FACULTAT DE QUÍMICA

Development of methods in Microemulsion Liquid Chromatography

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

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CERTIFICAN

Que la presente Memoria, "Development of methods in Microemulsion Liquid Chromatography", constituye la Tesis Doctoral de

Dña. NIKITABEN PANKAJKUMAR PATEL

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 catorce de abril de dos mil veintiuno.

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This PhD study would not have been possible without the support and guidance extended by many people. I want to take a moment to thank them.

The journey changed me, changed my life and perspective. I feel different. When I was in my sixteen, I made one of the most important decisions of my life: to study in Science field. I completed my Bachelor degree in 2013. After this, I decided not to study further because of my father´s financial condition. But my parents never gave up and motivated me to continue my studies. I started my Master in Analytical Chemistry and got my degree in 2015. Then I worked as an assistant professor in a private college for one year.

During this period, I met with most important person of my life, got married and came to Valencia in Spain to live with him. Then, I started thinking about getting a job, which seemed quite difficult for me. My husband suggested me to do a PhD. degree. We looked for getting admission in university and started a part time job in a flower shop. At the same time, I joined an English course in a private language school.

There, one day, I met with Professor Guillermo Ramis Ramos, who followed the same course as me. Without hesitation, I would say that he was a blessing in disguise for me, since it came into my life at a time when I had no clue. After listening all above, he handed me a piece of paper with a name, phone number and address and then I got a hope. I couldn't meet with him again but that day, and today I have this opportunity to thank him. So, I thank you so much for giving me the right way.

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whenever I showed her my work. Even though she was a busy person, she would always find time to meet with me, whether it was to discuss my research or just having lunch. Along the pandemics, our emails were our means of communication and way to keep in touch that gave me the motivation to work harder and make the best effort possible. Her effort and patience will never be forgotten. I am really thankful to her for giving me the wonderful opportunity to complete my PhD. under her supervision, which is truly an honor.

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Living and studying in another country is not easy, but with the help of all of you, this became easier. So, thank you so much all of you again.

ABBREVIATIONS

A: Effective hydrogen-bond acidity; also left peak half-width

A_0: Intercept of the left half-width plot

α(CH_2): Methylene (or hydrophobic) selectivity

ACN: Acetonitrile

AOT: Aerosol OT, Sodium bis(2-ethylhexyl) sulfosuccinate

B: Effective hydrogen-bond basicity; also right peak half-width

B_0: Intercept of the right half-width plot

BBB: Blood-brain-barrier

Brij-35: Polyoxyethylene(23)lauryl ether

C: Concentration of modifier

C_2C_1IM: 1-Ethyl-3-methylimidazolium

$[C_4C_1IM][C_8OSO_3]$: 1-Butyl- 3-methylimidazolium octylsulphate

$[C_6C_1IM]^+$: 1-Hexyl-3-methylimidazolium

$[C_6SO_4]^-$: Hexyl sulfate;

$[C_8C_1IM]^+$: 1-Octyl-3-methylimidazolium

$[C_8C_8IM]\ ]^+$: 1-3-dioctylimidazolium

$[C_{12}H_{25}SO_4]^+$: Dodecyl sulphate

$[C_{14}C_1IM]^+$: 1-Methyl-3-tetradecylimidazolium

$[C_{16}C_1IM][Br]$: 1-Hexadecyl-3-methylimidazolium bromide

$[C_{16}C_4IM][Br]$: 1-Hexadecyl-3-butylimidazolium bromide

$[C_nH_{2n+1}C_1IM][C_mH_{2m+1}SO_3]$: Alkylimidazolium alkylsulphate

CCD: Central composite design

C8: Octyl carbon chain

C18: Octadecyl carbon chain

CMC: Critical micellar concentration

CTAB: Cetyltrimethylammonium bromide

D: Apparent octanol-water partition coefficient

DTAB: Dodecyl trimethylammonium bromide

E: Excess molar refraction

E_r: Relative error

φ: Concentration of organic solvent

φ_0: Reference concentration of co-surfactant

ϕ: Concentration of oil

ϕ_0: Reference concentration of oil

Genapol X-080: Polyethylene glycol monoalkyl ether

GAs: Genetic algorithms

HLB: Hydrophilic-lipophilic balance

HPLC: High performance liquid chromatography

HSLC: High submicellar liquid chromatography

HTAB: Hexadecyl trimethylammonium bromide

ICH: International Conference of Harmonization

i.d.: Internal diameter

IL: Ionic liquid

IL/W: Ionic liquid-in-water

k: Retention factor

$k_{i,exp}$: Experimental retention factor

$k_{i,pred}$: Predicted retention factor

$k_{i,mean}$: Mean value of the experimental retention factors

k_w: Retention factor in water

K_1: Mobile/stationary phase distribution constant

K_2: Aqueous phase/oil drops distribution constant

K_3: Stationary phase/oil drops distribution constant

K_{AS}: Solute-stationary phase distribution equilibrium constant

K_{AM}: Solute-micelle distribution equilibrium constant

K_{AD}: Constant quantifying the shift of the distribution equilibria, when the organic solvent is added, in the direction of the mobile phase

K_{MD}: Constant quantifying the shift of the distribution equilibria, when the organic solvent is added, in the direction of the micelle

K_{SD}: Constant quantifying the shift of the distribution equilibria, when the organic solvent is added, in the direction of the stationary phase

LOD: Limit of detection

LOQ: Limit of quantification

LSER: Linear solvation energy relationship

LSS: Linear solvent strength

M: Molar concentration (mol/L)

μ: Concentration of surfactant monomers in the mobile phase forming micelles

μ_0: Reference concentration of surfactant

m_A: Slope left half-width plot

m_B: Slope right half-width plot

MEEKC: Microemulsion electrokinetic chromatography

MEKC: Micellar electrokinetic chromatography

MELC: Microemulsion liquid chromatography

ME: Microemulsion

MLC: Micellar liquid chromatography

MPa: Megapascal

MW: Molecular weight

N: Number of experimental points

n_C: Number of carbon atoms

nm: Nanometer

NTF_2^-: bis(Trifluoromethyl sulphonyl) imide

O/W: Oil-in-water

ODS: Octadecyl silane

OVAT: One-variable-at-a-time

PC: Phosphatidylcholine

PCA: Principal component analysis

pK_a: Acid dissociation constant

$P_{o/w}$: Octanol-water partition coefficient

PrOH: 1-Propanol

QRAR: Quantitative retention-activity relationships

R^2: Determination coefficient

RP: Reversed-phase

RPLC: Reversed-phase liquid chromatography

RSD: Relative standard deviation

S: Dipolarity/ polarizability; also elution strength parameter (Snyder's equation)

S_μ: Elution strength of surfactant

S_φ: Elution strength of co-surfactant

S_ϕ: Elution strength of oil

$S_{\mu\varphi}$: Interaction constant between surfactant and co-surfactant

$S_{\phi\varphi}$: Interaction constant between surfactant and oil

SAIL: Surface active ionic liquid

SB-12: N-dodecyl-N-N-dimethyl-3-ammonium-1-propanesulphonate

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SDOSS: Sodium dioctyl sulphosuccinate

SP: Solute property

STS: Sodium tetradecyl sulphate

TEA: Triethylamine

TFA: Trifluoroacetic acid

TCAs: Tricyclic antidepressants

t_R: Retention time

Triton-X 100: Polyethylene glycol

TTAB: Tetradecyl trimethylammonium bromide

Tween-20: Polyoxyethylene (20) sorbitan monolaurate

Tween-21: Polyoxyethylene (20) sorbitan monolaurate

Tween-80: Polyoxyethylene (20) sorbitan monooleate

UV: Ultraviolet

V: Volume; also McGowan characteristic volume

v/*v*: Volume/volume fraction

w: Peak width

w/*v*: Weight/volume fraction

w/*w*: Weight/weight fraction

W/O: Water-in-oil

RESUMEN

La Cromatografía Líquida de Microemulsiones (MELC, *Microemulsion Liquid Chromatography*) es un modo de la Cromatografía Líquida de Fase Inversa (RPLC, *Reversed-Phase Liquid Chromatography*), en el que las fases móviles contienen microemulsiones (MEs) de aceite en agua, lo que da lugar a nuevas interacciones de los solutos con el sistema cromatográfico. Las MEs se obtienen de forma espontánea mezclando dos líquidos inmiscibles (agua y aceite), en presencia de un surfactante. A menudo también se requiere un co-surfactante para estabilizarlas.

Una mezcla agua / aceite origina dos fases inmiscibles, debido a la gran tensión superficial que existe entre los dos líquidos. Sin embargo, en presencia de un surfactante, se obtiene un sistema líquido organizado, macroscópicamente homogéneo y termodinámicamente estable. Las moléculas del surfactante están formadas por una larga cola apolar y una cabeza polar. Al incorporar el surfactante en la mezcla agua / aceite, se forma una microestructura que proporciona un límite definido entre las fases oleosa y acuosa: el aceite penetra en la micela que forma el surfactante y se estabiliza en su interior en forma de gotas diminutas. La naturaleza compleja de las fases móviles en MELC da lugar a numerosas opciones de composición, en cuanto al tipo y concentración del surfactante, aceite y co-surfactante, que pueden dar lugar a separaciones muy satisfactorias, en comparación con otros modos cromatográficos.

Uno de los principales atractivos de las MEs de aceite en agua es su capacidad para solubilizar compuestos en una amplia gama de polaridades, desde compuestos polares a altamente hidrofóbicos, lo que es de gran interés en muchos campos. El efecto solubilizante sobre las matrices apolares de algunas muestras también es importante, Todo ello permite analizar mezclas de compuestos de diferentes polaridades y realizar la inyección directa de las

muestras, lo que en RPLC convencional requiere largos tratamientos previos. La mayoría de las aplicaciones en MELC corresponden al análisis de muestras que contienen compuestos hidrofóbicos en productos farmacéuticos apolares, tales como cremas, ungüentos y supositorios, además de fluidos fisiológicos y otras matrices biológicas. Sin embargo, la técnica presenta algunos inconvenientes que deben tenerse en cuenta: la adsorción del surfactante sobre las fases estacionarias de base sílice, la mayor viscosidad de la fase móvil que puede originar altas presiones, la necesidad de incrementar los cuidados de la columna y la correcta selección del tipo y concentración del disolvente orgánico apolar utilizado para formar las micro-gotas en la ME.

La Memoria de Tesis Doctoral incluye estudios fundamentales que incrementan el conocimiento sobre la MELC. También muestra la aplicación de la técnica al análisis de preparados farmacéuticos. Con este propósito, se utilizan fases móviles que contienen el surfactante aniónico dodecilsulfato sódico (SDS, *sodium dodecyl sulphate*), octano como disolvente apolar (aceite), y 1-butanol como co-surfactante. También se explora el uso de líquidos iónicos en las MEs, en lugar de octano. Los estudios se han realizado con varios compuestos: los parabenos butilparabeno, etilparabeno, metilparabeno y propilparabeno, y dos grupos de compuestos básicos, los antagonistas de los receptores β-adrenérgicos acebutolol, atenolol, celiprolol, carteolol, esmolol, labetalol, metoprolol, oxprenolol, pindolol, propranolol y timolol, y los antidepresivos tricíclicos (TCAs, *tricyclic antidepressants*) amitriptilina, clomipramina, imipramina, maprotilina y nortriptilina. Los estudios realizados sobre el comportamiento cromatográfico de estos analitos consideraron tanto el comportamiento de retención, como los perfiles de los picos. A continuación, se describe en detalle el contenido del trabajo realizado durante la Tesis Doctoral.

1. Elaboración de una guía práctica para el uso de la Cromatografía Líquida de Microemulsiones

Las investigaciones incluidas en la Memoria de Tesis Doctoral suponen una nueva línea de trabajo para el grupo investigador al que pertenecen las supervisoras del trabajo realizado. El grupo se interesó, a partir de 1988, en el uso de medios organizados, realizando una serie de estudios donde se utilizaban micelas de surfactante para aumentar la absortividad y fluorescencia en diversos métodos analíticos espectroscópicos. Tras los contactos mantenidos con el Profesor Alain Berthod de la Universidad Claude Bernard de Lyon (Francia), que había realizado investigaciones pioneras en el campo de la Cromatografía Líquida Micelar (MLC, *Micellar Liquid Chromatography*), el grupo inició en 1991 investigaciones en este modo cromatográfico. Posteriormente, a partir de 2008, las supervisoras del trabajo de Tesis Doctoral se interesaron en el uso de fases móviles en las que se añade una cantidad relativamente elevada de disolvente orgánico a una disolución de surfactante, para evitar la formación de micelas (HSLC, *High Submicellar Liquid Chromatography*). En esta Tesis Doctoral, el grupo ha iniciado estudios sobre el uso de MEs como fases móviles en cromatografía líquida. Ambos, medios submicelares y MEs poseen el interés de permitir la elución de compuestos fuertemente retenidos en MLC.

El número de artículos publicados en MELC es aún limitado, pero varios investigadores han ofrecido información muy valiosa. En cualquier caso, es evidente que este modo cromatográfico está recibiendo una atención creciente. Sin embargo, a pesar de la publicación de algunos artículos de revisión sobre MELC previos al inicio de esta Tesis Doctoral, la información proporcionada era contradictoria y confusa. Se debe considerar que las fases móviles preparadas con MEs son complejas, ya que requieren la mezcla de al menos

tres reactivos en medio acuoso, de los que se han propuesto una amplia variedad, especialmente para el disolvente apolar y el co-surfactante. Por lo tanto, antes de iniciar las investigaciones en MELC, se consideró necesario conocer en profundidad la información conocida hasta la fecha. Así, el trabajo de Tesis Doctoral se inició con la realización de un estudio detallado de la bibliografía existente, lo que dio lugar a la preparación de dos artículos de revisión que expusieron críticamente los aspectos fundamentales de la técnica y la selección de condiciones experimentales.

Los artículos de revisión realizados al inicio de la Tesis Doctoral constituyen una amplia recopilación de la información existente sobre los diferentes factores involucrados en la MELC, y actualiza los conocimientos sobre la técnica. Además, analiza los factores que conducen a una separación exitosa. Todo ello ha dado lugar a una guía introductoria, que puede ser útil para los investigadores interesados en esta modalidad cromatográfica e incentivar el desarrollo de procedimientos analíticos en MELC, que se muestra como una técnica competitiva frente al uso de otras modalidades de RPLC para el análisis de compuestos apolares. Finalmente, se ofrecen algunos consejos prácticos para preparar fases móviles preparadas con MEs estables, que conduzcan a resultados reproducibles.

Los aspectos más interesantes encontrados en la bibliografía, en relación al trabajo experimental en MELC, se refieren a la naturaleza y concentración de los reactivos para preparar las MEs utilizadas como fases móviles. Otros aspectos relevantes son el estudio de los mecanismos de retención, la selectividad y la práctica experimental para la determinación de fármacos en muestras clínicas y farmacéuticas. Las MEs ofrecen una selectividad única y tiempos de retención más bajos, con una eficacia equivalente o superior a la obtenida en la RPLC convencional, lo que conduce a separaciones isocráticas

satisfactorias. Se reduce así el problema existente en RPLC convencional relativo al incremento exponencial en la retención de compuestos de polaridad decreciente (el denominado problema general de elución cromatográfica), haciendo menos necesaria la elución con gradientes de disolvente orgánico.

Los investigadores que trabajan en MELC indican que el uso de MEs es competitivo para algunos analitos, frente a otras fases móviles preparadas en presencia o ausencia de surfactante. Además, se considera que las MEs son más respetuosas con el medio ambiente, ya que se reducen la contaminación y los residuos en los laboratorios. La información experimental relevante se resume a continuación:

- *Surfactante:* Es necesario para formar las MEs. El tamaño de las gotas y la carga de la fase estacionaria y de las micelas dependen del tipo de surfactante. Por lo tanto, la separación se ve muy afectada al cambiar la molécula del surfactante. Los surfactantes más habituales en la preparación de las fases móviles en MELC son el aniónico SDS y el no iónico Brij-35. La concentración de surfactante para preparar una fase móvil en MELC debe encontrarse, en cualquier caso, por encima de su concentración micelar crítica (CMC), puesto que se requieren micelas para poder obtener las gotitas de la ME.
- *Aceite:* La elección del disolvente apolar también es importante para formar las gotitas en la ME. Generalmente se utilizan alcanos y alcoholes, ambos de longitud de cadena variable. Uno de los aceites más habituales es el octano. Las concentraciones estudiadas para este disolvente se hallan, generalmente, en el intervalo entre el 0 y el 1.2 % *m*/*m*, pero para poder eluir compuestos insolubles más rápidamente, se utilizan MEs más apolares, lo que se consigue aumentando las concentraciones de surfactante y co-surfactante,

así como el contenido de aceite hasta el 2 % *m*/*m*. Recientemente se ha recomendado el uso de líquidos iónicos apolares (ILs, *ionic liquids*), en lugar del disolvente orgánico en MELC, con el fin de reducir los residuos en el laboratorio y su impacto ambiental.

- *Co-surfactante:* Se suele añadir un alcohol de longitud de cadena media, como co-surfactante, para reducir la tensión interfacial a valores prácticamente nulos y formar así una ME estable. Para conseguir una ME adecuada para MELC, la elección del co-surfactante parece más importante que la elección del aceite. Al cambiar el tipo de co-surfactante, se modifica la polaridad de la fase móvil lo que afecta a la retención de los solutos. Por su parte, al aumentar la concentración de co-surfactante en la fase móvil, se reduce la retención debido al incremento en la proporción de fase orgánica en el componente acuoso. Los co-surfactantes más habituales en MELC son el 1-propanol y el 1-butanol. Las concentraciones se encuentran generalmente en los intervalos entre el 5 y el 15 % *v*/*v* para 1-propanol, y entre el 6.6 y 16.5 % *v*/*v* para 1-butanol. Fuera de estos intervalos, las MEs no se forman, y por encima del 8.6 % *v*/*v*, la presión de la columna crece excesivamente.
- *Otros reactivos:* La fase continua de agua en las MEs generalmente contiene otros aditivos, como son los reactivos utilizados para preparar el tampón que controla el pH de la mezcla. Al igual que para otros modos de RPLC, la retención de los compuestos ionizables se ve fuertemente afectada por el pH de la fase móvil.
- *Columnas cromatográficas:* Las columnas más comúnmente utilizadas en RPLC, empaquetadas con fases estacionarias C18 o C8 químicamente enlazadas y con un tamaño de partícula de 3-5 µm, son también habituales

en MELC. Dado que las MEs pueden producir fases móviles de elevada viscosidad, se puede generar una presión excesiva que puede afectar a la columna. Para solucionar esta limitación, se ha recomendado el uso de columnas más cortas. Las columnas monolíticas pueden dar lugar también a un menor tiempo de análisis, incluso en elución isocrática con un elevado flujo de fase móvil.

La complejidad de la composición de una ME adecuada para cromatografía líquida, y el hecho de que los diferentes factores que afectan al comportamiento cromatográfico interactúan entre sí, pueden requerir muchas manipulaciones durante el desarrollo de un método hasta lograr una separación aceptable para mezclas complejas multi-analito. Éste es el motivo de la propuesta, por parte varios investigadores, del uso de una ME como punto de partida, a la hora de desarrollar un método para una nueva separación de la que no se posee información previa. En base a estas condiciones iniciales, varios investigadores han propuesto el uso de metodologías asistidas por ordenador para optimizar la composición de la fase móvil en MELC, lo que reduce significativamente el tiempo y el consumo de reactivos para el desarrollo de los métodos. A lo largo del trabajo desarrollado, recogido en la Memoria de Tesis Doctoral, se logró un alto dominio de la técnica, lo que condujo a nuevas recomendaciones para implementar procedimientos analíticos.

2. Comparación de modos cromatográficos que utilizan el surfactante dodecilsulfato sódico para el análisis de fármacos básicos

El grupo investigador en el que se integran las investigaciones recogidas en la Memoria de Tesis Doctoral posee una larga experiencia en el desarrollo de métodos para el análisis de compuestos básicos, que se iniciaron en 1996. Los compuestos básicos son de gran interés en farmacología, puesto que muchos principios activos poseen este carácter. Por otro lado, el control de estos compuestos se realiza principalmente mediante cromatografía líquida. Sin embargo, el análisis de compuestos básicos mediante RPLC convencional ha sido un desafío desde sus inicios, ya que en el intervalo de pH habitual de la fase móvil (por debajo de 7), estos compuestos dan lugar a especies protonadas catiónicas, que interactúan con los silanoles aniónicos libres existentes en las fases estacionarias de base sílice (las más usuales en la práctica experimental). Ello produce un rendimiento deficiente (mayor retención y formación de picos anchos que muestran colas). El perfil de los picos puede mejorarse significativamente añadiendo diversos reactivos (aditivos), que se adsorben sobre la fase estacionaria, como algunos surfactantes o líquidos iónicos, que recubren la columna, impidiendo el acceso de los solutos a los silanoles libres con carácter aniónico.

El grupo investigador ha demostrado que, además de las aminas convencionalmente utilizadas como aditivos en RPLC, la adición de SDS a las fases móviles en MLC es capaz de suprimir el efecto adverso de los silanoles. Las largas cadenas hidrofóbicas de los monómeros de SDS se asocian a las cadenas alquílicas de las fases estacionarias (generalmente C18), con el grupo sulfato orientado hacia el exterior de la superficie. Ello crea una fase estacionaria con carga negativa que enmascara los silanoles, pero que también atrae a los solutos catiónicos. Esto es un inconveniente, ya que da lugar a

tiempos de retención prolongados, lo que obliga a añadir a la fase móvil una cantidad de disolvente orgánico relativamente elevada, con el fin de aumentar la fuerza eluyente. En estudios realizados por el grupo, a partir de 2008, se encontró que cuando se utiliza SDS en concentración moderada en presencia de un alto contenido de disolvente orgánico, como 1-propanol, se obtienen procedimientos ventajosos de HSLC, a pesar de no formarse micelas. Al inicio del trabajo de Tesis Doctoral, se pensó que la MELC podría ser aún más ventajosa, ya que puede permitir reducir aún más la cantidad de disolvente orgánico, dando lugar a un método más ecológico.

No existía ninguna referencia previa que describiera la aplicación de la MELC a compuestos básicos. Por lo tanto, se consideró que podría tener interés realizar un estudio detallado a fin de examinar esta posibilidad, comparando exhaustivamente la MELC con los resultados obtenidos utilizando RPLC convencional, MLC y HSLC, las dos últimas técnicas haciendo uso también del surfactante aniónico SDS. Se realizó así un estudio exhaustivo del cambio de comportamiento en la retención y la forma de los picos cromatográficos, a medida que se modificaba el ambiente dentro de la columna (presencia de micelas o monómeros de surfactante y naturaleza de la fase estacionaria), al utilizar los distintos modos cromatográficos. La Memoria incluye una extensa discusión sobre el comportamiento observado cuando se modifica la concentración de SDS (en los modos de cromatografía líquida que utilizan surfactante), octano (en MELC), y los disolventes orgánicos acetonitrilo, 1-propanol y 1-butanol (en todas las técnicas estudiadas).

En las investigaciones realizadas, se utilizó un grupo de once antagonistas de los receptores β-adrenérgicos (acebutolol, atenolol, carteolol, celiprolol, esmolol, labetalol, metoprolol, oxprenolol, pindolol, propranolol, y timolol), como compuestos de prueba. Este grupo de compuestos es ideal para estudiar

el comportamiento de los compuestos básicos, ya que existe una gran cantidad de ellos comercializados con diversas polaridades, y la cinética de interacción con la fase estacionaria es similar, para diferentes compuestos. Se examinó el efecto producido al variar diferentes factores experimentales, observándose los cambios en la retención y perfil de los picos de los solutos, que afectan a la resolución. Otro factor examinado fue el consumo de disolvente orgánico.

El estudio mostró las posibilidades de la MLC, HSLC y MELC. Los tiempos de retención de los compuestos básicos, demasiado elevados cuando se utilizan fases móviles que contienen únicamente surfactante, pueden modularse a valores prácticos mediante la adición de diferentes cantidades de uno o dos disolventes orgánicos, lo que da lugar a procedimientos competitivos. Los medios submicelares (HSLC) redujeron la retención y mejoraron la eficacia cromatográfica, en la separación de los antagonistas de los receptores β-adrenérgicos, en comparación con la RPLC convencional y la MLC. Además, la MELC dio lugar a tiempos de análisis muy bajos, y necesitó una menor cantidad de disolvente orgánico para eluir los solutos más retenidos, en comparación con el modo submicelar. La obtención de picos más estrechos y simétricos en MLC, HSLC y MELC, respecto a la RPLC convencional, indica un enmascaramiento eficaz de los silanoles.

En MELC, los intervalos de concentración explorados fueron 0.104–0.173 M para SDS (lo que garantizaba la formación de micelas), 8.2–17.3 % para 1-butanol y 0.28–1.28 % para octano. Fuera de estos intervalos, las MEs no eran estables o no se formaron. La concentración de 1-butanol no se pudo incrementar por encima del 17.3 %, debido a la presión excesiva en la columna, que podría resultar dañada.

La modalidad de HSLC utilizando acetonitrilo ofrece los perfiles de pico más satisfactorios. Sin embargo, el alto volumen de disolvente orgánico que

requiere la HSLC para lograr tiempos de retención suficientemente cortos, para la mayoría de solutos hidrofóbicos, hace que este modo cromatográfico sea menos atractivo. Por el contrario, la MELC reduce significativamente los tiempos de retención utilizando cantidades muy pequeñas de los disolventes orgánicos (1-butanol y octano). Mediante una optimización adecuada, es posible conseguir resolución satisfactoria en la separación de mezclas de los antagonistas de los receptores β-adrenérgicos en tan sólo unos minutos.

En general, los picos cromatográficos se caracterizan por su altura, posición, anchura y asimetría, dependiendo las dos últimas de los valores de las semi-anchuras de pico izquierda y derecha. Hace una década, el grupo investigador confirmó que se obtienen correlaciones simples entre los valores de las semi-anchuras de pico y sus tiempos de retención, para diversos grupos de compuestos, a los que denominó gráficos de semi-anchura. Para la elución isocrática, las representaciones son en realidad parabólicas, aunque a menudo las parábolas se pueden aproximar a líneas rectas. Los gráficos se obtienen con los datos de las semi-anchuras de pico y tiempos de retención, para un conjunto de solutos que experimentan la misma cinética de interacción, cuando son eluidos con una misma fase móvil o fases móviles de composición variable. Cuando la resistencia a la transferencia de masa es diferente, los gráficos muestran una cierta dispersión. En esta Memoria, se muestra que los gráficos de semi-anchura constituyen una herramienta muy útil para la predicción de los perfiles de los picos en los cromatogramas. Además, revelan el grado de similitud de la cinética de interacción de los solutos, cuando se analizan en diferentes condiciones.

Los datos experimentales recogidos en el trabajo de Tesis Doctoral permitieron una comparación global del rendimiento de cuatro modos cromatográficos (RPLC convencional, MLC, HSLC y MELC). La reducción

del efecto silanol en los modos en los que se utiliza surfactante fue muy significativa. La característica más destacable es que las pendientes de los gráficos de semi-anchura derecha e izquierda son similares, debido a la formación de picos casi simétricos a diversos tiempos de retención. En RPLC convencional, los picos muestran una cola significativa mientras que en HSLC con 1-propanol, los picos pasan de tener cola a deformación frontal, a bajas concentraciones de SDS (0.02–0.04 M) y una concentración elevada de 1-propanol (25 %).

Las altas eficacias obtenidas en HSLC y MELC garantizaron una alta resolución. El orden de elución de los antagonistas de los receptores β-adrenérgicos en MLC y HSLC se modificó, respecto al encontrado en RPLC convencional, y también fue distinto entre HSLC y MELC. La resolución de los picos fue máxima con la fase móvil que contenía SDS y acetonitrilo al 35% (HSLC), debido a la mayor anchura de los picos en MELC, aunque a costa de un mayor consumo de disolvente orgánico.

3. Modelización de la retención en Cromatografía Líquida de Microemulsiones

La modelización de la retención, en función de la composición de la fase móvil, es una tarea frecuente en la práctica cromatográfica, de gran importancia en cromatografía líquida para encontrar las condiciones óptimas de separación y comprender el mecanismo de retención de los compuestos eluidos. El grupo investigador poseía experiencia previa en la modelización de la retención en RPLC convencional con fases móviles hidro-orgánicas, así como con fases móviles en presencia de aditivos, con excelentes resultados. Aunque existían algunos antecedentes en el campo de la optimización en

MELC, los métodos no eran de tipo interpretativo (i.e., basados en modelos). Por lo tanto, se consideró interesante desarrollar una ecuación matemática que permitiera la descripción del efecto, sobre la retención de compuestos de diversa naturaleza, de cada uno de los tres factores con los que se trabaja en MELC (concentraciones de SDS, octano y 1-butanol), considerando su interacción mutua.

El estudio se realizó utilizando dos grupos de compuestos de distinta naturaleza: parabenos y antagonistas de los receptores β-adrenérgicos. El uso de fases móviles micelares puras (i.e., sin disolvente orgánico) dio lugar a tiempos de retención extremadamente elevados, para ambas familias de compuestos, por lo que no resultaron prácticas para su análisis. Para obtener tiempos de retención suficientemente cortos para estos compuestos, fue necesario añadir un disolvente orgánico que ofreciera una elevada fuerza eluyente, tal como 1-butanol. En MELC, los tiempos de análisis de mezclas de parabenos y antagonistas de los receptores β-adrenérgicos se redujeron aún más mediante la adición de octano. Así, por ejemplo, el tiempo de análisis para parabenos y los compuestos básicos fue 5 y 6.5 min, respectivamente, utilizando una fase móvil que contenía SDS 0.10 M / 1 % de octano / 7 % de 1-butanol (MELC), y 4.4 y 5.7 min con SDS 0.18 M / 12 % de 1-butanol (MLC en presencia de una elevada cantidad de surfactante).

En la bibliografía sobre MELC, no se encontró ningún estudio riguroso sobre los intervalos de concentración de los reactivos que deben mezclarse para formar MEs estables. Para garantizar el éxito en la formación de MEs, no sólo es importante la elección de los reactivos, sino también su concentración en la fase móvil. Así, puesto que interesaba investigar la calidad de la modelización de la retención en amplios intervalos de concentración, era necesario asegurar previamente los intervalos que originaban una ME, en lugar

de una emulsión. Por lo tanto, con el objetivo de verificar la formación de un medio transparente, apto para ser utilizado en cromatografía líquida, se realizó previamente un estudio basado en la observación visual de las mezclas, en el que se establecieron los intervalos de concentraciones de SDS, octano y 1-butanol que es posible mezclar para formar una ME estable, evitando la formación de una emulsión que pudiera dañar al equipo o a la columna. El estudio mostró también el periodo de tiempo en el que las MEs permanecen estables. Tras mezclar los reactivos, se dejaron reposar las mezclas durante al menos 12 horas. Posteriormente se centrifugaron, y cuando inicialmente no dieron lugar a dos fases bien diferenciadas (i.e., una emulsión), se dejaron varias semanas en reposo para comprobar su estabilidad.

Se obtuvo un gráfico que representaba los límites de octano y 1-butanol en los que las MEs son estables, a dos concentraciones de SDS (0.10 M y 0.18 M). El gráfico de concentraciones indicó que con una cantidad relativamente baja de 1-butanol y SDS 0.10 M, y una concentración creciente de octano, las MEs son inestables, después de algunas semanas, pero al aumentar la cantidad de SDS a 0.18 M, no se observó la separación de las fases agua y octano.

A pesar del beneficio que el uso de una mayor concentración de 1-butanol podría suponer para solubilizar una mayor cantidad de octano, el límite superior de concentración de co-surfactante se limitó para evitar altas presiones, lo que podría producir daños a la columna e instrumento. Por otro lado, al añadir una cantidad baja de octano (0.2%), la separación de fases no fue visible para ninguna concentración de SDS ensayada, incluso a concentraciones muy bajas de 1-butanol. Esto indicó que el surfactante es capaz de solubilizar pequeñas cantidades de octano, sin la necesidad de co-surfactante. Finalmente, con el objetivo de preservar el rendimiento de la columna, evitar daños al instrumento y reducir el impacto ambiental, se fijó el

límite superior de 1-butanol a un 12 %, tanto en ausencia como en presencia de octano (MLC y MELC, respectivamente).

Se demostró la viabilidad de modelizar la retención en MELC, con una exactitud satisfactoria, considerando simultáneamente los tres componentes de la fase móvil (surfactante, alcohol y aceite), con errores en el intervalo entre el 1.1 y el 2.5 %. La ecuación obtenida se basó en un modelo previo que el grupo investigador propuso en 1996 para describir la retención, utilizando fases móviles micelares que contienen un co-surfactante (MLC híbrida). En general, los datos obtenidos en MELC ofrecieron un mejor ajuste del comportamiento de retención, en comparación con MLC, con errores de ajuste en el intervalo entre el 0.43 y 3.2 %. El estudio se realizó a un pH de la fase móvil ligeramente superior a 2, que se fijó con ácido trifluoroacético. Se utilizó una columna con alta tolerancia al pH (XTerra) para estos estudios, pero los resultados también son satisfactorios con fases estacionarias convencionales tamponadas a pH 3–3.5.

Al realizar el ajuste de los datos al modelo, con ayuda de la aplicación Solver de Microsoft Excel, se observó que el proceso de convergencia era problemático, ya que requería valores iniciales muy cercanos al óptimo para tener éxito. Para resolver este problema, la ecuación que describía la retención se transformó trasladando el origen a las coordenadas de la fase móvil que mostró la máxima retención (es decir, la fase con la menor fuerza eluyente). La influencia de cada modificador sobre la fuerza eluyente fue muy similar para todos los compuestos de prueba, con valores medios de 0.072, 0.119 y 0.98 para el surfactante, co-surfactante y aceite, respectivamente. Por lo tanto, el octano dio lugar a la fuerza eluyente más elevada, apreciablemente mayor que para SDS y 1-butanol. Por otro lado, el modelo propuesto para MELC reveló que, cuando se inserta octano dentro de la micela, ésta se modifica. Por

lo tanto, la interacción entre soluto y micela se modifica, como indican los valores de los parámetros del modelo, para MLC y MELC.

A pesar de que la fase móvil con SDS 0.18 M, 1 % de octano y 12 % de 1-butanol mostró los mejores resultados, en términos de tiempo de análisis, tanto para los parabenos como para los antagonistas de los receptores β-adrenérgicos, la resolución sólo fue totalmente satisfactoria para los parabenos. Para los compuestos básicos, los picos de atenolol y carteolol aparecían solapados; por lo tanto, para estos compuestos resultó más adecuada una fase móvil con menor concentración de octano (0.25 %).

4. Análisis de compuestos básicos apolares en preparados farmacéuticos

El trabajo realizado con los antagonistas de los receptores β-adrenérgicos (con un carácter básico y polaridad alta o intermedia) indicó la idoneidad de la MELC para el análisis de compuestos básicos. Tal como se ha explicado, el rendimiento cromatográfico de los compuestos básicos es muy deficiente en RPLC convencional, donde se obtienen largos tiempos de análisis y picos deformados, siendo el consumo de disolvente orgánico algo elevado. Los tiempos de retención en MLC con el surfactante aniónico SDS son también elevados, requiriéndose cantidades relativamente elevadas de disolvente orgánico para reducirlos. Esta situación es aún más problemática en el análisis de antidepresivos tricíclicos (TCAs), que son fármacos muy utilizados en la práctica médica, con un carácter básico y elevada hidrofobicidad. Por lo tanto, se pensó que el uso de MELC podría proporcionar resultados satisfactorios en estos análisis, por lo que se implementó un método analítico para el análisis de fármacos en preparados farmacéuticos que contienen TCAs, utilizando MELC con una fase móvil que contenía SDS 0.173 M, 1.42 % (*v*/*v*) de octano y

8.15 % (*v*/*v*) de 1-butanol, haciendo uso de detección UV. El método mostró ventajas respecto al tiempo de análisis y consumo de disolvente orgánico.

El grupo investigador se había interesado anteriormente en el control de estos compuestos en preparados farmacéuticos, para los que la RPLC convencional proporciona resultados muy insatisfactorios. El primer método publicado por el grupo data de 2003. Más tarde, en 2012, intentando mejorar estos análisis, se desarrollaron métodos en MLC con fases móviles acuosas de SDS y 1-pentanol, o de Brij-35 en ausencia de disolvente orgánico. De ahí, que se decidiera llevar a cabo un estudio comparativo del comportamiento cromatográfico de los TCAs en RPLC, cuando se utilizan como fases móviles mezclas hidro-orgánicas, medios micelares y MEs. En el estudio realizado, se comparó el rendimiento analítico en términos de linealidad, exactitud y precisión intra- e inter-día.

Se llevó a cabo una extensa validación, que incluyó cinco TCAs (amitriptilina, clomipramina, imipramina, maprotilina y nortriptilina) y cinco preparados farmacéuticos (cada una conteniendo un TCA), comercializados en España. Los resultados fueron muy satisfactorios, con buenas recuperaciones y una preparación de muestra muy sencilla, sin la necesidad de realizar ningún pre-tratamiento más que la solubilización de la muestra y su filtración antes de su inyección. Las recuperaciones se situaron en el intervalo del 80 al 120 %, lo que se encuentra dentro de los valores tolerados para productos farmacéuticos. Por lo tanto, el método desarrollado para MELC resultó útil para el control de calidad de preparados que contienen TCAs. Una ventaja del procedimiento de MELC es la reducción de los tiempos de retención, en comparación con RPLC convencional y MLC con SDS, incluso utilizando 1-butanol en MLC como co-surfactante. El procedimiento de MELC mantiene el perfil de los picos mejorado conseguido en MLC.

La validación del método se realizó de acuerdo con las directrices de la ICH (*International Conference of Harmonization*) y ofreció buenos resultados para los fármacos analizados:

- Las curvas de calibrado cumplieron los requisitos de linealidad, con coeficientes de determinación $R^2 > 0.990$. Las pendientes y ordenadas en el origen de las curvas de calibrado, obtenidas durante tres días no consecutivos y a lo largo de tres semanas diferentes, se mantuvieron estables, lo que indicó que el rendimiento de la columna se mantuvo con una buena capacidad de predicción de las concentraciones de los analitos, a partir de las rectas de regresión ajustadas.
- Las precisiones intra- e inter-día siempre estuvieron por debajo del 2.5 %, y las exactitudes intra- e inter-día se situaron en los intervalos entre el –0.9 % y +1.2 %, y –1.7 % y +0.5%, respectivamente.
- Los límites de detección y cuantificación se situaron, para los distintos analitos, generalmente por debajo de 0.09 µg/mL y 0.31 µg/mL, respectivamente, excepto para maprotilina, que fueron 1.15 µg/mL y 3.85 µg/mL.
- La robustez se evaluó modificando el caudal y las concentraciones de SDS, octano y 1-butanol en la fase móvil. Cada uno de estos factores se varió dentro de un intervalo en torno al valor utilizado para desarrollar el procedimiento analítico, siguiendo el método (OVAT, *one-variable-at-a-time*), donde se hacen variar los factores uno a uno, manteniendo todos los demás constantes en su valor original. La reproducibilidad (RSD) alcanzada para los tiempos de retención se situó por debajo del 2 %, correspondiendo los valores más altos a la concentración de octano, lo que confirma su

importante papel en la formación de las MEs. Se obtuvo una mayor variabilidad para las áreas de los picos.

Los resultados se compararon con los obtenidos con los procedimientos que utilizan fases móviles preparadas con 35 % (*v*/*v*) de acetonitrilo, SDS 0.075 M / 6 % (*v*/*v*) de 1-pentanol y Brij-35 0.02 M sin disolvente orgánico. La precisión más satisfactoria correspondió a los procedimientos de MELC y micelar con SDS y 1-pentanol, con valores generalmente por debajo del 2 %. Mientras tanto, para los modos hidro-orgánico y micelar puro con Brij-35, la precisión inter-día osciló entre el 0.65 % y 3.1 %. Los límites de detección y cuantificación fueron más bajos con el procedimiento de MELC, excepto para amitriptilina y maprotilina, para los que se obtuvieron valores más bajos con el procedimiento hidro-orgánico.

5. Interacciones de los solutos en sistema cromatográficos que utilizan surfactante y líquido iónico

Idealmente, el mejor disolvente es utilizar sólo agua en ausencia de disolvente orgánico, si se consideran los peligros para la salud, la generación de residuos y la economía. Sin embargo, la ausencia de disolvente orgánico no siempre es posible. Por ello, se han propuesto disolventes más ecológicos para sustituir a los disolventes orgánicos empleados convencionalmente, con el fin de disminuir el impacto medioambiental y el riesgo de exposición química. En este sentido, los líquidos iónicos, constituidos por sales frecuentemente en estado líquido a temperatura ambiente, conteniendo un catión orgánico voluminoso asociado a un anión inorgánico u orgánico generalmente más pequeño, han llamado mucho la atención en diversos campos científicos y tecnológicos.

En el campo de la MELC, es relevante la aparición de un artículo publicado por Peng *et al.* en 2017, en el que se propone el análisis de ácidos fenólicos neutros, utilizando una fase móvil con SDS y 1-butanol, en la que se reemplaza el octano como disolvente apolar por un líquido iónico (IL, *ionic liquid*) inmiscible en agua (hexafluorofosfato de 1-hexil-3-metilimidazolio, $[C_6C_1IM][PF_6]$). Cabe señalar que, en la bibiografía consultada, este tipo de ME suele contener un surfactante no iónico, tal como Brij-35 o Triton X-100, en lugar del surfactante aniónico SDS.

Como se ha comentado, durante el trabajo de Tesis Doctoral se investigó el análisis de un grupo de fármacos básicos catiónicos (antagonistas de los receptores β-adrenérgicos), utilizando una ME formada por la mezcla de SDS, octano y 1-butanol. A la vista de los resultados de Peng *et al.*, se consideró interesante evaluar la viabilidad de este tipo de MEs preparadas con IL, para evitar el uso de octano en estos análisis. El trabajo se inició utilizando el IL $[C_6C_1IM][PF_6]$ en la ME, pero la investigación se extendió a continuación a otros ILs de imidazolio con cadenas de alquilo de diversas longitudes (n = 2, 4 y 6), asociados a los aniones Cl^-, BF_4^-, o PF_6^-. Estos ILs poseen diversa solubilidad y capacidad de adsorción sobre las fases estacionarias de C18, y son los más comúnmente utilizados como aditivos en las fases hidro-orgánicas utilizadas en RPLC. El estudio permitió profundizar en el conocimiento sobre el efecto del catión y anión de un IL sobre el sistema cromatográfico, en presencia de aditivos iónicos, en base al comportamiento de retención y los perfiles de pico observados para los fármacos catiónicos analizados. Los resultados se interpretaron comparándolos con los obtenidos con fases móviles que contenían IL sin SDS, y acetonitrilo en lugar de 1-butanol como disolvente orgánico.

Los gráficos de retención, frente a la concentración de aditivo, mostraron que en las MEs estudiadas, el surfactante aniónico SDS compite con los aniones que forman el IL por la adsorción sobre la columna cromatográfica. El comportamiento observado (retención decreciente al aumentar la concentración del IL) es similar al encontrado en ausencia de SDS, para ILs formados por un catión fuertemente adsorbido asociado a un anión débilmente adsorbido, como es el caso de $[C_6C_1IM][BF_4]$ y $[C_6C_1IM][Cl]$. Por su parte, en ausencia de SDS, un IL con un catión que muestra una baja adsorción o un anión más fuertemente adsorbido, la retención se mantiene constante o se incrementa, dando lugar a un máximo a una concentración particular de IL, que depende de su naturaleza. Sin embargo, en todas las fases móviles con IL, los perfiles de los picos de los compuestos básicos mejoraron, en comparación al uso de mezclas hidro-orgánicas en RPLC, dando lugar a picos simétricos (o casi simétricos), con un efecto más intenso en presencia de SDS. La mejora de los perfiles de pico se explica por el enmascaramiento del efecto silanol por parte de los iones de los aditivos.

Cuando se reemplaza el octano por un IL, la presencia de 1-butanol es menos importante para la formación de mezclas transparentes y estables con SDS, útiles para RPLC. Además, el surfactante permite la obtención de disoluciones más concentradas de los ILs, lo que sugiere la formación de una estructura organizada de SDS / IL en la fase móvil. Puesto que el 1-butanol dio lugar a picos poco retenidos y una baja resolución para los compuestos básicos, se investigó la posibilidad de eliminarlo. En efecto, una fase móvil compuesta únicamente por el surfactante dodecilsulfato sódico (SDS) y el IL $[C_6C_1IM][Cl]$ dio lugar a resultados prometedores, con perfiles de pico satisfactorios, buena resolución y retención adecuada para los compuestos estudiados. Cabe señalar que, en estas mezclas, la retención de los compuestos básicos se puede modular

para alcanzar valores prácticos, modificando las concentraciones de SDS e IL, en base a la atracción de los compuestos básicos catiónicos por el anión del surfactante y la repulsión por el catión del IL, y sin la necesidad de añadir un disolvente orgánico. Esto puede dar lugar a una interesante “fase móvil verde”. Se espera que el comportamiento de retención dependa de la relación de concentraciones del IL y surfactante en la fase móvil, así como de la naturaleza del IL. Debe indicarse que una fase móvil con sólo IL requiere una cierta cantidad de disolvente orgánico para obtener tiempos de análisis adecuados y la retención en fases móviles que contienen únicamente SDS es excesiva. Es de esperar que el comportamiento de retención dependa de la relación de concentraciones del IL y tensioactivo en la fase móvil, así como de la naturaleza del IL añadido.

En la bibliografía, ha habido un gran interés en la síntesis de ILs con comportamiento de surfactante, en los que el catión del IL está asociado a un anión de un surfactante convencional. Se obtiene un ambiente similar en disolución acuosa con la mezcla ensayada del cloruro de alquil-imidazolio y la sal sódica de dodecilsulfato (SDS). La micela formada, posiblemente se halle compuesta por empalizadas alternas del catión del IL y anión del surfactante. Cabe señalar que no hay estudios previos publicados sobre el uso de esta combinación de aditivos en RPLC.

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OBJECTIVES AND
DEVELOPMENT OF THE RESEARCH

Microemulsion Liquid Chromatography (MELC) is a relatively new Reversed-Phase Liquid Chromatographic (RPLC) mode that utilises a microemulsion (ME) as mobile phase. This gives rise to a particular mechanism of retention and selectivity, and allows the solubilisation of compounds in a wide range of polarities. Unfortunately, the technique has a few issues, such as those associated to the high backpressure because of a more viscous mobile phase, the requirement of a more careful column care, the adsorption of surfactant on the stationary phase, and the need to select a suitable organic solvent and its composition to form stable ME droplets. It is fundamental to improve the knowledge on the appropriate composition of MEs as mobile phases, and on their mechanism of retention, and achieve the possibility of modelling their behaviour, since this will help the development of useful analytical methodologies, particularly in the field of the analysis of pharmaceuticals, physiological fluids and other biological materials. The PhD. work collected in this Project offers some proposals in this regard.

The supervisors of this PhD. have studied since 1995, in depth, the possibility of using micellar solutions with conventional surfactants of different character, as mobile phases in liquid chromatography, with a large number of publications in the so-called Micellar Liquid Chromatography (MLC) mode. The aim of this work is applying the gathered knowledge in MLC to MELC. The particular objectives are next summarised:

Objective 1. *Analysis of previous knowledge in MELC*

With this PhD. work, the research group has been initiated in the research in MELC. Therefore, an exhaustive literature survey and deep study of the previous knowledge was first needed. Although there were some published review articles, it was soon evident that there were wrong ideas with respect to the use

of microemulsions in liquid chromatography, still reported. Therefore, the published information should be studied very thoroughly, compared and organised. The purpose was to write a guide for future researchers.

