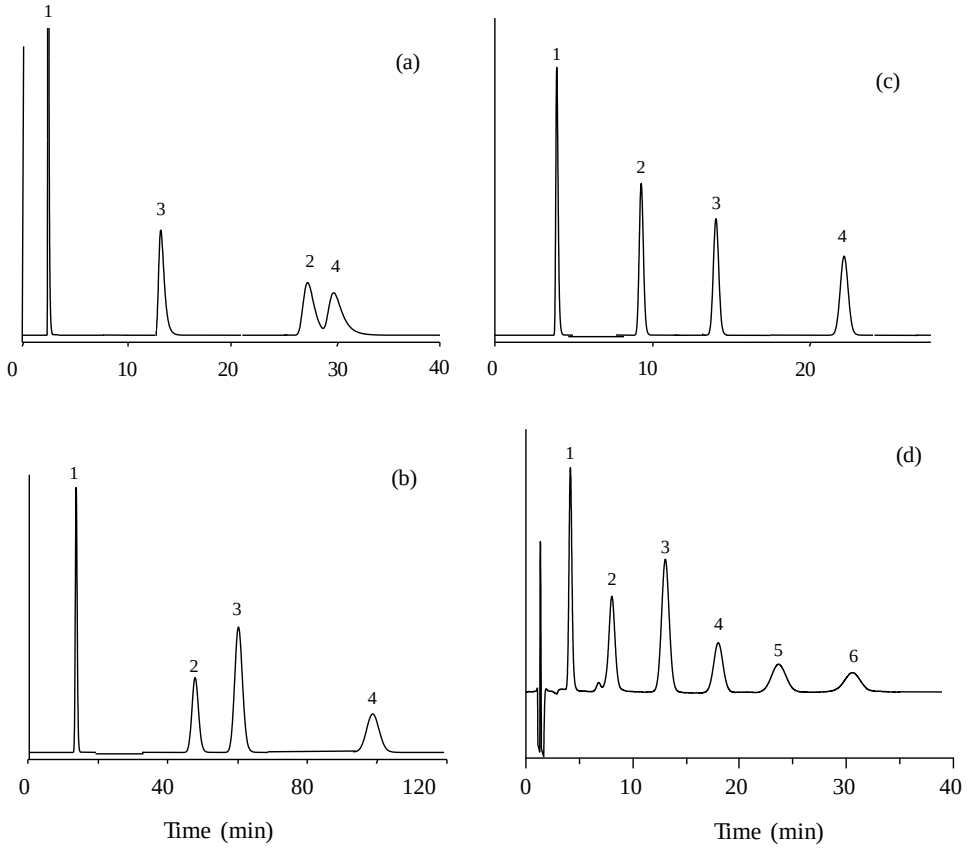


Figure 6.4. Chromatograms for a mixture of β-blockers, eluted with: (a) 15% v/v acetonitrile (Kromasil C18), (b) 0.1125 M SDS and 10% v/v acetonitrile (Kromasil C18), (c) 0.1125 M SDS and 45% (*v*/*v*) acetonitrile (Kromasil C18), and (d) mixed micellar system composed of 0.02 M Brij-35 and 0.15 M SDS (Zorbax Eclipse C18). Compound identity: (1) atenolol, (2) celiprolol, (3) metoprolol, (4) oxprenolol, (5) propranolol, and (6) alprenolol.

The retention times were smaller with mobile phases containing exclusively Brij-35. The apolar TCAs (with octanol-water partition coefficients, log $P_{o/w}$, ranging between 3.9 and 5.3) [36] were eluted at practical retention times under these conditions (Figure 6.3b). However, the retention of most β-blockers (with log $P_{o/w}$ between 0.25 and 3.4) [36] was excessively low. Thus, for example, the retention times for oxprenolol and propranolol (log $P_{o/w}$ = 2.4 and 3.4, respectively) with the mobile phase containing a small concentration of Brij-35 (0.01 M) were 2.7 and 11.8 min, respectively, and other more polar β-blockers eluted close to the dead time. Also, the retention of the most retained β-blockers decreased significantly with 0.02 M Brij-35.

The retention capability of the C18 stationary phase simultaneously modified with both Brij-35 and SDS, towards basic compounds (such as TCAs and β-blockers), is larger compared to a stationary phase exclusively modified with Brij-35, and significantly smaller with regard to a stationary phase exclusively modified with SDS. The increased retention with the mixed Brij-35/SDS system is not advantageous for TCAs (compare Figures 6.3b and c), but for β-blockers, it allows modulating the retention to practical values (Figure 6.4d), without the requirement of adding an organic solvent.

6.4.2. Solute-stationary phase and solute-mobile phase interactions

In the early development of MLC, a three-phase model (stationary phase, water and micelle) was proposed to understand the mechanism of retention. This model gave rise to equations that describe the changes in solute retention at increasing concentration of the modifiers (surfactant and organic solvent) [37,38]. The approach is valid for both ionic and non-ionic surfactants and considers two association equilibria between solute and stationary phase,

and solute and micelle. The theory is described in Chapter 5 (Section 5.4.3), giving rise to the following model:

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}[M] \quad (6.1)$$

Eq. (6.1) describes a 1/*k* *versus* surfactant concentration linear plot. The extrapolation of the linear segments gives a measurement of the strength of the interaction between the solute and stationary phase (K_{AS}), expressed as the inverse of the intercept. The slope combined with the value of K_{AS} indicates the interaction between the solute and mobile phase (K_{AM}).

To our knowledge, this equation has not been applied to measure the strength of the interaction of solutes with stationary phases modified by the simultaneous adsorption of two surfactants in the presence of mixed micelles. We have fitted to Eq. (6.1) the data obtained at increasing concentrations of SDS, in the presence of fixed Brij-35, and similarly, at increasing concentrations of Brij-35 in the presence of fixed SDS. The estimated association constants K_{AS} and K_{AM} are given in Table 6.1. For comparative purposes, the values obtained with the micellar system containing only Brij-35 are included. Owing to the strong solute-stationary phase interaction between TCAs and β-blockers with the sulphate group of SDS, which yield extremely long retention times, the estimation of these constants was not possible for purely micellar mobile phases of this surfactant. However, based on previous work, it is known that the intercept in Eq. (6.1) is practically null for the studied solutes eluted exclusively with SDS, indicating very high K_{AS} and K_{AM} values [23,24].

Table 6.1. Solute-stationary phase (K_{AS}) and solute-mobile phase (K_{AM}) association constants for the studied basic compounds eluted with mobile phases containing Brij-35 or mixtures of Brij-35 and SDS.

	Brij-35[a]		0.02 M Brij-35 / SDS[b]		0.15 M SDS / Brij-35[a]	
Compound	K_{AS}	K_{AM}	K_{AS}	K_{AM}	K_{AS}	K_{AM}
Alprenolol	–	–	138.9	34.0	39.1	32.4
Propanolol	–	–	108.7	36.1	30.0	34.3
Amitryptiline	35.7	148.9	185.2	50.7	30.7	18.7
Clomipramine	98.0	306.5	303.0	68.2	37.7	17.5
Doxepin	14.7	74.8	161.3	61.5	21.8	17.3
Imipramine	21.9	91.8	212.8	64.0	27.6	17.0
Maprotiline	62.9	204.6	232.6	45.7	43.7	21.6
Nortryptiline	57.5	188.9	227.3	46.9	43.7	23.7
Trimipramine	36.4	151.4	200.0	45.1	38.3	21.2

[a] Increasing concentration of Brij-35 from 0.01 to 0.05 M.

[b] Increasing concentration of SDS from 0.02 to 0.15 M.

As observed in Table 6.1, the set of runs where SDS was increased and Brij-35 was fixed yielded stronger solute-stationary phase interactions, whereas the runs where Brij-35 was increased with fixed SDS provided solute affinity to the stationary phase similar or smaller than that observed with only the presence of Brij-35. Thus, in a mixed Brij-35/SDS system, the interaction between the basic solutes and each surfactant in the modified stationary phase was different (stronger with SDS). Finally, the solute-micelle association constants (K_{AM}) in the mixed micellar systems were significantly smaller. This suggests that the affinity of the basic solutes to the mixed micelles is smaller, giving rise to a decreased elution strength.

6.4.3. Peak profiles in the mixed Brij-35/SDS micellar systems

As already explained in Chapter 5, the graphical representation of the left (*A*) and right (*B*) half-widths, measured at 10% peak height, *versus* the retention time, allows an overview of the changes that occur in the width and asymmetry of the chromatographic peaks obtained with a given column. The validity of these plots to compare the behaviour of different families of compounds, using different types of columns and mobile phases, has been demonstrated in previous work [26,39–42]. The construction of half-width plots is very simple, being represented by the following equations:

$$A = m_A\, t_R + A_0 \tag{6.2}$$

$$B = m_B\, t_R + B_0 \tag{6.3}$$

where m_A and m_B are the slopes of the linear correlations for the left and right half-widths, respectively, and A_0 and B_0 the corresponding intercepts representing the extra-column contribution to the peak broadening. Eqs. (6.2) and (6.3) allow for the prediction of the peak half-widths for compounds eluted

at different retention times, and the calculation of the apparent efficiencies associated to each compound. These parameters are also useful to characterise chromatographic columns. The sum of m_A and m_B represents the broadening rate of chromatographic peaks inside the column, and its ratio (m_B/m_A) indicates the peak asymmetry at high retention times. The study of the effect of the surfactant mediated systems on the peak profiles was performed based on the construction of plots at each mobile phase composition, using the half-widths for several probe compounds.

Figure 6.5 shows the half-width plots for the TCAs and β-blockers eluted with the Brij-35 and/or Brij-35/SDS systems. The slopes of the linear segments for the left (m_A) and right (m_B) half-widths, and their sum and ratio for the assayed mobile phases are given in Table 6.2. Figure 6.5a depicts the half-width plots for a mobile phase containing only 0.02 M Brij-35. The correlations were satisfactory for both half-widths. The larger slope for the right half-width indicates an appreciable peak tailing. Figures 6.5b and 6.5c show the half-width plots obtained for a mixed Brij-35/SDS system. The coincidence of the slopes of the linear segments for both half-widths ($m_B/m_A \approx 1.0$, which means highly symmetrical peaks) is remarkable (compare with Figure 6.5a). This indicates that SDS is able to protect the silanol groups in the column, hindering the access of the basic compounds. Although the peak asymmetry with Brij-35 ($m_B/m_A = 2.33$) is significantly larger with respect to the mixed Brij-35/SDS systems, it should be noted that when the basic compounds are eluted from C18 columns with aqueous-organic mobile phases, the peak asymmetry may be even larger ($m_B/m_A = 3.60$, see Figure 6.4a).

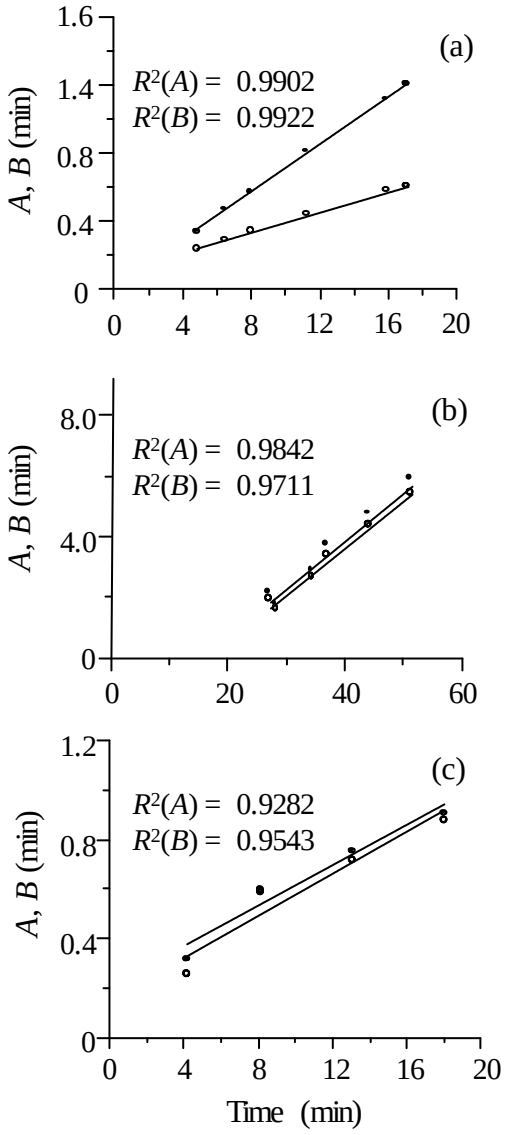


Figure 6.5. Half-width plots for mobile phases containing: (a) 0.02 M Brij-35, (b,c) 0.02 M Brij-35/0.15 M SDS. Left (A, ○) and right (B, ●) half-widths. Compounds: (a,b) TCAs, propranolol and alprenolol, and (c) atenolol, celiprolol, metoprolol and oxprenolol.

Table 6.2. Half-width plots parameters for TCAs and β-blockers eluted with different micellar mobile phases: slopes for the left (m_A) and right (m_B) half-width plots, sum of slopes and slopes ratio.

Mobile phase	m_A	m_B	$m_A + m_B$	m_B/m_A
0.02 M Brij-35 [a]	0.030	0.071	0.101	2.33
0.02 M Brij-35 / 0.02 M SDS [a]	0.083	0.082	0.165	0.99
0.02 M Brij-35 / 0.04 M SDS [a]	0.098	0.100	0.198	1.02
0.02 M Brij-35 / 0.08 M SDS [a]	0.141	0.150	0.291	1.06
0.02 M Brij-35 / 0.12 M SDS [a]	0.157	0.159	0.316	1.01
0.02 M Brij-35 / 0.15 M SDS [a]	0.123	0.119	0.242	0.96
0.01 M Brij-35 / 0.15 M SDS [a]	0.207	0.213	0.420	1.03
0.02 M Brij-35 / 0.15 M SDS [a]	0.123	0.119	0.242	0.96
0.03 M Brij-35 / 0.15 M SDS [a]	0.141	0.152	0.293	1.13
0.04 M Brij-35 / 0.15 M SDS [a]	0.160	0.180	0.340	1.07
0.05 M Brij-35 / 0.15 M SDS [a]	0.127	0.139	0.266	1.10
0.02 M Brij-35 / 0.15 M SDS [b]	0.0423	0.0410	0.0833	0.97

[a] TCAs, alprenolol and propranolol.

[b] Atenolol, celiprolol, metoprolol and oxprenolol.

The silanol masking capability of SDS has been extensively demonstrated using hybrid mobile phases of SDS and organic solvent [39–42]. As noted, the effect is similar for the mixed Brij-35/SDS system. However, $m_A + m_B$ values are appreciably larger (i.e., the peaks are broader) with respect to the mobile phases containing only Brij-35, probably due to the larger carbon contents when both surfactants are adsorbed.

6.4.4. Selectivity and resolution

In order to explore the selectivity achieved with the mixed micellar systems, the retention factors obtained for the TCAs, propranolol and alprenolol with a mobile phase containing only Brij-35 were correlated with those using mobile phases containing both Brij-35 and SDS (Figure 6.6a). The retention factors for pairs of mixed micellar mobile phases were also correlated (Figures 6.6b and c). The observed changes in relative retention can be explained by the changes in the stationary phase nature and elution strength with the mobile phase composition. Besides the significant changes in absolute retention in the presence and absence of SDS, and with changes in the concentration of both surfactants, the three plots show differences in selectivity. Similar results were obtained at other concentrations. We should here recall that more polar β-blockers elute close to the dead time with mobile phases containing only Brij-35.

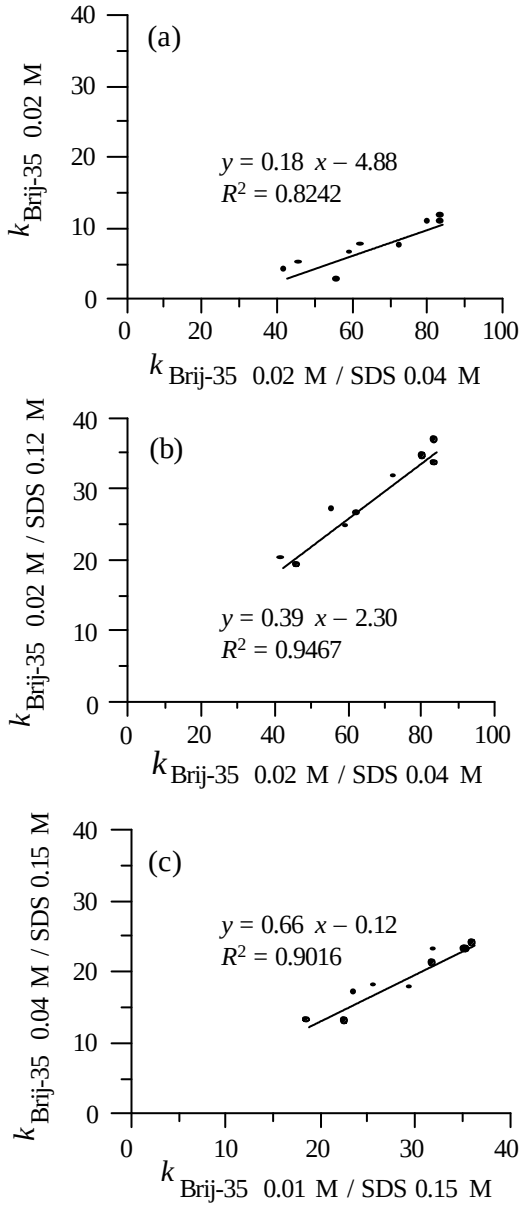


Figure 6.6. Comparison of the selectivity of chromatographic systems containing only Brij-35, and both Brij-35 and SDS (retention factors are plotted). The data correspond to the seven TCAs, propranolol and alprenolol.

The main goal in a chromatographic separation is to achieve the resolution of all peaks. In order to observe the resolution capability of a column simultaneously modified with Brij-35 and SDS, mixtures of the two sets of probe compounds (TCAs and β-blockers) were eluted with mixed micellar Brij-35/SDS mobile phases. Figure 6.3c shows a chromatogram corresponding to the separation of several TCAs. As observed, for these compounds, mixed Brij-35/SDS mobile phases do not offer any advantage with respect to the use of mobile phases containing Brij-35: in the presence of SDS the peaks are significantly broader and show longer retention. The TCAs remain unresolved in MLC, either with SDS/pentanol (the retention times with a more polar solvent are too high), and with Brij-35 or Brij-35/SDS without organic solvent. However, samples containing the individual TCAs can be analysed with good results using a green RPLC method with Brij-35 in the absence of organic solvent in sufficiently small analysis times. This procedure was demonstrated to be competitive against classical RPLC with an optimised mobile phase (32% acetonitrile) [26].

In contrast, the mixed Brij-35/SDS system is revealed as promising to succeed in the separation of mixtures of β-blockers, with a favourable effect on retention and resolution. The most polar β-blockers (such as atenolol, celiprolol, metoprolol, oxprenolol, with log $P_{o/w}$ values between 0.25 and 2.0), which are not sufficiently retained with mobile phases containing only Brij-35, and are excessively retained with mobile phases with only SDS, are eluted at practical retention times with the mixed Brij-35/SDS system. Figure 6.4d depicts the chromatogram for a mixture of six β-blockers, using an isocratic mobile phase containing 0.02 M Brij-35 and 0.15 M SDS. The mixed micellar mobile phase was able to separate the set of β-blockers with an analysis time below 35 min in the absence of an organic solvent.

A smaller analysis time will be obtained by optimising the mobile phase composition, which will depend on the particular analysed β-blocker or set of β-blockers. For comparison purposes, Figure 6.4a shows the chromatogram of the most polar β-blockers studied in this work, obtained in 15% acetonitrile. The retention of alprenolol and propranolol was above 60 min in these conditions.

The repeatability of the retention time, and the peak efficiency and area, performing ten-fold injections, are indicated in Table 6.3 for the six β-blockers at three concentrations. The results show that the analysis can be carried out successfully with a mobile phase only composed by water and two detergents at room temperature.

6.5. Conclusions

More than two-thirds of the reported applications in MLC employ the anionic surfactant SDS, with a special relevance in the pharmaceutical field. The references on the analytical use of Brij-35 in MLC are few, except in the field of QSAR studies. Although procedures using the Brij-35/SDS mixture are found in the MLC literature for several types of compounds, there are no previous descriptions on its application to basic compounds. Also, detailed comparisons between the mixed micellar systems and those using a single surfactant (as shown in this work) have not been carried out.

Table 6.3. Repeatability in retention times, area and efficiency at three different concentrations of β-blockers.

	2 µg/mL			7 µg/mL			14 µg/mL		
Compound	t_R (min)	Area	*N*	t_R (min)	Area	*N*	t_R (min)	Area	*N*
Atenolol	4.13 ± 0.01	1.45 ± 0.02	940 ± 30	4.14 ± 0.01	6.08 ± 0.02	880 ± 14	4.16 ± 0.01	11.19 ± 0.02	870 ± 12
Celiprolol	7.95 ± 0.02	1.57 ± 0.05	910 ± 60	8.00 ± 0.02	4.96 ± 0.02	865 ± 8	8.05 ± 0.01	9.41 ± 0.06	840 ± 12
Metoprolol	12.86 ± 0.02	2.92 ± 0.05	1450 ± 50	12.95 ± 0.04	9.31 ± 0.07	1400 ± 27	13.06 ± 0.02	17.30 ± 0.07	1400 ± 9
Oxprenolol	17.71 ± 0.05	1.37 ± 0.07	1820 ± 150	17.86 ± 0.07	4.19 ± 0.03	1900 ± 38	18.01 ± 0.04	7.84 ± 0.07	1840 ± 36
Propranolol	23.28 ± 0.12	0.89 ± 0.16	1130 ± 320	23.42 ± 0.09	3.89 ± 0.09	1300 ± 76	23.66 ± 0.07	6.97 ± 0.09	1290 ± 45
Alprenolol	30.04 ± 0.14	0.95 ± 0.17	1560 ± 470	30.26 ± 0.11	3.04 ± 0.10	1700 ± 160	30.60 ± 0.08	5.54 ± 0.22	1700 ± 83

This work shows that the separation of basic compounds of diverse polarity, with Brij-35/SDS mobile phases, yields retention times and peak profiles that are dominated by the strong association of the cationic solutes with the adsorbed SDS on the stationary phase. However, the simultaneous adsorption of Brij-35 confers the stationary phase higher polarity that decreases the retention times, which are significantly shorter than those obtained with mobile phases containing only SDS. This avoids the addition of an organic solvent.

The preference for the mixed Brij-35/SDS system against the single Brij-35 system depends on the polarity of the basic compounds. Thus, aqueous mobile phases containing only Brij-35 are preferable for analysing apolar basic compounds (as TCAs). Meanwhile, the retention of polar and moderately polar basic compounds (as β-blockers), which is too short with mobile phases containing only Brij-35, can be modulated to practical values by the addition of SDS to the mobile phase containing Brij-35, and may yield successful resolution. Therefore, the described methods with Brij-35 in the absence or presence of SDS can be the basis of successful "green" chromatographic analyses of basic compounds. The studies in this work should be used as a guideline to develop the analytical procedures.

6.6. References

[1] A. Berthod, M.C. García Álvarez-Coque, *Micellar Liquid Chromatography*, Marcel Dekker, New York, 2000.

[2] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, New insights and recent developments in micellar liquid chromatography, *Sep. Purif. Rev.* 38 (2009) 45–96.

[3] M.J. Ruiz Ángel, S. Carda Broch, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Retention mechanisms in micellar liquid chromatography, *J. Chromatogr. A* 1216 (2009) 1798–1814.

[4] E. Bonet Domingo, M.J. Medina Hernández, M.C. García Álvarez-Coque, On the direct injection of urine samples in micellar liquid chromatography, *Quím. Anal.* 12 (1993) 167–172.

[5] M.C. García Álvarez-Coque, S. Carda Broch, Direct injection of physiological fluids in micellar liquid chromatography, *J. Chromatogr. B* 736 (1999) 1–18.

[6] L. Zhu, L. Ding, Q. Zhang, L. Wang, F. Tang, Q. Liu, S. Yao, Direct analysis of cryptotanshinone and tanshinone IIA in biological samples and herbal medicinal preparations by a green technique of micellar liquid chromatography, *Green Chem.* 11 (2009) 132–137.

[7] M.F. Borgerding, W.L. Hinze, Characterization and evaluation of the use of nonionic polyoxyethylene(23)dodecanol micellar mobile phases in reversed-phase high-performance liquid chromatography, *Anal. Chem.* 57 (1985) 2183–2190.

[8] M.F. Borgerding, F.H. Quina, W.L. Hinze, J. Bowermaster, H.M. McNair, Investigation of the retention mechanism in nonionic micellar liquid chromatography using an alkylbenzene homologous series, *Anal. Chem.* 60 (1988) 2520–2527.

[9] M.F. Borgerding, W.L. Hinze, L.D. Stafford, G.W. Fulp Jr., W.C. Hamlin Jr., Investigations of stationary phase modification by the mobile phase surfactant in micellar liquid chromatography, *Anal. Chem.* 61 (1989) 1353–1358.

[10] L.J. Cline-Love, J.J. Fett, Optimization of selectivity in micellar chromatographic procedures for the determination of drugs in urine by direct injection, *J. Pharm. Biomed. Anal.* 9 (1991) 323–333.

[11] M.L. Marina, O. Jiménez, M.A. García, S. Vera, Study of the separation selectivity of a group of benzene and naphthalene derivatives in micellar liquid chromatography, *Microchem. J.* 53 (1996) 215–224.

[12] P. Menéndez Fraga, A. Blanco González, A. Sanz Medel, J.B. Cannata Andia, Micellar versus reversed phase liquid chromatography for the determination of desferrioxamine and its chelates with aluminium and iron in uremic serum, *Talanta* 45 (1997) 25–33.

[13] A. Berthod, S. Tomer, J.G. Dorsey, Polyoxyethylene alkyl ether nonionic surfactants: Physicochemical properties and use for cholesterol determination in food, *Talanta* 55 (2001) 69–83.

[14] M. Gil Agustí, L. Álvarez Rodríguez, L. Monferrer Pons, D. Bose, A. Durgbanshi, J. Esteve Romero, Chromatographic determination of carbaryl and other carbamates in formulations and water using Brij-35, *Anal. Lett.* 35 (2002) 1721–1734.

[15] N. Memon, M.I. Bhanger, M.Y. Khuhawer, Determination of preservatives in cosmetics and food samples by micellar liquid chromatography, *J. Sep. Sci.* 28 (2005) 635–638.

[16] W. Thogchai, B. Liawruangrath, Micellar liquid chromatographic determination of arbutin and hydroquinone in medicinal plant extracts and commercial cosmetic products, *Int. J. Cosmetic Sci.* 35 (2013) 257–263.

[17] Y.M. Dong, N. Li, Q. An, N.W. Lu, A novel nonionic micellar liquid chromatographic method for simultaneous determination of pseudoephedrine, paracetamol, and chlorpheniramine in cold compound preparations, *J. Liq. Chromatogr., Rel. Technol.* 38 (2015) 251–258.

[18] J.M. Sanchis Mallols, R.M. Villanueva Camañas, S. Sagrado, M.J. Medina Hernández, Quantitative retention-structure and retention-activity relationship studies of ionic and non-ionic catecholamines by micellar liquid chromatography, *Chromatographia* 46 (1997) 605–612.

[19] C.R. Yin, L.Y. Ma, J.G. Huang, L. Xu, Z.G. Shi, Fast profiling ecotoxicity and skin permeability of benzophenone ultraviolet filters using biopartitioning micellar chromatography based on penetrable silica spheres, *Anal. Chim. Acta* 804 (2013) 321–327.

[20] J. Nawrocki, The silanol group and its role in liquid chromatography, *J. Chromatogr. A* 779 (1997) 29–71.

[21] S. Bocian, B. Buszewski, Residual silanols at reversed-phase silica in HPLC: A contribution for a better understanding, *J. Sep. Sci.* 35 (2012) 1191–1200.

[22] B.K. Lavine, S. Hendayan, W.T. Cooper, Y. He, Selectivity in micellar liquid chromatography: Surfactant-bonded phase associations in micellar reversed phase liquid chromatography, *ACS Symp. Ser.* 740 (2000) 290–313.

[23] M.J. Ruiz Ángel, J.R. Torres Lapasió, M.C. García Álvarez-Coque, S. Carda Broch, Retention mechanisms for basic drugs in the submicellar and micellar reversed-phase liquid chromatographic modes, *Anal. Chem.* 80 (2008) 9705–9713.

[24] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 7082–7089.

[25] M.J. Ruiz Ángel, S. Carda Broch, E.F. Simó Alfonso, M.C. García Álvarez-Coque, Optimised procedures for the reversed-phase liquid chromatographic analysis of formulations containing tricyclic antidepressants, *J. Pharm. Biomed. Anal.* 32 (2003) 71–84.

[26] J.J. Fernández Navarro, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography without organic solvent for determination of tricyclic antidepressants, *J. Sep. Sci.* 35 (2012) 1303–1309.

[27] J.J. Baeza Baeza, Y. Dávila, J.J. Fernández Navarro, M.C. García Álvarez-Coque, Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC, *Anal. Bioanal. Chem.* 404 (2012) 2973–2984.

[28] K. Ogino, H. Uchiyama, M. Abe, *Mixed Surfactant Systems*, Marcel Dekker, New York, 1993.

[29] X. Li, J.S. Fritz, Mixed surfactants as mobile phase additives for the separations of organic compounds by HPLC, *Anal. Chem.* 68 (1996) 4481–4488.

[30] P. Ebrahimi, M.R. Hadjmohammadi, Simultaneous optimization of resolution and analysis time in mixed micellar liquid chromatography of coumarins by use of a utility function, *Anal. Bioanal. Chem.* 384 (2006) 851–858.

[31] J. Sun, J. Mao, X. Liu, Y. Wang, Y. Sun, Z. He, Separation and mechanism elucidation for six structure-like matrine-type alkaloids by micellar liquid chromatography, *J. Sep. Sci.* 32 (2009) 2043–2050.

[32] R. Nakao, C. Haldin, Mixed anionic and non-ionic micellar liquid chromatography for high-speed radiometabolite analysis of positron emission tomography radioligands, *J. Chromatogr. A* 1281 (2013) 54–59.

[33] O.R.T. Thomas, G.F. White, Metabolic pathway for the biodegradation of sodium dodecyl sulfate by Pseudomonas sp. C12B, *Biotechnol. Appl. Biochem.* 11 (1989) 318–327.

[34] M.T. Scott, M.N. Jones, The biodegradation of surfactants in the environment, *Biochim. Biophys. Acta* 1508 (2000) 235–251.

[35] H. Gao, S. Zhao, S. Mao, H. Yuan, J. Yu, L. Shen, Y. Du, Mixed micelles of polyethylene glycol (23) lauryl ether with ionic surfactants studied by proton 1D and 2D NMR, *J. Coll. Int. Sci.* 249 (2002) 200–208.

[36] C.J. Drayton (Ed.), *Comprehensive Medicine Chemistry*, Vol. 6, Pergamon Press, Oxford, 1990.

[37] D.W. Armstrong, F. Nome, Partitioning behavior of solutes eluted with micellar mobile phases in liquid chromatography, *Anal. Chem.* 53 (1981) 1662–1666.

[38] M. Arunyanart, L.J. Cline Love, Model for micellar effects on liquid chromatography capacity factors and for determination of micelle-solute equilibrium constants, *Anal. Chem.* 56 (1984) 1557–1561.

[39] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[40] J.J. Fernández Navarro, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, The role of the dual nature of ionic liquids in the reversed-phase liquid chromatographic separation of basic drugs, *J. Chromatogr. A* 1218 (2011) 398–407.

[41] S. Pous Torres, M.J. Ruiz Ángel, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Performance of a Chromolith RP-18e column for the screening of β-blockers, *J. Sep. Sci.* 32 (2009) 2841–2853.

[42] M.J. Ruiz Ángel, S. Pous Torres, S. Carda Broch, M.C. García Álvarez-Coque, Performance of different C18 columns in reversed-phase liquid chromatography with hydro-organic and micellar-organic mobile phases, *J. Chromatogr. A* 1344 (2014) 76–82.

CHAPTER 7

ANALYSIS OF BASIC DRUGS BY LIQUID CHROMATOGRAPHY WITH ENVIRONMENTALLY FRIENDLY MOBILE PHASES IN PHARMACEUTICAL FORMULATIONS

7.1. Abstract

Basic drugs are positively charged in the usual working pH (2–8) in reversed-phase liquid chromatography. This gives rise to a strong association with the residual ionised silanols in conventional silica-based stationary phases, which is translated in poor peak shape and high consumption of organic solvent to get appropriate retention times. Micellar mobile phases containing surfactants give rise to modified stationary phases, where silanols are masked, improving the peak shape. However, mobile phases containing the anionic surfactant sodium dodecyl sulphate (SDS) require a small amount of organic solvent to conveniently decrease the retention of cationic analytes. An alternative is the use of mixed micellar mobile phases prepared with SDS and the more polar non-ionic surfactant Brij-35, which modulates the retention of basic drugs to practical analysis times, eliminating the need of organic solvent. Two simple chromatographic procedures for the control of the β-blockers atenolol, celiprolol, metoprolol, oxprenolol and propranolol in pharmaceutical formulations were developed and compared, using 0.15 M SDS/15% 1-propanol and 0.15 M SDS/0.05 M Brij-35 at pH 3 with UV detection. Both methods were validated according to the ICH guideline. Satisfactory recoveries were achieved, with intra- and inter-day relative standard deviations usually below 2% for both micellar modes. Sample preparation was simple and only required solubilisation and filtration prior to injection.

7.2. Introduction

Pharmaceutical products containing β-blockers have been used for over 40 years in the remedy of angina, hypertension, heart failure and cardiac arrhythmias [1,2]. These drugs are also frequently indicated to prevent migraine, treat hyperthyroidism and control or overcome anxiety disorders. Considering that a World Health Organisation (WHO) report confirmed in 2012 that approximately 33.3% of the population suffers from cardiac problems (data from 194 countries) [3], it is not strange that β-blockers are widespread prescribed in large amounts every year in many countries [4]. Therefore, the monitoring of these drugs in biological, wastewater and pharmaceutical samples is essential. For these studies, effective, efficient, selective and reproducible analytical methods are required.

Chromatography and capillary electrophoresis are the most frequent analytical techniques for the analysis of β-blockers [5–9]. Among these, reversed-phase liquid chromatography (RPLC) is particularly useful due to its ease of operation, selectivity, rapidity and reproducibility [10,11]. However, the chemical nature of β-blockers, composed by at least one aromatic ring structure attached to a side alkyl chain possessing a secondary hydroxyl and amine functional group, confers them a basic character (pK_a close or higher than 9), which is the reason of several problems when alkyl-bonded silica based columns are used. At the working pH range of the mobile phases (2–8) in RPLC, β-blockers are mainly positively charged and able to interact with the negatively charged residual silanols, which are present in the octadecyl packages as a consequence of steric problems in the derivatisation process of silica supports [12,13]. As a consequence, the retention of cationic basic compounds increases and due to the slow kinetics of this process, tailed and broad peaks are obtained, affecting the selectivity and peak resolution.

Therefore, solutions are needed to obtain appropriate RPLC analytical methods for basic drugs.

Several strategies have been addressed to suppress or reduce the silanol activity. The most extended are buffering the pH at acidic values to partially protonate the residual silanols, or masking the silanol sites through the addition to the mobile phase of ionic (cationic or anionic), or even non-ionic reagents, able to be adsorbed on the stationary phase to form a sort of shield that avoids the electrostatic interaction with the silanols [14,15]. Bulky additives such as room temperature ionic liquids, amines or surfactants have yielded very satisfactory results in terms of peak symmetry [15–18]. When surfactants are selected as additives, they are usually added to the mobile phase at concentrations above their critical micellar concentration, which means that micelles are formed in the separation environment. For this reason, this RPLC mode is popularly known as micellar liquid chromatography (MLC) [19].

The anionic sodium dodecyl sulphate (SDS) is the most common surfactant in MLC and has revealed as one of the most effective silanol suppressors, giving rise to almost symmetrical peaks [19]. However, pure aqueous micellar mobile phases of SDS are too weak to elute cationic drugs of intermediate to high hydrophobicity and yield poor peak shape. The adsorption of the SDS monomers on the stationary phase, with the sulphate group oriented outside, confers it a negative charge that strongly attracts the cationic basic drugs to the anionic surfactant on the stationary phase, increasing significantly the retention. Only for highly polar compounds, such as thiazides, pure SDS mobile phases are appropriate to carry out the analyses [20]. Depending on the compound polarity, at least a small amount of organic solvent (frequently short-chain alcohols) is needed to get convenient analysis times. The addition of organic solvent also improves significantly the efficiency. Since the early years of

MLC, 1-propanol has become the solvent of choice for moderately polar compounds [21,22], including β-blockers [23], being 1-butanol and 1-pentanol more indicated for highly apolar compounds. However, although the concentration of organic solvent in MLC is frequently smaller with respect to conventional hydro-organic mobile phases in RPLC, the amount of organic solvent is still relatively important for some analytes (e.g., at least 20% 1-propanol is needed to elute the most hydrophobic β-blockers), so that alternative solutions have been proposed.

One of the most efficient approaches that keeps the benefits of mobile phases of SDS to improve the peak shape, and avoids the use of organic solvent, is the elution with micellar mobile phases containing SDS and the non-ionic surfactant Brij-35. Brij-35 monomers, the same as SDS, are adsorbed on the stationary phase with the hydrophilic polar end oriented away from the surface, increasing the polarity of the stationary phase without giving rise to a net charge [24]. Using mobile phases with only Brij-35, the β-blockers elute close to the dead time due to the higher polarity of the modified alkyl-bonded stationary phase. Instead, the combination of SDS and Brij-35 in the mobile phase allows the modulation of their retention behaviour, yielding convenient retention times [24]. Therefore, the use of organic solvent is avoided, being safer for the analyst.

In this work, a detailed comparison study of the chromatographic behaviour of five commonly prescribed β-blockers (atenolol, celiprolol, metoprolol, oxprenolol, and propranolol), considering retention, peak shape and resolution is carried out with micellar mobile phases containing both SDS and Brij-35, whose effect is compared with that observed with conventional MLC mobile phases of SDS and 1-propanol. With the achieved information, simple MLC procedures for the determination of the five β-blockers in pharmaceutical

formulations were established. The robustness of both procedures in routine analysis is demonstrated through an extensive validation according to the International Conference of Harmonisation (ICH) Guideline [25]. Even though the analysed drugs are not found together in the formulations, single procedures suitable for the control of several drugs are very useful for the pharmacological laboratory, since the maintenance of separate chromatographic conditions for each drug increases the cost of the analyses.

7.3. Experimental

7.3.1. Reagents

The β-blockers used in this study were: atenolol, celiprolol (chlorhydrate), metoprolol (tartrate), oxprenolol, and propranolol (chlorhydrate) (all from Sigma, St. Louis, MO, USA). Stock solutions of approximately 100 μg/mL of the drugs, which remained stable during at least two months at 4 ^{o}C, were prepared in a small amount of ethanol (Scharlab, Barcelona, Spain) and sonicated with an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany). The working solutions were diluted with mobile phases containing SDS (Scharlab), or SDS and Brij-35 (Acros Organics, Geel, Belgium). Uracil (Acros Organics) was used as dead time marker. Solutions were filtered through 0.45 μm polytetrafluoroethylene membranes (Análisis Vínicos, Tomelloso, Ciudad Real, Spain).

The mobile phases contained SDS and 1-propanol (Scharlab), or SDS and Brij-35, and were buffered at pH 3.0 with sodium dihydrogen phosphate (Fluka, Buchs, Switzerland) and HCl (Scharlab). The mobile phases, which were renewed several times along the validation process, were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA), and

degassed in an ultrasonic bath. Nanopure water (Thermo Scientific, Dubuque, IA, USA) was used throughout.

7.3.2. Apparatus and chromatographic conditions

An Agilent chromatographic system (Waldbronn, Germany), equipped with a quaternary pump (Series 1200), an autosampler (Series 1100), a thermostated column compartment (Series 1100) set at 25 ^{o}C, a UV-visible wavelength detector (Series 1260), and an HPChemStation (Agilent, B.04.03) for data acquisition, was used. The analytes were monitored at 225 nm. The mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were integrated with MICHROM [26]. All simulations and optimisations were also performed with this software.

Two Zorbax Eclipse XDB (Agilent) columns (one for each procedure) with the following characteristics were used: 150 mm × 4.6 mm i.d., 5 µm particle size, 10% carbon load, 180 m^2/g surface area, and 80 Å pore size. The analytical columns were connected to similar 30 mm pre-columns for protection. The flow rate was 1 mL/min. Duplicate injections of 20 µL were made.

The mobile phases used in the analysis of the pharmaceutical formulations were 0.15 M SDS/15% (v/v) 1-propanol and 0.15 M SDS/0.05 M Brij-35. These micellar mobile phases were continuously flushed through the chromatographic system along the working days to avoid surfactant and buffer precipitation on the stationary phase, being renewed each week. A small flow rate of 0.1 mL/min was used overnight to avoid daily cleaning and re-equilibration. Recycling through the chromatographic system reduced reagent consumption and wastes. Column cleaning was achieved with 15 mL of

pure water to reduce the amount of surfactant and buffer. Next, water was replaced by 100% methanol to remove the adsorbed surfactant on the stationary phase. In order to assure complete surfactant desorption, at least 15 mL of methanol should be passed through the column. During weekend, the column was kept with methanol.

7.3.3. Procedure

The analysed pharmaceuticals were commercialised as tablets. The average weight per tablet was calculated by weighting 10 units. The contents were ground and reduced to a homogeneous fine powder in a mortar. Several portions of powder were taken and treated with a small amount of ethanol, and then sonicated in the presence of 0.15 M SDS/15% 1-propanol or 0.15 M SDS/0.05 M Brij-35. The obtained solutions were made up to the mark in a volumetric flask with each mobile phase. The excipients were not soluble in the assayed media, hence the sample solutions should be filtered through 0.45 μm polytetrafluoroethylene membranes before injection into the chromatograph.

7.4. Results and discussion

7.4.1. Chromatographic behaviour of β-blockers in hybrid and mixed MLC

A comparative study of the chromatographic behaviour and analysis of the β-blockers was carried out, using two micellar mobile phases containing SDS, which differed in the second reagent added to the mobile phase: 1-propanol or Brij-35. The mobile phases were buffered at pH 3.0 to enhance the peak shape through the protonation of the residual-free silanol groups on the stationary phase. In order to follow in detail the change in the behaviour of the drugs with

mobile phase composition, chromatographic data (retention factors and peak half-widths) were modelled from experimental designs that involved five mobile phases. For the hybrid micellar mode, the experimental design covered a domain of 0.075–0.15 M SDS and 5–15% (*v*/*v*) 1-propanol. For the mixed micellar mode, the domains for the surfactants were 0.05–0.15 M SDS and 0.02–0.05 M Brij-35. The studied concentration ranges gave rise to enough retention for the β-blockers and guaranteed micelle formation. Two Zorbax columns of identical characteristics (type B columns made of highly purified silica) were used for each micellar mode.

Figures 7.1a and b show the chromatograms for the optimal conditions in both micellar modes: 0.15 M SDS/0.05 M Brij-35 and 0.11 M SDS/10% 1-propanol, respectively. The resolution was excellent in both cases but the retention time for oxprenolol and propranolol was larger in the hybrid micellar mode (> 20 min). It can be also observed that the peaks were quite symmetrical, although broader in the mixed mode (Figure 7.1a). The optimal mobile phases obtained in both micellar modes are appropriate for screening purposes of the five studied β-blockers. However, for the hybrid system a mobile phase composed by 0.15 M SDS and 15% 1-propanol was preferred to obtain more practical retention times for the two most retained β-blockers (oxprenolol and propranolol). Figure 7.1c shows the chromatogram obtained under these conditions.

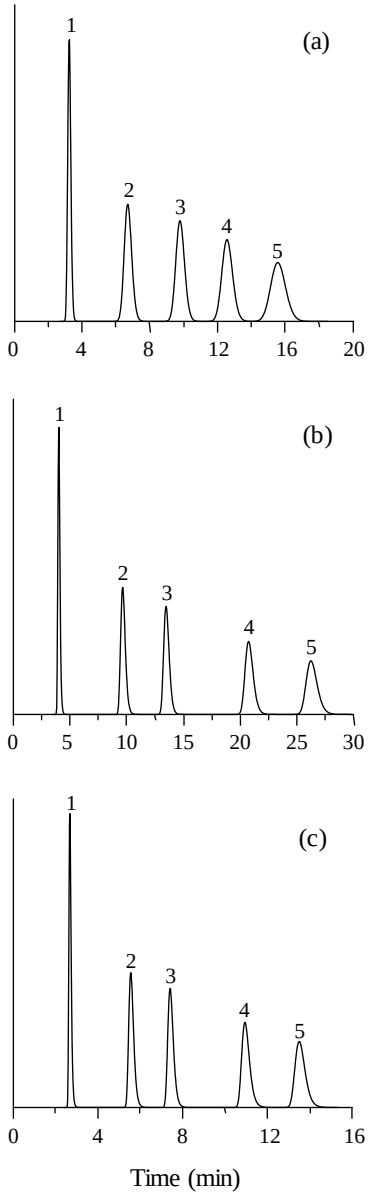


Figure 7.1. Chromatograms for the mixture of the five β-blockers eluted with: (a) 0.15 M SDS/0.05 M Brij-35, (b) 0.11 M SDS/10% 1-propanol, and (c) 0.15 M SDS/15% 1-propanol. Peak identity: (1) atenolol, (2) celiprolol, (3) metoprolol, (4) oxprenolol, and (5) propranolol.

The elution order for the β-blockers analysed in this work, using the SDS/1-propanol and SDS/Brij-35 micellar mobile phases was the same. However, the observed differences in peak distribution and analysis time suggest some differences in selectivity, owing to the different nature of the modified stationary phase (with only SDS in the hybrid mode, and with both SDS and Brij-35 in the mixed mode). A better evidence of this can be achieved by correlating the retention factors for different mobile phase compositions. Figures 7.2a and d, on the one hand, and Figures 7.2b and e, on the other, show that the selectivity does not change with mobile phase composition inside each micellar mode (similar plots were obtained for other assayed mobile phases). In contrast, significant differences were observed between the hybrid and mixed modes (Figures 7.2c and f).

7.4.2. Peak shape

The examination of efficiency and asymmetry changes with mobile phase composition was carried out through the construction of peak half-width plots, which represent the left (*A*) and right (*B*) half-widths of the chromatographic peaks (measured at 10% peak height to avoid baseline noise) *versus* the retention time:

$$A = m_A t_R + A_0 \quad (7.1)$$

$$B = m_B t_R + B_0 \quad (7.2)$$

where m_A and m_B are the slopes of the linear correlations, and A_0 and B_0 the intercepts. The validity of these plots to compare the behaviour of basic drugs analysed with different types of columns and mobile phase additives was demonstrated in previous reports [16,27], and have been commented in Chapters 5 and 6.

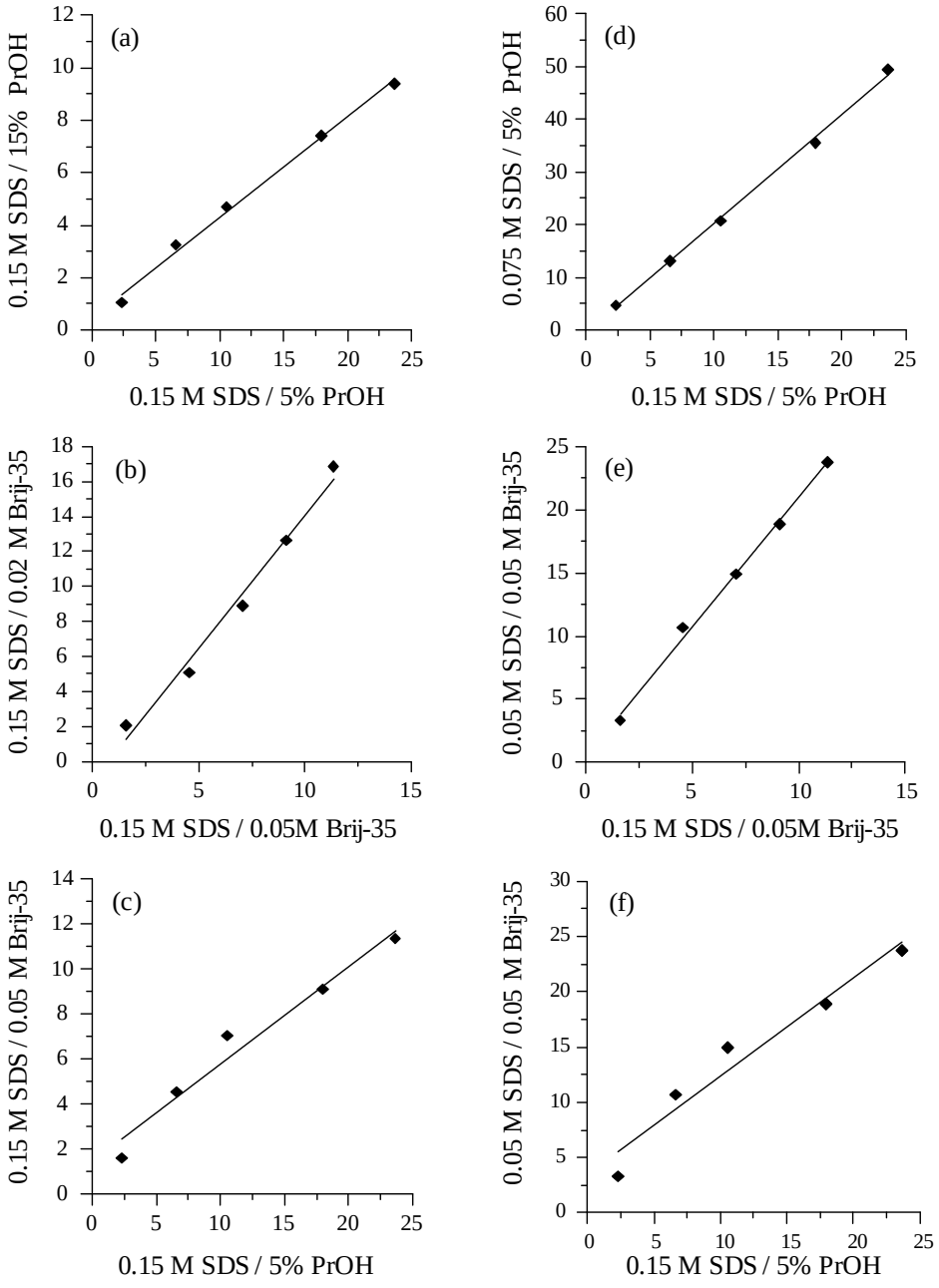


Figure 7.2. Selectivity of the chromatographic systems (retention factors for the five β-blockers are plotted).

The combination of the parameters calculated from the linear regressions allows the characterisation of a chromatographic system: the sum of slopes ($m_A + m_B$) represents the peak broadening rate inside the column, whereas the ratio (m_B/m_A) indicates the asymmetry of peaks eluting at a time where the extra-column contribution is non-significant. The extra-column contributions are associated to the intercepts ($A_0 + B_0$ and B_0/A_0).

In order to compare the peak performance, peak half-width plots were built considering the data obtained with all mobile phases in the experimental design. The plots are depicted in Figures 7.3a and b. The most remarkable characteristic is that the slopes of the right (B) and left (A) half-widths were similar, which indicates the elution of nearly symmetrical peaks with the micellar mobile phases. Peak asymmetry for β-blockers eluted from C18 columns with aqueous-organic mobile phases has been found to be 3.60 (see Figure 6.4 in Chapter 6).

Considering the whole experimental domain, the extra- and intra-column contributions to the peak broadening rate and peak asymmetry for both micellar SDS/1-propanol and SDS/Brij-35 modes with the Zorbax column were: $A_0 + B_0 = -0.14$ and 0.93, $B_0/A_0 = 0.55$ and 2.00, $m_A + m_B = 0.106$ and 0.110, and $m_B/m_A = 1.43$ and 1.31, respectively. It is observed that the peak shape at the column entrance was poorer in the presence of Brij-35 (the peaks were broader), but the intra-column contributions to the peak broadening rate were similar for both micellar chromatographic modes. Consequently, at short retention times, the peaks were wider with Brij-35 (Figure 7.3), but at increasing retention times the widths tended to become similar to those obtained with the SDS/1-propanol mixtures.

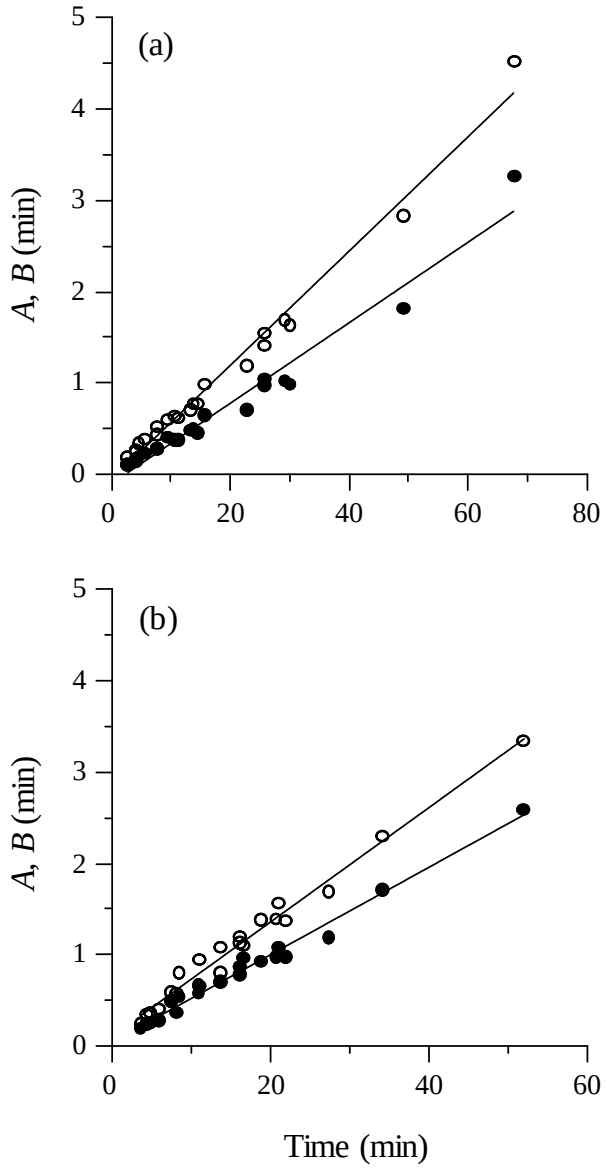


Figure 7.3. Half-width plots (left, A (●), and right, B (○) peak regions), for the five β-blockers, eluted with: (a) SDS/1-propanol, and (b) SDS/Brij-35. The whole set of mobile phases in the experimental design was considered.

7.4.3. Method validation

The performance of both micellar systems was further compared for the analysis of the β-blockers in formulations, making their validation according to the ICH Guideline [25]. The selected mobile phase compositions were: 0.15 M SDS/15% 1-propanol and 0.15 M SDS/0.05 M Brij-35 in the hybrid and mixed micellar modes, respectively. Calibration curves for each β-blockers were built using the areas of the chromatographic peaks from duplicate injections of standard solutions, at five concentrations in the 10–40 μg/mL range. The injected solutions were obtained from the stock aqueous solutions by dilution, and renewed every week. The assayed concentrations for the diluted solutions were uniformly distributed along the studied concentration range.

Calibration parameters were obtained during three non-consecutive days along three different weeks, and the curves were built as peak area *versus* the analyte concentration expressed as μg/mL. The parameters of the fitted straight lines (slope and intercept), and the corresponding absolute errors, are given in Table 7.1. As observed, all calibration curves met the linearity requirements, with determination coefficients $R^2 > 0.9990$. The slopes and intercepts were stable throughout the validation process and did not differ significantly between both micellar modes. This indicates a high prediction capability of the concentrations of the analytes from the fitted regression straight lines, and the fact that the chromatographic column performance is maintained.

Table 7.1. Day-to-day calibration parameters obtained for the β-blockers.

Compound		SDS/1-propanol			SDS/Brij-35		
		Slope	Intercept	R^2	Slope	Intercept	R^2
Atenolol	[a]	0.603 ± 0.006	–0.14 ± 0.06	0.9998	0.725 ± 0.005	–0.05 ± 0.19	0.9994
	[b]	0.606 ± 0.009	–0.21 ± 0.12	0.9999	0.733 ± 0.010	0.08 ± 0.20	0.9998
Celiprolol	[a]	1.00 ± 0.04	–0.97 ± 0.0.09	0.9997	1.00 ± 0.03	–0.99 ± 0.14	0.9999
	[b]	0.99 ± 0.06	–0.9 ± 0.3	0.9996	0.99 ± 0.04	–0.86 ± 0.25	0.9996
Metoprolol	[a]	0.525 ± 0.006	–0.23 ± 0.08	0.9993	0.55 ± 0.03	–0.3 ± 0.5	0.9996
	[b]	0.534 ± 0.012	–0.29 ± 0.11	0.9996	0.538 ± 0.014	–0.2 ± 0.4	0.9996
Oxprenolol	[a]	0.504 ± 0.006	–0.332 ± 0.013	0.9997	0.462 ± 0.009	–0.33 ± 0.03	0.9996
	[b]	0.502 ± 0.009	–0.27 ± 0.06	0.9997	0.470 ± 0.011	–0.4 ± 0.3	0.9998
Propranolol	[a]	2.788 ± 0.020	–5.35 ± 0.19	0.9991	2.370 ± 0.017	–1.4 ± 0.4	0.9998
	[b]	2.66 ± 0.11	–3.5 ± 1.5	0.9994	2.29 ± 0.06	–0.8 ± 0.8	0.9998

[a] Average for the same set of samples measured along three non-consecutive days during the same week.

[b] Average for different sets of samples measured along three days during three consecutive weeks.

The intra- and inter-day repeatability was evaluated by measuring the signals from solutions at three different concentrations: 10, 25, and 40 μg/mL, within the linear range of the calibration curves, for each analyte, performing five replicated measurements. Inter-day variation was performed during three non-consecutive days. The accuracy was also established using the calibration curve obtained each day. The results are summarised in Tables 7.2 and 7.3. The precision is expressed as relative standard deviation (RSD), and the accuracy as relative error (relative difference between the values found from the calibration and the concentration of the standards). As can be seen, the intra- and inter-day precisions were usually below 1% and 2% for the hybrid and mixed modes, respectively.

The limits of detection (LODs) and quantification (LOQs) were defined as the lowest concentration of the analytes that can be detected with signal-to-noise ratios greater than 3:1 and 10:1 (3- and 10-times the signal standard deviation), respectively. LODs and LOQs were obtained by injecting five solutions containing between 0.1 and 0.6 μg/mL of each β-blockers. For the micellar modes using SDS/1-propanol and SDS/Brij-35, LODs (μg/mL) were, respectively: atenolol (0.05, 0.08), celiprolol (0.04, 0.04), metoprolol (0.05, 0.14), oxprenolol (0.06, 0.07), and propranolol (0.02, 0.08), and LOQs were: atenolol (0.16, 0.26), celiprolol (0.13, 0.13), metoprolol (0.16, 0.46), oxprenolol (0.20, 0.23), and propranolol (0.07, 0.26).

Table 7.2. Intra-day and inter-day precision and accuracy for the hybrid SDS/1-propanol micellar mode.

		Intra-day			Inter-day		
Compound	Added (µg/mL)	Found (mean ± SD) (µg/mL)	RSD (%)	Accuracy (%)	Found (mean ± SD) (µg/mL)	RSD (%)	Accuracy (%)
Atenolol	10.00	10.01 ± 0.08	0.8	0.1	10.08 ± 0.08	0.8	0.8
	25.00	25.04 ± 0.04	0.2	0.2	25.01 ± 0.03	0.1	0.04
	40.00	40.27 ± 0.09	0.2	0.7	40.15 ± 0.11	0.3	0.4
Celiprolol	10.00	10.19 ± 0.06	0.6	1.9	9.94 ± 0.20	2.0	–0.6
	25.00	25.13 ± 0.05	0.2	0.5	24.71 ± 0.16	0.6	–1.2
	40.00	40.36 ± 0.06	0.4	0.9	40.05 ± 0.13	0.3	0.1
Metoprolol	9.90	9.75 ± 0.07	0.7	–1.5	9.78 ± 0.04	0.4	–1.2
	24.75	24.95 ± 0.12	0.5	0.8	24.87 ± 0.08	0.3	0.5
	39.60	39.73 ± 0.08	0.2	0.3	39.59 ± 0.13	0.3	–0.03
Oxprenolol	10.10	10.40 ± 0.04	0.4	3.0	10.28 ± 0.10	1.0	1.8
	25.25	25.19 ± 0.07	0.3	–0.2	25.16 ± 0.06	0.2	–0.4
	40.40	40.69 ± 0.09	0.2	0.7	40.683 ± 0.012	0.03	0.7
Propranolol	9.90	9.947 ± 0.025	0.3	0.5	9.92 ± 0.03	0.3	0.2
	24.75	24.88 ± 0.05	0.2	0.5	24.79 ± 0.08	0.3	0.2
	39.60	39.66 ± 0.11	0.3	0.2	39.59 ± 0.06	0.2	–0.03

Table 7.3. Intra-day and inter-day precision and accuracy for the mixed SDS/Brij-35 micellar mode.

		Intra-day			Inter-day		
Compound	Added (µg/ml)	Found (mean ± SD) (µg/mL)	RSD (%)	Accuracy (%)	Found (mean ± SD) (µg/mL)	RSD (%)	Accuracy (%)
Atenolol	10.50	10.39 ± 0.11	1.1	–1.0	10.44 ± 0.06	0.6	–0.6
	26.50	25.97 ± 0.11	0.4	–1.1	26.14 ± 0.16	0.6	–0.4
	42.00	41.97 ± 0.15	0.4	–0.1	42.0 ± 0.3	0.7	0.0
Celiprolol	9.90	9.69 ± 0.12	1.2	–2.1	9.80 ± 0.06	0.6	–1.0
	24.75	24.75 ± 0.12	0.5	0.0	24.84 ± 0.12	0.5	0.4
	39.60	39.27 ± 0.20	0.5	–3.4	39.0 ± 0.7	1.8	–1.5
Metoprolol	10.00	10.21 ± 0.08	0.8	2.1	10.09 ± 0.12	1.2	0.9
	25.00	25.24 ± 0.07	0.3	1.0	25.08 ± 0.23	0.9	0.3
	40.00	40.20 ± 0.06	0.1	0.5	40.08 ± 0.13	0.3	0.2
Oxprenolol	10.10	10.04 ± 0.11	1.1	–0.6	10.7 ± 0.4	3.7	0.6
	25.25	25.22 ± 0.12	0.5	–0.1	25.6 ± 0.5	2.0	0.1
	40.40	40.54 ± 0.11	0.3	0.3	40.67 ± 0.20	0.5	0.7
Propranolol	9.90	10.11 ± 0.10	1.0	2.1	10.00 ± 0.09	0.9	1.0
	24.75	25.08 ± 0.06	0.2	1.3	24.95 ± 0.14	0.6	0.8
	39.60	39.66 ± 0.08	0.2	0.2	39.657 ± 0.025	0.1	0.1

Robustness, which is defined as the effect of some experimental parameters on the retention time and peak areas of the analytes, was also investigated. The assayed parameters were the flow rate, and the pH and concentrations of SDS, 1-propanol and Brij-35 in the mobile phase. Each of these parameters was varied within a range around the value used to develop the analytical method. The parameters were modified following the one-variable-at-a-time (OVAT) method, where the variables are changed one by one, keeping all other parameters constant at their original value. In Table 7.4, the mean values, and absolute and relative standard deviations, obtained in the measurement of retention times and peak areas are provided.

For the retention times, the RSD values were sually in the 1–2% range for all assayed parameters, corresponding the highest RSD to the concentration of the two surfactants. A greater variability was observed for the peak areas. Note that the range of variation was larger for the concentration of Brij-35. Therefore, to carry out these micellar methods, the concentration of the two surfactants must be accurately controlled, especially for Brij-35.

7.4.4. Analysis of pharmaceutical formulations

Several formulations prescribed in Europe containing one of the five β-blockers studied in this work were analysed (Table 7.5). Parallel analyses of all formulations were carried out using 0.15 M SDS/15% 1-propanol and 0.15 M SDS/0.05 M Brij-35. Five samples of each formulation were analysed making duplicate injections to obtain average values of the drug concentrations. For this purpose, an appropriate amount of each sample was weighed to prepare solutions containing approximately 25 μg/mL of the drugs.

Table 7.4. Robustness of the proposed methods.

Compound	Parameter	Level	SDS/1-propanol		SDS/Brij-35	
			Retention time (min) (RSD, %)	Area (arbitrary units) (RSD, %)	Retention time (min) (RSD, %)	Area (arbitrary units) (RSD, %)
Atenolol	Flow rate (mL/min)	0.99 – 1.01	2.68 ± 0.03 (1.1)	15.2 ± 0.3 (2.0)	3.15 ± 0.04 (1.3)	13.38 ± 0.18 (1.3)
	SDS (M)	0.145 – 0.155	2.61 ± 0.05 (1.9)	15.18 ± 0.10 (0.7)	3.17 ± 0.07 (2.2)	13.07 ± 0.25 (1.9)
	Propanol (%)	14.9 – 15.1	2.63 ± 0.03 (1.1)	15.4 ± 0.3 (1.9)	–	–
	Brij-35 (M)	0.045 – 0.055	–	–	3.15 ± 0.07 (2.2)	12.9 ± 0.4 (3.1)
	pH	2.9 – 3.1	2.641 ± 0.021 (0.8)	15.24 ± 0.15 (1.0)	3.165 ± 0.019 (0.6)	13.11 ± 0.19 (1.4)
Celiprolol	Flow rate (mL/min)	0.99 – 1.01	5.30 ± 0.06 (1.1)	23.7 ± 0.7 (3.0)	6.25 ± 0.06 (1.0)	20.12 ± 0.08 (0.4)
	SDS (M)	0.145 – 0.155	5.14 ± 0.14 (2.7)	24.55 ± 0.06 (0.2)	6.27 ± 0.17 (2.7)	20.5 ± 0.8 (3.9)
	Propanol (%)	14.9 – 15.1	5.16 ± 0.07 (1.4)	24.4 ± 0.8 (3.3)	–	–
	Brij-35 (M)	0.045 – 0.055	–	–	6.24 ± 0.16 (2.6)	20.6 ± 1.1 (5.3)
	pH	2.9 – 3.1	5.20 ± 0.04 (0.8)	24.2 ± 0.9 (3.7)	6.29 ± 0.05 (0.8)	20.07 ± 0.18 (0.9)
Metoprolol	Flow rate (mL/min)	0.99 – 1.01	7.23 ± 0.10 (1.4)	12.9 ± 0.3 (2.3)	8.91 ± 0.12 (1.3)	11.21 ± 0.16 (1.4)
	SDS (M)	0.145 – 0.155	6.98 ± 0.21 (3.0)	12.5 ± 0.07 (0.6)	9.0 ± 0.3 (3.3)	11.0 ± 0.3 (2.7)
	Propanol (%)	14.9 – 15.1	7.02 ± 0.11 (1.6)	12.9 ± 0.4 (3.1)	–	–
	Brij-35 (M)	0.045 – 0.055	–	–	9.0 ± 0.3 (3.3)	11.0 ± 0.3 (2.7)
	pH	2.9 – 3.1	7.08 ± 0.07 (1.0)	12.7 ± 0.3 (2.4)	9.05 ± 0.10 (1.1)	10.95 ± 0.16 (1.5)
Oxprenolol	Flow rate (mL/min)	0.99 – 1.01	10.52 ± 0.15 (1.4)	12.4 ± 0.3 (2.4)	11.02 ± 0.12 (1.1)	10.07 ± 0.04 (0.4)
	SDS (M)	0.145 – 0.155	10.13 ± 0.3 (3.0)	12.26 ± 0.08(0.7)	11.1 ± 0.3 (2.7)	10.1 ± 0.3 (3.0)
	Propanol (%)	14.9 – 15.1	10.17 ± 0.18 (1.8)	12.43 ± 0.07 (0.6)	–	–
	Brij-35 (M)	0.045 – 0.055	–	–	11.1 ± 0.5 (4.5)	10.06 ± 0.15 (1.5)
	pH	2.9 – 3.1	10.26 ± 0.11 (1.1)	12.38 ± 0.12 (1.0)	11.14 ± 0.13 (1.2)	9.95 ± 0.20 (2.0)
Propranolol	Flow rate (mL/min)	0.99 – 1.01	13.39 ± 0.15 (1.1)	63.5 ± 1.0 (1.6)	13.15 ± 0.17 (1.3)	46.3 ± 0.7 (1.5)
	SDS (M)	0.145 – 0.155	12.9 ± 0.5 (3.9)	62.66 ± 0.23 (0.4)	13.3 ± 0.4 (3.0)	46.1 ± 0.8 (1.7)
	Propanol (%)	14.9 – 15.1	12.9 ± 0.3 (2.3)	63.3 ± 0.04 (0.6)	–	–
	Brij-35 (M)	0.045 – 0.055	–	–	13.3 ± 0.6 (4.5)	45.6 ± 1.1 (2.4)
	pH	2.9 – 3.1	13.04 ± 0.21 (1.6)	63.0 ± 0.5 (0.8)	13.33 ± 0.17 (1.3)	45.3 ± 0.5 (1.1)

Table 7.5. Analysis of several formulations containing β-blockers.

Formulation (laboratory)	Composition (mg)	SDS/1-propanol		SDS/Brij-35	
		Found (mg)	Label claim (%)	Found (mg)	Label claim (%)
Atenolol Alter (Alter Laboratories)	Per tablet: Atenolol (50), wheat starch, corn starch and other excipients	54.2 ± 1.9	108.4	53.4 ± 2.1	106.8
Cardem (Rorer)	Per tablet: Celiprolol chlorhydrate (200)	222 ± 22	111.0	217 ± 9	108.5
Beloken Retard (Astra Zeneca)	Per tablet: Metoprolol succinate (95) and excipients	107 ± 10	112.6	100 ± 11	105.3
Trasicor (Ciba-Geigy)	Per tablet: Oxprenolol chlorhydrate (80)	83.9 ± 1.5	104.9	86 ± 3	107.5
Propanolol Accord (Accord Healthcare)	Per tablet: Propanol hydrochloride (40), wheat starch, cellulose and other excipients	39.7 ± 1.1	99.3	40.2 ± 0.6	100.5

Figures 7.4 and 7.5 show chromatograms for the formulations. The excipients were eluted at the dead time or did not absorb at the measuring wavelength. Table 7.5 gives the found contents, together with the label claim percentages, which were usually around 100%. According to the ICH guideline, for the assay of a finished (drug) product, a range from 80 to 120% of the sample concentration is accepted [25].

7.5. Conclusions

The analysis of β-blockers requires the addition of silanol suppressing reagents to the mobile phase, in order to avoid the formation of tailed and broad peaks. In previous work, hybrid micellar mobile phases containing SDS and 1-propanol demonstrated their potential for the analysis of these basic drugs. The aim of this work was to check if reliable analyses of commonly prescribed β-blockers were possible with micellar mobile phases where the organic solvent is substituted by a second surfactant (Brij-35), giving rise to a mobile phase without organic solvent (i.e., composed only by water and detergents). Only a small amount of methanol (at least 15 mL) is needed to clean the chromatographic column before the pump is stopped.

The results with SDS/Brij-35 were compared with those obtained using SDS/1-propanol. With an appropriate ratio of SDS and Brij-35, the investigated drugs were eluted at sufficiently short analysis times with good performance, as is the case with a similar concentration of SDS in a 15% 1-propanol medium. It should be also highlighted that the mixed mobile phase of SDS and Brij-35 can be recycled throughout the system without affecting the chromatographic performance along repetitive analyses during several days. All these characteristics reduce the economic and environmental cost of the procedure.

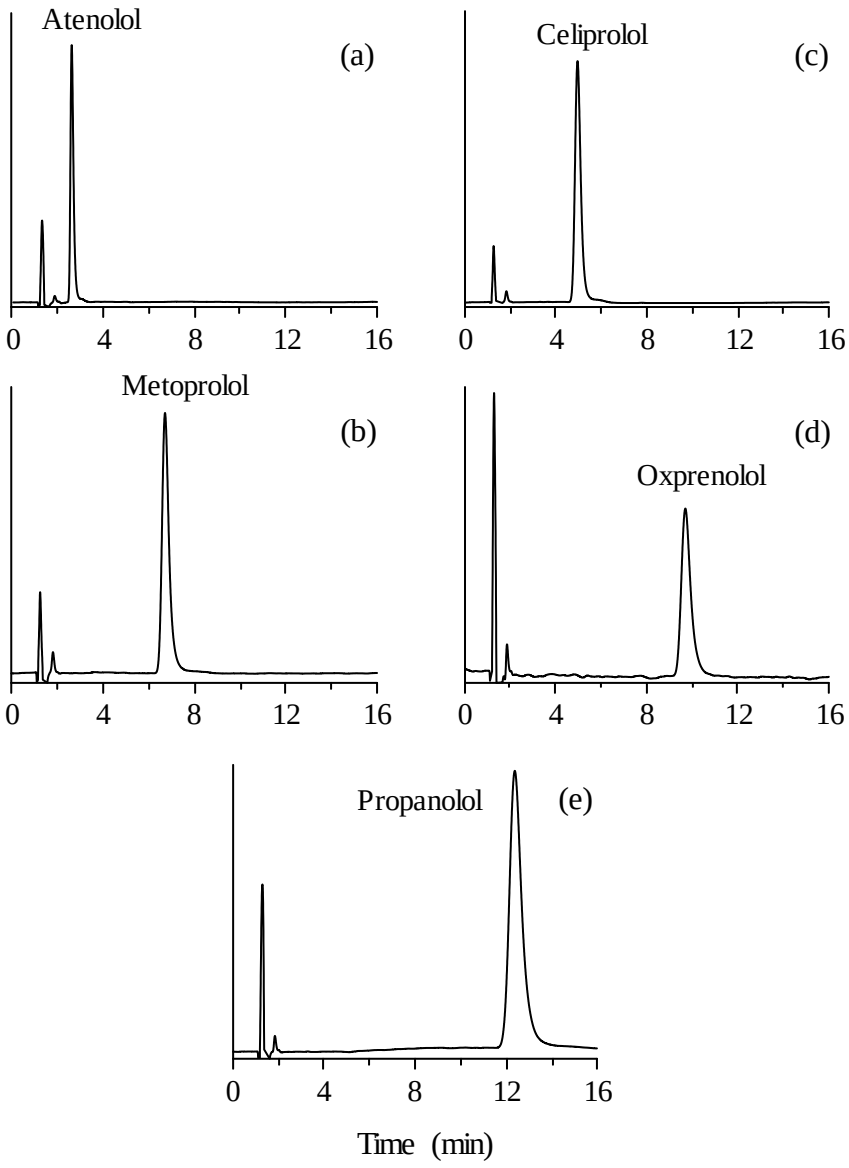


Figure 7.4. Chromatograms for the formulations containing the five β-blockers, eluted with 0.15 M SDS/15% 1-propanol: (a) Atenolol Alter, (b) Cardem, (c) Belokem Retard, (d) Trasicor, and (e) Propranolol Accord.

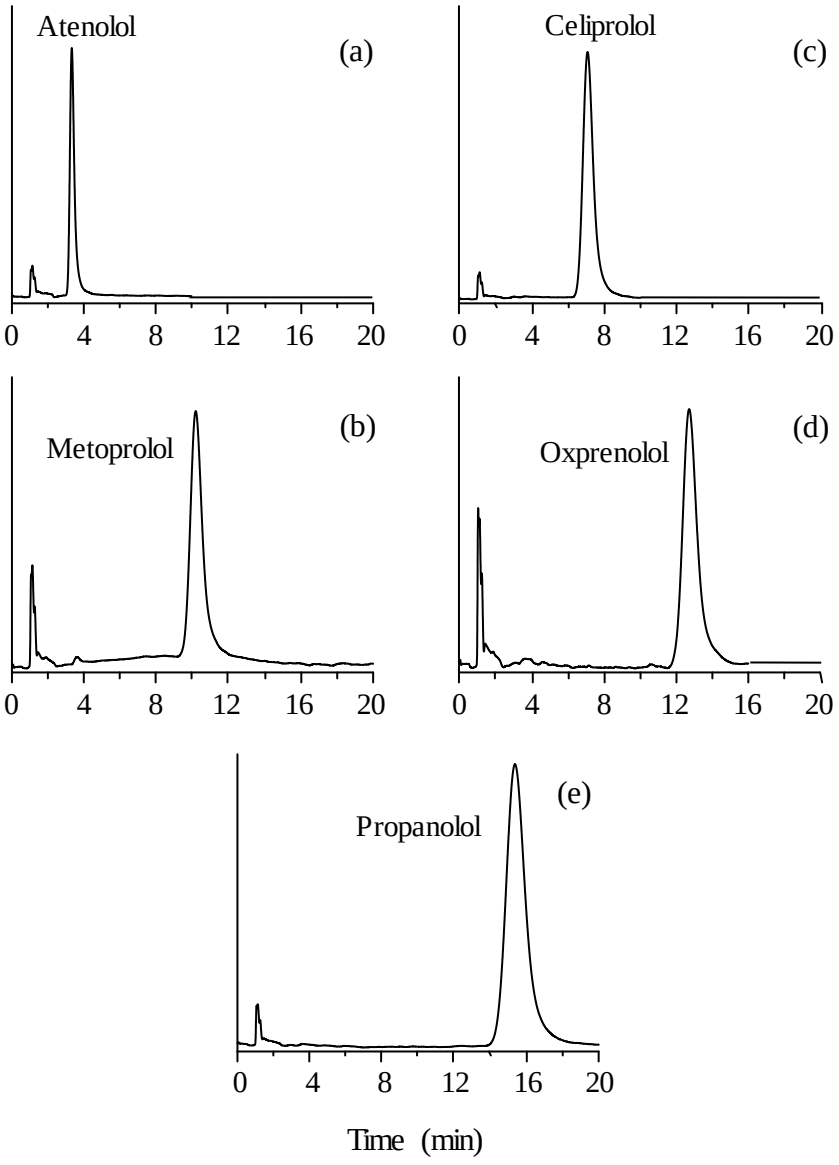


Figure 7.5. Chromatograms for the formulations containing β-blockers, eluted with 0.15 M SDS/0.05 M Brij-35: (a) Atenolol Alter, (b) Cardem, (c) Belokem Retard, (d) Trasicor, and (e) Propranolol Accord.

The chromatographic procedures applied to the control of commercialised formulations, yielded satisfactory results. The preparation of the samples (only solubilisation and filtration) was simple, without needing previous extraction. SDS and Brij-35 are biodegradable, being the described procedure an example of micellar “green” chromatographic analysis.

7.6. References

[1] J.R. Waller, D.G. Waller, Drugs for systemic hypertension and angina, *Medicine* 42 (2014) 538–543.

[2] J.R. Waller, D.G. Waller, Drugs for heart failure and arrhythmias, *Medicine* 42 (2014) 620–624.

[3] *World Health Statistics 2012 Report*, World Health Organization. Geneva, 2012.

[4] M. Scheurer, M. Ramil, C.D. Metcalfe, S. Groh, T.A. Ternes, The challenge of analyzing beta-blocker drugs in sludge and wastewater, *Anal. Bioanal. Chem.* 396 (2010) 845–856.

[5] H.Y. Aboul Enein, I. Ali, *Chiral Separations by Liquid Chromatography and Related Technologies*, Marcel Dekker, New York, 2003.

[6] K. Saleem, I. Ali, U. Kulsum, H.Y. Aboul-Enein, Recent developments in HPLC analysis of β-blockers in biological samples, *J. Chromatogr. Sci.* 51 (2013) 807–818.

[7] A.E. Bretnall, G.S. Clarke, Selectivity of capillary electrophoresis for the analysis of cardiovascular drugs, *J. Chromatogr. A* 745 (1996) 145–154.

[8] J.P. Landers, *Handbook of Capillary and Microchip Electrophoresis and Associated Microtechniques*, 3rd ed., CRC Press, Boca Raton, FL, 2007.

[9] I. Ali, H.Y. Aboul-Enein, V.K. Gupta, Nano chromatography and capillary electrophoresis, in: *Pharmaceutical and Environmental Analyses*, Wiley & Sons, Hoboken, NJ, 2009.

[10] L.R. Snyder, J.J. Kirkland, J.W. Dolan, *Introduction to Modern Liquid Chromatography*, 3rd ed., Wiley, New York, 2010.

[11] M.C. García Álvarez-Coque, J.J. Baeza Baeza, G. Ramis Ramos, Reversed phase liquid chromatography in: *Analytical Separation Science Series*, Vol. 1 (edited by J. Anderson, A. Berthod, V. Pino, A.M. Stalcup), Wiley, New York, 2015, pp. 159–198.

[12] H. Engelhardt, Ch. Blay, J. Saar, Reversed phase chromatography: The mystery of surface silanols, *Chromatographia* 62 (2005) S19–S29.

[13] U.D. Neue, K. Tran, A. Méndez, P.W. Carr, The combined effect of silanols and the reversed-phase ligand on the retention of positively charged analytes, *J. Chromatogr. A* 1063 (2005) 35–45.

[14] D.V. McCalley, The challenges of the analysis of basic compounds by high performance liquid chromatography: Some possible approaches for improved separations, *J. Chromatogr. A* 1217 (2010) 858–880.

[15] A. Martín Calero, V. Pino, J.H. Ayala, V. González, A.M. Afonso, Ionic liquids as mobile phase additives in high-performance liquid chromatography with electrochemical detection: Application to the determination of heterocyclic aromatic amines in meat-based infant foods, *Talanta* 79 (2009) 590–597.

[16] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[17] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, A. Berthod, S. Carda Broch, On the use of ionic liquids as mobile phase additives in high-performance liquid chromatography, *Anal. Chim. Acta* 883 (2015) 1–21.

[18] S. Calabuig Hernández, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, Performance of amines as silanol suppressors in reversed-phase liquid chromatography, *J. Chromatogr. A* 1465 (2016) 98–106.

[19] M.J. Ruiz Ángel, M.C. García Álvarez-Coque, A. Berthod, New insights and recent developments in micellar liquid chromatography, *Sep. Purif. Rev.* 38 (2009) 45–96.

[20] S. Carda Broch, J.S. Esteve Romero, M.C. García Álvarez-Coque, Liquid chromatographic determination of some thiazide diuretics in pharmaceuticals with a sodium dodecyl sulphate mobile phase, *Analyst* 123 (1998) 301–306.

[21] A. Aparicio, M.P. San Andrés, S. Vera, Separation and determination of phenolic antioxidants by HPLC with surfactant/n-propanol mobile phases, *J. High. Resolut. Chromatogr.* 23 (2002) 324–328.

[22] M.S. Jiménez, R. Velarte, J.R. Castillo, Direct determination of phenolic compounds and phospholipids in virgin olive oil by micellar liquid chromatography, *Food Chem.* 100 (2007) 8–14.

[23] J. Rodenas Montano, C. Ortiz Bolsico, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Implementation of gradients of organic solvent in micellar liquid chromatography using DryLab®: Separation of basic compounds in urine samples, *J. Chromatogr. A* 1344 (2014) 31–41.

[24] M.J. Ruiz Ángel, E. Peris García, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A method for the analysis of basic compounds, *Green Chem.* 17 (2015) 3561–3570.

[25] *ICH Harmonized Tripartite Guideline*: Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, CH, 2005, www.ich.org

[26] J.R. Torres Lapasió, *MICHROM Software*, Marcel Dekker, New York, 2000.

[27] J.J. Baeza Baeza, M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

Part 2

IONIC LIQUIDS AS MOBILE PHASE ADDITIVES IN LIQUID CHROMATOGRAPHY

CHAPTER 8

EFFECT OF BUFFER NATURE AND CONCENTRATION ON THE CHROMATOGRAPHIC PERFORMANCE OF BASIC COMPOUNDS IN THE ABSENCE AND PRESENCE OF 1-HEXYL-3-METHYLIMIDAZOLIUM CHLORIDE

8.1. Abstract

In reversed-phase liquid chromatography, the performance for basic compounds is affected by the interaction of the protonated (cationic) species with the anionic free silanols on the alkyl-bonded stationary phases. Using aqueous-organic mobile phases in the absence of additives, the retention may be too high, and the peaks be broad and asymmetric. The performance is improved by addition to the mobile phase of ionic liquids, from which 1-hexyl-3-methylimidazolium chloride (HMIM·Cl) has especially good characteristics. A recent report has also revealed that the use of the phosphate system as buffer, at varying concentration and pH, may have a significant role in the chromatographic performance of basic compounds, with effects on both retention and peak shape. In this work, this study has been extended to other three buffer systems (acetate, citrate, and formate), at increasing concentrations and pH 3 and 7, in the presence and absence of HMIM·Cl. The results have been compared with those obtained with the phosphate system. The retention increases by addition of larger concentration of all buffers, in both absence and presence of HMIM·Cl. Without additive, peak performance also is enhanced significantly. This effect is minimal in the presence of HMIM·Cl, which yields highly symmetrical peaks at all buffer concentrations, due to an effective blocking of the silanol activity.

8.2. Introduction

Ionic liquids (ILs) can be considered as salts or solvents. By essence, they are made of cations and anions and possess as special feature low melting temperature (usually below 100 ^{o}C). As solvents, ILs have non-molecular nature, and show mainly low volatility and flammability, together with high thermal stability [1,2]. Due to these properties, ILs started to be used as promising benign or green solvents that could replace pollutant organic solvents in the laboratories. Nevertheless, several reports have specified in the last years certain reticence to the term “green” [3,4], since some ILs are not as safe and non-toxic as it was thought, especially during their synthesis.

In the field of chromatographic analysis, ILs have been used in reversed-phase liquid chromatography (RPLC) as mobile phase additives to successfully enhance peak performance in the analysis of basic compounds, using conventional stationary phases [5–17]. It should be noted that in RPLC, basic compounds may experience additional ion-exchange interactions with residual anionic silanols on silica packings, which increase their retention and result in broad and asymmetrical peaks [18–21]. In this regard, ILs have become a serious alternative to the common reagents traditionally used to enhance chromatographic performance (e.g. amines and surfactants).

Ionic liquids of different nature have been considered in RPLC, starting with the alkylammonium-based ILs associated to nitrate and thiocyanate [22,23], or acetate and formate [24–28]. However, the use of these ILs was problematic due to their high viscosity, production of corrosion of the metallic parts of the HPLC systems after extensive use, poor baseline stability, weaker elution strength compared to common organic solvents, rapid deterioration of the silica-based packing, and poor efficiencies. For this reason, in the 90’s, imidazolium-based ILs associated to chloride, bromide, tetrafluoroborate or

hexafluorophosphate, were proposed as the best option to combine with acetonitrile-water mixtures, since they were able to overcome these initial drawbacks [5–17].

The interaction mechanism of ILs with RPLC stationary phases is more complex compared to the behaviour of other additives, such as amines or the anionic surfactant sodium dodecyl sulfate (which is also a good silanol suppressor [29,30]). Both cations and anions in the IL may be able to interact with the stationary phases and create an asymmetrical bilayer, positively or negatively charged, depending on the relative adsorption of the cation and the anion [31,32]. This means that these additives are dual modifiers, which enrich the nature of the interactions taking place simultaneously inside the column.

In order to gain more insight in the behaviour of ILs as silanol suppressors, a series of comprehensive studies on the silanol suppression effect of imidazolium-based ILs with long side chains was carried out, based on modelling the changes of retention and peak shape using increasing amounts of the ILs in the mobile phase [32–35]. The effect of ILs associated to tetrafluoroborate and hexafluorophosphate anions was also compared to that achieved with different amines [31,32]. Since the silanol suppression effect of imidazolium cations was dominant with respect to the interactions with the associated anion, the research was then extended to ILs associated to chloride (without affinity towards the stationary phase) [35]. 1-Hexyl-3-methylimidazolium chloride (HMIM·Cl) was revealed as an efficient peak profile enhancer of basic compounds in RPLC, using conventional stationary phases, since it produced narrower chromatographic peaks, combined with smaller retention and consumption of organic solvent.

Acetonitrile-water mixtures containing an ionic liquid as modifier in RPLC are usually buffered at fixed pH. It should be noted that changes in organic solvent concentration may affect the pH and pK_a of the acid-base buffer and solute [36], which would have influence on solute retention. However, the selection of the buffer nature and concentration, which may be critical, is not usually considered. A recent report with basic analytes revealed that the addition of phosphate buffer to the mobile phase at variable concentration had a significant role in the separation process, with effects on both the retention mechanism and peak shape [37]. Therefore, the performance of basic analytes under the addition of a specific buffer system deserves a more deep study.

In this work, the study was extended to buffers of different nature, in the absence and presence of the ionic liquid HMIM·Cl in acetonitrile-water mobile phases. The variation of retention times, and eventual changes in peak shape and selectivity, of a group of basic β-blockers were investigated with mobile phases containing four different buffer systems (acetic acid/acetate, citric acid/dihydrogen citrate, formic acid/formate and phosphoric acid/dihydrogen phosphate), at increasing concentrations at pH 3. Buffer solutions composed of hydrogen citrate/citrate and dihydrogen phosphate/hydrogen phosphate were also compared at pH 7. Conclusions about the effect of the buffer systems on the suppression of silanol activity, in the presence and absence of HMIM·Cl are addressed.

8.3. Experimental

8.3.1. Reagents

β-Blockers are commercialised and used for the treatment of various cardiac diseases [38]. Six of these drugs were used in this work as probe compounds: acebutolol, atenolol, metoprolol, nadolol, oxprenolol and timolol (all from Sigma, St. Louis, MO, USA). The drugs were dissolved in a small amount of acetonitrile, with the aid of an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany), and diluted with water up to a concentration of approximately 100 μg/mL. The solutions were stored at 4 ^{o}C, remaining stable during at least two months. These were diluted to obtain solutions of approximately 20 μg/mL, which were injected into the chromatograph. Uracil (Acros Organics, Geel, Belgium) was selected as dead time marker.

Mobile phases were prepared with acetonitrile (Scharlab, Barcelona, Spain), in the absence and presence of the ionic liquid HMIM·Cl (Sigma). Based on previous experience [32,35], the concentration of acetonitrile was fixed at 10 % (*v*/*v*), except when the citrate buffer was added, for which 15% acetonitrile was used.

The mobile phases were buffered at pH 3 with ammonium formate (Sigma), citric acid (Scharlab), sodium acetate (Sigma), and sodium dihydrogen phosphate (Sigma), and at pH 7 with citric acid and sodium dihydrogen phosphate, in both cases at concentrations in the range from 5 to 30 mM, to which HCl (Scharlab) or sodium hydroxide (Panreac, Barcelona) was added to get the desired pH. Measurement of pH was made with a Hach (Loveland, CO, USA) combined electrode 5202 (glass electrode and reference electrode containing a 3.0 M KCl solution in water as salt bridge), and a Crison micropH 2002 potentiometer with a precision of 0.1 mV. The standardisation of the

pH-meter was always carried out using aqueous buffers. The pH was measured in the aqueous solution before adding the organic solvent to the mobile phase, and afterwards, in the aqueous-organic mixture to get the final pH value [39].

Nylon membranes of 0.45 μm (Micron Separations, Westboro, MA, USA) were used to filter the drug solutions and mobile phases, which were also degassed in an ultrasonic bath. Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

8.3.2. Apparatus and columns

An Agilent chromatograph (Waldbronn, Germany), equipped with an isocratic pump (Series 1260), an autosampler (Series 1260), a thermostated column compartment (Series 1260) set at 25 ºC, a UV-visible wavelength detector (Series 1100), and an HPChemStation (Agilent, B.02.01) for data acquisition was used. β-Blockers were monitored at 254 nm, except timolol, for which the signal was measured at 300 nm. The mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were integrated with MICHROM [40].

The chromatographic column was a Zorbax Eclipse XDB C18 (Agilent), with the following characteristics: 150 mm × 4.6 mm i.d., 5 μm particle size, 10 % carbon load, 180 m^2/g surface area, and 80 Å pore size. A similar 30 mm pre-column was connected to the analytical column for protection. The flow-rate was 1 mL/min. Duplicate injections of 20 μL were made.

8.4. Results and discussion

8.4.1. pK_a and pH variation of the selected buffers with solvent composition

β-Blockers are ionisable compounds that can exist in the ionised (cationic) or non-ionised forms depending on the mobile phase pH. These compounds are basic (pK_a = 9–10), due to the presence of an aromatic ring attached to a side alkyl chain possessing secondary hydroxyl and amine functional groups [41]. This means that at the working pH range of conventional silica-based C18 stationary phases (2–8) they are positively charged. When fixing the mobile phase pH, it should be considered that the pH and also the pK_a for a specific acid-base buffer may vary with solvent composition. Methanol and acetonitrile are the most common organic solvents used for the preparation of mobile phases in RPLC. In a series of reports by Bosch and co-workers [36,39,42–46], it was found that buffered solutions prepared from anionic and neutral (uncharged) acids, such as those used in our study (e.g. acetic acid/acetate), increased their pH value when acetonitrile or methanol were added, whereas buffers prepared with cationic acids (e.g. ammonium/ammonia) showed the reverse trend. The pH changes were induced by the variation of the pK_a values of buffer components with solvent composition changes.

In Table 8.1, the ${}^{s}_{w}pK_a$ values obtained in aqueous-organic solutions containing acetonitrile in the 10–15% range are compared with the pK_a values in water [39]. Note that the ${}^{s}_{w}pK_a$ values were obtained in the ${}^{s}_{w}\mathrm{pH}$ scale (i.e. the electrode system was calibrated with aqueous buffers, and the pH measured in the mobile phase after mixing the aqueous buffer with the organic modifier). The ${}^{s}_{w}\mathrm{pH}$ scale is especially convenient for its simplicity of measurement, since it does not require pH standards for each aqueous-organic

composition. As observed, the pK_a values increased with a mean change of (${}^{s}_{w}\mathrm{p}K_a - \mathrm{p}K_a$) = +0.18 pH unities.

Table 8.1. Values of acid-base dissociation constants in water and acetonitrile-water, for the buffers used in this work.

	pK_a [a]	${}^{s}_{w}\mathrm{p}K_a$ [b]
Buffer	Water	10 – 15% (*v*/*v*) acetonitrile
Acetic / acetate	4.74	4.93
Citric /dihydrogen citrate	3.16	3.30
Hydrogen citrate / citrate	6.42	6.61
Formic / formiate	3.72	3.93
Phosphoric / dihydrogen phosphate	2.21	2.38
Dihydrogen phosphate / hydrogen phosphate	7.23	7.39

[a] Ref. [39].

[b] ${}^{s}_{w}\mathrm{p}K_a$ was obtained by calibrating the electrode system with aqueous buffers, and measuring the pH in the mobile phase after mixing the aqueous buffer with the organic modifier.

The pH of the mobile phases used throughout the study were fixed at 3, using the four buffer systems (acetate, citrate, formate and phosphate), and at pH 7 buffered with the citrate and phosphate systems. Table 8.2 shows the difference in pH between aqueous and aqueous-organic solutions containing 10 or 15% acetonitrile, at three buffer concentrations (5, 10 and 30 mM). The measurements were made in the absence and presence of 10 mM HMIM·Cl. As observed, in the studied range, the pH variation seemed not be affected by the buffer concentration (only a slight variation was observed for the formate system), or by the buffer nature, being hydrogen citrate/citrate the system showing greater differences: 0.56, 0.61 and 0.58 at 5, 10 and 30 mM, respectively. The addition of 10 mM HMIM·Cl to the mobile phases did not change these trends.

8.4.2. Dominant acid-base species at the pH of the assayed mobile phases

According to the information obtained from Tables 8.1 and 8.2, acetate and formate buffers exist mainly in solution in their protonated neutral forms at pH 3, whereas for the citrate and phosphate buffers (especially for the latter), the anionic species are dominant. Close to pH 7, the citrate and phosphate systems loss an additional hydrogen ion, which means that the anionic species of the buffer systems are clearly dominant in the aqueous-organic solutions.

Meanwhile, in theory, the described changes in pK_a and pH values of the buffer systems should not affect the retention behaviour of the β-blockers. The protonated cationic species of these compounds will be still dominant at both pH 3 and 7, since they have a strong basic character with p$K_a \geq 9$ [41]. The organic solvent is likely to cause a reduction in the pK_a of these compounds, but not enough to change significantly their ionisation state.

Table 8.2. Experimental ΔpH values between aqueous and acetonitrile-water solutions at different buffer concentrations, in the absence and presence of 10 mM HMIM·Cl.

Buffer	5 mM		10 mM		30 mM	
	No additive	HMIM·Cl	No additive	HMIM·Cl	No additive	HMIM·Cl
Acetic / acetate[a] (pH = 3)	0.47	0.48	0.44	0.42	0.35	0.35
Citric /dihydrogen citrate[b] (pH = 3)	0.31	0.34	0.30	0.34	0.30	0.35
Hydrogen citrate / citrate[b] (pH = 7)	0.56	0.58	0.61	0.60	0.58	0.64
Formic / formate[a] (pH = 3)	0.33	0.30	0.23	0.25	0.19	0.23
Phosphoric / dihydrogen phosphate[a] (pH = 3)	0.45	0.46	0.36	0.38	0.30	0.31
Dihydrogen phosphate/hydrogen phosphate[a] (pH = 7)	0.32	0.35	0.39	0.37	0.37	0.39

[a]Acetonitrile-water 10:90 (v/v)

[b]Acetonitrile-water 15:85 (v/v)

Therefore, the only relevant acid-base equilibria at the working pH values (3 and 7) will be that of silanols and buffers. Silanols are weakly acidic, with average acid-base constants ranging from ca. pK_a = 4.5 to ca. 7, depending on the type of silica [47]. This means that their degree of ionisation (from neutral to anionic) varies with the mobile phase pH, inducing changes in the cationic solute/anionic silanol slow interaction that is the reason of the broad and asymmetrical peaks of basic compounds. This interaction may be blocked by the addition of different types of reagents, such as ILs and buffers. A detailed study of the changes in retention and peak shape of β-blockers at pH 3 and 7, in the absence and presence of HMIM·Cl at varying buffer concentration, is described in Sections 8.4.3 to 8.4.5, which will confirm these observations.

8.4.3. Effect of ionic liquid on retention

In previous work [35], the chromatographic retention of a set of β-blockers on a C18 Kromasil column was compared upon addition, to mobile phases containing a fixed concentration of 15% acetonitrile, of the ILs 1-ethyl, 1-butyl and 1-hexyl-3-methylimidazolium associated to chloride as anion (EMIM·Cl, BMIM·Cl and HMIM·Cl, respectively), at concentrations ranging between 10 and 40 mM. It was observed that solute retention decreased dramatically after the first addition (10 mM) of the ILs, the effect with HMIM·Cl being larger. This IL also gave rise to better peak shape. Further addition of the ILs in the mobile phase resulted in minimal changes of the retention factors in all cases.

The larger effect induced by HMIM·Cl was explained by the more intense adsorption of the IL cation ($HMIM^+$) on the alkyl-bonded stationary phase with respect to $EMIM^+$ and $BMIM^+$. Also, in these experiments, the influence of the anion was minimised since in aqueous solution, chloride is strongly hydrated and has low affinity towards the stationary phase. Therefore, chloride is not (or

is only scarcely) involved in the chromatographic separation, allowing the isolation of the effect of the imidazolium cation. The low retention times and efficient peak profile obtained with HMIM·Cl make this IL a convenient additive to enhance the chromatographic performance of β-blockers. For this reason, it was selected for this work.