Objective 2. *Working ranges and cares in MELC*

Despite the previous work that has been published in MELC, it has been considered necessary to investigate the working ranges that make the formation of suitable MEs for liquid chromatography possible. The study of published reports and the careful work made in the laboratory along this work, with these mobile phases, should give some new light on their management.

Objective 3. *Study of the behaviour of basic compounds in MELC*

Basic compounds give rise to cationic protonated species, in the usual pH range used in liquid chromatography, which causes broad and asymmetric peaks due to their interaction with the negatively charged silanol groups that exist free in conventional alkyl-bonded stationary phases. The peak profile can be improved by adding diverse reagents, such as some surfactants or ionic liquids. In previous work, the supervisors of this PhD. work demonstrated that the anionic surfactant SDS improves the peak profile when added to the mobile phase, due to coating of the stationary phase, which masks the activity of silanols. However, this surfactant has the drawback of attracting cationic solutes, especially towards the stationary phase, resulting in long retention times. This forces the addition of a large amount of organic solvent to the mobile phase to increase the elution strength.

In this PhD. Project, we demonstrate that it is possible to decrease the amount of organic solvent in the mobile phase through the use of MELC, using mobile phases of SDS, octane and 1-butanol. In this way, a less polluting method is

obtained, which keeps the advantage of allowing very short retention times. It has been considered of interest to carry out detailed comparative studies of MELC with regard to other surfactant-related RPLC modes (MLC and High Submicellar Liquid Chromatography, HSLC).

Objective 4. *Modelling the chromatographic behaviour in MELC*

Modelling of retention, as a function of the composition of the mobile phase, is a common task in chromatographic practice, and is of big importance in liquid chromatography to be able to find the optimal separation conditions using interpretive optimisation methods, and understand the retention mechanism of the eluted compounds. The research group to which the supervisors of this PhD. work belong have previous experience in the modelling of retention in conventional chromatography with hydro-organic mobile phases, as well as in MLC and related techniques, with excellent results. Although there is some background in the field of optimisation in MELC, the is no proposal of a global model considering the three reagents used in MELC, simultaneously. Therefore, one of the purposes of this work is to develop a mathematical equation that describes the effect of each of the three factors that influence the MELC mobile phase (in this work, the concentrations of SDS, octane and 1-butanol), on the retention of compounds of different nature. Throughout the work, it will also be shown that the peak profile can be modelled. It is intended to show the advantages of such modelling.

Objective 5. *Exhaustive validation of an analytical procedure for the analysis of basic compounds in MELC*

After the above fundamental studies, it was considered convenient to develop an analytical method for the analysis of non-polar basic compounds, such as tricyclic antidepressants, whose determination has presented major drawbacks in conventional hydro-organic liquid chromatography and MLC. The method was applied to the analysis of pharmaceutical preparations and has involved an extensive validation.

Objective 6. *Studying the performance of MEs where the non-polar solvent is replaced by an ionic liquid*

In the last decades, ionic liquids (ILs) have attracted attention in liquid chromatography as mobile phase additives. The supervisors of this PhD work have been involved in a research, where the mechanism of retention using imidazolium-based ILs added to hydro-organic mobile phases has been studied in detail. Recently, such ILs have been used to replace the non-polar solvent in the MELC mobile phase to analyse neutral phenolic compounds. It was considered of interest to study the performance of aqueous IL-based MEs in the analysis of cationic solutes, and increase the knowledge on their interactions inside a modified column with ionic additives.

In general, the shown reports have implied a large experimental effort, designed to explore and extract information on the chromatographic behaviour of compounds of different nature (11 β-adrenoceptor antagonists, 5 tricyclic antidepressants and 5 parabens). A wide diversity of experimental conditions has been assayed, using aqueous mobile phases containing acetonitrile- or 1-propanol in conventional RPLC, SDS and acetonitrile or 1-propanol in MLC and HSLC, and SDS, octane or IL, and 1-butanol, in MELC.

Supervisors and research laboratory

The research work leading to the PhD. degree in Chemistry was started in November 2017. A Master degree on "Analytical Chemistry" was previously studied at the Sardar Patel University in India, offered by the Department of Analytical Chemistry.

The experimental work along the PhD. period was developed in the Department of Analytical Chemistry at the University of Valencia, under the supervision of Professors María Celia García Álvarez-Coque and María José Ruiz Ángel.

Publications

The publications included in this PhD. work 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, Nikitaben Pankajkumar Patel, Samuel Carda Broch, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Oil-in-water Microemulsion Liquid Chromatography
   Separation and Purification Reviews 49 (2020) 89–111.
   IF (2019): 5.324 (Analytical Chemistry: 12/86; 1st quartile) (Chapter 1).
2. Nikitaben 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
   Journal of Chromatography A 1592 (2019) 91–100.
   IF (2019): 4.049 (Analytical Chemistry: 14/86; 1st quartile) (Chapter 2).

3. Nikitaben Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Comparison of surfactant-mediated liquid chromatographic modes with sodium dodecyl sulphate for the analysis of basic drugs
   Analytical Methods 12 (2020) 2443–2452.
   IF (2019): 2.596 (Analytical Chemistry: 38/86; 2nd quartile) (Chapter 3).

4. Nikitaben Pankajkumar Patel, Ester Peris García, Olga Schiopu, María José Ruiz Ángel, Juan José Baeza Baeza, María Celia García Álvarez-Coque
   Performance and modelling the retention in Microemulsion Liquid Chromatography
   Journal of Chromatography A 1634 (2020) 461651
   IF (2019): 4.049 (Analytical Chemistry: 14/86; 1st quartile) (Chapter 4).

5. Nikitaben Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Analysis of tricyclic antidepressants in pharmaceuticals by Microemulsion Liquid Chromatography
   Microchemical Journal 160 (2020) 105659.
   IF (2019): 3.594 (Analytical Chemistry: 19/86; 1st quartile) (Chapter 5).

6. Nikitaben Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Solute interactions in Microemulsion Liquid Chromatography with ionic liquids

   In preparation (2021) (Chapter 6).

Congress communications

The developed research has been also presented in 4 scientific conferences in the period June 2018 to November 2020 (3 communications in an international conference and 3 in Spanish conferences, all as posters). The PhD. candidate has attended to one of these conferences: SEQA´2019 in Valladolid.

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

1. Ester Peris García, Nikitaben Pankajkumar Patel, Samuel Carda Broch, María José Ruiz Ángel, María Celia García Álvarez-Coque
   Microemulsion Liquid Chromatography: Main features and applications
   (Poster P108)

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

2. Ester Peris García, Nikitaben 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)
3. Nikitaben 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)

4. Nikitaben 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)

27th International Symposium on Electrophoretic and Liquid Phase Separation Techniques (ITP'2020), Virtual Conference, Nanjing (China), November 2020 (international)

5. María José Ruiz Ángel, Nikitaben Pankajkumar Patel, Ester Peris García, María Celia García Álvarez-Coque
   Retention and peak shape in RPLC with microemulsion for basic drugs
   (Poster)

6. María José Ruiz Ángel, Nikitaben Pankajkumar Patel, Ester Peris García, María Celia García Álvarez-Coque
   Modelling of retention in Microemulsion Liquid Chromatography (MELC)
   (Poster)

The research was funded by three national Research Projects:

1. Project CTQ2016-75644-P: *"Design of methodologies to optimise the separation quality in liquid chromatography"*, funded by the Ministry of Economy, Industry and Competitiveness (Spain), January 2017–December 2019. 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 PID2019-106708GB-I00: *"Fundamental studies and design of strategies in one- and two-dimensional chromatography to enhance the separation performance for complex samples"*, funded by Ministry of Science, Innovation and Universities (Spain), January 2020–December 2022. Main researchers: María Celia García Álvarez-Coque and José Ramón Torres Lapasió.

CHAPTER 1

OIL-IN-WATER
MICROEMULSION LIQUID CHROMATOGRAPHY

1.1. Abstract

Oil-in-water microemulsions (O/W MEs) are obtained spontaneously by mixing two immiscible liquids (water and oil), in the presence of a surfactant. A co-surfactant is also often needed for ME stabilisation. The surfactant provides a microstructure with a definite boundary between oil and water phases. O/W MEs are used as mobile phases in a chromatographic mode known as microemulsion liquid chromatography (MELC). One of the main appeals of O/W MEs is the ability to solubilise compounds in a wide range of polarities, from polar to hydrophobic. The solubilising effect on sample matrices is also noteworthy. The dual behaviour of O/W MEs offers unique selectivity and reduced retention times, with equivalent or superior efficiency compared to conventional reversed-phase liquid chromatography, giving rise to successful isocratic separations. The complex nature of MELC mobile phases allows numerous composition options (type and concentration of surfactant, oil and co-surfactant) that lead to good separation performance, when compared to other chromatographic modes. A thorough revision of the main topics concerning MELC, such as nature and properties of O/W MEs, mechanism of retention, selectivity and diverse aspects related to the experimental practice for the determination of drugs in clinical and pharmaceutical samples, is presented.

1.2. Introduction

The mobile phase nature governs the separations in reversed-phase liquid chromatography (RPLC) [1,2]. Although it is most often composed of a mixture of water and organic solvent, the addition of different reagents as modifiers has demonstrated to solve problems related to low or high retention, inappropriate selectivity, or poor efficiency, among others [3]. Armstrong and Henry [4] and other authors [5–7] have demonstrated that a micellar solution of surfactant can enhance some separations and reduce the organic solvent consumption in RPLC. Surfactants of different character (non-ionic, anionic, cationic, and even zwitterionic) have been used in RPLC as additives. Particularly interesting is the feasibility of performing the direct injection of physiological samples using anionic surfactants [5,6,8–10]. Owing to the presence of micelles in the mobile phase, the technique was referred as Micellar Liquid Chromatography (MLC). A capillary electrophoretic technique termed Micellar Electrokinetic Chromatography (MEKC), which employs a micellar solution as the running buffer, was developed parallel to MLC [11,12]. Hundreds of successful applications have been reported for both MLC and MEKC. However, some problems with the analysis of samples containing highly non-polar compounds still remained, which should be solved. This is the reason why the use of microemulsions (MEs) was explored based on their ability to solubilise and extract water-insoluble species.

MEs consist of a micellar phase surrounded by either an aqueous (oil in water MEs) or an organic (water in oil MEs) phase. In 1986, Hernandez-Torres *et al.* [13] first proposed water-in-oil (W/O) MEs as mobile phases for Normal Phase Liquid Chromatography (NPLC). These were composed of reversed micelles of sodium bis(2-ethylhexyl) sulphosuccinate (Aerosol OT or AOT) and water dispersed in hexane. After making a detailed work on the use of MEs containing

AOT and hexane using an unbonded silica column [14], Berthod *et al.* reported in 1992 the application of oil-in-water (O/W) MEs in RPLC with a C18 column, prepared by adding a small amount of heptane to a mixture of micelles of sodium dodecyl sulphate (SDS), 1-pentanol and water [15]. These reports gave birth to a new chromatographic mode, which is referred as Microemulsion Liquid Chromatography (MELC). One year before, Watarai had proposed the use of MEs in capillary electrophoresis, instead of micelles [16] that gave rise to Microemulsion Electrokinetic Chromatography (MEEKC) [17]. MEEKC underwent quickly great development, with multiple applications. In contrast, it was not until 10–12 years later that major interest appeared in MELC [18,19]. Owing to this later development, MELC took advantage of the previous knowledge gathered in MLC and MEEKC. Reviews describing the basis and applications of MELC were published in 2005 [20], 2007 [21], and 2008 [22].

Most reported applications in MELC refer to the analysis of drugs, both in pharmaceuticals (Table 1.1) [23–48], and in physiological fluids and other biological material (Table 1.2 and Figure 1.1) [49–62]. The number of reports is still limited, but this chromatographic mode seems promising and is receiving growing attention. This chapter updates the knowledge on MELC, and organises and describes in detail the different factors that should be considered to obtain successful separations. Some orientation on the experimental practice and field of application of this chromatographic mode is also offered.

Table 1.1. Experimental characteristics of MELC procedures for the analysis of pharmaceuticals.

Compounds	Sample (number of analytes) / Stationary phase / Mobile phase / Elution mode / Temperature	Ref.
Analgesics and related products: 2-acetamidophenol, 4-aminophenol, capsaicin, 4-chloroacetonilide, 4-hydroxyacetophenone, 4-nitrophenol, and paracetamol	Suppositories (6) / Symmetryshield C18 (150 × 4.6 mm i.d, 3.5 μm) / 33 g SDS, 66 g 1-butanol, 8 g octane, 0.05 % TFA in 3 % acetonitrile at pH 2.8 / gradient / 50 ºC	[23]
	Suppositories (6) / Symmetryshield C18 (150 × 4.6 mm i.d, 3.5 μm) / 41.6 g CTAB, 66 g 1-butanol, 8 g octane–0.05 % TFA, pH 2.8 / isocratic and gradient elution / 50 ºC	[24]
	Liniments (1) / Grace Smart RP 18 (150 × 4.6 mm i.d.) and Onyx monolithic C18 (100 × 3 mm) / 3.3 % SDS, 8 % 1-butanol, 1 % heptane, 0.05 % TFA / isocratic / room temperature	[25]
Antibiotics: norfloxacin and tinidazole	Tablets and eye drops (2) / Symmetry C18 (150 × 4.6 mm i.d., 5μm) / 3.5 % SDS, 10 % 1-propanol, 0.5 % octanol, 0.3 % trimethylamine (TEA) in 0.02 M phosphoric acid at pH 6.5 / isocratic / 25 ºC	[26]
Antihistamines: desloratidine and loratidine	Tablets and syrups (2) / Hibar-Lichrosorb cyanopropyl (250 × 4.6 mm i.d., 5 μm)/ 0.1 M SDS, 10 % 1-propanol, 1 % octanol, 0.3 % TEA in 02 M phosphoric acid at pH 3 / isocratic / 25 ºC	[27]
Antihypertensives: fosinoprilat and sodium fosinopril	Tablets (2) / XTerra C18 (50 × 4.6 mm i.d., 3.5 μm) / 2.2 % SDS, 8.0 % 1-butanol, 0.9 % cyclohexane, 88.9 % 25 mM disodium phosphate at pH 2.8 / isocratic / 30 ºC	[19]
Antihypertensives: enalapril, hydrochlorothiazide, irbesartan, losartan, and perindopril tert-butylamine	Tablets (5) / Nucleosil C4 (250 × 4.6 mm i.d., 5 μm) / 2.0 % SDS, 7.75 % 1-butanol, 0.24 % butyl acetate, 0.3 % ethyl acetate, water at pH 3.7 / isocratic / 40 ºC	[28,29]
	Tablets (2) / Cyano (150 × 4.6 mm i.d., 5 μm) / 0.2 M SDS, 10 % 1-propanol, 1 % octanol, 0.3 % TEA, 0.02 M phosphoric acid at pH 3.5 / isocratic / 25 ºC	[30]
	Tablets (2) / Grace Smart C18 (150 × 4.6 mm i.d., 5 μm) / 95 % (*v/v*) of 3.0 % SDS, 6.0% 1-butanol, 0.8 % octane, 90.2 % water and 5 % acetonitrile at pH 5 / isocratic / 30 ºC	[31]
	Tablets (2) / Cyano (150 × 4.6 mm i.d., 5 μm) / 0.2 M SDS, 10 % 1-propanol, 1 % octanol, 0.3 % TEA, 0.02 M phosphoric acid / isocratic / room temperature	[32]

Table 1.1 (continued).

Compounds	Sample (number of analytes) / Mobile phase / Stationary phase / Elution mode / Temperature	Ref.
Anti-inflammatories: ibuprofen	Oral suspension (1) / Eurospher II C18 (150 × 4.6 mm i.d., 5 μm) / 5.0 g SDS, 6.6 g 1-butanol, 0.8 g heptane, 0.05 % TFA at pH 3.4 / isocratic / 25 ºC	[33]
Bronchodilators: salbutamol and terbutaline	Inhaler (1) / Spherisorb C18 (250 × 4.6 mm i.d., 5 μm)/ 1.5 % Brij-35, 2.5 % 1-butanol, 0.5 % ethyl acetate, 95.5 % 20 mM orthophosphate buffer at pH 3 / isocratic / 25 ºC	[34,35]
Calcium channel blockers: felodipine and nifedipine	Capsules and tablets (2) / Spherisorb C18 (250 × 4.6 mm i.d., 5 μm) / 4.5 % SDS, 7.6 % 1-butanol, 0.8 % octane, 87.1 % 15 mM orthophosphate buffer at pH 3 / isocratic / 30 ºC	[36]
Cathecolamines: dopamine	Tablets (1) / XTerra C18 / 33 % SDS, 1.0 % 2-propanol, 66 % ethyl acetate, 0.2 % TFA / isocratic / 30 ºC	[37]
Cholesterol lowering drugs: acetate ester, anhydrosimvastatin hydroxy acid, ezetimibe, simvastatin, lovastatin, methyl-simvastatin, simvastatin-dimer	Tablets (7) / X-Terra C18 (50 × 4.6 mm i.d., 3.5 μm) / 1.7 % SDS, 7.0 % 1-butanol, 0.9 % diisopropyl ether, 90.4 % 25 mM di-sodium phosphate at pH 7.0 / 30 ºC	[38]
	Tablets (2) / Cyano (150 × 4.6 mm i.d., 5 μm) / 0.2 M SDS, 10 % 1-propanol, 1 % octanol, 0.3 % TEA, 0.02 M phosphoric acid at pH 5 / isocratic / 25 ºC	[39]
Diuretics: bumetanide	Tablets (1) / Hypersil silica (100 × 4.6 mm i.d., 5 μm) / 8.3 % SDS, 16.6 % 1-pentanol, 70 % heptane, 5 % 70 mM sodium acetate at pH 7.5 / isocratic / 25 ºC	[40]
Flunarizine and five degradation products	Capsules (6) / Lichrosorb cyanopropyl (250 × 4.6 mm i.d., 5 μm) / 0.15 M SDS, 10 % 1-propanol, 1 % octanol, 0.3 % TEA, 0.02 M phosphoric acid at pH 6.8 / isocratic / 25 ºC	[41]

Table 1.1 (continued).

Compounds	Sample (number of analytes) / Stationary phase / Mobile phase / Elution mode / Temperature	Ref.
Parabens and diverse drugs: beclamethasone, butylparaben, caffeine, ethylparaben, methylparaben, oxibendazole, paracetamol, and propylparaben	Tablets (5) / SymmetryShield C18 (150 × 4.6 mm i.d., 5 µm) and Luna C18 (100 × 4.6 mm i.d., 3 µm) / 3.3 % SDS, 6.6% 1-butanol, 0.8 % octane, 0.05 % TFA / isocratic / 60 ºC	[42]
	Tablets and nicotine lozenges (5) / Chromolith RP 18 (100 × 4.6 mm i.d.) / 3.3 % SDS, 6.6 % 1-butanol, 0.8 % octanol, 0.05 TFA / isocratic and gradient elution / 60 ºC	[43]
Steroids: boldenone, clostebol acetate, dydrogesterone, methandrostenolone, medroxyprogesterone, medroxyprogesterone acetate, methenolone enanthate, methyltestosterone, nandrolone, nandrolone decanoate, progesterone, stanozolol, testosterone enanthate, and testosterone propionate	Tablets, injections, suspensions and gels (14) / Spherisorb ODS-2 (120 × 4.6 mm i.d., 5 µm) / 0.1 M SDS, 7 % 1-pentanol / isocratic / room temperature	[44]
	Tablets (6) / Venusil ASB C18 (250 × 4.6 mm i.d., 5 µm) / 30 % SDS, 6.6 % 1-butanol, 0.8 % octane / isocratic / 35 ºC	[45]
Vitamins and other drugs: L-ascorbic acid (vitamin C), cholecalciferol (vitamin D_3), cobalamin (vitamin B12), folic acid, nicotinamide, phylloquinone (vitamin K_1), pyridoxine (vitamin B6), retinol palmitate (vitamin A), rivoflavin (vitamin B2), thiamine (vitamin B1), α-tocopherol acetate (vitamin E)	Syrup (4) /Zorbax Eclipse XDB C8 (150 × 4.6 mm i.d., 5 µm) / 73.6 mM SDS, 13.6 % 1-butanol, 0.48 % diethyl ether, 0.02 M phosphate buffer at pH 7 / isocratic / 32.5 ºC	[46]
	Tablets (4) / Venusil ASB C18 (150 × 4.6 mm i.d., 5 µm) / 98 % *v*/*v* of 50 % SDS, 10 % 1-butanol, 1.0 % octane, 2 % acetonitrile, 84 % water / isocratic / 40 ºC	[47]
	Pharmaceuticals and food (7) / TC C18 (250 mm × 4.6 mm, 5 µm) / 2 % SDS, 60 % Brij-35, 66 % 1-butanol, 80 % ethyl acetate, water / isocratic / 30 ºC	[48]

Table 1.2. Experimental characteristics of MELC procedures for the analysis of physiological fluids and biological material.

Compounds	Sample (number of analytes) / Stationary phase / Mobile phase / Elution mode / Temperature	Ref.
Alkaloids: berberine, coptisine, epiberberine, jatrorrhizine, and palmatine	Herbs (5) / Zorbax SB C18 (50 × 4.6 mm i.d., 1.8 μm) / 1.0 % SDS, 8.0 % 1-butanol, 0.8 % ethyl acetate, 0.1 % acetic acid, 10 % acetonitrile / isocratic / 35 ºC	[49]
Anesthetic: propofol	Human plasma (1) / Hypersil C18 / 3.0 % SDS, 0.8 % heptane, 6.0 % 1-butanol–0.5 % acetic acid / isocratic / room temperature	[50]
Antihypertensives: cilazapril, fosinoprilat, and fosinopropil.	Human plasma (2) / Bakerbond ENV C18 (150 × 4.6 mm i.d., 5 μm)/ 2.0 % SDS, 6.0 % 1-propanol, 1.0 % diisopropyl ether, 91.0 % 25 mM di-sodium hydrogen phosphate at pH 2.8 / isocratic / 25 ºC	[51]
Antihypertensives: hydrochlorothiazide, and irbesartan	Human plasma (2) / Cyano (150 × 4.6 mm i.d., 5 μm) / 0.2 M SDS, 10 % 1-propanol, 1 % octanol, 0.3 % TEA, 0.02 M phosphoric acid / isocratic / room temperature	[32]
Antihypertensives, diuretics and non steroidal anti-inflammatories: acebutolol, atenolol, bumetanide, furosemide, ibuprofen, nadolol, naphthalene, naproxen, and timolol	Plasma and urine (9) / Apex ODS C18 (120 × 4.6 mm i.d., 5 μm) / 2 % SDS, 10 % 1-butanol, 1 % octanol, 0.3 % TEA, 0.02 M phosphoric acid / isocratic / room temperature	[18]
Bronchodilators: terbutaline	Urine (1) /Spherisorb C18 (250 × 4.6 mm i.d., 5 μm) / 1.5 % Brij-35, 2.5 % 1-butanol, 0.5 % ethyl acetate, 1.1 % octanesulfonic acid, 94.4 % orthophosphate at pH 3 / isocratic / 30 ºC	[52]
Cholesterol lowering drugs : simvastatin and simvastatin acid	Human plasma (1) / C18 (150 × 4.6 mm i.d., 3.5 μm) / 1.0 % SDS, 4.0 % 1-butanol, 0.5 % diisopropyl ether, 94.5 % 25 mM disodium hydrogen phosphate at pH 7.0 / isocratic	[53]

Table 1.2 (continued).

Compounds	Sample (number of analytes) / Stationary phase / Mobile phase / Elution mode / Temperature	Ref.
Diverse drugs: Caffeic acid, cryptotanshinone, sodium danshensu, protocatechualdehyde, rosmarinic acid, salvianolic acid B, salvianolic acid C, tanshinone I, tanshinone II; acetaminophen, acetone, acetophenone, anisole, benzene, benzyl alcohol, caffeine, chloramphenicol, dexamethasone, diazepam, ethylbenzene, hydrocortisone, hydrocortisone-21-acetate, naphthalene, nifedipine, nitrobenzene, *m*-nitroaniline, *p*-nitroaniline, 2-octanone, 3-pentanone, phenol, prednisolone, prednisone, progesterone, propiophenone, resorcinol, and toluene	Danshen root (9) and standards (27) / Chromolith RP-18 (100 × 4.6 mm i.d., 2 μm) , Venusil ASB C8 (200 × 4.6 mm i.d., 5 μm), Odyssil C18 (150 × 4.6 mm i.d., 5 μm), Odyssil C18 (250 × 4.6 mm i.d., 5 μm) / 6.0 % Brij-35, 6.6 % 1-butanol, 0.8 % cyclohexane, 8 mM CTAB, 86.6 % sodium dihydrogen phosphate / isocratic / 30 ºC	[54]
Diverse drugs: Caffeic acid, cryototanshinone, lithospermic acid, procatechualdehyde, procatechuic acid, rosmarinic acid, sodium danshensu, salvianolic acid B, salvianolic acid C, tanshinone I, and tanshinone II A	Herbs (9) / Venusil ASB C8 (200 × 4.6 mm i.d., 5 μm), Chromolith RP 18 (100 × 4.6 mm i.d., 2 μm), Odyssil C18 (150 × 4.6 mm i.d., 5 μm), Odyssil C18 (250 × 4.6 mm i.d., 5 μm) / 6.7 % Brij-35, 6.9 % 1-butanol, 0.84 % cyclohexane, 8 mM CTAB, 85.6 % phosphate buffer at pH 6.6 / isocratic / 30 ºC	[55]
	Herbs (8) / Zorbax SB C18 (150 × 4.6 mm i.d., 5 μm) / 1.0 % SDS, 3.0 % 1-butanol, 0.2 % HMIM·PF6, 95.8 % water at pH 2.5 / isocratic / 30 ºC	[56]
Dopamine receptor antagonist: LE300, N-methyl metabolite	Mouse serum (2) / RP C18 Chromolith (100 × 4.6 mm i.d.) / 4.0 % SDS, 13.0 % 2-propanol, 1.0 % diisopropyl ether, 50 mM potassium dihydrogen phosphate at pH 3.5 / isocratic / 25 ºC	[57]

Table 1.2 (continued).

Compounds	Sample (number of analytes) / Mobile phase / Stationary phase / Elution mode / Temperature	Ref.
Flavonoids: astragalin, bergenin, hyperoside, isoquercitrin, quercetin, quercetin-3-O-sophoroside rutin, vitexin, and vitexin-2″-O-rhamnoside	Leaf extracts (7) / Zorbax Extend C18 (100 × 4.6 mm i.d., 3.5 μm) / 1.2 % Genapol X-080, 2.5 % 1-butanol, 0.5 % ethyl acetate, 95.8 % 20 mM phosphoric acid at pH 6.0 / isocratic / 25 ºC	[58]
	Leaf extracts (4) / Venusil ASB C18 (150 × 4.6 mm i.d., 5 μm) / 1.0 % Brij-35, 1.1 % 1-butanol, 0.1 % octanol , 0.3 % TEA, phosphoric acid at pH 2.5 / isocratic	[59]
Laxative: emodin	Rat serum (1) / Hypersil C18 (150 × 4.6 mm i.d., 5 μm) / 3.3 % SDS, 6.6 % 1-butanol, 1.0 % octane, water / isocratic	[60]
Phenylethanoid glycosides: acteoside, *p*-coumaric acid, echinacoside, isoacteoside, and tubuloside B	Rat plasma (5) / Zorbax Extend C18 (100 × 4.6 mm i.d., 3.5 μm) / 1.5 % Genapol X-080, 2.5 % 1-propanol, 0.8 % ethyl acetate, 0.3 % TEA, 20 mM phosphoric acid at pH 6.0 / isocratic / 25 ºC	[61]
Tetracycline antibiotics: doxycycline, oxytetracycline, and tetracycline	Milk (3) / Zorbax Eclipse XDB C18 (150 × 4.6 mm i.d., 5 μm) / 2 % SDS, 6 % 1-butanol, 0.6 % heptane, 0.01 M borate at pH 7.8 / isocratic / 50 ºC	[62]

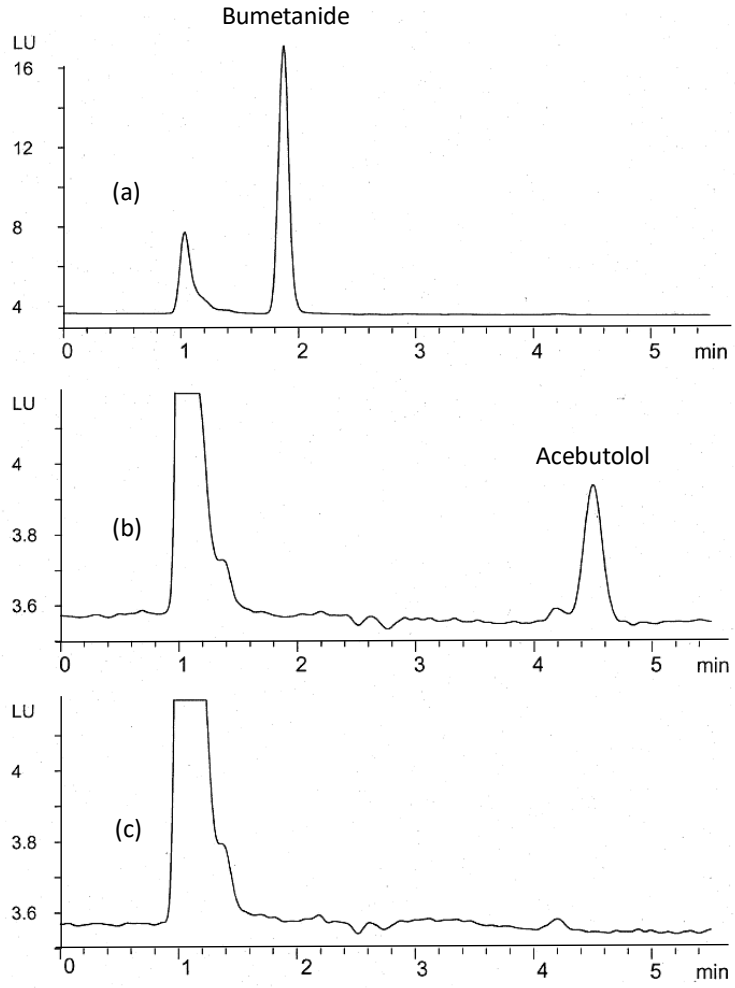


Figure 1.1. Chromatograms of plasma samples spiked with 5 μg/mL bumetanide (a), and acebutolol (b), and chromatogram of blank plasma (c). Experimental conditions: Zorbax Eclipse XDB-C8 column (150 × 4.6 mm i.d., 5 μm), 2 % SDS / 10 % 1-butanol / 1 % 1-octanol / 0.3 % TEA in 0.02 M phosphoric acid, 1 mL/min flow-rate, fluorescence detection with excitation and emission wavelengths of 344 and 431 nm, respectively (Adapted from El-Sherbiny *et al.* [18]).

1.3. Nature of microemulsions

MEs are obtained spontaneously by mixing two liquids (oil and water) with limited mutual solubility, in the presence of a micelle-forming surfactant. The oil and water components are totally immiscible. Without the surfactant, two phases would be formed. In the presence of surfactant, organised, macroscopically homogeneous and thermodynamically stable liquid systems are obtained [63–66]. The surfactant provides a microstructure with a definite boundary between the oil and water phases. Conventional surfactant molecules contain a polar head group and a non-polar tail with larger volume (particularly, for ionic surfactants). When incorporated into immiscible mixtures of oil and water, an interface film that separates the oil phase from the continuous aqueous phase is formed.

There are three types of MEs: O/W MEs (known as L1 phases), W/O MEs (known as L2 phases) and a continuous phase with a sponge-like structure containing a mixture of the two liquids (Figure 1.2) [14,67]. The formation of each structure depends on the nature and concentration of the components, and temperature. For liquid chromatography, only W/O and O/W MEs have been used. W/O MEs are constituted of spherical droplets of water, surrounded by a layer of surfactant, dispersed within the continuous oil phase (such as heptane or 1-octanol) (Figure 1.2). The surfactant polar head is faced inward concentrating the charge density in the droplet water core, while the surfactant hydrocarbon tail remains outward in the oil, forming reversed micelles.

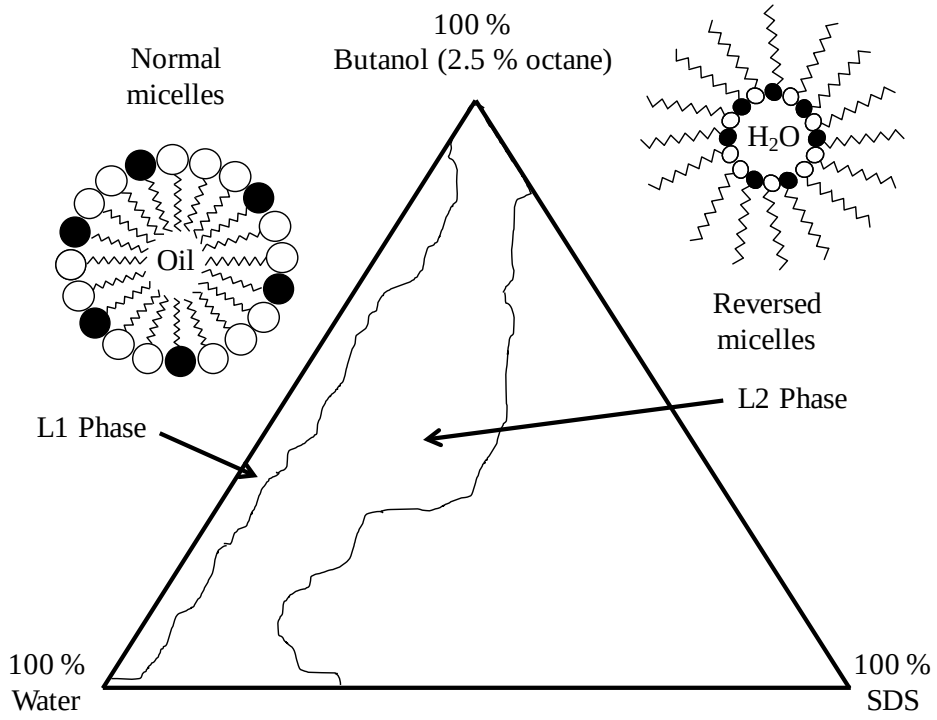


Figure 1.2. Schematic representation of a ternary phase diagram for SDS/1-butanol (2.5 % octane)/water system outlining the L1 (O/W) and L2 (W/O) phases, formed with normal and reversed micelles, respectively. The 1-butanol/octane ratio is 97.5/2.5. White = polar head group of surfactant; black = polar head group of co-surfactant. Percentages are expressed as *w/w*.

When the amount of oil in the system is very small, the interaction with the water phase of the hydrophilic (often charged) surfactant heads aggregates the surfactant into normal micelles (Figure 1.2). This is the basis of O/W MEs, which are formed by oil droplets dispersed in a continuous aqueous medium containing surfactant at a concentration well above its critical micelle concentration (CMC). In the O/W mixture, the oil phase is located inside the normal micelles, dissolved by the long hydrophobic carbon tails of the surfactant molecules. Often, in both O/W and W/O MEs, a medium chain alcohol acts as co-surfactant, fulfilling the geometric requirements to get the appropriate curvature in the interfacial region [68]. The carbon tail of a typical co-surfactant is located in the oil, while the hydrophilic group (e.g., hydroxyl) remains in the water, bridging the O/W interface.

The effect of the co-surfactant on lowering the intermolecular repulsion experienced by the hydrophilic surfactant head groups (especially, for ionic surfactants) depends on its ability to be packed between the surfactant monomers around the oil droplets. Since the tails of surfactant and co-surfactant are both aliphatic, their interfacial tensions against water are practically identical. This helps to get a stable structure, but too low or too high concentrations of co-surfactant will cause separation into two liquid phases. The surface tension in the ME droplets is also lowered by the addition of salt, as this reduces the electrostatic repulsion between the charged droplets [69].

The interfacial tension depends on the composition of the hydrocarbon domain of the interfacial layer. According to Nagarajan and Ruckenstein [70], an O/W droplet is characterised by three geometrical variables:

(i) the surface area of the droplet in contact with the surfactant,
(ii) the ratio of co-surfactant-to-surfactant molecules in the interfacial layer and
(iii) the ratio of oil-to-surfactant molecules.

In this work, we will refer only to mobile phases prepared with O/W MEs. These consist of submicron (nanometer-sized) spherical droplets of immiscible lipophilic organic solvent, enclosed in a surfactant/co-surfactant layer. This enables its dispersion throughout the aqueous phase. With a proper composition, MEs are stable for several weeks or months.

1.4. Properties of microemulsions useful for HPLC

The diameter of the oil droplets of O/W MEs used in MELC is less than 10 nm. Because of this size, some authors refer to them as nanoemulsions [34,56,66]. The nanometer-sized structure and number of droplets offer an extraordinarily large specific interfacial area to the ME system, able to yield strong interactions. The small droplet size is also an advantage with regard to absorbance detection, since fully optically transparent solutions with sufficiently low viscosity are obtained. Larger droplets would scatter white light. Meanwhile, the usual components in O/W MEs (surfactants, oils, co-surfactants and water) allow low UV detection wavelengths (down to 190 nm), which is extremely important for the sensitive detection of compounds containing weak chromophores.

The main interest of MEs is their ability to solubilise compounds in a wide range of polarities. The extent of solubilisation has a direct impact in the RPLC separation of compounds. The nanometer-sized droplets will be ionic or neutral depending on the charge of the surfactant heads that surrounds the droplets. This charge will affect the interaction of charged hydrophilic compounds with MEs.

The water solubilising capability of hydrophilic compounds is also strongly dependent on the surfactant/co-surfactant ratio. The most attractive feature of MEs is, however, their enhanced solubilising ability toward hydrophobic compounds. The interaction of the non-polar tail group of the surfactant with the oil droplet makes the micelle core more hydrophobic. The inclusion of highly water-insoluble compounds in the high concentration of oil contained in the micelle droplet is possible, as the surface of the droplet is not very rigid and the compounds can penetrate easily, while they would not penetrate a conventional micelle.

Using ME mobile phases, the analysis of complex mixtures of compounds in a wide range of polarities (from hydrophilic to hydrophobic) is possible. An example is given by the analysis of the active drug oxibendazole, which is far less water soluble than the paraben preservatives it is formulated with [42]. The dual behaviour of MEs allows affording this separation using isocratic conditions. This represents an advantage in comparison to the conventional RPLC mode, which requires a gradient of organic solvent for the successful analysis of such mixtures.

The solubilising effect on sample matrices is also remarkable. An interesting application of MELC is the analysis of samples containing water-soluble drugs present in non-polar pharmaceutical matrices, such as creams, ointments, or suppositories, carrying out the direct injection of the sample [23–25]. Usually, non-polar matrices should be treated with suitable solvents to extract the active compounds before making an RPLC analysis possible.

1.5. Partitioning behaviour

1.5.1. Retention mechanism in the polyphasic system

In RPLC, the stationary phase is non-polar, while the mobile phase is relatively polar. The separation in the conventional mode, where an aqueous-organic mixture with or without buffer is used, is governed by the interactions of solutes with both stationary and continuous mobile phases [1]. Solutes interacting more strongly with the less polar stationary phase will exhibit longer retention, compared with those solutes that are better solubilised in the more polar mobile phase. Retention will be significantly affected by the difference in polarity of mobile phase and stationary phase, and will decrease as more organic solvent is added, since this makes the mobile phase polarity closer to the stationary phase. The presence of reagents, which modify the nature of one or both phases, will also have significant influence on the chromatographic behaviour, because of the added interactions with the eluted compounds [3]. The physico-chemical properties of analytes, such as polarity, feasibility of ion-pairing, hydrogen-bonding, or electrostatic interaction with both mobile phase and stationary phase, among others, will also affect the retention. On the other hand, ionisable solutes will be strongly influenced by the mobile phase pH and ionic strength.

The high aqueous content in the reported procedures in MELC with O/W MEs makes the overall polarity quite high and very compatible with RPLC columns. However, the presence of surfactant, co-surfactant and oil in the mobile phase affects the partitioning process and, therefore, the separation behaviour. In O/W MELC, both mobile phase and stationary phase suffer significant changes in their characteristics, compared to a conventional aqueous-organic RPLC system.

Previous work carried out in MLC has shown that the surfactant monomers in the mobile phase have great ability to be adsorbed on the surface of the stationary phase [5,71]. The adsorbed surfactant monomers fill up part of the pore volume in the silica packings. This gives rise to changes in the stationary phase polarity, surface area and thickness, which affect drastically the thermodynamics and kinetics of the partitioning of solutes into the stationary phase, with consequences on retention, selectivity and efficiency. For SDS, a common surfactant in both MLC and MELC, the long hydrophobic chain of the monomers is associated to the alkyl-chains in the chromatographic column, with the sulphate group oriented outside from the surface [72]. This creates a stationary phase with a negative charge, able to attract cationic solutes, which increases their retention. The polarity of the stationary phase is also globally modified, with consequences in the retention of solutes.

The thickness of the surfactant layer on the stationary phase depends on the concentration of surfactant in the mobile phase. A thick layer yields poor efficiency, especially for low polar solutes. Co-surfactants (typically, medium chain alcohols) are added to compete with the surfactant for the adsorption sites. This reduces the surfactant layer and, therefore, the peak efficiency improves. In MELC, the hydrophobic organic solvent used to create the oil droplets may also distribute to some extent on the surface of the stationary phase. This results in an increase in the amount of stationary phase, which also affects solute selectivity and retention [20]. Meanwhile, the surfactant coated oil droplets in MELC act as a pseudo-stationary phase, providing a secondary partitioning mechanism with both the modified stationary phase and bulk mobile phase.

Three simultaneous partitioning equilibria are, therefore, established between: (i) ME droplets and bulk solvent, (ii) ME droplets and stationary phase, and (iii) bulk solvent and stationary phase (Figure 1.3). Several complex

interactions (hydrophobic, electrostatic, steric and hydrogen-bonding, among others) can be expected between solutes and both ME mobile phase and modified stationary phase. Altogether, the multiple factors that affect the partitioning equilibria lead to the potential separation ability of MELC. The complexity of behaviours makes, however, the optimisation of retention and selectivity rather complex.

The extent of solubilisation of the analytes in the O/W ME has an effect on the chromatographic behaviour. Water-insoluble (more lipophilic) compounds tend to reside in the core of the hydrophobic oil droplet, while highly water-soluble compounds will interact predominantly with the continuous aqueous phase. Consequently, the retention of the more lipophilic analytes will be primarily governed by the partitioning into the ME droplets. The molecular thermodynamic approach and lattice fluid self-consistent field theory were applied to the evaluation of some microstructural characteristics of the ME mobile phase, in the separation of simvastatin and its six impurities [68]. Fundamental interfacial properties of MEs were calculated from the properties of the pure components (surfactant, co-surfactant and oil). The best resolution was achieved with a ME containing a large number of small droplets, with the smallest film thickness and interfacial tension.

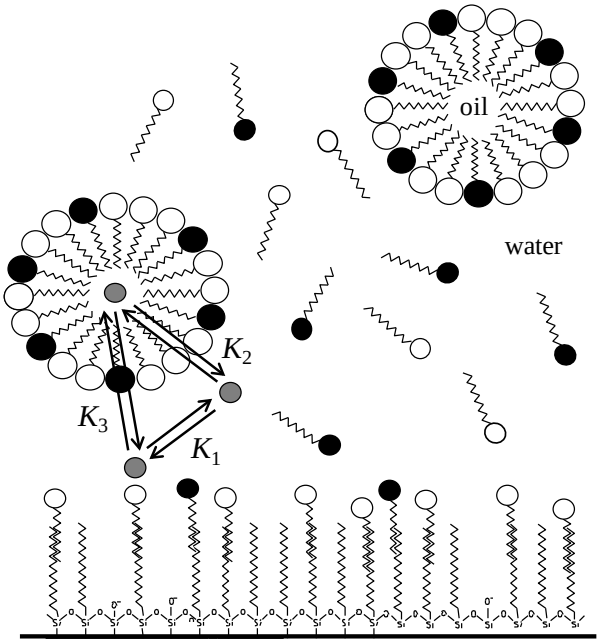


Figure 1.3. Behaviour of the distribution of a generic solute in MELC. Equilibrium constants of the distribution between: mobile phase and stationary phase (K_1), aqueous phase and oil drops (K_2), and stationary phase and oil drops (K_3). White = polar head group of SDS, black = polar head group of co-surfactant, and grey = solute.

1.5.2. Methylene selectivity

In an early MELC study, the separation of a series of alkylbenzene homologues from toluene to decylbenzene was studied to assess the effect of increasing the proportion of the organic solvents in the ME mobile phase containing AOT, heptane and 1-pentanol [15]. A larger concentration of the

organic solvents reduced the retention of the alkylbenzenes, and this effect was more pronounced for compounds with longer alkyl chains. This revealed the unique solubilising power of MEs for hydrophobic compounds. The behaviour was confirmed in a study, where drugs illegally used for sports were analysed [73].

Methylene (or hydrophobic) selectivity, $\alpha(CH_2)$, is one of the main parameters used to characterise the capability of a chromatographic system to separate homologues, which is of fundamental importance for RPLC, because the alkyl chains are the main surface active functional groups of the stationary phase. Methylene selectivity is calculated from the retention factors (k) of two homologues differing in only one methylene group (compound A = $R–R_1$) and (compound B = $R–CH_2–R_1$), as $\alpha(CH_2) = k(B)/k(A)$) [74]. In RPLC with aqueous organic mobile phases, log $\alpha(CH_2)$ is linearly related to the number of carbon atoms in the normal chain of the homologues, n_C, as follows:

$$\log k = \log \alpha(CH_2)\, n_C + \log \beta \qquad (1.1)$$

where the intercept (log β) measures the specific interactions between the functional groups of the homologues with both the mobile phase and stationary phase. Eq. (1.1) indicates that the retention of the homologues increases notably with an increase in the number of methylene groups in the alkylbenzenes.

Several reports on the retention of homologues have provided information about the differences in retention mechanism between conventional RPLC and MLC [74,75]. It was not until 2015 that Sokolova *et al.* carried out a detailed comparative study with several alkylbenzenes between conventional RPLC and MELC [76].