To investigate the effect of HMIM·Cl on the chromatographic behaviour of the Zorbax Eclipse C18 column, a similar experiment at increasing concentrations of this IL, ranging between 10 and 40 mM was developed. In previous work [48], it was checked that this column yielded shorter retention times with respect to the Kromasil C18 column. For this reason, the concentration of acetonitrile was fixed mainly at 10% instead of 15%. In this study, 10 mM phosphoric acid/dihydrogen phosphate was added to buffer the pH at 3. The retention behaviour is shown in Figure 8.1. As before with the Kromasil column, the addition of a bulky imidazolium cation to the mobile phase, such as $HMIM^+$, gave rise to a significant decrease in the retention of the β-blockers, reaching quickly column saturation where changes in retention are minimal. For this reason, HMIM·Cl was added to the mobile phase at a fixed concentration of 10 mM in further studies. The selected concentrations for HMIM·Cl and acetonitrile guaranteed the elution of the assayed drugs in practical analysis times.

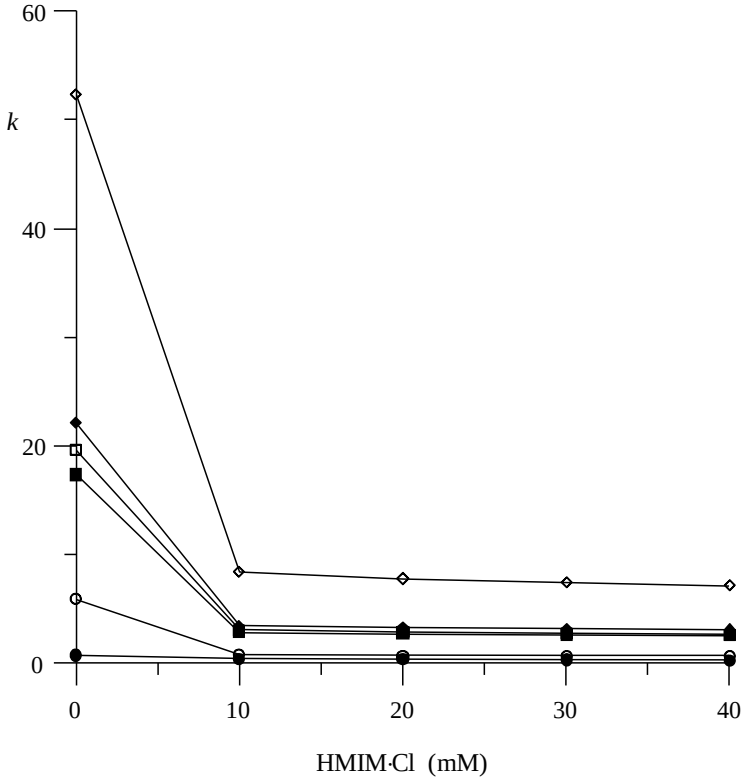


Figure 8.1. Retention behavior of the assayed β-blockers eluted from a C18 column with mobile phases containing 10% acetonitrile, 10 mM phosphate at pH 3, and increasing concentrations of 1-hexyl-3-methylimidazolium chloride. Solute identities: atenolol (●), nadolol (○), timolol (■), metoprolol (□), acebutolol (♦), and oxprenolol (◇).

8.4.4. Effect of buffer on retention

In previous work [37], it was observed that phosphate buffer may have an important role in the separation process. The results obtained showed that increasing amounts of this buffer system at low pH (pH 3), in the absence of any mobile phase additive, increased the retention times. Also, the peak shape was improved. The change in retention and peak shape suggested a possible adsorption of the phosphate anion on the stationary phase, which attracts the cationic solutes and blocks the activity of residual silanols in the C18 column. However, the effect of increasing concentration of phosphate buffer on the chromatographic performance was minimal when an IL was added to the mobile phase.

In order to extend this previous research, four buffer systems composed of acetic acid/acetate, formic acid/formate, citric acid/dihydrogen citrate, and phosphoric acid/dihydrogen phosphate, were added at increasing concentrations to mobile phases containing 10% acetonitrile at pH 3, in the absence and presence of HMIM·Cl. In another series of experiments with hydrogen citrate/citrate and dihydrogen phosphate/hydrogen phosphate the pH was also fixed at 7. It should be noted that citrate buffers gave rise to longer retention times for all compounds (e.g. > 120 min for oxprenolol at pH 3 and 10% acetonitrile), which forced the use of 15% acetonitrile in the mobile phases to reduce the retention times to more practical values.

The effect of buffer concentration on retention, in the absence of HMIM·Cl, can be observed in Figure 8.2. As observed, phosphate buffer (Figure 8.2b) gave rise to somewhat lower retention. The effect of formate (Figure 8.2a) and acetate (Figure 8.2d) on retention was similar.

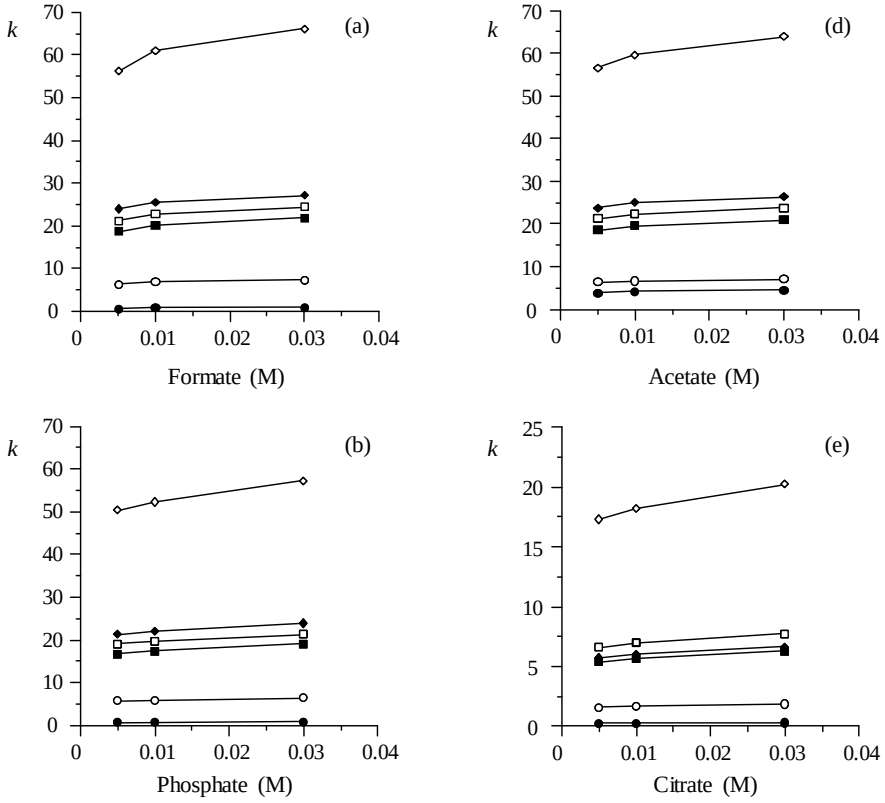


Figure 8.2. Retention behaviour of the β-blockers eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile (without ILC), and different buffer concentrations at pH 3 (a, b, d and e) and pH 7 (c and f). Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). Solute identities: atenolol (•), nadolol (○), timolol (■), metoprolol (□), acebutolol (♦), and oxprenolol (◇).

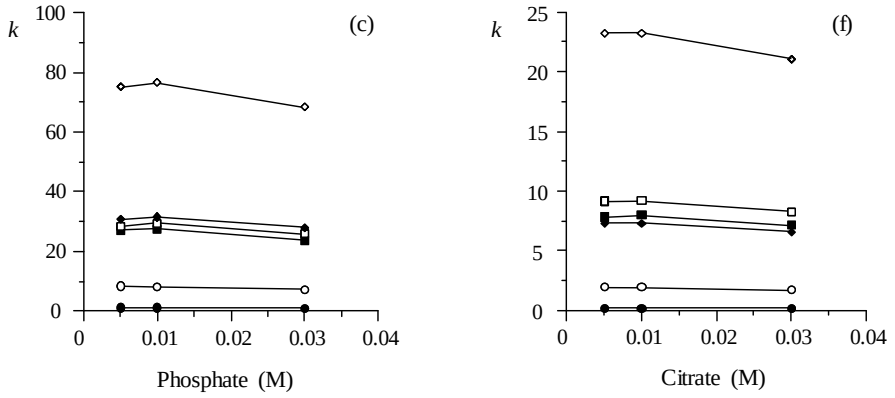


Figure 8.2 (continued).

The retention factors in the presence of increasing concentration of the buffers showed an increasing trend at pH 3, whereas the retention decreased for 30 mM phosphate and citrate buffers at pH 7 (Figures 8.2c and f). In previous work [37], the changes in retention with buffer concentration was attributed to a combination of interactions that included hydrophobic association of solutes with the alkyl-bonded stationary phase, and competitive attraction of the cationic solutes to the anionic silanols and phosphate ion weakly adsorbed to the stationary phase. It should be noted, that although the dominant species for the acetate and formate systems in the mobile phase, at pH 3, are not mainly the basic (anionic) species, the change in retention was also more pronounced at increasing concentration of these buffers. At pH 7, using the phosphate and citrate systems as buffers (Figures 8.2c and f), the retention factors were larger with respect to pH 3, probably due to the interaction of the cationic solutes with the anionic silanols more strongly ionised at pH 7.

In the presence of 10 mM HMIM·Cl, the chromatographic behaviour of the cationic solutes upon addition of the buffers to the mobile phase differed from the behaviour observed without IL (Figure 8.3).

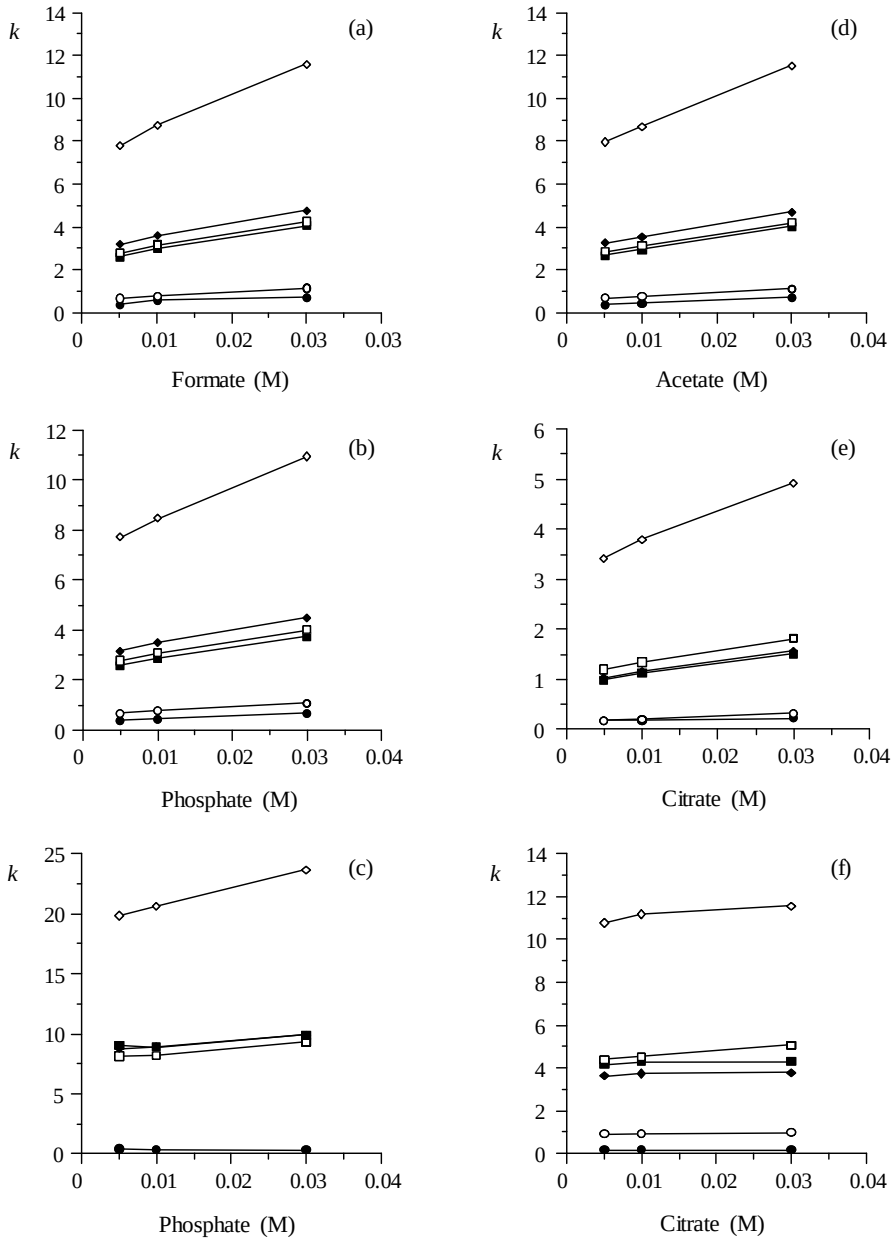


Figure 8.3. Retention behaviour of the β-blockers, in the presence of 10 mM HMIM·Cl. Other conditions and compound identities are given in Figure 8.2.

The retention factors increased almost linearly at increasing buffer concentration, except for citrate at pH 7. The changes were more significant at pH 3. Note that the retention factors were significantly shorter in the presence of HMIM·Cl, which is a consequence of the repulsion of the basic compounds by the $HMIM^+$ cation adsorbed on the alkyl chains of the stationary phase. At constant concentration of this IL, ionic interactions derived from the adsorption of buffer anion on the stationary phase may be primarily responsible of the observed changes in retention.

8.4.5. Effect of ionic liquid and buffer on peak shape

The plots of the left and right half-widths at 10% peak height versus the retention time have demonstrated to be a practical tool to evaluate the effect on the peak shape of basic drugs of the silanol activity inside a chromatographic column [29,30]. The plots can be assimilated to linear trends:

$$A = m_A t_R + A_0 \quad (8.1)$$

$$B = m_B t_R + B_0 \quad (8.2)$$

In these equations, A and B are the left and right half-widths, m_A and m_B are the slopes of the linear correlations, and A_0 and B_0 represent the extra-column contributions to the peak broadening. The peak broadening rate inside the column is given by the sum of slopes ($m_A + m_B$), whereas the ratio (m_B/m_A) indicates the asymmetry of peaks eluting at a time where the extra-column contributions are non-significant. The half-width plots can be built from the data for: (i) all analytes eluted at each mobile phase composition, (ii) each analyte eluted at several mobile phase compositions, or (iii) all analytes eluted at all assayed mobile phase compositions (global plots).

Figures 8.4 and 8.5 depict the half-widths plots obtained for the set of β-blockers, in the absence and presence of HMIM·Cl, respectively, with 10 mM buffer concentration at pH 3 and 7. Similar plots were obtained for 5 and 30 mM buffer system. As observed, the correlations for the individual plots were highly satisfactory in all cases ($r^2 > 0.99$). In the absence of additive (Figure 8.4), the slope of the right half-width (*B*) was significantly larger with regard to the left half-width (*A*), which was indicative of asymmetrical peaks seemingly produced by the slow interaction equilibrium between solutes and silanols in the stationary phase, especially at pH 7 (Figures 8.4c and f). When HMIM·Cl was added, the angle between the straight-lines for the half-widths was smaller (i.e. the peaks reduced significantly their asymmetry), both at pH 3 (Figures 8.5a, b, d and e) and 7 (Figures 8.5c and f).

The parameters obtained from the fitting of the half-width plots in the absence of HMIM·Cl are shown in Table 8.3, at increasing concentration of the buffers. The trends for the width and asymmetry of the peaks of β-blockers, are shown in Figures 8.6 and 8.7, respectively). As observed, an increasing buffer concentration in the range 5 to 30 mM resulted in narrower and more symmetrical peaks for the basic compounds in the presence of all buffer systems. The changes in peak shape were especially notorious for the formate and citrate buffers (m_B/m_A values changed from 4.7 to 1.7, and 6.0 to 2.4, respectively). This again suggests that, in the absence of IL, the access of the cationic basic compounds to the silanols on the column is more efficiently hindered at higher buffer concentration, due to the increasing amount of the anionic species of the buffer adsorbed on the stationary phase.

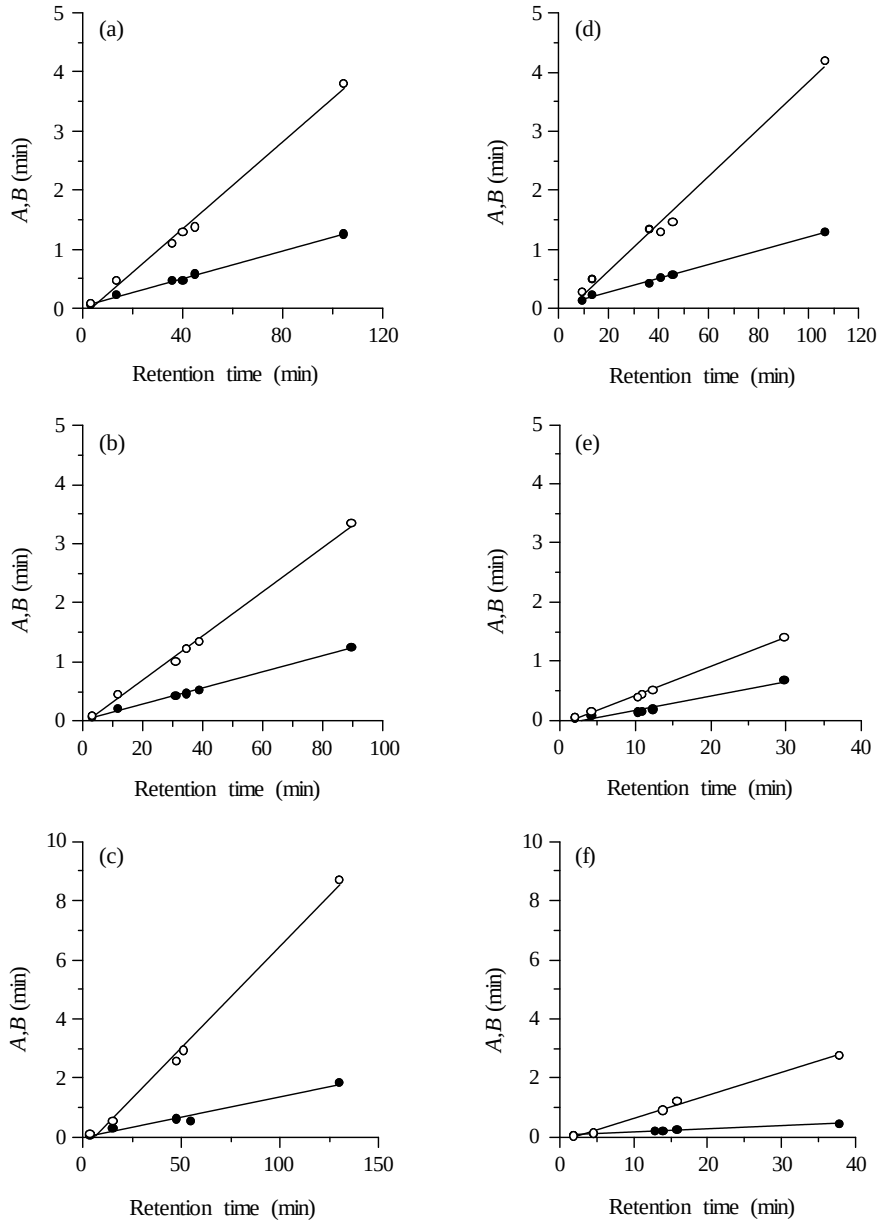


Figure 8.4. Plots depicting the left (●) and right (○) peak half-widths versus the retention times for the assayed β-blockers, in the absence of ionic liquid. Other conditions are given in Figure 8.2.

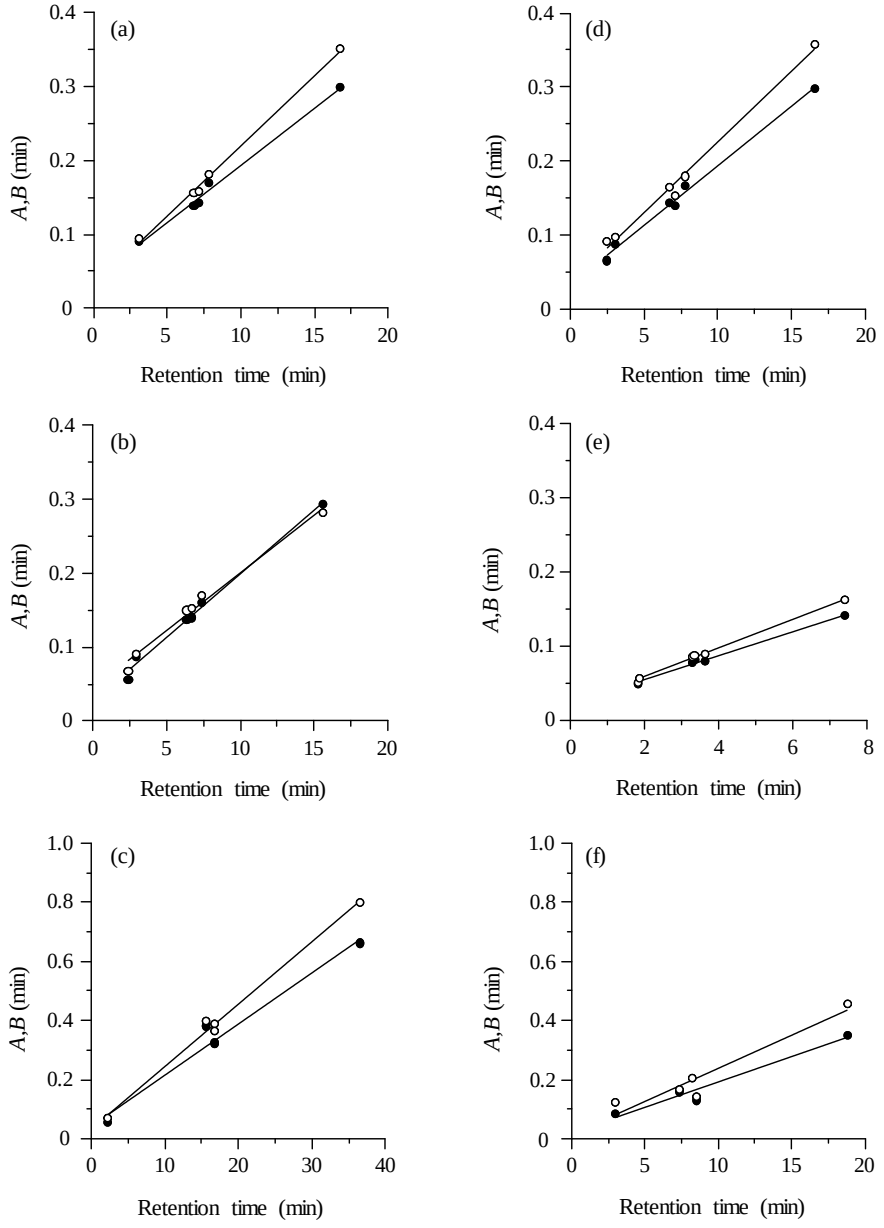


Figure 8.5. Plots depicting the left (●) and right (○) peak half-widths versus the retention times for the assayed β-blockers, in the presence of 10 mM HMIM·Cl. Other conditions are given in Figure 8.2.

Table 8.3. Half-widths plots parameters for the assayed β-blockers at increasing concentration of the buffer systems, at 10% or 15% acetonitrile without additive.[a]

		Acetate		Formate		Citrate		Phosphate	
	Buffer (mM)	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A
pH 3	5	0.061	3.99	0.064	4.70	0.077	5.99	0.066	2.98
	10	0.052	3.39	0.048	3.16	0.073	2.06	0.051	2.78
	30	0.044	2.20	0.042	1.70	0.042	2.45	0.043	1.94
pH 7	5	–	–	–	–	0.106	7.69	0.065	3.13
	10	–	–	–	–	0.088	7.37	0.083	5.07
	30	–	–	–	–	0.067	4.56	0.064	3.53

[a] m_A and m_B are the slopes of the half-widths plots (see Eqs. (8.1) and (8.2)).

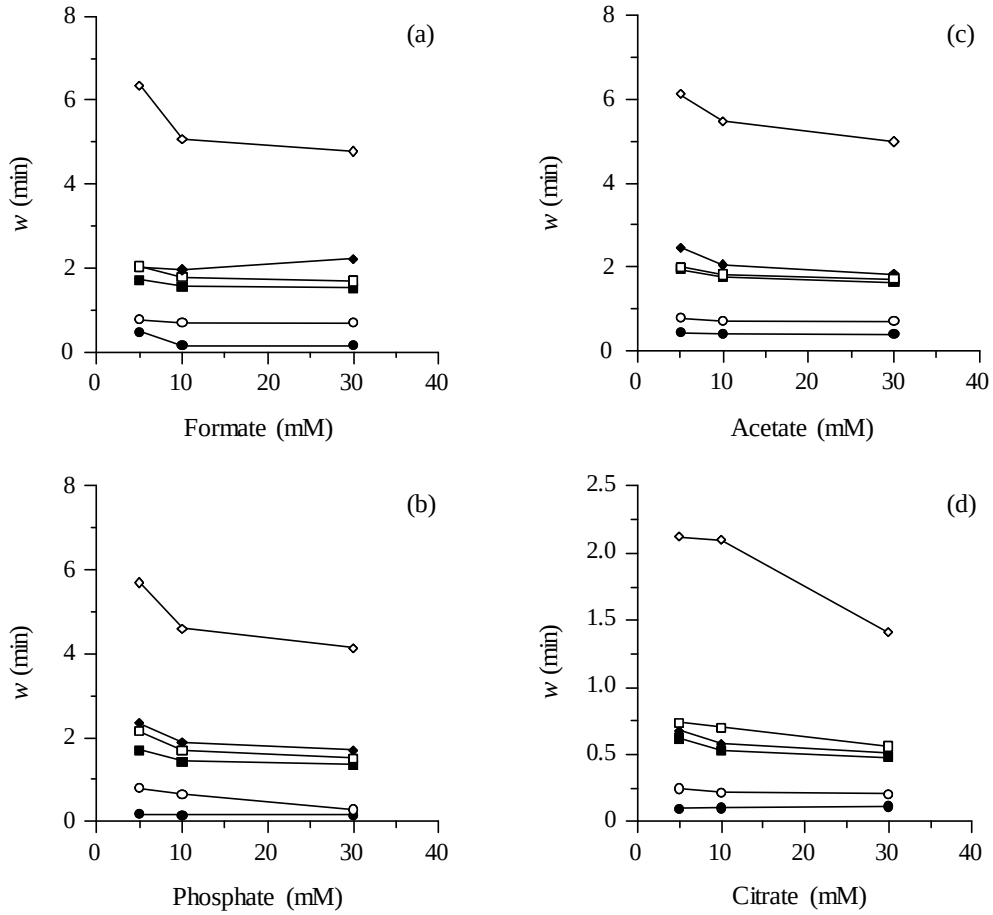


Figure. 8.6. Peak widths for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and different concentrations of: (a) formate, (b) phosphate at pH 3, (c) acetate, and (d) citrate at pH 3. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). Solute identities are given in Figure 8.1.

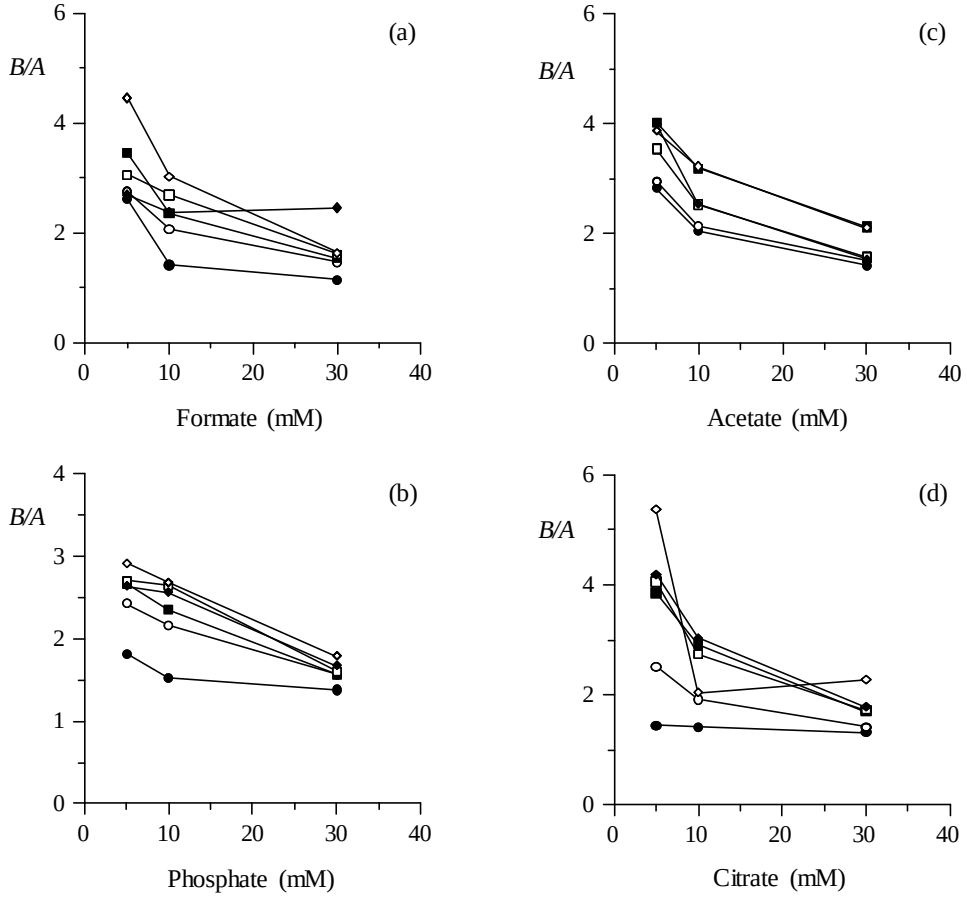


Figure 8.7. Peak asymmetries for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and different concentrations of the buffer systems. Other details are described in Figures 8.1 and 8.6.

Table 8.4 shows the parameters for the half-widths plots obtained at increasing buffer concentration, in the presence of HMIM·Cl in the mobile phase. Note that upon addition of the IL, the peak symmetry was significantly improved (with slope ratios, m_B/m_A, mostly close to one) and the peaks were narrower, even compared to those obtained with 30 mM buffer systems in the mobile phase without IL. Also, in the presence of IL, there is no apparent effect of the buffer concentration on both peak width and symmetry for all the assayed systems. Thus, at increasing buffer concentration, the peaks varied only slightly their slopes ratio between 1.3 and 1.1. The more symmetrical peaks, obtained in the presence of HMIM·Cl at any buffer concentration, indicate an effective column protection of the IL, which coat the surface of the C18 stationary phase.

At pH 7, silanols are significantly deprotonated, which is the reason of the poorer peak shape, especially at low buffer concentration (Table 8.3). However, at increasing buffer concentration, the peak shape improves. Again, the addition of HMIM·Cl at this pH gave rise to better performance, although it was slightly poorer compared to pH 3 (Table 8.4). The efficiency values (number of theoretical plates), obtained for the β-blockers using all assayed buffer systems, in the absence and presence of HMIM·Cl are given in Tables 8.5 and 8.6. Note that the information given by the efficiencies do not reveal the trends in the behaviour of chromatographic peaks as clearly as the half-width plots do, owing to the contribution of the extra-column tubing to peak broadening which is more significant for the less retained compounds.

Table 8.4. Half-widths plots parameters for the assayed β-blockers at increasing concentration of the buffer systems, in the presence of 10 mM HMIM·Cl at 10% or 15% acetonitrile (see Table 8.1).[a]

		Acetate		Formate		Citrate		Phosphate	
	Buffer (mM)	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A
pH 3	5	0.034	1.13	0.036	1.17	0.037	1.26	0.036	1.16
	10	0.035	1.18	0.035	1.23	0.036	1.20	0.033	0.91
	30	0.034	1.21	0.032	1.13	0.036	1.14	0.035	1.17
pH 7	5	–	–	–	–	0.042	1.40	0.045	1.56
	10	–	–	–	–	0.039	1.32	0.040	1.20
	30	–	–	–	–	0.039	1.31	0.039	1.13

[a] m_A and m_B are the slopes of the half-widths plots (see Eqs. (8.1) and (8.2)).

Table 8.5. Peak efficiencies observed for the β-blockers in acetonitrile-water mixtures at pH 3, without ionic liquid, at several buffer concentrations.

	Acetic acid / acetate (mM)			Formic acid / formate (mM)			Phosphoric acid / dihydrogen phosphate (mM)			Citric acid / dihydrogen citrate (mM)		
Compound	5	10	30	5	10	30	5	10	30	5	10	30
Atenolol	3800	6000	4600	3600	6300	7600	3500	5200	6000	6000	5500	4900
Nadolol	2700	4200	5900	2700	4400	6100	2300	3800	5900	2900	4700	7000
Timolol	2500	3800	6700	3300	6000	9500	3200	5300	9100	2000	3700	7800
Metoprolol	3300	5500	9400	3300	5300	9300	2500	4500	9000	2000	3200	8000
Acebutolol	2500	5400	10200	4600	6000	5200	2700	4600	8600	1900	3400	7400
Oxprenolol	2200	3500	6400	1700	4100	8000	2300	4000	7700	1100	2500	6400

Table 8.6. Peak efficiencies observed for the β-blockers in acetonitrile-water mixtures at pH 3, containing 10 mM HMIM·Cl, at several buffer concentrations.

	Acetic acid / acetate (mM)			Formic acid / formate (mM)			Phosphoric acid / dihydrogen phosphate (mM)			Citric acid / dihydrogen citrate (mM)		
Compound	5	10	30	5	10	30	5	10	30	5	10	30
Atenolol	6000	3800	6300	6200	9100	6600	6100	6500	6300	7100	6000	7900
Nadolol	4500	4800	4200	5400	5000	4600	4800	4900	4500	6600	3600	4900
Timolol	8500	8400	9100	8600	9400	9500	8500	8700	9500	7000	7200	7800
Metoprolol	9400	10400	9300	8300	9900	9400	9300	9400	9500	6400	8200	7800
Acebutolol	8700	9100	9500	9000	9100	10000	9000	9100	9800	6900	7100	7600
Oxprenolol	11400	10900	11900	11000	11400	13300	10800	11100	11500	10000	10300	10800

8.4.6. Selectivity and resolution

In Sections 8.4.3 and 8.4.4, the effect on retention of the addition of IL, and of the nature and concentration of the four buffer systems, on the retention is commented. The addition of IL gives rise to a significant reduction in retention. In contrast, in the presence and absence of IL, retention increases when larger concentration of buffer is added, especially in the case of the citrate system. Besides these changes in retention, it is convenient to check possible changes in selectivity (i.e. relative retention). In order to explore this, the retention factors of β-blockers were correlated for different conditions. Figures 8.8 and 8.9 depict the correlations achieved by comparing the retention factors with 30 mM buffer, in the absence and presence of IL, and without IL in the presence of 5 and 30 mM buffer, respectively. In all examined situations, and for all buffer systems, a high correlation was observed between the retention factors in the different conditions. This suggests similar selectivity when either IL or a larger concentration of the buffers is added to the mobile phase, although the retention times are different.

Chromatograms monitored at 254 nm for mixtures of the probe compounds eluted with mobiles phases containing 10% or 15% acetonitrile, and 30 mM buffer concentration in the absence and presence of 10 mM HMIM·Cl, are shown in Figures 8.10 and 8.11. The peaks obtained for timolol does not appear in the chromatograms, owing to its very low absorption at 254 nm (its absorption maximum is at 300 nm). The retention times for timolol in the absence and presence of 10 mM HMIM·Cl were 38.40/8.67 (formate at pH 3), 38.42/8.56 (acetate at pH 3), 33.57/7.84 (phosphate at pH 3), 41.29/18.45 (phosphate at pH 7), 11.26/3.88 (citrate at pH 3) and 12.67/8.19 (citrate at pH 7). Note that for citrate the acetonitrile content was 15% instead of 10%.

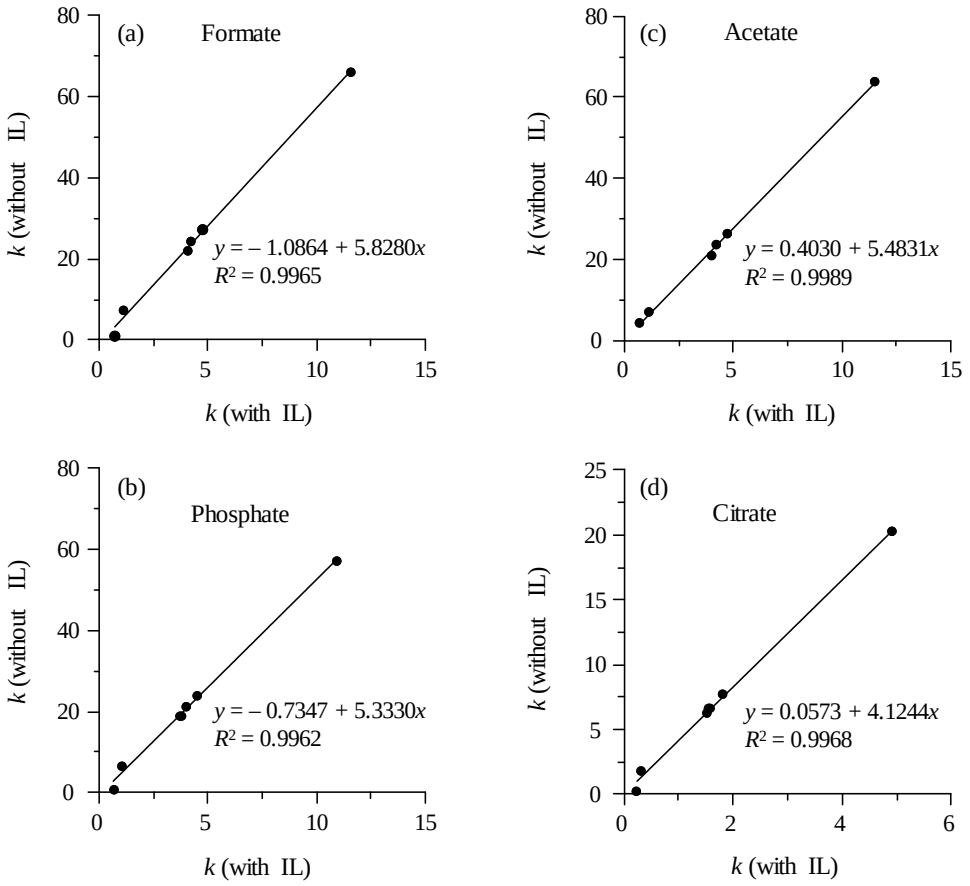


Figure 8.8. Selectivity comparison for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and 30 mM buffer system at pH 3. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). The plotted data are the retention factors in the absence and presence of HMIM·Cl for the six probe compounds.

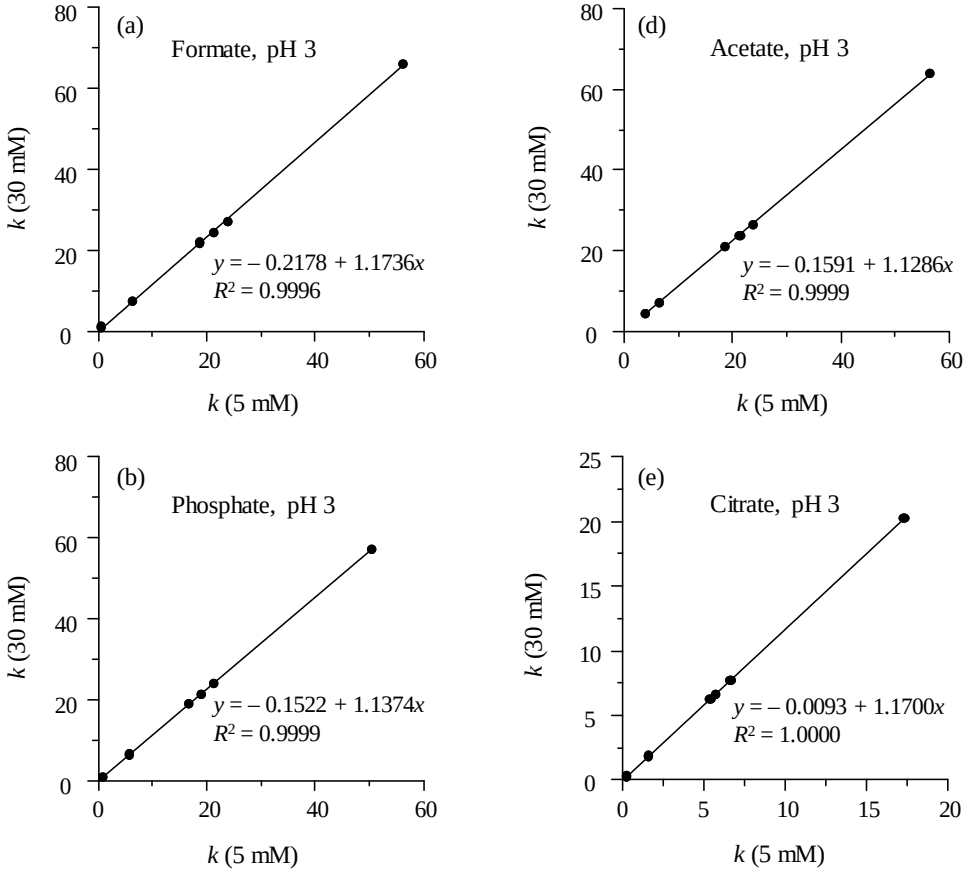


Figure 8.9. Selectivity comparison for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile in the absence of ionic liquid. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). The plotted data are the retention factors for the six probe compounds, with mobile phases that contained 5 and 30 mM buffer.

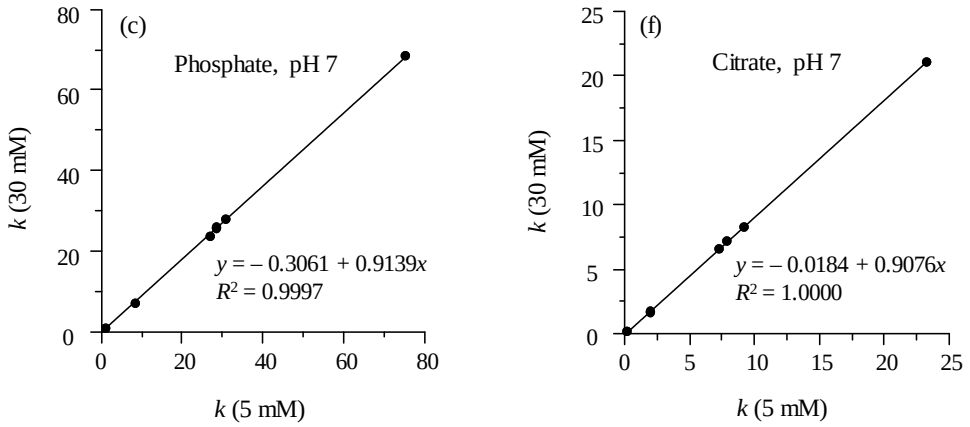


Figure 8.9 (continued).

The elution order of the mixture of β-blockers did not change by addition of the different buffer systems assayed in this work. As commented above, the retention times were significantly shorter and the behaviour of the peaks was enhanced in the presence of HMIM·Cl. The resolution was quite satisfactory, with baseline resolved peaks, except for the partial overlapping between metoprolol and acebutolol with the dihydrogen phosphate/hydrogen phosphate system at pH 7 (Figure 8.11c), and atenolol and nadolol with the citric/dihydrogen citrate system at pH 3 (Figure 8.11e).

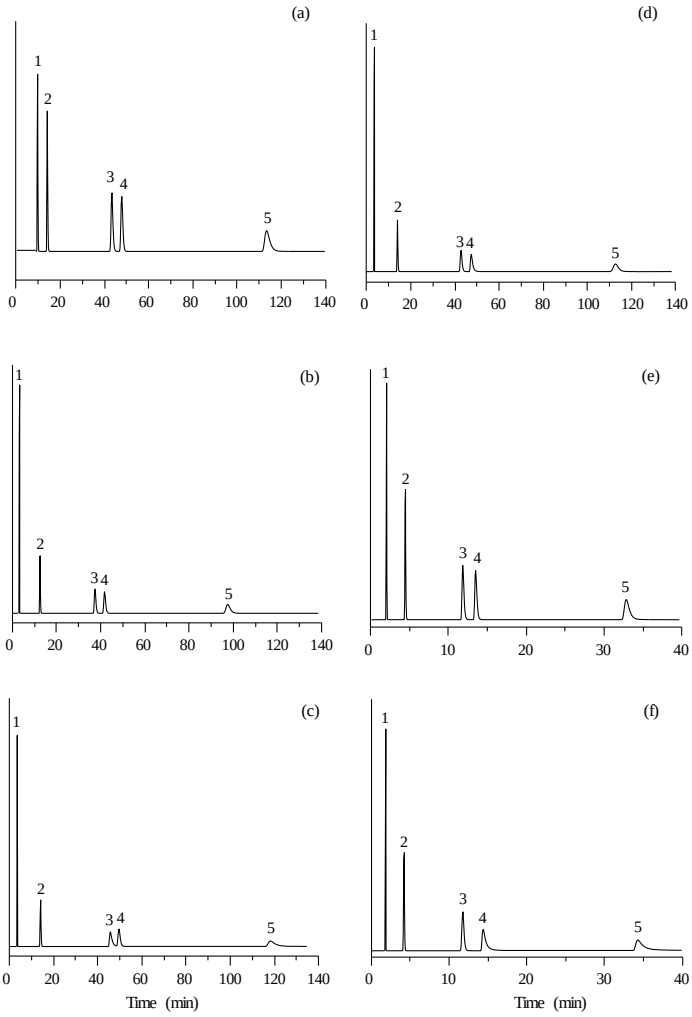


Figure 8.10. Chromatograms for a mixture of β-blockers detected at 254 nm, eluted with mobile phases containing a fixed concentration of acetonitrile and 30 mM buffer: (a) formate, (b) phosphate at pH 3, (c) phosphate at pH 7, (d) acetate, (e) citrate at pH 3, and (f) citrate at pH 7. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). Solute identity: (1) atenolol, (2) nadolol (3) metoprolol, (4) acebutolol, and (5) oxprenolol.

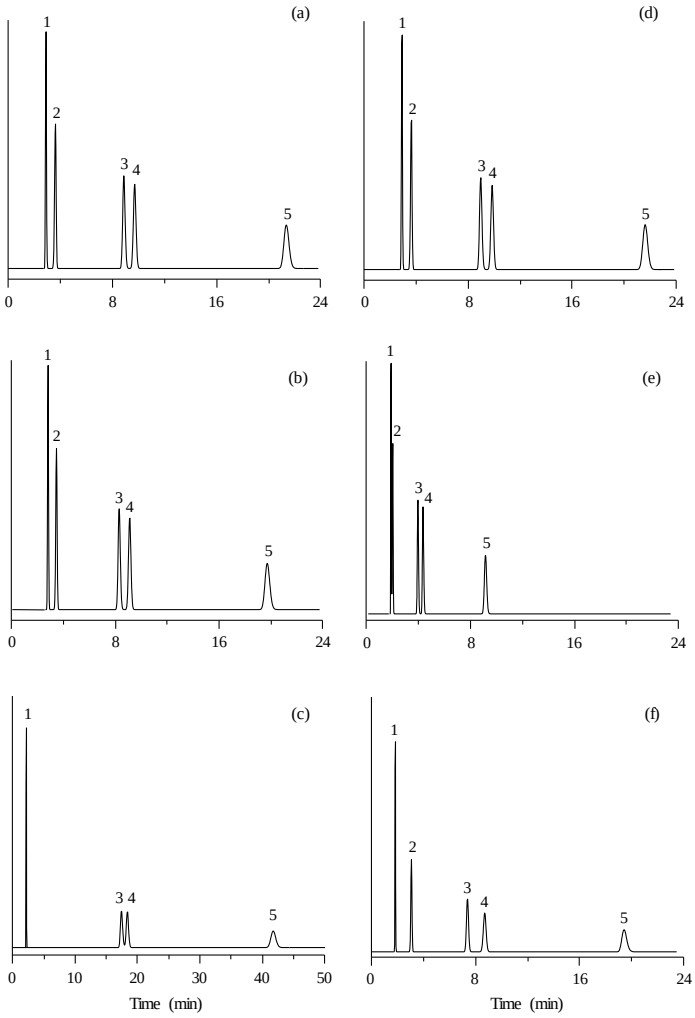


Figure 8.11. Chromatograms for a mixture of β-blockers detected at 254 nm, in the presence of 10 mM HMIM·Cl. Other details are given in Figure 8.10.

8.5. Conclusions

Protonated (cationic) basic compounds give rise to broad and asymmetrical peaks, due to the slow sorption-desorption kinetics on silanols. The adsorption on the stationary phase of different types of additives in the aqueous-organic mobile phase may avoid this interaction, but the retention behaviour is also affected. Thus, depending on the charge of the adsorbed additive, the retention may decrease (repelled by a cationic additive), or increase (attracted by an anionic additive).

The positive effect on the chromatographic performance of basic compounds when using ILs as mobile phase additives is well-known. However, these studies normally perform without considering in-depth the effect that the buffer can be also exerting. The $HMIM^+$ cation is highly adsorbed on these columns, whereas the chloride anion shows scarce adsorption. This gives rise to a significant reduction in the retention times of basic compounds. Also, the adsorption of $HMIM^+$ is able to suppress the silanol activity giving rise to highly symmetrical peaks.

On the other hand, the retention times increased when larger concentrations of the buffers acetic acid/acetate, citric acid/dihydrogen citrate, formic acid/formate and phosphoric acid/dihydrogen phosphate were added to the mobile phase, both in the absence and presence of HMIM·Cl. In spite of the different nature of the four acid-base systems, they have the common feature of forming anionic species at the working pH values (3 and 7). At increasing buffer concentration, the retention factors followed a concave trend in the absence of IL for all acid-base systems, whereas the trend was almost linear in the presence of HMIM·Cl at both pH 3 and 7. Although the change in retention was larger in the absence of IL, it was relatively more significant in the presence of the additive.

The observed behaviour for the retention reveals a small adsorption of the buffer anions on the stationary phase. The adsorbed buffer anions attract the cationic basic compounds to the stationary phase, giving rise to larger retention times at increasing buffer concentration. In the series without IL, the buffer anions are directly adsorbed on the alkyl bonded chains in the stationary phase, and the observed concave trend in the retention indicates column saturation for the buffer anions. In the presence of IL, the buffer anions are probably attracted to the IL cation. The linear trends observed for the retention of the cationic compounds, at increasing concentration of buffer anions, indicate their increasing interaction by electrostatic attraction to the stationary phase covered by the cationic IL. Finally, the similar selectivity observed in the presence and absence of IL, and at varying concentration of the buffer anions, indicates that the observed effects should be primarily explained by the electrostatic interaction of the cationic solutes with the cationic IL and/or the anionic species of the buffer, adsorbed on the stationary phase.

In the absence of IL, the peak shape for basic compounds was improved (the peaks were narrower and more symmetrical) at larger concentration of the buffers. This also suggests the adsorption of the buffer anions on the stationary phase and their blocking effect on the activity of residual silanols in the C18 column, although this effect is weaker compared to that observed for $HMIM^+$. The kinetics of the electrostatic association of the basic compounds with the buffer anions seems to be faster than the kinetics for the ion-exchange processes involving the anionic silanols on the silica surface. In contrast to this behaviour, in the presence of HMIM·Cl, the effect on the peak shape of increasing concentrations of the buffers was non-significant, since the adsorbed IL already produces an effective blocking of the silanol activity with highly symmetrical peaks at all buffer concentrations.

Among the studied buffers, the citrate system seemed to show more affinity for the stationary phase, based on the larger retention times and better peak symmetries. Phosphate buffer yielded somewhat smaller retention, than the formate and acetate buffers (which are only partially deprotonated in solution), whose effect on retention was similar. The increased retention observed with these two buffers suggests an induced deprotonation of the monoprotic acid-base systems due to anion adsorption, which would give rise to a larger concentration of the anionic species in the aqueous-organic mobile phases. In any case, in the absence and presence of additive, the studies in this work make attention on the relevance of controlling the buffer concentration on the analytical procedures.

5.6. References

[1] U. Domanska, General review of ionic liquids and their properties, in: M. Koel (Ed.), *Ionic Liquids in Chemical Analysis*, CRC Press, New York, 2009, pp. 1–71.

[2] T.D. Ho, C. Zhang, L.W. Hantao, J.L. Anderson, Ionic liquids in Analytical Chemistry: Fundamentals, advances, and perspectives, *Anal. Chem.* 86 (2014) 262–285.

[3] D. Zhao, Y. Liao, Z. Zhang, Toxicity of ionic liquids, *Clean* 35 (2007) 42–48.

[4] G. Cevasco, C. Chiappe, Are ionic liquids a proper solution to current environmental challenges?, *Green Chem.* 16 (2014) 2375–2385.

[5] L. He, W. Zhang, L. Zhao, X. Liu, S. Jiang, Effect of 1-alkyl-3-methylimidazolium-based ionic liquids as the eluent on the separation of

ephedrines by liquid chromatography, *J. Chromatogr. A* 1007 (2003) 39–45.

[6] W. Zhang, L. He, Y.L. Gu, X. Liu, S. Jiang, Effect of ionic liquids as mobile phase additives on retention of catecholamines in reversed-phase high-performance liquid chromatography, *Anal. Lett.* 36 (2003) 827–838.

[7] X. Xiao, L. Zhao, L. Xia, S. Jiang, Ionic liquids as additives in high performance liquid chromatography: Analysis of amines and the interaction mechanism of ionic liquids, *Anal. Chim. Acta* 519 (2004) 207–211.

[8] R. Kaliszan, M.P. Marszałł, M.J. Markuszewski, T. Baczek, J. Pernak, Suppression of deleterious effects of free silanols in liquid chromatography by imidazolium tetrafluoroborate ionic liquids, *J. Chromatogr. A* 1030 (2004) 263–271.

[9] M.P. Marszałł, T. Baczek, R. Kaliszan, Reduction of silanophilic interactions in liquid chromatography with the use of ionic liquids, *Anal. Chim. Acta* 547 (2005) 172–178.

[10] M.J. Ruiz Ángel, S. Carda Broch, A. Berthod, Ionic liquids versus trimethylamine as mobile phase additives in the analysis of β-blockers, *J. Chromatogr. A* 1119 (2006) 202–208.

[11] A.V. Herrera Herrera, J. Hernández Borges, M.A. Rodríguez Delgado, Ionic liquids as mobile phase additives for the high-performance liquid chromatographic analysis of fluoroquinolone antibiotics in water samples, *Anal. Bioanal. Chem.* 392 (2008) 1439–1446.

[12] A. Martín Calero, J.H. Ayala, V. González, A.M. Afonso, Ionic liquids as desorption solvents and memory effect suppressors in heterocyclic aromatic amines determination by SPME-HPLC fluorescence, *Anal. Bioanal. Chem.* 394 (2009) 937–946.

[13] A. Martín Calero, V. Pino, J.H. Ayala, V. González, A.M. Afonso, Ionic liquids as mobile phase additives in high-performance liquid chromatography with electrochemical detection: Application to the determination of heterocyclic aromatic amines in meat-based infant foods, *Talanta* 79 (2009) 590–597.

[14] A. Martín Calero, G. Tejral, J.H. Ayala, V. González, A.M. Afonso, Suitability of ionic liquids as mobile-phase additives in HPLC with fluorescence and UV detection for the determination of heterocyclic aromatic amines, *J. Sep. Sci.* 33 (2010) 182–190.

[15] A. Petruczynik, Effect of ionic liquid additives to mobile phase on separation and system efficiency for HPLC of selected alkaloids on different stationary phases, *J. Chromatogr. Sci.* 50 (2012) 287–293.

[16] Y. Tang, A. Sun, R. Liu, Y. Zhang, Simultaneous determination of fangchinoline and tetrandrine in Stephania tetrandra S. Moore by using 1-alkyl-3-methylimidazolium-based ionic liquids as the RP-HPLC mobile phase additives, *Anal. Chim. Acta* 767 (2013) 148–154.

[17] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, A. Berthod, S. Carda Broch, On the use of ionic liquids as mobile phase additives in high-performance liquid chromatography, Anal. Chim. Acta 883 (2015) 1–21.

[18] J. Nawrocki, The silanol group and its role in liquid chromatography, *J. Chromatogr. A* 779 (1997) 29–71.

[19] M. Reta, P.W. Carr, Comparative study of divalent metals and amines as silanol-blocking agents in reversed-phase liquid chromatography, *J. Chromatogr. A* 855 (1999) 121–127.

[20] U.D. Neue, K. Tran, A. Méndez, P.W. Carr, The combined effect of silanols and the reversed-phase ligand on the retention of positively charged analytes, *J. Chromatogr. A* 1063 (2005) 35–45.

[21] H. Engelhardt, Ch. Blay, J. Saar, Reversed phase chromatography: The mystery of surface silanols, *Chromatographia* 62 (2005) S19–S29.

[22] C.F. Poole, B.R. Kersten, S.S.J. Ho, M.E. Coddens, K.G. Furton, Organic salts, liquid at room temperature, as mobile phases in liquid chromatography, *J. Chromatogr.* 352 (1986) 407–425.

[23] P.H. Shetty, P.J. Youngberg, B.R. Kersten, C.F. Poole, Solvent properties of liquid organic salts used as mobile phases in microcolumn reversed-phase liquid chromatography, *J. Chromatogr.* 411 (1987) 61–79.

[24] M. Waichigo, T.L. Riechel, N.D. Danielson, Ethylammonium acetate as mobile phase modifier in liquid chromatography, *Chromatographia* 61 (2005) 17–23.

[25] M.M. Waichigo, N.D. Danielson, Ethylammonium formate as an organic solvent replacement for ion-pair reversed-phase liquid chromatography, *J. Chromatogr. Sci.* 44 (2006) 607–614.

[26] M.M. Waichigo, N.D. Danielson, Comparison of ethylammonium formate to methanol as a mobile-phase modifier for reversed-phase liquid chromatography, *J. Sep. Sci.* 29 (2006) 599–606.

[27] M.M. Waichigo, B.M. Hunter, T.L. Riechel, N.D. Danielson, Alkylammonium formate ionic liquids as organic mobile phase replacements, for reversed-phase liquid chromatography, *J. Liq. Chromatogr. Relat. Technol.* 30 (2007) 165–184.

[28] S. Grossman, N.D. Danielson, Methylammonium formate as a mobile phase modifier for totally aqueous reversed-phase liquid chromatography, *J. Chromatogr. A* 1216 (2009) 578–3586.

[29] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[30] J.J. Baeza Baeza, M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

[31] A. Berthod, M.J. Ruiz Ángel, S. Huguet, Nonmolecular solvents in separation methods: Dual nature of room temperature ionic liquids, *Anal. Chem.* 77 (2005) 4071–4080.

[32] J.J. Fernández Navarro, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, The role of the dual nature of ionic liquids in the reversed-phase liquid chromatographic separation of basic drugs, *J. Chromatogr. A* 1218 (2011) 398–407.

[33] J.R. Fernández Navarro, J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Silanol suppressing potency of alkyl-imidazolium ionic liquids on C18 stationary phases, *J. Chromatogr. A* 1232 (2012) 166–175.

[34] J.J. Fernández Navarro, J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, 1-Hexyl-3-methylimidazolium tetrafluoro-borate: An efficient column enhancer for the separation of basic drugs by reversed-phase liquid chromatography, *J. Chromatogr. A* 1258 (2012) 168–174.

[35] M.T. Úbeda Torres, C. Ortiz Bolsico, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, Gaining insight in the behaviour of imidazolium-based ionic liquids as additives in reversed-phase liquid chromatography for the analysis of basic compounds, *J. Chromatogr. A* 1380 (2015) 96–103.

[36] E. Bosch, S. Espinosa, M. Rosés, Retention of ionizable compounds on high-performance liquid chromatography: III. Variation of p*K* values of

acids and pH values of buffers in acetonitrile-water mobile phases, *J. Chromatogr. A* 824 (1998) 137–146.

[37] S. Carda Broch, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, Extent of the influence of phosphate buffer and ionic liquids on the reduction of the silanol effect in a C18 stationary phase, *J. Chromatogr. A* 1559 (2018) 112–117.

[38] R. Mehvar, D.R. Brocks, Stereospecific pharmacokinetics and pharmacodynamics of beta-adrenergic blockers in humans, *J. Pharm. Sci.* 4 (2001) 185–200.

[39] S. Espinosa, E. Bosch, M. Rosés, Retention of ionizable compounds on HPLC: XII. The properties of liquid chromatography buffers in acetonitrile-water mobile phases that influence HPLC retention, *Anal. Chem.* 74 (2002) 3809–3818.

[40] J.R. Torres-Lapasió, *MICHROM Software*, Marcel Dekker, New York, 2000.

[41] C.J. Drayton (Ed.), *Comprehensive Medicine Chemistry*, Vol. 6, Pergamon Press, Oxford, 1990.

[42] I. Canals, J.A. Portal, E. Bosch, M. Rosés, Retention of ionizable compounds on HPLC. 4. Mobile-phase pH measurement in methanol/ water, *Anal. Chem.* 72 (2000) 1802–1809.

[43] S. Espinosa, E. Bosch, M. Rosés, Retention of ionizable compounds in high-performance liquid chromatography: IX. Modelling retention in reversed-phase liquid chromatography as a function of pH and solvent composition with acetonitrile-water mobile phases, *J. Chromatogr. A* 947 (2002) 47–58.

[44] X. Subirats, E. Bosch, M. Rosés, Retention of ionisable compounds on high-performance liquid chromatography: XV. Estimation of the pH

variation of aqueous buffers with the change of the acetonitrile fraction of the mobile phase, *J. Chromatogr. A* 1059 (2004) 33–42.

[45] X. Subirats, E. Bosch, M. Rosés, Retention of ionisable compounds on high-performance liquid chromatography: XVII. Estimation of the pH variation of aqueous buffers with the change of the methanol fraction of the mobile phase, *J. Chromatogr. A* 1138 (2007) 203–215.

[46] X. Subirats, E. Bosch, M. Rosés, Retention of ionisable compounds on high-performance liquid chromatography: XVIII. pH variation in mobile phases containing formic acid, piperazine, tris, boric acid or carbonate as buffering systems and acetonitrile as organic modifier, *J. Chromatogr. A* 1216 (2009) 2491–2498.

[47] A. Méndez, E. Bosch, M. Rosés, U.D. Neue, Comparison of the acidity of residual silanol groups in several liquid chromatography columns, *J. Chromatogr. A* 986 (2003) 33–44.

[48] M.J. Ruiz Ángel, S. Pous Torres, S. Carda Broch, M.C. García Álvarez-Coque, Performance of different C18 columns in reversed-phase liquid chromatography with hydro-organic and micellar-organic mobile phases, *J. Chromatogr. A* 1344 (2014) 76–82.

CHAPTER 9

SUITABILITY OF 1-HEXYL-3-METHYLIMIDAZOLIUM IONIC LIQUIDS FOR THE ANALYSIS OF PHARMACEUTICAL FORMULATIONS CONTAINING TRICYCLIC ANTIDEPRESSANTS

9.1.Abstract

The reversed-phase chromatographic behaviour of six tricyclic antidepressants (amitryptiline, clomipramine, doxepin, imipramine, nortryptiline and maprotiline) was examined in this work with acetonitrile-water mobile phases, in the absence and presence of the ionic liquids 1-hexyl-3-methyl imidazolium chloride and 1-hexyl-3-methylimidazolium tetrafluoroborate, which have interesting features for the separation of basic compounds, in terms of peak shape combined with reduced retention. Tricyclic antidepressants (TCAs) are low polarity drugs that strongly associate to the alkyl chains of conventional stationary phases. They are also positively charged in the usual working pH range (2–8) in reversed-phase liquid chromatography, due to their strong basic character. In consequence, TCAs may interact with the residual ionised silanols present in conventional silica-based stationary phases, which is translated in stronger retention, and tailed and broad peaks. A simple chromatographic procedure for the control of TCAs in pharmaceutical formulations was developed using a C8 column and a mobile phase containing 30% acetonitrile/10 mM 1-hexyl-3-methyl-imidazolium chloride at pH 3, with UV detection. Intra- and inter-day precisions were usually below +1.0%, and intra- and inter-day bias (accuracy) ranged usually between –2.1% and +2.4%, and between –3.0% and +2.3%, respectively. Sample preparation was simple and only required solubilisation and filtration previous to injection.

9.2. Introduction

Basic drugs are positively charged at the usual working pH range (2–8) in reversed-phase liquid chromatography (RPLC). This means that, besides the interaction with the alkyl chains of conventional C18 stationary phases, this type of drugs are attracted to the negatively charged residual silanols present in the octadecyl columns, as a consequence of steric problems in the derivatisation process of silica supports [1,2]. Since the interaction with silanols suffers slow kinetics, the whole process results in stronger retention and tailed and broad peaks.

In the last decade, ionic liquids (ILs) have received special attention as efficient peak profile enhancers of basic drugs in RPLC, using conventional stationary phases. Among the numerous ILs used in HPLC, several authors have reported that those formed by the 1-hexyl-3-methylimidazolium (HMIM) cation and the anions chloride and tetrafluoroborate have interesting features for the separation of basic compounds: narrower chromatographic peaks, combined with smaller retention and lower consumption of organic solvent [3–8]. This can be explained considering that both the cation and anion of ILs are able to interact with the stationary phase [9]. The adsorption of HMIM on a C18 stationary phase is significantly stronger with regard to the cations 1-ethyl-and 1-butyl-3-methylimidazolium (EMIM and BMIM, respectively), and the anions chloride and tetrafluoroborate. In fact, a better enhancer would be the 1-octyl-3-methylimidazolium cation (OMIM), due to stronger adsorption with respect to $HMIM^+$ [10], but this reagent is not used owing to its poor solubility.

There is a wide literature on the use of ILs as mobile phase additives in RPLC. A recent review gathers 130 references published up to 2015 [11]. Most studies are focused on fundamental aspects of the chromatographic separation

in the presence of ILs [12–16]. However, the developed procedures are not usually validated and/or applied to real samples. This can leave out the influence of the ILs on essential aspects, such as linearity, repeatability and robustness.

The goal of this work was to develop a simple RPLC procedure with a mobile phase containing an IL, and evaluate its robustness in routine analysis through an extensive validation. For this study, six tricyclic antidepressants (TCAs) were selected. The procedure was applied to the analysis of the TCAs in pharmaceutical formulations. The validation followed the International Conference of Harmonization (ICH) Guideline [17].

TCAs are frequently prescribed for the treatment of depressive disorders owing to their efficiency in elevating the mood of patients, even with children and adolescents [18–20]. The availability of efficient, selective and reproducible analytical procedures for these drugs is of critical importance in biological samples, but also routine methods are required for pharmaceutical samples. Dynamic separation techniques including RPLC [20–25], capillary electrophoresis (CE) [26–28], and gas chromatography (GC) [29–32] have been used for the analysis of TCAs. Among these techniques, RPLC is considered as the most efficient and robust, due to its simple operation, strong separation ability, short analysis time, reproducibility, and wide sample application.

TCAs possess a chemical structure that consists of three rings with a side chain of *N*-alkylmethylamine or *N*-alkyldimethylamine. The ring structure determines a relatively low polarity with octanol-water partition coefficients (log $P_{o/w}$) in the range 3.9–5.3, whereas the amine groups confer them a basic character with pK_a values in the range 9.0–9.7 [33].

The low polarity of TCAs gives rise to strong association with the alkyl chains of conventional C18 stationary phases, which requires high contents of organic solvent in the mobile phase to get sufficiently short analysis times. Also, their basic character favours the interaction with residual silanols, which yields tailing peaks, whose magnitude depends on the kind of column [34]. To solve this issue, ionic and non-ionic surfactants have been occasionally added to the mobile phases to improve the chromatographic performance of TCAs, but with not totally satisfactory results [35–38]. For this reason, it was expected that the addition to the mobile phase of ILs containing the HMIM cation could show interesting performance for the analysis of TCAs by RPLC.

A recent study that compared the chromatographic performance of a group of TCAs, using $BMIM^+$ associated to chloride or hexafluorophosphate as mobile phase modifier [39], should be here commented. The BMIM cation gives rise to longer retention with respect to $HMIM^+$, especially when associated to a chaotropic anion such as hexafluorophosphate, which has a strong affinity for the stationary phase attracting electrostatically TCAs to the stationary phase [40].

9.3. Experimental

9.3.1. Reagents

Stock solutions of approximately 100 μg/mL amitryptiline, clomipramine, doxepin, imipramine, maprotiline and nortryptiline (all from Sigma, St. Louis, MO, USA) were prepared in a small amount of acetonitrile (Scharlab, Barcelona, Spain) with the aid of an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany). The solutions were kept at 4 ºC and remained stable during

at least two months. The injected solutions were diluted with water. Uracil (Acros Organics, Geel, Belgium) was used as dead time marker.

The mobile phases contained acetonitrile and 1-hexyl-3-methylimidazolium chloride (HMIM·Cl) or 1-hexyl-3-methylimidazolium tetrafluoroborate (HMIM·BF_4), both from Sigma. For comparison purposes, mobile phases in the absence of IL were also prepared. All mobile phases were buffered at pH 3.0 with sodium dihydrogen phosphate (Fluka, Buchs, Switzerland) and HCl (Scharlab), before the addition of the organic solvent. The drug solutions and the mobile phases, which were renewed several times along the validation process, were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA), and degassed in an ultrasonic bath. Nanopure water (Thermo Scientific, Dubuque, IA, USA) was used throughout.

9.3.2. Apparatus and chromatographic conditions

An Agilent instrument (Waldbronn, Germany), equipped with an isocratic pump (Series 1100), an autosampler (Series 1260), a thermostated column compartment (Series 1260) fixed at 25 ºC, a UV-visible wavelength detector (Series 1100), and an HPChemStation (Agilent, B.04.03) for data acquisition, was used. The TCAs were monitored at 254 nm, except maprotiline, which was detected at 278 nm. The mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were integrated with MICHROM [41]. The simulation of chromatograms and optimisation of the mobile phase were also performed with this software.

Zorbax Eclipse XDB C18 and C8 (Agilent) columns with the following characteristics were used: 150 mm×4.6 mm i.d., 5 μm particle size, 180 m^2/g surface area, 80 Å pore size, and 10% and 7.6% carbon load for the C18 and C8 columns, respectively. The analytical columns were connected to similar

30 mm guard columns. The recommended analysis of the pharmaceutical preparations was carried out with the C8 column and 30% (*v*/*v*) acetonitrile/ 10 mM HMIM·Cl, buffered at pH 3.0. The flow rate was 1 mL/min.

9.3.3. Procedure

The pharmaceuticals analysed in this work were tablets. The average weight per tablet was calculated from the weight of 10 units. The contents were ground and reduced to a homogeneous fine powder in a mortar. Several portions of powder were taken and sonicated with a small amount of acetonitrile or 30% acetonitrile/10 mM HMIM·Cl (the selected mobile phase). Dilution was also made with the mobile phase. The excipients were not soluble in the assayed media, hence the sample solutions had to be filtered through 0.45 μm Nylon membranes before injection into the chromatograph.

9.4. Results and discussion

9.4.1. Experimental design

ILs formed by the HMIM cation and anions of low or moderate affinity towards the stationary phase, such as chloride and tetrafluoroborate, have revealed as excellent peak shape enhancers for basic drugs, such as β-blockers, since they combine narrower chromatographic peaks with smaller retention, using conventional C18 columns [8,40]. However, once column saturation is reached, minimal changes in retention are observed at increasing concentration of the additives.

On view of these results, a Zorbax Eclipse XDB C18 column (type B, made of highly purified silica) was initially selected, and the ILs HMIM·Cl and HMIM·BF_4 were added to acetonitrile-water mobile phases at a fixed concentration (10 mM) to elute the TCAs. The mobile phases were buffered at pH 3.0 to favour good peak shape, due to the protonation of the residual-free silanol groups on the stationary phase. In order to follow in detail the change in behaviour of the TCAs with the concentration of acetonitrile in the mobile phase, chromatographic data (retention factors and peak half-widths) were simulated based on the data from experimental designs involving three mobile phases, covering a domain between 20% and 30% (*v*/*v*) acetonitrile in the presence of HMIM·Cl, and between 25% and 35% acetonitrile in the presence of HMIM·BF_4. For comparison purposes, mobile phases in the absence of ILs were prepared containing 30–60% acetonitrile. The concentrations of acetonitrile and IL were selected to guarantee the elution of the solutes in practical analysis times.

A similar study was performed with a C8 column (also type B) and acetonitrile/water mixtures in the absence and presence of HMIM·Cl and HMIM·BF_4, with the following acetonitrile concentrations: 25–35% with HMIM·Cl, and 30–40 % with HMIM·BF_4 and without IL. In all these studies, duplicate injections of 20 µL were made, with both columns (C18 and C8).

With the information obtained by observing the chromatographic behaviour of the peaks for each compound, their retention and peak shape (peak width and asymmetry) was modelled. With the assistance of the MICHROM software, the profile of chromatograms containing the six TCAs at any mobile phase composition inside the studied range was predicted and optimised.

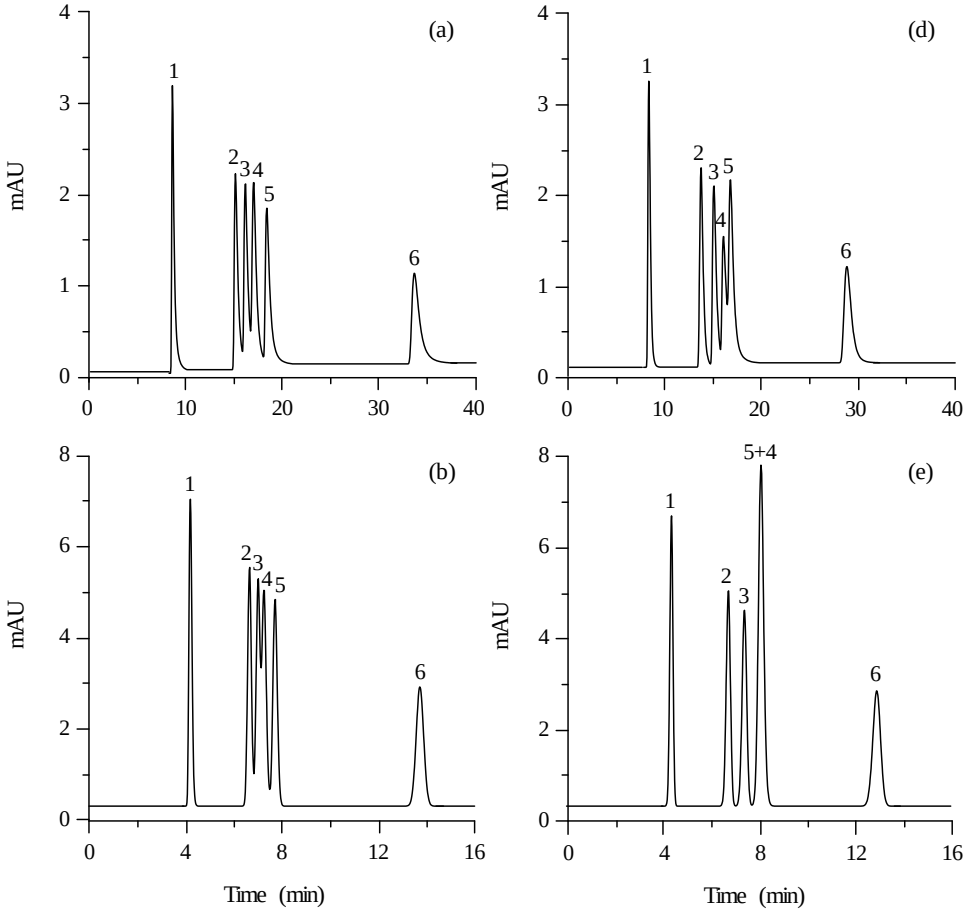


Figure 9.1. Simulated chromatograms for mixtures of the six TCAs using the C18 (a to c) and C8 (d to f) columns. Mobile phase composition: (a and d) 30% acetonitrile, (b and e) 30% acetonitrile/10 mM HMIM·Cl, and (c and f) 30% acetonitrile/10 mM HMIM·BF_4. Peak identity: (1) doxepin, (2) imipramin, (3) nortryptiline, (4) maprotiline, (5) amitryptiline, and (6) clomipramine.

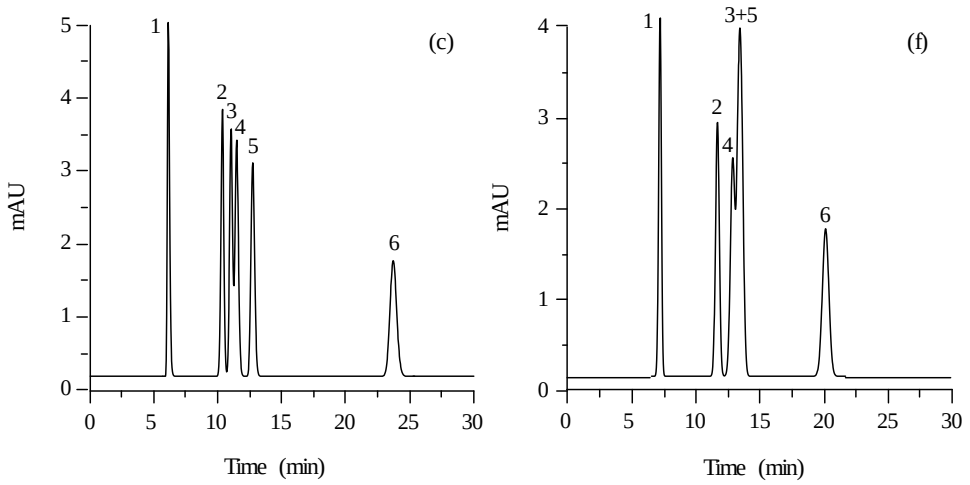


Figure 9.1 (continued).

9.4.2. Effect of ionic liquids on retention and peak profile of TCAs

Figures 9.1b and c show simulated chromatograms for mixtures of the six TCAs analysed with the C18 column, and Figures 9.1e and f, chromatograms obtained with the C8 column. For both columns, the mobile phases contained 30% acetonitrile and 10 mM HMIM·Cl or 10 mM HMIM·BF_4. For comparison purposes, a chromatogram obtained with 30% acetonitrile without IL is depicted for the two columns (Figures 9.1a and d).

The retention times were not significantly different between both columns, being somewhat smaller for the C8 column (as expected). The retention decreased remarkably with the addition of HMIM·Cl to the hydro-organic mobile phase. For the most retained TCA, clomipramine, the retention time decreased from 34 min to 13.9 min for the C18 column, and from 29 min to 12.9 min for the C8 column, owing to the repulsion between the cationic TCAs

and $HMIM^+$, the latter strongly adsorbed on the column (the chloride anion is weakly adsorbed). The addition of $HMIM{\cdot}BF_4$ to the hydro-organic mobile phase produced a smaller reduction of the retention: from 34 min to 23.5 min for the C18 column, and from 29 min to 20 min for the C8 column for clomipramine, due to the attraction of TCAs to the tetrafluoroborate anion.

Differences in selectivity were observed between both columns (C18 and C8), in the absence and presence of ILs. Nevertheless, the resolution was incomplete in all assayed conditions, with the best resolution corresponding to the C18 column in the absence of IL (Figure 9.1a), but with significantly longer analysis times. Thus, nortryptiline and maprotiline were unresolved with the C18 column, especially in the presence of ILs. Maprotiline and amitryptiline were unresolved with the C8 column both without IL and in the presence of HMIM·Cl. The peaks of nortryptiline, maprotiline and amitryptiline were highly overlapped with the C8 column in the presence of $HMIM{\cdot}BF_4$.

As expected, the chromatographic peaks in the presence of both ILs were almost symmetrical and narrower than those obtained with the hydro-organic mixtures. Without IL, significant tailing was observed (Figures 9.1a and d).

9.4.3. Selection of stationary phase and mobile phase additive

In previous section, the results have shown the advantage of using HMIM·Cl for the analysis of samples containing TCAs. There was no significant difference between the resolution and analysis time obtained with the C18 and C8 columns. However, the C8 column was finally selected for these analyses, based on the observation that several injections carried out in different consecutive days, and even during the same day, yielded increasing retention times in both the absence of IL and presence of HMIM·Cl and $HMIM{\cdot}BF_4$, especially for the C18 column. The gradual shift in retention times

may have important consequences in a validation process. It should be observed that this effect had not been observed in previous reports with β-blockers.

Figures 9.2a to c show the behaviour of two TCAs (doxepin and imipramine), when analysed with the C18 column without IL and in the presence of HMIM·Cl and HMIM·BF_4. Owing to their high hydrophobicity, the TCAs seemingly are accumulated on the column head, which increases the retention times owing to an "overloading" effect. The effect was stronger with mobile phases containing ILs, especially when the affinity of the anion for the stationary phase was larger (case of tetrafluoroborate). As commented, chloride is weakly adsorbed on the alkyl-bonded stationary phases, whereas tetrafluoroborate shows moderate affinity [9], and attracts electrostatically the cationic solutes to the stationary phase.

Figures 9.2d to f shows that the increasing retention times were minimised using the C8 column without IL and in the presence of HMIM·Cl. The overloading effect still remained with HMIM·BF_4, although in a lesser extent compared to the C18 column. These results indicate that the best conditions for analytical purposes should combine the use of the C8 column with mobile phases containing HMIM·Cl as additive.

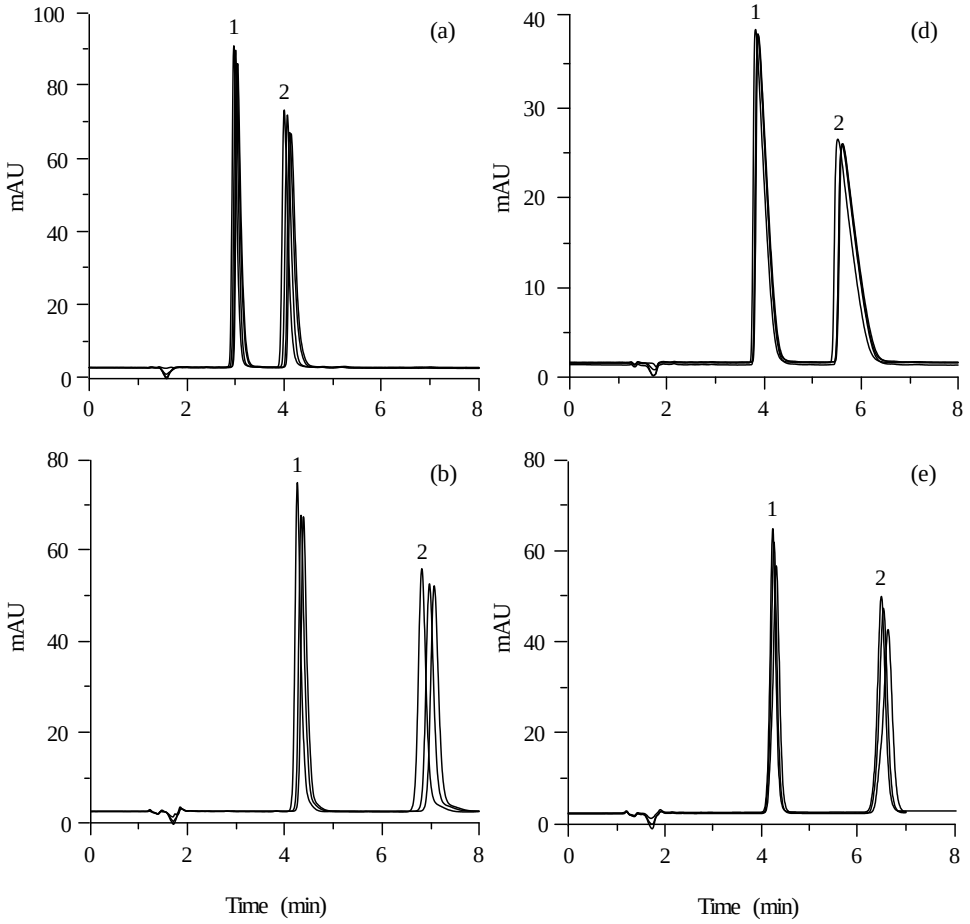


Figure 9.2. Overloading effect on the C18 (a to c), and C8 (d to f) columns. Mobile phase composition: (a) 40% acetonitrile, (b and e) 30% acetonitrile/10 mM HMIM·Cl, (c and f) 30% acetonitrile/10 mM HMIM·BF_4, and (d) 35% acetonitrile. Injections were carried out along four consecutive days. Compounds: (1) doxepin, and (2) imipramine.

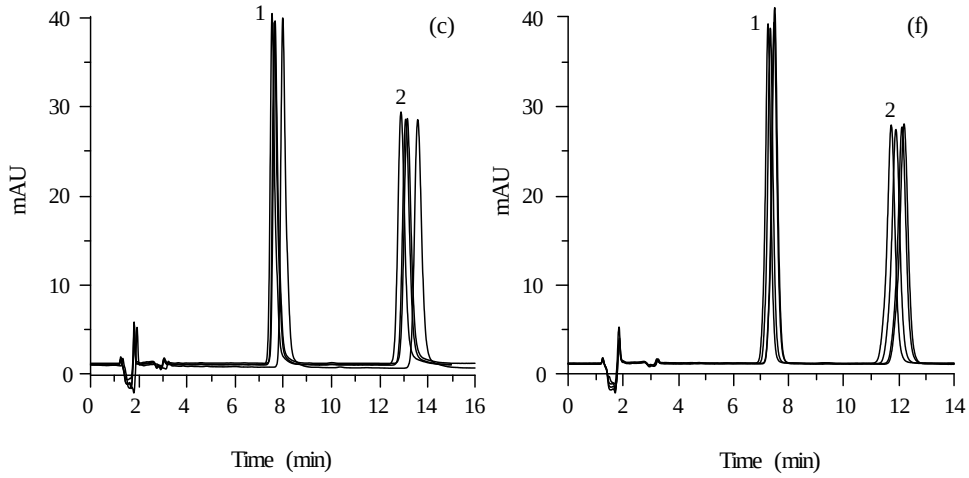


Figure 9.2 (continued).

9.4.4. Method validation

Calibration straight-lines for the TCAs, using the C8 column and 30% acetonitrile/10 mM HMIM·Cl, were built with the areas of the chromatographic peaks *versus* analyte concentration, expressed as μg/mL. The peaks were obtained from duplicate injections of standard solutions at five concentrations in the range 10–40 μg/mL, except for maprotiline (which showed lower molar absorptivity), for which the concentration range was 50–150 μg/mL. The working solutions were obtained by dilution of the stock aqueous solutions, and renewed every week. The assayed concentrations for the injected solutions were uniformly distributed along the studied concentration range. The calibration parameters (slope and intercept) were obtained during three non-consecutive days along three different weeks.

The parameters of the calibration straight-lines are given in Table 9.1. As observed, the linearity requirements were always met, with determination coefficients $R^2 > 0.9964$. The slopes and intercepts were stable throughout the validation process, which indicates a high prediction capability of the concentrations of analytes from the regression straight-lines, and the preservation of the chromatographic column performance.

Table 9.1. Day-to-day calibration parameters for the TCAs.

Compound		Slope	Intercept	R^2
Amitryptiline	[a]	0.434 ± 0.004	0.09 ± 0.04	0.9993
	[b]	0.430 ± 0.003	0.19 ± 0.12	0.9964
Clomipramine	[a]	0.446 ± 0.005	–0.34 ± 0.03	0.9984
	[b]	0.4410 ± 0.0017	–0.46 ± 0.13	0.9994
Doxepin	[a]	0.4680 ± 0.0021	0.57 ± 0.03	0.9990
	[b]	0.48 ± 0.01	0.3 ± 0.3	0.9982
Imipramine	[a]	0.480 ± 0.005	1.134 ± 0.007	0.9991
	[b]	0.50 ± 0.03	0.2 ± 0.8	0.9990
Maprotiline	[a]	0.02134 ± 0.00007	0.04 ± 0.03	0.9999
	[b]	0.02170 ± 0.00024	–0.03 ± 0.06	0.9993
Nortryptiline	[a]	0.440 ± 0.005	0.33 ± 0.06	0.9994
	[b]	0.455 ± 0.014	–0.1 ± 0.4	0.9989

[a] Average for the same set of calibration standards measured along three non-consecutive days during the same week.

[b] Average for different sets of calibration standards measured along three days during three consecutive weeks.

The intra- and inter-day repeatabilities were evaluated by measuring the signals for peaks obtained from solutions at three different concentrations inside the linear range of the calibration straight-lines: 10, 25 and 40 μg/mL for each analyte, except for maprotiline, which was assayed at 50, 100 and 150 μg/mL. Six replicated measurements were always performed. The inter-day variation was controlled during three non-consecutive days along a week. The accuracy was also established using the calibration straight-line obtained each day. The results are summarised in Table 9.2. The precision is expressed as relative standard deviation (RSD), and the accuracy as relative error (relative difference between the values found from the calibration and the concentration of the standards). As can be seen, the intra- and inter-day precisions were usually below +1.0%, and intra- and inter-day bias (accuracy) ranged usually between –2.1% (doxepin) and +2.4% (imipramine), and between – 3.0% (amitryptiline) and +2.3% (imipramine), respectively.

The limits of detection (LODs) and quantification (LOQs) were defined as the lowest concentration of the analytes that can be detected with signal-to-noise ratios greater than 3:1 and 10:1, respectively. LODs and LOQs were calculated from ten-fold injections of solutions containing 0.5 μg/mL of each drug. The obtained values were (LOD and LOQ expressed as μg/mL): amitryptiline (0.025, 0.084), clomipramine (0.019,0.064), doxepin (0.016, 0.054), imipramine (0.015, 0.05), maprotiline (0.168, 0.56), and nortryptiline (0.024, 0.08).

Figure 9.3 shows chromatograms of the sample blank (Figure 9.3a), and of solutions of the analytes prepared with the mobile phase at their LOD values (Figures 9.3b to d).

Table 9.2. Intra- and inter-day precision and accuracy.

		Intra-day			Inter-day		
Compound	Added (µg/ml)	Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)	Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)
Amitryptiline	10.16	9.95 ± 0.09	0.9	–2.1	9.86 ± 0.08	0.8	–3.0
	25.15	25.44 ± 0.05	0.2	1.2	25.42 ± 0.03	0.1	1.1
	40.24	39.86 ± 0.12	0.3	–0.9	39.92 ± 0.07	0.2	–0.80
Clomipramine	10.16	10.31 ± 0.05	0.5	1.5	10.31 ± 0.06	0.6	1.5
	25.40	25.18 ± 0.12	0.5	–0.9	25.04 ± 0.12	0.5	–1.4
	40.64	40.6 ± 0.3	0.7	–0.1	40.50 ± 0.09	0.2	–0.34
Doxepin	10.00	10.03 ± 0.07	0.7	0.3	10.00 ± 0.05	0.5	0.00
	25.00	24.47 ± 0.06	0.2	–2.1	24.45 ± 0.06	0.2	–2.2
	40.00	39.95 ± 0.10	0.3	–0.1	39.943 ± 0.006	0.0	–0.14
Imipramine	9.96	10.06 ± 0.03	0.3	1.0	9.96 ± 0.09	0.9	0.00
	24.90	25.50 ± 0.11	0.4	2.4	25.47 ± 0.07	0.3	2.3
	39.84	39.74 ± 0.03	0.6	–0.3	39.76 ± 0.10	0.3	–0.20

Table 9.2 (continued).

Compound	Added (µg/ml)	Intra-day			Inter-day		
		Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)	Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)
Maprotiline	49.90	49.6 ± 0.7	1.4	–0.6	49.9 ± 0.4	0.8	0.00
	99.80	100.47 ± 0.17	0.2	0.7	100.0 ± 0.4	0.4	0.20
	149.70	149.9 ± 0.7	0.5	0.1	150.0 ± 0.3	0.2	0.20
Nortryptiline	10.00	10.02 ± 0.06	0.6	0.2	10.11 ± 0.11	1.1	1.1
	25.00	25.32 ± 0.20	0.8	1.3	25.24 ± 0.07	0.3	0.96
	40.00	40.09 ± 0.07	0.2	0.2	40.01 ± 0.10	0.2	0.02

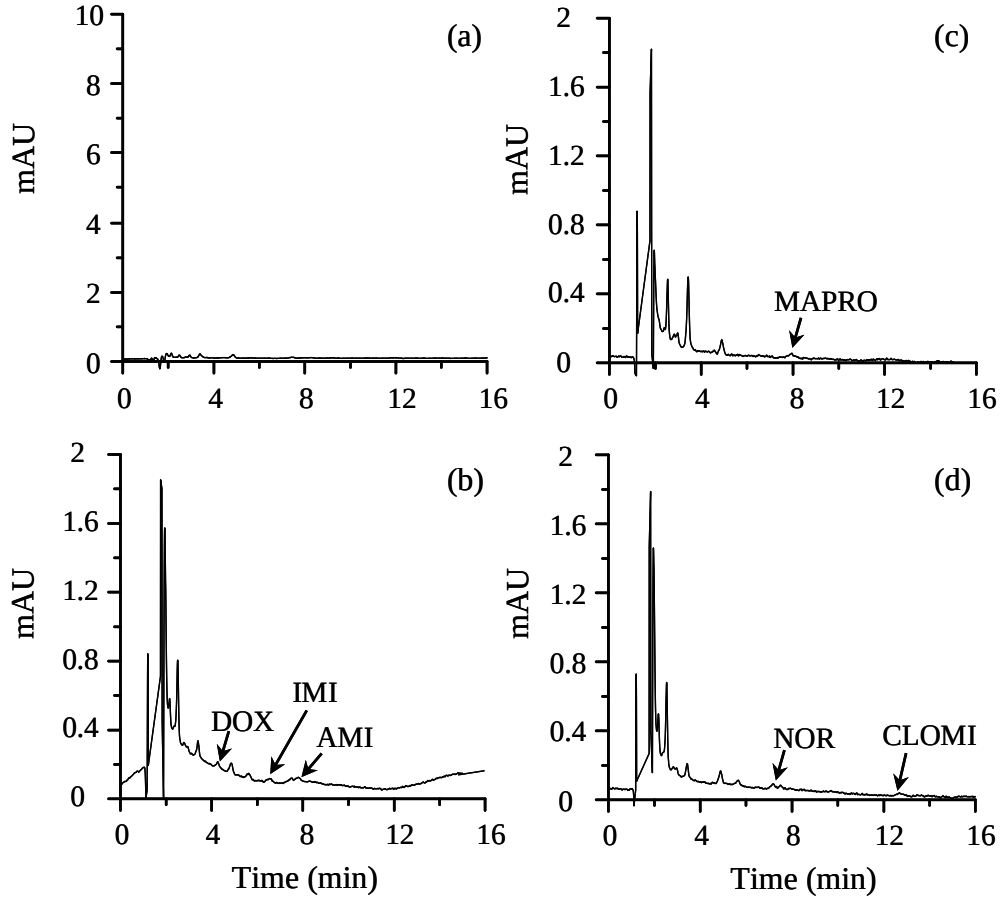


Figure 9.3. Chromatograms of: (a) sample blank (mobile phase: 30% acetonitrile/10 mM HMIM·Cl), (b) mixture of solutions of doxepin (DOX), imipramine (IMI) and amitryptiline (AMI), (c) solution of maprotiline (MAPRO), (d) mixture of nortryptiline (NOR) and clomipramine (CLOMI), all prepared with the mobile phase at their limits of detection (see Table 9.4).

The effect of some experimental parameters on the retention time and peak areas of analytes was also investigated, to evaluate the robustness of the analytical procedure. The assayed parameters were the flow-rate, the pH, and the concentrations of acetonitrile and HMIM·Cl in the mobile phase (Table 9.3). Each of these parameters was varied within a range around the value used to develop the procedure. The parameters were modified following the one-variable-at-a-time (OVAT) method, where the variables are changed one by one, keeping all other parameters constant at their original value. In Table 9.3, mean values, and absolute and relative standard deviations, obtained in the measurement of retention times and peak areas are provided. For the retention times, the RSD values ranged between 0.4 and 1.6% for the flow-rate, acetonitrile concentration and pH, but the variation range was larger for the concentration of HMIM·Cl (2.8–4.8%). For the peak areas, the variability was significantly smaller for the concentration of HMIM·Cl (0.4–0.9%), and similar for the other assayed parameters.

The main analytical figures of this work are compared in Tables 9.4 and 9.5 (see also Table 9.2) with the results obtained with previously published methods using a C8 column and mobile phases of different nature at pH 3.0: 32% acetonitrile without additive [38], 0.02 M Brij-35 without organic solvent [38], and 6% pentanol/0.075 M sodium dodecyl sulphate (SDS) [36].

Table 9.3. Robustness of the proposed procedure.

Compound	Parameter	Level	Retention time (min) (RSD, %)	Area (arbitrary units) (RSD, %)
Amitryptiline	Flow rate (mL/min)	0.99 – 1.01	7.40 ± 0.06 (0.8)	10.36 ± 0.16 (1.5)
	HMIM·Cl (M)	0.009 – 0.011	7.7 ± 0.3 (3.9)	11.73 ± 0.10 (0.9)
	Acetonitrile (%)	29.9 – 30.1	7.49 ± 0.04 (0.5)	10.23 ± 0.05 (0.5)
	pH	2.9 – 3.1	7.38 ± 0.05(0.7)	10.24 ± 0.09 (0.9)
Clomipramine	Flow rate (mL/min)	0.99 – 1.01	12.01 ± 0.13 (1.1)	10.20 ± 0.15 (1.5)
	HMIM·Cl (M)	0.009 – 0.011	12.5 ± 0.6 (4.8)	10.32 ± 0.07 (0.7)
	Acetonitrile (%)	29.9 – 30.1	12.15 ± 0.07 (0.6)	10.27 ± 0.05 (0.5)
	pH	2.9 – 3.1	12.05 ± 0.19 (1.6)	10.30 ± 0.04 (0.4)
Doxepin	Flow rate (mL/min)	0.99 – 1.01	4.08 ± 0.03 (0.7)	11.39 ± 0.23 (2.0)
	HMIM·Cl (M)	0.009 – 0.011	4.24 ± 0.12 (2.8)	12.19 ± 0.09 (0.7)
	Acetonitrile (%)	29.9 – 30.1	4.121 ± 0.016 (0.4)	11.13 ± 0.13 (1.1)
	pH	2.9 – 3.1	4.08 ± 0.04 (1.0)	11.34 ± 0.10 (0.9)
Imipramine	Flow rate (mL/min)	0.99 – 1.01	6.24 ± 0.05 (0.8)	12.03 ± 0.16 (1.3)
	HMIM·Cl (M)	0.009 – 0.011	6.48 ± 0.23 (3.5)	13.01 ± 0.09 (0.7)
	Acetonitrile (%)	29.9 – 30.1	6.31 ± 0.03 (0.5)	12.09 ± 0.06 (0.5)
	pH	2.9 – 3.1	6.23 ± 0.04 (0.6)	11.89 ± 0.13 (1.1)

Table 9.3 (continued).