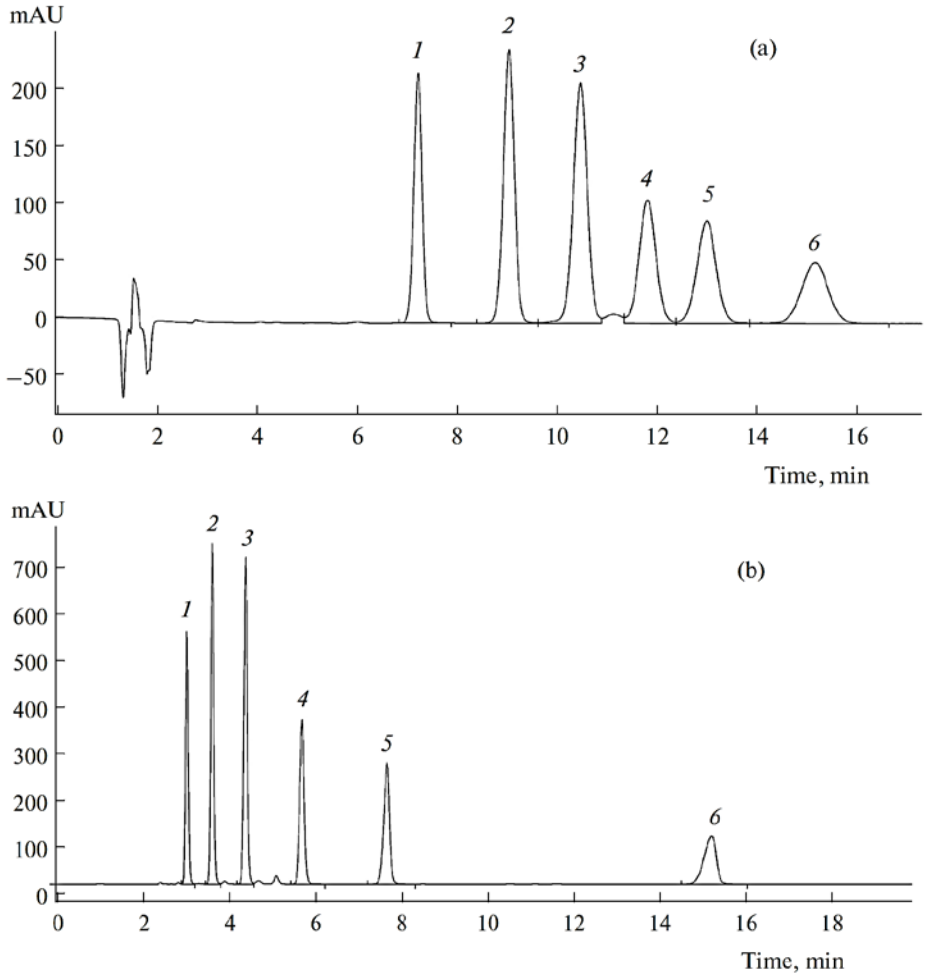


Figure 1.4. Chromatograms of a test mixture of alkylbenzenes using: (a) MELC with 3.3 % SDS / 8 % 1-butanol / 0.8 % heptane, and (b) conventional RPLC with 60 % acetonitrile. Other experimental conditions: Grace Smart C18 column (150 × 4.6 mm i.d., 5 μm), 1 mL/min, UV detection at 254 nm. Peak identity: (1) benzene, (2) toluene, (3) ethylbenzene, (4) propylbenzene, (5) butylbenzene, and (6) hexylbenzene (Reprinted from Sokolova *et al.* [76], with permission from Springer).

As previously observed for MLC, the dependence of log *k* versus n_C for MELC was found to be convex (instead of linear) during the transition from benzene to hexylbenzene. This convex dependence was noted for surfactants of different type and implies that a larger number of compounds eluted per unit time (Figure 1.4). According to the authors, the interaction between the ME droplets and the stationary phase represents the main contribution to this behaviour.

1.5.3. Binding behaviour

As described already, using O/W ME mobile phases, elution is not only governed solely by solute-stationary phase interactions, but also by the association of solutes with the ME droplets [42]. The theory proposed for MLC was again revised to understand the observed behaviour. For micellar mobile phases, Armstrong and Stine [77] proposed a classification of compounds into three groups as: (i) compounds binding to micelles, (ii) non-binding compounds, and (iii) anti-binding compounds. A similar classification was proposed for compounds in MELC as: (i) droplet-binding, (ii) droplet-non-binding, and (iii) droplet anti-binding [22,42].

The strength of the interactions with the ME droplets depends on the surfactant concentration, since the available surfactant affects their formation. The nature of the stationary phase is also relevant. Droplet-binding solutes would experience greater attraction to the swollen micelles in the mobile phase and elute more quickly at higher surfactant concentration. Anti-binding solutes would be, in contrast, forced into the stationary phase due to electrostatic repulsion from the droplets, thus increasing the retention time with increased surfactant concentration. However, the behaviour in the reported MELC procedures has been always droplet-binding. In MLC, most

anti-binding compounds with anionic micelles are negatively charged and the most binding solutes are positively charged. This behaviour cannot be observed with stationary phases that adsorb an appreciable amount of surfactant. This is the case of C8 and C18 columns, usual in the MELC reports.

1.6. Characterisation of MELC based on the solvation parameter model and measurement of lipophilicity

As commented, the retention of compounds in RPLC systems depends on hydrophobic, electrostatic, steric and hydrogen bond interactions between solutes and the chromatographic system. The solvation parameter model is a useful approach to characterise the observed behaviour. This approach was applied in a comparison study involving four ME mobile phases, two micellar mobile phases and a biological process (drug penetration across blood-brain barrier, BBB) (Figure 1.5) [78]. ME mobile phases were composed of SDS or Brij-35 (polyoxyethylene(23)lauryl ether), 1-butanol, heptane and phosphate buffer at pH 7. Micellar systems were prepared similarly without heptane.

The general solvation parameter model is expressed as:

$$SP = c + eE + sS + aA + bB + vV \quad (1.2)$$

where *SP* is a property for a series of solutes in a solvent system, such as log *k* or log BBB [79]. The capitals on the right of Eq. (1.2) correspond to solute descriptors: excess molar refraction (*E*), dipolarity/polarisability (*S*), effective hydrogen-bond acidity (*A*) and basicity (*B*), and McGowan characteristic volume (*V*).

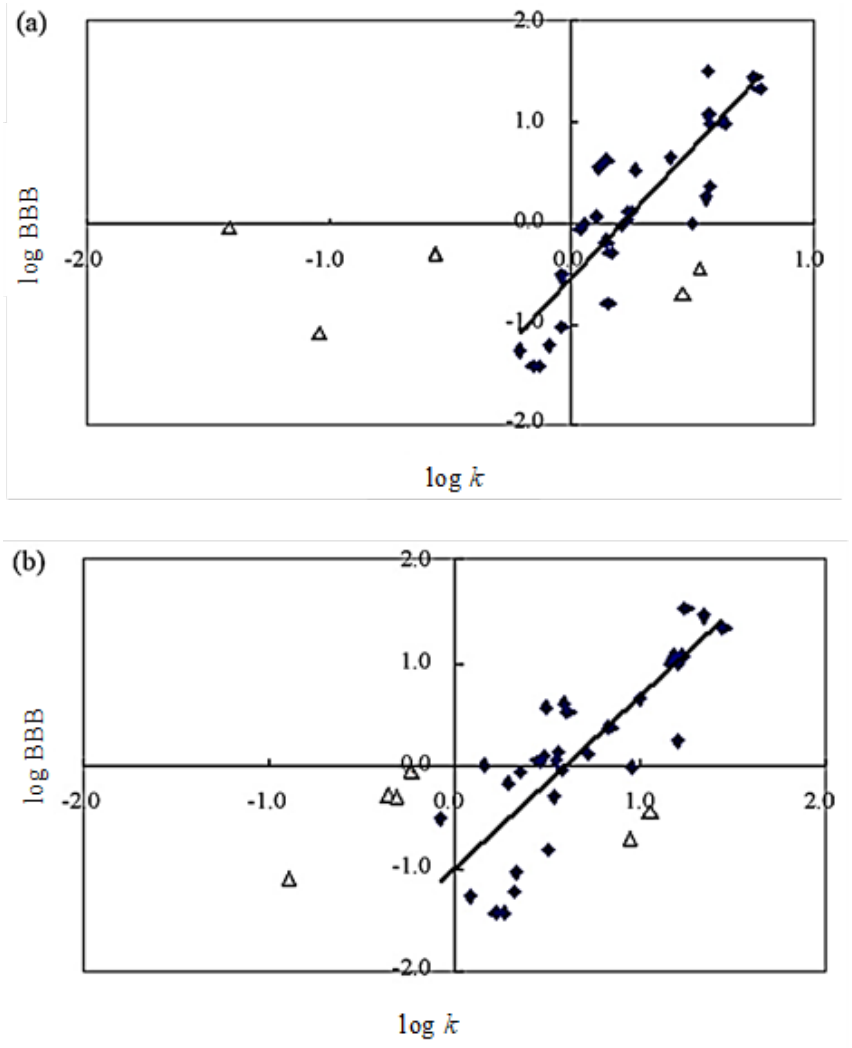


Figure 1.5. Plot of log BBB (drug penetration across blood-brain barrier) versus log *k* for two MELC systems: (a) 3.3 % SDS / 6.6 % 1-butanol / 1.6 % heptane / 88.5 % potassium dihydrogen phosphate solution, and (b) 3.3 % SDS / 6.6 % 1-butanol / 90.1 % potassium dihydrogen phosphate solution (Adapted from Liu *et al.* [78]).

The coefficients in Eq. (1.2) are obtained by multiple linear regression and reflect the differences between the two phases where the solute is being transferred, with regard to the capability of the environment to interact with solute *n*- and π-electron pairs (*e*), dipole-dipole and dipole-induced dipole interactions (*s*), hydrogen-bond basicity (*a*) and acidity (*b*), and relative ease of solute to form a cavity or solute hydrophobicity (*v*). The four terms, *e*, *s*, *a* and *b*, correspond to the polar contributions to the considered solute property, and the last term, *v*, to the hydrophobic contribution. The term *c* in Eq. (1.2) is the intercept of the regression.

The absolute values of coefficients *v* and *b* were the largest for all systems investigated [78]. This means that solute volume and hydrogen-bond basicity generally have maximal influence on the retention. The stationary phase modified by SDS appeared to have stronger capacity to interact with solute *n*- or π-electrons than a phase modified by Brij-35. The approach showed that the value of *a* decreased when the heptane content increased. Because the stationary phase in MELC systems can be modified by the adsorption of both surfactant and oil molecules, and the oil is a weaker hydrogen-bond acceptor compared to the surfactants (SDS and Brij-35), the stationary phase modified by ME has lower basicity than when modified by a micellar solution. On the other hand, it was observed that *s* decreased slightly by increasing the oil content. This means that the stationary phase was less dipolar than the mobile phase when more heptane replaced SDS or Brij-35. The coefficient *v* was more positive, and *s* and *a* less negative in the systems modified with Brij-35, compared to SDS. Therefore, the stationary phase modified with Brij-35 was more dispersive, less dipolar and less basic than when modified with SDS.

MELC has been reported to be useful for the rapid and reliable prediction of the partitioning behaviour of drug compounds in a 1-octanol/water system

(log $P_{o/w}$), being a possible alternative to the shake-flask method for high throughput lipophilicity measurement. A ME composed of 6.0 % Brij-35, 6.6 % 1-butanol, 0.8 % 1-octanol and 86.6 % 0.05 M phosphate buffer at pH 7.0 (percentages are given as *w/w*, unless otherwise indicated) was suggested for non-congeneric neutral and basic drugs [80]. A linear solvation energy relationship (LSER)-based method was also applied to compare MELC and MLC systems, as well as other biochemical systems, and identify the optimal system to measure the lipophilicity. The best MELC system consisted of 3.0 % SDS, 6.0 % 1-butanol, 0.8 % 1-octanol and 90.2 % water at pH 6.4 [81]. A more complex system employed a biomembrane-mimetic RPLC method using a C8 stationary phase and a ME mobile phase modified with phosphatidylcholine (PC) [82]. The optimal ME composition was 3.0 % SDS, 0.2 % PC, 6.0 % 1-butanol, 0.8 % ethyl acetate and 90.0 % water at pH 7.0. PC is the major molecular constituent of human biomembranes, and is able to be introduced in both stationary phase and mobile phase in a ME system. The polar head group of PC contacts the polar group of SDS and the hydrophobic tail points to the ethyl acetate phase, surrounded by the molecules of SDS. This system was used to estimate the lipophilicity of neutral and ionised drugs. The interaction between the MELC system and drugs was observed to be more similar to a biological membrane than the 1-octanol/water partition system.

Recently, biopartitioning liquid chromatography is gaining importance as a non-cellular system for the estimation of biological properties in early stages of drug development. MEs are suitable mobile phases, because of their ease of formulation, stability and adjustability to a large number of compositions to mimic biological structures. Several MELC systems have been characterised by means of the solvation parameter model, in order to assess their suitability as BBB distribution or permeability surrogates [51,78,83,84]. Principal component

analysis (PCA) and a distance parameter were used to compare the similarity of MLC and MELC, and select the most suitable chromatographic system to model a biological process. MELC with SDS containing 1.6 % heptane was proved to be superior to MLC with the same surfactant, and parallel to an MLC system with Brij-35, to predict the capability of BBB. In another report, the composition of the ME mobile phase was optimised to model the BBB by MELC [85]. During that study, a continuous increase in retention along the operation time was found for all assayed drugs. The authors suggested that the retention times of compounds in MELC should be corrected for long-term operations if the content of heptane in the ME mobile phase was as high as 1.6 %. For this purpose, methyl paraben was proposed as internal standard for acid and neutral drugs, and propranolol for basic drugs. The corrected retention factors were applied satisfactorily to develop the predictive model.

Biopartitioning MELC was also proposed to facilitate high-throughput drug screening and generate fingerprints for biological samples with multiple constituents in traditional Chinese medicine [86]. The parallel artificial membrane permeability assay model was used to determine the effective permeability of drugs, so that quantitative retention-activity relationships (QRAR) could be established, which were used to optimise the MELC method. The correlation between the pharmacokinetic parameters of several danshen constituents that were derived from the QRAR, and the corresponding retention data, were then used to predict their biological effectiveness.

1.7. Components used to build microemulsions in MELC

1.7.1. The surfactant

Surfactants play a key role in the stability of MEs and the separation quality in MELC. Both the formation of a stable ME and solute partitioning between mobile phase and stationary phase are extremely dependent on the surfactant nature. Also, the thickness of the layer of surfactant monomers adsorbed onto the stationary phase surface, which modifies significantly solute retention, selectivity and peak efficiency, depends on surfactant concentration. The surfactant interacts through its alkyl tail with the alkyl bonded groups of the usual stationary phases. This leaves the head groups oriented away from its surface forming a hydrophilic layer in contact with the ME mobile phase (Figure 1.3). The head groups of cationic surfactants may also interact with residual silanol groups, being incorporated into the bonded phase. This leaves the surfactant alkyl tails uppermost, which retains the hydrophobicity of the stationary phase.

The size of the droplets and the charge of the stationary phase and droplets depend on the type of surfactant. Therefore, substitution of a surfactant molecule by another, with a different head group or chain length, will affect the separation by altering the partitioning between solutes and ME droplets and the surfactant modified stationary phase. The effect of anionic, cationic and non-ionic surfactants with hydrocarbon chains of diverse lengths (12 to 16 carbons) has been investigated in MELC, and some of these surfactants have been used to implement analytical procedures (Tables 1.1 and 1.2). Charged solutes will interact electrostatically (attracted or repelled) with charged droplets and stationary phase in a ME system formed with an ionic surfactant. Altering the charge, solute association with both phases will change significantly.

The surfactants employed in MELC are presented in Table 1.3. Anionic MEs have been used in most MELC applications, SDS being the surfactant of choice. This can be explained by the previous experience and extensive use of this surfactant in MLC [5–7], partially due to its commercial availability, high purity and relatively low cost. An interesting feature of SDS is that it efficiently dissolves proteins, allowing the direct injection of physiological samples in the chromatographic system without any other treatment than filtration [9]. Apparently, it is difficult to find a better anionic surfactant for both MLC and MELC. However, Marsh *et al*. found that the separation quality for a probe mixture using a ME formed by sodium tetradecyl sulphate was similar to that obtained with SDS [42]. This observation needs, however, a deeper study.

Cationic surfactants are not useful for the direct injection of physiological samples, but some advantages were found with hexadecyl trimethylammonium bromide (HTAB) in the analysis of suppositories [24], as it offered greater solubilising power than SDS (five times more sample was dissolved), and the MEs formed were more stable due to the longer alkyl chain length of this cationic surfactant.

Since the MELC system with the anionic SDS could not separate highly hydrophilic compounds with very similar chemical properties, SDS was replaced by non-ionic surfactants [18,87]. The retention of some carboxylic acids increased with Tween surfactants, due to either the reduced distribution of the compounds into the ME droplets and/or stronger interaction with the stationary phase.

Table 1.3. Structure, molecular weight, critical micelle concentration (CMC), and hydrophile-lipophile balance (HLB) of the surfactants used for preparing microemulsions.

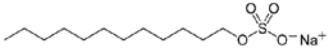
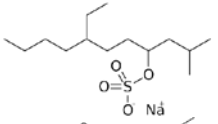
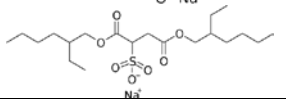
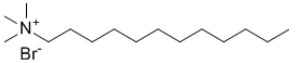
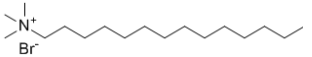
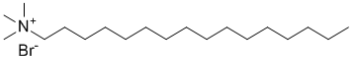
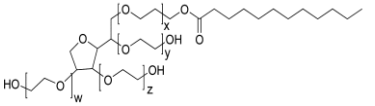
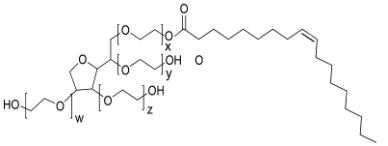
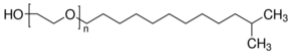
Surfactant	Structural formula	MW, g/mol [a]	CMC, mM	HLB
Anionic				
Sodium dodecyl sulfate (SDS)		288.4	8.2 [b]	40 [d]
Sodium tetradecyl sulfate (STS)		316.4	2.1 [b]	–
Sodium docusate (Sodium dioctyl sulfosuccinate, SDOSS or AOT)		444.6	2.5 [c]	40 [d]
Cationic				
Dodecyl trimethylammonium bromide (DTAB)		308.3	15.0 [b]	26 [e]
Tetradecyl trimethylammonium bromide (TTAB)		336.4	3.5 [b]	27 [e]
Hexadecyl or cetyl trimethylammonium bromide (HTAB, or CTAB)		364.4	0.9 [b]	24 [e]

Table 1.3 (continued).

Surfactant	Structural formula	MW, g/mol [a]	CMC, mM	HLB
Non-ionic				
Brij-35 (Polyoxyethylene (23) lauryl ether)		1198	0.06 [b]	16.9 [f]
Tween 21 (Polyoxyethylene (20) sorbitan monolaurate)		–	–	13.3 [g]
Tween 80 (Polyoxyethylene (20) sorbitan monooleate)		1310	0.012 [b]	15.0 [f]
Genapol X-080 (Polyethylene glycol monoalkyl ether)	w+x+y+z=20 	552	0.06-0.15 [a]	–

[a] https://www.sigmaaldrich.com; [b] Ref. [89];
[c] https://lib.dr.iastate.edu/cgi/viewcontent.cgi?referer=https://www.google.es/&httpsredir=1&article=11721&context=rtd; [d] Ref. [76];
[e] Ref. [90]; [f] Ref. [91]; [g] https://pharmlabs.unc.edu/labs/emulsions/hlb.htm

Recently, the non-ionic Genapol X-080 was reported to offer the best results in the separation of flavonoids [58], and phenylethanoid glycosides [61]. SDS has also been replaced, with different success, by mixtures of SDS and either the anionic surfactant AOT, or the non-ionic surfactants Brij-35, Tween-21 and Tween-80 [18,24,88]. The mixture of SDS and Brij-35, or SDS and Tween-21, did not offer any improvement in the separation of simvastatin [88], or paracetamol and its impurities [24]; instead, low retention and deterioration of the peak profiles for all analytes were observed. A mixture of SDS and AOT yielded increased retention for both sodium fosinopril and fosinoprilat, especially for the former, while in the presence of Brij-35 and Tween-21 no MEs were formed, at least at the assayed concentrations of the surfactants [19]. In the separation of pramipexole and its impurities, the SDS/Brij-35 mixture gave rise to a small increase in the retention times of all analytes, which was advantageous to avoid low elution close to the dead time, or to resolve some impurities [87].

In another work, SDS was able to separate the lipophilic diterpenoids tanshinone I, cryototanshinone and tanshinone II A [55]. In contrast, it failed in the separation of hydrophilic analytes. With Brij-35, the order of the peaks of tanshinone I and cryototanshinone changed and the resolution was decreased, but the separation of hydrophilic analytes improved. The SDS/Brij-35 mixture did not increase, however, the selectivity. Therefore, Brij-35 was finally selected as the unique surfactant.

Table 1.4. Solubility in water and polarity of the oils used in MELC.

Oil	Solubility (g/100 g H_2O)[a]	Polarity	
		Snyder[b]	Reichardt[c]
Pentane	0.004	0.0	0.009
Hexane	0.0014	0.0	0.009
Heptane	0.0003	0.0	0.012
Octane	< 0.0003	0.0	0.012
Cyclohexane	0.005	0.0	0.006
Toluene	0.05	2.3	0.099
1-Propanol	Miscible	3.9	0.617
1-Butanol	7.7	3.9	0.586
1-Pentanol	2.2	–	0.568
1-Hexanol	0.59	–	0.559
1-Heptanol	0.17	–	0.549
1-Octanol	0.096	3.2	0.537
Di-isopropyl ether	0.2	1.8	0.105
Ethyl acetate	8.7	4.3	0.228

[a] https://sites.google.com/site/miller00828/in/solvent-polarity-table

[b] Snyder's global polarity, Ref. [2].

[c] Relative polarity, Ref. [92].

1.7.2. Use of non-polar organic solvents as oils

In MELC, the choice of oil (the internal organic phase needed to form the O/W ME droplets) has an obvious effect on solute partitioning and selectivity. Several organic solvents of different nature in a relatively wide range of polarities have been assayed. In general, the hydrophobicity of the solvents was high, since this characteristic is indispensable for the formation of an O/W ME. The reported solvents in MELC, and their solubilities and polarities in water, are indicated in Table 1.4. Most often, alkanes and alcohols (the latter with higher water-solubility) of varying chain length have been selected. Other water-insoluble solvents used in analytical reports are cyclohexane, toluene, ethyl acetate, butyl acetate, di-isopropyl ether and 2-octanone.

As the chain length in the alkanes and alcohols added to MELC mobile phases increases, the retention times for hydrophobic analytes are reduced. Peak-to-peak resolution may also increase [42]. Alcohols seem to offer better peak efficiencies compared to alkanes, at the expense of longer analysis times, since their solubilising power is smaller. In the group of alkanes, hexane offers the best peak efficiency, but the analysis times are longer compared to octane. The molecular volume of the oil, relative to the hydrophobic chain of the surfactant, affects the extent to which it penetrates the surfactant tails of the O/W interface layer [18,88]. Oils of small molecular volume are usually not able to form a core in the centre of the ME droplet. They instead remain in the surfactant monolayer, altering the head region of the micelle and facilitating to a certain extent the solubilisation of some compounds in this region. This is the case of ethyl and butyl acetate [28]. In contrast, oils of large molecular volume, as heptane or octane, tend to form an oil core inside the droplets. This provides an extra region to solubilise hydrophobic compounds [93]. All this explains why replacement of

octane with an oil of smaller molecular volume, such as ethyl acetate, results in longer retention times.

In an early report, several organic solvents representing a wide range of polarities were compared as internal organic phase for forming the oil droplets in MELC: octane, 1-octanol, butyl acetate, di-isopropyl ether and 2-octanone [18]. Replacement of 1-octanol by di-isopropyl ether, butyl acetate or 2-octanone, resulted usually in slight retention increases for all assayed compounds, as the solutes do not partition as fully into the oil droplets. The use of octane with its higher lipophilicity, compared to 1-octanol, resulted in decreased retention, except for naphthalene. This was explained by the fact that octane may also partially coat the hydrophobic bonded phase, which would result in an increase in the amount of stationary phase.

In several other studies, the use of di-isopropyl ether or ethyl acetate (with higher polarity) instead of heptane or cyclohexane also increased the retention of all analytes [19,88]. Structurally similar substances (simvastatin, lovastatin and methylsimvastatin) were poorly resolved using heptane or cyclohexane as oil; di-isopropyl ether yielded better performance and was chosen for further studies [88]. In contrast, for a mixture of sodium fosinopril and fosinoprilat, cyclohexane was the best choice [19]. A mixture of parabens, oxibendazole and beclamethasone dipropionate, chlorobutane and ethyl acetate showed increased retention times and resolution, but with peak fronting and tailing [42].

The separation of several drugs in pharmaceuticals with either 1-octanol, butyl acetate or di-isopropyl ether as oils was found successful by other authors [27,30,39]. Di-isopropyl ether provided shorter retention when compared with 1-octanol. However, this was found optimal, since it offered the highest efficiency and resolution in still reasonable analysis times. Ethyl acetate, butyl acetate, di-isopropyl ether, 1-octanol and hexane were also investigated for the

separation of pramipexole and five impurities [87]. Even though these solvents did not show a strong impact on the analysis times, butyl acetate provided slightly better separation and peak profiles. Also, use of 1-octanol, butyl acetate or 2-octanone, instead of di-isopropyl ether in the separation of dopamine receptor antagonist LE300 and its N-methyl metabolite, resulted in increased selectivity and efficiency, but optimal performance was still achieved with di-isopropyl ether [57]. Meanwhile, replacing di-isopropyl ether with the more lipophilic octane yielded broad peaks. The separation of hydrochlorothiazide and losartan using either heptane, octane, 1-octanol or ethyl acetate was successful, but octane provided the shortest retention times [31].

Additional manipulation of the internal phase composition of the ME droplet structure was thought to improve the separation. It was found that the simultaneous presence of ethyl acetate and butyl acetate had very positive influence on the separation of perindopril and its four impurities [28]. The appearance of negative peaks in the chromatograms, due to differences in the densities of 1-butanol (used as co-surfactant), butyl acetate and ethyl acetate, did not affect the separation of the critical pairs. Micellar solutions of SDS with 1-pentanol as co-surfactant have been used for the separation of steroids in isocratic elution [44], and a range of basic drugs in gradient elution with a fixed amount of 1-pentanol [94]. This alcohol is dissolved in the micelle core. Therefore, the mixture of SDS and 1-pentanol can be considered either as a swollen micelle, or a ME with 1-pentanol playing the role of oil.

Finally, some oils have been described as not been able to form MEs with some surfactants. This is the case of the linear alkanes hexane, heptane and octane in the presence of Brij-35 [34], and hexane, heptane, toluene and 1-octanol in the presence of Genapol X-080 [58,61]. For the latter, ethyl acetate was selected as the best oil.

1.7.3. Use of ionic liquids as oils

The oil phase in MELC is usually constituted of alkanes, such as octane or cyclohexane, besides the less hydrophobic solvents used as co-surfactants (usually 1-propanol or 1-butanol). However, there is great concern to decrease the consumption of organic solvents in the laboratories, as a need to reduce wastes and their environmental impact. Therefore, designing an MELC system with less organic solvent in the mobile phase, but still providing high separation performance, has been considered for the analysis of real samples.

With this purpose, the use of ionic liquids (ILs) as the oil phase in MELC has been explored [56]. ILs are environmental friendly molten salts, composed entirely of large and dissymmetrical nitrogen or phosphorous heterocyclic rings (imidazolium, pyridinium or pyrrolidinium), and quaternary ammonium or phosphonium cations, all attaching different alkyl chains, combined with a variety of inorganic or organic anions. This results in a significant decrease in melting temperatures, which may be below room temperature [95,96].

Formation of ME droplets and the separation performance of several phenolic compounds was inspected, using SDS as surfactant and six hydrophobic ILs containing octyl-, hexyl- or butyl-3-methylimidazolium ($[C_8C_1IM]^+$, $[C_6C_1IM]^+$ or $[C_4C_1IM]^+$, respectively), as cation, and PF_6^-, BF_4^- or bis(trifluoromethyl sulphonyl) imide (NTf_2^-) as anion (Figure 1.6) [56]. It was found that all these ILs are able to yield MEs to be used as mobile phases, which are stable within at least two weeks. The retention times of all analytes increased at increasing alkyl chain in the imidazolium cation (from butyl to octyl), using the same anion.

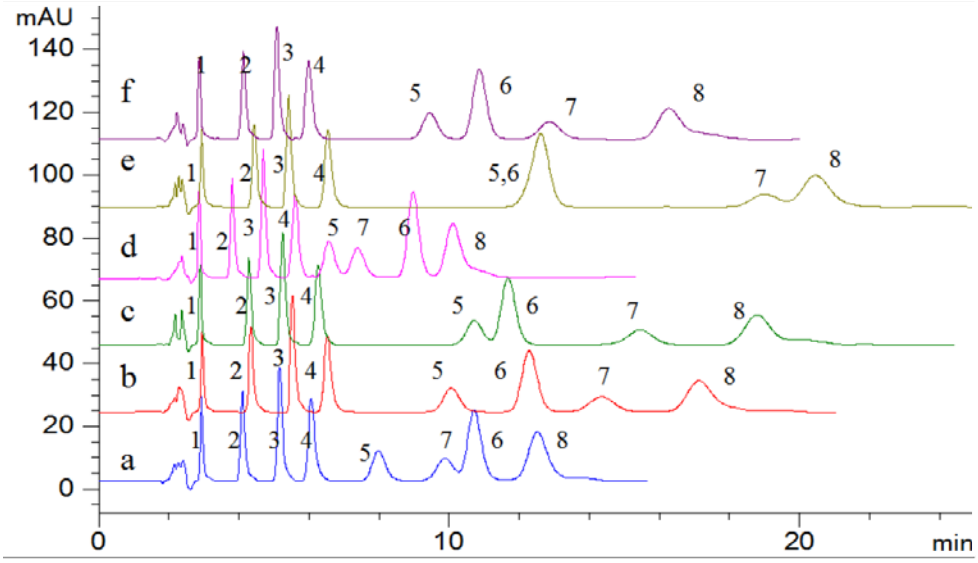


Figure 1.6. Effect of several ILs used as oil for the MELC separation of eight phenolic compounds, using SDS and 1-butanol with UV detection: (a) [C_4C_1IM][PF_6], (b) [C_6C_1IM][PF_6], (c) [C_8C_1IM][PF_6], (d) [C_4C_1IM][TF_2N], (e) [C_6C_1IM][BF_4], and (f) [C_8C_1IM][TF_2N]. Peak identity: (1) sodium danshensu, (2) protocatechuic acid, (3) protocatechuic aldehyde, (4) caffeic acid, (5) lithospermic acid, (6) rosmarinic acid, (7) salvianolic acid B, and (8) salvianolic acid A. Mobile phase composition: 0.2 % *w*/*v* IL / 1 % *w*/*v* SDS / 3 % *w*/*v* 1-butanol at pH 2.5. Other experimental conditions: SB-C18 column (150 × 4.6 mm i.d., 5 μm), 0.4 mL/min flow-rate (Reprinted from Peng *et al.* [56], with permission from Elsevier).

Replacing PF_6^- by NTF_2^-, the retention times were shorter and the resolution poorer. In contrast, BF_4^- yielded longer retention, but peak overlapping increased, indicating smaller distribution of analytes in the $[C_6C_1IM][BF_4]$ droplets. The elution order of some analytes depended on the IL nature, due to differences in solubility, and association and distribution of analytes. ILs were also adsorbed on the surface of the stationary phase to different extent, and this affected the retention and separation selectivity of analytes.

Based on its excellent separation selectivity and short retention times for all analytes, $[C_6C_1IM][PF_6]$ was selected to form the oil phase in an MELC green procedure [56]. The resolution achieved with this IL as oil was improved with regard to the use of ethyl acetate. The performance was even worse using dichloromethane and octane as oils.

1.7.4. Addition of co-surfactant to stabilise the microemulsion

In most cases, single-chain surfactants alone are unable to reduce sufficiently the interfacial tension between oil and water and, thus, facilitate the spontaneous formation of MEs. A more hydrophilic organic solvent than the oil, such as a medium chain length alcohol, is usually added as co-surfactant to further decrease the interfacial tension to nearly zero, resulting in a stable ME. The addition of co-surfactant to the mobile phase also solves the major problem present with mobile phases containing a surfactant: the formation of a layer of surfactant monomers adsorbed on the stationary phase, which makes solute mass transfer slower with negative consequences in peak efficiency [97]. The co-surfactant solves this problem by desorbing the surfactant from the stationary phase, in a greater or lesser extent, producing thus faster solute mass transfer [71,98,99]. The co-surfactant may significantly influence the solubility properties of the aqueous and oil phases, owing to its partitioning between both

phases [93]. It also increases the fluidity of the surfactant hydrocarbon tail and allows greater penetration into the central region [19]. The co-surfactant is, therefore, a very important component in a ME-forming system, with a great influence on solute partitioning [100].

The choice of co-surfactant appears to be more important than the choice of oil. Changing the type of co-surfactant, retention times and selectivity can be significantly altered [18,29,41,42,88]. Medium chain alcohols, such as 1-propanol and 1-butanol, are commonly used as co-surfactants in MELC. Other assayed solvents are the alcohols 2-propanol and 1-pentanol, besides acetonitrile and tetrahydrofuran. Some of these solvents are common in MLC [5]. 1-Butanol is, perhaps, the most common co-surfactant in the optimised MELC procedures, but authors often employ 1-propanol (the most usual in MLC) as first option. 1-Butanol penetrates easily into the oil core, giving rise to swollen droplets [17]. This alcohol increases the ME stability and the exchange rate of solutes between the aqueous medium and ME droplets. 1-Propanol, which is shorter, is miscible with water, giving rise to a more hydrophilic mixture of alcohol and surfactant [50]. Both alcohols (especially 1-butanol) are also adsorbed onto the stationary phase, replacing partially the surfactant monomers. This increases the stationary phase polarity. The efficiency is also improved, since the thickness of the surfactant layer is reduced.

By changing the co-surfactant, the polarity of the mobile phase is modified, which affects the separation. Through a proper selection of co-surfactant, the selectivity can be tuned as needed. The behaviour of several co-surfactants has been compared in several MELC reports. Although definitive conclusions cannot be extracted, certain trends are recognised. The experience of several authors in such comparison studies is next described.

To study the effect of the co-surfactant nature on the selectivity and efficiency in the separation of simvastatin and ezetimibe, 1-propanol (first selected) was replaced with 1-butanol, acetonitrile and tetrahydrofuran [39]. 1-Propanol provided the best behaviour with more appropriate retention times, higher efficiency and resolution. The separation with acetonitrile was insufficient, while 1-butanol resulted in band broadening and peak retardation. Tetrahydrofuran also provided smaller efficiency and poor resolution. 1-Propanol, 1-butanol and 1-pentanol were also compared in the separation of pramipexole and its five impurities [87]. Mobile phases containing 1-propanol or 1-butanol yielded good resolution, but 1-butanol provided significantly shorter analysis times and better performance. It was not possible to form a stable ME with 1-pentanol as co-surfactant. 1-Pentanol is a more hydrophobic alcohol and penetrates deeper into the ME droplets, making these to grow. By dissolving 1-pentanol in the oil-rich phase, the mixture of oil and alcohol becomes less hydrophobic. Also, since 1-pentanol is partially miscible with water, the mixture of alcohol and surfactant solution is less hydrophilic [101].

When tetrahydrofuran, 1-butanol, acetonitrile or methanol were considered to separate loratadine and desloratadine, only methanol could not be used as alternative to 1-propanol, since it did not provide a steady baseline chromatogram [27]. 1-Butanol and tetrahydrofuran offered reasonable resolution, while acetonitrile resulted in overlapped peaks. When 1-propanol was replaced with 1-butanol, tetrahydrofuran or ethanol to separate flunarizine and its degradation products, 1-butanol and tetrahydrofuran did not allow the elution of the analyte of interest [41]. Only ethanol could be used as alternative to 1-propanol. In contrast, when 1-propanol was replaced with 1-butanol, tetrahydrofuran, ethanol or acetonitrile to analyse the dopamine receptor antagonist LE300 and its N-methyl metabolite, the ME stability was affected only by ethanol or

acetonitrile [57]. For the determination of enalapril and hydrochlorothiazide, poor selectivity and resolution was found using tetrahydrofuran as co-surfactant, while acetonitrile and 1-butanol yielded good separation but with poorer efficiency compared to 1-propanol, which provided the highest separation efficiency and resolution [30]. Finally, the use of 2-propanol as co-surfactant resulted in decreased peak efficiency in the determination of potassium losartan [31]. The addition of acetonitrile was preferred.

1.7.5. Other reagents added to the microemulsions

The water continuous phase in MEs usually contains other additives to provide optimal separation conditions. As in any RPLC procedure, buffer reagents such as phosphate salts, or acids such as trifluoroacetic acid (TFA) and formic acid, are added to control the pH. These reagents may affect the separation. The retention of protonated basic analytes (which are positively charged) decreases with increased concentration of buffer, whether the mobile phase contains MEs or not. This is explained by the electrostatic attraction of the protonated analytes (which are cationic) to the buffer anions, forming less retained ion pairs. To this effect, the electrostatic interaction between the charged analytes and residual anionic silanol groups should be added. All these effects were observed in the MELC separation of terbutaline and bamethane [34], and nifedipine [36], using phosphate buffer at different concentration levels.

Ion pair reagents have been also added to MEs to change the charge on the droplets and stationary phase and, thus, alter solute retention and selectivity. These reagents can mask the silanol effect, which is the reason of poor efficiency and peak tailing for basic compounds. This is the case of triethylamine (TEA), which is a common cationic ion pair reagent. TEA facilitated fine tuning in the separation of closely eluting peaks [87]. The effect of the anionic octane

sulphonic acid and cationic tributylammonium hydrogen sulphate, added to a ME, was also investigated for the analysis of a mixture of parabens, oxibendazole and beclamethasone dipropionate [42]. Octane sulphonic acid increased the retention times and tailing with large broadening. This produced poorer peak resolution. Meanwhile, tributylammonium increased the retention times and altered the selectivity, without any effect on peak tailing. It should be highlighted that an adsorbed surfactant layer covers the stationary phase and avoids the interaction of basic compounds with silanol groups, improving the efficiency [71].

The addition of cyclodextrins was also explored in an early work in MELC. These reagents are cone shaped molecules of linked glucose residues, which form inclusion bodies with solutes. This molecular encapsulation is used to increase the solubilisation of poorly soluble solutes, and alter chromatographic partitioning. α- and β-Cyclodextrins were added to the ME mobile phase to analyse a mixture of parabens and oxibendazole [42]. γ-Cyclodextrin (which has a bigger cavity) was not used, as it was insoluble in the ME. Peak retention increased with α-cyclodextrin and even more with β-cyclodextrin. Selectivity was also altered, being beneficial to resolve some peak pairs. The observed effect was explained by the incorporation of cyclodextrins onto the stationary phase, which increased solute retention.

1.7.6. Sample dissolving solvent

The nature of the solvent used to dissolve the sample seems to be also relevant to get good resolution. Working standard solutions for the analytical applications are often prepared by appropriate dilution with the ME mobile phase [18,25]. The ME droplet core offers a hydrophobic environment, with the capability of

dissolving compounds of low solubility in aqueous medium. Dissolution is facilitated by stirring in an ultrasonic bath during a few minutes.

It was found that changing the sample solvent from the ME mobile phase to a diluted ME had no effect on the separation. However, retention times decreased for the most retained compounds with improvements in peak efficiency using a methanol/water mixture to dissolve the sample [102].

1.8. Columns

1.8.1. Types of columns

The compatibility of the O/W MEs with RPLC columns allows a wide variety of bonded phases. Most common columns in RPLC packed with chemically bonded C18 (or even C8), such as Zorbax Extend-C18, Spherisorb C18 and Zorbax-Eclipse XDB-C8, all with particle size of 3–5 μm, are usual in MELC [18,40,61]. Successful separations have been reported at room temperature, for a wide range of basic, neutral and acidic drugs, and a variety of excipients (Tables 1.1 and 1.2). In a recent work, a comparison study was performed to choose the most appropriate column among the following [33]: Phenomenex Luna C18, Supelco Discovery C18, Supelco Discovery HS C18, Waters Symmetry C18, and Knauer Eurospher II C18. A ME mobile phase, formed with SDS, heptane, 1-butanol and TFA, was used. The ME composition was optimised for the selected column (Eurospher II). A column packed with cyano bonded phase was also tested for MELC [30,39]. The cyano column yielded better separation than a conventional C18 column, with symmetrical peaks and reasonable resolution.

The main issue with ME mobile phases is their relatively high viscosity, which affects flushing through the chromatographic columns. MEs generate relatively high back-pressure inside the columns, which can limit the maximal

flow-rate and, therefore, the ability to reduce the analysis time by increasing the flow. To solve this limitation, shorter columns have been used successfully in MELC without loss of resolution or efficiency [42]. Also, a 50 mm long sub-2 μm SB-C18 column has been tested [49].

Monolithic columns have been also proposed as a solution to reduce the back-pressure [20,25,33,43,57]. Columns built with silica monolith rods are made of a continuous interconnected skeleton with large through-pores, typically 2 μm in diameter, that reduce the diffusion path and provide high permeability. The material contains another pore structure of shallow diffusive mesopores, typically 13 nm in diameter that provide additional surface area for chromatographic activity. This allows the operation at high flow-rate with reduced back-pressure. With a conventional column, ME mobile phases can generate back-pressure of 120 bar (12 MPa) at a flow-rate of 1 mL/min. Using a monolithic column, ca. 3-fold lower back-pressure is achieved with the same ME, which allows flow-rates of up to 4 mL/min, without exceeding 80 bar (8 MPa). All these features yielded rapid separations in isocratic and gradient elution, with good chromatographic performance in terms of peak efficiency (Figure 1.7). Recently, MELC has been extended to ultrahigh-pressure RPLC using a monolithic column [43].

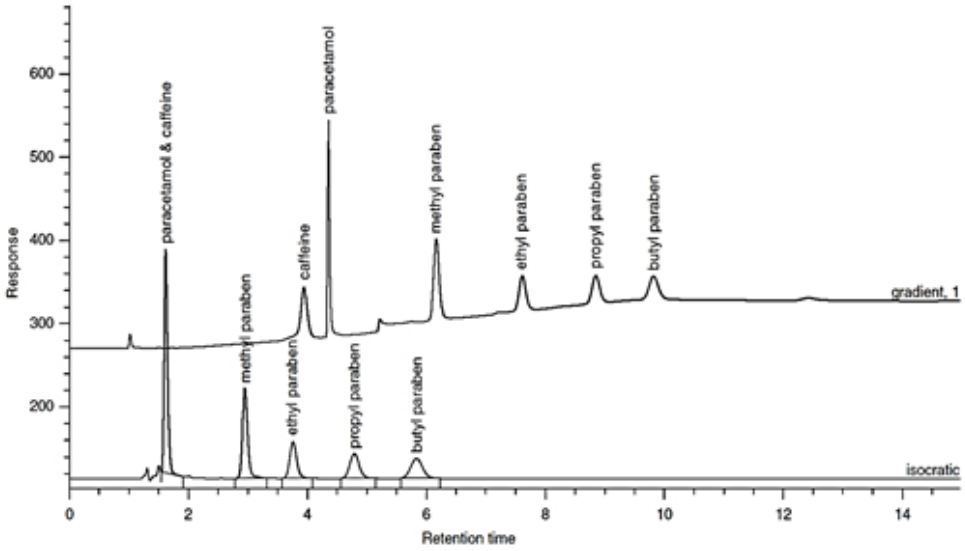


Figure 1.7. Gradient and isocratic separation of a mixture of paraben preservatives and paracetamol (0.1 mg/mL in ME), using a Hypersil BDS C18 column (150 × 4.6 mm i.d., 5 μm). Gradient conditions: reservoir A with 0.05 % *v/v* TFA in water; reservoir B with 3.3 % SDS / 6.6 % 1-butanol / 0.8 % octane / 0.05 % TFA, gradient started with 95 % A ramping to 100 % B in 7 min and held. Isocratic conditions: 100 % B, 1 mL/min, 30 ºC and 215 nm UV detection (Adapted from Marsh *et al.* [102]).

1.8.2. Column cares

As commented, in an MELC system, surfactant molecules not only allow the formation and stabilisation of the ME oil droplets, they are also adsorbed into the stationary phase modifying its nature. Although the co-surfactant competes with the surfactant for adsorption, and dissolves the surfactant layer on the stationary phase at least partially, some surfactants (especially those non-ionic) are difficult to desorb completely. Since the column is permanently modified, it should be kept for the exclusive use of an MELC procedure.

In RPLC, chromatographic columns require conditioning with the mobile phase before use. With conventional RPLC solvents, up to 30 column volumes may be needed to reach equilibrium. However, MEs are more viscous and contain less organic solvent. Thus, equilibration can take much longer (one to several hours) to achieve a steady baseline [22]. Reproducible separations have been described with columns completely equilibrated with MEs, getting a constant adsorbed layer on the packing [24]. It should be noted that with conventional columns, mobile phases containing surfactant should be filtered through 0.45 μm Nylon membranes before being used for chromatography. With sub-2-micron particle stationary phase, filtration should be carried out through 0.2 μm membranes [49].

Finally, the relatively high concentration of surfactant that MEs contain can be an issue if the surfactant is allowed to precipitate and/or accumulate within the column or equipment. Therefore, there is a higher need (with regard to conventional RPLC) to maintain cleanliness inside the chromatographic system with extra cares. Basic recommendations given to keep the column and equipment in good performance for MLC are also useful for MELC [103].

1.9. Comparison with Micellar Liquid Chromatography

The main difference between MLC and MELC is the presence of dispersed oil droplets in MELC, which are stabilised by the surfactant and co-surfactant molecules. An O/W ME is, in fact, a modification of a micellar system where a lipophilic organic solvent has been dissolved inside the micelles. For that reason, MEs are usually treated as solvent-modified micellar solutions, and MELC is viewed as an extension of the principles of MLC. However, more complex interactions with solutes are expected in MELC.

Several authors investigating the possibilities of MELC have compared its performance with that achieved in an MLC method using the same surfactant, but without the internal phase [18,27,30,39,42,58,61,94]. To make a fair comparison of conventional RPLC, MLC and MELC, the same column (Zorbax Eclipse XDB-C8) was used for the analysis of a mixture of neutral compounds (phenols and alkylbenzenes) [18]. In Figure 1.8, chromatograms for the three chromatographic modes are shown. The retention of all probe compounds in the MELC system was decreased significantly, compared to conventional RPLC. MELC provided also shorter retention than MLC, as the pseudo stationary phase (the ME droplets) is increased in size and hydrophobicity compared to the MLC system. The retention for the more lipophilic compounds was reduced due to increased partitioning into the ME droplets, while for those more hydrophilic it was primarily governed by the stationary phase. Hence, a more compressed chromatogram was obtained. Peak efficiency was similar in the three systems, whereas the selectivity was influenced by the composition of the mobile phase.

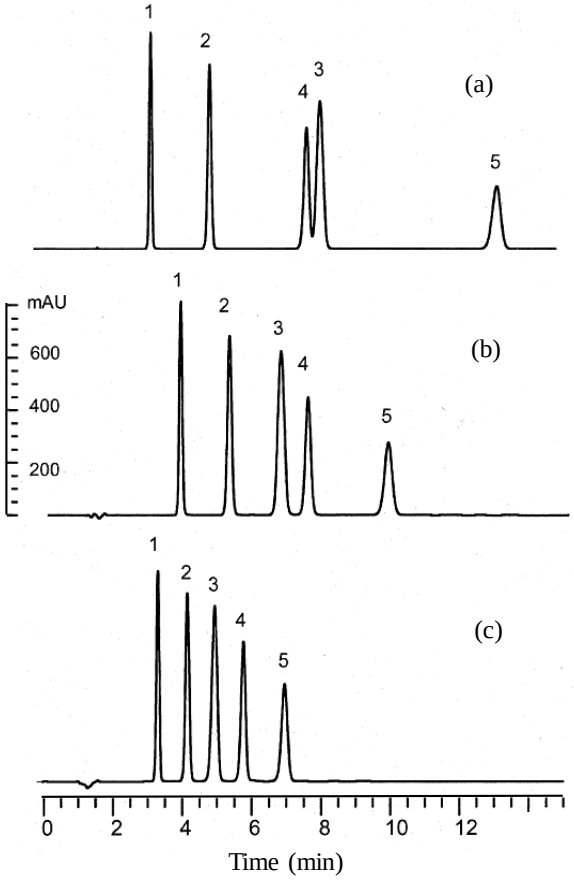


Figure 1.8. Comparison of RPLC modes: (a) conventional RPLC, methanol/water 50:50 *v/v*; (b) MLC, 2 % SDS / 10 % 1-butanol / 0.3 % triethylamine in 0.02 M phosphoric acid, and (c) MELC, 2 % SDS / 10 % 1-butanol / 1 % 1-octanol / 0.3 % triethylamine in 0.02 M phosphoric acid. Experimental conditions: Zorbax Eclipse XDB C8 column (150 × 4.6 mm i.d., 5 μm); 1 mL/min; 250 nm UV detection. Peak identity: (1) phenol, (2) *p*-methyl-phenol, (3) 3,5-dimethyl-phenol, (4) methoxybenzene, and (5) ethoxybenzene (Adapted from El-Sherbiny *et al.* [18]).