Compound	Parameter	Level	Retention time (min) (RSD, %)	Area (arbitrary units) (RSD, %)
Maprotiline	Flow rate (mL/min)	0.99 – 1.01	7.46 ± 0.06 (0.8)	2.115 ± 0.022 (1.0)
	HMIM·Cl (M)	0.009 – 0.011	7.7 ± 0.3 (3.9)	2.076 ± 0.014 (0.7)
	Acetonitrile (%)	29.9 – 30.1	7.56 ± 0.05 (0.7)	2.14 ± 0.02 (0.9)
	pH	2.9 – 3.1	7.53 ± 0.04 (0.5)	2.110 ± 0.021 (1.0)
Nortryptiline	Flow rate (mL/min)	0.99 – 1.01	6.82 ± 0.07 (1.0)	10.67 ± 0.14 (1.3)
	HMIM·Cl (M)	0.009 – 0.011	7.1 ± 0.3 (4.2)	10.98 ± 0.04 (0.4)
	Acetonitrile (%)	29.9 – 30.1	6.88 ± 0.03 (0.4)	10.71 ± 0.07 (0.7)
	pH	2.9 – 3.1	6.82 ± 0.09 (1.3)	10.80 ± 0.07 (0.6)

Table 9.4. Comparison of limits of detection (μg/ml), using mobile phases of different nature.

Compound	C_8/Acetonitrile-HMIM·Cl [a]	C_8/Acetonitrile-water [b]	C_8/Brij-35 [c]	C_8/Pentanol-SDS [d]
Amitryptiline	0.025	0.05	0.16	0.54
Clomipramine	0.019	0.31	0.38	0.18
Doxepin	0.016	0.02	0.10	0.24
Imipramine	0.015	0.09	0.25	–
Maprotiline	0.168	0.21	1.53	1.7
Nortryptiline	0.024	0.62	0.29	0.40

[a] 30% acetonitrile/10 mMHMIM·Cl.

[b] 32% acetonitrile without additive (Ref. 38).

[c] 0.02 M Brij-35 without organic solvent (Ref. 38).

[d] 6% pentanol/0.075 M sodium dodecyl sulphate (Ref. 36).

9.4.5. Analysis of pharmaceutical formulations

Several formulations prescribed in Europe containing one of the six TCAs studied in this work were analysed (Table 9.6 and 9.7). The analyses were carried out with five portions of powder for each formulation (see also Section 9.3.3). The weight of the portions was appropriate to prepare five solutions containing approximately 25 μg/mL of the drugs (100 μg/mL for maprotiline). Duplicate injections of these solutions were made.

Figure 9.4 shows chromatograms of the formulations analysed with 30% acetonitrile/10 mM HMIM·Cl. The excipients were eluted at the dead time or did not absorb at the detection wavelength. The found contents are indicated in Tables 9.6 and 9.7, together with the label claim percentages, which were around 100% for amitrytiline, maprotiline and nortryptiline, and above 100% for clomipramine, doxepin, imipramine. The results are compared with those obtained with 32% acetonitrile, 0.02 M Brij-35, and 6% pentanol/0.075 M SDS. It should be noted that according to the ICH guideline, for the assay of a finished (drug) product, a range from 80% to 120% of the sample concentration is accepted [17].

Table 9.5. Intra- and inter-day precision for alternative mobile phases (see Table 9.4).

Compound	C_8/Acetonitrile-water		C_8/Brij-35			C_8/Pentanol-SDS	
	Added (µg/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)	Added (µg/mL)	Intra-day RSD (%)	Inter-day RSD (%)
Amitryptiline	20	0.30	1.20	0.30	1.90	30	0.27	1.10
	30	0.30	0.20	0.40	0.90	50	0.17	1.30
	40	0.16	0.70	0.16	0.90	70	0.13	0.54
	50	0.30	1.10	0.30	1.60			
Clomipramine	20	1.50	2.00	0.50	1.70	30	0.40	1.20
	30	1.70	0.50	2.20	1.70	50	0.25	1.1
	40	0.40	1.10	0.40	2.10	70	0.63	0.54
	50	0.30	1.60	0.30	1.10			
Doxepin	20	0.60	1.90	0.14	1.00	30	0.18	1.40
	30	0.03	0.70	0.17	1.00	50	0.09	1.60
	40	0.30	0.60	0.20	1.20	70	0.09	0.58
	50	0.09	0.60	0.10	1.10			

Table 9.5 (continued).

Compound	Added (µg/mL)	C_8/Acetonitrile-water Intra-day RSD (%)	C_8/Acetonitrile-water Inter-day RSD (%)	C_8/Brij-35 Intra-day RSD (%)	C_8/Brij-35 Inter-day RSD (%)	Added (µg/mL)	C_8/Pentanol-SDS Intra-day RSD (%)	C_8/Pentanol-SDS Inter-day RSD (%)
Imipramine	20	0.14	4.00	0.60	2.10	30	–	–
	30	0.30	3.10	0.60	1.90	50	–	–
	40	0.50	2.90	1.10	2.30	70	–	–
	50	0.40	2.70	0.17	2.20			
Maprotiline	20	1.80	1.00	1.60	1.80	30	0.19	0.65
	30	1.60	2.60	0.80	2.90	50	0.16	0.90
	40	1.70	1.00	1.40	1.80	70	0.29	0.98
	50	1.10	1.20	0.60	0.90			
Nortryptiline	20	0.90	2.80	0.20	1.50	30	0.30	0.59
	30	3.00	1.50	0.60	1.20	50	0.20	1.30
	40	2.10	1.40	0.80	1.80	70	0.14	0.38
	50	1.80	1.50	0.70	1.70			

Table 9.6. Analysis of several formulations containing tricyclic antidepressants, using mobile phases of different nature. [a]

Formulation (laboratory)	Composition (mg)	C_8/Acetonitrile-HMIM·Cl		C_8/Acetonitrile-water	
		Found (mg)	Label claim (%)	Found (mg)	Label claim (%)
Tryptizol (ROVI)	Per tablet: amitryptiline hydrochloride (25), lactose, corn starch and other excipients	24.95 ± 0.21	99.8	22.3 ± 1.6	89.2
Anafranil (Sigma-tau)	Per tablet: clomipramine hydrochloride (25), saccharose, lactose, and other excipients	28.6 ± 1.8	114.4	23.9 ± 0.3	95.6
Sinequan (Farmasierra)	Per tablet: doxepin (25), granulated starch, magnesium stearate and other excipients	30.3 ± 0.9	121.2	24.1 ± 0.9	96.4
Tofranil (Amdipharm)	Per tablet: imipramine hydrochloride (25), colloidal silica, glycerol and other excipients	28.2 ± 2.3	112.8	23.6 ± 0.5	94.4
Ludiomil / Mutabase (Amdipharm)	Per tablet: maprotiline hydrochloride (25), magnesium stearate and other excipients	26.6 ± 0.9	106.4	25.1 ± 0.6	100.0
Patxibi / Norfenazin (BIOMED)	Per tablet: nortryptiline hydrochloride (25), lactose and other excipients	26.55 ± 0.17	106.2	19.7 ± 0.9	78.8

[a] See Table 9.4 for mobile phase compositions.

Table 9.7. Analysis of several formulations containing tricyclic antidepressants, using mobile phases of different nature. [a]

Formulation (laboratory)	Composition (mg)	C_8/Brij-35		C_8/Pentanol-SDS	
		Found (mg)	Label claim (%)	Found (mg)	Label claim (%)
Tryptizol (ROVI)	Per tablet: amitryptiline hydrochloride (25), lactose, corn starch and other excipients	22.5 ± 0.7	90.0	24.5	98.0
Anafranil (Sigma-tau)	Per tablet: clomipramine hydrochloride (25), saccharose, lactose, and other excipients	23.76 ± 0.16	95.0	24.8	99.2
Sinequan (Farmasierra)	Per tablet: doxepin (25), granulated starch, magnesium stearate and other excipients	24.0 ± 1.0	96.0	27.5	110.0
Tofranil (Amdipharm)	Per tablet: imipramine hydrochloride (25), colloidal silica, glycerol and other excipients	23.7 ± 0.6	94.8	–	–
Ludiomil / Mutabase (Amdipharm)	Per tablet: maprotiline hydrochloride (25), magnesium stearate and other excipients	24.5 ± 0.5	98.0	25.4	101.6
Patxibi / Norfenazin (BIOMED)	Per tablet: nortryptiline hydrochloride (25), lactose and other excipients	18.8 ± 0.5	75.2	24.4	97.6

[a] See Table 9.4 for mobile phase compositions.

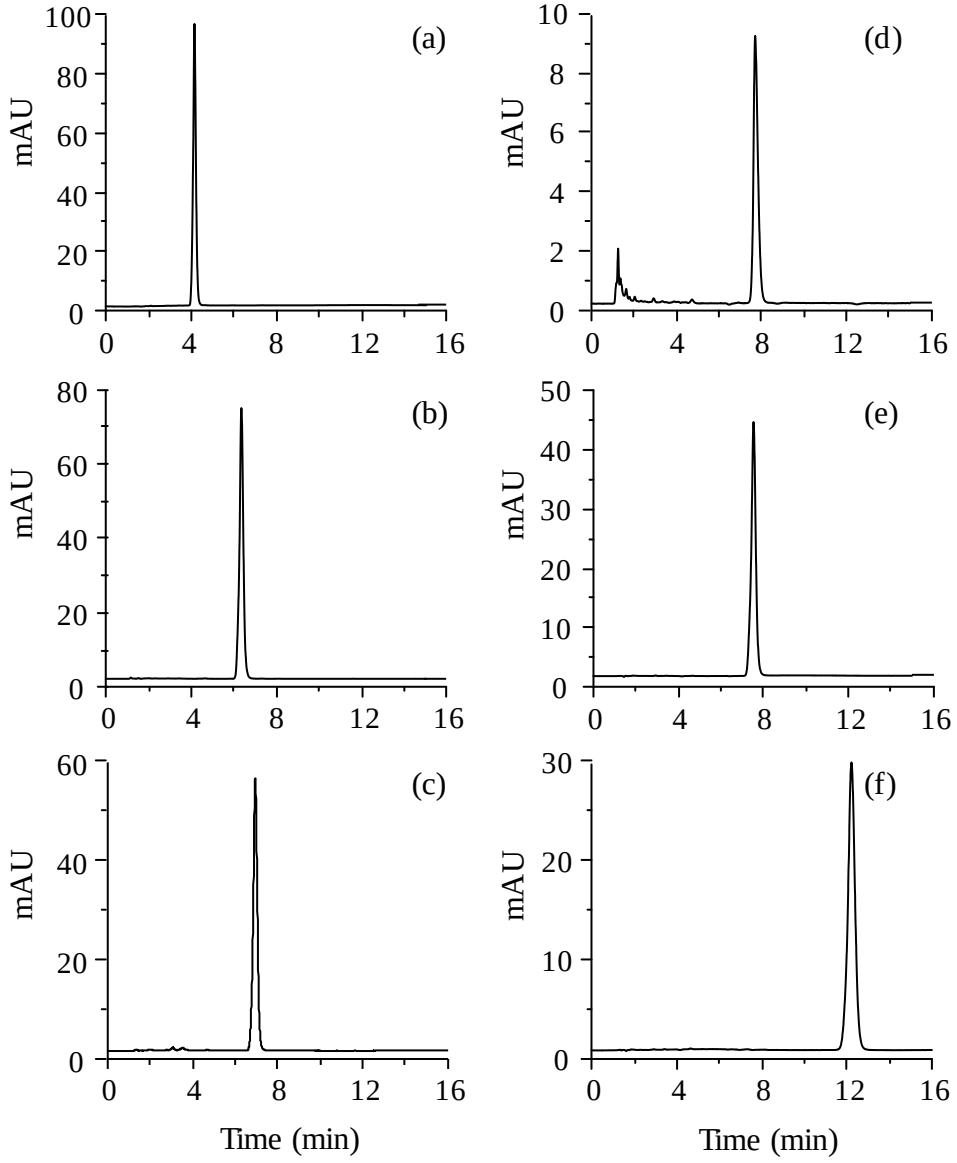


Figure 9.4. Chromatograms of some formulations containing TCAs, analysed with 30% acetonitrile/10 mM HMIM·Cl. Pharmaceuticals: (a) Sinequan (doxepin), (b) Tofranil (imipramine), (c) Patxibi (nortryptiline), (d) Ludiomil (maprotiline), (e) Tryptizol (amitryptiline), and (f) Anafranil (clomipramine).

9.5. Conclusions

The aim of this work was to check if reliable analyses of highly apolar basic drugs were possible in chromatographic procedures, where the analytes are eluted with acetonitrile/water mobile phases containing an IL formed by the HMIM cation and the anions chloride or tetrafluoroborate. TCAs were selected due to their interest in pharmaceutical analysis and wide prescription in the clinical field.

The ILs HMIM·Cl and HMIM·BF_4 yield narrow and symmetrical peaks for basic compounds, when added to hydro-organic mobile phases, using conventional C18 columns. However, when the TCAs were analysed using a C18 column with acetonitrile/water mobile phases, an overloading effect was observed. This was more pronounced when the mobile phase contained HMIM·Cl and HMIM·BF_4, especially for the latter, due to the higher affinity of tetrafluoroborate for the stationary phase. The overloading effect was smaller with a C8 column, but was still observed in the presence of HMIM·BF_4. The results indicate that the selected IL as mobile phase additive to develop an analytical procedure, followed by method validation, could limit its applicability. Several aspects should be considered in this selection: the IL capability to effectively block the silanol activity, the associated anion affinity for the stationary phase, and the hydrophobic character of the analytes.

The recommended procedure employs a mobile phase containing 30% acetonitrile in the presence of 10 mM HMIM·Cl, and was applied to the analysis of commercialised pharmaceutical formulations with satisfactory results. The procedure has the advantage of producing highly symmetrical peaks, requiring smaller organic solvent consumption, and a simple sample preparation without previous extraction of the drugs (only solubilisation and

filtration). The resolution was, however, poor, which limits its applicability in screening studies.

Method validation indicated that the proposed procedure offers an excellent linearity and satisfactory intra- and inter-day accuracy and precision. The method is also robust, but requires a rigorous control of the concentration of HMIM·Cl.

9.6. References

[1] H. Engelhardt, Ch. Blay, J. Saar, Reversed phase chromatography: The mystery of surface silanols, *Chromatographia* 62 (2005) S19–S29.

[2] U.D. Neue, K. Tran, A. Méndez, P.W. Carr, The combined effect of silanols and the reversed-phase ligand on the retention of positively charged analytes, *J. Chromatogr. A* 1063 (2005) 35–45.

[3] L.J. He, W.Z. Zhang, L. Zhao, X. Liu, S.X. Jiang, Effect of 1-alkyl-3-methylimidazolium-based ionic liquids as the eluent on the separation of ephedrines by liquid chromatography, *J. Chromatogr. A* 1007 (2003) 39–45.

[4] A. Martín-Calero, V. Pino, J.H. Ayala, V. González, A.M. Afonso, Ionic liquids as mobile phase additives in high-performance liquid chromatography with electrochemical detection: Application to the determination of heterocyclic aromatic amines in meat-based infant foods, *Talanta* 79 (2009) 590–597.

[5] A. Martín-Calero, J.H. Ayala, V. González, A.M. Afonso, Ionic liquids as desorption solvents and memory effect suppressors in heterocyclic aromatic amines determination by SPME-HPLC fluorescence, *Anal. Bioanal. Chem.* 394 (2009) 937–946.

[6] W. Bi, M. Tian, K.H. Row, Chiral separation and determination of ofloxacin enantiomers by ionic liquid-assisted ligand-exchange chromatography, *Analyst* 136 (2011) 379–387.

[7] J.J. Fernández Navarro, J.R. Torres Lapasió, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, 1-Hexyl-3-methylimidazolium tetrafluoroborate: An efficient column enhancer for the separation of basic drugs by reversed-phase liquid chromatography, *J. Chromatogr. A* 1258 (2012) 168–174.

[8] M.T. Úbeda Torres, C. Ortiz Bolsico, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, Gaining insight in the behaviour of imidazolium-based ionic liquids as additives in reversed-phase liquid chromatography for the analysis of basic compounds, *J. Chromatogr. A* 1380 (2015) 96–103.

[9] A. Berthod, M.J. Ruiz Ángel, S. Huguet, Nonmolecular solvents in separation methods: Dual nature of room temperature ionic liquids, *Anal. Chem.* 77 (2005) 4071–4080.

[10] M.P. Marszałł, T. Baczek, R. Kaliszan, Evaluation of the silanol-suppressing potency of ionic liquids, *J. Sep. Sci.* 29 (2006) 1138–1145.

[11] M.C. García Álvarez-Coque, M.J. Ruiz Ángel, A. Berthod, S. Carda Broch, On the use of ionic liquids as mobile phase additives in high-performance liquid chromatography, A review, *Anal. Chim. Acta* 883 (2015) 1–21.

[12] Y. Polyakova, Y.M. Koo, K.H. Row, Application of ionic liquids as mobile phase modifier in HPLC, *Biotechnol. Bioprocess Eng.* 11 (2006) 1–6.

[13] B. Buszewski, S. Studzinska, A review of ionic liquids in chromatographic and electromigration techniques, *Chromatographia* 68 (2008) 1–10.

[14] D. Han, M. Tian, D.W. Park, D.K. Choi, K.H. Row, Application of ionic liquids as mobile phase additives and surface-bonded stationary phase in liquid chromatography, *Korean J. Chem. Eng.* 26 (2009) 1353–1358.

[15] J. Flieger, Application of ionic liquids in liquid chromatography, in: *Ionic Liquids: Applications and Perspectives* (edited by A. Kokorin), Intech, Croatia, 2011, pp. 243–272.

[16] Y. Tang, A. Sun, R. Liu, Y. Zhang, Simultaneous determination of fangchinoline and tetrandrine in *Stephaniatetrandra S. Moore* by using 1-alkyl-3-methylimidazolium-based ionic liquids as the RP-HPLC mobile phase additives, *Anal. Chim. Acta* 767 (2013) 148–154.

[17] *ICH Harmonized Tripartite Guideline*: Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, CH, 2005 (Accessed 7 April 2017) www.ich.org.

[18] S. Akhondzadeh, H. Faraji, M. Sadeghi, K. Afkham, H. Fakhrzadeh, A. Kamalipour, Double-blind comparison of fluoxetine and nortriptyline in the treatment of moderate to severe major depression, *J. Clin. Pharm. Ther.* 28 (2003) 379–384.

[19] D. Bailly, Benefits and risks of using antidepressants in children and adolescents, *Expert Opin. Drug Saf.* 7 (2008) 9–27.

[20] V.F. Samanidou, M.K. Nika, I.N. Papadoyannis, HPLC and its essential role in the analysis of tricyclic antidepressants in biological samples, in *Recent Advances in Medicinal Chemistry* (edited by Atta ur Rahman, M.I. Choudhary, G. Perry), Elsevier, Amsterdam, Vol. 1, 2014, pp. 332–380.

[21] V.F. Samanidou, M.K. Nika, I.N. Papadoyannis, Development of an HPLC method for the monitoring of tricyclic antidepressants in biofluids, *J. Sep. Sci.* 30 (2007) 2391–2400.

[22] M. Wozniakiewicz, R. Wietecha-Poluszny, A. Garbacik, P. Koscielniak, Microwave-assisted extraction of tricyclic antidepressants from human serum followed by high performance liquid chromatography determination, *J. Chromatogr. A* 1190 (2008) 52–56.

[23] M.N. Uddin, V.F. Samanidou, I.N. Papadoyannis, Bio-sample preparation and analytical methods for the determination of tricyclic antidepressants, *Bioanalysis* 3 (2011) 97–118.

[24] M. Rezazadeh, J. Emami, A simple and sensitive HPLC method for analysis of imipramine in human plasma with UV detection and liquid-liquid extraction: Application in bioequivalence studies, *Res. Pharm. Sci.* 11 (2016) 168–176.

[25] M. Shamsipur, M. Mirmohammadi, High performance liquid chromatographic determination of ultratraces of the two tricyclic antidepressant drugs imipramine and trimipramine in urine samples after their dispersive liquid-liquid microextraction coupled with response surface optimization, *J. Pharm. Biomed. Anal.* 100 (2014) 271–278.

[26] C. Dell'Aquila, Separation of tricyclic antidepressants by capillary zone electrophoresis with N,N,N′,N′-tetramethyl-1,3-butanediamine (TMBD) as an effective electrolyte additive, *J. Pharm. Biomed. Anal.* 30 (2002) 341–350.

[27] M. Delmar Cantu, S. Hillebrand, M.E. Costa Queiroz, F.M. Lancas, E. Carrilho, Validation of non-aqueous capillary electrophoresis for simultaneous determination of four tricyclic antidepressants in pharmaceutical formulations and plasma samples, *J. Chromatogr. B* 799 (2004) 127–132.

[28] H.F. Wu, S.K. Kailasa, J.Y. Yan, Ch.Ch. Chin, H.Y. Ku, Comparison of single-drop microextraction with microvolume pipette extraction directly coupled with capillary electrophoresis for extraction and separation of tricyclic antidepressant drugs, *J. Ind. Eng. Chem.* 20 (2014) 2071–2076.

[29] R. Ito, M. Ushiro, Y. Takahashi, K. Saito, T. Ookubo, Y. Iwasaki, H. Nakazawa, Improvement and validation of the method using dispersive liquid-liquid microextraction with in situ derivatization followed by gas chromatography-mass spectrometry for determination of tricyclic antidepressants in human urine samples, *J. Chromatogr. B* 879 (2011) 3714–3720.

[30] S.S.H. Davarani, A.M. Najarian, S. Nojavan, M. Tabatabaei, Electromembrane extraction combined with gas chromatography for quantification of tricyclic antidepressants in human body fluids, *Anal. Chim. Acta* 725 (2012) 51–56.

[31] S. Seidi, Y. Yamini, M. Rezazadeh, Combination of electromembrane extraction with dispersive liquid-liquid microextraction followed by gas chromatographic analysis as a fast and sensitive technique for determination of tricyclic antidepressants, *J. Chromatogr. B* 913–914 (2013) 138–146.

[32] R. Xu, H.K. Lee, Application of electro-enhanced solid phase micro-extraction combined with gas chromatography-mass spectrometry for the determination of tricyclic antidepressants in environmental water samples, *J. Chromatogr. A* 1350 (2014) 15–22.

[33] C.J. Drayton (Ed.), *Comprehensive Medicine Chemistry*, Vol. 6, Pergamon Press, Oxford, 1990.

[34] M.J. Ruiz Ángel, S. Pous Torres, S. Carda Broch, M.C. García Álvarez-Coque, Performance of different C18 columns in reversed-phase liquid chromatography with hydro-organic and micellar-organic mobile phases,*J. Chromatogr. A* 1344 (2014) 76–82.

[35] J.M. Bermúdez Saldaña, C. Quiñones Torrelo, S. Sagrado, M.J. Medina Hernández, R.M. Villanueva Camañas, A micellar liquid chromatographic method for quality control of pharmaceutical preparations containing tricyclic antidepressants, *Chromatographia* 56 (2002) 299–306.

[36] M.J. Ruiz Ángel, S. Carda Broch, E.F. Simó Alfonso, M.C. García Álvarez-Coque, Optimised procedures for the reversed-phase liquid chromatographic analysis of formulations containing tricyclic antidepressants, *J. Pharm. Biomed. Anal.* 32 (2003) 71–84.

[37] D. Bose, A. Durgbanshi, A. Martinavarro Domínguez, M.E. Capella Peiró, S. Carda Broch, J. Esteve Romero, M. Gil Agustí, Amitriptyline and nortriptyline serum determination by micellar liquid chromatography, *J. Pharmacol. Toxicol. Met.* 52 (2005) 323–329.

[38] J.J. Fernández Navarro, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Reversed-phase liquid chromatography without organic solvent for determination of tricyclic antidepressants, *J. Sep. Sci.* 35 (2012) 1303–1309.

[39] M. Caban, P. Stepnowski, The antagonistic role of chaotropic hexafluorophosphate anions and imidazolium cations composing ionic liquids applied as phase additives in the separation of tricyclic antidepressants, *Anal. Chim. Acta* 967 (2017) 102–110.

[40] J.J. Fernández Navarro, M.C. García Álvarez-Coque, M.J. Ruiz Ángel, The role of the dual nature of ionic liquids in the reversed-phase liquid chromatographic separation of basic drugs, *J. Chromatogr. A* 1218 (2011) 398–407.

[41] J.R. Torres Lapasió, *MICHROM Software*, Marcel Dekker, New York 2000.

Part 3

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

CHAPTER 10

PROTOCOL TO COMPARE COLUMN PERFORMANCE APPLIED TO HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

10.1. Abstract

The lack of retention of highly polar solutes in liquid chromatography with reversed phase (RPLC) columns can be solved through the use of the hydrophilic interaction liquid chromatographic (HILIC) mode. Due to the complexity of the separation mechanisms in HILIC and the different factors that may have significant influence, the selection of the appropriate stationary phase plays a fundamental role in the development of analytical procedures to obtain good performance. In this work, the chromatographic performance of bare silica and six polar stationary phases with different functionalised groups (with neutral, cationic, anionic and zwitterionic character) are investigated. The behaviour is compared in terms of retention, selectivity, peak shape, and resolution, using acetonitrile-water mobile phases buffered at pH 3, and some correlation tools previously developed for RPLC. Six nucleosides and uracil were used as probe compounds. The results revealed significant differences among the columns regarding all inspected parameters. Some insight on the interactions established between solutes, and both stationary and mobile phases, is also given.

10.2. Introduction

The term hydrophilic interaction liquid chromatography (HILIC) was coined by Alpert in 1990 [1], although the use of mixtures of acetonitrile and buffered aqueous solutions in normal-phase liquid chromatography had been early assayed in 1975 for the chromatographic separation of saccharides and sugars [2,3]. Since then, the use of HILIC has extensively grown, especially in the last decade, owing to its potential in separating highly polar compounds [4]. HILIC is a complex chromatographic mode where the retention of polar compounds is the result of the contribution of multiple modes of interaction, which depend on the combination of the nature of both the stationary phase and mobile phase, and the physico-chemical properties of the analytes.

It is widely accepted that the aqueous component of the mobile phase gives rise to a hydrophilic environment through the formation of a water-rich adsorbed layer on the surface of the stationary phase, which allows partitioning of analytes between mobile phase and stationary phase [5–8]. This hydrophilic interaction governs mainly the retention mechanism in HILIC, which offers a complementary selectivity to RPLC. Secondary interactions involving hydrogen bonding, dipole-dipole and ion-exchange (electrostatic attraction and repulsion) can also take place between the polar analytes and stationary phase.

The complexity of the separation process, and the multiple factors that affect solute retention in HILIC, confer big importance to the stationary phase. During years, normal phase columns (e.g., bare silica, amino, and cyano) were almost exclusively used in HILIC. At present, although these phases are still popular for this chromatographic mode, new stationary phases with diverse functionalities, exclusively developed for HILIC, are commercially available with satisfactory results [9].

The stationary phases can be classified based on the charge characteristics of the functional groups, as neutral, charged (cationic and anionic), and zwitterionic. The offer is so wide that the selection of the most convenient stationary phase for a specific separation in HILIC may make the development of chromatographic methods rather complex. This is especially true considering that the chromatographic behaviour is additionally influenced by parameters such as pH, temperature and flow rate.

In the literature, several authors have examined the chromatographic behaviour of columns of different nature for the analysis of several types of compounds. The differences among the stationary phases have been usually established based on the influence of the water/acetonitrile content, pH, buffer concentration and temperature on retention factors, selectivity and resolution [10–38]. Van Deemter plots have been built to compare the stationary phases according to the efficiencies [13,23,25,26,34], but peak asymmetry has been scarcely discussed [12,21,26]. Occasionally, hierarchical clustering radar plots and principal component analysis have been used to evidence the different separation behaviour among stationary phases [12,15,22,39]. Several authors have also used frontal chromatography to investigate the amount of adsorbed water on the stationary phase to compare the role of different functionalities [40,41]. The amount of ligand on the particle surface [42], the establishment of quantitative-structure retention relationships [19], and the characterisation of stationary phases using the hydrophilic-subtraction retention model [43], have been less usual approaches for column comparison in the HILIC mode.

Commercially available HILIC stationary phases with different functionalities (bare silica, diol, cyano, alkyl amino, carbamoyl, pentafluorophenyl, and zwitterionic) have been usually compared. Some authors have also studied the performance of stationary phases prepared in their

laboratories [16,20,22,28,42]. The probe compounds used in these studies usually involve collections of solutes with acidic or basic character, nucleosides being the most popular compounds for column testing [13,22–25,29,31,34,43]. These compounds are biochemical precursors of nucleotides, and have biomedical and pharmaceutical interest, since they are involved in the regulation and modulation of diverse physiological processes [44,45]. The hydrophilic character of these compounds makes RPLC unsuitable to achieve their chromatographic separation, whereas HILIC has revealed as a promising alternative [46–49].

In this work, the chromatographic behaviour of seven columns commercially available for HILIC separations was compared based on the separation of a group of six nucleosides and uracil, using acetonitrile-water mobile phases buffered at pH 3. The comparison was made in terms of retention, selectivity, peak shape, and resolution, using a protocol developed in previous work for RPLC columns, in the absence and presence of secondary equilibria [50–53]. The observed behaviour gives some insight on the interactions established between solutes, and both stationary and mobile phases.

10.3. Experimental

10.3.1. Reagents

Six nucleosides (adenosine, cytidine, guanosine, thymidine, uridine and xantosine, all from Sigma, St. Louis, MO, USA), and uracil (Acros Organics, Geel, Belgium), were used as probe compounds. Their structures, acidity constants and octanol-water partition coefficients are given in Table 10.1.

The compounds were dissolved and diluted in nanopure water (Barnstead, Sybron, Boston, MA, USA), to a concentration of approximately 100 μg/mL. The final solutions contained a small amount of acetonitrile (VWR Chemicals, Radnor, PA, USA) to guarantee their stability during storing, which was extended during at least two months at 4 ^{o}C. Working solutions were also diluted with nanopure water to a final concentration approximately 20 μg/mL, before injection into the chromatograph.

Eight mobile phases were prepared with acetonitrile and increasing concentrations of an aqueous buffered solution prepared with 0.01 M ammonium formate (Sigma) (10, 15, 20, 25, 30, 35, 40 and 45% (*v*/*v*)). In all cases, the mobile phase was adjusted at pH 3 with formic acid (Acros Organics), referred to the aqueous-organic mixture, although the standardisation of the pH-meter was always carried out using aqueous buffers. The selected mobile phase compositions guaranteed the elution of the assayed compounds in practical analysis times. Nylon membranes of 0.45 μm (Micron Separations, Westboro, MA, USA) were used to filter the solutions of the probe compounds and mobile phases, which were degassed in an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany).

Table 10.1. Acidity constants (pK_a), octanol-water partition coefficients (log $P_{o/w}$), and apparent value (log D) at pH 3 for the probe compounds.

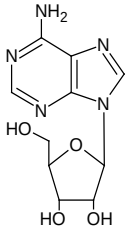
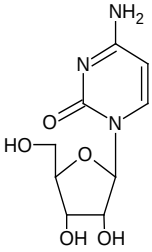
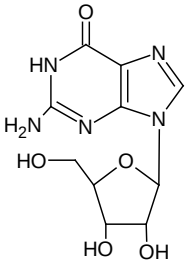
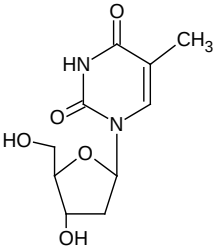
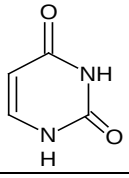
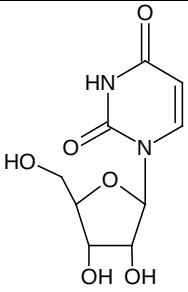
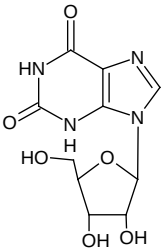
Compound	Structure	pK_a [a]	log $P_{o/w}$ [b]	log D [c]
Adenosine		3.7	–1.0 ± 0.5	–1.8
Cytidine		4.22; 12.5	–1.9 ± 0.4	–3.1
Guanosine		2.1; 9.2	–1.7 ± 0.6	–1.8
Thymidine		9.72	–1.1 ± 0.5	–7.8
Uracil		9.5	–0.7 ± 0.3	–7.2

Table 10.1 (continued).

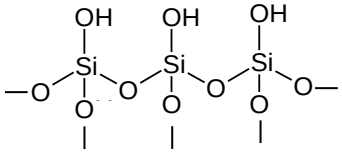
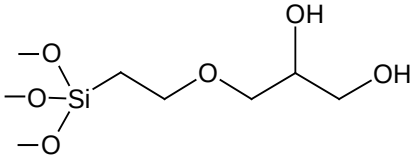
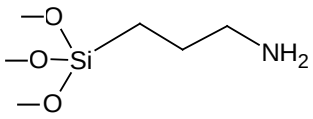
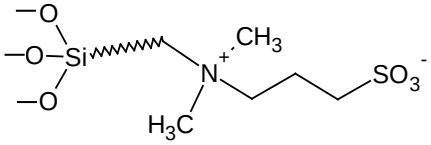
Compound	Structure	pK_a [a]	log $P_{o/w}$ [b]	log D [c]
Uridine		8.5	–1.6 ± 0.4	–7.1
Xantosine		5.5; 12.85	–2.1 ± 0.6	–4.6

[a] Reference [46]. [b] Calculated with ChemSketch, ACD Labs, 2012 version. [c] Calculated with Eqs. (10.1) and (10.2) (Section 10.4.1) [55].

10.3.2. Apparatus and columns

An Agilent chromatograph (Waldbronn, Germany), equipped with a quaternary pump (Series 1200), an autosampler (Series 1100), a thermostated column compartment (Series 1260) set at 25 ºC, and a UV-visible detector of variable wavelength (Series 1100), controlled by an OpenLAB CDS LC ChemStation (Agilent, C.01.07 SR3) for data acquisition, was used. The nucleosides and uracil were detected at 260 and 254 nm, respectively. Triplicate injections of 20 µL were made. The chromatographic peaks were integrated with the MICHROM software [54], whereas mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA).

Table 10.2. Assayed HILIC columns.

Column	Type	Structure
ACE Excel 5 SIL	Bare silica	 (low ion exchange capability)
Betasil Diol-100	Neutral	
ACE Excel 5 NH_2	Charged	 (anion exchange capability)
Sequant ZIC-HILIC	Zwitterionic	
ACE HILIC-A	Charged	NA[a] (cation exchange capability)
ACE HILIC-B	Charged	NA[a] (anion exchange capability)
ACE HILIC-N	Neutral	NA[a] (low ion exchange capability)

[a] Not available

Seven ACE (Aberdeen, UK) chromatographic columns (150 mm × 4.6 mm i.d. and 5 μm particle size) were selected, whose character and functional groups are indicated in Table 10.2. Triplicate injections of 20 μL were made. The relative standard deviations (%) of retention times for each column varied as follows: ACE Excel 5 SIL (0.02–0.14), Betasil Diol-100 (0.03–0.25), ACE Excel 5 NH_2 (0.02–0.13), ZIC-HILIC (0.02–0.11), HILIC-A (0.02–0.08), HILIC-B (0.02–0.08), and HILIC-N (0.02–0.06). The flow rate was 1 mL/min for bare silica, diol, amino and zwitterionic columns, and 0.5 mL/min for the columns identified as HILIC-A, HILIC-B and HILIC-N (ACE proprietary columns recently commercialised). According to the manufacturer, HILIC-A and HILIC-B are bonded acidic and basic phases with an ionisable negative and positive surface charge, respectively, depending on the mobile phase pH. HILIC-N is a polyhydroxy phase with a neutral character. Column regeneration was achieved following the manufacturer's recommendations.

10.4. Results and discussion

10.4.1. Retention capability of HILIC columns

Seven HILIC columns with different chemical behaviour were evaluated with mobile phases buffered at pH 3.0. All of them were silica-based, with the same dimensions (150 mm × 4.6 mm i.d.) and particle size (5 μm). Bare silica, diol and HILIC-N columns may show negative charge at mobile phase pH above 4–5 due to the deprotonation of residual silanols, while amino and HILIC-B columns (anion exchangers) should show positive charge, and HILIC-A (cation exchanger), negative charge. The zwitterionic column has a functionalised packing with a ligand conferring simultaneously positive

(quaternary ammonium group) and negative (sulphonate group) charge in a 1:1 ratio. Therefore, it has no net charge. However, the negative charge on the sulphonate group is capable of inducing electrostatic interactions with charged solutes, due to its position at the distal end of the ligand [9]. These characteristics can lead to different chromatographic behaviours, depending on the nature of the studied compounds.

Nucleosides and uracil are hydrophilic compounds with log $P_{o/w}$ values ranging from –0.7 to –2.1 (Table 10.1), which would allow appreciable retention on HILIC stationary phases. To understand the chromatographic behaviour of these compounds it is, however, necessary to examine the differences in their ionisation state. Thus, the pK_a values of adenosine, cytidine, guanosine and xantosine are in the range 2.1–5.5. Therefore, they are partially protonated at pH 3.0 giving rise to cationic solutes, and presumably will be well retained on cation exchange stationary phases. The pK_a values of thymidine, uracil and uridine are higher, ranging from 8.5 to 9.7. Consequently, these compounds exist mainly in cationic form at acidic pH.

Due to the different acid-base behaviour of the probe compounds, it is more useful to compare, instead of log $P_{o/w}$, the pH dependent distribution or apparent log $P_{o/w}$ (log D) values at pH close to that of the mobile phases used. These were calculated according to the equation proposed by Xing and Glen [55]:

$$\log D = \log P_{o/w} - \log [1 + 10^{(pH - pKa)}] \quad (10.1)$$

for acids, and

$$\log D = \log P_{o/w} - \log [1 + 10^{(pKa - pH)}] \quad (10.2)$$

for bases. The log D values were calculated using the pK_a values in aqueous solution (Table 1). It should be noted that pK_a values for nucleosides may vary

with solvent composition, therefore the correction considering the acid-base behavior may be only partial.

Figures 10.1 and 10.2 depict the changes in retention factor, *k*, at increasing water content in the acetonitrile-water mixture, for the seven probe compounds, eluted with all assayed columns. The retention factors gradually decreased at increasing water content, following an exponential trend, the more important changes being in the 10–20% water content range.

Cytidine (with log *D* = –3.1) showed the largest retention in bare silica, diol, zwiterionic and HILIC-A columns (with *k* values 1.94, 2.34, 7.34 and 1.24 respectively), whereas guanosine (log *D* = –1.8) was the nucleoside showing highest retention in the amino, HILIC-N and HILIC-B columns (with *k* = 4.39, 4.80 and 6.45, respectively). Uracil was the least retained compound for most assayed columns (*k* values in the range 0.27 to 1.14).

Considering that, in HILIC, the retention behaviour is mainly governed by hydrophilic partitioning of solutes on the adsorbed aqueous layer on the stationary phase, the observed differences in retention for the studied compounds suggest additional mechanisms involving specific interactions. Thus, ion exchange may happen in those columns where the stationary phase could be charged at pH 3. Zwitterionic and amino columns retained stronger all studied compounds, which reveals a more hydrophilic character for these stationary phases, where the adsorbed aqueous layer should be thicker [6,35].

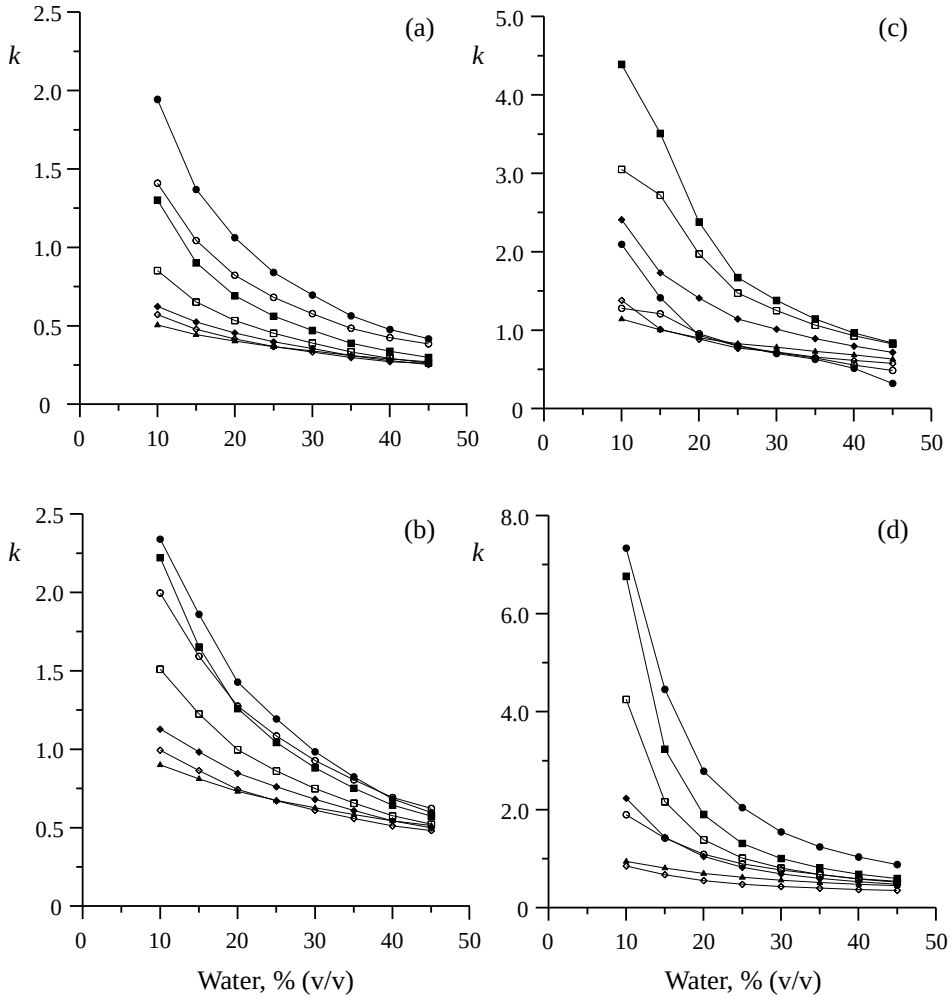


Figure 10.1. Effect of increasing water contents in the mobile phase on the retention of nucleosides and uracil, using different HILIC columns: (a) bare silica, (b) diol, (c) amino, and (d) zwitterionic. Compound identities: cytidine (●), guanosine (■), adenosine (○), xantosine (□), uridine (◆), thymidine (◇), and uracil (▲). Flow rate was 1 mL/min.

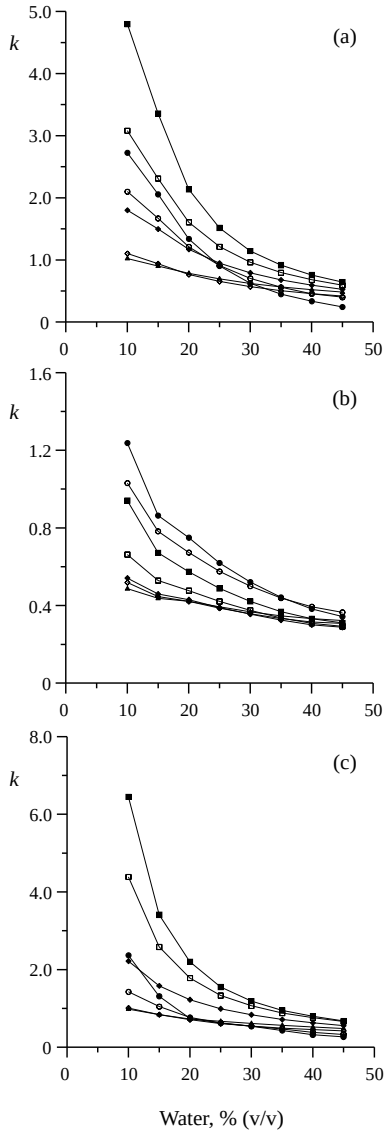


Figure 10.2. Effect of increasing water contents in the mobile phase on the retention of nucleosides and uracil, using the ACE HILIC columns: (a) HILIC-N, (b) HILIC-A, and (c) HILIC-B. See Figure 10.1 for compound identity. Flow rate was 0.5 mL/min.

Meanwhile, the diol column has a neutral character with no ion exchange capability for anions and cations. The ionisation of silanols would provide acidic sites to bare silica giving rise to larger retention for the protonated bases. This does not seem the case, since the nucleosides showed low retention times when eluted from both bare silica and diol columns.

The nucleosides were weakly retained on the HILIC-A stationary phase, which forced the use of a smaller flow rate of 0.5 mL/min for this column to increase the accuracy in the measurement of retention times. For comparison purposes, this flow rate was also used with the HILIC-N and HILIC-B columns. The retention with HILIC-N was unexpectedly strong for nucleosides (see Figure 10.2a). This indicated high polarity for this column, in spite of its presumably low ion exchange capability. The HILIC-A column showed also an unexpected behaviour (Figure 10.2b). According to the manufacturer's information, this column possesses the ability to form a negative charge and shows high cation exchange capability. Therefore, cationic solutes should have been strongly retained. However, the HILIC-A column gave rise to the lowest retention factors.

Therefore, the largest and smallest elution strengths were obtained for the zwitterionic and HILIC-A columns, respectively. The observed differences in retention behaviour, among the studied columns, gave rise to differences in selectivity and resolution, as will be shown in the next sections.

10.4.2. Selectivity

In order to explore, with more detail, the similarities and differences between columns, the retention factors of nucleosides were correlated for different pairs of columns, considering all assayed mobile phase compositions. A high correlation between the retention factors of the studied compounds suggests similar selectivity (i.e., relative retention), although the analysis times can differ, whereas the higher the scatter in the plots the larger the differences in selectivity between column pairs.

In this study, bare silica was selected as reference for columns showing different character (neutral, cationic, anionic and zwitterionic). Figure 10.3 shows some examples of the correlations obtained for mobile phases containing 10% water. As observed, bare silica, diol and HILIC-A (Figures 10.3a and c), on the one hand, and HILIC-B, amino and HILIC-N columns (Figures 10.3d and e), on the other, showed similar selectivity. The selectivity of HILIC-A was closer to bare silica (Figure 10.3c) and diol, whereas HILIC-N appreciably differed from these columns (see Figures 10.3a and b), in spite of its supposedly similar nature.

The zwitterionic column showed particular selectivity, since retention with this column showed poor correlation with all other columns (results not shown). The differences in selectivity for HILIC-N and zwitterionic columns (the columns that showed the poorest correlations) could be partially explained by the significantly different amount of adsorbed water on the columns.

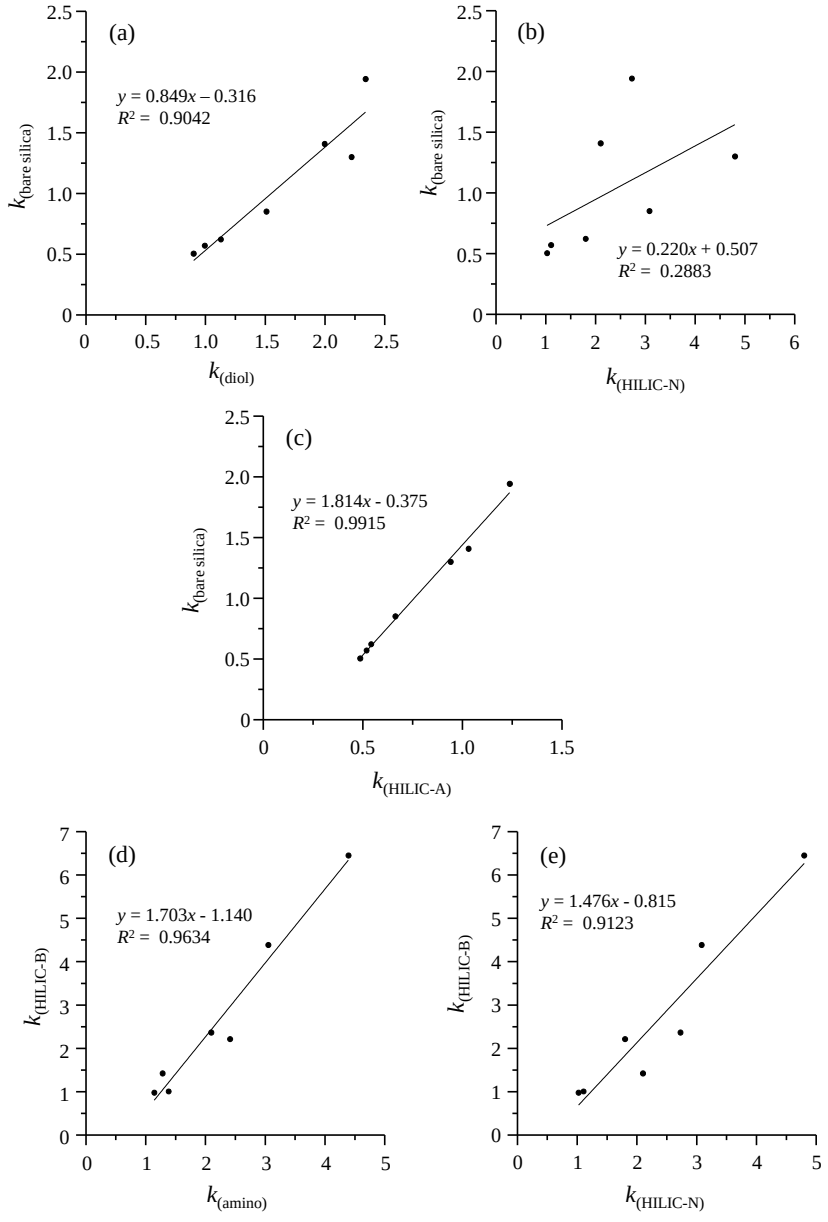


Figure 10.3. Comparison of the selectivity for the studied HILIC columns, using a mobile phase composed of 10% water in acetonitrile (retention factors for the seven probe compounds are plotted).

10.4.3. Column performance based on peak shape

As shown in previous chapters, the plots of the left (*A*) and right (*B*) half-widths, or alternatively the width (*A* + *B*), versus the retention time, give global information in a single plot about the peak shape behaviour of a group of compounds eluted from a given column [56,57]. The plots follow an almost linear trend, which can be represented according to the following equations:

$$A = m_A t_R + A_0 \quad (10.3)$$

$$B = m_B t_R + B_0 \quad (10.4)$$

where m_A and m_B are the slopes of the linear correlations, and the intercepts A_0 and B_0 indicate the extra-column contributions to the peak broadening. Half-widths plots depict directly the raw information obtained in the chromatograms, being illustrative of the broadening rate and asymmetry degree of chromatographic peaks. The sum of slopes ($m_A + m_B$) indicates the peak broadening rate inside the column, whereas their ratio (m_B/m_A) is the asymmetry of peaks eluting at a time where the extra-column contribution is non-significant.

Half-width or width plots can be constructed for several compounds eluted with a given mobile phase composition, or for one compound eluted with mobile phases at several compositions. Also, global plots where all studied compounds and mobile phases are considered (i.e., using all available data), can be built (as made in this work, Figures 10.4 and 10.5). The plots reveal similarities in behaviour among compounds and conditions with regard to the interaction kinetics with the stationary phase.

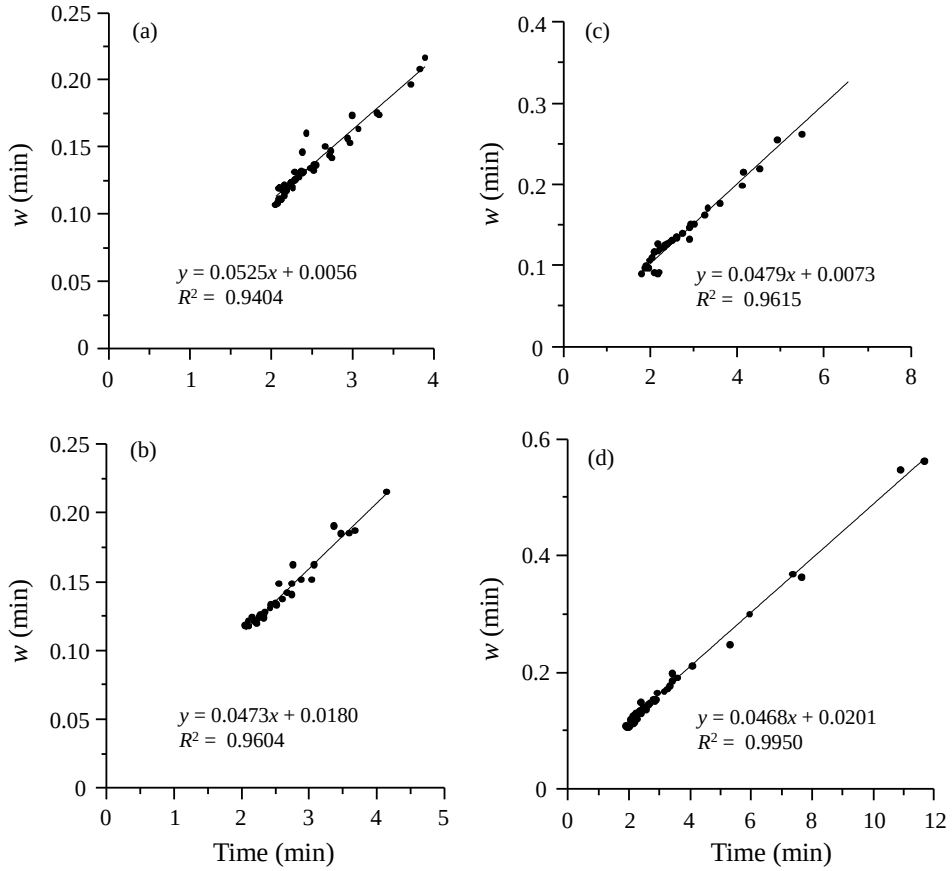


Figure 10.4. Widths (*A* + *B*) of chromatographic peaks for nucleosides and uracil, analysed with different columns at increasing water content, in the assayed experimental range. Columns: (a) bare silica, (b) diol, (c) amino, and (d) zwitterionic. Flow rate: 1 mL/min.

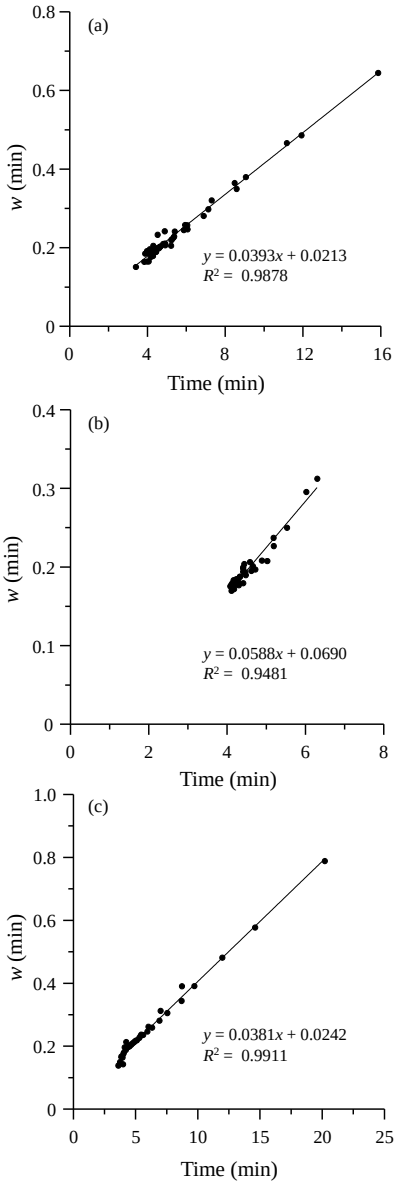


Figure 10.5. Widths (*A* + *B*) of chromatographic peaks for nucleosides and uracil, analysed with different columns at increasing water content, in the assayed experimental range. Columns: (a) HILIC-N, (b) HILIC-A, and (c) HILIC-B. Flow rate: 0.5 mL/min.

The similarities among compounds are not so evident by representing the efficiency (number of theoretical plates) instead (compare with Figures 10.6 and 10.7), due to the contribution of the extra-column tubing to peak broadening, which is more significant for less retained compounds. Therefore, the construction of width plots is a useful tool to compare columns. The plots also facilitate the simulation of realistic chromatograms for diverse purposes, such as the optimisation of separation conditions [58]. To our knowledge, this tool has not been applied before to the comparison of HILIC columns.

As recommended by Dorsey and Foley [59], measurement of half-widths was made at 10% peak height to appraise better the peak asymmetry, avoiding the baseline noise at lower height. However, for symmetrical peaks, the measurements can be made at larger height.

Half-width plots showed highly satisfactory correlations for all columns, with determination coefficients (R^2) usually in the 0.94–0.99 range (see Table 10.3). The lowest R^2 value corresponded to the right half-width plot built for the zwitterionic column. The lack of scattering of the plotted data indicates that, for most columns, the interaction kinetics for the compounds analysed with the studied mobile phases was similar. For the zwitterionic column, the smaller R^2 value is explained by the narrower peaks obtained for uracil and thymidine.

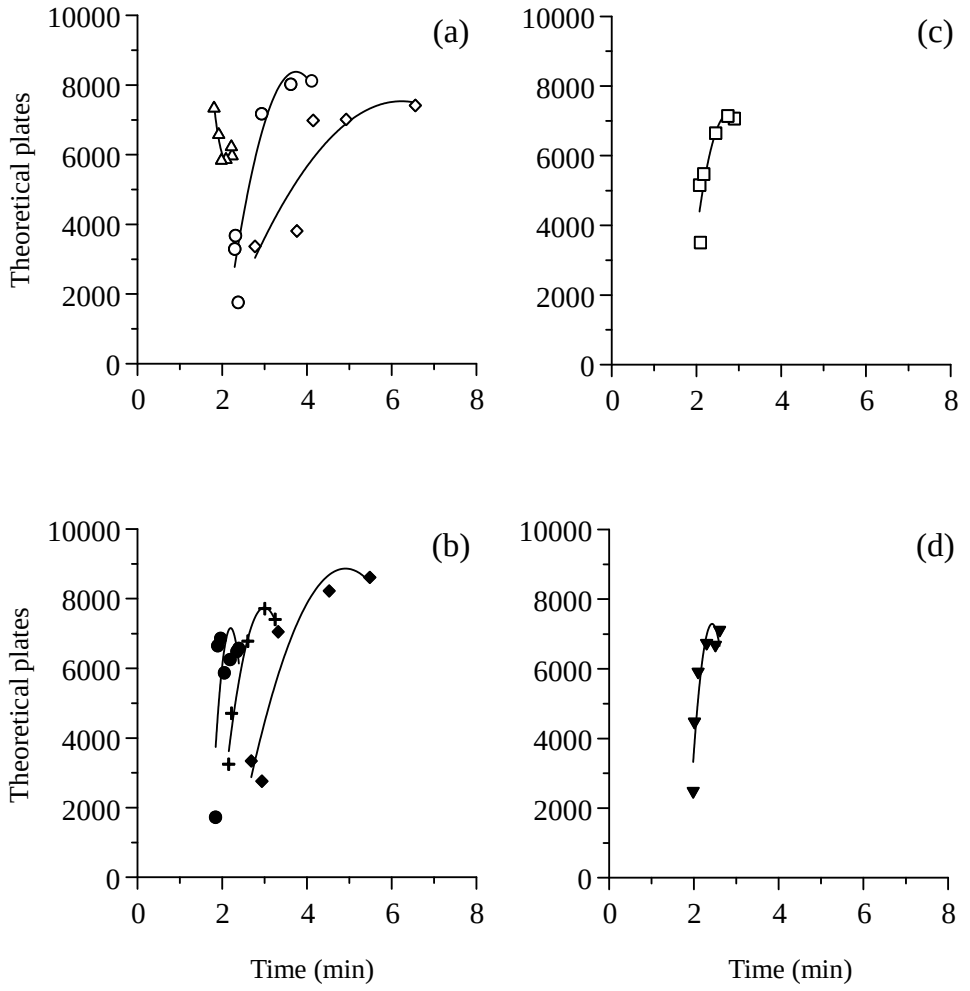


Figure 10.6. Efficiency (number of theoretical plates, *N*) as a function of retention time (t_R) for the amino column. The data correspond to the peaks of the probe compounds eluted with different water contents (%): 10 (◊), 15 (♦), 20 (○), 25 (+), 30 (□), 35 (▼), 40 (●), and 45 (Δ).

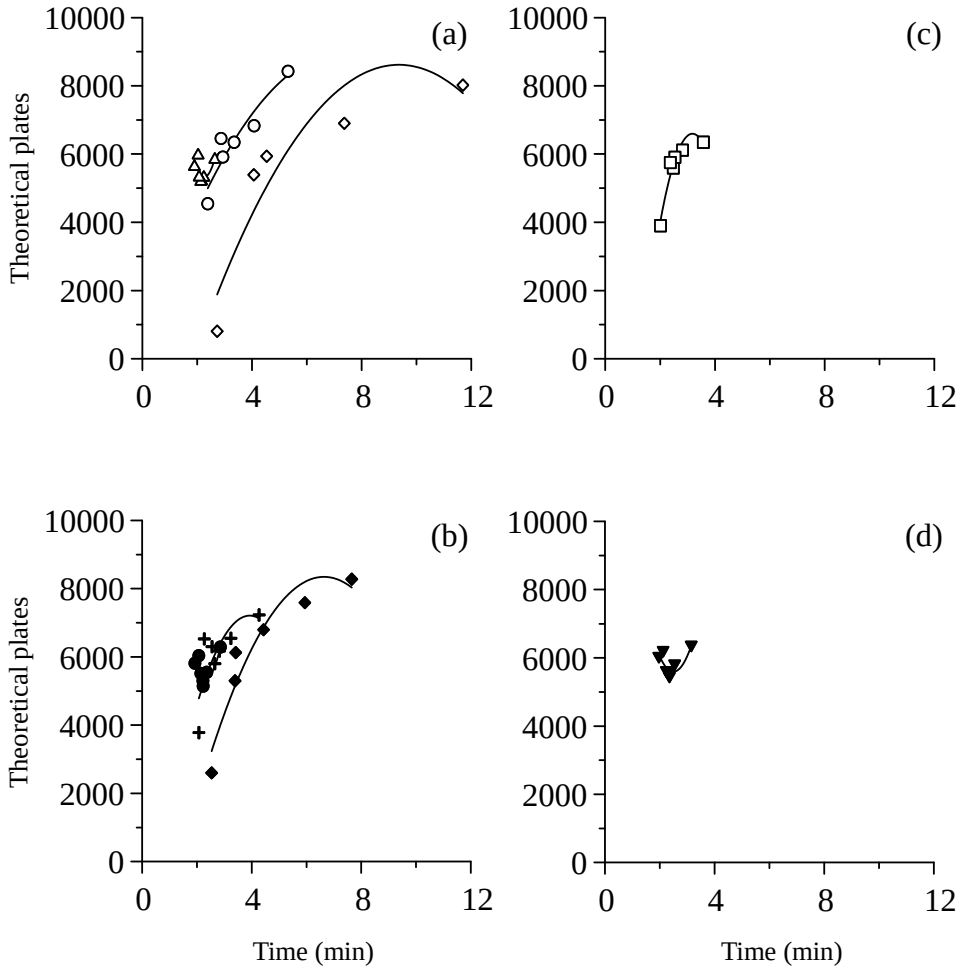


Figure 10.7. Efficiency (number of theoretical plates, *N*) as a function of retention time (t_R) for the zwitterionic column. Other details are given in Fig. 10.6.

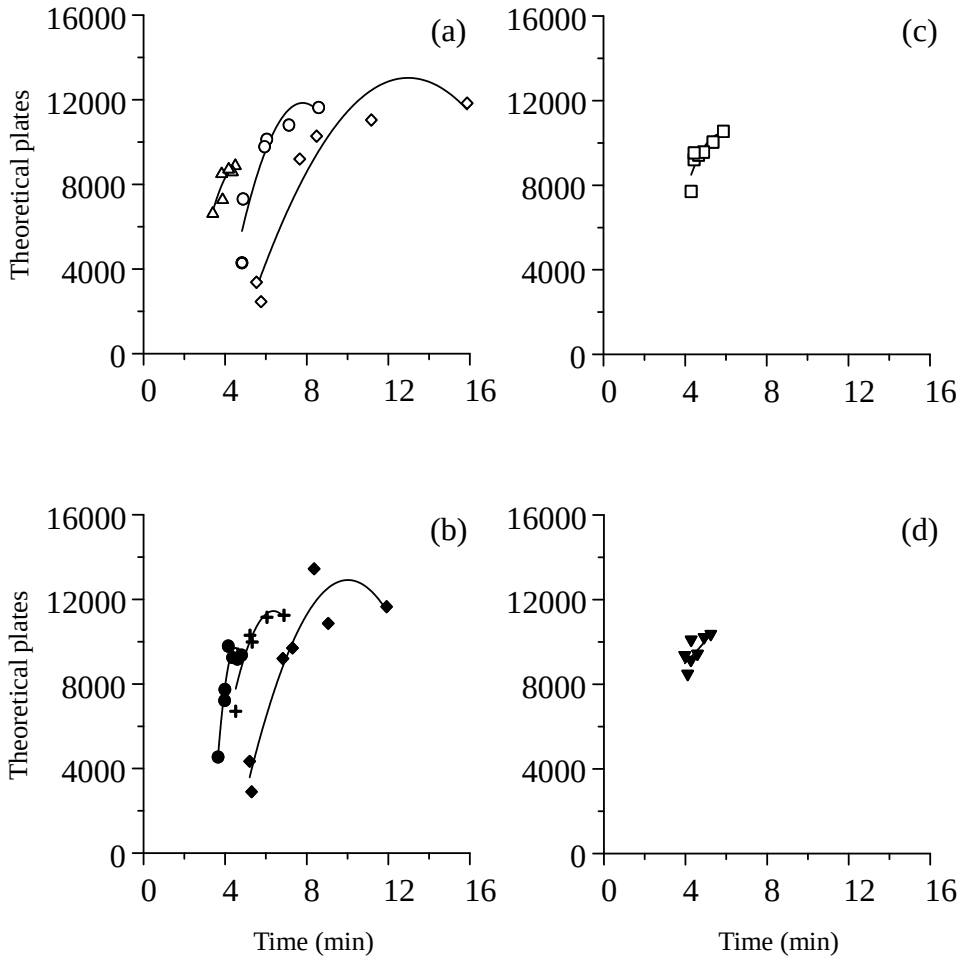


Figure 10.8. Efficiency (number of theoretical plates, *N*) as a function of retention time (t_R) for the HILIC-N column. Other details are given in Fig. 10.6.

Table 10.3. Parameters for the half-width plots (Eqs. (10.3) and (10.4)) for the assayed HILIC columns, considering all compounds and the whole range of water contents.

Column	m_A [a]	R^2 [b]	m_B [a]	R^2 [b]	$m_A + m_B$	m_B / m_A
Bare silica	0.027	0.9116	0.025	0.9337	0.053	0.92
Diol	0.024	0.9622	0.023	0.9153	0.048	0.97
Amino	0.022	0.9534	0.027	0.9584	0.050	1.20
Zwitterionic	0.024	0.9934	0.022	0.8312	0.047	0.93
HILIC-A	0.027	0.9596	0.029	0.9088	0.056	1.07
HILIC-B	0.019	0.9870	0.019	0.9541	0.039	1.00
HILIC-N	0.017	0.9700	0.022	0.9493	0.039	0.80

[a] m_A and m_B are the slopes of the left and right half-width plots.

[b] R^2 is the determination coefficient for the half-width plots.

On the other hand, for all columns, the slope of the straight-line that represented the right half-width (B) was similar to the slope representing the left half-width (A). Therefore, the peaks of the probe compounds were almost symmetrical in the assayed range. The sum of slopes for the left and right half-width plots was larger for bare silica ($m_A + m_B = 0.053$) and HILIC-A (0.056) columns, which indicates that in these two columns the peaks are more significantly wider. For the other columns, $m_A + m_B$ was 0.048 (diol), 0.050 (amino) and 0.047 (zwitterionic), and 0.039 for both HILIC-B and HILIC-N columns. Meanwhile, the ratio of slopes was always close to unity, with the most asymmetrical peaks corresponding to the amino ($m_B/m_A = 1.20$) column,

with tailing peaks, and bare silica (0.92), zwitterionic (0.93), and HILIC-N (0.80) columns, with fronting peaks. For the diol, HILIC-A and HILIC-B columns, the ratio of slopes was 0.97, 1.07, and 1.00, indicating highly symmetrical peaks.

The high symmetry achieved for the peaks for all compounds using all the assayed columns can be explained by the presence of the adsorbed water layer on all stationary phases, which would mask the activity of residual silanols. Note that highly asymmetrical peaks are obtained in RPLC when cationic solutes are chromatographed on silica-based columns. Considering that the probe compounds are positively charged at the mobile phase pH (pH 3), the lack of significant scattering in the width plots suggests the absence of interactions between the compounds and the anionic silanol groups on all stationary phases studied in this work.

10.4.4. Resolution

The chromatograms obtained for mixtures of the selected compounds eluted with a mobile phase containing 10% water, using the seven HILIC columns, are depicted in Figures 10.9 and 10.10. It should be first observed that the elution order of the mixture of compounds changed significantly for some columns, especially for adenosine and cytidine. As commented above, the retention times were larger for the zwitterionic column. The resolution was only rather satisfactory for the zwitterionic column (Figure 10.9d), with baseline resolved peaks except for the partial overlapping between thymidine and uracil.

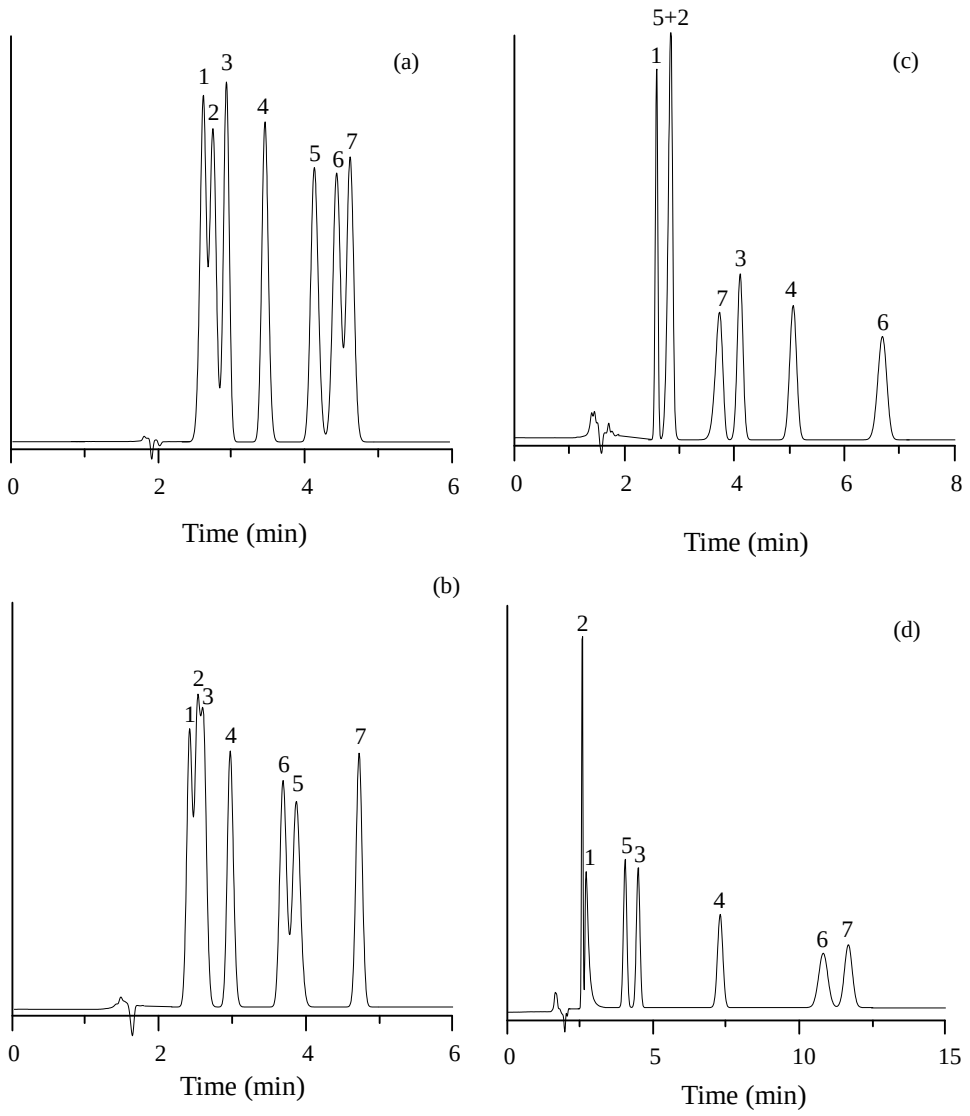


Figure 10.9. Chromatograms obtained for the set of nucleosides and uracil, eluted with a mobile phase containing 10% water from: (a) bare silica, (b) diol, (c) amino, and (d) zwitterionic columns. Compounds: (1) uracil, (2) thymidine, (3) uridine, (4) xantosine, (5) adenosine, (6) guanosine, and (7) cytidine. Flow rate: 1 mL/min.

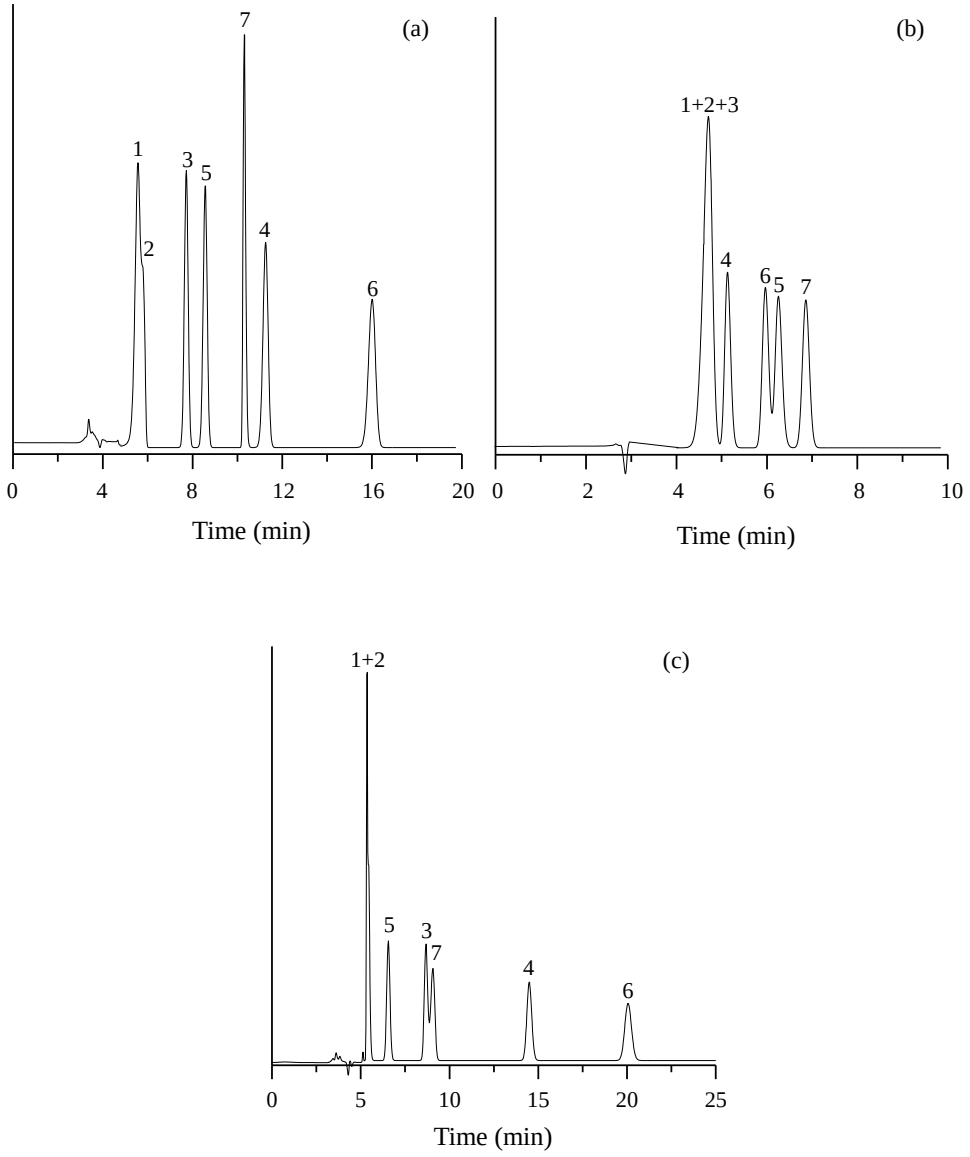


Figure 10.10. Chromatograms obtained for the set of nucleosides and uracil, eluted with a mobile phase containing 10% water from: (a) HILIC-N, (b) HILIC-A, and (c) HILIC-B columns. See Figure 10.9 for compound identity. Flow rate: 0.5 mL/min.

10.5. Conclusions

The seven HILIC stationary phases assayed in this work showed different retention capability for the group of nucleosides. The comparison study gives more details on the behaviour of the recently developed ACE HILIC-N, HILIC-A and HILIC-B columns, for which the manufacturer has provided scarce information. The zwitterionic, amino, HILIC-N and HILIC-B columns gave rise to higher retention times compared to bare silica, diol, and HILIC-A columns. This may be related partially to the thickness of the adsorbed water layer on the stationary phase, which depends on the packing nature.

On the other hand, at pH 3.0, the elution order was nearly identical for bare silica, diol and HILIC-A columns, but differed for the other assayed columns. The reason of this difference in terms of selectivity is the existence of secondary retention mechanisms in the chromatographic process, mainly related to the electrostatic interaction between the negative charged surface present on some columns and the positive charge of nucleosides at the working pH. The separation of the group of nucleosides, with a mobile phase containing 10% water, showed that the less retentive columns (bare silica, diol and HILIC-A) provided poor resolution. The resolution improved appreciably with more retentive columns (zwitterionic, amino and HILIC-N), especially in the case of the zwitterionic column.

Finally, the study showed that the chromatographic peaks were almost symmetrical for all HILIC columns, even when cationic solutes were analysed. This may be explained by the presence of the adsorbed water layer on the stationary phase, which masks the activity of residual silanols, responsible of the broad and asymmetrical peaks in RPLC for this type of compounds.

The implementation of an analytical procedure in HILIC will depend significantly on the selected type of column. This study recommends some

simple tools to help analysts in the selection of the more convenient column for a particular analysis.

10.6. References

[1] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, *J. Chromatogr. A* 499 (1990) 177–196.

[2] J.C. Linden, C.L. Lawhead, Liquid chromatography of saccharides, *J. Chromatogr. A* 105 (1975) 125–133.

[3] J.K. Palmer, A versatile system for sugar analysis via liquid chromatography, *Anal. Lett.* 8 (1975) 215–224.

[4] B. Buszewski, S. Noga, Hydrophilic liquid chromatography (HILIC): A powerful separation technique, *Anal. Bioanal. Chem.* 402 (2012) 231–247.

[5] Y. Guo, S. Gaiki, Retention behavior of small polar compounds on polar stationary phases in hydrophylic interaction chromatography, *J. Chromatogr. A* 1074 (2005) 71–80.

[6] P. Jandera, P. Janás, Recent advances in stationary phases and understanding of retention in hydrophilic interaction chromatography: A review, *J. Chromatogr. A* 967 (2017) 12–32.

[7] A. Cavazzini, M. Catani, A. Felinger, Hydrophilic interaction liquid chromatography, *in Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, M.L. Riekkola), Elsevier, Amsterdam, 2017, pp. 147–169.

[8] D.V. McCalley, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, *J. Chromatogr. A* 1523 (2018) 49–71.

[9] Y. Guo, S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography, *J. Chromatogr. A* 1218 (2011) 5920–5938.

[10] B.A. Olsen, Hydrophilic interaction chromatography using amino and silica columns for the determination of polar pharmaceuticals and impurities, *J. Chromatogr. A* 913 (2001) 113–122.

[11] J. Wu, W. Bicker, W. Lindner, Separation properties of novel and commercial polar stationary phases in hydrophilic interaction and reversed-phase liquid chromatography mode, *J. Sep. Sci.* 31 (2008) 1492–1503.

[12] S. Van Dorpe, V. Vergote, A. Pezeshki, C. Burvenich, K. Peremans, B. de Spiegeleer, Hydrophilic interaction LC of peptides: Column comparison and clustering, *J. Sep. Sci.* 33 (2010) 728–739.

[13] B. Chauve, D. Guillarme, P. Cléon, J.L. Veuthey, Evaluation of various HILIC materials for the fast separation of polar compounds, *J. Sep. Sci.* 33 (2010) 752–764.

[14] L. Nováková., I. Kaufmannová, R. Jánská, Evaluation of hybrid hydrophilic interaction chromatography stationary phases for ultra-HPLC in analysis of polar pteridines, *J. Sep. Sci.* 33 (2010) 765–772.

[15] R.I. Chirita, C. West, A.L. Finaru, C. Elfakir, Approach to hydrophilic interaction chromatography column selection: Application to neurotransmitters analysis, *J. Chromatogr. A* 1217 (2010) 3091–3104.

[16] H. Qiu, L. Loukotková, P. Sun, E. Tesarová, Z. Bosáková, D.W. Armstrong, Cyclofructan 6 based stationary phases for hydrophilic interaction liquid chromatography, *J. Chromatogr. A* 1218 (2011) 270–279.

[17] Z. Aturki, G. D'Orazio, A. Rocco, K. Si-Ahmed, S. Fanali, Investigation of polar stationary phases for the separation of sympathomimetic drugs with nano-liquid chromatography in hydrophilic interaction liquid chromatography mode, *Anal. Chim. Acta* 685 (2011) 103–110.

[18] J. Köster, R. Shi, N. Von Wirén, G. Weber, Evaluation of different column types for the hydrophilic interaction chromatographic separation of iron-citrate and copper-histidine species from plants, *J. Chromatogr. A* 1218 (2011) 4934–4943.

[19] R.I. Chirita, C. West, S. Zubrzycki, A.L. Finaru, C. Elfakir, Investigations on the chromatographic behaviour of zwitterionic stationary phases used in hydrophilic interaction chromatography, *J. Chromatogr. A* 1218 (2011) 5939–5963.

[20] J. Li, Y. Li, T. Chen, L. Xu, X. Liu, X. Zhang, H. Zhang, Preparation, chromatographic evaluation and comparison between linear peptide- and cyclopeptide-bonded stationary phases, *Talanta* 109 (2013) 152–159.

[21] J.C. Heaton, J.J. Russell, T. Underwood, R. Boughtflower, D.V. McCalley, Comparison of peak shape in hydrophilic interaction chromatography using acidic salt buffers and simple acid solutions, *J. Chromatogr. A* 1347 (2014) 39–48.

[22] R. Zhang, D.G. Watson, L. Wang, G.D. Westrop, G.H. Coombs, T. Zhang, Evaluation of mobile phase characteristics on three zwitterionic columns in hydrophilic interaction liquid chromatography mode for liquid chromatography-high resolution mass spectrometry

based untargeted metabolite profiling of Leishmania parasites, *J. Chromatogr. A* 1362 (2014) 168–179.

[23] M.D. Dolzan, D.A. Spudeit, Z.S. Breitbach, W.E. Barber, G.A. Micke, D.W. Armstrong, Comparison of superficially porous and fully porous silica supports used for a cyclofructan 6 hydrophilic interaction liquid chromatographic stationary phase, *J. Chromatogr. A* 1365 (2014) 124–130.

[24] H. Vlčková, K. Ježková, K. Štětková, H. Tomšíková, P. Solich, L. Nováková, Study of the retention behavior of small polar molecules on different types of stationary phases used in hydrophilic interaction liquid chromatography, *J. Sep. Sci.* 37 (2014) 1297–1307.

[25] H. Song, E. Adams, G. Desmet, D. Cabooter, Evaluation and comparison of the kinetic performance of ultra-high performance liquid chromatography and high-performance liquid chromatography columns in hydrophilic interaction and reversed-phase liquid chromatography conditions, *J. Chromatogr. A* 1369 (2014) 83–91.

[26] J.C. Heaton, D.V. McCalley, Comparison of the kinetic performance and retentivity of sub-2 μcore-shell, hybrid and conventional bare silica phases in hydrophilic interaction chromatography, *J. Chromatogr. A* 1371 (2014) 106–116.

[27] E.Y. Santali, D. Edwards, O.B. Sutcliffe, S. Bailes, M.R. Euerby, D.G. Watson, A comparison of silica C and silica gel in HILIC mode: The effect of stationary phase surface area, *Chromatographia* 77 (2014) 873–881.

[28] Q. Wang, J. Li, X. Yang, L. Xu, Z. Shi, L. Xu, Investigation on performance of zirconia and magnesia-zirconia stationary phases in hydrophilic interaction chromatography, *Talanta* 129 (2014) 438–447.

[29] D.V. McCalley, Study of retention and peak shape in hydrophilic interaction chromatography over a wide pH range, *J. Chromatogr. A* 1411 (2015) 41–49.

[30] Q. Wang, Z. Luo, M. Ye, Y. Wang, L. Xu, Z. Shi, L. Xu, Preparation, chromatographic evaluation and application of adenosine 5-monophosphate modified ZrO_2/SiO_2 stationary phase in hydrophilic interaction chromatography, *J. Chromatogr. A* 1383 (2015) 58–69.

[31] M. Skoczylas, S. Bocian, B. Buszewski, Dipeptide-bonded stationary phases for hydrophilic interaction liquid chromatography, *RSC Adv.* 6 (2016) 96389–96397.

[32] A. Sentkowska, M. Biesaga, K. Pyrzynska, Retention study of flavonoids under different chromatographic modes, *J. Chromatogr. Sci.* 54 (2016) 516–522.

[33] Q. Zhang, C. Xiao, W. Wang, Q. Mingrong, J. Xu, H. Yang, Chromatography column comparison and rapid pretreatment for the simultaneous analysis of amantadine, rimantadine, acyclovir, ribavirin, and moroxydine in chicken muscle by ultra-high performance liquid chromatography and tandem mass spectrometry, *J. Sep. Sci.* 39 (2016) 3998–4010.

[34] H. Li, C. Liu, Q. Wang, H. Zhou, Z. Jiang, The effect of charged groups on hydrophilic monolithic stationary phases on their chromatographic properties, *J. Chromatogr. A* 1469 (2016) 77–87.

[35] M. Bagheri, M. Taheri, M. Farhadpour, H. Rezadoost, A. Ghassempour, H. Aboul-Eneim, Evaluation of hydrophilic interaction liquid chromatography stationary phases for analysis of opium alkaloids, *J. Chromatogr. A* 1511 (2017) 77–84.

[36] C. Oellig, W. Schwack, Comparison of HILIC columns for residue analysis of dithiocarbamate fungicides, *J. Liq. Chromatogr. Rel. Technol.* 40 (2017) 415–418.

[37] S. Arase, S. Kimura, T. Ikegami, Method optimization of hydrophilic interaction chromatography separation of nucleotides using design of experiment approaches: I. Comparison of several zwitterionic columns, *J. Pharm. Biomed. Anal.* 158 (2018) 307–316.

[38] L. Rastegar, H. Mighani, A. Ghassempour, A comparison and column selection of hydrophilic interaction liquid chromatography and reversed-phase high-performance liquid chromatography for detection of DNA methylation, *Anal. Biochem.* 557 (2018) 123–130.

[39] B. Buszewski, M. Skoczylas, Multi-parametric characterization of amino acid- and peptide-silica stationary phases, *Chromatographia* 82 (2019) 153–166.

[40] E. Bartó, A. Felinger, P. Jandera, Investigation of the temperature dependence of water adsorption on silica-based stationary phases in hydrophilic interaction liquid chromatography, *J. Chromatogr. A* 1489 (2017) 143–148.

[41] J. Soukup, P. Jandera, Adsorption of water from aqueous acetonitrile on silica-based stationary phases in aqueous normal-phase liquid chromatography, *J. Chromatogr. A* 1374 (2014) 102–111.

[42] S. Dinç, S.S. Olmez, A. Tuncel, Comparison of newly developed hydroxyl-functionalized monodisperse HILIC columns, *J. Liq. Chromatogr. Rel. Technol.* 40 (2017) 649–655.

[43] J. Wang, Z. Guo, A. Shen, L. Yu, Y. Xiao, X. Xue, X. Zhang, X. Liang, Hydrophilic-subtraction model for the characterization and comparison of

hydrophilic interaction liquid chromatography columns, *J. Chromatogr. A* 1398 (2015) 29–46.

[44] J.L. Rideout, D.W. Henry, L.W. Beacham (Eds.), *Nucleosides, Nucleotides and their Biological Applications*, Elsevier, Amsterdam, 1983.

[45] J.S. Choi, A.J. Berdis, Nucleoside transporters: Biological insights and therapeutic applications, *Fut. Med. Chem.* 4 (2012) 1461–1478.

[46] G. Marrubini, B.E. Castillo Mendoza, G. Massolini, Separation of purine and pyrimidine bases and nucleosides by hydrophilic interaction chromatography, *J. Sep. Sci.* 33 (2010) 803–816.

[47] N.L. Padivitage, M.K. Dissanayake, D.W. Armstrong, Separation of nucleotides by hydrophilic interaction chromatography using the FRULIC-N column, *Anal. Bioanal. Chem.* 405 (2013) 8837–8848.

[48] D. García Gómez, E. Rodríguez Gonzalo, R. Carabias Martínez, Stationary phases for separation of nucleosides and nucleotides by hydrophilic interaction liquid chromatography, *Trends Anal. Chem.* 47 (2013) 111–128.

[49] M. Mateos Vivas, E. Rodríguez Gonzalo, J. Domínguez Álvarez, D. García Gómez, R. Carabias Martínez, Determination of nucleosides and nucleotides in baby foods by hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents, *Food Chem.* 211 (2016) 827–835.

[50] J.J. Baeza Baeza, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, Prediction of peak shape in hydro-organic and micellar-organic liquid chromatography as a function of mobile phase composition, *J. Chromatogr. A* 1163 (2007) 119–127.

[51] J.J. Baeza Baeza, S. Pous Torres, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Approaches to characterise chromatographic column performance based on global parameters accounting for peak broadening and skewness, *J. Chromatogr. A* 1217 (2010) 2147–2157.

[52] S. Pous Torres, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Comparison of the performance of Chromolith Performance RP-18e, 1.8-μm Zorbax Eclipse XDB-C18 and XTerra MS C18, based on modelling approaches, *Anal. Bioanal. Chem.* 405 (2013) 2219–2231.

[53] M.J. Ruiz Ángel, S. Pous Torres, S. Carda Broch, M.C. García Álvarez-Coque, Performance of different C18 columns in reversed-phase liquid chromatography with hydro-organic and micellar-organic mobile phases, *J. Chromatogr. A* 1344 (2014) 76–82.

[54] J.R. Torres Lapasió, *MICHROM Software*, Marcel Dekker, New York, 2000.

[55] L. Xing, R.C. Glen, Novel methods for prediction of logP, pKa, and logD, *J. Chem. Inf. Comput. Sci.* 42 (2002) 796–805.

[56] M.J. Ruiz Ángel, S. Carda Broch, M.C. García Álvarez-Coque, Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography, *J. Chromatogr. A* 1217 (2010) 1786–1798.

[57] J.J. Baeza Baeza, M.J. Ruiz Ángel, M.C. García Álvarez-Coque, S. Carda Broch, Half-width plots, a simple tool to predict peak shape, reveal column kinetics and characterise chromatographic columns in liquid chromatography: State of the art and new results, *J. Chromatogr. A* 1314 (2013) 142–153.

[58] J.A. Navarro Huerta, J.R. Torres Lapasió, M.C. García Álvarez-Coque, Estimation of peak capacity based on peak simulation, *J. Chromatogr. A* 1574 (2018) 101–113.

[59] J.P. Foley, J.G. Dorsey, Equations for calculation of chromatographic figures of merit for ideal and skewed peaks, *Anal. Chem.* 55 (1983) 730–737.

CHAPTER 11

RETENTION MODELLING WITH HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COLUMNS

11.1. Abstract

Hydrophilic interaction liquid chromatography (HILIC) provides an approach to separate from polar to highly polar solutes using similar eluents to RPLC, but with a polar stationary phase that allows the adsorption of water onto its surface. Although HILIC has been extensively applied during the last decade, the discussion concerning the retention mechanisms involved inside the column is still open. It seems to be widely accepted that multiple modes of interaction, including partitioning into the adsorbed water-rich layer, and polar and electrostatic (ion-exchange) interactions take place in a HILIC environment. The complexity of the separation process may affect the prediction of retention. In this work, the performance of several retention models proposed for RPLC, NPLC and HILIC is studied for seven HILIC columns (underivatised silica, and silica containing diol, amino and sulfobetaine functional groups, together with three new columns manufactured by ACE), for a group of six polar nucleosides (adenosine, cytidine, guanosine, thymidine, uridine and xanthosine) and uracil. The behaviour was compared with that offered by two reversed-phase liquid chromatography (RPLC) columns, using five diuretics and four sulphonamides. The elution strength capability of the columns is also examined in detail, which highlights the differences in the interaction of the solutes with the assayed stationary phases.

11.2. Introduction

Since the introduction of bonded phases, high performance liquid chromatography is one of the most important analytical techniques due to its reliability, separation capability and versatility. However, the analysis of highly polar compounds in RPLC has been a challenge due to their lack of retention [1,2]. One solution has been the addition to the mobile phase of additives, such as surfactants or ionic liquids, which modify the stationary phase increasing its polarity [3]. This allows both increasing the retention of polar compounds and reducing the retention of those highly apolar. However, this strategy has the disadvantage of reducing the efficiency of chromatographic columns and increasing the equipment pressure. Another way to solve the problem has been replacing the conventional C18 bonded phases with more polar ones, which has led to the birth of hydrophilic interaction liquid chromatography (HILIC) as an alternative to succeed in the separation of polar compounds [4–10].

It is possible to differentiate between reversed phase liquid chromatography (RPLC) and HILIC by the new polar interactions that appear between the solute and the stationary phase, giving rise to more complex separation mechanisms in HILIC compared to RPLC. On the other hand, although non-bonded phases, such as pure silica, which are conventional in normal phase liquid chromatography (NPLC), are also used in HILIC, the use of mobile phases with an aqueous-organic nature (more similar to those in RPLC), clearly differentiates HILIC from NPLC. Additionally, in HILIC, the mobile phase forms a water-rich layer on the surface of the polar stationary phase, which interacts with a mobile phase deficient in water, creating thus a liquid/liquid extraction system. The analyte is distributed between these two layers.

HILIC has many specific advantages over the conventional NPLC and RPLC modes for the analysis of highly polar compounds. Thus, polar samples

always show good solubility in the aqueous-organic mobile phase used in HILIC, which overcomes the disadvantage of the poor solubility often found in NPLC. On the other hand, the expensive ion pair reagents, necessary to separate ionic compounds in RPLC, are not required in HILIC, and HILIC can be conveniently coupled to mass spectrometry (MS), especially in the electrospray ionisation (ESI) mode.

HILIC separations are performed in the isocratic mode with a high percentage of organic solvent, or in the gradient mode starting with a high percentage of organic solvent and ending with a high proportion of aqueous solvent. Ionic additives, such as ammonium acetate and ammonium formate, are commonly used to control the pH of the mobile phase and the ionic strength. These reagents can also contribute to change the polarity of stationary phase and mobile phase, resulting in changes in retention. For ionisable analytes, pH must be adjusted to guarantee the analyte is in a single ionic form.

A number of authors have proposed mathematical models to describe the retention in HILIC on the light of different theoretical basis [11–18]. In this work, the retention properties of seven HILIC columns (underivatised silica, and silica containing diol, amino and sulfobetaine functional groups of different nature, together with three new columns manufactured by ACE) are studied. The ability to fit and predict the retention times is investigated, using six polar nucleosides (adenosine, cytidine, guanosine, thymidine, uridine and xanthosine [19]) and uracil as probe compounds. The accuracy of seven retention models proposed for RPLC, NPLC and HILIC, as well as models that consider mixed retention mechanisms [11], was studied for the HILIC columns. The behaviour was compared with that offered by two reversed-phase liquid chromatography (RPLC) columns, using four polar sulphonamides and five diuretics, with low

retention in RPLC. A detail study on the elution strength capability is also included.

11.3. Theory

Modelling of retention based on the composition of the mobile phase is a common task in the chromatographic practice [11,15,18,20–31]. The accurate description of retention is of great importance in liquid chromatography to find the optimal conditions of separation, and understand the retention mechanisms of solutes. In RPLC, when the retention mechanism is partitioning, the change in retention with the concentration of organic modifier is usually described by the linear solvent strength (LSS) model [30]:

$$k = e^{\ln k_w - S\varphi} \tag{11.1}$$

where φ is the volumetric fraction of organic modifier in the mobile phase, k_w is the extrapolated value of the retention factor at $\varphi = 0$, and S is a constant that describes the elution strength of the modifier, which is particular for each solute. The retention factor is calculated according to:

$$k = \frac{t_R - t_0}{t_0 - t_{ext}} \tag{11.2}$$

t_R, t_0 and t_{ext} being the retention time, dead time, and extra-column time, respectively. Given its small value, t_{ext} is often neglected in the calculation of the retention factor.

The LSS model usually works well for sufficiently small modifier ranges, but for large ranges, more complex models are needed, such as the quadratic logarithmic model [20], which is an extension of the LSS model:

$$k = e^{\ln k_W - a\varphi + b\varphi^2} \tag{11.3}$$

Another model, which accurately describes the retention, considers the polarities of solute, mobile phase and stationary phase [25]. In this case, a logarithmic-hyperbolic equation is obtained:

$$k = e^{\ln k_W - a\frac{\varphi}{1+b\varphi}} \tag{11.4}$$

This model can be simplified by assigning a value of 1.42 to parameter *b*, for mobile phases of acetonitrile-water, and 0.47 for methanol-water mixtures [29].

When the predominant mechanism is adsorption, models similar to those used in NPLC have been proposed [18,23,31]:

$$k = (a\varphi)^{-m} \tag{11.5}$$

$$k = (a + b\varphi)^{-m} \tag{11.6}$$

where *a*, *b* and *m* are model parameters, which should be fitted. Eq. (11.5) is a two-parameter model and Eq. (11.6), a more flexible three-parameter model. The latter equation is also known as Jandera-Churácek or ABM model.

Combined models have been proposed when mixed retention mechanisms are assumed [11,15,28]:

$$k = (\varphi)^{-m} \mathrm{e}^{a+b\varphi} \tag{11.7}$$

$$k = (1 + b\varphi)^{-m} \mathrm{e}^{a+c\varphi} \tag{11.8}$$

The pH is another experimental factor that must be considered when the solute has acid-base properties. In this case, the retention factor can be obtained by applying a weighted sum of the properties of the basic and acidic species [3]:

$$k = \frac{k_A + k_{HA} Kh}{1 + Kh} \tag{11.9}$$

where k_A and k_{HA} are the retention factors of the basic and acidic species, respectively, and K is the protonation constant of the solute. Therefore, the uncontrolled variation of the pH can introduce serious errors in a chromatographic separation. To avoid this, in this work the pH was kept constant at a value of 3.

11.4. Experimental

11.4.1. Reagents

In the HILIC studies, six nucleosides (adenosine, cytidine, guanosine, thymidine, uridine and xantosine), all from Sigma (St. Louis, MO, USA), and uracil (Acros Organics, Geel, Belgium) were used as probe compounds. In the RPLC studies, the probe compounds for the Zorbax column were five diuretics (furosemide, althiazide, and trichloromethiazide from Sigma, ethacrynic acid, kindly donated by Merck, Sharp & Dohme, Madrid, Spain, and xipamide kindly donated by Lacer, Barcelona, Spain). For the Chromolith column four sulphonamides (sulphamerazine, sulphachloropyridazine, sulphisoxazole, and sulphaquinoxaline, all from Sigma) were used. The structures, acidity constants (pK_a) and octanol-water partition coefficients (log $P_{o/w}$) of the probe compounds are shown in Table 11.1. The range in log $P_{o/w}$ is –2.1 to –0.7 for nucleosides, 1.00 to 2.20 for diuretics and 0.11 to 1.45 for sulphonamides.