The MLC system yielded poorer efficiency and considerably longer retention time, compared to MELC, in the separation of 11 neutral aromatic compounds [94]. The elution order was the same in both systems. Also, using SDS as surfactant, the MLC method yielded smaller resolution and efficiency [27,30,39]. In the presence of the non-ionic surfactant Genapol X-080, the addition of oil again increased the elution strength compared to the micellar solution. Thus, in MLC, longer retention was obtained for a mixture of six flavonoids [58] and four phenylethanoid glycosides [61], compared to the ME mobile phase.

1.10. Conclusions

The words of Marsh *et al.* [42] are very illustrative of the advantages of MELC:

"using a ME as the mobile phase offers an additional capability to separate mixtures of components compared to other modes of RPLC. The hydrophobic ME core is able to solubilise hydrophobic compounds, while hydrophilic compounds are compatible with the aqueous continuous phase. For complex separations involving mixtures of hydrophilic and hydrophobic compounds, other RPLC modes often require a gradient for successful chromatography. Using a ME mobile phase they can be separated isocratically, owing to its extensive solubilising capability".

In O/W MEs, oil is dispersed into nano-droplets in the continuous aqueous phase, through the assistance of a surfactant and a co-surfactant that reside on the O/W interface. Conventional RPLC methods may cause an environmental problem because of the large amount of organic solvent needed (usually, acetonitrile or methanol), especially to elute hydrophobic compounds, which increases the waste-disposal burden of laboratories. The mobile phases used in

MELC can overcome this problem, because of the smaller amount of organic solvent (the ranges of water content, co-surfactant and oil concentrations of ME mobile phases are usually 90–95 %, 5–10 % and 0.5–2.0 %, respectively). Besides, the surfactants used in MELC are biodegradable [104,105]. Therefore, ME systems are considered as environmental friendly alternatives to the traditional solvents used in RPLC, giving rise to useful greener analytical methods.

MLC is a mature technique with hundreds (or even thousands) of reported applications since the 80s. MELC has a more recent development, with most reports published in the last 10–15 years. The field of application of MELC seems to be reserved to samples containing hydrophobic compounds, for which higher efficiencies, shorter analysis times and better solubilisation are obtained with regard to MLC. These samples normally require high levels of organic solvent, or normal phase conditions to be eluted.

The double nature of MEs (aqueous component and oil droplets) can make both hydrophilic and hydrophobic samples be easily dissolved. The micelle structure itself assists in the solubilisation of moderate to highly hydrophobic compounds, because of the variety of possible interactions with the surfactant molecules. Therefore, MELC seems capable of separating in the same run a quite complex range of acidic, basic and neutral compounds in a wide range of polarities, which are quite poorly separated by conventional RPLC, or even in MLC. This is useful for a variety of applications with reductions in method complexity, organic solvent consumption and cost. Several authors have reported selectivity and efficiency values for MELC, comparable to or larger than those obtained with conventional RPLC systems. Moreover, the ability to dissolve sample matrices in the hydrophobic oil core reduces the pre-treatment needed in the analysis of complex samples using conventional RPLC. This is especially

useful for samples with high oil content, and in clinical analysis where protein precipitation is avoided.

Separations using MEs are often faster than those implemented in the conventional RPLC mode. Therefore, MELC is considered as a high-speed separation technique using conventional equipment. Besides the control of mobile phase factors, the analysis time can be further decreased, without loss of resolution, by using shorter columns, or monolithic columns that allow increasing the flow-rate. Several issues of the technique, as those associated to the relatively high backpressure in the chromatographic system caused by the more viscous mobile phase, or the need of increased column cares, or the adsorption of surfactant on the stationary phase, and the use of a suitable hydrophobic organic solvent to form the ME droplets, seem be solved.

The complexity of the interactions and operating parameters in MELC needs a more systematic investigation. There is a need to better understand the mechanism of retention, which will help in the development of applications. The number of reports on the technique should continue to expand, in order to consider MELC as a viable alternative to conventional RPLC for routine analysis.

1.11. References

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CHAPTER 2

MODULATION OF RETENTION AND SELECTIVITY IN OIL-IN-WATER MICROEMULSION LIQUID CHROMATOGRAPHY

2.1. Abstract

Microemulsions (MEs) are stable, isotropically clear (transparent) solutions consisting of an oil and water stabilised by a surfactant and a co-surfactant. Oil-in-water Microemulsion Liquid Chromatography (MELC) is a relatively new chromatographic mode, which uses an O/W ME as mobile phase. Retention, selectivity and efficiency can be modified by changing the concentration of the ME components and the ratio between the aqueous and oil phases. This work makes a critical survey on the information found in the literature about the mobile phase compositions that lead to the creation of successful O/W ME mobile phases, as well as the effect of pH for ionisable compounds and temperature. The viability of performing the analyses using isocratic and gradient elution is also considered. The complexity of the composition of a successful ME, and the fact that the different factors interact each other, may require many manipulations during method development to achieve an acceptable separation for complex mixtures. This is the reason of the proposal from several authors of a standard ME as starting point when developing a method for a new separation with no previous reports. Based on these initial conditions, the interest of several authors in applying computer-assisted approaches to optimise the composition of ME mobile phases, and reduce significantly the time and reagent consumption for method development, is described. Some practical tips are given to prepare stable ME mobile phases that yield reproducible results.

2.2. Introduction

Microemulsions (MEs) are thermodynamically stable, transparent, optically clear, and isotropic liquid dispersions comprising oil phase, surfactant, co-surfactant (usually a medium chain-length alcohol), and water phase. Both hydrophilic and hydrophobic compounds can be dissolved in MEs, which are extensively applied in different fields. MEs have been used as pseudo-stationary phases in capillary electrophoresis, and as mobile phases in High-Performance Liquid Chromatography (HPLC), in the techniques called Microemulsion Electrokinetic Chromatography (MEEKC) [1], and Microemulsion Liquid Chromatography (MELC) [2–6], respectively.

There are three types of MEs: oil-in-water (O/W), water-in-oil (W/O), and a continuous phase with a sponge-like structure containing a mixture of the two liquids [7,8]. The formation of each type depends on the nature and concentration of the components, and temperature. However, in HPLC, O/W MEs are mostly used combined with conventional Reversed-Phase Liquid Chromatography (RPLC) stationary phases, due to their high water content, low viscosity and solubilizing power, and certainly, the popularity of RPLC. In MELC, solutes are partitioned between ME droplets, the stationary phase modified by adsorption of surfactant and co-surfactant, and bulk solvent (Figure 2.1). Separations are usually carried out using isocratic elution.

MELC has been developed based on the principles of Micellar Liquid Chromatography (MLC), where the mobile phase is composed of micellar solutions [9,10]. An O/W ME is, in fact, a modification of a micellar system where a lipophilic organic solvent is incorporated inside the micelles. Since the eighties, several hundreds of successful reports have been published in MLC. However, the analysis of samples containing highly non-polar compounds has

been a problem. Some authors thought MELC could be the solution. MELC presents unique selectivity and several advantages compared to MLC and conventional RPLC, for the analysis of water-insoluble species, even in mixtures with hydrophilic compounds, owing to the more complex interactions with solutes expected in MELC. The reduced retention times, equivalent or superior efficiency with implications in the resolution, and unique solubilizing power of MEs, are the most remarkable features of MELC.

Although the first report on O/W MELC appeared in 1992 [11], major interest in this chromatographic mode began only after 2003. There are three early reviews published in 2005 [2], 2007 [3], and 2008 [4]. Recently, we were interested in developing procedures in MELC, but found that the big amount of published information should be revised thoroughly, and especially, needed to be organised. We first published a review that analyses the mechanisms of retention in MELC with O/W MEs, and describes the great variety of reagents suitable to act as ME oils, surfactants and co-surfactants. The published analytical procedures were also summarised [6]. Most applications in MELC refer to the analysis of hydrophobic compounds, both in pharmaceuticals, physiological fluids and other biological materials. The methods include the analysis of alkaloids, antihypertensive agents, cholesterol lowering drugs, steroids, tetracycline antibiotics, and vitamins, among other compounds. The number of reports is still limited, but MELC seems promising, and nowadays it is receiving growing attention.

Simple variation in the polarity of the internal phase (typically, heptane, octane, cyclohexane, di-isopropyl ether, and ethyl acetate), as long as changes in the nature of surfactants (mainly sodium dodecyl sulphate, SDS, and polyoxyethylene(23)lauryl ether also known as Brij-35), and co-surfactants (mainly 1-propanol, 1-butanol, and 1-pentanol), affect the absolute and relative

retention of analytes. It is noteworthy that in almost every MELC report, the concentration ranges of the ME reagents are systematically investigated. We found, however, an enormous variability of experimental conditions, which should be inspected in detail to be useful. The discussion of the modulation of retention, selectivity and efficiency by varying the concentration of the reagents (oil, surfactant and co-surfactant) that form a ME, as well as the ratio between the aqueous and oil phases, needed a dedicated work. In this chapter, we give some orientations on the compositions leading to the creation of successful O/W ME mobile phases. The final choice will depend on the required separation speed, selectivity and efficiency. The viability of performing the analyses using isocratic and gradient elution, as well as the optimisation of mobile phase composition, is also commented.

2.3. Concentration ranges of the microemulsion reagents in MELC

Changes in the ME reagents (nature and concentration) affect not only the formation of stable oil droplets and elution strength, but also the stationary phase nature, since oil, surfactant and co-surfactant molecules all can be adsorbed onto the stationary phase [11,12]. Any variation in the ME composition can alter the equilibrium between the two phases, and consequently, the chromatographic behaviour of solutes in MELC. With a proper selection of the concentrations of surfactant and co-surfactant, the ME will be able to solubilise a greater proportion of oil. This in turn will enable the development of procedures suitable for the analysis of particularly water insoluble compounds [13]. It is, thus, very important to select a proper mobile phase composition with regard to all ME components, in order to achieve satisfactory separations.

The complex nature of MEs yields numerous composition options for getting good separation performance, when compared to other chromatographic modes.

The differences in the types and concentrations of oil, surfactant and co-surfactant could result in a great diversity of results, as will be next shown.

2.3.1. Concentration of surfactant

The critical micellar concentration (CMC) is the concentration of surfactant molecules in solution at which micelles start to form. Further addition of surfactant will increase the number of micelles, while the amount of free surfactant molecules in solution will remain constant. The CMC values in aqueous medium for SDS and Brij-35 (the most usual surfactants in reported MELC methods) are 8.2 mM and 0.06 mM, respectively [9].

When an organic solvent is added to the aqueous medium, the CMC values are altered. A study carried out for SDS micellar mobile phases, in the presence of different co-surfactants at increasing concentration, is very illustrative in this regard [14]. The CMC of SDS increased with methanol and acetonitrile, whereas it decreased with 1-propanol, 1-butanol and 1-pentanol. For 1-butanol and 1-pentanol, which partition into the micelle, the CMC barely changed for alcohol concentrations above 4 % and 1.5 % *v*/*v*, respectively. This behaviour indicates that the micelle is mainly modified at lower alcohol concentrations by introduction of the molecule of 1-butanol and 1-pentanol into the micelle palisade. At high concentration of these alcohols, the molecules probably are dissolved in the micelle core.

It should be noted that the inserted oil in the ME droplets can affect micelle formation. According to Marsh *et al.* [13], the CMC of SDS in an SDS/octane/1-butanol ME was reached at 18 mM (0.5 % *w*/*w*), instead of 8.3 mM (0.24 % *w*/*w*) for aqueous medium. In all cases, the concentration of surfactant used for preparing a ME should be well above its CMC. This will

produce enough micelles and ensure the system be dominated by stable oil droplets.

The effect of the concentration of SDS on solute retention and peak performance has been investigated by several authors. Some representative studied ranges are: 0.05–0.15 M (1.4–4.3 % *w*/*w*) [15] (a range usual in MLC [9]), 1.75–5 % *w*/*w* [4], 2.5–4.5 % *w*/*w* [16], and 2.9–7.2 % *w*/*w* [17]. The concentration finally selected was often relatively high inside the studied range, which is explained by the high lipophilicity of the analytes [18]. Thus, for example, for the 2.9–7.2 % *w*/*w* range, the selected concentration for routine use was 5.8 % *w*/*w*, since it provided good peak efficiency and highest resolution [17]. Although well above the CMC of SDS, the system could not solubilise the oil below 1.75 % *w*/*w*, and thus, the ME could not be formed. Also, MELC was found unstable below 3 % *w*/*w* SDS [13]. In another report, it was commented that no ME was formed below 2.5 % *w*/*w* SDS, and high back-pressure was generated above 4.5 % *w*/*w* [16].

In the separation of isoquinoline alkaloids [19] (Figure 2.1A) and phenolic compounds [20], 0.6 to 1.8 % *w*/*w* SDS was the best range. The resolution decreased when the concentration of SDS in the mobile phase increased from 0.6 to 1.8 % *w*/*w*. Decreasing the concentration down to 0.5 % *w*/*w* dramatically increased the retention of the analytes, which was too large to be measured.

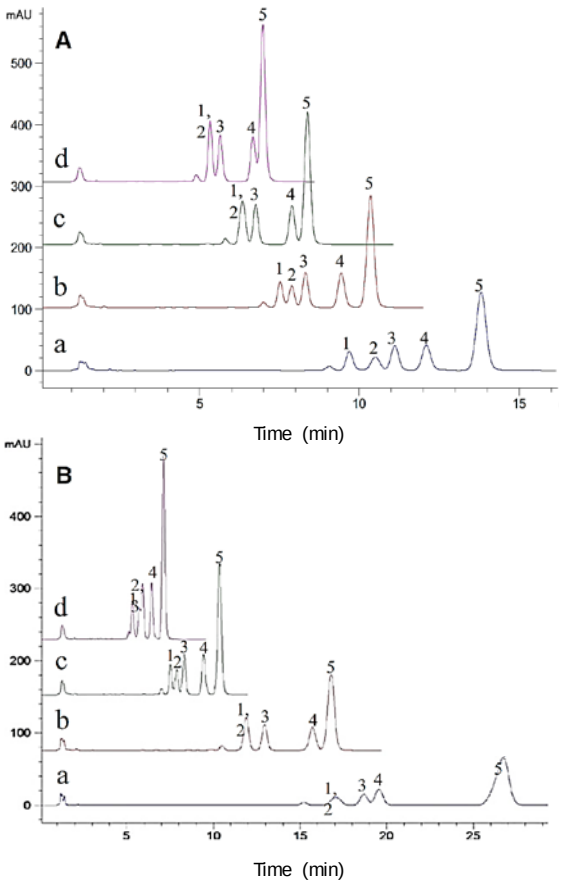

Figure 2.1. Effect of the concentration of surfactant (A) and co-surfactant (B) on the separation of five alkaloids from *R. coptidis* sample. (A) SDS concentration (%, *w*/*v*): (a) 0.6, (b) 1.0, (c) 1.4, and (d) 1.8; other experimental conditions were 0.8 % *w*/*v* ethyl acetate, 8.0 % *w*/*v* 1-butanol, 0.1 % *v*/*v* acetic acid and 10 % *v*/*v* acetonitrile. (B) 1-Butanol concentration: (a) 4.0, (b) 6.0, (c) 8.0, and (d) 10.0; other experimental conditions were 0.8 % *w*/*v* ethyl acetate, 1.0 % *w*/*v* SDS, 0.1 % *v*/*v* acetic acid and 10 % *v*/*v* acetonitrile. Analytes: (1) epiberberine, (2) jatrorrhizine, (3) palmatine, (4) coptisine, and (5) berberine (reprinted from Ye *et al.* [19] with permission from Wiley).

Authors found that, all over the investigated range, an increased concentration of SDS yielded shorter retention times for all analytes, or at least for some of them. This was explained by the distribution of solutes into the increased volume of the ME droplets (which act as pseudo-stationary phase), or the association of solutes at the droplets surface [21]. This made them travel with the speed of the mobile phase flowing towards the detector. These effects were more pronounced for lipophilic solutes, which have a high affinity for the oil droplets.

The improved efficiency has been also attributed to the higher distribution of solutes into the ME droplets [22]. It seems that in the presence of oil and co-surfactant, an increased surfactant concentration increases the number of micelles in the mobile phase, but does not alter the layer of surfactant adsorbed on the stationary phase, and so its effect on solute retention. Therefore, the increased surfactant only affects the interaction of solutes with the ME droplets [13].

Some recommendations on the most appropriate concentration ranges for other less common surfactants can be found in the literature. Thus, the non-ionic Brij-35 was studied in the 0.5–2 % *w*/*w* range. It was found that the retention was shorter with increased concentration in the 0.5–1 % *w*/*w* range, which was explained by the modification of the stationary phase surface by the surfactant [23,24]. However, a further increase in the amount of Brij-35 had very small effect on the retention of analytes.

The non-ionic Genapol X-080 was investigated in the 0.4–2.0 % *v*/*v* range for the separation of a group of flavonoids [25], and 0.5–2.5 % *v*/*v* for phenylethanoid glycosides [26]. It was found that the retention decreased with increasing concentration of Genapol X-080 up to 1.2 and 1.5 % *v*/*v*, respectively. Below 0.4–0.5 % *v*/*v*, the ME was hardly formed or was unstable. Above 2–2.5 % *v*/*v*, there was very little effect on the retention of analytes.

2.3.2. Oil content

When the concentration of oil is zero, the mobile phase containing surfactant will have conventional micelles. The addition of oil to the micellar mobile phase decreases the retention, due to the increased mobile phase hydrophobicity. However, the observed effects upon changing the oil content in the ME mobile phase will depend on the nature of analytes. The effect will be stronger for lipophilic compounds. This can be explained by considering that an increase in oil leads to more oil droplets, where lipophilic compounds can partition. In contrast, hydrophilic compounds will have higher affinity towards the ME continuous phase, and therefore, they are not partitioned as fully into the oil droplets [13,24].

Different types of oil have been used to build the mobile phase in MELC (Figure 2.2). Most often, alkanes (hexane, heptane and octane) and alcohols (mainly 1-pentanol and 1-octanol, with higher water solubility than alkanes), of varying chain length, have been used. Other water-insoluble solvents used in analytical reports are cyclohexane, dichloromethane, ethyl acetate and di-isopropyl ether. Some authors have indicated that changing the oil content had no significant impact on improving the separation performance. This is the case of a report using SDS/1-butanol/cyclohexane MEs, where the oil content was finally fixed at 0.9 % *w*/*w* [27]. Ethyl acetate was investigated in the 0.5-1.0 % *w*/*w* range, but only a slight decrease in retention of analytes was observed on increasing its concentration above 0.5 % *w*/*w* [19,23,25].

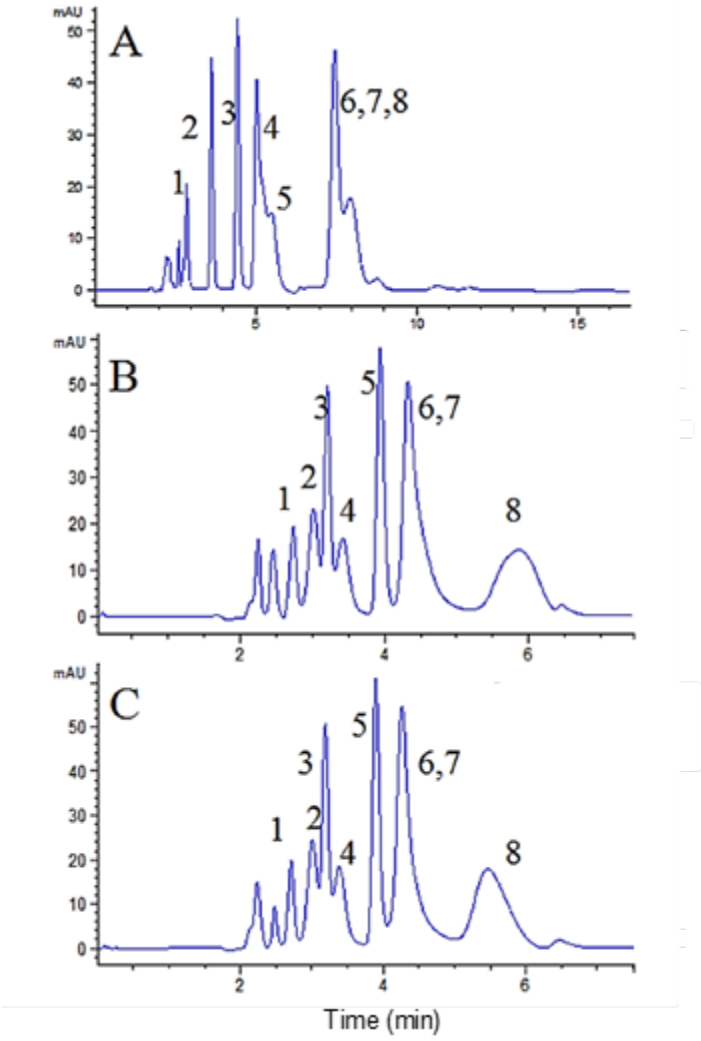

Figure 2.2. Effect of the oil type (0.2 % *w*/*v* oil) on the separation of phenolic compounds with UV detection: (A) ethyl acetate, (B) dichloromethane, and (C) octane. Other experimental conditions were 1 % *w*/*v* SDS and 3 % *w*/*v* 1-butanol at pH 2.5. Analytes: (1) sodium danshensu, (2) protocatechuic acid, (3) protocatechuic aldehyde, (4) caffeic acid, (5) lithospermic acid, (6) rosmarinic acid, (7) salvianolic acid B, and (8) salvianolic acid A (reprinted from Peng *et al.* [20], with permission from Elsevier).

However, other authors have reported significant effects on retention, selectivity and efficiency, and consequently, on the resolution of analytes by changing the oil content [28]. In general, the concentration range examined for oils is narrow. Thus, it was observed that increasing the di-isopropyl ether content from 0.25 % to 1 % *w*/*w*, the efficiency and resolution increased, but these decreased slightly above 1 % *w*/*w* [16,28]. On the other hand, for octane, the oil content was varied from 0 to 1.2 % *w*/*w*, no more oil being solubilised by SDS. When the mobile phase contained 1 % *w*/*w* or more oil, poor reproducibility was obtained, indicating that the system was unstable. An oil content of 0.8 % was selected [13]. A more hydrophobic SDS/1-butanol/octane ME, capable of eluting insoluble compounds more quickly, was prepared by increasing the concentrations of surfactant and co-surfactant. This enabled raising the oil content from 0.8 to 2 % *w*/*w*, so that the optimal oil : surfactant : co-surfactant ratio was kept, without compromising the ME stability [13].

One of the most recent advances in MELC is the use of ionic liquids as oils. The effect of different concentrations of the ionic liquid 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6C_1IM][PF_6]$) was investigated in the 0.1–0.4 % *w*/*v* range [20]. The optimal separation of several phenolic acids was achieved with a ME consisting of 1 % *w*/*v* SDS, 3 % *w*/*v* 1-butanol, 0.2 % *w*/*v* $[C_6C_1IM][PF_6]$, and 95.8 % *v*/*v* water at pH 2.5, buffered with phosphoric acid and 10 % *v*/*v* ammonia.

2.3.3 Concentration of co-surfactant

The co-surfactant has a very important role in the stability of MEs. It also influences the chromatographic behaviour. By increasing the concentration of co-surfactant in the ME mobile phase, the proportion of organic phase in the aqueous component increases, and therefore, the solubilisation effect. This reduces solute retention, especially for water-insoluble compounds [13,29,30]. Concomitantly, the elution speed for hydrophilic solutes is decreased [20].

According to some authors, the type and concentration of co-surfactant in MELC may have the biggest influence on the separation, but this again will depend on the analytes [21,31]. The most usual co-surfactants are 1-propanol and 1-butanol. Consequently, most studies on the effect of the concentration of co-surfactant on the chromatographic behaviour in MELC correspond to these two alcohols.

An illustrative example of such studies refers to the determination of paracetamol in suppository formulations [32]. The addition of 1-propanol up to 5 % *v*/*v* decreased only slightly the retention of this drug. However, the peak efficiency was significantly enhanced. No effect on retention and efficiency was found above 5 % *v*/*v* 1-propanol. Also, it was found that above 3.8 % *v*/*v* acetonitrile the ME became very cloudy and prolonged sonication was required to re-form it.

The concentration range for 1-propanol in a study to optimise the separation of four phenylethanoid glycosides was limited to 1–3 % *v*/*v* [26]. The retention decreased with increasing concentration of 1-propanol from 1 % to 2.5 % *v*/*v*, but a further increase did not yield significant changes. Therefore, 2.5 % *v*/*v* 1-propanol was used in subsequent experiments.

In other reports, the effect of co-surfactant concentration on the chromatographic behaviour of a variety of compounds, using 1- and 2-propanol was investigated in the 5–15 % *v*/*v* range. An increase in co-surfactant concentration resulted in decreased retention times [17,28,33]. Concentrations below 5 % *v*/*v* yielded broad peaks and reduced sensitivity. Optimal performance (highest efficiency and best resolution) was obtained at relatively high concentration of 10–13 % *v*/*v*. A higher concentration greatly increased the backpressure, due to the high mobile phase viscosity.

In the optimisation of the separation of several drugs, the concentration of 1-butanol was varied in the 6.6–16.5 % *v*/*v* range [13]. Outside this range, the ME was unstable. The authors indicated that the ME system was able to accommodate increases in 1-butanol within the oil droplets up to a concentration of 9 % *v*/*v*, with little effect on the separation. Above this value, 1-butanol remained in the aqueous component, increasing the organic proportion in the mobile phase, which yielded faster elution for hydrophobic solutes. The chromatogram was thus compressed: the retention of the least retained compound remained constant, while it was decreased for the other compounds. This reduced the resolution.

In the implementation of a method for nifedipine in pharmaceutical formulations, an increase in the 5.6–8.6 % *v*/*v* range for 1-butanol was observed to have no marked effect on the retention, whereas below 5.6 % *v*/*v* an unstable ME system was obtained [16]. Concentrations above 8.6 % *v*/*v* were not viable due to the increased column back-pressure. Experiments performed with different concentrations of 1-butanol showed that the retention of five isoquinoline alkaloids decreased noticeably along the 4–10 % *v*/*v* range (Figure 2.1 B) [19]. However, with the highest assayed concentrations, overlapping of some peaks was visible. Concentrations below 8.0 % *v*/*v* 1-butanol resulted in

broad peaks and reduced sensitivity. Therefore, 8.0 % *v*/*v* was selected as optimal, as it yielded the best separation with short analysis time. Other authors investigated a narrower 1-butanol range (0.5–3.5 % *v*/*v*) to optimise the separation of a variety of drugs [23–25]. Changing the concentration of 1-butanol from 0.5 to 2.5 % *v*/*v*, the retention times decreased. Since a further increase showed no marked effect on the retention, 2.5 % *v*/*v* 1-butanol was selected in subsequent experiments.

2.4. Selection of pH

In aqueous-organic RPLC, retention of compounds is correlated with their polarity: more hydrophobic solutes will be longer retained. When compounds are ionised, they become more polar and retention decreases. Consequently, the mobile phase pH will strongly affect the retention of ionisable compounds. The same behaviour is expected in MELC: retention of ionisable compounds will be influenced by the mobile phase pH, the behaviour being more complex with respect to conventional RPLC since the interaction of the acid-base species with both ME droplets and modified stationary phase (especially for charged solutes interacting with charged surfactants) will shift the acid-base equilibria. One of the acid-base species will be stabilised changing the value of the dissociation constant (pK_a), as is also the case in MLC [34,35]. Therefore, the pH range affecting the retention of ionisable compounds will be probably different in MELC and conventional RPLC. Meanwhile, for non-ionisable compounds, retention will be unaffected over the entire pH working range.

The effect, in MELC, of the mobile phase pH on retention, selectivity, and especially resolution, has been studied by several authors. In an early report, the behaviour of carboxylic acids (furosemide, bumetanide and naproxen), and basic compounds (atenolol, nadolol and timolol) was studied [21]. The pK_a values of

carboxylic acids in aqueous medium are in the 3.5–5 range, and therefore, these compounds become increasingly ionised as the pH increases in acidic medium. As they become ionised, their retention decreases due to the weaker interaction with the stationary phase. The low affinity of carboxylate ions towards the ME droplets makes partitioning towards the pseudo-stationary phase neither possible. Similar behaviour was found for naproxen (with pK_a = 4.4), which was examined above pH = 1.5 [13], and fosinoprilat [31], whose retention times were almost doubled by decreasing the mobile phase pH from 4.5 to 2.5. The pH was adjusted to 2.8 to attain good selectivity in a reasonable analysis time. In contrast, basic compounds are fully protonated in the 3.5–5 pH range, and thus their retention does not depend on the pH [21].

The behaviour of hydrochlorothiazide and losartan potassium was studied at diverse pH values (Figure 2.3) [22]. The two drugs differed in hydrophobicity (octanol/water partition coefficient, log $P_{o/w}$) and acidity constants (pK_a). For hydrochlorothiazide, log $P_{o/w}$ = –0.5 and pK_a = 7.9, while for losartan, log $P_{o/w}$ = 3.3 and pK_a = 5.5. Therefore, the retention time of losartan increased as the pH of the mobile phase was decreased below pH = 7. Meanwhile, there was no significant effect on the retention of hydrochlorothiazide. It was also observed that at pH 7 the peak profile of losartan was poor and baseline separation could not be achieved between both compounds. A pH value of 5.0 was finally considered as optimal for the separation and detection of both analytes (hydrochlorothiazide and losartan potassium), in a single run.

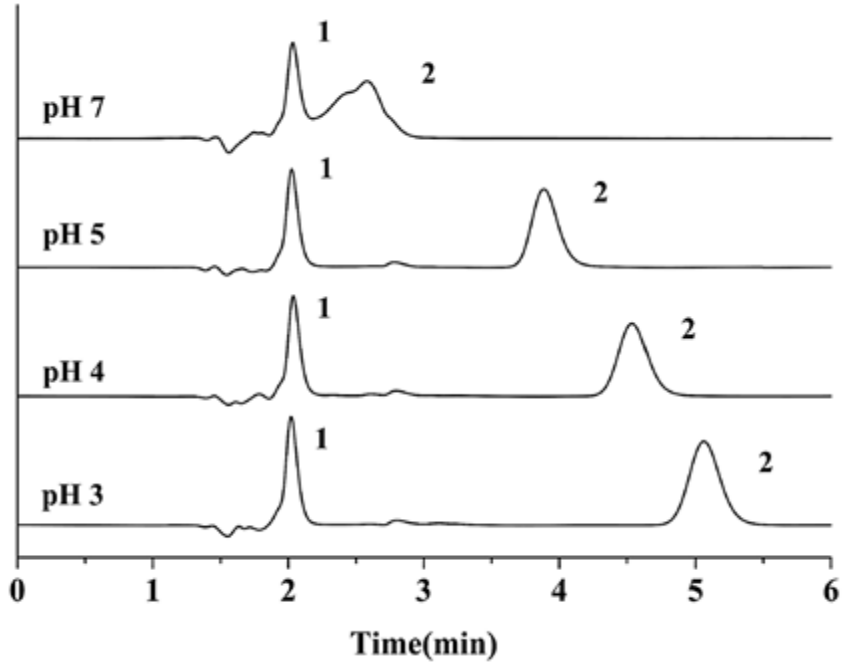

Figure 2.3. Effect of pH on the chromatographic behaviour of: (1) hydrochlorothiazide, and (2) losartan potassium (reprinted from Li *et al.* [22], with permission from Oxford Academic).

Dopamine receptor antagonist LE300 and its N-methyl metabolite were investigated using ME mobile phases in the 2.5–7.5 pH range [28]. Increasing the pH from 2.5 to 3.5 improved the efficiency and resolution. A value of 3.5 was selected, since it yielded well resolved peaks of high efficiency with reasonable analysis time. At pH > 3.5, the separation was poorer.

The effect on the MELC separation of six flavonoids in leaf extracts [25] and four phenylethanoid glycosides in rat plasma [26], was investigated in the 2–6 pH range, using phosphoric acid and increasing amounts of trimethylamine to adjust the pH. The retention time of the analytes decreased with increasing pH, indicating a weak acidic character. A pH value of 6 was found optimal to get separation in sufficiently short analysis time. A similar recent study was carried out with eight phenolic compounds (also weak acids), in the 1.5–5.5 pH range using phosphoric acid and ammonium hydroxide to adjust the pH [20]. Strong co-elution was observed at pH 1.5–2.0 and above 5. Therefore, pH 2.5 was chosen as the most appropriate, since it provided good resolution within reasonable analysis time.

2.5. Effect of temperature

The effect of temperature on the chromatographic behaviour of solutes in MELC has been investigated by several authors. In an early study, Marsh *et al.* [13] injected a test mixture of parabens, oxibendazole and beclamethasone dipropionate at each of the following temperatures: 20, 30, 40, 50 and 60 ^{o}C. It was found that the separation was robust to temperature changes, since increasing the operating temperature had little effect on the retention times. However, peak-to-peak resolution between the last two peaks was enhanced. It was explained by the increase in solute mass transfer from stationary phase to bulk mobile phase, droplet to stationary phase, and droplet to bulk mobile phase, at higher temperature. This resulted in a reduction in band broadening, especially for the most hydrophobic compounds. Average improvement of peak efficiency of 22 % was observed for all peaks when raising the temperature from 40 to 60 ^{o}C.

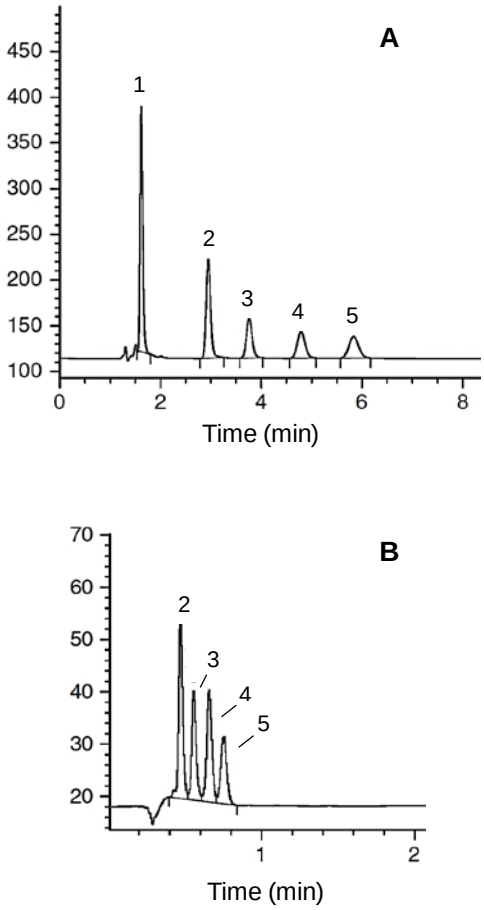

Figure 2.4. Isocratic separation of a mixture of paraben preservatives and paracetamol, using: (A) Hypersil BDS C18 column (150 × 4.6 mm i.d., 5 µm) at 1 mL/min, and (B) Chromolith RP-18e (100 × 4.6 mm i.d.) at 4 mL/min. Experimental conditions: 3.3 % *w*/*w* SDS, 6.6 % *w*/*w* 1-butanol, 0.8 % *w*/*w* octane, and 0.05 % *v*/*v* TFA, 60 ºC, and UV detection at 215 nm. Analytes: (1) paracetamol, (2) methyl paraben, (3) ethyl paraben, (4) propyl paraben, and (5) butyl paraben (adapted from Altria *et al.* [36]).

In two reports with a test mixture of parabens, caffeine and paracetamol, the same results were found [36,37]. Raising the temperature from 20 to 60 ºC reduced the retention time by only 0.2 min for the early eluting compounds and by 1 min for those most retained. Instead, an important effect on the efficiency was found (Figure 2.4), with improvements in the 20–70 % range. A similar behaviour was found for salbutamol and an internal standard from 20 to 50 ºC [24], and nifedipine from 25 to 45 ºC [16]. In another work, both peak retention and efficiency for paracetamol were affected by temperature, while peak asymmetry was unaffected [32].

2.6. Isocratic and gradient elution

One of the main strengths of the RPLC methods with mobile phases containing surfactant (MLC or MELC) lies in the capability of performing isocratic separations of mixtures of compounds exhibiting a relatively wide range of polarities, in convenient analysis times. In these chromatographic modes, the retention of the least retained compounds is often larger compared to conventional RPLC (which is interesting with regard to the direct injection of physiological samples). Meanwhile, the retention of the most lipophilic compounds is shorter (which has the advantage of the applicability of a single set of experimental conditions to a wide range of analytes, without needing gradient elution) [10,21].

However, several authors have also suggested that gradient elution in both MLC and MELC could be useful to reduce the analysis times for complex mixtures, with more fine control of retention than the isocratic methods. Gradient elution may offer benefits and enhance separation selectivity for hydrophilic and closely-eluting compounds. It was already proposed in MLC in the 90's for both the surfactant [38] and organic solvent [39]. Gradients of acetonitrile, methanol and 2-propanol, at constant micelle concentration, were shown to provide efficient separations. Among other applications, a gradient where both micelle and organic solvent were simultaneously increased, using SDS and 1-pentanol (where 1-pentanol played the role of oil) is worth to comment (Figure 2.5) [40]. Initially, 0.05 % *v*/*v* formic acid solution was pumped through the column and the amount of SDS/1-pentanol was increased. Successful separations of basic drugs (Figure 2.5A), and neutral aromatics (Figure 2.5B), were achieved in 14 and 4 min, respectively.

Other studies showed that MELC is suitable to carry out separations using gradient elution for a wide range of compounds. The gradients were usually formed with an aqueous starting eluent, which was mixed with increasing amounts of ME.

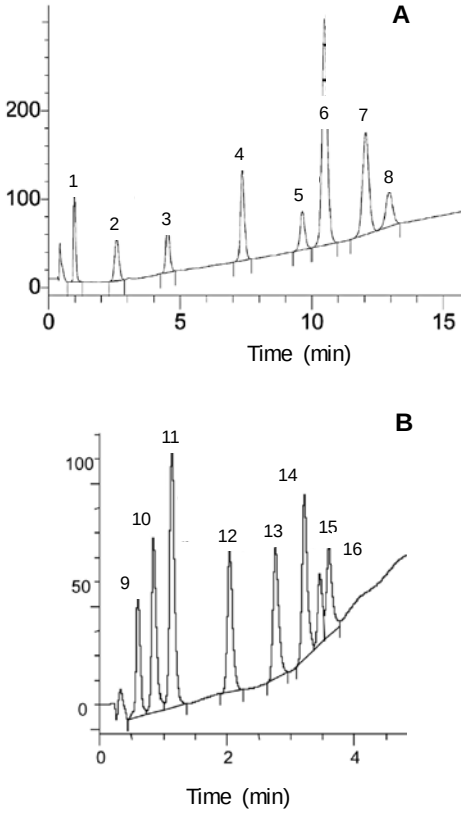

Figure 2.5. Separation by gradient MELC with SDS and 1-pentanol (Reservoir A: water / 0.05 % *v*/*v* formic acid; reservoir B: 33 g SDS and 100 g 1-pentanol dissolved in 1 litre of water with 0.05 % *v*/*v* formic acid; detection at 240 nm): (A) Basic compounds (flow-rate, 0.5 mL/min; gradient was 5 % *v*/*v* B for 1 min, reaching 70 % *v*/*v* B at 15 min). (B) Neutral aromatics (flow-rate, 0.8 mL/min; gradient was 10 % *v*/*v* B, reaching 100 % at 3 min). Analytes: (1) norepinephrine, (2) isoproterenol, (3) atenolol, (4) pindolol, (5) lignocaine, (6) salmeterol, (7) labetalol, (8) bupivacaine, (9) phenacetin, (10) acetanilide, (11) acetophenone, (12) propiophenone, (13) butyrophenone, (14) valerophenone, (15) hexanophenone, and (16) heptanophenone (adapted from Bryant *et al.* [40]).

A successful separation of 12 neutral aromatic compounds was obtained using a gradient formed by mixing from 5 % *v*/*v* ME (SDS / 1-butanol / octane / 0.05 % formic acid) up to 70 % *v*/*v* ME at 15 min [40]. A gradient separation in less than 1 min was also reported for a mixture of paraben preservatives, using a monolithic column and a flow-rate of 4 mL/min [36]. For these analyses, a linear gradient ramp was used starting with 5 % ME / 95 % *v*/*v* TFA up to 100 % ME. Also, an optimised MELC gradient method resolved, in 7 min, paracetamol and five related compounds potentially present in formulations [32]. The optimal gradient was started with 50 % ME / 50 % *v*/*v* 0.05 % TFA aqueous solution and ramped up to 100 % ME in 8 min.

According to the authors, the methods gave superior peak efficiency and faster elution than MLC for the same test mixture, and superior selectivity than the MELC isocratic method. The MELC gradient methods were also superior to those in the conventional RPLC mode, in terms of analysis time, selectivity and efficiency. A remarkable characteristic was that there was no need for column re-equilibration between injections, since increasing the concentration of the ME droplets in the mobile phase does not change the structure and composition of the modified stationary phase, which is the case for hydro-organic gradients. The methods resulted in considerable time savings, compared to conventional RPLC. The ME gradient can be extended up to 100 %, which is needed to elute highly hydrophobic compounds. This is not possible in conventional RPLC, due to column dehydration and difficulties in restarting the gradient.

In spite of the above benefits of MELC with gradient elution, there are also some negative comments in the literature that should be indicated. Thus, Bryant and Altria found that when a high proportion of water (at the start of the gradient) was tried to be mixed with an O/W ME, this was disrupted (demixed) in the

pump, giving rise to an immiscible two phase suspension [40]. This made analysis impossible because the cloudy, unstable ME resulted in a very high and noisy UV signal. This problem was solved by increasing the ionic strength of the aqueous component by addition of NaCl. The presence of salt allowed easier formation of the ME at high water contents. Thus, the gradient was built by mixing 0.5 M NaCl solution (solvent A) with 3.3 % *w*/*w* SDS, 6.6 % *w*/*w* 1-butanol, 8 % *w*/*w* octane and 10 mM sodium tetraborate (solvent B). The gradient started at 95 % *v*/*v* of solvent A for 2 min, and decreased linearly to 25 % *v*/*v* at 10 min. Under these conditions, the solutes were retained on the column until a sufficiently high ME composition was formed and each solute was eluted in turn.

Marsh *et al.* commented that, initially, they did not observe ME demixing in the course of the experiments, and the addition of NaCl was not required for a successful gradient [37]. However, later they found the ME/water problem, and concluded that the pumping and mixing properties of the chromatographic system affected the compatibility of the two mixed eluents.

According to McEvoy *et al.*, peak retention times and resolution were irreproducible in MELC gradients. These authors indicated that reproducible separations can only be achieved using isocratic MELC [41]. The irreproducible retention times in gradient MELC were attributed to the nature of the adsorbed layer on the column packing and the possible breakdown of the unstable ME produced during gradient elution. The authors affirmed that reproducibility could be only achieved by allowing the column to equilibrate with the ME mobile phase to get a constant adsorbed layer on the packing. When a concentration gradient is employed, equilibration is not possible since the nature of the adsorbed layer is constantly changing during gradient runs. While MEs containing the cationic surfactant cetyltrimethyl ammonium bromide (CTAB) remained stable during

gradient runs, the problem of column equilibration still remained owing to the changes in the adsorbed CTAB layer [41].

As O/W MEs are composed mainly of water, performing gradient elution by ramping up the concentration of the aqueous component would not reverse the polarity of the eluent, and may succeed in the separation of co-eluting compounds. Trying to exploit this idea, gradient conditions were obtained by diluting a ME prepared with SDS, from 100 % to 0 % *v*/*v* with its aqueous component (0.05 % TFA), in order to simulate a concentration ramp of the components during gradient elution [41]. The diluted ME samples were visually examined to assess their stability, based on the appearance of turbidity. Decreases in stability were also monitored through the observation of possible increases in surface tension. At concentrations between 100 % and 80 % *v*/*v* ME, the system remained stable. When the ME was diluted to below 77 % *v*/*v*, it became turbid and the surface tension increased linearly up to the point where the CMC of SDS was reached (approximately 8 % *v*/*v* ME). Below the CMC, the surface tension of the system rapidly approached that of water.

2.7. Optimisation strategies

As commented, most published work in MELC corresponds to isocratic elution. Accordingly, optimisation strategies have been developed for this elution mode. Factors usually investigated include the type and concentration of surfactant and co-surfactant, the type of oil (less often its concentration), and pH in the mobile phase. As commented in previous sections, using adequate concentrations of surfactant and co-surfactant is essential to form a stable ME. If the oil : surfactant (or co-surfactant) ratio is incorrect, the ME will either fail or decompose into separate oil and water layers within a short period. Usually, the

elution ability of the ME increases with increasing surfactant and co-surfactant concentrations. In general, all factors can alter the selectivity and efficiency.

The complexity of the composition of MEs will require many manipulations during method development [42], in order to achieve an acceptable separation for complex mixtures (i.e., sufficiently short analysis time and good resolution for the peaks of interest). In fact, one main problem of MELC is the many factors that should be optimised. This also makes robustness testing more demanding [43,44]. It is also the reason of the proposal from several authors of a selected ME as starting point, when developing a method for a new separation with no previous reports. For this purpose, a typical ME used in MEEKC was proposed in an early MELC work by El-Sherbiny *et al.*, consisting in 3.3 % *w*/*w* SDS, 6.6 % *w*/*w* 1-butanol, 0.8 % *w*/*w* octane and 89.3 % *w*/*w* aqueous solution of 0.05 % TFA [21]. This ME, referred in MELC reports as standard ME (or standard conditions), is appropriate to separate mixtures containing compounds in a wide range of polarities. The same composition has been used in several reports [13,30,32,36,37,40,41]. Another suggested starting ME composition (2 % *w*/*w* SDS, 10 % *w*/*w* 2-propanol, 1 % *w*/*w* 1-octanol and 0.3 % *w*/*w* triethylamine in 0.02 M phosphoric acid) was inspired in a typical MLC mobile phase [21]. From these initial compositions, several modifications (one factor at a time) are usually performed to tune the analysis time and resolution in a controlled way.

Method development with trial and error approaches can be very time-consuming (especially when the number of factors is large). Also, these approaches often do not lead to the best experimental conditions. On the other hand, varying all factors affecting an MELC separation can generate a vast amount of data that further should be analysed and interpreted. Therefore, it is desirable to get the best conditions by performing a limited number of experiments in a minimal time, using computer-assisted approaches. Several

authors have been interested on the use of diverse Chemometrics tools to study the influence of the experimental factors in MELC, determine which are of primary importance, and find the optimal conditions for the selected factors. The approaches take benefit of the proper modelling of the main factors affecting the retention (concentrations of oil, surfactant and co-surfactant, to which pH should be added for ionisable compounds) [29,45–48].

Experimental design techniques have been applied in several reports to optimise the composition of ME mobile phases. The several factors that affect the separation interact each other. Thus, their simultaneous optimisation is needed. Using experimental design, the number of involved experiments is significantly reduced, and the risk of missing non-linear relationships is minimised. However, some previous experiments should be performed to choose the suitable factors and determine the experimental domain. More often, 2^3 full factorial designs (where all input factors are set at two levels) have been applied [27,29,48]. In a more complex study including the column type, a 2^4 full factorial design with four central point replications was developed [49].

New extreme values (star points) were also added to the full factorial designs, for each factor, to build a central composite design (CCD) [27,44,48,50]. This was used to create the matrix of experiments for mapping the chromatographic response surface to optimise the separation conditions. CCD produces a detailed quantitative model which can be used for the prediction of how a response relates to the values of several factors. This tool was applied combined with artificial neural networks in optimisation and robustness testing [43].