Table 11.1. Acidity constants (pK_a) and octanol-water partition coefficients (log $P_{o/w}$), for the probe compounds

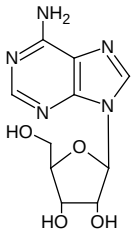
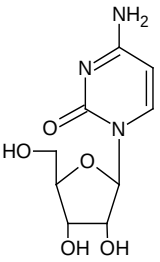
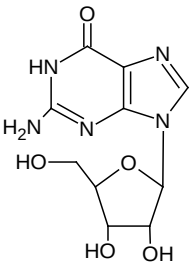
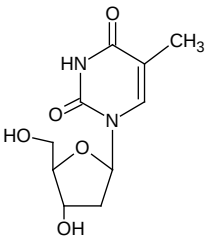
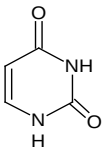
Compound	Structure	pK_a	log $P_{o/w}$
Adenosine		3.7[a]	–1.0 ± 0.5[b]
Cytidine		4.22; 12.5[a]	–1.9 ± 0.4[b]
Guanosine		2.1; 9.2[a]	–1.7 ± 0.6[b]
Thymidine		9.72[a]	–1.1 ± 0.5[b]
Uracil		9.5[a]	–0.7 ± 0.3[b]

Table 11.1 (continued)

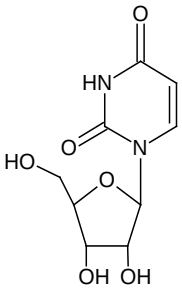
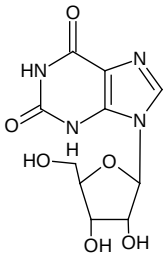
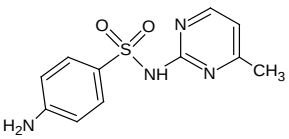
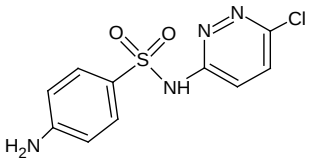
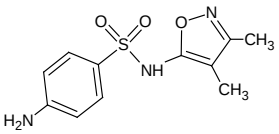
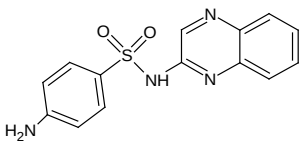
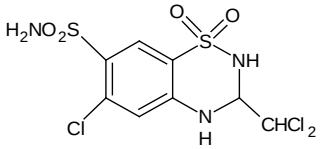
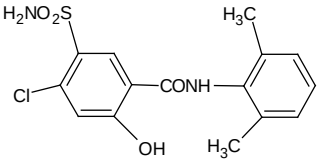
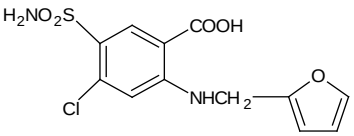
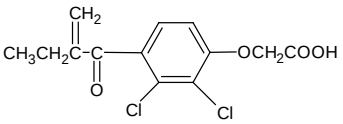
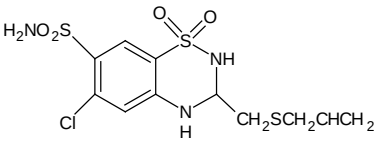
Compound	Structure	pK_a	log $P_{o/w}$
Uridine		8.5[a]	–1.6 ± 0.4[b]
Xantosine		5.5; 12.85[a]	–2.1 ± 0.6[b]
Sulphamerazine		2.2, 7.0[c]	0.11[c]
Sulphachloropyridazine		1.9, 5.1[c]	0.71[c]
Sulphisoxazole		1.8, 5.0[d]	0.81[c]
Sulphaquinoxaline		5.5[c]	1.45[c]

Table 11.1 (continued)

Compound	Structure	pK_a	log $P_{o/w}$
Trichloromethiazide		10.6, 8.6, 7.3[e]	1.00[f]
Xipamide		10.0, 4.8[e]	2.19[f]
Furosemide		7.5, 3.8[e]	1.81[f]
Ethacrynic acid		3.5[e]	2.20[f]
Althiazide		-	1.17[f]

[a] Reference [32]. [b] Calculated with ChemSketch, ACD Labs, 2012 version, [c] From Ref. [33], [d] From Ref. [34], [e] From Ref. [35,36], [f] From Ref. [37].

Stock solutions of approximately 100 μg/mL of the drugs were prepared in a small amount of acetonitrile (VWR Chemicals, Radnor, PA, USA), with the aid of an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany), and diluted in nanopure water (Barnstead, Sybron, Boston, MA, USA). The solutions, kept at 4 ^{o}C, remained stable during at least two months. In HILIC, the injected solutions were diluted with acetonitrile to get 20 μg/mL solutions, whereas in RPLC, the injected solutions were diluted with nanopure water to get 10 μg/mL solutions.

In HILIC, mobile phases were prepared with acetonitrile and increasing concentrations of an aqueous buffered solution (10, 15, 20, 25, 30, 35, 40 and 45% *v*/*v*), prepared with 0.01 M ammonium formate (Sigma). The mobile phases were adjusted at pH 3 with formic acid (Acros Organics), referred to the aqueous-organic mixture, although the standardisation of the pH-meter was always carried out using aqueous buffers. In the RPLC mode, mobile phases contained acetonitrile in the range 10 to 45% *v*/*v* for the Chromolith column, and 28 to 58% *v*/*v* for the Zorbax column, and were buffered with 0.01 M sodium dihydrogen phosphate (Fluka, Buchs, Switzerland), adjusting the pH at 3 with HCl and NaOH (Scharlab), before the addition of the organic solvent.

All drug solutions and mobile phases were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA), and degassed in an ultrasonic bath.

11.4.2. Apparatus and columns

The Agilent chromatographic system (Waldbronn, Germany), used for this study, was equipped with a quaternary pump (Series 1200), an autosampler (Series 1100), a thermostated column compartment (Series 1260) set at 25 ºC, and a UV-visible detector of variable wavelength (Series 1100). Nucleosides were detected at 260 nm, sulphonamides at 254 nm, and diuretics at 274 nm. Triplicate injections of 20 µL were made.

Data acquisition was controlled by an OpenLAB CDS LC ChemStation (Agilent, C.01.07 SR3). The mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA).

For the HILIC studies, seven ACE (Aberdeen, United Kingdom) columns (150 mm × 4.6 mm i.d. and 5 µm particle size) were used: bare silica (ACE Excel 5 SIL), neutral (Betasil Diol-100 and ACE HILIC-N), anionic (ACE Excel 5 NH_2 and ACE HILIC-B), cationic (ACE HILIC-A), and zwitterionic with a sulphobetaine group (Sequant ZIC-HILIC). The zwitterionic column does not have net charge, but the negative charge on the sulphonate group is capable of inducing electrostatic interactions with charged solutes, due to its position at the distal end of the ligand [4]. According to the manufacturer, HILIC-A, HILIC-B and HILIC-N are stationary phases based on ultra-pure silica. HILIC-A has an acidic character with an ionisable negative surface charge depending upon mobile phase pH. At pH 3, the retention of the cationic nucleosides was low, therefore the acidic group should be protonated. HILIC-B is a basic phase with an ionisable positive charge depending also on the mobile phase pH, and HILIC-N is a polyhydroxy phase. The flow rate was 1 mL/min for bare silica, diol, amino and zwitterionic columns, and 0.5 mL/min for the columns identified as HILIC-A, HILIC-B and HILIC-N.

In RPLC, a Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm i.d. and 5 μm particle size) from Agilent (Waldbronn, Germany), and a silica based monolithic column Chromolith Speed ROD C18 (50 mm × 4.6 mm i.d.) from Merck (Darmstadt, Germany) were used. The flow rate was 1 mL/min.

11.4.3. Column conditioning and regeneration

When the work is started with a HILIC column, it must be kept in contact with the mobile phase for a while so that the interparticular spaces and the pores of the stationary phase are filled with liquid and thus stabilised to achieve reproducible retention. The conditioning (equilibration) of the column is usually carried out experimentally by visual inspection of the chromatograms, checking the absence of changes in the baseline, or measuring the retention time or column efficiency from consecutive injections of one or more compounds, up to get reproducible results.

The time necessary to consider that a column is conditioned is usually longer in HILIC than in RPLC, due to the need to maintain stable the water layer adsorbed on the stationary phase surface. This time can be variable depending on the stationary phase nature, column length, and flow rate, as well as the type of organic solvent used in the mobile phase or the buffer system, so that its determination in HILIC acquires relevance in the design of analytical procedures. As a general rule, in RPLC, a column of standard dimensions (150 mm × 4.6 mm) requires at least 10 volumes of mobile phase for conditioning. Thus, working at a flow rate of 1 mL/min, the equilibration time will be ca. 15 min. For HILIC, this amount is increased to between 60 and 80 volumes of mobile phase to achieve complete equilibration [38].

In this work, column conditioning was checked from measurements of the retention time of a probe compound (cytidine). It was observed that previous use of the column influenced the conditioning time. The columns in this work were brand-new, so an initial equilibration time of at least 12 h (with a flow rate of 1 mL/min) was needed for conditioning. However, after the columns had been regenerated after use, it was also decided to perform the conditioning for about a 12 h period. The equilibration of the columns was checked by making cycles of five consecutive injections of cytidine repeated every 60 min, until checking that the retention time reached stable values. In order to check the reproducibility of the behavior, cytidine was also injected at the beginning and end of each working day.

It should be noted that, once a column has been conditioned with a certain mobile phase, it is possible to change directly to another mobile phase with higher water content without the need to regenerate the column. In this case, it was found that the conditioning time was clearly shorter (around 30 min), which indicates that the water layer, once formed, evolves rapidly towards the new working conditions, which may be of interest to face gradient elution.

On the other hand, after use, the HILIC column was first flushed with 70:30 *v*/*v* acetonitrile:water to remove all buffer salts, and afterwards, with 100% isopropanol at low flow rate for storage.

11.5. Results and discussion

11.5.1. Retention behaviour in HILIC and RPLC columns

Representative chromatograms of the probe compounds, with similar elution time ranges, are given in Figures 11.1 to 11.3, which correspond to the seven nucleosides analysed with the HILIC columns, the five diuretics analysed with the C18 Zorbax column, and the four sulphonamides analysed with the C18 Chromolith column. Figure 11.1 corresponds to the diol, silica, amino and zwitterionic columns, and Figure 11.2 to the HILIC-A, HILIC-B and HILIC-N columns. It is observed that the seven nucleosides show, in general, different behaviour in each HILIC column, with changes in the elution order. As already indicated, in HILIC, other interactions, different from those based exclusively on polarity may appear, due to the participation of electrostatic interactions, and interactions between dipoles and hydrogen bonding.

The retention times in HILIC varied considerably between the different columns. The measured dead times were 1.383, 1.613, 1.213, 1.408, 3.102, 2.71, and 2.736 min for the diol, silica, amino, zwitterionic, HILIC-A, HILIC-B, and HILIC-N columns, respectively, whereas the elution ranges for the nucleosides were 1.87–4.62, 1.80–4.75, 1.61–6.77, 1.90–11.70, 3.99–6.97, 3.43–20.21, and 3.40–15.86, respectively. For the RPLC Zorbax and Chromolith columns, the dead times were 1.18 and 0.81 min, respectively, and the elution ranges: 1.55–65.72 and 1.11–29.83 min, respectively.

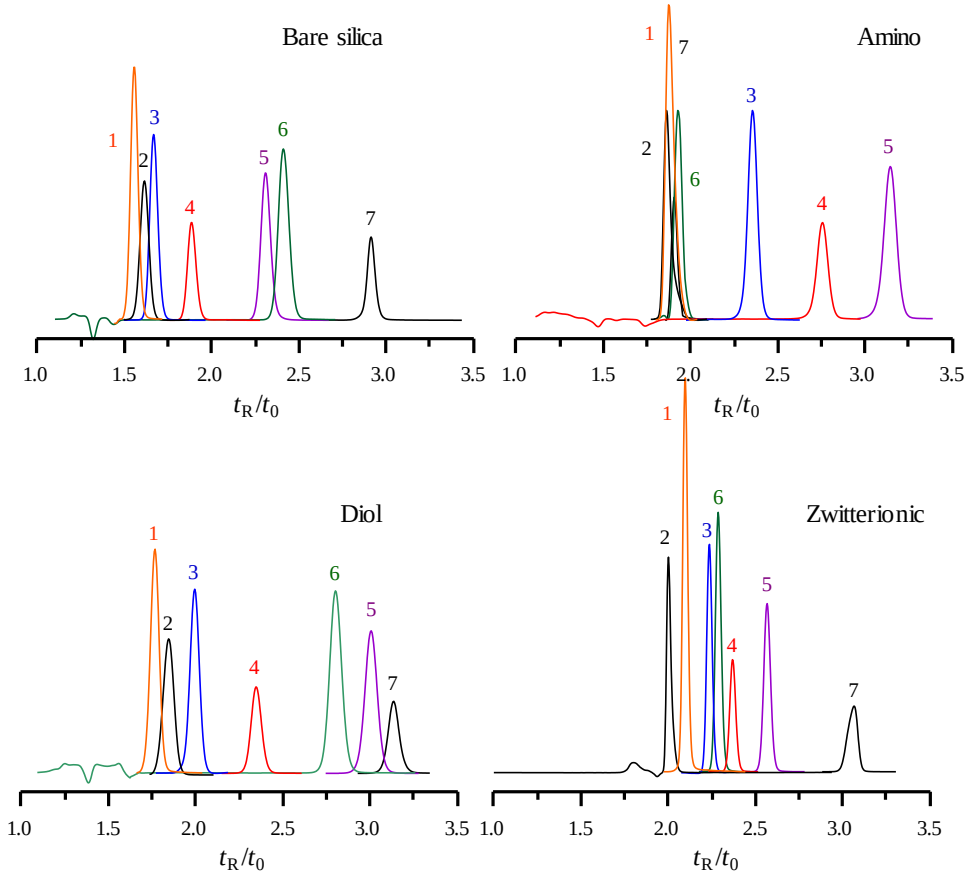


Figure 11.1. Chromatographic peaks obtained for the seven nucleosides eluted from HILIC columns with a mobile phase containing 10% water for bare silica, 11% water for diol, 20% water for amino, and 25% water for the zwitterionic column. The probe compounds (according to their elution order with bare silica) are: (1) Uracil, (2) thymidine, (3) uridine, (4) xanthosine, (5) guanosine, (6) adenosine, and (7) cytidine.

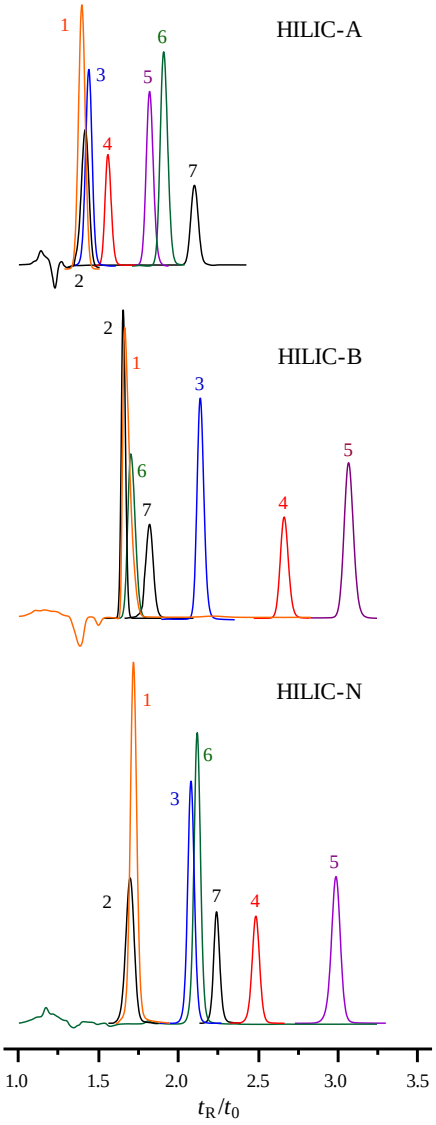


Figure 11.2. Chromatographic peaks obtained for the seven nucleosides eluted from HILIC columns. Chromatographic peaks obtained for the seven nucleosides eluted from HILIC columns with a mobile phase containing 10% water for HILIC A, and 20% water for HILIC B and HILIC N. Other details are given in Fig. 11.1.

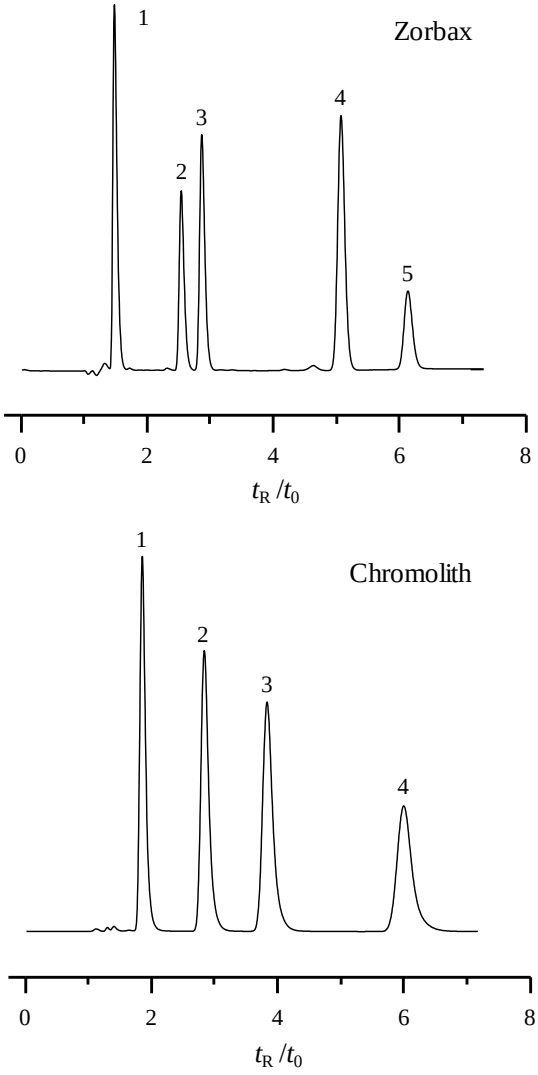


Figure 11.3. Chromatographic peaks obtained for diuretics and sulphonamides, eluted from Zorbax and Chromolith columns with mobile phases containing 46% and 20% acetonitrile, respectively. Compound identities for the Zorbax column: (1) trichloromethiazide, (2) althiazide, (3) furosemide, (4) xipamide, and (5) ethacrynic acid. For the Chromolith column: (1) sulphamerazine, (2) sulphachloropyridazine, (3) sulphisoxazole, and (4) sulphaquinoxaline.

In all assayed HILIC columns, uracil and thymidine were eluted most rapidly. The most retained compound is more variable: guanosine shows a high retention in most assayed columns, and cytidine in most columns except in the amino, HILIC-N and HILIC-A columns. Xanthosine shows an intermediate retention in all columns.

In Figures 11.4 to 11.6, the variation of ln *k* at increasing modifier concentration (water in HILIC and acetonitrile in RPLC) is depicted. In both cases, a non-linear behaviour is observed versus the percentage of modifier in the mobile phase, more pronounced in the C18 columns (i.e., the variation in retention is closer to the linear behaviour for the HILIC columns). For the RPLC columns, the elution order is always the same (the lines do not cross each other). In HILIC, this behaviour is also frequent, but with some exceptions being the most noteworthy behaviour found for cytidine eluted from the amino, HILIC-B and HILIC-N columns, where its retention is strongly reduced by increasing the percentage of water, giving rise to several peak reversals.

11.5.2. Performance of retention models applied to HILIC columns

As indicated, a large number of models have been proposed to predict the retention of compounds eluted in RPLC. The purpose of this work has been checking if these models are able to describe the retention behaviour of nucleosides by HILIC, and compare their performance, in comparison to the same models used for C18 columns in RPLC.

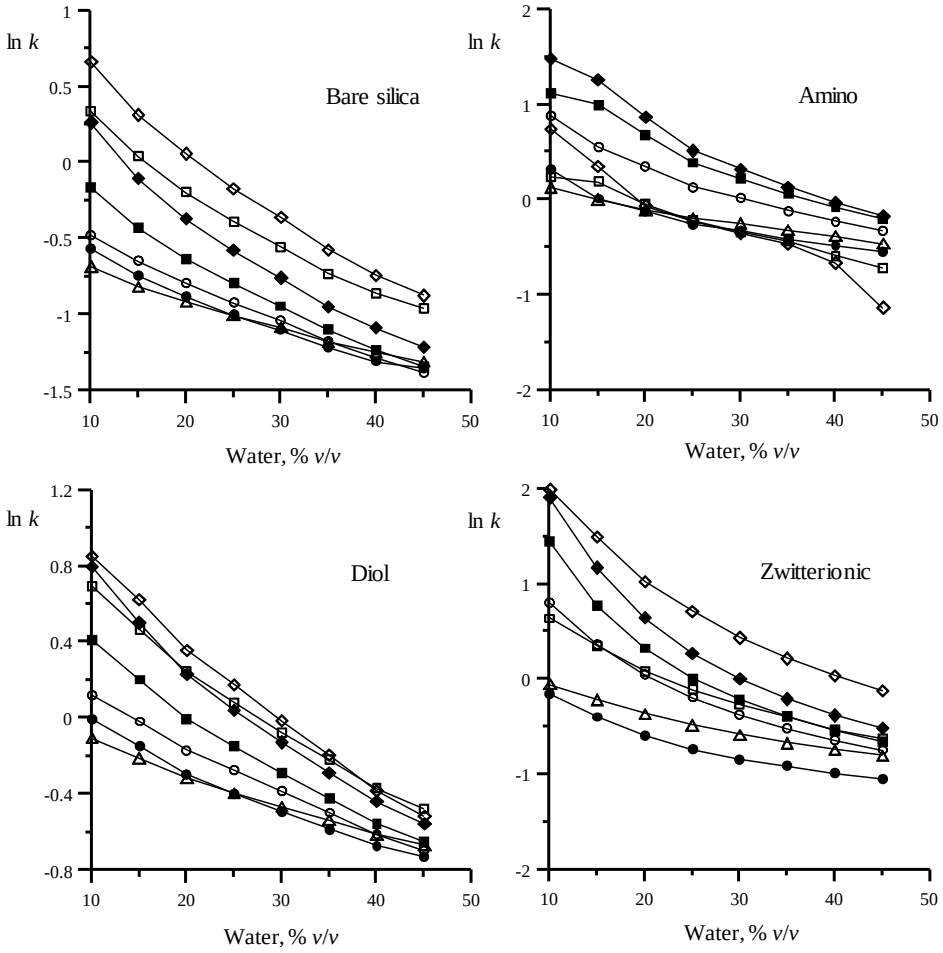


Figure 11.4. Retention behaviour for the nucleosides eluted from HILIC columns at increasing concentration of water. Compound identities (according to their elution order): Uracil (Δ), thymidine (●), uridine (○), xantosine (■), guanosine (♦), adenosine (□), and cytidine (◊).

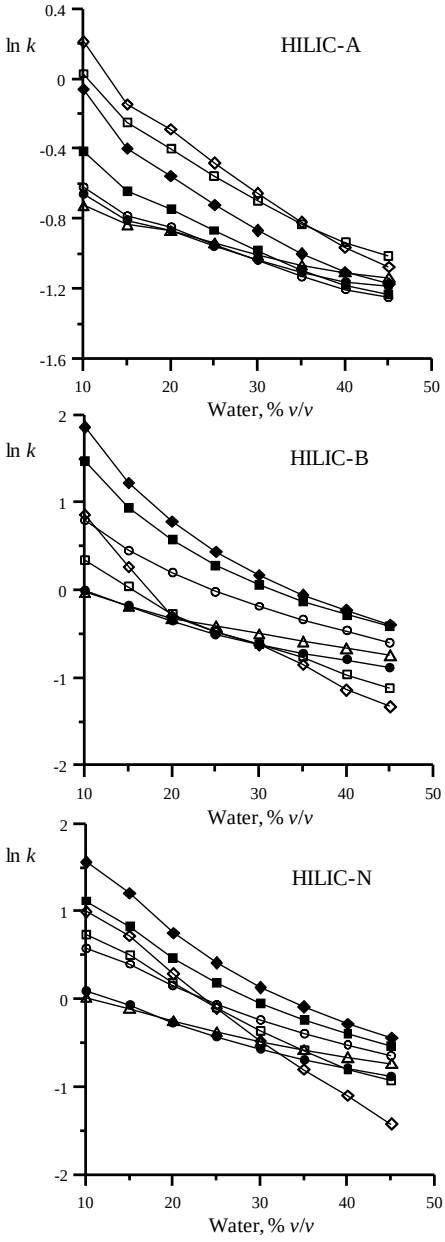


Figure 11.5. Retention behaviour for the nucleosides eluted from HILIC columns at increasing concentration of water. Compound identities are given in Figure 11.4.

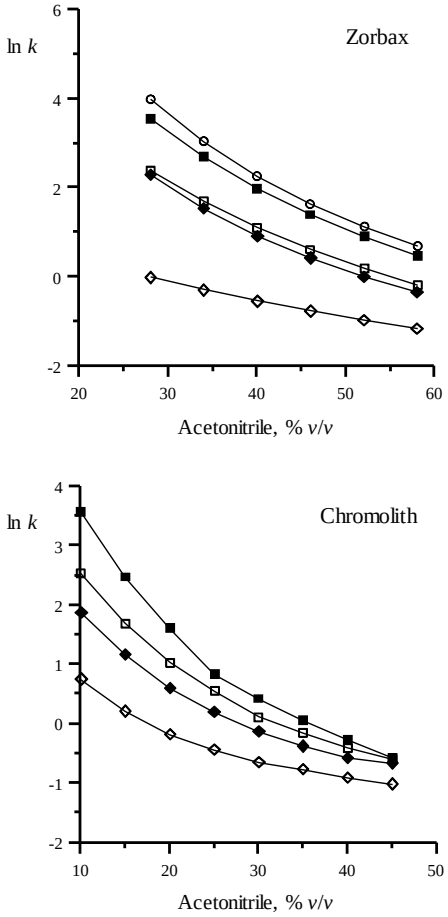


Figure 11.6. Retention behaviour for the diuretics and sulphonamides, eluted from the RPLC columns at increasing concentration of acetonitrile. Compound identities for the Zorbax column: trichloromethiazide (◊), althiazide (♦), furosemide (□), xipamide (■), and ethacrynic acid (○). For the Chromolith column: sulphamerazine (◊), sulphachloropyridazine (♦), sulphisoxazole (□), and sulphaquinoxaline (■).

It should be noted that the use of the Solver tool of Excel and the original models (Eqs. (11.1) and (11.3) to (11.8)) made convergence of the models rather difficult, requiring initial values very close to the optimum. Therefore, it was thought that the practical use of the studied models could be highly improved by transforming the equations, so that the coordinates for the maximal retention in the experimental design is taken as the starting point, instead of the data for $\varphi = 0$ (absence of modifier). In this way, the non-linear fitting of the model parameters to the experimental data was much easier, since convergence was facilitated. The reason is that the initial value of ln k (ln k_o) is reliably known, as it agrees with the value of the retention factor for the mobile phase of lowest elution strength, φ_o, in the experimental design.

In Table 11.2, the modified models are given. The transformation of the equations is next explained. Thus, for Eq. (11.1):

$$\begin{aligned} \ln k &= \ln k_w - S\varphi = \ln k_w - S(\varphi_o + \Delta\varphi) = \ln k_w - S\varphi_o - S\Delta\varphi \\ &= \ln k_o - S\Delta\varphi \end{aligned} \tag{11.10}$$

For Eq. (11.3):

$$\ln k = \ln k_w - a\varphi + b\varphi^2 = \ln k_w - a(\varphi_o + \Delta\varphi) + b(\varphi_o + \Delta\varphi)^2 \tag{11.11}$$

$$\ln k = \ln k_w - a(\varphi_o + \Delta\varphi) + b(\varphi_o^2 + \Delta\varphi^2 + 2\varphi_o\Delta\varphi)^2 \tag{11.12}$$

$$\ln k = \ln k_w - a\varphi_o + b\varphi_o^2 - a\Delta\varphi + 2b\varphi_o\Delta\varphi + b\Delta\varphi^2 \tag{11.13}$$

$$\ln k = \ln k_o - (a - 2b\varphi_o)\Delta\varphi + b\Delta\varphi^2 \tag{11.14}$$

For Eq. (11.4):

$$\ln k = \ln k_w - a\frac{\varphi_o + \Delta\varphi}{1 + b(\varphi_o + \Delta\varphi)} \tag{11.15}$$

$$\ln k = \ln k_{\mathrm{w}} - a\frac{\varphi_{\mathrm{o}}}{1+b\varphi_{\mathrm{o}}} + a\frac{\varphi_{\mathrm{o}}}{1+b\varphi_{\mathrm{o}}} - a\frac{\varphi_{\mathrm{o}}+\Delta\varphi}{1+b\varphi_{\mathrm{o}}+b\Delta\varphi} \tag{11.16}$$

$$\ln k = \ln k_{\mathrm{o}} + a\frac{\varphi_{\mathrm{o}}+b\varphi_{\mathrm{o}}^2+b\varphi_{\mathrm{o}}\Delta\varphi-\varphi_{\mathrm{o}}-\Delta\varphi-b\varphi_{\mathrm{o}}^2-b\varphi_{\mathrm{o}}\Delta\varphi}{(1+b\varphi_{\mathrm{o}})(1+b\varphi_{\mathrm{o}}+b\Delta\varphi)} \tag{11.17}$$

$$\ln k = \ln k_{\mathrm{o}} - a\frac{\Delta\varphi}{(1+b\varphi_{\mathrm{o}})(1+b\varphi_{\mathrm{o}}+b\Delta\varphi)} \tag{11.18}$$

$$\ln k = \ln k_{\mathrm{o}} - \frac{a}{1+b\varphi_{\mathrm{o}}} \times \frac{\Delta\varphi}{1+b\varphi_{\mathrm{o}}+b\Delta\varphi} \tag{11.19}$$

$$\ln k = \ln k_{\mathrm{o}} - \frac{\dfrac{a}{(1+b\varphi_{\mathrm{o}})^2}\Delta\varphi}{1+\dfrac{b}{1+b\varphi_{\mathrm{o}}}\Delta\varphi} \tag{11.20}$$

For Eq. (11.5):

$$k = \frac{(a\varphi_{\mathrm{o}})^{-m}}{(a\varphi_{\mathrm{o}})^{-m}}(a\varphi)^{-m} = e^{\ln k_{\mathrm{o}}}\left(\frac{a\varphi}{a\varphi_{\mathrm{o}}}\right)^{-m} = e^{\ln k_{\mathrm{o}}}\left(\frac{\varphi}{\varphi_{\mathrm{o}}}\right)^{-m} \tag{11.21}$$

where the following was considered:

$$k_{\mathrm{o}} = e^{\ln k_{\mathrm{o}}} = (a\varphi_{\mathrm{o}})^{-a} \tag{11.22}$$

For Eq. (11.6):

$$k = \left(a + b\left(\varphi_{\mathrm{o}} + \Delta\varphi\right)\right)^{-m} = (a + b\varphi_{\mathrm{o}} + b\Delta\varphi)^{-m} = (a_{\mathrm{o}} + b\Delta\varphi)^{-m} \tag{11.23}$$

For Eq. (11.7):

$$k = \left(\frac{\varphi}{\varphi_{\mathrm{o}}}\right)^{-m} \varphi_{\mathrm{o}}^{-m}\mathrm{e}^{a+b\left(\varphi_{\mathrm{o}}+\Delta\varphi\right)} = \left(\frac{\varphi}{\varphi_{\mathrm{o}}}\right)^{-m} \mathrm{e}^{\ln k_{\mathrm{o}} + b\Delta\varphi} \tag{11.24}$$

For Eq. (11.8):

$$k = \left(1 + b(\varphi_o + \Delta\varphi)\right)^{-m} e^{a + c(\varphi_o + \Delta\varphi)} = (1 + b\varphi_o + b\Delta\varphi)^{-m} e^{a + c(\varphi_o + \Delta\varphi)} = \\ = \left(1 + \frac{b}{1 + b\varphi_o}\Delta\varphi\right)^{-m} (1 + b\varphi_o)^{-m} e^{a + c(\varphi_o + \Delta\varphi)} \quad (11.25)$$

Since:

$$k_o = (1 + b\varphi_o)^{-m} e^{a + c\varphi_o} \quad (11.26)$$

the following is obtained:

$$k = (1 + d\,\Delta\varphi)^{-m} e^{\ln k_o + c\,\Delta\varphi}$$

(11.27)

The quality of the models to describe the retention was assessed, in each chromatographic mode, by performing the non-linear least-squares fitting of the experimental retention times and the retention times predicted by each model as follows:

$$\chi = \sum_{i=1}^{N} (t_{Ri,exp} - t_{Ri,pred})^2 \quad (11.28)$$

where N is the number of experimental points, $t_{Ri,exp}$ is the experimental retention time, and $t_{Ri,pred}$ the predicted retention time, obtained from Eq. (11.29):

$$t_{Ri,pred} = t_0\,(1 + k_{i,pred}) \quad (11.29)$$

t_0 being the column dead time and $k_{i,pred}$ the retention factor predicted by the particular model (see Table 11.2).

Table 11.2. Modified retention models and fitted model parameters.

Equations	Models	Parameters
	Two-parameter models	
Eq. (11.1) / Eq. (11.10)	$k = e^{\ln k_o - S\Delta\varphi}$	ln k_o, S
Eq. (11.5) / Eq. (11.21)	$k = e^{\ln k_o}\left(\frac{\varphi}{\varphi_0}\right)^{-m}$	ln k_o, m
	Three-parameter models	
Eq. (11.3) / Eq. (11.14)	$k = e^{\ln k_o - c\Delta\varphi + b\Delta\varphi^2}$	ln k_o, c, b
Eq. (11.4) / Eq. (11.20)	$k = e^{\ln k_o - c\frac{\Delta\varphi}{1 + d\,\Delta\varphi}}$	ln k_o, c, d
Eq. (11.6) / Eq. (11.23)	$k = (a_o + b\Delta\varphi)^{-m}$	a_o, b, m
Eq. (11.7) / Eq. (11.24)	$k = \left(\frac{\varphi}{\varphi_o}\right)^{-m} e^{\ln k_o + b\Delta\varphi}$	ln k_o, a, m
	Four-parameter model	
Eq. (11.8) / Eq. (11.27)	$k = [1 + d\Delta\varphi]^{-m} e^{\ln k_o + c\Delta\varphi}$	ln k_o, m, c, d

The fitting performance was measured by the determination coefficient R^2 and the mean fitting relative error:

$$\varepsilon_r(\%) = \frac{\sum_{i=1}^{N} \left| t_{Ri,\mathrm{exp}} - t_{Ri,\mathrm{pred}} \right|}{\sum_{i=1}^{N} t_{Ri,\mathrm{exp}}} \times 100 \qquad (11.30)$$

In Tables 11.3 and 11.4, the mean relative errors obtained in the fitting of the retention of the probe compounds (the seven nucleosides, five diuretics and four sulphonamides), for each model and column are gathered for the HILIC and RPLC columns. In general, it can be seen how the RPLC columns gave rise to larger errors with the LSS model, which is at least partially due to the larger range of variation of the retention (Figures 11.4–11.6). Thus, for example, for the Chromolith column, the range of k values for sulphisoxazole is between 12.8 and 0.69 min for acetonitrile percentages of 10 and 40%, respectively, while the retention times of cytidine, analysed with the zwitterionic column (which showed the weakest elution strength), varied between 7.3 and 1.03 min, for 10 and 40% water, respectively. The low errors obtained with the ACE HILIC-A column, which shows the weakest retention (for cytidine the retention factor varied between 1.2 and 0.38 between 10 and 40% of water) confirmed this trend.

The models represented by Eqs. (11.14) and (11.20) show a slightly more reliable behaviour, with average errors of 1.0% and 0.7%, respectively. It is observed that the fitting performance is enhanced by increasing the number of model parameters, with a global mean error for the HILIC columns of 2.3% for the models with two parameters, 0.8% for those with three parameters, and 0.6% for those with four parameters. For the RPLC columns, the global mean error was 3.6, 0.7 and 0.3%, for models with two, three and four parameters, respectively.

Finally, it is important to note that the HILIC columns show slightly better fitting behaviour than the RPLC columns, with average errors of 1.2% and 1.5%, respectively. This can be attributed to the greater linearity of the ln k versus modifier plots.

Table 11.3. Mean relative errors (%) obtained in the fitting of the retention of the probe compounds, for each assayed HILIC column.

	Silica	Diol	Amino	Zwitterionic	HILIC-A	HILIC-B	HILIC-N	Mean
Two-parameter models								
Eq. (11.10)	1.68	1.54	3.23	5.97	1.39	4.90	2.21	3.0±1.8
Eq. (11.21)	0.96	1.62	2.65	1.32	0.65	0.91	3.56	1.7±1.1
Three-parameter models								
Eq. (11.14)	0.44	0.38	1.93	1.22	0.60	1.35	1.07	1.0±0.6
Eq. (11.20)	0.28	0.30	1.18	0.34	0.54	0.59	1.22	0.7±0.5
Eq. (11.23)	0.23	0.29	1.17	0.63	0.48	0.46	1.21	0.6±0.4
Eq. (11.24)	0.19	0.33	1.79	0.52	0.47	0.56	1.49	0.8±0.6
Four-parameter model								
Eq. (11.27)	0.16	0.29	1.54	0.28	0.30	0.49	1.09	0.6±0.5

Table 11.4. Mean relative errors (%) obtained in the fitting of the retention of the probe compounds, for each assayed RPLC column.

	Zorbax	Chromolith	Mean
Two-parameter models			
Eq. (11.10)	4.62	8.26	6.4±2.6
Eq. (11.21)	0.83	1.35	1.1±0.4
Three-parameter models			
Eq. (11.14)	0.47	1.45	1.0±0.7
Eq. (11.20)	0.10	0.70	0.4±0.4
Eq. (11.23)	0.22	1.11	0.7±0.6
Eq. (11.24)	0.24	1.08	0.7±0.6
Four-parameter model			
Eq. (11.27)	0.20	0.37	0.3±0.1

11.5.3. Correlation between the parameters of the LSS model

Figures 11.4 to 11.6, where ln *k* vs modifier percentage data are represented, show a positive curvature, which indicates that the elution strength decreases with increasing modifier concentration. In this section, the elution strength of each probe compound is evaluated according to the LSS model. Due to its simplicity, this model has served as the basis for the study of RPLC retention. In Figures. 11.7 to 11.9, the values of elution strength (*S*) versus ln k_o (i.e. retention factor for the mobile phase of weakest elution strength) are depicted, as measured by the LSS model for each probe compound and column. A certain positive correlation can be observed for the assayed columns: compounds with smaller retention (smaller ln k_o value) show a smaller elution strength, that is, they are less affected by the increase in the concentration of modifier in the mobile phase.

The *S* values obtained in the fitting of the LSS model can be considered as an average measurement of the elution strength for each compound in the studied elution range. Each point in Figures 11.7 to 11.9 corresponds to the behaviour of a given compound. For this reason, the observed trend can be considered as a description of the general column behaviour. This behaviour is summarised in Table 11.5, where the parameters for the fitted straight-lines ($S = n + m \ln k_o$) are given. It is interesting to remark that the intercept of the line (*n*) is the elution strength predicted for a compound with $k_o = 1$ (the retention factor for the weakest mobile phase). Meanwhile, the slope describes the reduction in elution strength at decreasing ln k_o. The smaller the *m* value the smaller the effect of the modifier on the elution strength. If the slope would be $m = 0$, all compounds would exhibit the same elution strength, at all modifier concentrations.

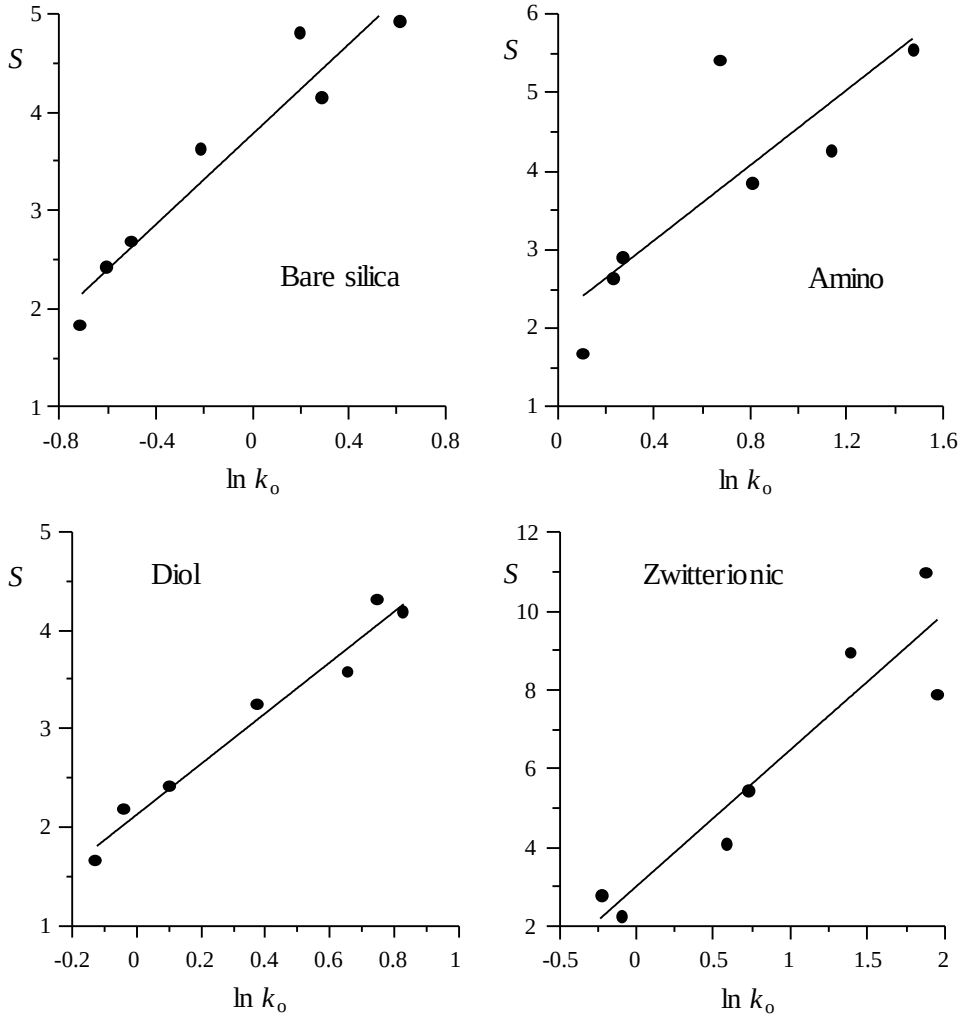


Figure 11.7. Linear fitting of the LSS elution strength parameter, *S*, versus ln k_o (predicted retention factor for the mobile phase of weakest elution strength), for bare silica, diol, amino and zwitterionic columns.

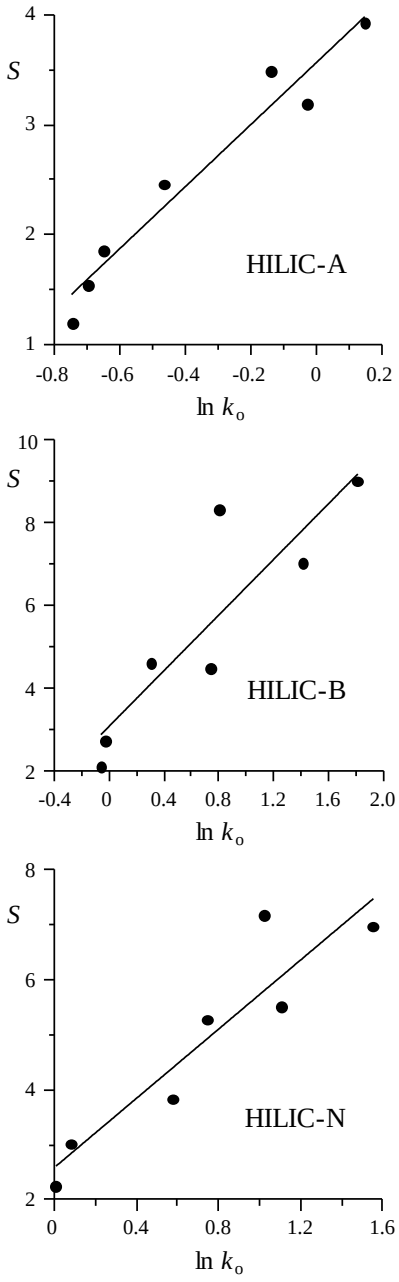


Figure 11.8. Linear fitting of the LSS elution strength parameter, *S*, versus ln k_o for HILIC-A, HILIC-B and HILIC-C columns.

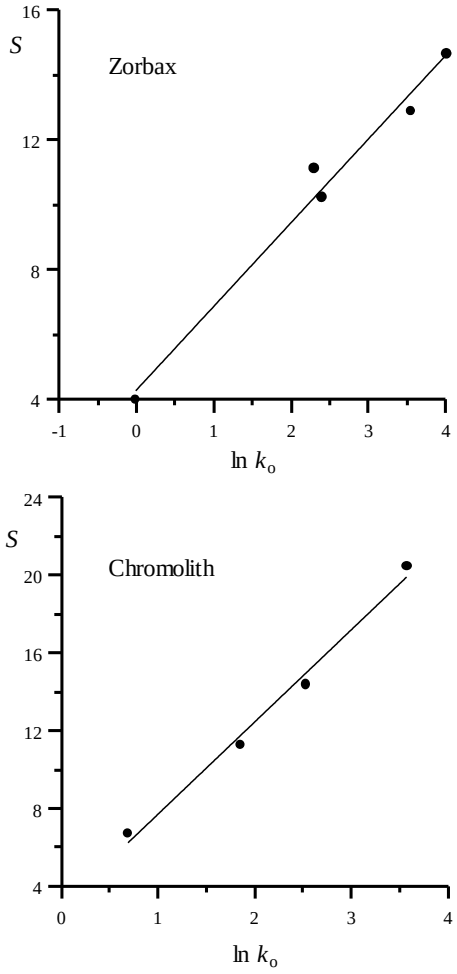


Figure 11.9. Linear fitting of the LSS elution strength parameter, *S*, versus ln k_o for the RPLC Zorbax and Chromolith columns.

Tabla 11.5. Results of the linear fitting of the parameters of the LSS model ($S = n + m \ln k_o$) (see Figures 11.7 to 11.9).

Column	*m*	*n*	R^2
Silica	2.289	3.777	0.923
Diol	2.570	2.135	0.969
Amino	2.378	2.168	0.706
Zwitterionic	3.494	2.981	0.883
HILIC-A	2.836	3.571	0.952
HILIC-B	3.387	3.058	0.787
HILIC-N	3.150	2.592	0.860
Zorbax	2.578	4.339	0.981
Chromolith	4.724	3.098	0.989

It can be observed that the best correlations between S and $\ln k_o$ were found for silica, diol and HILIC-A columns in the HILIC mode, and Zorbax and Chromolith in RPLC, indicating a smaller dispersion in the behaviour of the set of compounds. The greater dispersion corresponded to the amino, zwitterionic, HILIC-B and HILIC-N columns. The similarity of behaviour between all assayed columns is, however, surprising: the slopes of the adjusted straight-lines are in the interval between 2.29 and 3.39 for the HILIC and Zorbax columns, being somewhat larger for the zwitterionic (3.49) and Chromolith

(4.72) columns, which indicates a larger decrease in the elution strength with the reduction in ln k_o.

The LSS model is, certainly too simple, and therefore, it was found convenient to further study how the elution strength varies for each compound with increasing modifier concentration.

11.5.4. Variation of the elution strength with the modifier concentration

It is interesting to quantify the sensitivity of solute retention to changes in the modifier concentration. As commented, traditionally, the elution strength has been calculated, in RPLC, as the slope of the classical linear retention model that relates ln k with the concentration of the modifier (i.e., the LSS model). However, it is not easy to make use of a simple general retention model to describe the retention, due to the complexity of the involved interactions in the chromatographic process. For this reason, the variation of the elution strength was obtained numerically using directly the experimental data, calculated according to Eq. (11.31):

$$S(\varphi) = -\frac{d \ln k}{d\varphi} \cong -\frac{\ln k_u - \ln k_l}{\varphi_u - \varphi_l} \tag{11.31}$$

where the subscripts u and l indicate the upper and lower values of the experimental range. The modifier units are expressed as volumetric fraction to get more convenient values.

Eq. (11.31) gives a numerical estimate of the elution strength, and therefore, should be used with caution because, for the same initial value of retention factor (k_l), the elution capability depends on the modifier range ($\varphi_u - \varphi_l$) taken for the calculation. Therefore, in order to compare columns, the same modifier range should be used. Obviously, the elution strength also depends on the

compound. Tables 11.6 and 11.7 shows the calculated values of elution strength for the seven HILIC columns and two RPLC columns, respectively. The ranges of modifier concentration are indicated for each type of column. As observed, parameter $S(\varphi)$ was always larger for cytidine and guanosine, which were usually the most retained compounds. Also, the largest and smallest values were obtained for the zwitterionic and HILIC-A columns, respectively.