In the optimisation studies, only the resolution or both resolution and analysis time were taken into consideration. Multiple regression analysis of data was carried out to calculate the coefficients of quadratic polynomial equations, which

correlate the response (such as the resolution) to the different factors (usually, the concentrations of oil, surfactant, and co-surfactant), as follows:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (2.1)$$

where y represents the estimated response, and x_1, x_2 and x_3 are three experimental factors. The model is mapped against two of these factors, while the third factor is held constant at the central value. As an example, Figure 2.6 shows the response surfaces using different pairs of two factors in the separation of nine hydrophilic and hydrophobic components in *Salviae miltiorrhizae Radix et Rhizoma* (Danshen) [48]. The diagrams visualise the optimal conditions, but these can be finely obtained using software designed for optimisation. Figure 2.6 depicts also the chromatogram obtained at the optimal conditions. Addition to the ME of the cationic reagent CTAB below its CMC increased the retention and selectivity of acidic analytes. This surfactant couples with the anionic species to form a neutral compoubd that increases the hydrophobicity of these analytes.

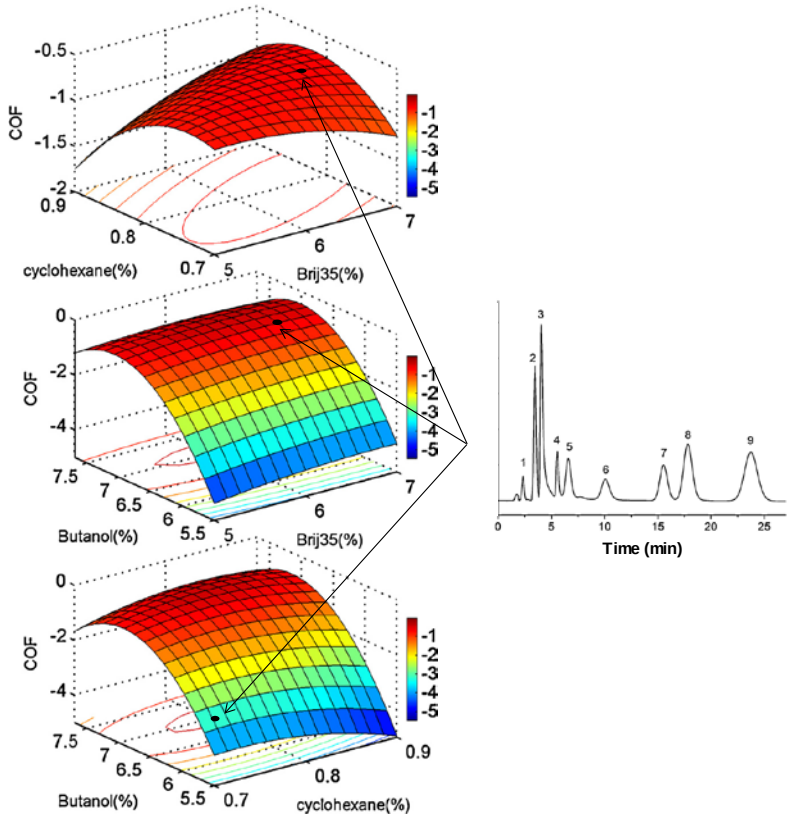

Figure 2.6. Three-dimensional resolution surface diagrams (left) for the separation of nine hydrophilic and hydrophobic components in Danshen with an Odyssil C18 column, considering the concentrations (*w*/*w*) of Brij-35, cyclohexane and 1-butanol, as factors. In each diagram, a factor was kept constant at the central level. Other experimental conditions were 85.6 % phosphate buffer (pH 6.6), 8 mM CTAB, 30 ºC, 0.8 mL/min flow-rate, and UV detection at 270 nm. Experimental chromatogram for the optimal conditions (right): 6.68 % Brij-35, 0.84 % cyclohexane and 6.92 % 1-butanol. Analytes: (1) sodium danshensu, (2) caffeic acid, (3) protocatechualdehyde, (4) rosmarinic acid, (5) salvianolic acid C, (6) salvianolic acid B, (7) tanshinone I, (8) cryototanshinone, and (9) tanshinone II A (adapted from Huang *et al.* [48]).

In view of the complex ME composition and the requirement of simultaneous optimisation of antagonistic objectives, some authors have employed multi-objective techniques using desirability functions (multi-criteria decision making tools) [16,48,51,52]. Genetic algorithms (GAs) were also applied to speed up the simultaneous optimisation of the different factors involved in MELC separations [53]. GAs emulate the biological evolutionary theory and allows finding a global, true optimised condition among several possible local alternatives. In that work, the studied factors were the concentrations of oil, surfactant and co-surfactant, temperature and pH.

2.8. Preparation of microemulsion mobile phases

The methodology used to prepare ME mobile phases is similar to that followed in MLC for hybrid micellar mobile phases, except for the fact that an oil is also added. First, an adequate amount of surfactant, most often SDS, is dissolved in a precisely measured volume of a solution of buffer, such as potassium dihydrogen phosphate. The pH is usually adjusted in the aqueous medium with NaOH or HCl [54]. Alternatively, the surfactant is first dissolved in water, and a buffer reagent is then added to fix the pH [13,55]. To prepare the ME, the co-surfactant (such as 1-butanol) and oil (such as heptane) are added to the surfactant solution, in this or the reversed order. After incorporating these and other additional reagents to the mixture, the solution should be stirred and sonicated in an ultrasonic bath during a few minutes (5–10 min). Diverse authors have also recommended sonication of the final mixture during 20–45 min [22,29,32,48,56]. Heating at 45 ^{o}C has also been suggested to obtain a stable ME [55]. Addition of each solvent should be carried out slowly while applying sonication to prevent disruption of the ME [32].

Mixing of oil, surfactant and co-surfactant before adding the aqueous component was also suggested [41]. The authors indicated that the ME was formed spontaneously upon addition of the aqueous phase to the other components, and remained unchanged during method development. In another procedure, ultrasonication was claimed unnecessary during the preparation of a ME containing SDS, 1-butanol, heptane and phosphate buffer [57]. According to the authors, standing for enough time with occasional shaking is favourable for the formation of a transparent ME. The length of standing time depended on the composition of the ME and temperature.

In general, the successive steps of addition of the reagents, alternated with stirring in an ultrasonic bath, are important to get stable MEs. The mixture of oil, surfactant, co-surfactant, and water should be stored in a closed container, and left at room temperature for more than 12 h (or overnight), to make sure that a stable optically transparent ME is formed. If the mixed solution is not clear after this period, the composition is unsuitable to form a stable ME.

In many reports, the authors claimed a sufficiently long period of good stability. Thus, at room temperature (around 25 ºC), ME mobile phases have been described to be stable for at least 2 months [25,26], or even several months [31]. However, MEs formed with SDS, 1-propanol and di-isopropyl ether, or SDS, 1-butanol, heptane and TFA have been indicated to be stable for only one week at room temperature [55,58]. The MEs were made finally stable at 4 ºC, yielding reproducible results as verified for at least one month.

2.9. Conclusions

ME mobile phases have been considered as environmental friendly alternatives to the traditional solvents used in RPLC, owing to the low amount of organic solvent needed, and the reduced volume of mobile phase required because of the short analysis times. A great variety of different reagents suitable to form O/W MEs have been reported, together with a number of compositional choices that lead to the creation of a successful mobile phase. Selectivity can be altered by the type of selected oil, surfactant and co-surfactant.

A single set of experimental conditions has been shown to be applicable to the analysis of a wide range of different compounds. This recommendation helps in method development, with time and cost savings compared to the need to optimise conventional RPLC and MLC conditions. If required, analysis time and resolution can be finely tuned by altering any of the several involved experimental factors. In this case, the number of operating parameters could make the task very laborious. It should be noted that retention times are often decreased by increasing the oil, surfactant and co-surfactant concentrations, but such changes can also affect negatively peak resolution. Changes in the mobile phase pH have similar effect on solute retention to that observed for MLC.

Gradient MELC has been recommended as a convenient method for drug stability studies. It offers the possibility of rapid determination of degradants and impurities in very hydrophobic formulations. However, there is some concern on the stability of MEs along the gradients and reproducibility of the results. Therefore, their implementation needs much deeper investigation.

Published work contains valuable information on how an MELC method should be implemented. However, the number of factors involved in this chromatographic mode can make such information rather misleading. This

chapter was written based on a detailed study of the published work. The reported information has been compared and organised to facilitate analysts in the implementation of their MELC methods. MELC is an ideal technique to separate mixtures of compounds in a wide range of polarities, from hydrophilic to hydrophobic, being especially interesting for the analysis of highly water-insoluble samples.

2.10. References

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CHAPTER 3

COMPARISON OF SURFACTANT-MEDIATED LIQUID CHROMATOGRAPHIC MODES WITH SODIUM DODECYL SULPHATE FOR THE ANALYSIS OF BASIC DRUGS

3.1. Abstract

In Reversed-Phase Liquid Chromatography (RPLC), basic drugs are positively charged at the usual working pH range and may interact with free anionic silanols present in conventional silica-based stationary phases. This is translated in stronger retention, and tailed and broadened peaks. This problem can be solved by the addition of reagents to the mobile phase that are adsorbed on the stationary phase, avoiding the access of solutes to silanols. Among these additives, surfactants used under micellar conditions have provided good silanol suppressing potency through the technique known as Micellar Liquid Chromatography (MLC). The most common surfactant is the anionic sodium dodecyl sulphate (SDS). When SDS is at moderate concentration, in the presence of high organic solvent content, micelles are not formed and the chromatographic mode is known as High Submicellar Liquid Chromatography (HSLC). Meanwhile, the addition of an oil to an aqueous solution of SDS containing micelles gives rise to microemulsions in a chromatographic mode known as Microemulsion Liquid Chromatography (MELC). A comprehensive comparison of the chromatographic behaviour of a set of basic β-adrenoceptor antagonists analysed by MLC, HSLC and MELC is carried out in this work, in terms of retention, peak profile and organic solvent consumption. The study shows that HSLC reduce the retention and enhance the efficiency, with respect to conventional RPLC and MLC, and MELC allows reduced analysis times with less organic solvent with respect to HSLC. The narrower and almost symmetrical peaks in MLC, HSLC and MELC, with respect to conventional RPLC, reveal the presence of silanol masking.

3.2. Introduction

Positively charged basic compounds, analysed by Reversed-Phase Liquid Chromatography (RPLC) with conventional alkyl-bonded stationary phases, are able to establish ion-exchange interactions with residual anionic silanols present on silica packings. These interactions are the reason of two undesirable effects: long retention times owing to the attraction of the cationic solutes to the anionic silanols, and formation of broad and asymmetrical peaks owing to the slow desorption kinetics of solute molecules associated to the silanols [1–5]. Numerous attempts to reduce the so-called "silanol effect" have been reported during the development of RPLC. Due to its simplicity, one practical and extended practice is the addition of reagents to the mobile phase able to be adsorbed on the stationary phase to cover the silanols. Amines [6,7], surfactants [8,9], and ionic liquids [10–12], are typical examples of mobile phase additives used to modify the stationary phase. Mobile phases containing these reagents have been shown to effectively minimise the interaction of cationic solutes with residual silanols, remarkably enhancing the peak efficiency and symmetry of basic compounds. Masking of silanols may also decrease the long retention times of basic compounds obtained with conventional RPLC columns.

Surfactants are usually added to the RPLC mobile phase above their critical micellar concentration (CMC) to allow the formation of micelles. Under these experimental conditions, the chromatographic mode is known as Micellar Liquid Chromatography (MLC) [13–15]. Although surfactants of diverse nature can be used in MLC, the anionic sodium dodecyl sulphate (SDS) has been selected in most reports. The long hydrophobic chain of SDS monomers covers the stationary phase, with the sulphate group oriented towards the mobile phase, resulting in a negatively charged stationary phase [16]. Aqueous solutions

containing only SDS are weak eluents that yield poor efficiencies. For this reason, the addition of a small amount of organic solvent (i.e., co-surfactant) was suggested in the early days of the technique to enhance the elution strength and peak efficiency [17]. The presence of organic solvent reduces the amount of surfactant adsorbed on the stationary phase and alters micelle formation (i.e., CMC and aggregation number), giving rise to its eventual disaggregation in the presence of large amounts of organic solvent. However, such mobile phases (containing surfactant monomers, but no micelles) have been demonstrated to provide good resolution and efficiency in the analysis of a variety of compounds, giving rise to a new chromatographic mode that has been called High Submicellar Liquid Chromatography (HSLC) [18]. The main drawbacks of HSLC are the higher organic solvent consumption with respect to MLC and the increased column back-pressure.

Another chromatographic mode using a surfactant in the mobile phase that has gained some relevance is oil-in-water (O/W) Microemulsion Liquid Chromatography (MELC) [19–22]. Microemulsions (MEs) are clear (transparent) colloidal solutions, thermodynamically stable, in which water and a non-polar solvent (two immiscible liquids) can coexist thanks to the presence of a surfactant [21, 23–25]. An organic solvent (such as 1-propanol, 1-butanol or 1-pentanol) is often needed as co-surfactant to stabilise the microscopic oil droplets. The surfactant plays a major role in the stability of the ME and the separation performance, but the oil choice is also important with respect to the distribution of the solutes between mobile phase and stationary phase, and the chromatographic selectivity.

Different applications have been reported in MLC/HSLC and MELC, the analysis of drugs in the pharmaceutical field and physiological fluids being the most common [15,20,21,26–28]. Although there is previous work on the

chromatographic analysis of basic drugs using MLC and HSLC, in this work, MELC is applied for the first time to these compounds, specifically eleven β-adrenoceptor antagonists used in the treatment of diverse cardiac diseases [29]. Mobile phases containing aqueous solutions of SDS and low or high content of acetonitrile or 1-propanol are used in MLC and HSLC, whereas SDS, water, octane and 1-butanol are mixed to prepare MEs to be used as mobile phases in MELC. A comprehensive study of the change of behaviour (retention and peak profile) is reported as the environment inside the column is changed, using surfactant-mediated liquid chromatographic modes. The modifications inside the column that give rise to such behaviour are discussed.

3.3. Experimental

3.3.1. Reagents

The β-adrenoceptor antagonists used in this study (ordered according to their polarity, see Table 3.1 [30,31]) were: (1) atenolol, (2) acebutolol, (3) carteolol, (4) pindolol, (5) metoprolol, (6) timolol, (7) celiprolol, (8) oxprenolol, (9) esmolol, (10) labetalol, and (11) propranolol, all from Sigma (St. Louis, MA, USA). These are basic drugs with p$K_a \geq 9$, which means that at the usual acidic working pH of the mobile phases in RPLC, the cationic species are dominant. Stock solutions of 200 μg/mL of the drugs were prepared in 1 mL of methanol (VWR, International, France), diluted with water in RPLC, MLC and HSLC, and with mobile phase in MELC, and then sonicated with an Elmasonic S 15-H ultrasonic bath from Elma (Singen, Germany).

Table 3.1. Structures, dissociation constants (pK_a) in water, and octanol-water partition coefficients (log $P_{o/w}$) of the β-adrenoceptor antagonists.

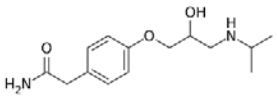
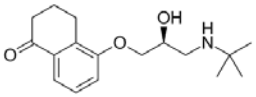
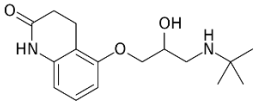
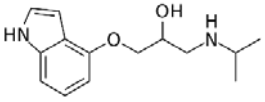
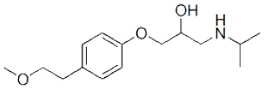
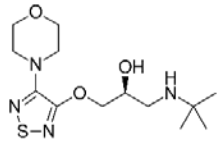
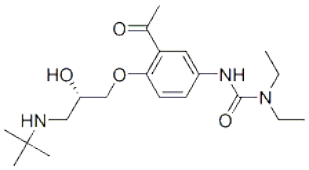
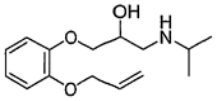
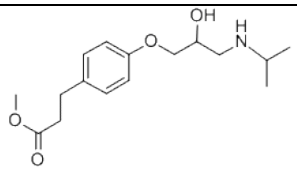
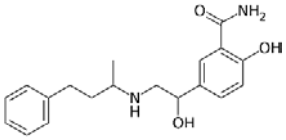
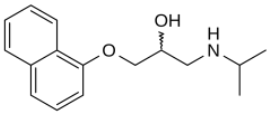
Compound	Code	Structure	pK_a [a]	log $P_{o/w}$ [b]
Atenolol	1		9.6	-0.026
Acebutolol	2		9.2	1.19
Carteolol	3		NA	1.42
Pindolol	4		8.8, 9.7	1.48
Metoprolol	5		9.7	1.69
Timolol	6		9.2	1.75
Celiprolol	7		NA	1.93
Oxprenolol	8		9.5	1.83

Table 3.1 (continued).

Compound	Code	Structure	pK_a [a]	log $P_{o/w}$ [b]
Esmolol	9		NA	2.00
Labetalol	10		7.4, 8.7	2.41
Propranolol	11		9.5	2.60

[a] Ref. [30]. [b] Ref. [31]. Values calculated from the structure by applying the on-line interactive LOGKOW program of the Environmental Science Centre of Syracuse Research Corporation. NA: not available

The solutions were kept at 4 ºC, and remained stable for at least 2 months. These were again diluted to 40 μg/mL with water in RPLC, MLC and HSLC, and with mobile phase in MELC, prior to injection. Chromatograms of mixtures of the β-adrenoceptor antagonists were also obtained at concentrations of *ca*. 10 μg/mL. Uracil from Acros Organics (Geel, Belgium) was used as dead time marker.

The aqueous mobile phases contained acetonitrile or 1-propanol in conventional RPLC; SDS and acetonitrile or 1-propanol in MLC and HSLC; and SDS, octane and 1-butanol in MELC. The reagents used to prepare the mobile

phases were SDS from Merck (99 % purity, Darmstadt, Germany), acetonitrile, 1-propanol and 1-butanol from Scharlab (Barcelona, Spain), and octane from Alfa Aesar (Kandel, Germany). An acidic pH is needed to obtain the cationic species of the basic drugs. In RPLC, MLC and HSLC, the pH was adjusted to 3.0±0.1 with 0.01 M citric acid monohydrate and sodium hydroxide from Panreac (Barcelona). Working at 1.33 ± 0.07, fixed with 0.05 % trifluoroacetic acid (TFA) from Fisher Scientific (UK) was preferred for the XTerra column used in MELC (see Section 3.2), since these conditions are recommended by several authors in this chromatographic mode; however, pH 3.0 should be used with other columns.

The concentration ranges for the assayed mobile phases were as follows:

(i) Conventional RPLC: 15–30 % *(v/v)* acetonitrile, and 5–15 % (*v*/*v*) 1-propanol.

(ii) MLC: 0.075–0.15 M SDS / 5–20 % (*v*/*v*) acetonitrile, and 0.02–0.1125 M SDS / 5–15 % (*v*/*v*) 1-propanol.

(iii) HSLC: 0.075–0.15 M SDS / 17.5–50 % (*v*/*v*) acetonitrile, and 0.02–0.15 M SDS / 20–35 % (*v*/*v*) 1-propanol.

(iv) MELC: 0.104–0.173 M SDS / 0.28–1.28 % (*v*/*v*) octane / 8.15–17.3 % (*v*/*v*) 1-butanol.

Solutions of the β-adrenoceptor antagonists and mobile phases were filtered through 0.45 μm Nylon membranes from Micron Separations (Westboro, MA, USA). Nanopure water from Barnstead, Sybron (Boston, MA, USA) was used throughout.

3.3.2. Apparatus and columns

The chromatograph consisted of a modular Agilent system (Waldbronn, Germany), equipped with quaternary pump (Series 1200), autosampler (Series 1260 Infinity II), thermostated column compartment (Series 1290 Infinity II), and diode array detector (Series 1100). The flow-rate was 1 mL/min. The β-adrenoceptor antagonists were monitored at 225 nm, except for timolol and uracil, which were detected at 300 nm and 254 nm, respectively. Duplicate injections of 20 µL were made to carry out the chromatographic separations.

An HP Chemstation (Agilent, C.01.07) was used for data acquisition. The mathematical treatment was performed with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were integrated with MICHROM [32].

The chromatographic column used in conventional RPLC, MLC and HSLC was a Kromasil C18 from Scharlab, with the following characteristics: 125 mm × 4.6 mm i.d., 5 µm particle size, 100 Å average pore diameter, 340 m^2/g surface area and 20 weight % total carbon [33]. In MELC, an XTerra-MS C18 column from Waters (MA, USA) was used: 150 mm × 4.6 mm i.d., 5 µm particle size, 120 Å average pore diameter, 175 m^2/g surface area and 12 % total carbon. XTerra MS C18 combines the best properties of silica and polymeric bonded phases to enable working at pH < 2 without damage. It also replaces one out of every three silanols with a methyl group during particle synthesis. For comparison purposes, the XTerra column was also used in conventional RPLC. In all cases, the columns were connected to C18 guard columns (30 mm × 4.0 mm i.d., 5 µm particle size) from Scharlab for protection.

The mobile phases were recycled between runs and also during the analysis to reduce the consumption of reagents. This increased the sustainability of the

procedure. In the chromatographic modes that used surfactant, the column was periodically rinsed with pure water and methanol (around 30 mL) to remove the surfactant from the stationary phase. During weekend, the column was maintained with methanol. In MELC, the column was also flushed with 60 mL of 50:50 methanol:water prior to start a run with a new mobile phase containing less surfactant, octane and/or 1-butanol. The procedure applied to regenerate the column after use depended on the chromatographic mode. In MLC and HSLC, in order to avoid buffer precipitation, 30 mL of pure water was flushed into the system, before 30 mL of methanol used to remove the surfactant from the column. In the MELC mode, 30 mL of 50:50 methanol: water followed by 30 mL of methanol was needed to regenerate the column.

3.4. Results and discussion

3.4.1. Retention behaviour of the basic drugs in mobile phases containing surfactant

The addition of surfactants to an aqueous-organic mobile phase in RPLC produces significant changes in the chromatographic behaviour. Particularly interesting is the use of ionic surfactants, such as SDS. This is significantly adsorbed on the surface of the non-polar stationary phase, creating a charged layer, which acts as a dynamic ion-exchanger for ionic analytes. Oppositely charged solute ions are attracted to the adsorbed surfactant ions and may reach high retention. This is the case of basic drugs analysed with SDS mobile phases at acidic pH, where the drugs are protonated with a positive charge. To reduce the high retention obtained with pure micellar solutions (containing only surfactant), the addition of different amounts of one or two organic solvents is needed: co-surfactant in MLC and HSLC, and oil and co-surfactant in MELC.

Most published work in these surfactant-mediated chromatographic modes corresponds to isocratic elution, which yields chromatograms with solutes regularly distributed along the chromatogram, similar to those obtained in gradient elution with conventional RPLC. In the presence of surfactant, less polar solutes are more easily eluted owing to their stronger solubilisation in the surfactant media, which gives rise to a linear relationship between the retention factor (k) and the octanol-water partition coefficient ($P_{o/w}$), instead of the usual linear relationship between log k and log $P_{o/w}$ in conventional RPLC [13].

Experimental factors usually investigated in MLC, HSLC and MELC include the type and concentration of surfactant, and the type and concentration of the added organic solvent(s) (less often, the pH of the mobile phase and temperature are studied). Once the surfactant and organic solvents have been selected, the effect of their concentration on the chromatographic behaviour should be evaluated, and eventually, optimised.

3.4.1.1. Modulation of the retention in MLC and HSLC

Protonated basic drugs interact with free anionic silanols present in conventional silica-based stationary phases, giving rise to stronger retention with aqueous-organic mobile phases. This can be observed in Figures 3.1a and b, which depict the changes in retention when acetonitrile-water or 1-propanol-water mixtures are used to elute the β-adrenoceptor antagonists (with retention factor ranges of 0.7–60.8 and 0.8–36.2 for 15 % acetonitrile and 5 % 1-propanol, respectively).

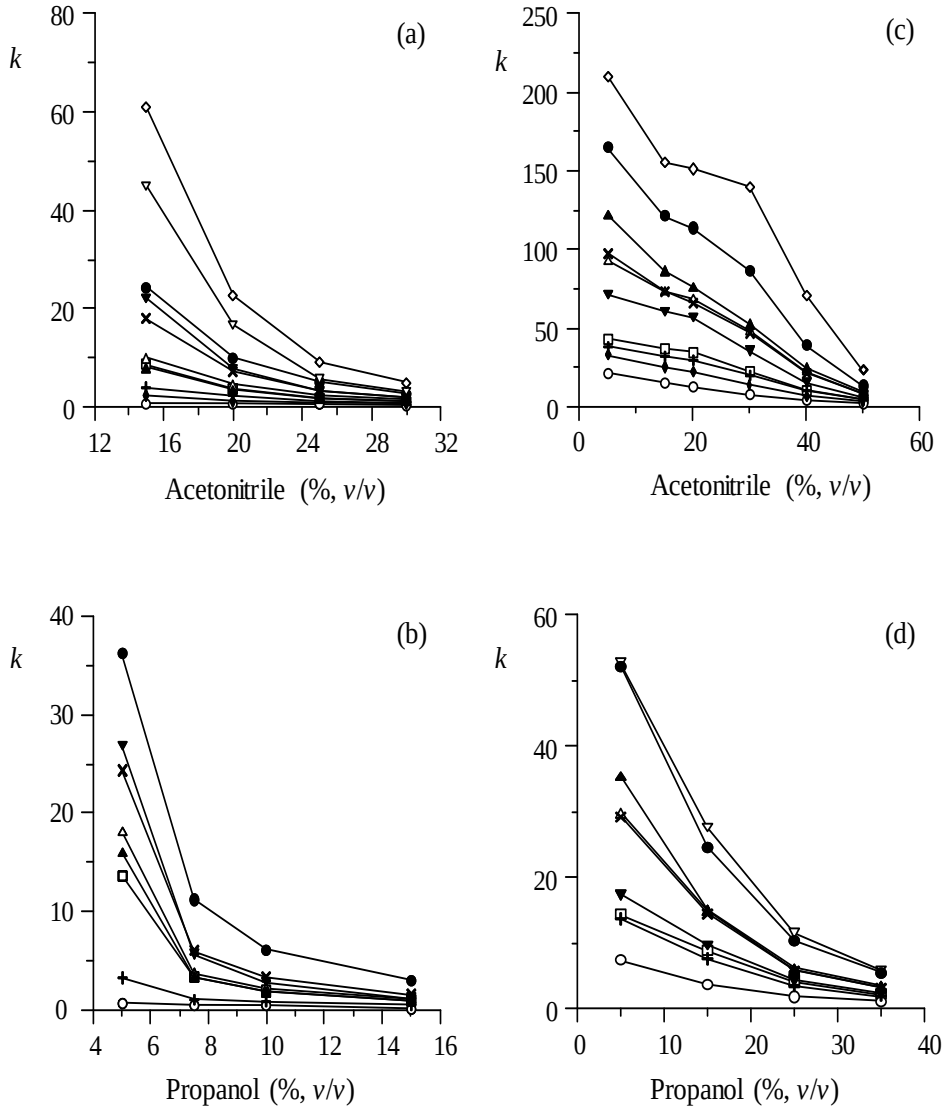


Figure 3.1. RPLC (a,b) and MLC/HSLC with 0.075 M SDS (c,d), at increasing concentrations of acetonitrile (a,c), and 1-propanol (b,d). Solute identity: (□) acebutolol Variation of retention in: (○) atenolol, (♦) carteolol, (▼) celiprolool, (×) esmolol, (▽) labetalol, (Δ) metoprolol, (●) oxprenolol, (+) pindolol, (◊) propranolol, and (▲) timolol.

In the presence of SDS in the mobile phase, the retention of the basic drugs was even stronger, due to the electrostatic attraction of the cationic solutes to the stationary phase modified with the anionic surfactant (Figures 3.1c and d), with retention factor ranges of 21.4–209.9 and 7.5–52.7, for 5 % acetonitrile and 5 % 1-propanol, respectively. Therefore, the analysis of these compounds by MLC requires the addition of a greater amount of organic solvent to reduce the retention. Several solvents have been used as co-surfactant. However, since the first published reports, 1-propanol has been the most common [13]. More recently, acetonitrile (with somewhat weaker elution strength) has been proposed [34]. The organic solvent molecules can bind the micelles and modify their shape. Acetonitrile and short-chain alcohols (ethanol and 1-propanol) interact with the micelle surface, reducing the repulsion among the ionic heads of the surfactant monomers in the micelle, whereas 1-butanol and 1-pentanol are inserted into the non-polar micelle core [35].

The minimal amount of SDS to form micelles in aqueous solution (CMC) is 8×10^{-3} M [13]; however, in the presence of acetonitrile this value is appreciably increased. Thus, for 20 % acetonitrile, it is ~3×10^{-2} M. In contrast, in the presence of 1-propanol and 1-butanol, the CMC decreases. For example, for 20 % 1-propanol and 5 % 1-butanol, the CMC is ~3×10^{-3} M and ~2.5×10^{-3} M, respectively [36]. However, beyond these values (~30 % acetonitrile, ~22 % 1-propanol and ~10 to 12 % 1-butanol), micelles are not formed [37,38] (i.e., the micellar phase is converted into a hydro-organic phase containing surfactant monomers).

In order to preserve the micelles, analysts working in MLC avoid large amounts of organic solvent in the mobile phase. However, in some reports with SDS solutions containing a relatively large concentration of organic solvent

where micelles cannot be formed (i.e., submicellar conditions), satisfactory performance (i.e., smaller retention times and enhanced peak profile) was found [18]. This gave rise to a chromatographic mode (HSLC) that can be considered as a bridge between MLC and conventional RPLC, since the concentration of the surfactant is similar to that used in MLC, but the mobile phase contains a high concentration of organic solvent, which does not allow the formation of micelles. As larger amounts of co-surfactant are used in HSLC, acetonitrile is preferable to 1-propanol and 1-butanol to avoid excessive back-pressure. In fact, 1-butanol is not recommended in this chromatographic mode.

Figures 3.1c and d depict the retention factors for several β-adrenoceptor antagonists, eluted with mobile phases containing 0.075 M SDS and acetonitrile or 1-propanol as co-surfactants at varying concentration. The mobile phases cover both MLC and HSLC conditions, with a transition in the 20–30 % range for acetonitrile and 15–25 % for 1-propanol. The retention factors decreased to 2.2–22.9 and 1.1–5.9, for 50 % acetonitrile and 35 % 1-propanol, respectively. The elution strength for 1-propanol was stronger, and allowed more adequate analysis times. However, at the highest concentrations of 1-propanol, the back-pressure was too high.

3.4.1.2 Modulation of the retention in MELC

As noted above, the attraction of cationic solutes to the stationary phase covered with anionic surfactant gives rise to long retention times. In the previous section, we have seen that the addition of an organic solvent to the mobile phase containing a surfactant reduces the retention, especially when used at high concentration. MELC is another option to get similar results. This chromatographic mode is applied for the first time in this work to the analysis of basic compounds.

MELC makes use of O/W MEs, which are formed by oil droplets dispersed in a continuous aqueous medium containing surfactant at a concentration well above its CMC. In these MEs, where the amount of oil in the liquid phase is very small, the interaction of the heads of the charged surfactant with water aggregates the surfactant into normal micelles. In these micelles, the oil phase is inserted in their core, dissolved by the long hydrophobic carbon tails of the surfactant molecules. In order to stabilise the droplets, a medium-chain alcohol (co-surfactant) is needed. The elution ability of the ME increases with increasing concentrations of surfactant and co-surfactant. However, if the oil/surfactant (or co-surfactant) ratio is not adequate, the ME will either fail or decompose into separate oil and water layers within a short period.

Figure 3.2 shows the effect of the use of mobile phases containing SDS, octane and 1-butanol on the chromatographic behaviour of the β-adrenoceptor antagonists. As MELC mobile phases contain three reagents, the optimisation of the mobile phase composition is more complex with respect to MLC/HSLC [22].

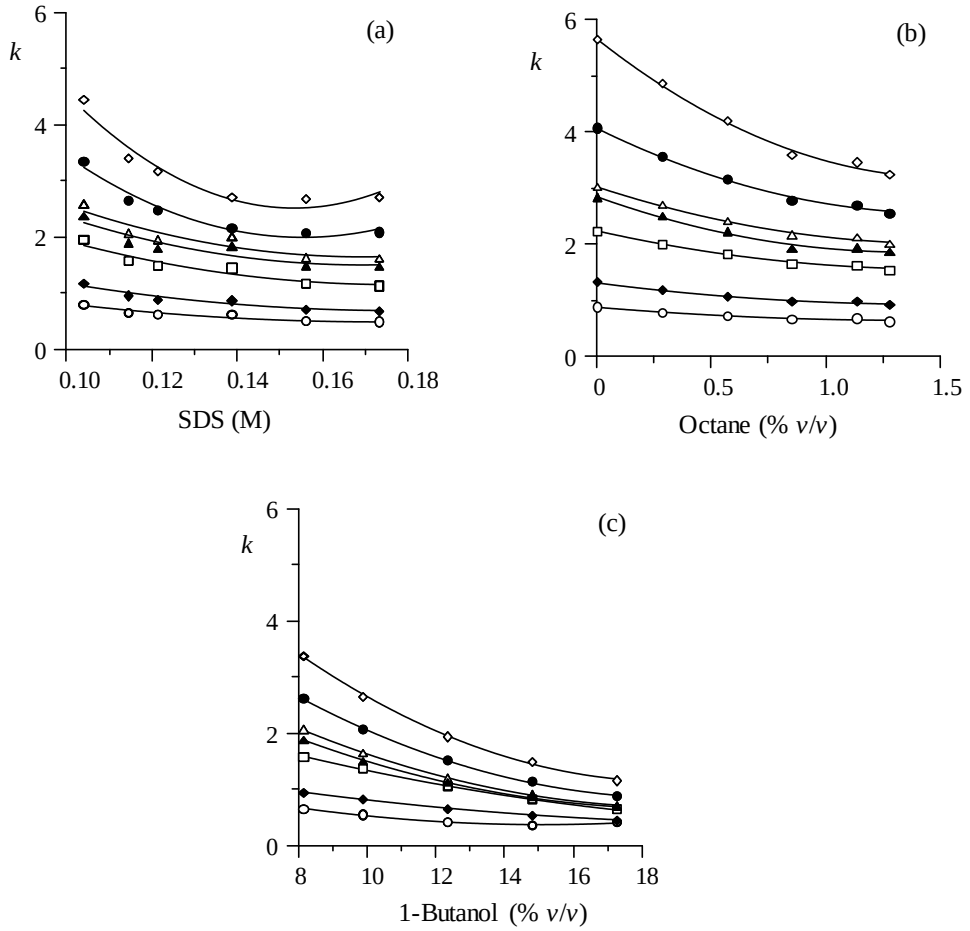


Fig. 3.2. Variation of retention in MELC at increasing concentration of: (a) SDS, (b) octane, and (c) 1-butanol. SDS concentration in (b) and (c) was 0.114 M, octane concentration in (a) and (c) was 1.14 %, and 1-butanol concentration in (a) and (b) was 8.15 %. Solute identity: (□) acebutolol, (○) atenolol, (♦) carteolol, (Δ) metoprolol, (●) oxprenolol, (◊) propranolol, and (▲) timolol.

It is for this reason that a recommended mobile phase composition is selected as starting point when developing the method for a new, previously unreported separation. In this work, we selected a ME recommended by Altria *et al.* [39,40], containing 0.114 M SDS, 1.14 % octane, and 8.15 % 1-butanol, which has been called "standard MELC mobile phase". Other compositions were obtained by modifying the concentration of only one reagent (surfactant, oil, or co-surfactant). The concentration ranges explored were 0.104 to 0.173 M for SDS (which guaranteed the formation of micelles), 0.28 to 1.28 % for octane, and 8.15 to 17.3 % for 1-butanol. Outside these ranges, the ME was not stable or could not be formed.

The chromatographic behaviour was tested at several temperatures (20, 40 and 60 ^{o}C). However, the effect of temperature was found to be minimal for the β-adrenoceptor antagonists examined in this work (see Figure 3.3).

As observed in Figure 3.2a, only a small decrease in retention was obtained at increasing SDS concentration in the ME. The reduction was larger for the most hydrophobic compounds (propranolol and oxprenolol), owing to their assumed higher affinity for the oil droplets, which are formed in a larger amount as the concentration of micelles in the mobile phase increases. The gradual addition of octane also yielded smaller retention times (Figure 3.2b). In the absence of octane, the hybrid micellar system contained only conventional micelles, but when octane was added, oil droplets were formed. The most hydrophobic compounds were again more affected by an increase in the concentration of oil, due to the progressive increase in the number of oil droplets in the mobile phase, which interact by partitioning with these solutes.

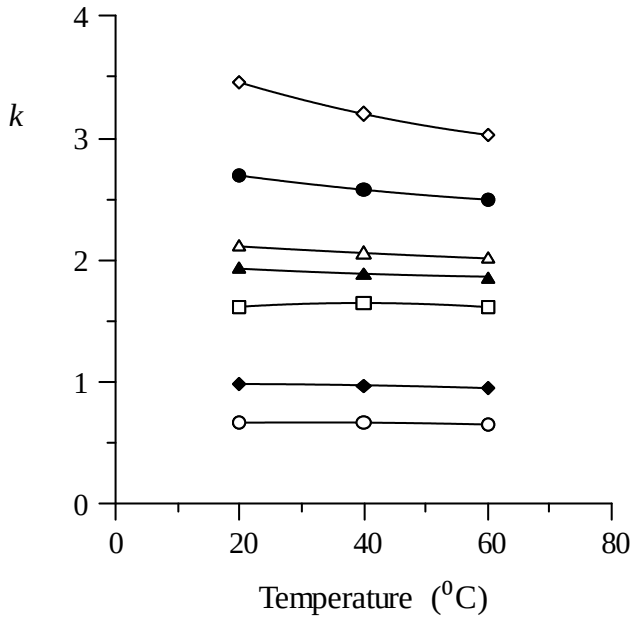


Figure 3.3. Variation of retentions factors at increasing temperature, using the composition of the standard ME mobile phase: 0.114 M SDS / 1.14 % (*v*/*v*) octane / 8.15 % (*v*/*v*) 1-butanol / 0.5 % (*v*/*v*) TFA. See Figure 3.1 for solute identity.

Surfactant and oil play an important role in the separation performance, but the co-surfactant also contributes to the formation of the oil droplets and the strength of the interactions with the solutes. Although the surfactant is predominant in the stability of MEs, the co-surfactant can help in reducing the superficial tension. Also, the co-surfactant has solubilizing properties that give rise to shorter retention times. In fact, increased concentrations of 1-butanol had a significant effect on the retention. As shown in Figure 3.2c, the concentration of 1-butanol was increased up to 17.3 %. However, the back-pressure was

excessive for the largest concentrations. It was even noted that the column had suffered damage. Interestingly, in a previous work on the effect of alcohols on the chromatographic behaviour of basic compounds in MLC/HSLC [41], only the increase in the concentration of methanol, ethanol and 1-propanol could be studied, since the column back-pressure was excessive with 1-butanol.

We considered interesting to observe the effect of 1-butanol in the absence of octane (Figure 3.2b), in order to compare the behaviour of MLC and MELC with this co-surfactant. As expected, a hybrid micellar mobile phase with 8.15 % 1-butanol produced significant reduction in the retention with respect to conventional RPLC and MLC, both using acetonitrile as modifier and co-surfactant, respectively (Figures 3.1a and c), and similar retention to RPLC with 15 % 1-propanol (Figure 3.1b) and HSLC with 35 % 1-propanol (Figure 3.1d). The addition of 1.14 % octane to form a ME with SDS and 8.15 % 1-butanol produced a further decrease in the retention from k = 5.65 to 3.45 for propranolol, 2.83 to 1.94 for timolol, and 0.88 to 0.67 for atenolol (Figures 3.2b and c). A further increase in 1-butanol, in the absence of octane, would presumably reduce the retention still further, but as noted, micelles break-down at ca. 10–12 % 1-butanol.

3.4.2. Effect of mobile phase composition on the peak profile

3.4.2.1. Half-width plots

The addition of surfactant and organic solvent(s) to the mobile phase alters not only the retention of the basic drugs, but also their peak profile. As previously noted, the broad and asymmetric peaks for basic compounds, obtained in RPLC with conventional columns using aqueous-organic mixtures have been explained by the slow desorption kinetics when the basic solutes (cationic) interact with free silanols (anionic). The adsorption of SDS on the stationary phase avoids this interaction and yields peaks with enhanced behaviour (narrower and more symmetrical).

To investigate the global changes in peak profile in MLC, HSLC and MELC, the construction of plots where the half-widths of chromatographic peaks are represented *versu*s the retention times is very convenient. These plots can be described by simple linear models [42]:

$$A = m_A\, t_R + A_0 \quad (3.1)$$

$$B = m_B\, t_R + B_0 \quad (3.2)$$

where A and B represent the left and right peak half-widths, respectively, which were measured at 10 % peak height to avoid baseline noise in the chromatograms. Similar plots can be built with the peak width. However, representing the half-widths, additional information is obtained related to peak asymmetry.

The parameters of the fitted plots allow the characterisation of the chromatographic system, since they indicate the rate of broadening for peaks eluting at increasing retention times (measured by the sum of slopes: $m_A + m_B$). Peaks will be symmetrical or nearly symmetrical if the lines depicting the right and left half-widths are nearly coincident. If the y-intercepts are very similar, the

ratio between the slopes (m_B / m_A) will indicate the asymmetry of peaks measured at a time where the extra-column contribution is non-significant. Finally, the extra-column contributions to the peak profile are illustrated by the intercepts ($A_0 + B_0$ and B_0/A_0). In all cases, the half-width plots give a full picture of the characteristics of the chromatographic peaks that are obtained with a particular column and conditions, since the values of widths and asymmetries with the retention times are represented.

To evaluate the influence of surfactant and organic solvent(s) on the peak profile, several half-width plots were drawn. Satisfactory correlations were obtained in all cases. Figure 3.4 depicts the half-widths plots showing the chromatographic behaviour in RPLC, MLC and HSLC, for mobile phases containing acetonitrile in the following ranges: 15–30 % for RPLC (Figure 3.4a), 5–15 % for MLC (Figure 3.4b), and 17.5–50 % for HSLC (Figure 3.4c). Figure 3.5 shows the results for the three chromatographic modes for mobile phases containing 1-propanol: 5–15 % for RPLC and MLC (Figures 3.5a and b), and 20–35 % for HSLC (Figure 3.5c). These plots give complementary information to that offered in Figure 3.1, in which the reduction of retention for each compound is represented at increasing concentrations of organic solvent and fixed concentration of surfactant. The data plotted in Figures 3.4 and 3.5 were obtained using mobile phases containing different amounts of SDS at varying organic solvent concentrations.

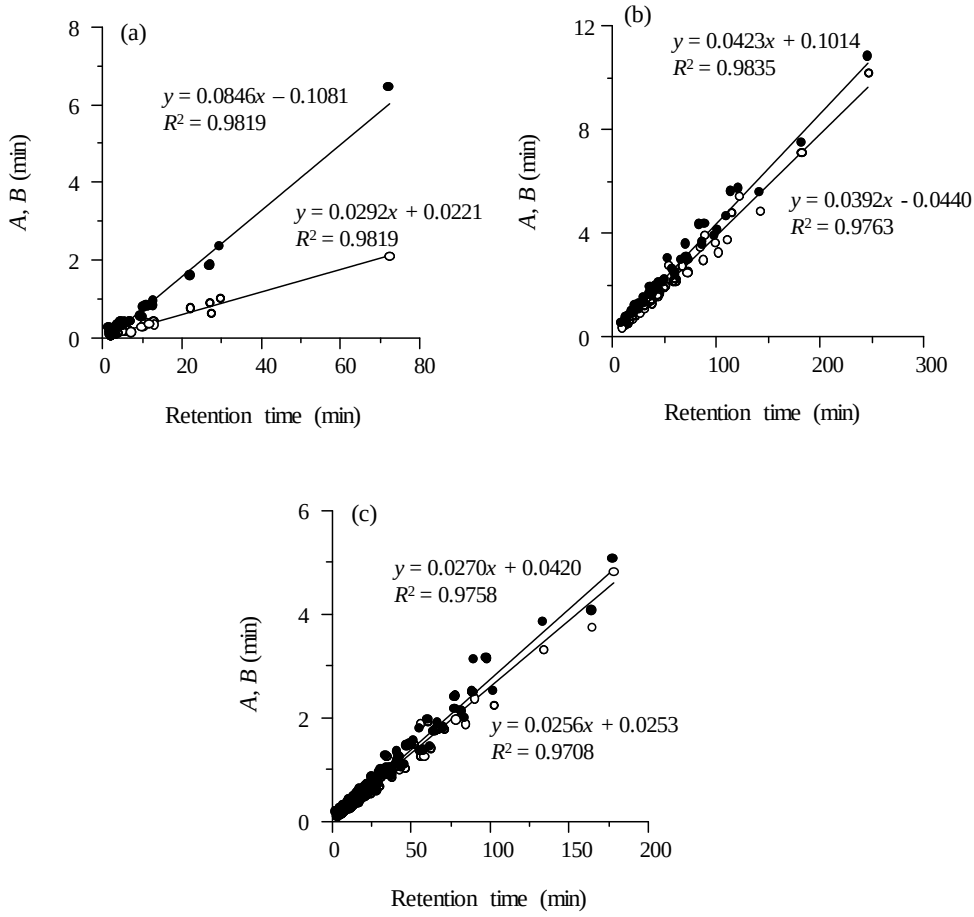


Figure 3.4. Half-width plots (left (*A*, ○) and right (*B*, ●)) for the β-adrenoceptor antagonists eluted with several mobile phase compositions, in the presence of acetonitrile inside the following ranges: (a) RPLC (15–30 % acetonitrile), (b) MLC (0.075–0.15 M SDS, 5–15 % acetonitrile), and (c) HSLC (0.075–0.15 M SDS, 17.5–50 % acetonitrile).

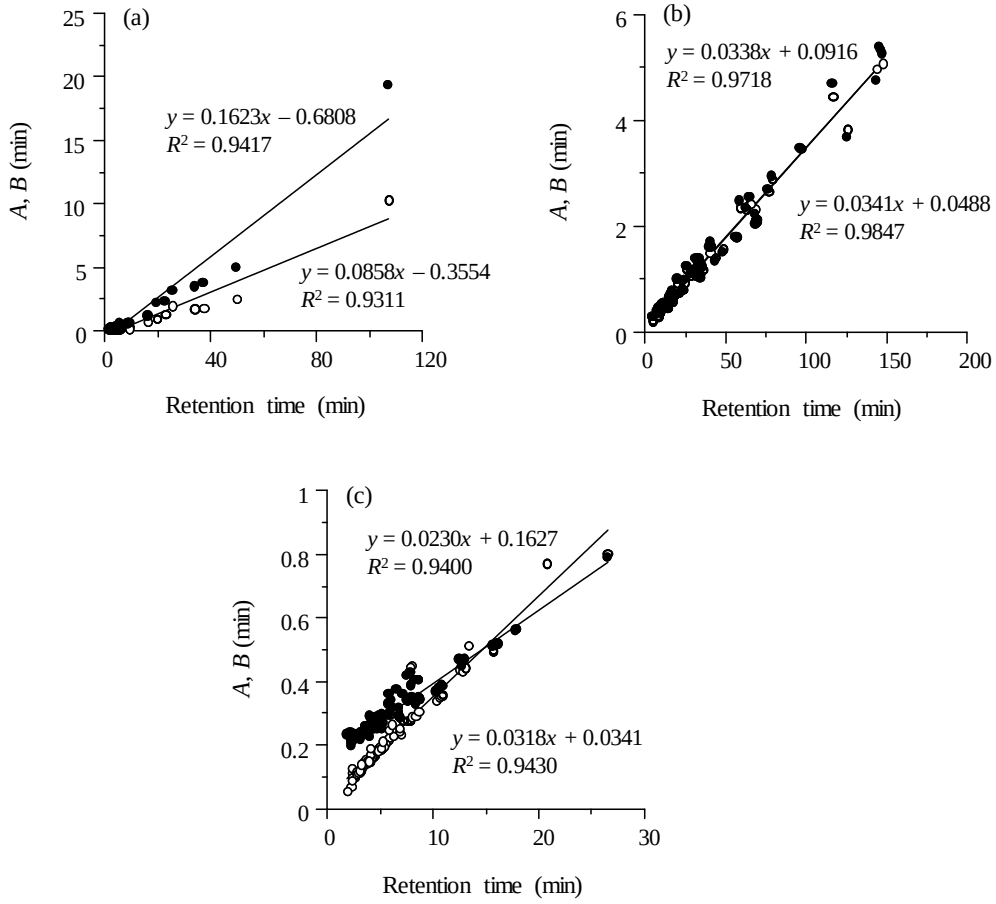


Figure 3.5. Half-width plots (left (*A*, ○) and right (*B*, ●)) for the β-adrenoceptor antagonists eluted with several mobile phase compositions in the presence of 1-propanol, inside the following ranges: (a) RPLC (5–15 % 1-propanol), (b) MLC (0.02–0.1125 M SDS, 5–15 % 1-propanol), (c) HSLC (0.02–0.15 M SDS, 20–35 % 1-propanol).

The plots in Figures 3.4 and 3.5 allow a global comparison of the three chromatographic modes. The significant reduction of the silanol effect in MLC and HSLC with respect to conventional RPLC is evident, in view of the much narrower and symmetrical peaks. The most remarkable characteristic is that in Figures 3.4b and c, and 3.5b, the slope of the right half-width (*B*) is very similar to the slope of the left half-width (*A*), which indicates the formation of almost symmetrical peaks, at diverse retention times, for the chromatographic modes that employ the surfactant SDS. In RPLC (Figures 3.4a and 3.5a), the peaks were significantly tailing, while in HSLC with 1-propanol (Figure 3.5c) the peaks changed from tailing to fronting when the concentration of SDS was low (0.02–0.04 M) and the concentration of 1-propanol, high (25 %).

The less ideal peak profiles, when higher concentrations of organic solvent are employed in the mobile phase, are due to a reduced masking effect by SDS molecules on the stationary phase due to their smaller steady-state concentration on the stationary phase.

The half-width plots in Figure 3.6 depict the characteristics of the peaks obtained in MELC, at diverse compositions (0.104–0.173 M SDS, 0.25–1.28 % octane, 8.15–17.3 % 1-butanol), where it is again evident that the peaks are fairly symmetrical at all assayed conditions. It was found that:

(i) The increase in the concentration of SDS had practically no influence on peak symmetry and width.

(ii) The increase in octane only gave rise to a slight increment in the width, while the asymmetry did not suffer remarkable changes.

(iii) The gradual increment in 1-butanol produced wider peaks, probably due to desorption of SDS from the stationary phase at high concentration of organic solvent.

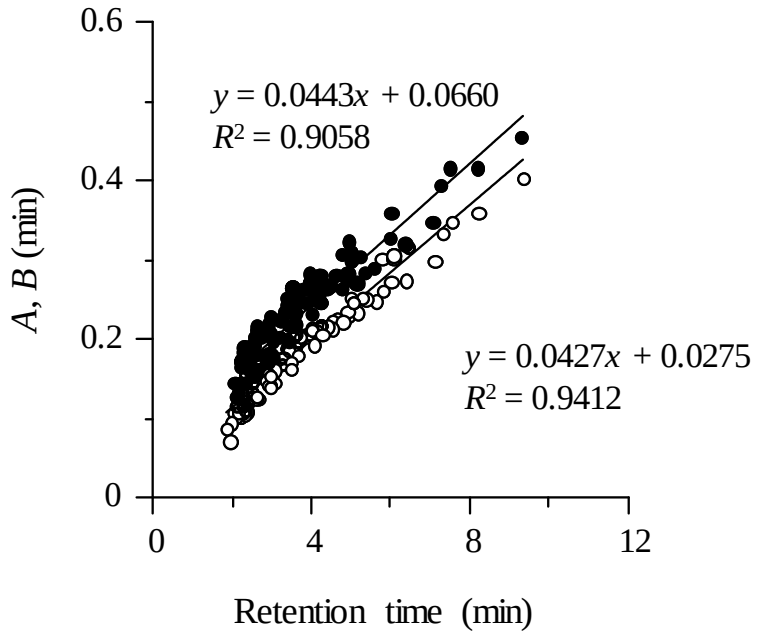


Figure 3.6. Half-width plots (left (*A*, ○) and right (*B*, ●)) for the β-adrenoceptor antagonists eluted in MELC, using several mobile phase compositions in the ranges: 0.104–0.173 M SDS, 0.25–1.28 % octane, and 8.15–17.3 % 1-butanol.

It should be also noted that the short retention times make the extra-column contributions relatively more significant. Finally, the changes in peak profile were minimal with the increase in temperature.

3.4.2.2. Peak efficiencies

Half-width plots give a picture of the changes that are produced when the environment inside the column is modified in surfactant-mediated chromatographic modes. These changes are translated into different efficiency values. Tables 3.2, 3.3 and 3.4 present the efficiencies for the β-adrenoceptor antagonists, analysed with different mobile phases in RPLC, MLC and HSLC with acetonitrile and 1-propanol, and in MELC with octane and 1-butanol at different concentrations. The efficiencies were calculated with the same information used to build the half-width plots (i.e., retention times, and left and right half-widths), according to the equation proposed by Foley and Dorsey [43].

The mean values of efficiencies are the following:

RPLC: 1500 ± 300 for 20 % acetonitrile, and 2000 ± 900 for 15 % 1-propanol.

MLC: 1800 ± 500 for 0.15 M SDS / 5 % acetonitrile, 2100 ± 300 for 0.15 M SDS / 15 % acetonitrile, and 2500 ± 500 for 0.1125 M SDS / 10 % 1 propanol.

HSLC: 5000 ± 1000 for 0.15 M SDS / 30 % acetonitrile, 4700 ± 2300 for 0.15 M SDS / 50 % acetonitrile, 1400 ± 400 for 0.15 M SDS / 25 % 1-propanol, and 900 ± 300 for 0.15 M SDS / 35 % 1-propanol).

MELC: 1100 ± 190 for 0.114 M SDS / 1.14 % octane / 8.15 % 1-butanol, 1130 ± 170 for 0.173 M SDS / 1.14 % octane / 8.15 % 1-butanol, 1600 ± 400 for 0.114 M SDS / 0.28 % octane / 8.15 % 1-butanol, and 1500 ± 400 for 0.114 M SDS / 1.14 % octane / 17.3 % 1-butanol.

Table 3.2. Efficiencies in RPLC and MLC.[a]

	RPLC		MLC		
Mobile phase	20 % ACN	15 % PrOH	0.15 M SDS 5 % ACN	0.15 M SDS 15 % ACN	0.1125 M SDS 10 % PrOH
Acebutolol	1577	2202	1811	2040	2205
Alprenolol	1328	–	1676	2219	–
Atenolol	997	1976	2588	1552	1711
Carteolol	943	–	2103	1915	–
Celiprolol	2139	2209	1879	2184	2171
Esmolol	1848	2129	1577	2384	2868
Labetalol	1604	1021	1711	1839	2160
Metoprolol	1797	1806	683	2499	2930
Nadolol	986	–	–	1457	–
Oxprenolol	1411	1069	2080	2571	3081
Pindolol	1670	4030	2068	2157	2337
Propranolol	1228	–	974	2023	–
Timolol	1679	1995	2464	2457	3001
Mean	1477 ± 368	2049 ± 871	1801 ± 549	2100 ± 346	2496 ± 483

[a] Calculated according to Foley and Dorsey ([43]).

Table 3.3. Efficiencies in HSLC.[a]

Mobile phase	0.15 M SDS 30% ACN	0.15 M SDS 50% ACN	0.15 M SDS 25% PrOH	0.15 M SDS 35% PrOH
Acebutolol	3919	2672	1160	770
Alprenolol	5664	8486	–	–
Atenolol	2598	1711	704	338
Carteolol	3525	2196	–	–
Celiprolol	4377	3221	1147	787
Esmolol	5209	4692	1459	904
Labetalol	5075	5907	1696	1092
Metoprolol	5449	5296	1635	1013
Nadolol	4024	2612	–	–
Oxprenolol	6125	6830	2123	1350
Pindolol	4187	2719	1110	822
Propranolol	5285	7997	–	–
Timolol	5459	6165	1628	1084
Mean	4684 ± 1003	4654 ± 2300	1407 ± 420	907 ± 282

[a] Calculated according to Foley and Dorsey ([43]).

Table 3.4. Efficiencies in MELC.

Mobile phase	0.114 M SDS / 1.14% octane / 8.15 % 1-butanol	0.173 M SDS / 1.14% octane / 8.15 % 1-butanol
Acebutolol	977	973
Atenolol	1022	1266
Carteolol	874	831
Metoprolol	1256	1271
Oxprenolol	1393	1138
Propranolol	1315	1279
Timolol	1162	1145
Mean	1100 ± 190	1130 ± 170
	0.114 M SDS / 0.28% octane / 8.15 % 1-butanol	0.114 M SDS / 1.14% octane / 17.3% 1-butanol
Acebutolol	1421	1328
Atenolol	1107	2218
Carteolol	1266	1061
Metoprolol	1871	1366
Oxprenolol	2053	1457
Propranolol	1987	1403
Timolol	1644	1446
Mean	1600 ± 400	1500 ± 400

[a] Calculated according to Foley and Dorsey ([43]).

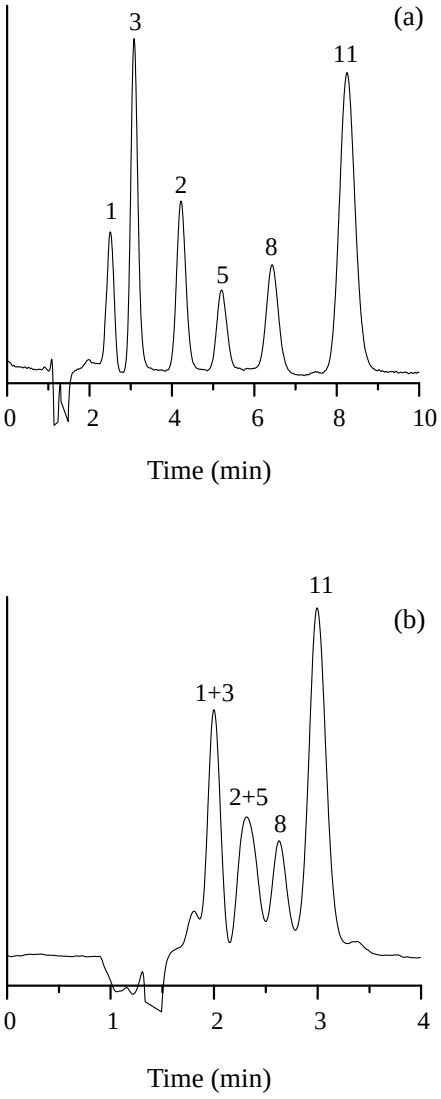


Figure 3.7. Experimental chromatograms obtained for mixtures of β-adrenoceptor antagonists in MELC with: (a) 0.114 M SDS / 0.28 % octane / 8.15 % 1-butanol, and (b) 0.114 M SDS / 1.14 % octane / 17.3 % 1-butanol, using the XTerra column. See Section 3.3.1 for peak identity.

Therefore, the efficiencies were higher when the amount of acetonitrile increased from 5 to 15 % (MLC), and were similar for 30 and 50 % acetonitrile (HSLC). In contrast, the efficiencies decreased when the amount of 1-propanol increased from 10 % (MLC) to 25 % and 35 % (HSLC). It was also found that the best efficiencies in MELC were obtained using a smaller amount of octane (0.114 M SDS / 0.28 % octane / 8.15 % 1-butanol), or a larger amount of 1-butanol (0.114 M SDS / 1.14 % octane / 17.3 % 1-butanol). However, MELC with a large amount of 1-butanol dramatically reduced the analysis time and resolution (Figure 3.7). It also gave rise to high back-pressure.

3.4.3. Separation performance in MLC, HSLC and MELC

In Figure 3.8, optimal chromatograms are depicted for conventional RPLC (15 % acetonitrile, Figures 3.8a and b), MLC (0.1125 M SDS / 17.5 % acetonitrile, Figure 3.8c), and HSLC (0.1125 M SDS / 35 % acetonitrile, Figure 3.8d, and 0.15 M SDS / 35 % 1-propanol, Figure 3.8e). These chromatograms offered maximal resolution as indicated by the software MICHROM [32]. The chromatogram for the “standard mobile phase” (0.114 M SDS / 1.14 % octane / 8.15 % 1-butanol, Figure 3.8f) is also shown for MELC.

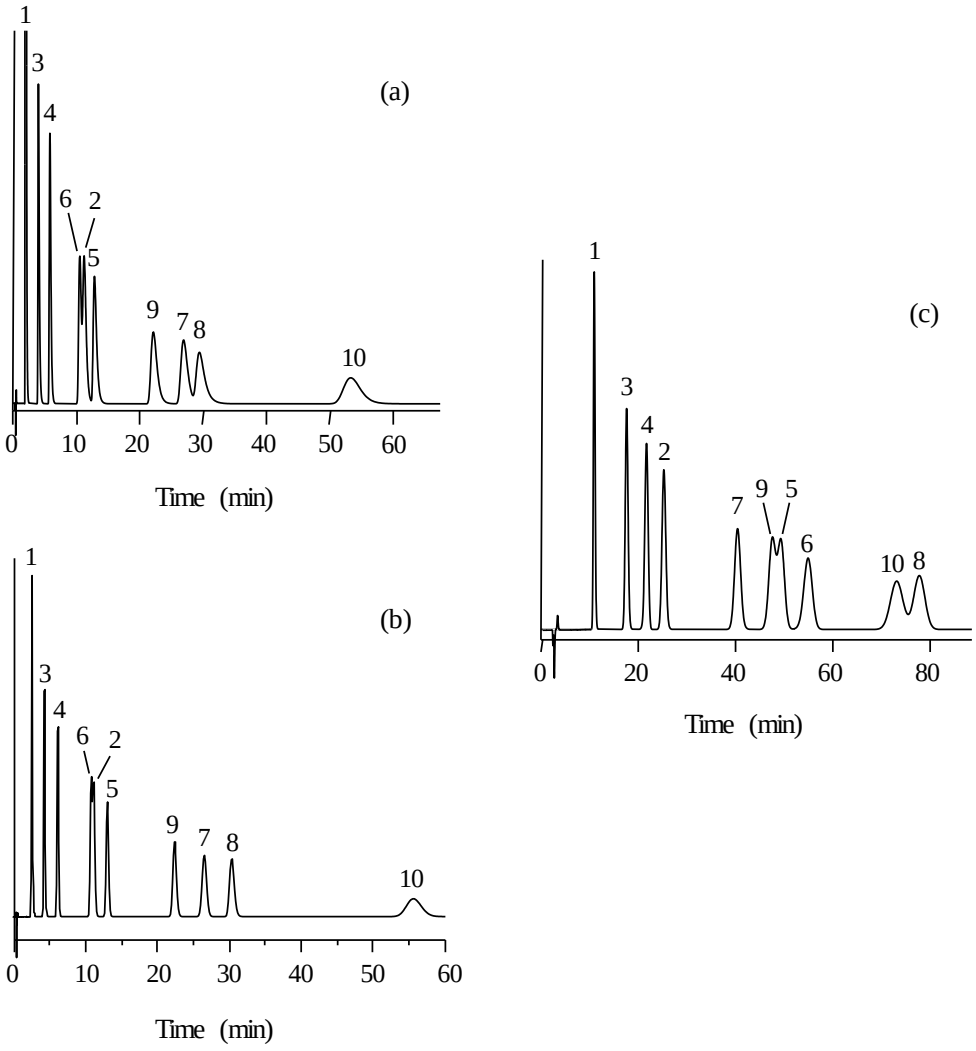


Figure 3.8. Experimental chromatograms obtained for mixtures of β-adrenoceptor antagonists with Kromasil C18 (a,c), and XTerra (b) columns, in: (a,b) RPLC (15 % acetonitrile), and (c) MLC (0.1125 M SDS / 17.5 % acetonitrile). See Section 3.3.1 for peak identity.

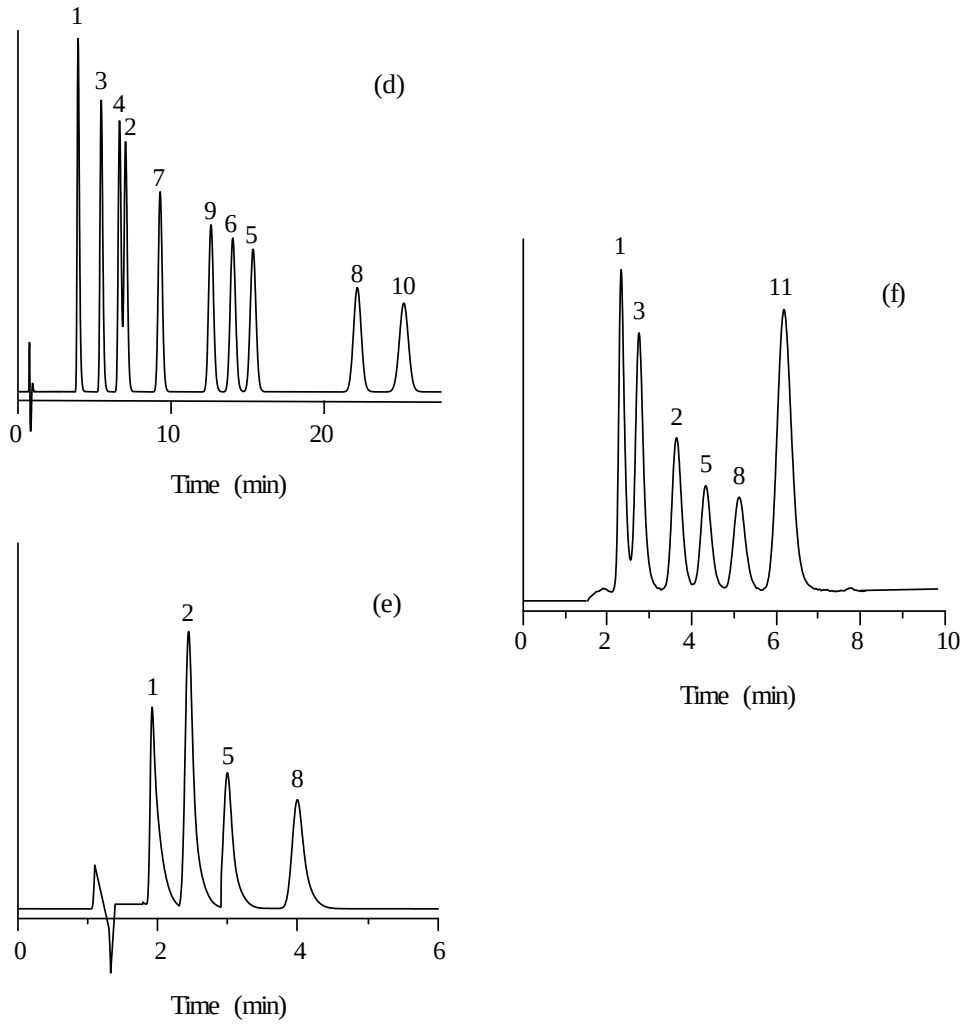


Figure 3.8 (continued)**.** Experimental chromatograms obtained for mixtures of β-adrenoceptor antagonists with Kromasil C18 (a,c), and XTerra (b) columns, in: (d) HSLC (0.1125 M SDS / 35 % acetonitrile), (e) HSLC (0.15 M SDS / 35 % 1-propanol), and (f) MELC (0.114 M SDS / 1.14 % octane / 8.15 % 1-butanol). See Section 3.3.1 for peak identity.

The chromatograms in Figures 3.8a and b correspond to a mixture of ten β-adrenoceptor antagonists eluted with aqueous-organic mixtures from Kromasil and XTerra C18 columns, respectively. As previously noted, in the XTerra column the amount of silanols on the stationary phase has been reduced. In effect, it can be observed that the use of this column results in narrower and more symmetrical peaks, in comparison to the conventional Kromasil column. However, the retention times were similar for both columns, using the same mobile phase composition (15 % acetonitrile), with no change in the elution order.

The experimental chromatograms in Figure 3.8c and d correspond to the best separations obtained in MLC and HSLC with the Kromasil column, respectively, using acetonitrile as modifier. The concentration of SDS in both cases is the same (0.1125 SDS). The increase in the amount of acetonitrile (17.5 % to 35 %) significantly decreased the analysis times, from *ca*. 80 min for MLC (Figure 3.8c) to *ca*. 25 min for HSLC (Figure 3.8d). In both modes, the peaks were almost symmetrical, which indicates silanol groups were effectively masked by the surfactant. As observed, the elution order of the β-adrenoceptor antagonists in MLC and HSLC changed with respect to RPLC, and it was also different between the two surfactant-mediated modes. Peak resolution was maximal with the mobile phase containing SDS and 35 % acetonitrile (Figure 3.8d).

As shown in Figure 3.1, the elution strength of 1-propanol was higher for MLC / HSLC, compared with acetonitrile. With a large amount of 1-propanol (35 %), the β-adrenoceptor antagonists were eluted at very short retention times (Figure 3.8e). However, the peaks were considerably tailing, which can be explained again by the desorption of SDS from the stationary phase.

The chromatogram in Figure 3.8f was obtained with the “standard MELC mobile phase”, using the XTerra column. It can be seen that the β-adrenoceptor antagonists were eluted in shorter times compared to HSLC with 35 % acetonitrile, even for the most hydrophobic compound (propranolol), while the retention times were similar (somewhat smaller) to those obtained in HSLC with 35 % 1-propanol (Figure 3.8e). Note that the peaks in MELC were almost symmetrical (see also Figure 3.8d). However, it is evident that the reduction in peak retention is not followed by sufficient reduction in peak width. The chromatograms in Figures 3.8e and f were obtained with a small number of compounds with the aim of appraising better the peak profile. With more compounds in the injected mixture, the peaks would overlap. A smaller amount of organic solvent (1-propanol in HSLC, and octane or 1-butanol in MELC) would increase the retention and resolution. In fact, the use of a ME of 0.114 M SDS / 0.28 % octane / 8.15 % 1-butanol (Figure 3.7) yielded more satisfactory resolution, with a short analysis time (9 min).

To give an additional perspective on the performance of each chromatographic mode, the half-widths plots obtained from the data of the peaks in chromatograms for different mobile phase compositions (MLC and HSLC, in the presence of 15, 30 and 50 % acetonitrile, and 10 and 35 % 1-propanol, and MELC using the standard mobile phase) are compared in Figure 3.9 and Table 3.5, where the parameters for the fitted lines according to Equations (3.1) and (3.2) are given.

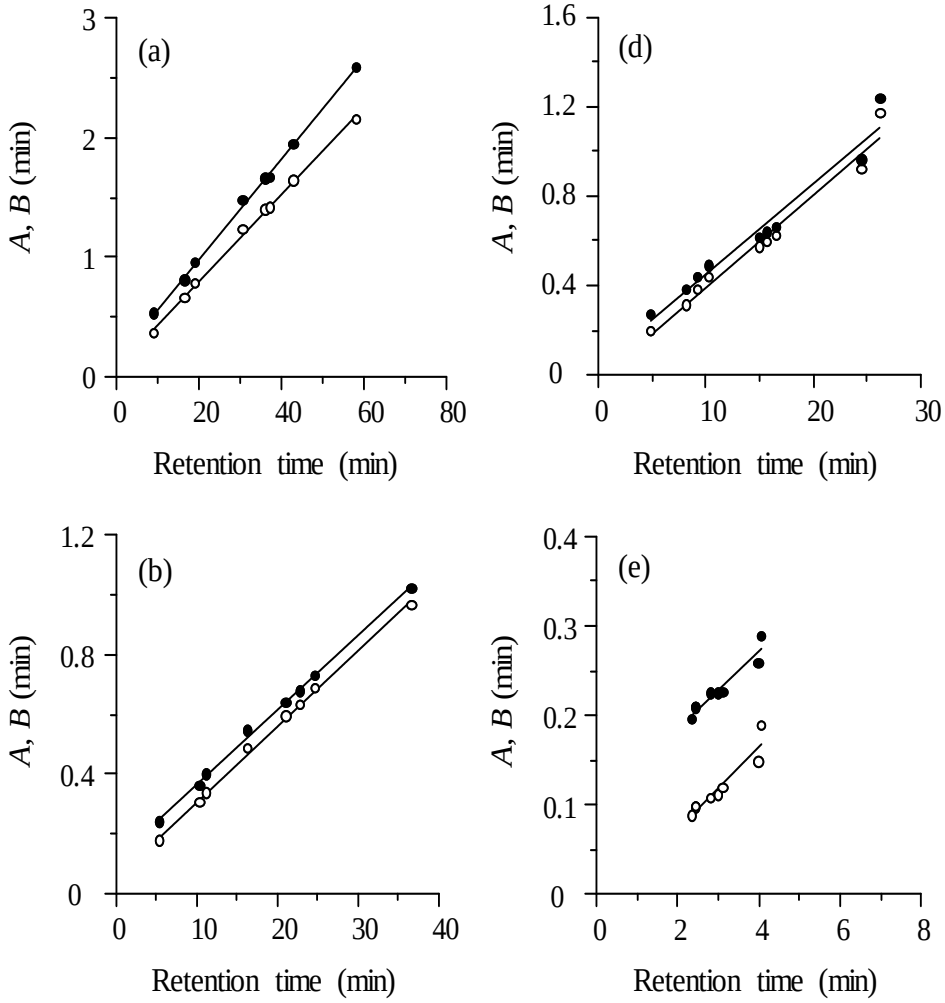


Figure 3.9. Half-width plots (left (*A*, ○) and right (*B*, ●)) for the β-adrenoceptor antagonists eluted with: (a) MLC (0.15 M SDS/15 % acetonitrile), (b) HSLC (0.15 M SDS/30 % acetonitrile), (d) MLC (0.1125 M SDS / 10 % 1-propanol), and (e) HSLC (0.15 M SDS / 35 % 1-propanol).

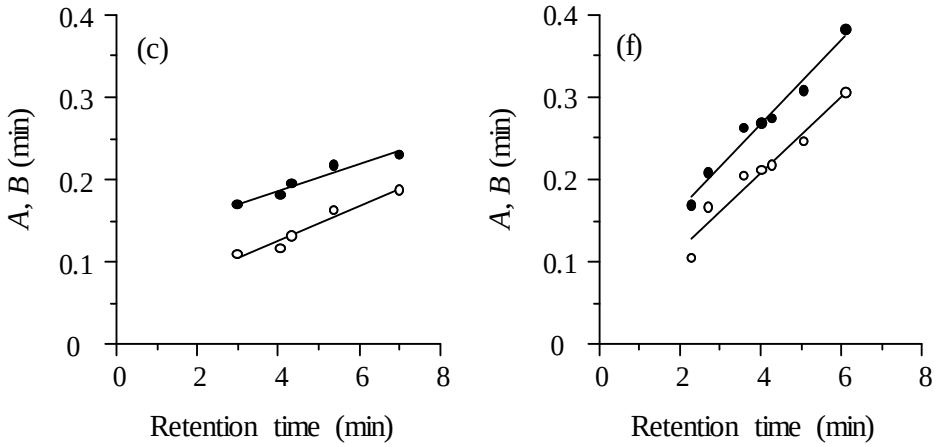


Figure 3.9 (continued). Half-width plots (left (*A*, ○) and right (*B*, ●)) for the β-adrenoceptor antagonists eluted with: (c) HSLC (0.15 M SDS/50 % acetonitrile), and (f) MELC (0.114 M SDS/1.14 % octane/8.15 % 1-butanol).

The plots in Figure 3.9 show that, in all cases, the peaks are narrower and more symmetrical with respect to RPLC (Figures 3.4a and 3.5a). The most symmetrical peaks (with m_B/m_A close to one) were obtained in HSLC with 30 % acetonitrile (Figure 3.9b), and MLC with 10 % 1-propanol (Figure 3.9d) (see also Table 3.5). When the concentration of both solvents was increased (50 % acetonitrile and 35 % 1-propanol) (Figures 3.9c and e), the peak profile was poorer. Note that the plots in Figures 3.9c, e and f were drawn using the same scale. When comparing the peaks, it should be observed that the intercepts of the lines differ, probably due to the different extra-column contributions. For this reason, the m_B/m_A ratio does not give clear information regarding the peak asymmetry. As noted above, the information given by this ratio is only valid for peaks eluted at sufficiently long times, where the extra-column contributions can be neglected.

Table 3.5. Parameters of fitted peak half-width plots according to Equations (3.1) and (3.2). [a]

	m_a	r^2	m_b	r^2	$m_a + m_b$	m_b / m_a
Micellar liquid chromatography						
0.15 M SDS / 15 % acetonitrile	0.036	0.9979	0.042	0.9985	0.078	1.167
0.1125 M SDS / 10 % 1-propanol	0.041	0.9708	0.041	0.9587	0.082	1.000
High submicellar liquid chromatography						
0.15 M SDS / 30 % acetonitrile	0.025	0.9976	0.025	0.9977	0.050	1.000
0.15 M SDS / 50 % acetonitrile	0.022	0.9746	0.016	0.9731	0.038	0.727
0.15 M SDS / 35 % 1-propanol	0.046	0.8934	0.042	0.9161	0.088	0.913
Microemulsion liquid chromatography						
0.114 M SDS / 1.14 % octane / 8.2 % 1-butanol	0.046	0.9468	0.051	0.9768	0.097	1.109

[a] The fitted values correspond to the plots in Figure 3.9.

However, it can be concluded that the peaks are narrower in HSLC with 50 % acetonitrile ($m_a + m_b = 0.038$) with respect to MELC ($m_a + m_b = 0.097$). On the other hand, as noted for Figure 3.5c, in HSLC using large amount of organic solvent, there is a trend to produce fronting peaks for the most retained compounds ($m_b/m_a = 0.727$ for HSLC with 50 % acetonitrile and $m_b/m_a = 0.913$ for HSLC with 35 % 1-propanol).

3.5. Conclusions

The use of mobile phases in RPLC containing SDS and different types of organic solvent, in different amounts, gives rise to diverse microenvironments that affect the chromatographic behaviour (retention and peak profile). This work gives a comprehensive overview of the possibilities of three chromatographic modes (MLC, HSLC and MELC) that employ SDS in the mobile phase for the analysis of a group of basic compounds (β-adrenoceptor antagonists). The differences, advantages and disadvantages of each mode are discussed.

Despite the high retention times in MLC, this chromatographic mode is attractive due to its high solubilizing power, which allows the analysis of non-polar samples and the direct injection of physiological fluids, due to the solubilisation of the proteins in the micellar medium, which elute at the beginning of the chromatograms [15]. However, the retention times for basic drugs in MLC are too high.

This work shows that both HSLC and MELC yield reduced retention times in the separation of basic compounds. Also, the chromatographic peaks are narrower and more symmetrical with respect to conventional RPLC, which indicates that the suppressing silanol activity by SDS observed for MLC is kept. This gives rise to competitive procedures. HSLC with acetonitrile offers better

peak profile and control of the experimental conditions. However, the high volume of organic solvent that this mode requires to achieve sufficiently small retention times for most hydrophobic solutes may make HSLC less attractive. In contrast, MELC makes a significant reduction of retention times using very small amounts of organic solvents. Thus, by using a small amount of the non-polar solvent (octane) and an adequate optimisation, it is possible to get satisfactory resolution in the separation of mixtures of β-adrenoceptor antagonists in just a few minutes.

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CHAPTER 4

MODELLING THE RETENTION IN MICROEMULSION LIQUID CHROMATOGRAPHY

4.1. Abstract

The capability of liquid chromatography with microemulsions (MEs), as mobile phases, was studied for the analysis of four parabens (butylparaben, ethylparaben, methylparaben, and propylparaben), and seven β-adrenoceptor antagonists (acebutolol, atenolol, carteolol, metoprolol, oxprenolol, propranolol, and timolol). MEs were formed by mixing aqueous solutions of the anionic surfactant sodium dodecyl sulphate, the alcohol 1-butanol that played the role of co-surfactant, and octane as oil. In order to guarantee the formation of stable MEs, a preliminary study was carried out to determine the appropriate ranges of concentrations of the three components. For this purpose, mixtures of variable composition were prepared, and the possible separation of two phases (formation of an emulsion) was visually detected. The advantage offered by the addition of octane to micellar mobile phases, inside the concentration range that allows the formation of stable MEs, was evaluated by comparing the retention behaviour, peak profile and resolution of mixtures of the probe compounds, in the presence and absence of octane. The final aim of this work was the proposal of a mathematical equation to model the retention behaviour in Microemulsion Liquid Chromatography (MELC). The derived global model that considered the three factors (surfactant, alcohol and oil) allowed the prediction of retention times at diverse mobile phase compositions with satisfactory accuracy (in the 1.1–2.5 % range). The behaviour was compared with that found with mobile phases without octane. The model also yielded information about the retention mechanism and revealed that octane, when inserted inside the micelle, modifies the interactions between solutes and micelles.

4.2. Introduction

The use of surfactants of diverse nature (anionic, cationic and neutral) has expanded during the last decades, in the so-called Micellar Liquid Chromatography (MLC), to analyse multi-component mixtures in a wide range of polarities and different types of samples [1–3]. The anionic surfactant sodium dodecyl sulphate (SDS) is the most widely used in MLC, due to its low cost, availability, high purity and ability to dissolve proteins, allowing the direct injection of physiological samples into the chromatograph, without the need to perform a pre-treatment of the sample except filtration [4–7]. In this chromatographic mode, the surfactant is adsorbed on the stationary phase, modifying its nature. In the mobile phase, it is at a concentration above the critical micellar concentration (CMC), being thus organised forming micelles, while the excess remains as free monomers (Figure 4.1). The presence of surfactant allows both stationary and mobile phases to establish additional interactions with solutes and other modifiers, and modulates the chromatographic behaviour, which expands the possibilities of separation. A small amount of organic solvent (usually a short or medium chain alcohol) is also added. The role of the alcohol is to increase the elution strength and enhance the peak profile [8,9].

Another more recent chromatographic mode, which uses a surfactant, is oil-in-water Microemulsion Liquid Chromatography (MELC) [10–12]. A mixture of oil and water gives rise to two immiscible phases, due to the high surface tension between the two liquids. However, in the presence of micellised surfactant (and often a co-surfactant), an organised, macroscopically homogeneous and thermodynamically stable liquid system, a microemulsion (ME), is formed [13]. In this system, a small amount of oil can penetrate the micelle, being stabilised in its core as small droplets.

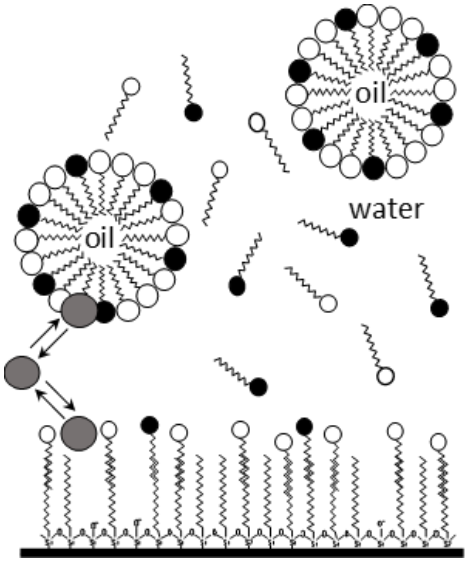


Figure 4.1. Simplified representation of the interior of a column, when using a mobile phase in MLC and MELC. The micelle, surfactant monomers (○), and co-surfactant (●) are dissolved in an aqueous medium. In MELC, an oil is stabilised inside the micelle, associated with the hydrocarbon chains of the surfactant. The solute appears with a larger circle in gray (the equilibria that take place with mobile phase and stationary phase are shown).

The main interest of these MEs is their ability to solubilise lipophilic compounds, in a wide range of polarities, which affects their separation by Reversed-Phase Liquid Chromatography (RPLC) when MEs are used as mobile phases [14–16]. Most applications in MELC refer to the analysis of samples containing compounds with low polarity in non-polar pharmaceuticals, such as creams, ointments and suppositories, in physiological fluids and other biological matrices [10,17–24].

In liquid chromatography, the description of retention has allowed better understanding of the behaviour of solutes, with benefits in the development of the technique. It also gives insight on the influence of the experimental factors, determining which are of primary importance, and finding the optimal conditions. Although there is extensive work to describe the retention in MLC [25], there is no comprehensive work for MELC that considers simultaneously the effect of the three experimental factors involved in the formation of a ME (concentrations of surfactant, co-surfactant and oil) [26–28]. The main purpose of this work was, thus, to check the performance of a global model that takes into account the three factors. The retention model is expected to yield some information on the mechanism of the processes that take place inside the chromatographic column, which modulate the retention. It can also allow interpretive predictions of retention to facilitate the search of the best separation conditions in liquid chromatography, using MEs as mobile phases. For prediction and optimisation purposes, the proposed model was modified to get better convergence, ensuring a correct optimal solution.

In order to generalise the conclusions, two groups of probe compounds were selected for this study: four parabens (which are preservatives and antimicrobial agents used in cosmetics [29]), and seven β-adrenoceptor antagonists (which are drugs used to treat angina pectoris, hypertension, heart failure, and cardiac arrhythmias) [30]. The chromatographic behaviour of these compounds was compared using SDS micellar media containing 1-butanol, in the absence and presence of octane. It should be indicated that a mixture of 0.10 M SDS, 0.85 % (*v*/*v*) octane and 8.2 % (*v*/*v*) 1-butanol was proposed as a standard ME for MELC by El-Sherbiny *et al.* [16], being since then extensively used. The study covered a wide range of conditions, and considered not only the retention behaviour, but

also the profile of chromatographic peaks. Along the manuscript, the stability range for MEs containing SDS, octane and 1-butanol is studied in detail.

4.3. Theory: Description of retention in mobile phases containing surfactant

Modelling of retention, depending on the composition of the mobile phase, is a common task in chromatographic practice [25,31,32]. When governed by partitioning, in mixtures of organic solvent and water, the variation in retention with the concentration of the organic solvent (φ) is usually described using the linear solvent strength (LSS) logarithmic model [33]:

$$\ln k = \ln k_w - S\varphi \quad (4.1)$$

where k_w is the extrapolated value of the retention factor when $\varphi = 0$ (i.e., the mobile phase with the lowest elution strength, consisting only of water), and S is a parameter that describes the strength of the modifier to elute a particular solute. This model usually works well for sufficiently small intervals of modifier, but more complex models including at least one quadratic term are needed for larger intervals.

When the retention mechanism is dominated by solute adsorption on the stationary phase, as is the case of MLC, where the surfactant is immobilised on its surface, a non-logarithmic equation is preferable to describe the retention [25]:

$$k = \frac{K_{AS}}{1 + K_{AM}\,\mu} \quad (4.2)$$

where μ is the concentration of surfactant monomers in the mobile phase forming micelles. Equation (4.2) can be rearranged as follows:

$$\frac{1}{k} = \frac{1 + K_{AM}\,\mu}{K_{AS}} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}\,\mu = c_0 + c_1\,\mu \tag{4.3}$$

In the presence of organic solvent (hybrid MLC), a more complex equation has been proposed [34,35]:

$$k = \frac{K_{AS}\,\dfrac{1 + K_{SD}\,\varphi}{1 + K_{AD}\,\varphi}}{1 + K_{AM}\,\dfrac{1 + K_{MD}\,\varphi}{1 + K_{AD}\,\varphi}\,\mu} \tag{4.4}$$

where K_{AS} and K_{AM} are constants related to the solute-stationary phase and solute-micelle distribution equilibria, respectively, and K_{SD}, K_{AD} and K_{MD} quantify the shift of the distribution equilibria, when the organic solvent is added, in the direction of the stationary phase (K_{SD}), mobile phase (K_{AD}), and micelle (K_{MD}). The K_{SD} coefficient is only significant for non-polar compounds and can be eliminated for other compounds, which is frequent in practice [34–36]. Therefore, for low or intermediate polarity, Equation (4.4) can be reformulated assuming $K_{SD} \approx 0$. This gives rise to the following simplified model:

$$\begin{aligned} \frac{1}{k} &= \frac{1}{K_{AS}}(1 + K_{AD}\,\varphi) + \frac{K_{AM}}{K_{AS}}(1 + K_{MD}\,\varphi)\,\mu = \\ &= c_0 + c_1\,\mu + c_2\,\varphi + c_{12}\,\mu\varphi \end{aligned} \tag{4.5}$$

which can be rewritten as:

$$k = \frac{1}{c_0 + c_1\,\mu + c_2\,\varphi + c_{12}\,\mu\varphi} \tag{4.6}$$

Equation (4.4) has been extended to describe sub-micellar conditions, in the presence of both high concentrations of surfactant and organic solvent [37]:

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\,\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\,\varphi}{1+K_{AD}\,\varphi}\,\mu + K_{\varphi}\,\varphi^{2}} \tag{4.7}$$

The φ^2 term is added to account the greater impact of the organic solvent in the mobile phase, when its concentration is high (compare with Equation (4.4)). Rearranging the terms in Equation (4.7), the following is obtained:

$$\begin{aligned}\frac{1}{k} &= \frac{1}{K_{AS}}(1+K_{AD}\,\varphi) + \frac{K_{\varphi}}{K_{AS}}(1+K_{AD}\,\varphi)\,\varphi^{2} + \frac{K_{AM}}{K_{AS}}(1+K_{MD}\,\varphi)\,\mu \\ &= c_0 + c_1\,\mu + c_2\,\varphi + c_3\,\varphi^{2} + c_4\,\varphi^{3} + c_5\,\mu\varphi\end{aligned} \tag{4.8}$$

which can be simplified by removing the cubic term to:

$$\begin{aligned}\frac{1}{k} &= \frac{1}{K_{AS}}(1+K_{AD}\,\varphi) + \frac{K_{\varphi}}{K_{AS}}\varphi^{2} + \frac{K_{AM}}{K_{AS}}(1+K_{MD}\,\varphi)\,\mu \\ &= c_0 + c_1\,\mu + c_2\,\varphi + c_3\,\varphi^{2} + c_4\,\mu\varphi\end{aligned} \tag{4.9}$$

All this knowledge about the description of retention in MLC and High Sub-micellar Liquid Chromatography (HSLC) will be used, in this work, to propose a retention model in MELC.

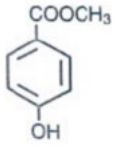
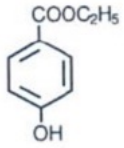
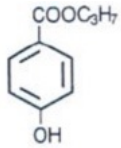
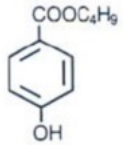
4.4. Experimental

4.4.1. Reagents

Two groups of probe compounds were used: four parabens (butylparaben, ethylparaben, methylparaben and propylparaben) from Fluka (Buchs, Switzerland) and seven β-adrenoceptor antagonists (acebutolol, atenolol, carteolol, metoprolol, oxprenolol, propranolol and timolol), from Sigma (St. Louis, MO, USA). Their structures, acidity constants and polarities, measured as the logarithm of the partition coefficient between octanol and water, are given in Tables 4.1 and 4.2. Stock solutions of 100 μg/mL of the probe compounds were prepared by dissolving the solid reagents in 1 mL of acetonitrile from Scharlab (Barcelona, Spain), with the help of an ultrasonic bath Elmasonic IT-H from Elma (Singen, Germany), and then diluted with water. The solutions, stored at 4 ºC, remained stable for at least two months. These solutions were diluted with water to a final concentration of 20 μg/mL, prior to injection into the chromatograph. Uracil from Acros Organics (Geel, Belgium) was used as hold-up time marker.

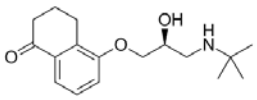
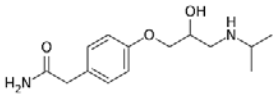
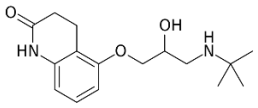
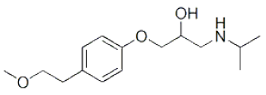
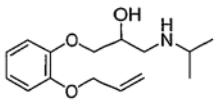
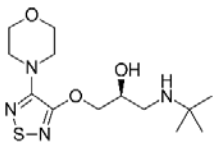
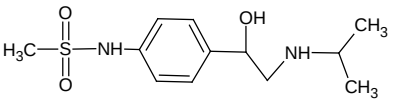
The mobile phases contained sodium dodecyl sulphate (99 % purity) from Merck (Darmstadt, Germany) and 1-butanol from Scharlab, in MLC, and sodium dodecyl sulphate, octane and 1-butanol from Alfa Aesar (Kandel, Germany), in MELC. Molar concentrations were used for the surfactant, and volumetric fraction (expressed as percentage) for octane and 1-butanol.

Table 4.1. Structures, acidity constants and octanol-water partition coefficients for parabens.

Compound	Structure	pK_a [a]	log $P_{o/w}$ [b]
Methylparaben		8.47	1.91
Ethylparaben		8.50	2.34
Propylparaben		8.47	2.94
Butylparaben		8.47	3.50

[a] [38]. [b] [39].

Table 4.2. Structures, acidity constants and octanol-water partition coefficients for β-adrenoceptor antagonists.[a]

Compound	Structure	pK_a	log $P_{o/w}$
Acebutolol		9.2 [a]	1.19 [a]
Atenolol		9.6 [a]	-0.026 [a]
Carteolol		NA [a]	1.42 [a]
Metoprolol		9.7 [a]	1.69 [a]
Oxprenolol		9.5 [a]	1.83 [a]
Timolol		9.2 [a]	1.75 [a]
Propranolol		9.5 [a]	2.60 [a]

[a] [40]. NA: not available.

In all cases, 0.1 % trifluoroacetic acid from Fisher Scientific (UK) was added, resulting pH = 2.25 ± 0.14 in MLC, and 2.15 ± 0.09 in MELC. The pH was controlled within ±0.002 units, using a Crison pH meter (Model MicropH 2002, Barcelona), and an Crison Orion combined glass electrode (Model 8102), which contained Ag/AgCl reference electrodes with a saline bridge filled with a 3.0 M KCl solution. The pH meter was calibrated with aqueous buffers, while the pH of the mobile phases was always adjusted in the presence of the organic solvent.

All solutions were filtered through 0.45 µm Nylon membranes from Micron Separations (Westboro, MA, USA). The mobile phases were degassed in an ultrasonic bath, after filtration.

4.4.2. Apparatus and chromatographic conditions

An Agilent instrument (Waldbronn, Germany), equipped with a quaternary pump (Series 1200), autosampler (Series 1260 Infinity II), thermostatic column compartment (Series 1290 Infinity II), and UV-visible diode array detector (Series 1100), all controlled with an Agilent OpenLAB CDS LC ChemStation (version C.01.07), was used. The signal was monitored at a wavelength of 215 nm for parabens and 225 nm for β-adrenoceptor antagonists, except for timolol which was detected at 300 nm (its absorption maximum). The dead time marker (uracil) was detected at 254 nm.

The chromatographic column was an XTerra MS C18, with the following characteristics: 150 mm length, 4.6 mm internal diameter, 5 µm particle size, 15.2 % carbon load, 177 m^2/g surface area, and 127 Å pore size. The mobile phase flow-rate was set at 1 mL/min. Duplicate injections of the working solutions were made at a fixed temperature of 25 °C, using a volume of 20 µL.