Figures 11.10 to 11.12 depict the elution strength calculated according to Eq. (11.31) versus the logarithm of the initial k value (k_l) for several ranges of organic modifier. Each compound is depicted with a particular symbol to appraise the differences in their behaviour. It should be again noted that the observed correlation is clearly better for the RPLC columns, which should be explained because the interaction with the stationary phase is mainly hydrophobic. Meanwhile, the HILIC columns show a range of behaviours. The maximal dispersion in HILIC was observed for the amino and HILIC-N columns, and the smallest dispersion for bare silica. The trend observed for each compound in Figures 11.10 to 11.12 is somewhat similar to the trend among compounds in Figures 11.7 to 11.9. Table 11.8 shows the parameters for the fitted line ($S(\varphi)$) versus ln k_{init}) in Figures 11.10 to 11.12.

The RPLC columns show a very homogeneous behavior, which gives rise to similar trends for all compounds (Figure 11.12). Meanwhile, the amino and HILIC-N columns show a particularly heterogeneous behavior. The global behavior is shown in Table 11.8 for the HILIC and RPLC columns. The slope of the $S(\varphi)$ versus ln k_l, in the considered range, varies between 1.66 and 4.97 (Table 11.8). Chromolith is the column that shows the highest slope, while the Zorbax column has a behavior more consistent with the HILIC columns.

Table 11.6. Elution strength according to Eq. (11.31), in the range between 10 and 20% water, for the HILIC columns and nucleosides.

Column	Silica		Diol		Amino		Zwitterionic	
Compound	k_1	$S(\varphi)$	k_1	$S(\varphi)$	k_1	$S(\varphi)$	k_1	$S(\varphi)$
Cytidine	1.94	6.05	2.34	4.94	2.09	8.09	7.34	9.68
Guanosine	1.30	6.33	2.22	5.67	4.39	6.12	6.76	12.69
Adenosine	1.41	5.39	2.00	4.48	1.28	2.94	1.90	5.54
Xantosine	0.85	4.69	1.51	4.15	3.05	4.36	4.25	11.21
Uridine	0.62	3.16	1.13	2.87	2.41	5.36	2.234	7.58
Thymidine	0.57	3.16	0.99	2.90	1.38	4.43	0.85	4.31
Uracil	0.50	2.29	0.90	2.08	1.14	2.37	0.94	3.01

	HILIC-A		HILIC-B		HILIC-N	
	k_1	$S(\varphi)$	k_1	$S(\varphi)$	k_1	$S(\varphi)$
Cytidine	1.24	5.01	2.37	11.57	2.73	7.12
Guanosine	0.94	4.94	6.45	10.75	4.80	8.09
Adenosine	1.03	4.26	1.43	6.2	2.10	5.51
Xantosine	0.66	3.29	4.39	9.01	3.08	6.52
Uridine	0.54	2.31	2.21	5.94	1.80	4.31
Thymidine	0.52	2.08	1.01	3.51	1.10	3.67
Uracil	0.49	1.44	0.98	3.03	1.02	2.65

Table 11.7. Elution strength according to Eq. (11.31) for the RPLC columns diuretics and sulphonamides.

Column	Zorbax (34–46%)[a]		Zorbax (46–58%)[a]	
Compound	k_l	$S(\varphi)$	k_l	$S(\varphi)$
Trichloromethiazide	0.751	3.96	0.467	3.35
Althiazide	4.66	9.29	1.53	6.36
Furosemide	5.48	9.03	1.85	6.74
Xipamide	14.78	10.76	4.07	7.66
Ethacrynic acid	20.94	11.72	5.13	7.87
	Chromolith (10–20%)[a]		Chromolith (20–30%)[a]	
	k_l	$S(\varphi)$	k_l	$S(\varphi)$
Sulphamerazine	2.12	9.31	0.837	4.69
Sulphachloropyridazine	6.51	12.72	1.83	7.34
Sulphisoxazole	12.64	14.98	2.83	9.18
Sulphaquinoxaline	35.7	19.65	5.01	11.87

[a] Upper and lower values of the experimental range considered in the calculation of $S(\varphi)$.

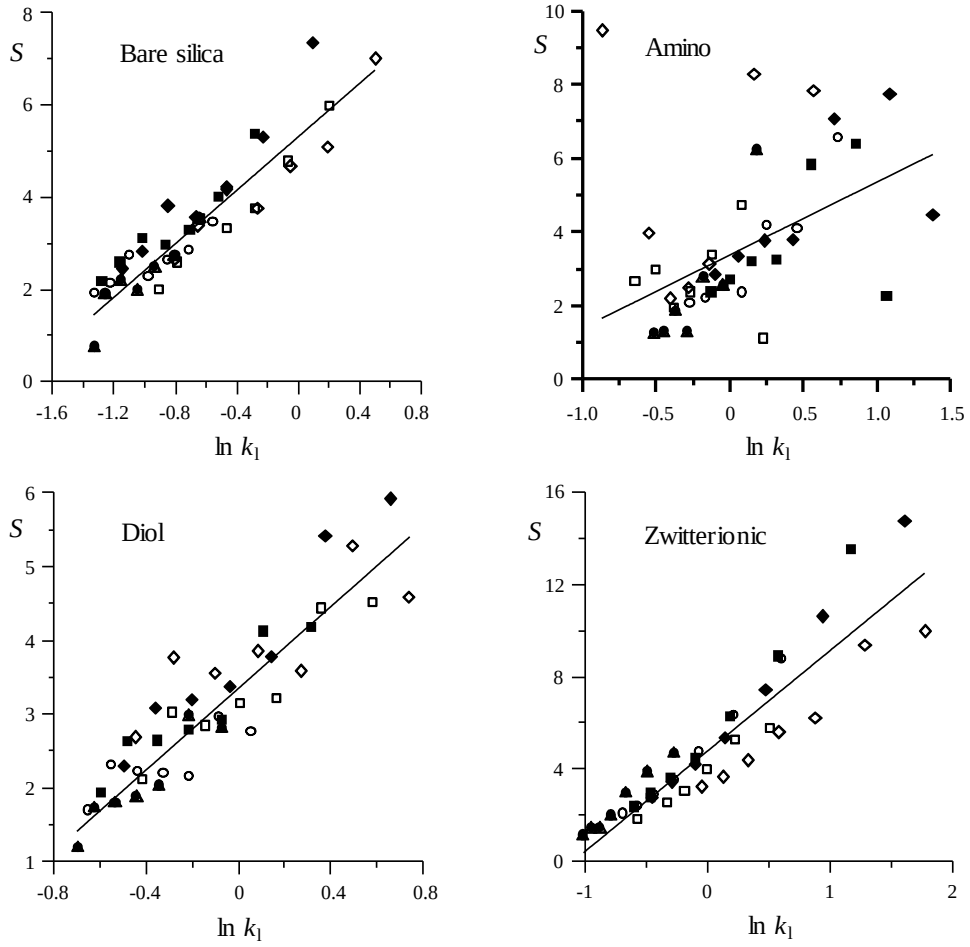


Figure 11.10. Behaviour of the experimental elution strength as a function of the retention factors for the mobile phase of lower elution strength, for bare silica, diol, amino and zwitterionic columns. The fitting line for all experimental data is depicted. The elution strength values were calculated for the following water ranges (*v*/*v*): 10–15%, 15–20%, 20–25%, 25–30%, 30–35%, 35–40%, and 40–45%. Compound identities are given in Figure 11.4.

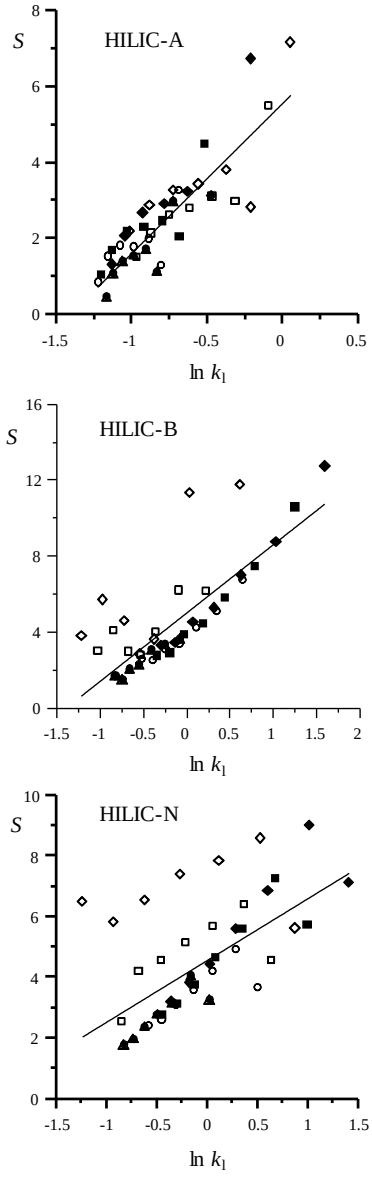


Figure 11.11. Behaviour of the experimental elution strength as a function of the retention factor for the mobile phase of lower elution strength, for HILIC-A, HILIC-B and HILIC-N columns. Other details are given in Figures 11.4 and 11.10.

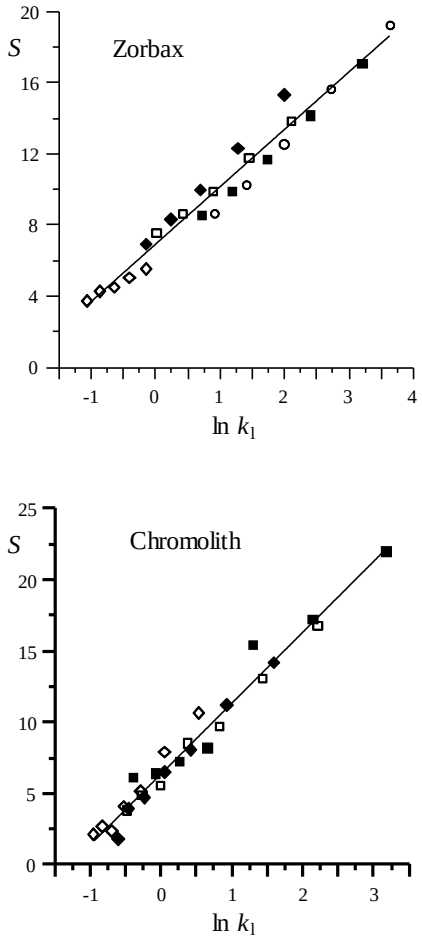


Figure 11.12. Behaviour of the experimental elution strength as a function of the retention factor for the mobile phase of lower elution strength, for the Zorbax and Chromolith columns. The fitting line for all experimental data is depicted. For the Zorbax column, the elution strength values were calculated for the following acetonitrile ranges (*v*/*v*): 28–34%, 34–40%, 40–46%, 46–52%, and 52–58%. For the Chromolith column (*v*/*v*): 10–15%, 15–20%, 20–25%, 25–30%, 30–35%, 35–40%, and 40–45%. Compound identities are given in Figure 11.6.

Table 11.8. Results of the linear fitting of $S(\varphi)$ versus k_l (lower value in the experimental range considered for the calculation) (see Figures 11.10 to 11.12).

Column	*m*	*n*	R^2
Silica	2.76	3.34	0.809
Diol	2.90	5.31	0.846
Amino	1.97	3.42	0.202
Zwitterionic	4.35	4.78	0.853
HILIC-A	3.97	5.59	0.735
HILIC-B	3.59	4.99	0.602
HILIC-N	1.66	3.86	0.432
Zorbax	3.24	6.87	0.962
Chromolith	4.97	6.51	0.969

11.6. Conclusions

This work is focused on the modelling of the retention and study of the elution strength of HILIC columns of different nature, used to separate highly polar compounds. The comparison with RPLC columns using polar compounds is also presented. The research has been structured in three sections: comparison of the quality of seven retention models, study of the LSS model, and measurement of the elution strength capability of each column, at varying mobile phase composition. The final conclusions are summarised next:

(i) In general, the HILIC data have yielded better fittings of the experimental retention factors versus organic solvent content in the mobile phase, compared to the RPLC data, probably due to the smaller variation of retention observed with the HILIC columns.

(ii) The non-linear fitting of the model parameters to the experimental data was much easier by transforming the equations, so that the coordinates for the maximal retention in the experimental design is taken as the starting point.

(iii) The fitting quality increases with the number of model parameters. Among the two- and three-parameter models, Eqs. (11.14) and (11.20) showed more reliable behaviour with mean errors of 1.0% and 0.7%, respectively.

(iv) A strong correlation is observed between the values of the S parameter in the LSS model (Eq. (11.1)) (which quantifies the mobile phase elution strength on each solute) and ln k_o (which quantifies the retention level of the solute). The most outstanding conclusion is that the correlation is very similar for all columns.

(v) The similar behaviour in elution capability ($S(\varphi)$, which takes into account the change in elution strength with the mobile phase composition) of the HILIC and RPLC columns is surprising, considering the different retention mechanisms. A rather good correlation of $S(\varphi)$ versus the initial retention factor, for a series of ranges of solvent content, was obtained for the RPLC columns, since the interactions of solutes are mostly hydrophobic. For the HILIC columns, the smallest dispersion of behaviours was observed for bare silica, whereas the amino and HILIC-N gave rise to a range of behaviours for the different nucleosides.

10.7. References

[1] M.C. García Álvarez-Coque, J.J. Baeza Baeza, G. Ramis Ramos, Reversed phase liquid chromatography, in *Analytical Separation Science Series* (edited by J.L Anderson, A. Stalcup, A. Berthod, V. Pino), Wiley-VCH, New York, Vol. 1, 2015, pp. 159–197.

[2] C.F. Poole, N. Lenca, Reversed phase liquid chromatography, in *Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, M.L. Riekkola), 2nd ed., Elsevier, Amsterdam, 2017, pp. 91–123.

[3] M.C. García Álvarez-Coque, J.A. Navarro Huerta, J.R. Torres Lapasió, Secondary chemical equilibria in reversed-phase liquid chromatography, in *Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, M.L. Riekkola), 2nd ed., Elsevier, Amsterdam, 2017, pp. 125–146.

[4] Y. Guo, S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography, *J. Chromatogr. A* 1218 (2011) 5920–5938.

[5] B. Buszewski, S. Noga, Hydrophilic liquid chromatography (HILIC): A powerful separation technique, *Anal. Bioanal. Chem.* 402 (2012) 231–247.

[6] A. Periat, I.S. Krull, D. Guillarme, Applications of hydrophilic interaction chromatography to amino acids, peptides and proteins, *J. Sep. Sci.* 38 (2015) 357–367.

[7] Q. Zhang, F.Q. Yang, L. Ge, Y.J. Hu, Z.N. Xia, Recent applications of hydrophilic interaction liquid chromatography in pharmaceutical analysis, *J. Sep. Sci.* 40 (2017) 49–80.

[8] P. Jandera, P. Janás, Recent advances in stationary phases and understanding of retention in hydrophilic interaction chromatography, *J. Chromatogr. A* 967 (2017) 12–32.

[9] A. Cavazzini, M. Catani, A. Felinger, Hydrophilic interaction liquid chromatography, in *Liquid Chromatography: Fundamentals and Instrumentation* (edited by S. Fanali, P. Haddad, C.F. Poole, M.L. Riekkola), 2nd ed., Elsevier, Amsterdam, 2017, pp. 147–169.

[10] D.V. McAlley, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, *J. Chromatogr. A* 1523 (2018) 49–71.

[11] G. Jin, Z. Guo, F. Zhang, X. Xue, Y. Jin, X. Liang, Study on the retention equation in hydrophilic interaction liquid chromatography, *Talanta* 76 (2008) 522–527.

[12] D.V. McCalley, Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography, *J. Chromatogr. A* 1217 (2010) 3408–3417.

[13] A.E. Karatapanis, Y.C. Fiamegos, C.D. Stalikas, A revisit to the retention mechanism of hydrophilic interaction liquid chromatography using model organic compounds, *J. Chromatogr. A* 1218 (2011) 2871–2879.

[14] E. Tyteca, A. Périat, S. Rudaz, G. Desmet, D. Guillarme, Retention modeling and method development in hydrophilic interaction chromatography, *J. Chromatogr. A* 1337 (2014) 116–127.

[15] M.R. Euerby, J. Hulse, P. Petersson, A. Vazhentsev, K. Kassam, Retention modelling in hydrophilic interaction chromatography, *Anal. Bioanal. Chem.* 407 (2015) 9135–9152.

[16] Y. Guo, Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC), *Analyst* 140 (2015) 6452–6466.

[17] P. Česla, N. Vaňková, J. Křenkováb, J. Fischer, Comparison of isocratic retention models for hydrophilic interaction liquid chromatographic separation of native and fluorescently labeled oligosaccharides, *J. Chromatogr. A* 1438 (2016) 179–188.

[18] P. Jandera, T. Hájek, Z. Sromová, Mobile phase effects in reversed-phase and hydrophilic interaction liquid chromatography revisited, *J. Chromatogr.* A 1543 (2018) 48–57.

[19] M. Mateos Vivas, E. Rodríguez Gonzalo, J. Domínguez Álvarez, D. García Gómez, R. Carabias Martínez, Determination of nucleosides and nucleotides in baby foods by hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents, *Food Chem.* 211 (2016) 827–835.

[20] P.J. Schoenmakers, H.A.H. Billiet, L. de Galan, Description of solute retention over the full range of mobile phase compositions in reversed-phase liquid chromatography, *J. Chromatogr. A* 282 (1983) 107–121.

[21] R.M. López Marques, P.J. Schoenmakers, Modelling retention in reversed-phase liquid chromatography as a function of pH and solvent composition, *J. Chromatogr. A* 592 (1992) 157–182.

[22] K. Valkó, L.R. Snyder, J.L. Glajch, Retention in reversed-phase liquid chromatography as a function of mobile-phase composition, *J. Chromatogr. A* 656 (1993) 501–520.

[23] P. Jandera, M. Kucerová, J. Holíková, Description and prediction of retention in normal-phase high performance liquid chromatography with binary and ternary mobile phases, *J. Chromatogr. A* 762 (1997) 15–26.

[24] M.C. García Álvarez-Coque, J.R. Torres Lapasió, J.J. Baeza Baeza, Modelling of retention behaviour of solutes in micellar liquid chromatography, *J. Chromatogr. A* 780 (1997) 129–148.

[25] U.D. Neue, C.H. Phoebe, K. Tran, Y. Cheng, Z. Lu, Dependence of reversed-phase retention of ionizable analytes on pH, concentration of organic solvent and silanol activity, *J. Chromatogr. A* 925 (2001) 49–67.

[26] L.G. Gagliardi, C.B. Castells, C. Ràfols, M. Rosés, E. Bosch, Modeling retention and selectivity as a function of pH and column temperature in liquid chromatography, *Anal. Chem.* 78 (2006) 5858–5867.

[27] M.C. García Álvarez-Coque, J.R. Torres Lapasió, J.J. Baeza Baeza, Models and objective functions for the optimisation of selectivity in reversed-phase liquid chromatography, *Anal. Chim. Acta* 579 (2006) 125–145.

[28] P. Nikitas, A. Pappa Louisi, Retention models for isocratic and gradient in reversed phase liquid chromatography, *J. Chromatogr. A* 1216 (2009) 1737–1735.

[29] M. Rosés, X. Subirats, E. Bosch, Retention models for ionizable compounds in reversed-phase liquid chromatography: Effect of variation of mobile phase composition and temperature, *J. Chromatogr. A* 1216 (2009) 1756–1775.

[30] L.R. Snyder, J.J. Kirkland, J.W. Dolan, *Introduction to Modern Liquid Chromatography*, 2nd ed., John Wiley & Sons, New York, 2011.

[31] P. Jandera, T. Hájek, M. Růžičková, Retention models on core-shell columns, *J. AOAC International* 100 (2017) 1636–1646.

[32] N.L. Padivitage, M.K. Dissanayake, D.W. Armstrong, Separation of nucleotides by hydrophilic interaction chromatography using the FRULIC-N column, *Anal. Bioanal. Chem.* 405 (2013) 8837–8848.

[33] S. Carda Broch, A. Berthod, Countercurrent chromatography for the measurement of the hydrophobicity of sulfonamide amphoteric compounds, *Chromatographia* 59 (2004) 79–87.

[34] A. Białk Bielinska, S. Stolte, M. Matzke, A. Fabianska, J. Maszkowska, M. Kołodziejska, B. Liberek, P. Stepnowski, J. Kumirska, Hydrolysis of sulphonamides in aqueous solutions, *J. Hazard. Mater.* 221–222 (2012) 264–274.

[35] C. Hansch, *Comprehensive Medicinal Chemistry* (edited by R.G. Sammes, J.B. Taylor), Pergamon Press, Oxford, 1990, Vol. 6.

[36] R. Ventura, J. Segura, Detection of diuretic agents in doping control. *J. Chromatogr. B* 687 (1996) 127–144.

[37] A. Berthod, S. Carda Broch, M.C. García Álvarez-Coque, Hydrophobicity of ionizable compounds: A theoretical study and measurements of diuretic octanol-water partition coefficients by countercurrent chromatography, *Anal. Chem.* 71 (1999) 879–888.

[38] D.V. McCalley, A study of column equilibration time in hydrophilic interaction chromatography, *J. Chromatogr. A* 1554 (2018) 61–70.

SUMMARY AND CONCLUSIONS

Reversed phase liquid chromatography (RPLC) is usually the technique of choice for the analysis of a wide range of organic compounds, the separation mechanism being mainly based on solute polarity. However, ionised compounds, which are polar, show little or no retention. Also, there are serious problems to analyse basic cationic compounds, due to their interaction with the anionic free silanols on the silica-based columns (the most usual). This gives rise to long retention times, and broad and asymmetrical peaks, due to the slow sorption-desorption kinetics of the cationic solutes on silanols. This PhD. work gathers several studies carried out to solve these problems in three fields of interest.

- Micellar liquid chromatography (MLC) with Brij-35
- Ionic liquids as mobile phase additives in liquid chromatography
- Hydrophilic interaction liquid chromatography (HILIC)

The PhD. work includes both fundamental studies and the application of the methodologies to the analysis of physiological fluids and pharmaceutical formulations. The fundamental studies have the purpose of comparing the chromatographic behaviour of different groups of analytes with diverse polarity, acid-base character and charge (sulphonamides, β-blockers, tricyclic antidepressants, flavonoids, diuretics and nucleosides). Peak retention and shape are examined. These are properties of interest with regard to the achievement of appropriate analysis times and resolution. Diverse graphs have been built, which revealed the decay behaviour for retention times, the kinetic interaction of the solutes with the stationary phase (through the observation of the so-called half-width plots), the selectivity (through the correlation of retention times), and the maximal resolution in the chromatograms of mixtures of analytes.

In the next pages, the general conclusions from each chapter are outlined.

C.1. Micellar Liquid Chromatography with non-ionic surfactants

Polyoxyethylene(23)lauryl ether (known as Brij-35) is a non-ionic surfactant, considered in micellar liquid chromatography as an alternative to the extensively used anionic surfactant sodium dodecyl sulphate (SDS), for the analysis of a variety of compounds. However, in spite of its relevance in quantitative structure-activity relationships (QSAR), its use is still minor. The purpose of Part 1 in this work was to study the possibilities of the use of Brij-35, the most suitable conditions for isocratic and gradient elution, the possible use of other non-ionic surfactants, and the advantages of using mixed mobile phases of Brij-35 and SDS. The studies have the purpose to encourage the development of applications with non-ionic surfactants.

C.1.1. Isocratic and gradient elution in Micellar Liquid Chromatography with Brij-35

- The possibility of using pure aqueous solutions of the non-ionic surfactant Brij-35 (i.e. water and soap), as mobile phases in RPLC in both isocratic and gradient elution modes, was examined. These solutions can be an interesting “green” strategy in an effort to minimise pollution and wastes, increasing thus sustainability.

- It is shown that purely micellar mobile phases of Brij-35 are suitable for the chromatographic separation of compounds of high and intermediate polarity, or polar compounds which interact with the hydroxyl end group of the non-ionic surfactant by hydrogen bonding with the ethoxylated chain of the non-ionic surfactant. This is the case of flavonoids and sulphonamides, which contain hydroxyl and amino

groups, respectively. In contrast, the polarity of the assayed β-blockers and the formation of internal hydrogen bonds between the adjacent hydroxyl and amino groups, in these compounds, do not allow a sufficiently strong interaction with the stationary phase modified with Brij-35.

- Although it was possible to elute flavonoids and sulphonamides with purely micellar mobile phases of Brij-35, hybrid mobile phases containing a low concentration of Brij-35 and acetonitrile improved significantly the performance (the peaks were narrower and the analysis times shorter).

- The chromatographic peaks obtained with Brij-35 in isocratic elution are almost symmetrical. The construction of half-width plots revealed the different kinetics of the interaction of flavonoids and sulphonamides with the stationary phase modified by Brij-35. Also, flavonoids exhibit significant dispersion, which indicate the differences in the kinetics for each flavonoid. This behaviour depends on the position of the hydroxyl substituents on the benzene rings of these compounds.

- In gradient elution, significant reduction in the peak width was observed at fixed concentration of Brij-35 and increasing concentration of acetonitrile, or increasing concentration of both Brij-35 and acetonitrile. The peak width was also reduced at high temperature (50 ^{o}C), enhancing the chromatographic performance. The high efficiencies guarantee high resolution, while the presence of Brij-35 offers particular selectivity, and also affects global retention.

- Working with gradients of acetonitrile at fixed concentration of Brij-35 is especially interesting for the analysis of drugs in physiological fluids, making the direct injection of the sample. In this approach, a purely micellar medium of Brij-35 can be used at the start of the gradient, and once the proteins are eluted from the sample, a gradient of acetonitrile should be applied to increase the elution strength, in order to achieve short retention times for all analytes.

C.1.2. Effect of sodium dodecyl sulphate and Brij-35 on the analysis of sulphonamides in physiological samples using direct injection and acetonitrile gradients

- The isocratic elution of a mixture of 15 sulphonamides did not allow their screening in either hydro-organic, or hybrid micellar modes with SDS or Brij-35, using acetonitrile as modifier, due to the poor resolution and high analysis times. However, the amount of organic solvent needed in the micellar mode is significantly smaller, and allows the analysis of particular compounds in a kind of "green chromatography".

- The observed behaviour for sulphonamides eluted with mobile phases of Brij-35 may be explained due to a combination of a more polar modified stationary phase, and the interaction of the amine groups in sulphonamides with the polyoxyethylene tail of the non-ionic surfactant by formation of hydrogen bonds. The higher polarity of a stationary phase modified with the non-ionic surfactant was checked comparing the retention behaviour of the homologous series of alkyl-benzenes in the absence and presence of Brij-35.

- The implementation of acetonitrile gradients in the micellar mode using SDS allowed the screening of sulphonamides at practical analysis times. Using Brij-35, the analysis time decreased considerably with respect to the isocratic elution, but the resolution was insufficient. Although the peaks appeared more evenly distributed in the chromatogram, these were broad and strongly overlapped for different sulphonamides. Nevertheless, the use of gradient elution with acetonitrile in the presence of Brij-35 can be useful to analyse simple mixtures of sulphonamides.

- Column re-equilibration took less than 2 min for the hydro-organic and hybrid micellar mode in the presence of SDS, and 3 min with Brij-35. These times are sufficiently short to be practical.

- The implementation of gradient elution in MLC can be useful for the analysis of physiological samples (milk and urine), using direct injection, as a consequence of its simplicity, the small re-equilibration time, and the elimination of any pre-treatment.

- The analysis of fortified milk and urine samples was carried out using gradients of acetonitrile, in the presence of SDS or Brij-35, revealing no interference from endogenous components in the matrix sample of milk for both surfactants. An isocratic step of 2 min was added before the gradient to facilitate the solubilisation of the proteins, increasing column protection.

C.1.3. Search of non-ionic surfactants suitable for Micellar Liquid Chromatography

- There are few references on the use of MLC with non-ionic surfactants, Brij-35 being the most usual. Hence the interest of investigating the application of other non-ionic surfactants. The study showed that polyoxyethylene(10)tridecyl ether (C13E10) and polyoxyethylene(10) lauryl ether (C12E10) can be used in MLC, due to their high solubility in water, combined with null absorption in the UV range. Other non-ionic surfactants were less suitable because of their low solubility (Brij-L4, TritonX-114 and Span 20), or high UV absorption (Triton X-100 and IGEPAL CO-630).

- An advantage of using surfactants like SDS or the non-ionic from the Brijs family is its biodegradable character. Also, the adsorption of Brij-35, C12E10 and C13E10 on the stationary phase confers higher polarity that decreases the retention times, for most solutes, avoiding in many cases the need of an organic solvent, which increases the greenness of MLC.

- The elution strength of Brij-35, C12E10 and C13E10 was strong enough to elute sulphonamides, β-blockers and tricyclic antidepressants (TCAs), without the need of organic solvent. It was also possible to analyse flavonoids, but the addition of a relatively small amount of acetonitrile to the non-ionic micellar mobile phases was needed to decrease the retention times of these compounds to practical values.

- The basic β-blockers and TCAs (which are cationic at the mobile phase pH) were mainly retained according to their polarity. This was evidenced by the high correlation between the octanol-water partition coefficient (log $P_{o/w}$) and the logarithmic values of the association constants (K_{AS} and K_{AM}), which measure the interactions of solutes with the stationary phase and mobile phase, respectively.

- The higher interactions of the acidic sulphonamides with both, stationary phase and mobile phase, and consequently their higher retention times (compared to the basic compounds), can be explained by the interaction of the sulphonamide group with the ethoxylated chain of the non-ionic surfactants. This is also the case for flavonoids, which interact more strongly with the non-ionic surfactants through their hydroxyl groups.

- The chromatographic peaks were narrower and more symmetrical with C12E10 and C13E10, compared to Brij-35. The addition of an organic solvent, such as acetonitrile, decreased the retention times of flavonoids, but peaks were broader and asymmetrical.

- The chromatographic behaviour of the probe compounds in the presence of C12E10 and C13E10 is similar and depend on the solute nature. However, among the three non-ionic surfactants (Brij-35, C12E10 and C13E10), only C13E10 is seemingly non-irreversibly adsorbed on the stationary phase.

C.1.4. Reversed-Phase Liquid Chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A green method for the analysis of basic compounds

- The retention times of basic compounds (such as β-blockers and TCAs) on stationary phases modified with micellar mobile phases of the anionic SDS are too high to be practical, forcing the addition of a high amount of organic solvent. The high retention is produced by the electrostatic interaction of the protonated compounds (cationic) with the anionic surfactant adsorbed on the stationary phase. Meanwhile, most β-blockers are not sufficiently retained using mobile phases with Brij-35, since this surfactant is neutral, the polarity of the stationary phase is decreased, and there are no specific interactions between the basic compounds and the modified stationary phase. Also, with Brij-35, the efficiencies are poorer. A mixed mobile phase containing both SDS and Brij-35 can solve all these limitations.

- For basic compounds, the retention capability of the stationary phase modified with the mixed mobile phase of Brij-35 and SDS is larger compared to a stationary phase exclusively modified with Brij-35, and significantly smaller with regard to a stationary phase exclusively modified with SDS.

- The interactions between the basic solutes, the modified stationary phase and the micelles of Brij-35 (measured by the association constants K_{AS} and K_{AM}, respectively) were different to the interactions with the mixed Brij-35/SDS system. The interactions with the SDS system were so strong that the association constants could not be measured.

- Highly symmetrical peaks were obtained (with asymmetry factor ≈ 1.0, similar to that obtained with only SDS). The asymmetry factor with Brij-35 (2.33) was significantly larger with respect to the mixed Brij-35/SDS systems. This indicates that the effect of SDS in the mixed mobile phase is kept, hindering the access of the basic compounds to silanols in the stationary phase, and giving rise to a sufficiently rapid interaction kinetics.

- The retention of polar and moderately polar basic compounds (β-blockers), which is too short with mobile phases containing only Brij-35, can be modulated to practical values by the addition of SDS to the mobile phase containing Brij-35. In the mixed micellar system, the analysis is carried out with good resolution and adequate analysis time. For TCAs, mobile phases with only Brij-35 are preferable.

- Peak performance was enhanced using mobile phases that contain both Brij-35 and SDS, without the need of the addition of organic solvent, giving rise to successful "green" RPLC procedure.

C.1.5. Analysis of basic drugs by Liquid Chromatography with environmentally friendly mobile phases in pharmaceutical formulations

- Mixed mobile phases of Brij-35 and SDS allow the elution of β-blockers in relatively short analysis times, avoiding the need of an organic solvent added to the pure mobile phases of SDS, on the one hand, and the short times obtained with mobile phases that only contain Brij-35, on the other. The mixed mobile phases yield symmetrical peaks and good resolution for the analysis of β-blockers.

- A simple chromatographic procedure for the control of the β-blockers atenolol, celiprolol, metoprolol, oxprenolol and propranolol in pharmaceutical formulations was developed, using 0.15 M SDS/0.05 M Brij-35 at pH 3 as mobile phase and UV detection, with label claims in the range of 80–120%. The method agreed with the results obtained with 0.15 M SDS/15% 1-propanol, with the advantage of avoiding the use of an organic solvent. Sample preparation was simple and only required solubilisation and filtration prior to injection.

- The validation of the method with the mixed mobile phase was made according to the ICH guideline and offered good results for the tested drugs:
  (a) All calibration curves met the linearity requirements, with determination coefficients $R^2 > 0.9990$. The slopes and intercepts of the calibration curves, during three non-consecutive days and along three different weeks, were stable throughout the validation process, indicating column performance was maintained, and a good prediction capability of the concentrations of the analytes from the fitted regression straight-lines.
  (b) Intra and inter-day precision and accuracy were usually below 2%.
  (c) Limits of detection (LODs) and quantification (LOQs) were below 0.14 μg/mL and 0.26 μg/mL, respectively.
  (d) Robustness assays, considering flow rate, pH and concentrations of Brij-35 and SDS in the mobile phase yielded RSD values for the retention times usually in the 1–2% range. A greater variability was observed for Brij-35, and for the peak areas considering all studied factors.

C.2. Ionic liquids as mobile phase additives in Liquid Chromatography

In the last decade, the interest in the use of ionic liquids (ILs) as additives in RPLC has grown. Most work with ILs deals with the separation of basic compounds, trying to eliminate the silanol problem with conventional silica-based columns and improve chromatographic performance. The interactions of ILs with the analytes may be more complex than those observed with surfactants, since both cation and anion in the IL can be adsorbed on the stationary phase. Also, the added buffer systems may affect the chromatographic performance. This is here investigated. The development of a procedure to analyse TCAs in pharmaceutical formulations is also reported.

C.2.1. Effect of buffer nature and concentration on the chromatographic performance of basic compounds in the absence and presence of 1-hexyl-3-methylimidazolium chloride

- Protonated (cationic) basic compounds interact with residual anionic silanols on silica packings, which produces poor performance. The adsorption on the stationary phase of different types of additives in the aqueous-organic mobile phase may avoid this interaction, but the retention behaviour is also affected. Thus, depending on the charge of the adsorbed additive, the retention may decrease (repelled by a cationic additive, which is the case of IL cations), or increase (attracted by an anionic additive, which is the case of the anionic surfactant SDS). It is here shown that the anionic species of buffers added to the mobile phase are also adsorbed on the stationary phase. This modifies the retention.

- The retention and peak shape of six β-blockers in RPLC using mobile phases containing four different buffer systems (acetic acid/acetate, formic acid/formate, phosphoric acid/dihydrogen phosphate and citric acid/dihydrogen citrate), at increasing concentration in the presence and absence of the IL HMIM·Cl, was studied. The cation of this IL ($HMIM^+$) is significantly adsorbed on C18 columns, while chloride has no affinity towards the stationary phase. Retention increased by addition of larger concentration of all buffer systems, in both the absence and presence of HMIM·Cl, which indicates the importance of controlling the buffer concentration on the analytical procedures.

- In the absence of IL, the buffer anions are directly adsorbed on the alkyl bonded chains in the stationary phase, the observed trend in retention indicating column saturation at the highest concentration. The adsorbed buffer anions should attract the cationic basic compounds to the stationary phase. Meanwhile, in the presence of IL, the buffer anions should be attracted by the IL cation. The linear trends observed in the retention of the cationic basic compounds, at increasing concentration of the buffer anions, indicate an increasing interaction of these anions (and consequently, the analytes), due to electrostatic attraction to the stationary phase covered by $HMIM^+$.

- The changes in retention by addition of the cationic $HMIM^+$ were significantly larger with regard to those observed by addition of larger concentration of the anionic buffer species. In spite of this, a high correlation was observed between the retention factors in the presence and absence of IL, and at varying concentration of the buffer anions. The elution order of the mixture of β-blockers did not change in the

presence of the different buffer systems, in the absence and presence of IL. All this suggests similar selectivity, which should be explained mainly by the similar nature of the interactions (electrostatic) of the cationic solutes with the IL cation and buffer anions adsorbed on the stationary phase.

- The generation of narrow and almost symmetrical peaks, in the presence of HMIM·Cl, at any concentration of buffer, indicates an effective protection of the column by the IL, which covers the surface of the C18 stationary phase. In the absence of IL, the access to silanols seems to be also more efficiently hindered at higher buffer concentration, but the effect is again smaller to that observed for the IL. In fact, in the presence of IL, there is no apparent effect of the buffer concentration on both peak width and symmetry for all assayed buffer systems.

- Finally, citrate ion seemed to show more affinity for the stationary phase, based on the larger retention times and better peak symmetries. Phosphate buffer yielded somewhat smaller retention, than formate and acetate buffers, whose effect on retention was similar. Although at pH 3 the dominant species for the acetate and formate systems in the mobile phase were not the basic (anionic) species, the increased retention for the β-blockers by addition of larger concentration of these buffers suggests an induced deprotonation of the monoprotic systems due to anion adsorption.

C.2.2. Suitability of 1-hexyl-3-methyl imidazolium ionic liquids for the analysis of pharmaceutical formulations containing tricyclic antidepressants

- Tricyclic antidepressants are low polarity drugs that strongly associate to the alkyl chains of conventional stationary phases. Also, these compounds interact with the residual ionised silanols present in conventional silica-based stationary phases, which is translated in stronger retention, and tailed and broad peaks. It was thought that the addition of an IL could improve the chromatographic performance of TCAs. However, a carryover effect (increased retention times) was observed with a C18 column, in the presence of the imidazolium ILs. This was explained by the high hydrophobicity of TCAs accumulated on the column head. The effect was stronger with HMIM·BF_4, due to the high affinity of the anion (tetrafluoroborate) towards the stationary phase, which attracts electrostatically the cationic analytes to the alkyl-bonded stationary phase.

- The carryover effect was minimised using a C8 column, in the presence of HMIM·Cl. In these conditions, a significantly smaller amount of acetonitrile compared to hydro-organic mobile phases was needed, with reasonable analysis times and good peak shape. The ILs HMIM·Cl and HMIM·BF_4 yield narrow and symmetrical peaks for basic compounds, when added to hydro-organic mobile phases. The method improves a previous method developed with a mobile phase containing the IL 1-butyl-3-methylimidazolium associated to chloride or hexafluoro-phosphate.

- To perform the analysis, the sample was just solubilised in the mobile phase and filtrated (no previous extraction of the drugs was needed). However, the resolution was still poor, which limits the applicability of the procedure for screening studies.

- Method validation showed good results for all analytes with the following results:
  (a) All calibration curves met the linearity requirements, with determination coefficients $R^2 > 0.9964$. The slopes and intercepts were stable throughout the validation process, indicating high prediction capability and preservation of the chromatographic column performance.
  (b) Intra- and inter-day precisions were usually below +1.0%, and intra- and inter-day accuracies in the ranges –2.1% to +2.4%, and –3.0% to +2.3%, respectively.
  (c) Limits of detection and limits of quantification were usually below 0.25 μg/mL and 0.09 μg/mL.
  (d) Considering the retention times, the RSD values in the robustness studies ranged between 0.4% and 1.6% for the flow-rate, acetonitrile concentration and pH, but the variation range was larger for HMIM·Cl concentration (2.8–4.8%). For the peak areas, the variability was significantly smaller for the HMIM·Cl concentration (0.4–0.9%), and similar for the other assayed parameters. A rigorous control of the concentration of HMIM·Cl is recommended.

C.3. Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography allows the chromatographic analysis of highly polar compounds. Initially, normal phase columns were used for this chromatographic mode. However, new stationary phases have been developed to get appropriate performance for a variety of compounds. In this section, the chromatographic behaviour of seven columns of different nature commercially available for HILIC separations is compared in terms of retention, selectivity, peak shape, and resolution. For this purpose, a protocol developed in previous work for RPLC columns was used. The observed behaviour gives some insight on the interactions established between the solutes and stationary phases, some of them similar to those discussed in previous sections. A mathematical approach to model the retention behaviour of the HILIC columns is also developed.

C.3.1. Protocol to compare column performance applied to Hydrophilic Interaction Liquid Chromatography

- The chromatographic performance of seven HILIC columns of different nature was studied. The columns were the neutral ACE Excel 5 SIL and Betasil Diol-100, the anion exchanger ACE Excel 5 NH_2, the zwitterionic ZIC-HILIC, and the recently commercialised HILIC-A (cation exchanger), HILIC-B (anion exchanger), and HILIC-N (neutral). Nucleosides, which are often used for column testing in HILIC, were used as probe compounds.

- The ionisation state of nucleosides was kept in mind to understand the diverse column behaviour: adenosine cytidine, guanosine and xantosine are partially protonated at pH 3 giving rise to cationic solutes.

Meanwhile, thymidine, uracil and uridine exist mainly in cationic form at acidic pH. All these compounds should be well retained on cation exchange stationary phases

- Zwitterionic and amino columns retained stronger all studied compounds, which reveals a more hydrophilic character for these stationary phases, where the adsorbed aqueous layer should be thicker. Although bare silica and the diol column have a neutral character with no ion exchange capability for anions and cations, the ionisation of silanols can provide free anionic species that would increase the retention of protonated (cationic) bases. However, this does not seem the case, since the analysed nucleosides showed low retention times when eluted from both bare silica and diol columns. This indicates that the silanol effect is masked by the adsorbed aqueous layer on the HILIC columns.

- The retention with HILIC-N (neutral) and HILIC-A (cationic) also showed unexpected behaviours. The retention with HILIC-N was significantly stronger than expected for nucleosides, indicating high polarity for this column, in spite of its presumably low ion exchange capability. Meanwhile, in spite of the high cation capability, claimed by the manufacturers, HILIC-A gave rise to the lowest retention factors. The reason of this performance may be related to the working pH, where the stationary phase could be still protonated. The elution order of the mixture of nucleosides, especially for adenosine and cytidine, depended significantly on the column nature.

- The high correlations of the retention factors between bare silica, diol and HILIC-A, on the one hand, and HILIC-B, amino and HILIC-N, on the other hand, indicated similar selectivity. The zwitterionic column showed instead particular selectivity. The differences in selectivity for this column and HILIC-N could be partially explained by the significantly different amount of adsorbed water on the columns.
- In general terms, peaks were symmetrical for all columns, even with the cationic solutes. This also indicates that the activity of residual silanols is masked by the adsorbed aqueous layer. However, peaks were wider for bare silica and HILIC-A columns and more asymmetrical for the amino (with tailing peaks), followed by bare silica, zwitterionic and HILIC-N (with fronting peaks). The best results in terms of symmetry were obtained for diol, HILIC-A and HILIC-B.
- The lack of scattering of the half-width plots for all HILIC columns (except for the zwitterionic) indicated similar interaction kinetics for the group of nucleosides in the studied conditions. For the zwitterionic column, the larger dispersion can be explained by the narrower peaks obtained for uracil and thymidine, which should exhibit particular kinetics.
- The best resolution was achieved with the zwitterionic column. Resolution was also good for the amino and HILIC-N columns.

C.3.2. Retention modelling with Hydrophilic Interaction Liquid Chromatography columns

- The modelling of the retention and study of the elution strength of seven HILIC columns of different nature (underivatized silica, and silica containing diol, amino and sulfobetaine functional groups of different nature, together with three new columns manufactured by ACE), used to separate highly polar compounds (nucleosides and uracil), was studied. The comparison of the performance of the HILIC columns, with regard to two RPLC columns using polar compounds (sulphonamides and diuretics), is also presented.
- Column conditioning and regeneration was checked making cycles of five consecutive injections of cytidine repeated each 60 min, until getting stable values of retention time. To check the reproducibility, cytidine was also injected at the beginning and end of each working day. Initial equilibration of at least 12 hours at 1 mL/min was needed for conditioning, while once the column has been conditioned, only 30 min was required to change to a higher water content mobile phase.
- The accuracy of seven retention models was studied, including models describing mixed retention mechanisms (partitioning into the adsorbed water-rich layer, and polar and electrostatic interactions). In general, the HILIC data yielded better fitting of retention compared to the RPLC data, probably due to the smaller variation of retention observed with the HILIC columns. The fitting quality increases with the number of model parameters. The two- and three-parameter models showed thus more reliable behaviour, with mean errors of 1.0% and 0.7%, respectively.

- A strong correlation was found for the LSS model between the values of the *S* parameter (which quantifies the mobile phase elution strength on each solute) and the intercept (which quantifies the retention level of the solute). Best correlations corresponded to silica, diol, HILIC-A in HILIC, and Zorbax and Chromolith in RPLC, indicating a smaller dispersion in the behaviour of the set of compounds. However, the most outstanding conclusion is that the correlation is very similar for all columns.
- The similar behaviour in elution capability of the HILIC and RPLC columns is surprising, considering the different retention mechanisms.

CONTRIBUTION OF THE PhD WORK TO PUBLICATIONS

The chapters in this PhD. work correspond to the following publications, listed according to the publication date. The percentage of contribution of Ester Peris García as PhD. student is indicated with each article.

- Ester Peris García, Casandra Ortiz Bolsico, Juan José Baeza Baeza, María Celia García Álvarez-Coque
  Isocratic and gradient elution in micellar liquid chromatography with Brij-35.
  Journal of Separation Science 38 (2015) 2059–2067 (Chapter 3).
  Contribution: 100% Ester Peris García

- María José Ruiz Ángel, Ester Peris García, María Celia García Álvarez-Coque
  Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A method for the analysis of basic compounds.
  Green Chemistry 17 (2015) 3561-3570 (Chapter 6).
  Contribution: 100% Ester Peris García

- Ester Peris García, María Teresa Úbeda Torres, María José Ruiz Ángel, María Celia García Álvarez-Coque
  Effect of sodium dodecyl sulphate and Brij-35 on the analysis of sulphonamides in physiological samples using direct injection and acetonitrile gradients.
  Analytical Methods 8 (2016) 3941–3952 (Chapter 4).
  Contribution: 70% Ester Peris García; 30% María Teresa Úbeda Torres

- Ester Peris García, María José Ruiz Ángel, Samuel Carda Broch, María Celia García Álvarez-Coque
  Analysis of basic drugs by liquid chromatography with environmentally friendly mobile phases in pharmaceutical formulations.
  Microchemical Journal 134 (2017) 202–210 (Chapter 7).
  Contribution: 100% Ester Peris García

- Sonia Calabuig Hernández, Ester Peris García, María Celia García Álvarez-Coque, María José Ruiz Ángel
  Suitability of 1-hexyl-3-methylimidazolium ionic liquids for the analysis of pharmaceutical formulations containing tricyclic antidepressants.
  Journal of Chromatography A 1559 (2018) 118–127 (Chapter 9).
  Contribution: 70% Ester Peris García; 30% Sonia Calabuig Hernández

- Ester Peris García, Jorge Rodríguez Martínez, Juan José Baeza Baeza, María Celia García Álvarez-Coque, María José Ruiz Ángel
  Search of new non-ionic surfactants for micellar liquid chromatography.
  Analytical and Bioanalytical Chemistry 410 (2018) 5043–5057
  (Chapter 5).
  Contribution: 100% Ester Peris García

- María Celia García Álvarez-Coque, María José Ruiz Ángel, Ester Peris García
  Micellar liquid chromatography
  Encyclopedia of Analytical Science (edited by P.J. Worsfold, A. Townshend, C. Poole, M. Miró), Elsevier, Waltham, MA, 3rd ed., Vol. 6, (2019) 133–142 (chapter written by invitation) (Chapter 2).
  Contribution: 100% Ester Peris García

- Raquel Burgos Gil, Ester Peris García, María José Ruiz Ángel, Juan José Baeza Baeza, María Celia García Álvarez-Coque
  Protocol to compare column performance applied to hydrophilic interaction liquid chromatography.
  Microchemical Journal 149 (2019) 103973 (Chapter 10).
  Contribution: 70% Ester Peris García; 30% Raquel Burgos Gil

- Ester Peris García, Samuel Carda Broch, María Celia García Álvarez-Coque, María José Ruiz Ángel
  Effect of buffer nature and concentration on the chromatographic performance of basic compounds in the absence and presence of 1-hexyl-3-methylimidazolium chloride
  Journal of Chromatography A 1602 (2019) 397–408 (Chapter 8).
  Contribution: 100% Ester Peris García

- Ester Peris García, María José Ruiz Ángel, Juan José Baeza Baeza, María Celia García Álvarez-Coque
  Study on the retention modelling using hydrophilic interaction liquid chromatography column
  Article in preparation (2019) (Chapter 11).
  Contribution: 100% Ester Peris García

The supervisors (or at least one of them) of this PhD. work (María Celia Álvarez-Coque, María José Ruiz Ángel and Samuel Carda Broch) appear as co-authors. All of them belong to the FUSCHROM group, which is directed by María Celia Álvarez-Coque, who is also main researcher of the projects which have funded the research. María José Ruiz Ángel have proposed the main lines

of several works. Samuel Carda Broch contributed to the articles in Chapters 7 and 8.

The articles in Chapters 3, 5, 10 and 11 were carried out in collaboration with Juan José Baeza Baeza, member of the research group, who contributed developing empirical models to describe the retention behaviour.

Casandra Ortiz Bolsico participated in the initial period of the PhD. work training, supervising the experimental work of the PhD candidate (preparation of solutions, experimental technique, handling of instruments, data processing, and interpretation of the results). In addition, some research work contributed to the training of the degree students María Teresa Úbeda Torres and Raquel Burgos Gil, and Master students Sonia Calabuig Hernández and Jorge Rodríguez Martínez.