The MICHROM software was used to obtain the retention times, efficiencies, and half-widths of chromatographic peaks, as well as simulate chromatograms and optimise the mobile phase [41]. Other mathematical treatments was carried out with the Solver application of Excel (Microsoft Office 2010, Redmond, WA, USA).

4.4.3. Column care

Throughout the experimental work, a series of safety measures were taken with the aim of prolonging the useful life of the columns and equipment, and increasing environmental sustainability:

- The mobile phases were recirculated between runs, as well as during the analyses, to reduce the consumption of reagents and amount of residues.
- A flow-rate of 0.2 mL/min was used overnight, in order to avoid cleaning and frequent re-conditioning of the column.
- Before moving to a mobile phase with lower concentrations of SDS, octane and 1-butanol, the column was cleaned by flushing 60 mL of a 50:50 methanol:water mixture, at a flow-rate of 1 mL/min, followed by 30 mL of pure methanol.
- Cleaning was not required when moving to a mobile phase with higher concentration of the mobile phase components, which was verified by the repeatability of the retention times.
- The column was stored in methanol over the weekend.

4.5. Results and discussion

4.5.1. Nature and concentration of the mobile phase components in MELC

The mobile phases in MELC contain three key components for the formation of a ME: surfactant, oil and co-surfactant, all three dissolved in an aqueous phase (water solution containing a reagent at fixed pH). The surfactant provides stability to the ME. On the other hand, as in MLC, the surfactant monomers are adsorbed on the surface of the stationary phase and coat it homogeneously. This adsorption produces an increase in the thickness of the stationary phase, which results in a pseudo stationary-phase that modifies the retention, selectivity and efficiency. The alkyl tails of the surfactant interact with the alkyl chains of the stationary phase, whereas the polar part of the adsorbed surfactant monomers is oriented towards the aqueous phase. In this way, a hydrophilic layer is formed which is in contact with the mobile phase. On the other hand, a concentration above the CMC guarantees the formation of micelles in the mobile phase, which in MELC will allow the existence of oil droplets dispersed in the aqueous phase, creating a transparent medium. The droplets size, as well as the charge of the droplets and pseudo stationary-phase will vary depending on the type of surfactant used (cationic, anionic or neutral).

The choice of oil has also a large effect on the selectivity and distribution of solutes between mobile phase and stationary phase. Various organic solvents, with different nature and polarity, have been explored to be used as oils in MELC, although alkanes (hexane, heptane and octane) and long-chain alcohols (1-pentanol and 1-octanol) are usually used. Other water-insoluble solvents are also usual, such as cyclohexane, toluene, ethyl acetate, butyl acetate, di-isopropyl ether and 2-octanone.

In most cases, the surfactant molecules are sufficient to reduce the interfacial tension between the oil and water, facilitating the formation of a ME. However, to reduce the interfacial tension, and thus achieve a more stable ME, the addition of a more hydrophilic organic solvent is usual, which acts as a co-surfactant. It is common to use the medium-sized alkyl chain alcohols 1-propanol and 1-butanol. The co-surfactant influences the solubilisation properties of the oil in the aqueous phase, since it is distributed between both phases. It also increases the fluidity of the hydrocarbon tails of the surfactant and allows better penetration of the solutes into the micelle core. In addition to the three main components, other reagents are usually added to the mobile phase to control the pH, such as phosphate salts, and acids as trifluoroacetic acid (TFA) or formic acid.

To ensure the formation of MEs, not only must the nature of each of the components of the mobile phase be taken into account, but also their concentration, since retention, selectivity and efficiency in MELC can be modified by changing the concentration of the three components. The surfactant CMC is highly important, since below this value, micelles do not form, and therefore, MEs cannot also be formed. In the case of SDS, the CMC in aqueous medium is 8.3×10^{-3} M. However, below 6.1×10^{-2} M SDS, the system does not solubilise the oil, and therefore, MEs are not formed. Furthermore, MEs are unstable below 0.10 M SDS [17]. Some authors have indicated that MEs does not form below 8.7×10^{-2} M SDS, and that above 0.156 M SDS high pressure is produced, which can damage the column [26].

Regarding the oil, which is the component that allows a micellar system to be converted into a ME, several studies have been carried out to evaluate the appropriate concentration for its formation. Marsh *et al.* examined the octane (used as oil) concentration ranges, from 0 to 1.7 % (*v*/*v*), since above this concentration, SDS is not capable of dissolving it [17]. According to several

authors, the type and concentration of co-surfactant in MELC has also a very notable influence on the separation of compounds. The co-surfactant concentration is limited by the pump pressure. However, this component improves the chromatographic profile, so a compromise must be found between the pressure and the width and asymmetry of chromatographic peaks.

Finally, to optimise a procedure in MELC, the retention behaviour must be taken into account. This is regulated by the composition of the mobile phase. An increase in the concentration of SDS, oil and co-surfactant leads to shorter retention times, depending on the type of interaction that the analytes establish with each component.

4.5.2. Adequate range of concentrations to form microemulsions

When reviewing the published literature on the analysis of compounds in MELC, no rigorous study of the concentration ranges of the reagents that must be mixed to form MEs was found. Outside these ranges, emulsions (non-transparent media) would be obtained, which prevents the detection of the eluted compounds and the preservation of the integrity of the chromatographic column. Hence, first, a detailed study was designed to know the mixtures that lead to the formation of MEs, in the presence of SDS. Based on the information obtained from the literature, and previous assays, octane was selected as oil, and 1-butanol as co-surfactant.

The formation of MEs is determined by the composition of the mobile phase. A change in the nature of the surfactants (anionic, cationic or neutral), as well as a simple variation in the polarity of the oil, or an alteration in the chain length of the co-surfactant, notably affects the absolute and relative retention of the analytes in RPLC, but especially the formation of MEs. The choice of

components is not only important, the concentration in the mobile phase of each of the components is essential to guarantee the success in its formation.

Therefore, with the aim of verifying the formation of a transparent medium, suitable to be used in RPLC, or the possible appearance of two well differentiated phases (the formation of an emulsion), a preliminary study was carried out where several mixtures containing different amounts of the three components (SDS, octane and 1-butanol) were prepared. The importance of this study can be explained by the need of preparing mobile phases of MEs in a wide range of compositions to modulate the retention of the probe compounds. It was, thus, needed to confirm previously the range of compositions that led to homogeneous mixtures.

The effect of the concentration of octane and 1-butanol, on the preparation of a ME, was studied at two concentrations of SDS (0.10 M and 0.18 M), which guaranteed the formation of micelles. The reagents were mixed and the mixture was allowed to stand for at least 12 hours. After this, it was centrifuged (stress test), and when it did not initially give rise to two well-differentiated phases, it was left several weeks at rest to check the ME stability.

Two mixtures were used as references to observe the formation of a stable ME (a completely transparent mixture, Figure 4.2, right), and an emulsion (where the appearance of a whitish phase in the upper region was observed, Figure 4.2, left). The reference ME consisted of 0.10 M SDS, 0.85 % octane and 8.2 % 1-butanol, while the reference emulsion contained only 0.10 M SDS and 0.85 % octane.

Figure 4.2. Visual appearance of a reference ME (a fully transparent mixture composed of 0.10 M SDS, 8.2 % 1-butanol and 0.85 % octanol, right) and an emulsion (appearance of a whitish phase in the upper region of a mixture of 0.10 M SDS and 0.85 % 1-butanol, left).

In this study, the concentration of octane was increased in the range between 1.2 % and 6.0 %, keeping 1-butanol fixed at 8.2 % or 12 %. Meanwhile, the concentration of 1-butanol was varied in the range 0.5 % to 6.0 %, keeping octane fixed at 0.85 % or 0.2 %. The composition of all tested mixtures, prepared at different concentrations of their components, are represented in Figure 4.3. This figure contains the information of the mixtures that after several weeks remained transparent (those that visually did not show phase separation), indicating that the ME was stable, and the mixtures that yielded the formation of an emulsion (where two phases were observed), and consequently, were not suitable for liquid chromatography.

At higher concentration of surfactant and co-surfactant, MEs can solubilise higher amount of octane. Thus, as can be seen in Figure 4.3, for a fixed concentration of 12 % 1-butanol using 0.18 M SDS, a stable ME is formed with a maximal octane concentration of 4.5 %, while with 0.10 M SDS a stable ME is only possible with up to 2.5 % octane. Meanwhile, at lower concentration of 1-butanol (8.2 %), the increase in surfactant only allowed a small increase in octane to get a stable ME: up to 1.8 % octane for 0.10 M SDS and up to 2.3 % for 0.18 M SDS. Despite the benefit that the use of a higher concentration of 1-butanol could bring over the solubilisation of a higher amount of octane, the upper concentration limit of co-surfactant was limited due to the high pump back-pressure, which could damage the chromatographic column and apparatus.

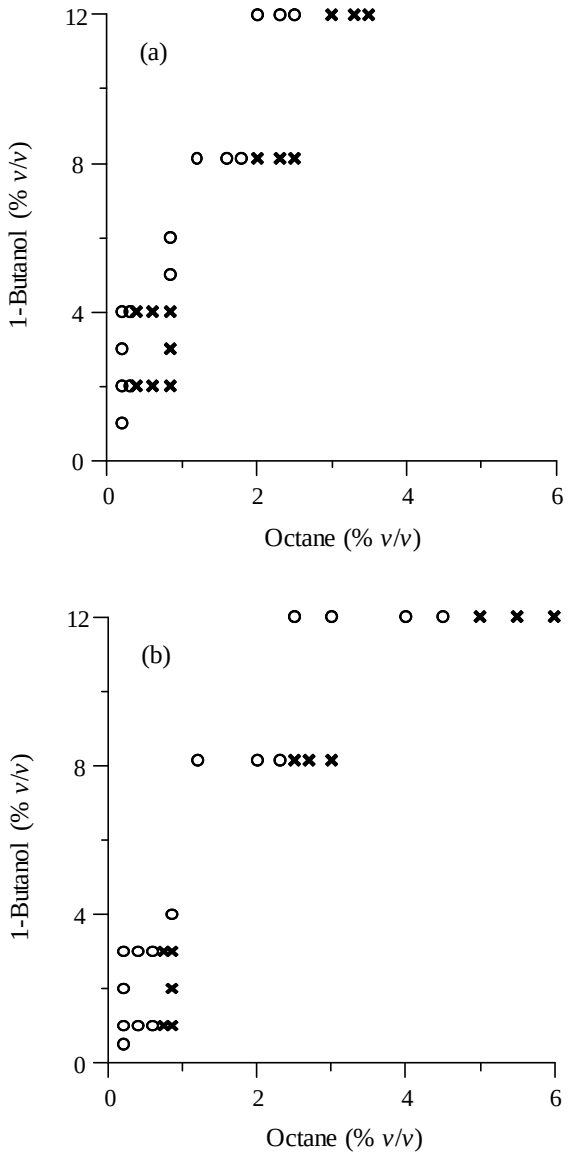


Figure 4.3. Concentrations of octane and 1-butanol that give rise to a ME (○) and an emulsion (×), in the presence of: (a) 0.10 M SDS, and (b) 0.18 M SDS.

The minimal concentration of 1-butanol required to form a ME was also studied, using a fixed octane concentration of 0.85 % and 0.2 %, at the two SDS concentrations tested (0.10 M and 0.18 M). It was observed that for 0.85 % octane, at least 5 % 1-butanol was required to get a ME for 0.10 M SDS, and 4 % 1-butanol for 0.18 M SDS. When a low amount of octane (0.2 %) was added, phase separation was not visible at either SDS concentration, even at very low concentration of 1-butanol (less than 1 %, Figure 4.4). This indicated that the surfactant was capable of solubilising small amounts of octane without the need of co-surfactant.

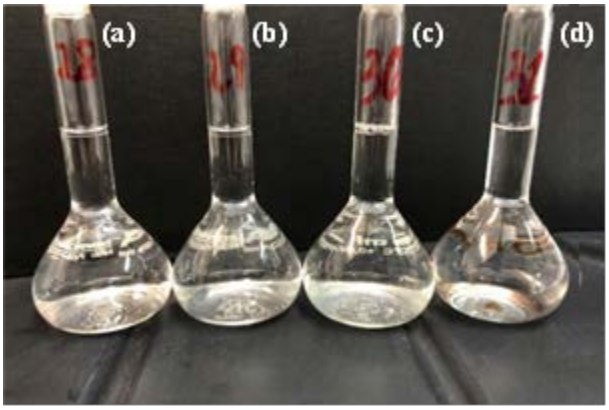


Figure 4.4. Visual appearance of mixtures containing 0.18 M SDS, 0.2 % octane, and a variable content of 1-butanol: (a) 3 %, (b) 2 %, (c) 1 %, and (d) 0.5 %.

The effect of increasing octane (Figure 4.5), or decreasing 1-butanol (Figure 4.6) in the mixtures, gave rise to an upper phase that increased in thickness and turned whitish. This effect was larger using a smaller concentration of surfactant

(0.10 M SDS; compare Figures 4.5 c and d, and Figures 4.5 g and h). On the other hand, with the addition of a relatively high percentage of 1-butanol (12 %), the whitish layer of the upper phase disappeared (Figure 4.7).

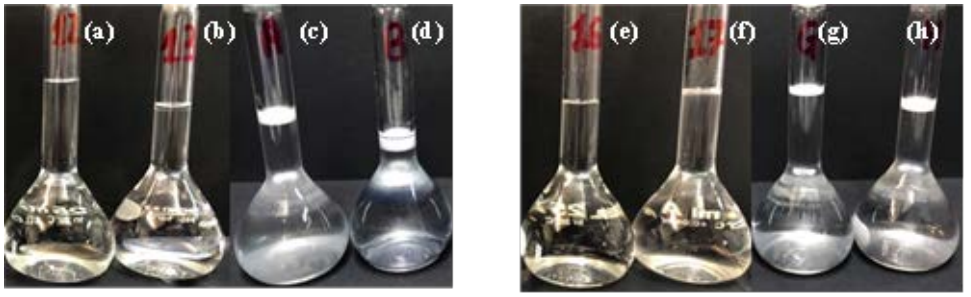


Figure 4.5. Visual appearance of the mixtures for an increasing amount of octane, in the presence of 8.2 % 1-butanol, 0.10 M SDS (a to d) and 0.18 M SDS (e to h). Octane concentration: (a) 1.8 %, (b) 2.0 %, (c) 2.3 %, (d) 2.5 %, (e) 2.3 %, (f) 2.5 %, (g) 2.7 %, and (h) 3 %.

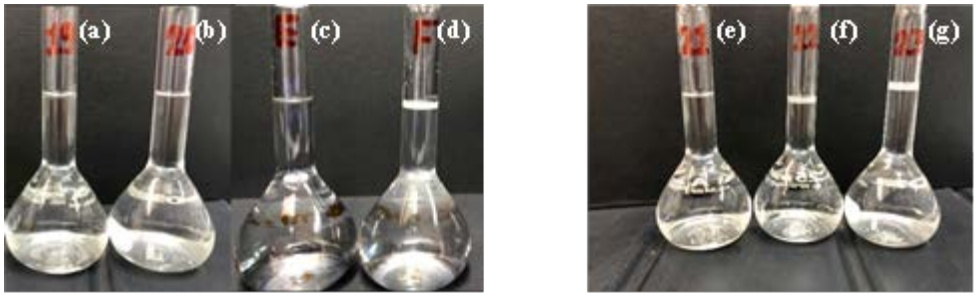


Figure 4.6. Visual appearance of the mixtures when 1-butanol was decreased in the presence of 0.85 % octane, with 0.10 M SDS (a to d) or 0.18 M SDS (e to g). 1-Butanol concentration: (a) 5 %, (b,e) 4 %, (c,f) 3 %, and (d,g) 2 %.

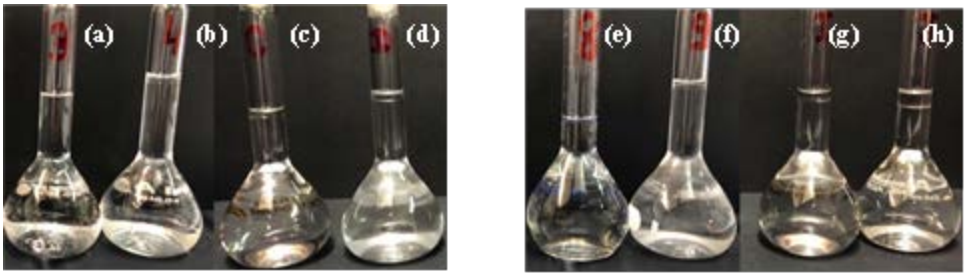


Figure 4.7. Visual appearance of mixtures with increasing octane content, in the presence of 12 % 1-butanol, and 0.10 M SDS (a to d) or 0.18 M SDS (e to h). Octane concentration: (a) 2.5 %, (b) 3 %, (c) 3.3 %, (d) 3.5 %, (e) 4.5 %, (f) 5 %, (g) 5.5 %, and (h) 6 %.

The mixtures that did not show phase separation remained stable for at least one month, except those that contained 0.10 M SDS with a high concentration of 1-butanol (12 %). These mixtures, after several weeks, formed an emulsion (Figure 4.8). In these conditions, micelles are not formed, which prevents the solubilisation of octane by the surfactant. By increasing the SDS concentration to 0.18 M, phase separation was not observed, even after several weeks.

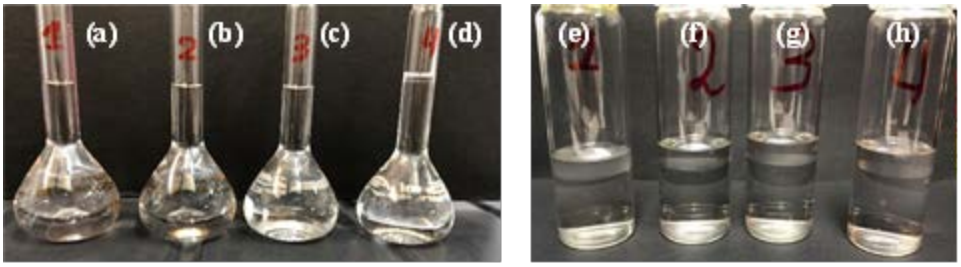


Figure 4.8. Visual appearance of the mixtures when octane was increased, in the presence of 12 % 1-butanol and 0.10 M SDS, after 12 hours (a to d), and after several weeks (e to h). Octane concentration: (a,e) 2.0 %, (b,f) 2.3 %, (c,g) 2.5 %, and (d,h) 3.0 %.

The study above allowed establishing the appropriate limits where MEs were stable, and consequently, the valid composition of the three components (SDS, octane and 1-butanol) needed to prepare a mobile phase to avoid possible damage to the equipment or column, due to the formation of emulsions. The study also showed the time period MEs remain stable. Based on the visual observation of the prepared mixtures and assisted by Figure 4.3, Figure 4.9 was prepared. The plot depicts the limits of the three components, needed to prepare stable MEs, useful to be used as mobile phases in MELC. Only those mixtures containing concentrations of octane and 1-butanol in the region above each line are suitable for liquid chromatography.

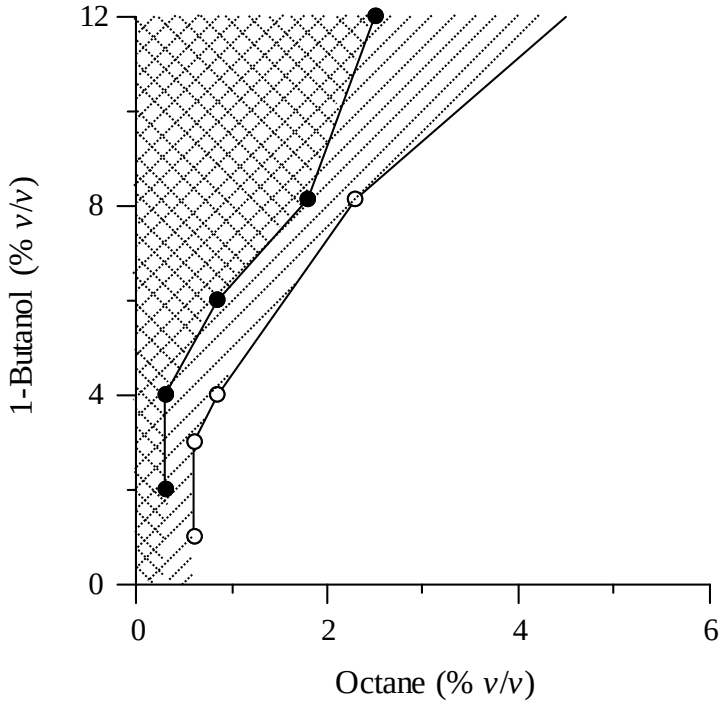


Figure 4.9. Concentration range for octane and 1-butanol, suitable for the preparation of MEs, in the presence of 0.10 M (●), and 0.18 M (○) SDS. The regions above the curves correspond to the compositions that lead to the formation of stable MEs at the two assayed SDS concentrations.

4.5.3. Comparison of the retention in MELC versus MLC

The selection of the composition of the mobile phase in MELC is quite laborious, due to the complexity of the nature of MEs, where there are three components to optimise, which interact each other. The type of surfactant, co-surfactant and octane (in our study, SDS, octane and 1-butanol) is important, but also their concentration, to obtain proper retention of solutes. MELC has been developed based on the principles of MLC, where the mobile phase is composed of a micellar solution. In fact, MELC can be considered as a modification of MLC, where an organic solvent with lipophilic character is incorporated into the micelle. Therefore, it is interesting to compare the retention in both chromatographic modes (MLC and MELC), to appraise the advantages of the addition of an oil.

Figures 4.10 and 4.11 show the variation in retention for parabens and β-adrenoceptor antagonists, respectively, in MLC (mobile phases in the absence of octane) and MELC with mobile phases containing increasing amounts of octane (0.25, 0.5 and 1 %), at two concentrations of SDS (0.10 M and 0.18 M) and 1-butanol (5 % and 12 %). By adding octane to the micellar system, MLC moves to MELC, resulting usually in decreased retention times, even using a small amount of octane (0.2 %). This reduction was significantly larger at higher octane concentration (1 %), especially for the most retained compounds (butylparaben in the group of parabens, and propranolol in the group of β-adrenoceptor antagonists). Here, it should be noted that the mobile phase with 1 % octane, at both SDS concentrations, required higher concentration of 1-butanol (7 %) for a ME to be formed (Figures 4.10a and b, and Figures 4.11a and b).

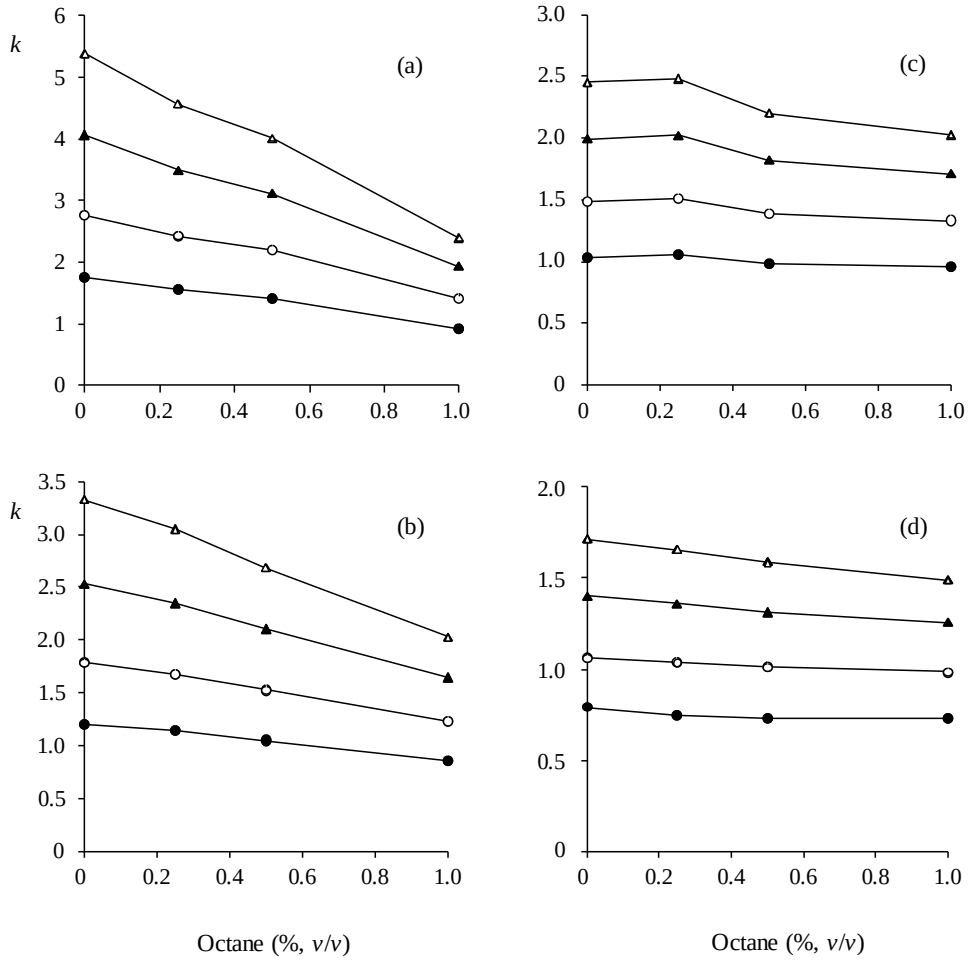


Figure 4.10. Variation of retention with increasing octane concentration in mobile phases containing: 0.10 M (a,c) and 0.18 M (b,d) SDS, and 5 % (a, b) and 12 % (c,d) 1-butanol. At 1 % octane and 0.10 M SDS, the concentration of 1-butanol was increased to 7 %. Compounds: (●) methylparaben, (○) ethylparaben, (▲) propylparaben, and (Δ) butylparaben.

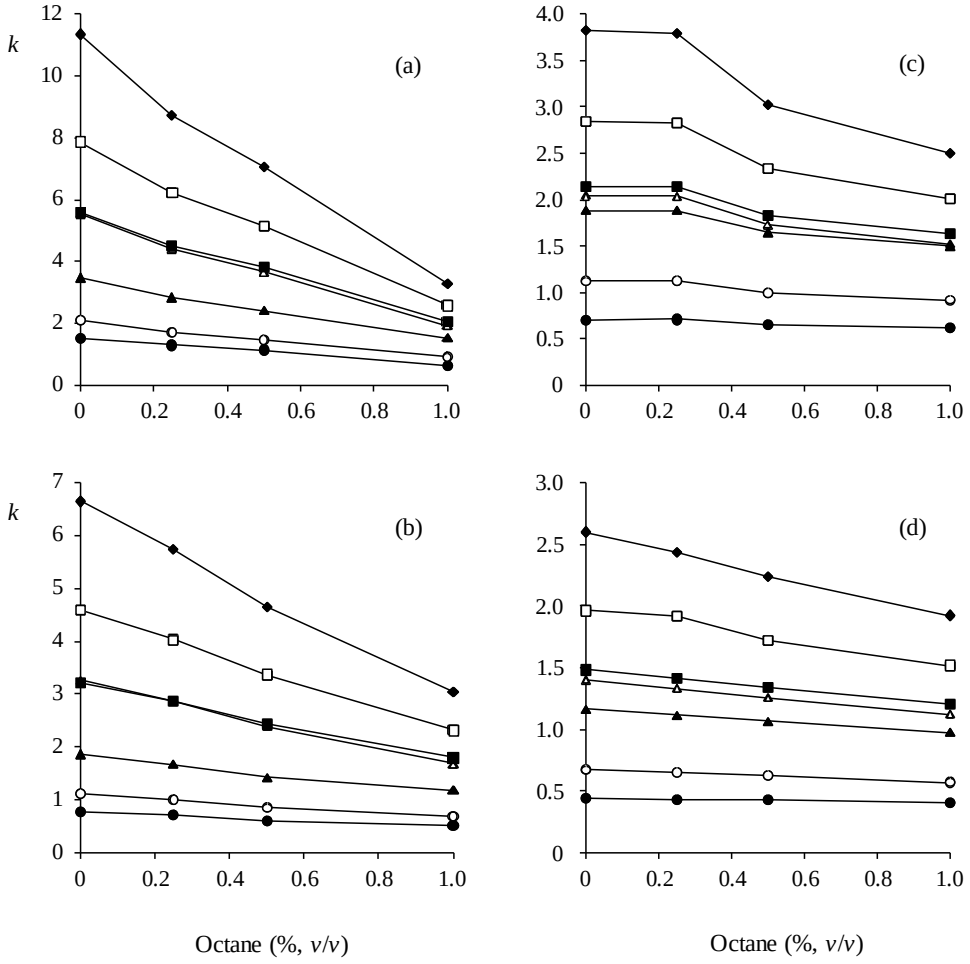


Figure 4.11. Variation of retention with increasing octane concentration in mobile phases containing: 0.10 M (a,c) and 0.18 M (b,d) SDS, and 5 % (a,b) and 12 % (c,d) 1-butanol. At 1 % octane and 0.10 M SDS, the concentration of 1-butanol was increased to 7 %. Compounds: (●) atenolol, (○) cartelolol, (▲) acebutolol, (Δ) timolol, (■) metoprolol, (□) oxprenolol, and (♦) propranolol.

On the other hand, at low concentrations of both, SDS and 1-butanol (Figures 4.10a and 4.11a), the reduction in retention was large when adding the oil, whereas at high concentration of both SDS (0.18 M) and 1-butanol (12 %), practically no variation in retention was observed (Figures 4.10d and 4.11d). The highest pressures were obtained with mobile phases containing 0.18 M SDS and 12 % 1-butanol, for both MLC and MELC, with values between 226 and 260 bar, without octane and with 1 % octane, respectively (see Table 4.3). Therefore, with the aim of preserving the column performance, avoid damage to the apparatus and reduce the environmental impact, the upper limit of 1-butanol was set at 12 %.

The decrease in retention was larger at increasing 1-butanol (from 5 % to 12 %), compared to increasing SDS (from 0.10 M to 0.18 M), for both parabens (compare Figure 4.10a with Figures 4.10b an c), and β-adrenoceptor antagonists (compare Figure 4.11a with Figures 4.11b and c). The reduction in retention was always larger for the β-adrenoceptor antagonists, due to the higher initial retention produced by the attraction towards the stationary phase (coated by the anionic surfactant) of the cationic species formed at the acidic pH of the mobile phase.

Table 4.3. Pressures measured with the MLC and MELC mobile phases used in this work.

	SDS (M)	Octane (%)	1-Butanol (%)	Pressure (bar)
MLC	0.10	–	5	154
	0.10		12	190
	0.14		8.5	188
	0.18		5	183
	0.18		12	226
MELC	0.10	0.25	5	156
	0.10		12	194
	0.14		8.5	187
	0.18		5	185
	0.18		12	236
	0.10	0.5	5	158
	0.10		12	-
	0.14		8.5	203
	0.18		5	195
	0.18		12	251
	0.10	1.0	7	180
	0.10		12	216
	0.14		8.5	202
	0.18		7	230
	0.18		12	260

Figures 4.12 and 4.13 depict chromatograms obtained with the mobile phase that yielded the largest and smallest elution strength among those assayed, for MLC, and MELC containing 0.2 and 1 % octane. As previously mentioned, the analysis time was more significantly reduced with the mobile phases of smaller elution strength (Figures 4.12a, b and c and 4.13a, b and c): from 10 min in MLC to 8.5 and 5 min in MELC, for parabens, and from 20 min in MLC to 16 and 6.5 min in MELC for β-adrenoceptor antagonists. In contrast, the mobile phases with the highest elution strength (Figures 4.12d, e and f, and 4.13d, e and f) only gave rise to small changes in the analysis time. For both types of compounds, the elution order was the same in both MLC and MELC.

Despite the fact that the mobile phase with 0.18 M SDS, 12 % 1-butanol and 1 % octane showed the best results in terms of analysis time, for both parabens and β-adrenoceptor antagonists (Figures 4.12f and 4.13f), peak resolution was only satisfactory for parabens, making this mobile phase the most suitable. For β-adrenoceptor antagonists, the peaks of atenolol and carteolol were overlapped; therefore, a mobile phase with smaller octane concentration (0.25 %) was more convenient (Figure 4.13e).

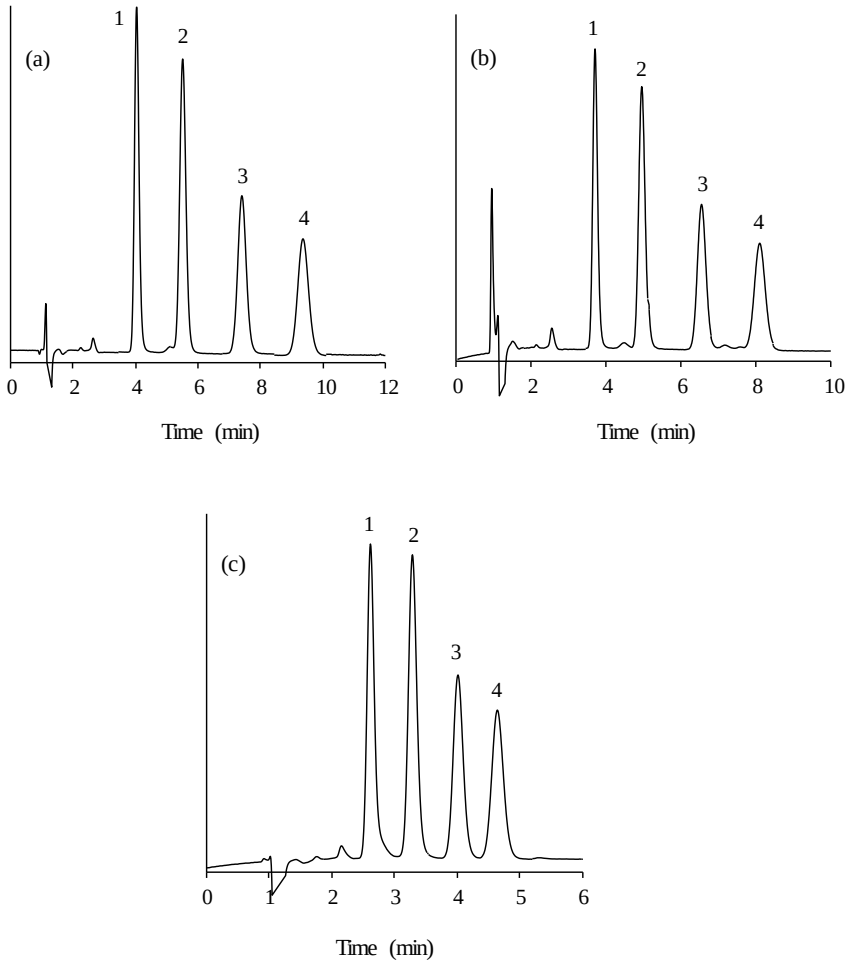


Figure 4.12. Chromatograms obtained with: 0.10 M SDS (a to c), and with 5 % (a and b) and 7 % (c) 1-butanol, and 0.25 % (b) and 1 % (c) octane. Chromatogram "a" corresponds to micellar mobile phases in the absence of octane. Compounds: (1) methylparaben, (2) ethylparaben, (3) propylparaben, and (4) butylparaben.

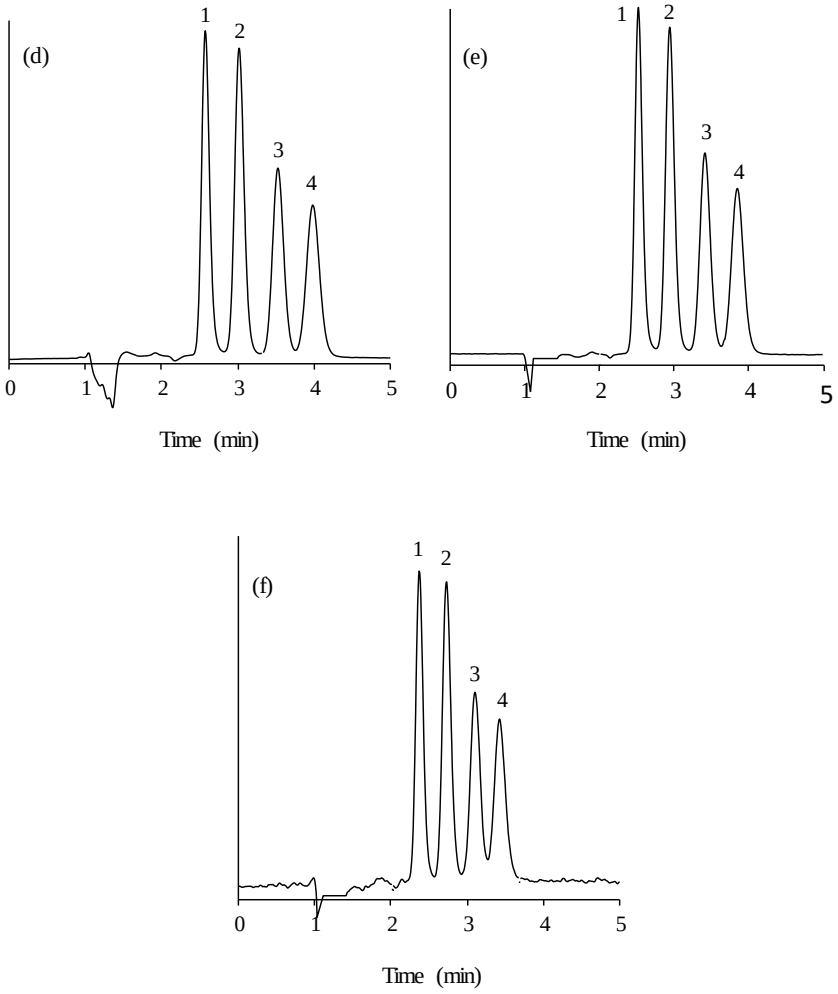


Figure 4.12 (continued). Chromatograms obtained with: 0.18 M SDS (d to f), 12 % (d to f) 1-butanol, and 0.25 % (e) and 1 % (f) octane. Chromatogram "d" corresponds to micellar mobile phases in the absence of octane. Compounds: (1) methylparaben, (2) ethylparaben, (3) propylparaben, and (4) butylparaben.

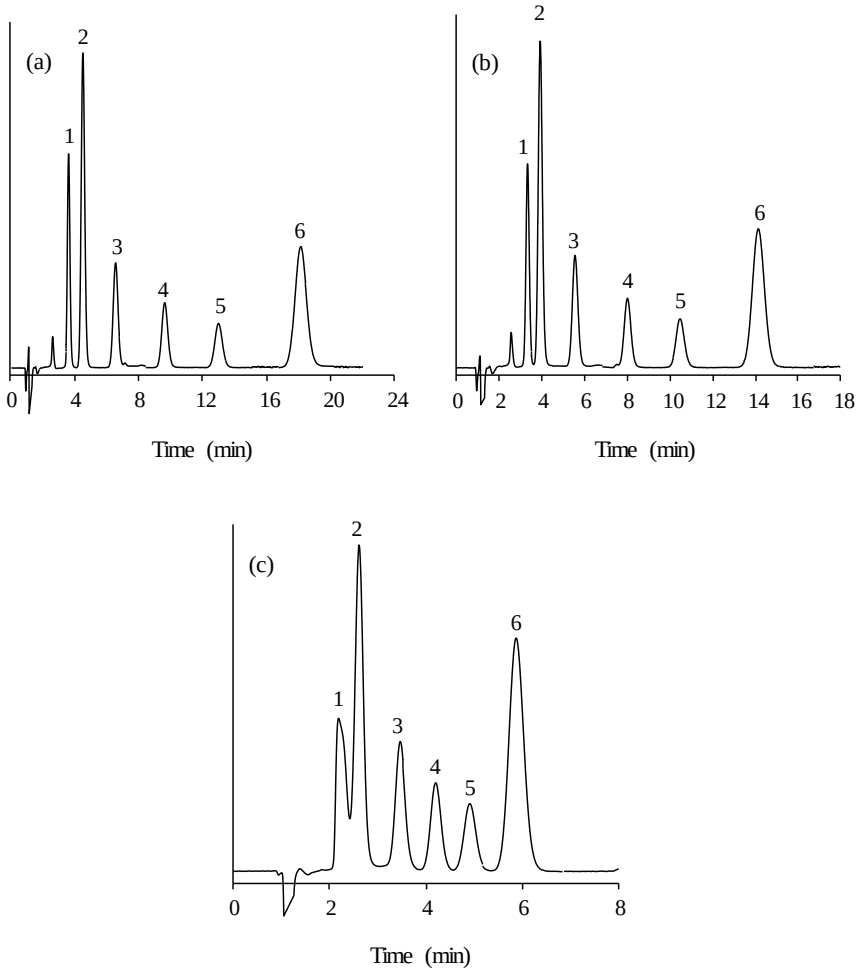


Figure 4.13. Chromatograms obtained with: 0.10 M SDS (a to c), 5 % (a and b) and 7 % (c) 1-butanol, and 0.25 % (b) and 1 % (c) octane. Chromatogram "a" corresponds to micellar mobile phases in the absence of octane. Compounds: (1) atenolol, (2) carteolol, (3) acebutolol, (4) metoprolol, (5) oxprenolol, and (6) propranolol. Timolol is not shown in the chromatograms because it absorbs at a different wavelength.

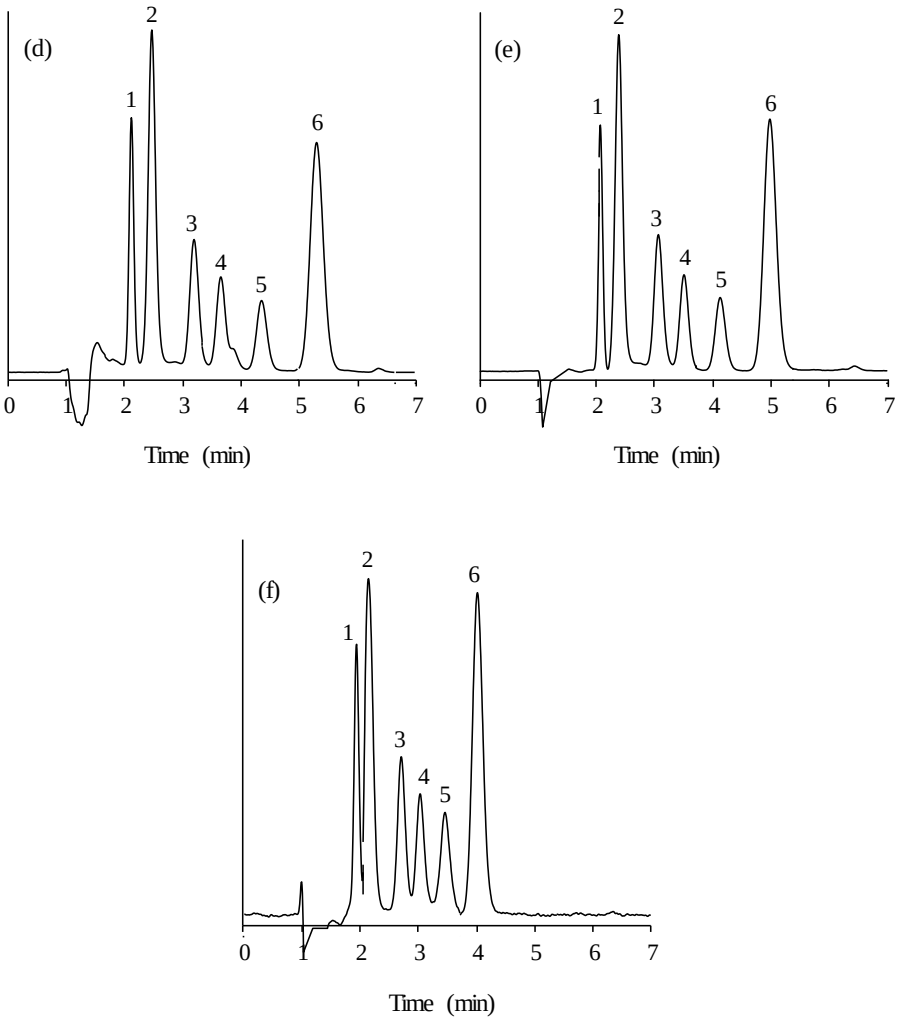


Figure 4.13 (continued). Chromatograms obtained with: 0.18 M SDS (d to f), 12 % (d to f) 1-butanol, and 0.25 % (e) and 1 % (f) octane. Chromatogram "d" corresponds to micellar mobile phases in the absence of octane. Compounds: (1) atenolol, (2) carteolol, (3) acebutolol, (4) metoprolol, (5) oxprenolol, and (6) propranolol. Timolol is not shown in the chromatograms because it absorbs at a different wavelength.

4.5.4. Chromatographic profiles in MELC and MLC

A very desirable feature of chromatographic peaks is being narrow and symmetric. However, in conventional RPLC, this is not often the case. The peaks are especially wide and asymmetric in the analysis of basic compounds, such as β-adrenoceptor antagonists, due to the electrostatic interaction between the cationic species at acidic pH in the mobile phase and the residual anionic silanols in the stationary phases of silica, which causes slow mass transfer kinetics. A solution to avoid the silanol effect is adding to the mobile phase a reagent that adsorbs on the stationary phase, such as SDS [42], which hinders the access of basic compounds to the silanol groups.

A practical way to visualise the profile of chromatographic peaks is the construction of plots that represent the left (*A*) and right (*B*) half-widths of the peaks against their corresponding retention times [43]. The plots follow a practically linear behaviour:

$$A = m_A\, t_R + A_0 \tag{4.10}$$

$$B = m_A\, t_R + B_0 \tag{4.11}$$

where m_A and m_B are the slopes of the correlations of the left and right half-widths, respectively, and A_0 and B_0 the corresponding intercepts, which include the extra-column contribution to peak broadening. To avoid the baseline noise, the peaks were measured at 10 % peak height. The sum of the slopes (m_A + m_B) represents the broadening rate of the peaks at increasing retention times, while the m_B/m_A ratio represents the asymmetry of the peaks at times where the extra-column contribution is not significant.

The plots in Figure 4.14 were built with the peak data obtained for the whole set of mobile phases, for parabens and β-adrenoceptor antagonists, in either MLC or MELC modes. Symmetric peaks were obtained in both cases, indicating that the capability of SDS as silanophilic suppressor in MLC is maintained in MELC.

The values of the parameters that define the half-width plots are given in Table 4.4. As observed, the successive addition of octane did not yield a significant change in the peak profiles, for both types of compounds. There is only a small increase in width (m_A + m_B), especially for the β-adrenoceptor antagonists, but the symmetry improves. MELC has also the advantage of reducing the analysis times, compared to MLC. In order to appraise the enhancement in the peak profiles of the β-adrenoceptor antagonists, when using micellar mobile phases and MEs (thanks to the presence of SDS adsorbed on the stationary phase), it is convenient to compare the asymmetry and width parameters with those obtained in conventional RPLC with a water/acetonitrile mixture (m_A+ m_B = 0.114 and m_B/m_A = 2.90 [44]). These values indicate that the peaks are much broader and asymmetric when hydro-organic mobile phases are used.

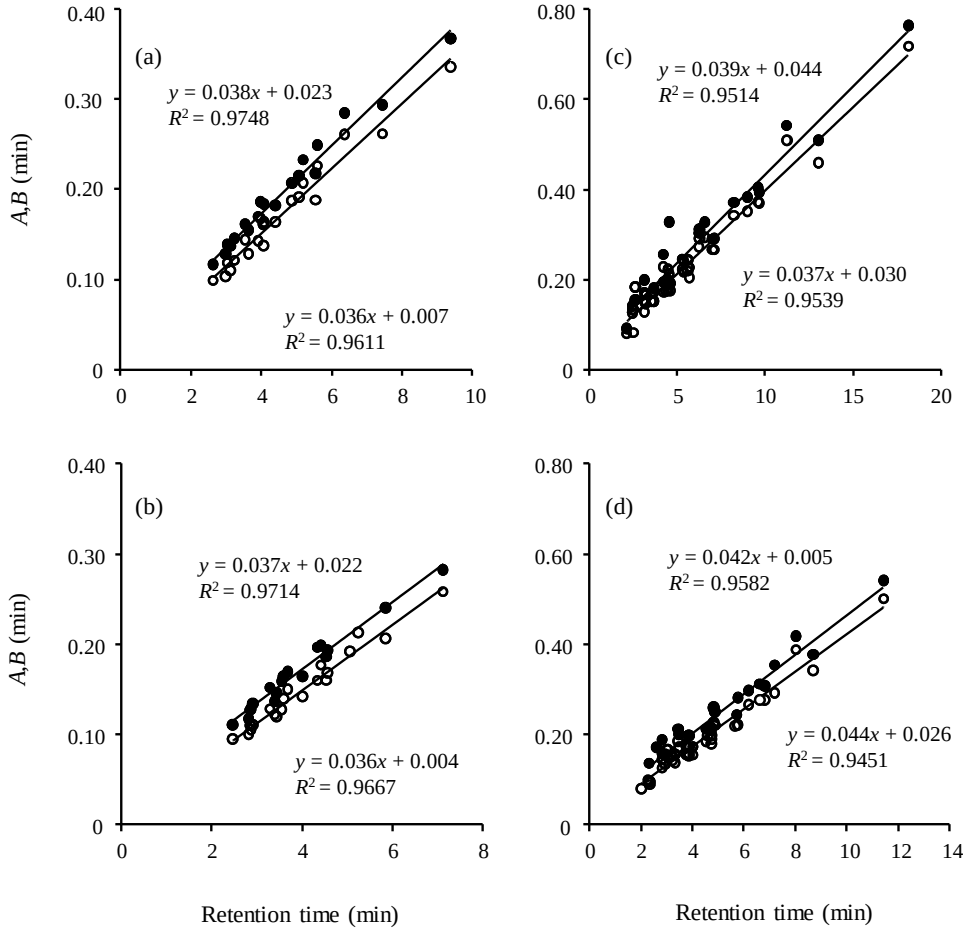


Figure 4.14. Half-width plots (left, *A* (○) and right, *B* (●)), built with the data obtained for all mobile phases used in: (a and c) MLC with SDS and 1-butanol in the 0.10–0.18 M and 5–12 % intervals, respectively, and (b and d) MELC with 0.5 % octane. Compounds: (a and b) parabens, and (c and d) β-adreno-ceptor antagonists.

Table 4.4. Parameters for the regressed lines in the half-width plots for the peaks obtained in MLC and MELC, for parabens and β-adrenoceptor antagonists.

	Octane (%, *v*/*v*)	m_A	m_B	$m_A + m_B$	m_B/m_A
			Parabens		
MLC	–	0.0361	0.0376	0.074	1.042
MELC	0.25	0.0347	0.0364	0.071	1.049
	0.5	0.0363	0.0374	0.074	1.030
	1.0	0.0394	0.0408	0.080	1.036
			β-Adrenoceptor antagonists		
MLC	–	0.0368	0.0389	0.076	1.057
MELC	0.25	0.0382	0.0397	0.078	1.039
	0.5	0.0418	0.0438	0.086	1.048
	1.0	0.0473	0.0490	0.096	1.036

4.5.5. Modelling the retention behaviour in MELC and MLC

Figure 4.15 illustrates how the change in concentration of each modifier (SDS, octane and 1-butanol), keeping fixed the other two components in the ME, affects the inverse of the retention factor. The assayed mobile phases were:

- 0.11 M, 0.12 M, 0.14 M, 0.16 M and 0.17 M SDS, in the presence of 1.14 % octane and 8.2 % 1-butanol (Figure 4.15a)
- 0.28 %, 0.57 %, 0.85 %, 1.14 % and 1.28 % octane, in the presence of 0.11 M SDS and 8.2 % 1-butanol (Figure 4.15b)
- 8.2 %, 9.9 %, 12.4 % and 14.8 % 1-butanol, in the presence of 0.11 M SDS and 1.14 % octane (Figure 4.15c)

As observed, the linearity was met for the three modifiers, as follows:

$$\frac{1}{k} = a + bC \tag{4.12}$$

where C is the concentration of each modifier. Based on the observation of the data in Figure 4.15, and considering that the behaviour in MELC should be similar to that described in Equation (4.6), the following model is proposed to describe the retention in MELC, in the three-factor space:

$$k = \frac{1}{c_0 + c_1\,\mu + c_2\,\varphi + c_3\,\phi + c_{12}\,\mu\varphi + c_{23}\,\phi\varphi} \tag{4.13}$$

where μ, φ and ϕ refer to the concentrations of surfactant, co-surfactant and oil.

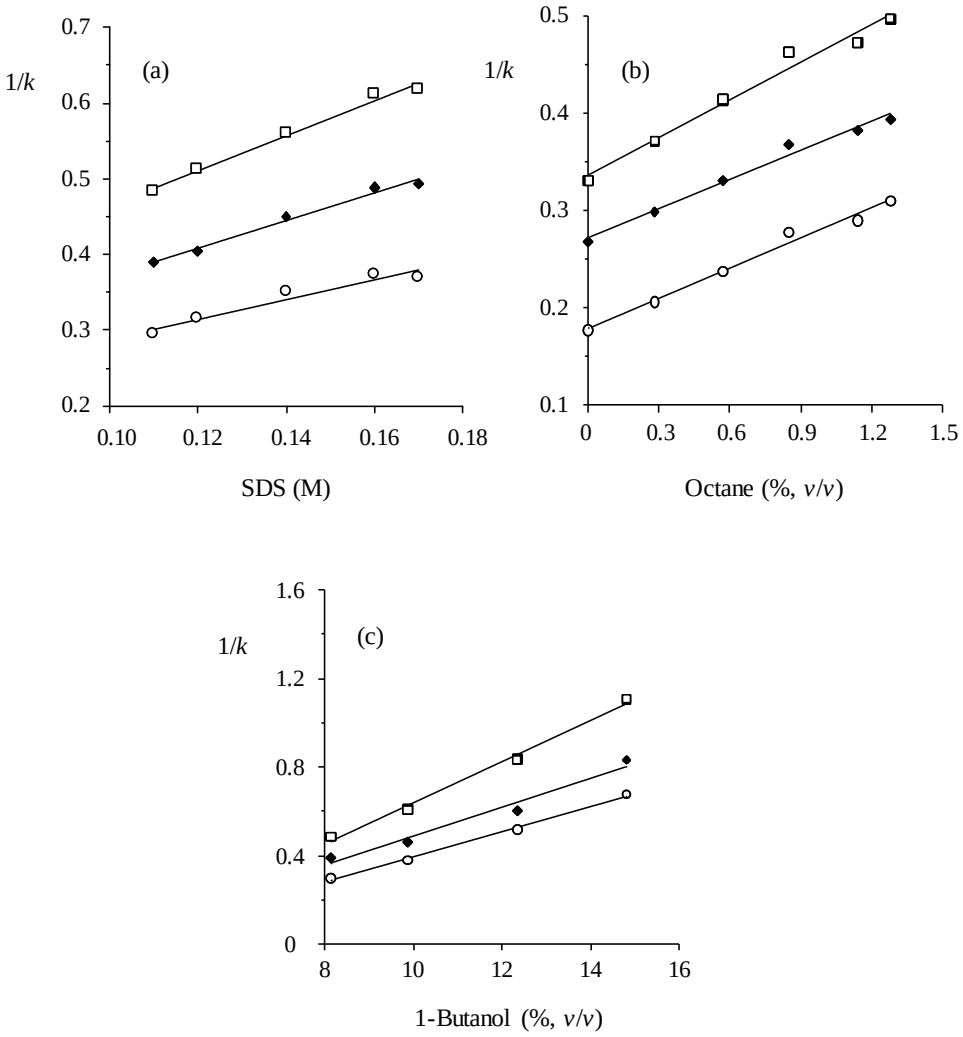


Figure 4.15. Dependence of the reversed retention factors on the concentration of the three reagents in the ME, for: (a) SDS in the presence of 1.14 % octane and 8.2 % 1-butanol, (b) octane in the presence of 0.11 M SDS and 8.2 % 1-butanol, and (c) 1-butanol in the presence of 0.11 M SDS and 1.14 % octane. Compounds: (◆) butylparaben, (○) propranolol, and (□) metoprolol.

In order to facilitate the evaluation of the interaction coefficients between the three modifiers (c_{12} and c_{23} in Equation (4.13)), a new experimental design was prepared (shown in Table 4.3), constituted of 15 mobile phases. In this design, three SDS concentration levels (0.10 M, 0.14 M and 0.18 M) were examined, and for each level, one or two concentrations of 1-butanol (among the following: 5 %, 7 %, 8.5 % and 12 %), all at three octane concentrations (0.25 %, 0.50 % and 1.0 %). The fitted retention factors were the mean values obtained from duplicate injections.

Model performance, in each chromatographic mode, was evaluated by non-linear least squares fitting of the difference between the experimental retention times and the retention times predicted by each model:

$$\chi = \sum_{i=1}^{N} \left(k_{i,\mathrm{exp}} - k_{i,\mathrm{pred}} \right)^2 \tag{4.14}$$

where N is the number of experimental points, and $k_{i,\mathrm{exp}}$ and $k_{i,\mathrm{pred}}$ the experimental and predicted retention factors, respectively. The fitting quality was evaluated through the determination coefficient R^2, as follows:

$$R^2 = 1 - \frac{\sum_{i=1}^{N} \left(k_{i,\mathrm{exp}} - k_{i,\mathrm{pred}} \right)^2}{\sum_{i=1}^{N} \left(k_{i,\mathrm{exp}} - k_{i,\mathrm{mean}} \right)^2} \tag{4.15}$$

$k_{i,\mathrm{mean}}$ being the mean value of the experimental retention factors.

The mean relative fitting error was obtained as:

$$E_{\mathrm{r}}(\%) = \frac{\sum_{i=1}^{N} \left| k_{i,\mathrm{exp}} - k_{i,\mathrm{pred}} \right|}{\sum_{i=1}^{N} k_{i,\mathrm{pred}}} \times 100 \qquad (4.16)$$

All calculations were carried out with the Microsoft Excel Solver application. The convergence process was problematic when fitting the experimental data to Equation (4.13), since it required initial values very close to the optimum to succeed. To solve this problem, the equation was transformed moving the origin to the mobile phase that showed maximal retention (the phase with the smallest elution strength). In previous reports [45,46], the advantage of this transformation has been demonstrated. It makes the non-linear fitting of the experimental data much easier. The final fitted equation was as follows:

$$k = \frac{k_0}{1 + S_{\mu}\Delta\mu + S_{\varphi}\Delta\varphi + S_{\phi}\Delta\phi + S_{\mu\varphi}\Delta\mu\,\Delta\varphi + S_{\phi\varphi}\Delta\phi\,\Delta\varphi} \qquad (4.17)$$

The parameters S_{μ}, S_{φ} and S_{ϕ} in Equation (4.17) measure the elution strength, and $S_{\mu\varphi}$ and $S_{\phi\varphi}$ are interaction coefficients. Instead of the absolute value of each factor, the equation contains the difference between each experimental concentration and the value corresponding to the mobile phase with the smallest elution strength, which was taken as reference (in our study: μ_0 = 10 cM: φ_0 = 5 % and ϕ_0 = 0):

$$\Delta\mu = \mu - \mu_0 = \mu - 10 \qquad (4.18)$$

$$\Delta\varphi = \varphi - \varphi_0 = \varphi - 5 \qquad (4.19)$$

$$\Delta\phi = \phi - \phi_0 = \phi \qquad (4.20)$$

Operating in this way, k_0 in Equation (4.17) was perfectly defined, which benefited the convergence and reliability of the fitting. In order to make the concentration ranges for the three ME components more similar, centimolar concentrations were used for SDS, and *v*/*v* percentages for the two organic solvents (octane and 1-butanol). This facilitated the interpretation of the coefficients.

Table 4.5 shows the model parameters and performance of the fitting of the experimental data to Equation (4.17). As observed, the fittings were very satisfactory, with relative fitting errors in the 1.1–2.5 % range. It can be seen that the influence of each modifier on the elution strength is very similar for all probe compounds, with mean values of 0.072 ± 0.017, 0.119 ± 0.045, and 0.98 ± 0.20 for S_{μ}, S_{φ} and S_{ϕ}, respectively. Therefore, octane has the highest elution strength, appreciably above that of SDS and 1-butanol. The influence of the interaction constants ($S_{\mu\varphi}$ and $S_{\phi\varphi}$) was minor, although still significant.

Table 4.5. Fitting of the experimental data in MELC to Equation (4.17).

Compound	k_0	S_μ	S_φ	S_ϕ	$S_{\mu\varphi}$	$S_{\phi\varphi}$	R^2	E_r (%)
Methylparaben	1.563 ± 0.037	0.0434 ± 0.0058	0.0636 ± 0.0083	0.686 ± 0.11	0.00353 ± 0.0016	0.00 ± 0.027	0.9646	1.10
Ethylparaben	2.458 ± 0.049	0.0557 ± 0.0054	0.0852 ± 0.0081	0.714 ± 0.096	0.00361 ± 0.0016	0.00 ± 0.025	0.9785	1.31
Propylparaben	3.559 ± 0.066	0.0617 ± 0.0053	0.1057 ± 0.0087	0.794 ± 0.097	0.00404 ± 0.0017	0.00 ± 0.027	0.9837	1.53
Butylparaben	4.657 ± 0.085	0.0636 ± 0.0053	0.1229 ± 0.0095	0.879 ± 0.10	0.0047 ± 0.0019	0.004 ± 0.029	0.9859	1.69
Propranolol	8.89 ± 0.19	0.0686 ± 0.0066	0.187 ± 0.016	1.29 ± 0.15	0.0069 ± 0.0034	0.130 ± 0.056	0.9864	2.53
Oxprenolol	6.32 ± 0.13	0.0699 ± 0.0066	0.170 ± 0.015	1.16 ± 0.14	0.0047 ± 0.0030	0.088 ± 0.049	0.9854	2.31
Atenolol	1.324 ± 0.081	0.104 ± 0.024	0.113 ± 0.030	1.06 ± 0.37	0.0010 ± 0.0068	0.00 ± 0.10	0.8647	2.42

Table 4.5 (continued).

Compound	k_0	S_μ	S_φ	S_ϕ	$S_{\mu\varphi}$	$S_{\phi\varphi}$	R^2	E_r (%)
Acebutolol	2.871 ± 0.072	0.0866 ± 0.0087	0.0690 ± 0.0091	0.98 ± 0.14	0.0035 ± 0.0023	0.00 ± 0.034	0.9711	1.96
Metoprolol	4.59 ± 0.10	0.0733 ± 0.0072	0.157 ± 0.014	1.06 ± 0.15	0.0040 ± 0.0029	0.046 ± 0.046	0.9817	2.23
Carteolol	1.729 ± 0.041	0.0900 ± 0.0085	0.0702 ± 0.0087	1.04 ± 0.14	0.0049 ± 0.0023	0.00 ± 0.034	0.9754	1.53
Timolol	4.489 ± 0.089	0.0698 ± 0.0062	0.167 ± 0.013	1.16 ± 0.13	0.0059 ± 0.0028	0.055 ± 0.044	0.9867	1.97
Mean value	–	0.072 ± 0.017	0.119 ± 0.045	0.98 ± 0.20	0.0043 ± 0.0015	0.029 ± 0.045		

To observe better the influence of each variable on the retention, it is convenient to rewrite Equation (4.17). Dividing numerator and denominator by $(1 + S_\varphi \Delta\varphi)$, and rearranging the terms:

$$k = \frac{\dfrac{k_0}{1+S_\varphi\Delta\varphi}}{\dfrac{1+S_\varphi\Delta\varphi}{1+S_\varphi\Delta\varphi} + \dfrac{S_\mu\Delta\mu + S_{\mu\varphi}\Delta\mu\Delta\varphi}{1+S_\varphi\Delta\varphi} + \dfrac{S_\phi\Delta\phi + S_{\phi\varphi}\Delta\phi\Delta\varphi}{1+S_\varphi\Delta\varphi}} \tag{4.21}$$

Equation (4.21) yields to:

$$k = \frac{\dfrac{k_0}{1+S_\varphi\Delta\varphi}}{1 + \dfrac{1+K_{\mu\varphi}\Delta\varphi}{1+S_\varphi\Delta\varphi} S_\mu\Delta\mu + \dfrac{1+K_{\phi\varphi}\Delta\varphi}{1+S_\varphi\Delta\varphi} S_\phi\Delta\phi} \tag{4.22}$$

where $K_{\mu\varphi} = S_{\mu\varphi} / S_\mu$ and $K_{\phi\varphi} = S_{\phi\varphi} / S_\phi$. In Equation (4.22), both surfactant and octane follow the same pattern (compare with Equation (4.2)), while 1-butanol is a modifier that conditions the equilibrium constants of the solute with the stationary phase and micelles (compare with Equation (4.4)).

Finally, with comparative purposes, the data obtained in MLC with mobile phases containing SDS and 1-butanol (without octane), and the same column, were fitted using the classical experimental design of five mobile phases [25], with 0.10 M, 0.14 M and 0.18 M SDS, each level at one or two concentrations of 1-butanol (among the following: 5 %, 8.5 % and 12 %) (see Table 4.3). The following equation was used (compare with Equation (4.6)):

$$k = \frac{k_0}{1 + S_\mu\Delta\mu + S_\varphi\Delta\varphi + S_{\mu\varphi}\Delta\mu\Delta\varphi} \tag{4.23}$$

Table 4.6. Fitting of the experimental data in MLC to Equation (4.23).

Compound	k_0	S_μ	S_φ	$S_{\mu\varphi}$	R^2	E_r (%)
Methylparaben	1.814 ± 0.010	0.0564 ± 0.0018	0.0980 ± 0.0027	0.0008 ± 0.0006	0.99959	0.43
Ethylparaben	2.841 ± 0.053	0.0674 ± 0.0066	0.120 ± 0.010	0.0024 ± 0.0025	0.9965	1.49
Propylparaben	4.161 ± 0.094	0.0738 ± 0.0084	0.143 ± 0.014	0.0030 ± 0.0035	0.9955	1.87
Butylparaben	5.502 ± 0.055	0.0762 ± 0.0038	0.1673 ± 0.0073	0.0049 ± 0.0018	0.99923	0.86
Propranolol	11.59 ± 0.32	0.087 ± 0.012	0.267 ± 0.033	0.0073 ± 0.0081	0.9956	2.66
Oxprenolol	8.03 ± 0.25	0.087 ± 0.013	0.237 ± 0.032	0.0052 ± 0.0079	0.9939	2.93
Atenolol	1.532 ± 0.035	0.110 ± 0.011	0.155 ± 0.015	0.0030 ± 0.0042	0.9962	1.99

Table 4.6 (continued).

Compound	k_0	S_μ	S_φ	$S_{\mu\varphi}$	R^2	E_r (%)
Acebutolol	3.56 ± 0.13	0.103 ± 0.017	0.116 ± 0.019	0.0024 ± 0.0054	0.9887	3.03
Metoprolol	5.70 ± 0.20	0.089 ± 0.015	0.215 ± 0.032	0.0035 ± 0.0077	0.9921	3.18
Carteolol	2.151 ± 0.059	0.104 ± 0.013	0.119 ± 0.015	0.0041 ± 0.0044	0.9937	2.32
Timolol	5.65 ± 0.15	0.085 ± 0.011	0.232 ± 0.027	0.0058 ± 0.0067	0.9955	2.49
Mean value	–	0.086 ± 0.016	0.170 ± 0.058	0.0039 ± 0.0018		

Table 4.6 shows the model parameters for Equation (4.23) and the errors obtained when fitting the data in MLC. Relative fitting errors were in the 0.43–3.2 % range. As observed, the values of S_μ (elution strength for the surfactant), and the interaction term ($S_{\mu\varphi}$) were very similar in MELC and MLC, while S_φ (elution strength for 1-butanol) was significantly higher in MLC. Figure 4.16 depicts the accuracy of the predictions for both MLC and MELC.

4.6. Conclusions

The usefulness of MEs in liquid chromatography with mobile phases containing SDS, octane (as oil), and 1-butanol (as co-surfactant) has been once more demonstrated. The three modifiers give rise to a reduction in the retention times of solutes, when their concentration is increased. In MLC, pure micellar mobile phases (i.e., without organic solvent) provide too long retention times for parabens and β-adrenoceptor antagonists, to be practical in analysis. To obtain sufficiently low retention times for these compounds, it is necessary to add an organic solvent with high elution strength, such as 1-butanol. In MELC, the analysis times of mixtures of parabens and β-adrenoceptor antagonists decreased further down to 4–5 min by addition of octane, which offered an elution strength stronger than 1-butanol. Thus, for example, the analysis time for parabens and β-adrenoceptor antagonists was 5 and 6.5 min, respectively, using a mobile phase containing 0.10 M SDS, 1 % octane and 7 % 1-butanol (MELC), similar to that obtained in MLC with 0.18 M SDS and 12 % 1-butanol (4.4 and 5.7 min).

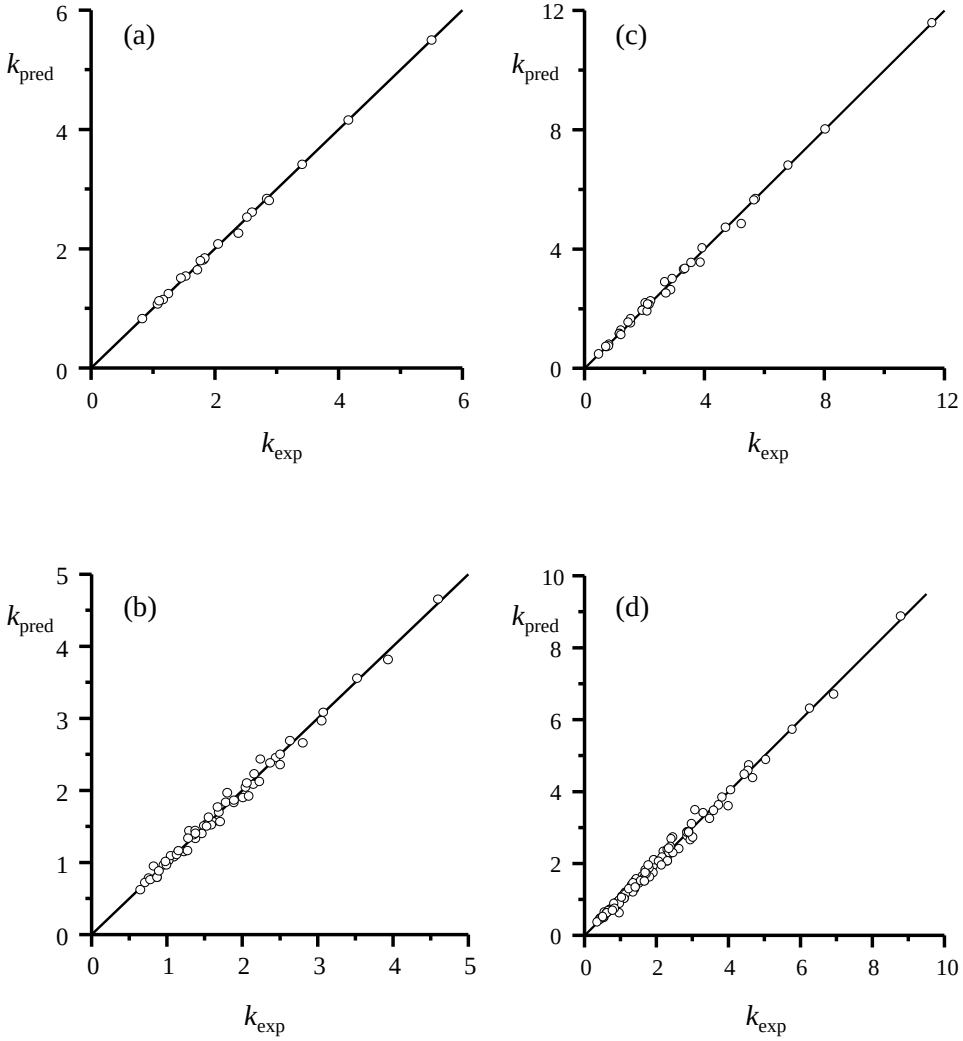


Figure 4.16. Accuracy of the predictions for: (a,c) MLC (Equation (4.23), 5 mobile phases) and (b,d) MELC (Equation (4.17), 15 mobile phases). Compounds: (a,b) Parabens, and (c,d) β-adrenoceptor antagonists.

Satisfactory peak profiles were obtained using MEs as mobile phases, which is especially important for the analysis of basic compounds, such as the studied β-adrenoceptor antagonists, which in conventional RPLC yield wide and asymmetric peaks. This means that MEs keep the feature of suppressing the silanol effect, observed previously in MLC with SDS as surfactant. The advantages of the reduced retention times and enhanced peak profiles must be added to the capability of MEs to dissolve compounds in a very wide range of polarities and allow the direct injection of biological samples for the analysis of non-polar compounds.

This chapter demonstrates the feasibility of modelling the retention in MELC, considering altogether the three components in the mobile phase, with very good accuracy (fitting errors below 2.5 %). The derived equations are similar to those used in MLC in the presence of an organic solvent (hybrid MLC). Modelling the retention is interesting for the optimisation of the best experimental conditions and also offers information on the retention mechanisms. The proposed model for MELC revealed that, when octane is inserted inside the micelle, this is modified. Therefore, the interactions between the solutes and the micelle are changed, as indicated by the values of the model parameters in both MLC and MELC.

Modelling of retention was preceded by a study of the range of concentrations of SDS, octane and 1-butanol that can be mixed to form stable MEs, avoiding the formation of an emulsion. An increase in the concentration of SDS and 1-butanol allowed a larger amount of octane be stabilised inside the micelles. The high number of runs carried out throughout this work has been possible due to the simplicity in the preparation of the MEs and the short analysis times using MEs as mobile phases.

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CHAPTER 5

ANALYSIS OF TRICYCLIC ANTIDEPRESSANTS IN PHARMACEUTICALS BY MICROEMULSION LIQUID CHROMATOGRAPHY

5.1. Abstract

Basic compounds yield long retention times and broad and asymmetric peaks in Reversed-Phase Liquid Chromatography, due to interaction with residual silanols in the columns. The addition of the anionic surfactant sodium dodecyl sulphate in the so called Micellar Liquid Chromatography can enhance the efficiency, but long retention is achieved because of electrostatic attraction of the cationic species to the sulphate group of this surfactant. This forces the addition of a strong organic solvent to get appropriate analysis times. An alternative is the use of a microemulsion (ME), formed by mixing surfactant, oil and an alcohol as co-surfactant. Association of hydrophobic compounds with the oil droplets increases the elution strength, which is translated in short retention. The advantages of using MEs in the analysis of tricyclic antidepressants, compared to the use of hydro-organic mixtures and micellar mobile phases, are here studied. A method with a ME containing 0.173 M SDS, 1.42 % (*v*/*v*) octane and 8.15 % (*v*/*v*) 1-butanol was developed and validated for the analysis of amitryptiline, clomipramine, imipramine, maprotiline and nortryptiline in pharmaceutical formulations. Satisfactory results were obtained, with intra- and inter-day precisions below 2.5 %, and intra- and inter-day accuracy between -1.7 % and 1.2 %. Good recoveries were obtained with simple sample preparation.

5.2. Introduction

Tricyclic antidepressants (TCAs) are drugs usually prescribed for the treatment of depressive disorders, due to its efficiency in changing the mood of the patients, even with children, teenagers, and prenatal women [1,2]. The importance of these drugs because of its wide use, and possible secondary effects, makes their analysis using simple and practical analytical techniques, such as Reversed-Phase Liquid Chromatography (RPLC), necessary.

The molecules of TCAs contain three rings and an amino group that confers them a basic character, with pK_a values in the 9.0–9.7 range. This is the reason of the broad and asymmetric peaks obtained in conventional RPLC, due to their interaction with the residual silanols present in the C18 columns [3]. Moreover, these compounds have low polarity, with log $P_{o/w}$ values between 3.9 and 5.3 [4]. All this yields long retention, forcing the addition of a high amount of organic solvent in the mobile phase to get practical analysis times. In order to solve both problems (long retention and broad and asymmetric peaks), our research group has suggested the addition of different types of reagents to the mobile phase, such as surfactants and ionic liquids [5–7].

Sodium dodecyl sulphate (SDS) is added above its critical micellar concentration (CMC) in the so-called Micellar Liquid Chromatography (MLC), where it acts as silanol suppressor in the analysis of basic compounds [8]. Monomers of surfactant adsorbed onto the stationary phase, with the sulphate group oriented away from its surface, modifies the retention behaviour, which is modulated by the micelles formed in the mobile phase. On the other hand, the formation of a pseudo-stationary phase, with adsorbed surfactant, masks the residual silanols, hindering the access of basic compounds, which enhances the peak profiles. However, the sulphate group confers a negative charge to the

modified stationary phase, which is translated into an increased retention owing to the interaction with the cationic species of the basic compounds. To overcome this problem, an organic solvent of high elution strength, such as 1-butanol or 1-pentanol, is needed [5].

In order to minimise the high demand of organic solvent needed with SDS in the analysis of basic compounds, the suitability of pure micellar mobile phases of the non-ionic surfactant Brij-35 (with a neutral character) was also investigated [6]. We report here another alternative to get practical retention times for TCAs: the use of a microemulsion (ME) in the mobile phase, in the so-called Microemulsion Liquid Chromatography (MELC).

MEs are transparent colloidal solutions, thermodynamically stable, where water and a non-polar solvent can coexist thanks to the presence of a surfactant. A co-surfactant (an organic solvent, such as 1-butanol or 1-pentanol) is usually added in order to stabilise the oil droplets [9–13]. In MELC, the separation performance can be modulated by changing the nature and concentration of surfactant, oil and co-surfactant. Most reported applications in MELC refer to the analysis of drugs in pharmaceuticals [14–18], physiological fluids, and other biological materials [19–21].

The aim of this work is investigating the suitability of the MELC mode for the analysis of five TCAs in pharmaceutical formulations. The optimisation of the concentration of the components in the mobile phase was carried out by examining the changes in retention and peak profile with the concentration of SDS, octane and 1-butanol. Based on these results, a procedure using a ME containing 0.173 M SDS, 1.42 % (*v*/*v*) octane and 8.15 % (*v*/*v*) 1-butanol, as mobile phase, was developed and validated according to the International Conference of Harmonization (ICH) Guideline [22]. In order to evaluate the advantages of using this chromatographic mode, the results were compared with

those obtained in previous reports using a hydro-organic mixture (with acetonitrile) [5], and a micellar medium with SDS / 1-pentanol, or Brij-35 [6], as mobile phases. The advantages of using MELC are discussed.

5.3. Experimental

5.3.1. Reagents

Stock solutions of approximately 500 mg/mL of the TCAs amitryptiline, clomipramine, imipramine, maprotiline and nortryptiline from Sigma (St. Louis, MO, USA) were prepared by adding 1 mL of methanol from VWR International (France), and water, and sonicating with an Elmasonic S 15-H ultrasonic bath from Elma (Singen, Germany). The solutions were stable during at least two months at 4 ºC. In order to optimise the experimental conditions, these solutions were diluted with water to get a concentration of 20 µg/mL for the injected solutions. Uracil from Acros Organics (Geel, Belgium) was used as dead time marker.

The ME used as mobile phase was prepared with SDS (99 % purity) from Merck (Darmstadt, Germany), octane from Alfa Aesar (Kandel, Germany), 1-butanol from Scharlab (Barcelona), and 0.05 % trifluoroacetic acid from Fisher Scientific (UK) to get an acidic medium (pH = 1.3). This pH guaranteed the protonation of silanol groups, which reduced their interaction with the amine groups of TCAs and enhanced the efficiency. The reagents were mixed and the mixture was allowed to stand for at least 12 h. When it did not initially give rise to two well differentiated phases, it was left several weeks at rest to assure the ME stability.

The drug solutions and mobile phases were filtered through 0.45 µm Nylon membranes from Micron Separations (Westboro, MA, USA) and degassed in an

ultrasonic bath. Nanopure water from Barnstead Sybron (Boston, MA, USA) was used throughout.

5.3.2. Apparatus and columns

An Agilent instrument (Waldbronn, Germany) was used, equipped with quaternary pump (Series 1200), an autosampler (Series 1260 Infinity II), thermostated column compartment (Series 1290 Infinity II) set at 20 °C, diode array detector (Series 1100), and HPChemstation (Agilent, C.01.07) for data acquisition. The signal was monitored at 254 nm, except for maprotiline, which was detected at 278 nm. Uracil was detected at 254 nm. The chromatographic peaks were integrated with MICHROM [23].

An XTerra MS C18 column from Waters (Milford, MA, USA), with a useable 1-12 pH range and the following characteristics was used: 150 mm × 4.6 mm i.d, 5 µm particle size, 15.2 % total carbon content, 177 m^2/g surface area and 127 Å average pore diameter. The flow-rate was set at 1 mL/min. Duplicate injections of 20 µL were made. A small flow rate of 0.2 mL/min was used overnight to avoid daily cleaning and re-equilibration of the column. Recycling the mobile phase through the chromatographic system reduced reagent consumption and wastes. When required, column cleaning was done with a mixture of 50:50 pure water and methanol to remove the absorbed surfactant on the stationary phase. During weekend, the column was kept with methanol.

5.3.3. Procedure

The pharmaceuticals were commercialised as tablets. The average weight per tablet was calculated from 10 units. The contents were ground and reduced to a homogeneous fine powder in a mortar. The appropriate amount of powder to get around 65 μg/mL of the drugs was taken and sonicated in the presence of approximately 1 mL of methanol, which was enough to facilitate the solution of the active ingredient. Dilution was made with water. The excipients were not soluble in the assayed media, hence the sample solutions should be filtered through 0.45 μm Nylon membranes, before injection into the chromatograph. The reproducibility assays indicated a high recovery of the drugs.

5.4. Results and discussion

5.4.1. Influence of the mobile phase composition on the retention behaviour of TCAs

The selection of the mobile phase composition in MELC is rather laborious, due to the complexity of the ME nature. The choice of surfactant, oil and co-surfactant is important to form a ME, instead of an emulsion, and get appropriate retention for the analytes. The concentration of the three components have also relevance in the ME formation [24]. Variation of the concentration of the components allows the modulation of the retention behaviour, but out of the adequate range, the ME cannot be formed or will not be even stable (the separation of two phases is visually detected, or in case a clear solution is obtained, the retention times are not reproducible), or even the back-pressure will be too high.

In previous work, several authors investigated the most appropriate reagents for MELC [13,24]. The most useful surfactant is SDS, which has also been extensively studied in MLC. 1-Butanol is often recommended as co-surfactant. In fact, in the MELC literature, a ME prepared with 0.114 M SDS, 1.14% octane, 8.15% 1-butanol, and 0.05% trifluoroacetic acid (called "standard ME") is highly recommended, as starting point, when developing a method for a new separation with no previous reports. Based on these recommendations, we carried out a detail study of the chromatographic behaviour of β-adrenoceptor antagonists, which are basic compounds appreciably more polar than TCAs [25]. The assayed mobile phases were obtained by varying the concentrations of SDS, octane, and 1-butanol in the "standard ME", to get appropriate retention and column back-pressure, and ensure the stability of MEs. We thought that a similar MELC mobile phase could be useful for TCAs which, in RPLC with hydro-organic mixtures, yield very high retention.

Figure 5.1 shows the retention behaviour for the five TCAs eluted with mobile phases containing different concentrations of the three reagents (SDS, octane and 1-butanol). The surfactant has a remarkable effect on the selectivity of the method, due to its capability to modify the stationary phase by coating. It also allows the formation of oil droplets in the mobile phase with a remarkable effect on the retention of the analytes. It was found that the retention times of the TCAs decreased upon increasing the SDS concentration from 0.104 to 0.208 M, M, with a minor effect above 0.173 M (Figure 5.1a). For this reason, this concentration was selected to perform the analysis.

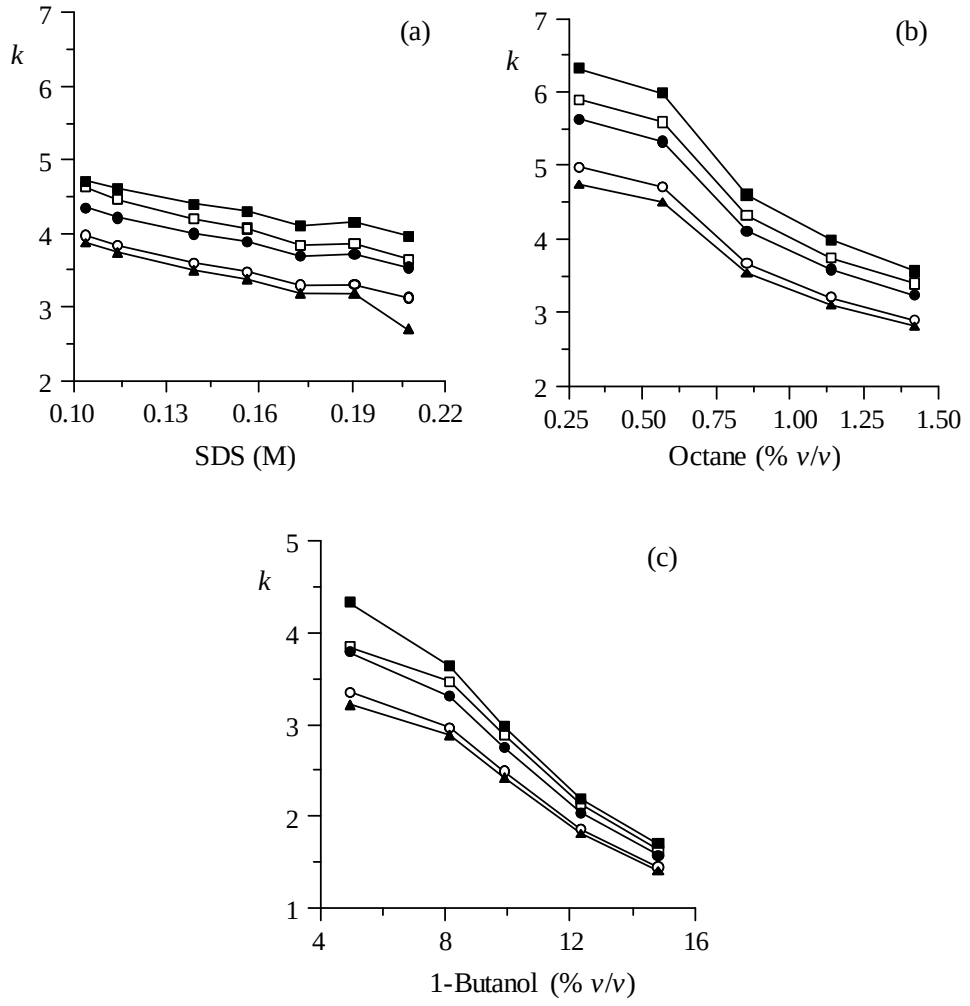

Figure 5.1. Effect on retention in MELC of increasing concentration of: (a) SDS, (b) octane, and (c) 1-butanol. SDS concentration in (b) and (c) was 0.173 M, octane concentration (*v*/*v*) in (a) and (c) was 1.14 % and 1.42 %, respectively, and 1-butanol concentration (*v*/*v*) in (a) and (b) was 8.15 %. Solute identity: (▲) imipramine, (○) amitryptiline, (●) nortryptiline, (□) clomipramine, and (■) maprotiline.

Figure 5.1b depicts the change in retention at increasing octane concentration, in the 0.28–1.42 % (*v*/*v*) range. This range was selected to ensure that, with the lowest concentration, a stable microemulsion was formed, and with the highest, micelles would not breakdown. A decrease in the retention times was observed, which can be explained by the higher interactions between the TCAs (which are lipophilic) and the oil droplets. A concentration of 1.42 % (*v*/*v*) octane was selected to perform the analysis of the pharmaceutical formulations, since it provided short retention times, still guaranteeing the formation of oil droplets. A higher concentration of octane would be detrimental for the ME stability.

The co-surfactant (1-butanol) also played an important role in the formation of oil droplets, and therefore, in the stability of the ME. To obtain the optimal concentration of 1-butanol, the 4.9–14.8 % (*v*/*v*) range was investigated (Figure 5.1c). It was found that an increase in the concentration of 1-butanol reduced significantly the retention times of TCAs, with a similar relative effect similar to octane. Despite the benefit that the use of a higher concentration of 1-butanol could bring over the solubilisation of a higher octane concentration and reduction of retention, concentrations above 15 % (*v*/*v*) 1-butanol were not possible due to the high back-pressure. A ME with 8.15 % (*v*/*v*) 1-butanol gave rise to sufficiently short retention times, being selected for the analysis of the pharmaceuticals. The inter-day reproducibility studies in Section 5.4.4 indicated the formation of a stable ME along weeks.

5.4.2. Advantage of MELC vs MLC

The incorporation of an oil to a micellar system, in MELC, forms oil droplets from which hydrophobic compounds undergo partitioning to the modified stationary phase. The presence of this new interaction allows the reduction of retention times with regard to MLC, due to the enhanced solving effect of the oil on such compounds.

In this work, mobile phases with the same surfactant and co-surfactant contents (0.173 M SDS and 8.15 % (*v*/*v*) 1-butanol) were prepared in MLC and MELC, with the purpose of observing the effect on retention of the addition of an oil, using the same column (XTerra C18). Table 5.1 indicates the changes in the retention times, for the TCAs under study, at increasing concentration of octane (*v*/*v*) from 0 % (MLC) to 0.28 %, and further to 1.42 % (*v*/*v*) (MELC). As can be seen, the transition from MLC to MELC with only 0.28 % (*v*/*v*) yields a reduction of approximately 2.5 min in the case of the most retained compounds (clomipramine and maprotiline). A higher concentration of octane (1.42 % (*v*/*v*)) had a more significant effect on the retention times, which decreased to half those found in MLC.

Table 5.1. Effect of the addition of oil on retention times (min).[a]

Compound	MLC	MELC	
	0 % octane	0.28 % octane	1.42 % octane
Amitryptiline	10.23	8.39	5.47
Clomipramine	12.22	9.68	6.16
Imipramine	9.81	8.06	5.35
Maprotiline	12.76	10.27	6.40
Nortryptiline	11.58	9.31	5.94

[a] In all cases, an XTerra C18 column was used, and the mobile phase contained 0.173 M SDS and 8.15% (*v*/*v*) 1-butanol, without or with octane.

5.4.3. Peak profiles

Changes in efficiency and asymmetry were evaluated assisted by the construction of plots that represent the left (*A*) and right (*B*) half-widths of the chromatographic peaks, for the group of TCAs injected at similar concentration, versus the retention time for each compound. The plots in Figure 5.2 illustrate the peak profiles in the chromatograms for the TCAs considered in this study.

The plots follow an almost linear behaviour [26]:

$$A = m_A t_R + A_0 \quad (5.1)$$

$$B = m_B t_R + B_0 \quad (5.2)$$

where, m_A and m_B are the slopes of the correlations for the left and right half-widths, and A_0 and B_0 the corresponding intercepts, which include the extra column contribution to peak broadening. In this work, the half-widths were measured at 10 % peak height to avoid the baseline noise. The sum of the slopes ($m_A + m_B$) represents the peak broadening rate as the analytes travel along the column, whereas the m_B/m_A ratio indicates the asymmetry of peaks that elute at a time where the extra-column contribution is non-significant.

Basic compounds, such as TCAs, interact with residual silanols in the silica stationary phases, giving rise to broad and asymmetric peaks in RPLC with conventional C18 columns. However, the addition of surfactants of different nature to the mobile phase in the so called MLC, has been shown to have an effective silanol suppressor effect [25,26]. Surfactants are adsorbed on the stationary phase making the access of basic compounds to residual silanols difficult. This enhances the peak profile as obtained in RPLC.

The half-width plots in MELC are compared in Figure 5.2 with those obtained in previous reports, where TCAs were analysed using conventional RPLC with 35 % acetonitrile (Figure 5.2a), and with MLC using SDS (Figure 5.2b) and Brij-35 (Figure 5.2c) [5,6]. The results in MELC are depicted in Figure 5.2d. In order to achieve better peak profiles in conventional RPLC (Figure 5.2a), a special column with low concentration of silanols was used (an XTerra C18, also used in this work for MELC, see Section 5.3.2).

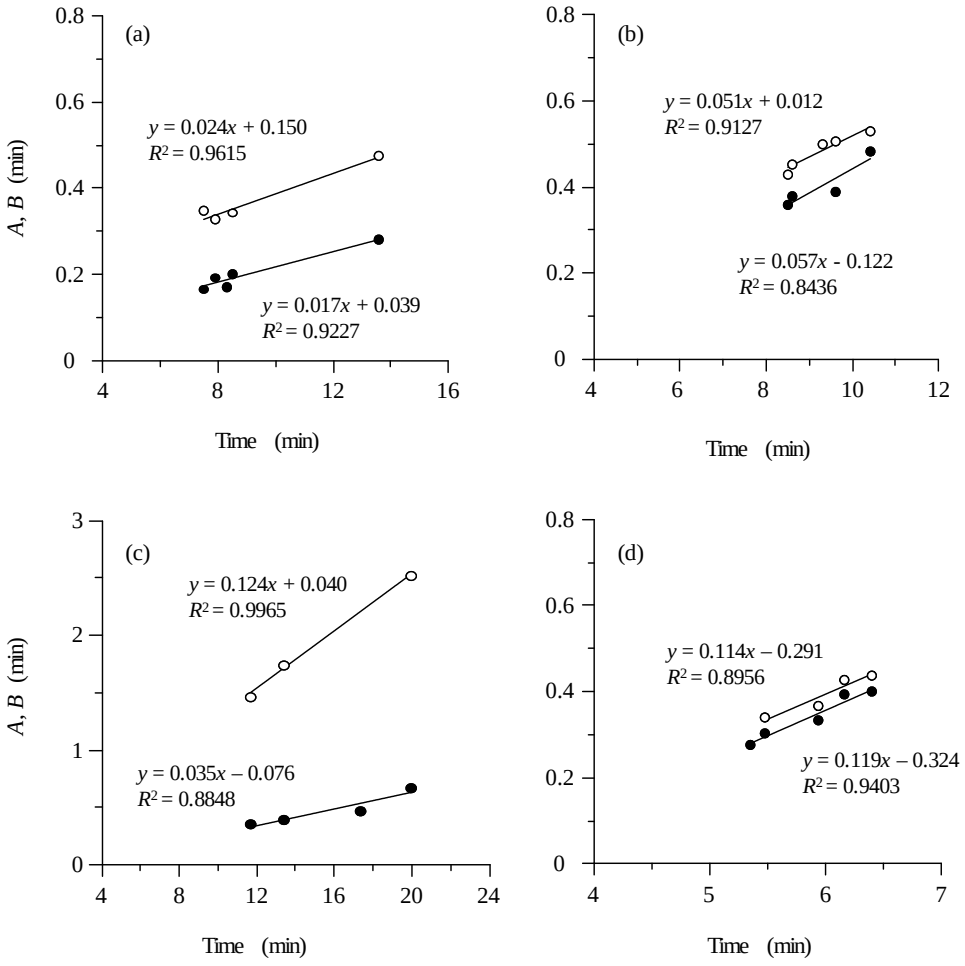

Figure 5.2. Half-width plots (left, *A* (●) and right, *B* (○)) for the TCAs, eluted with: (a) 35 % (*v*/*v*) acetonitrile (XTerra C18 column), (b) 0.072 M SDS / 6% (*v*/*v*) 1-pentanol (C8 column), (c) 0.02 M Brij-35 (C18 column), and (d) 0.173 M SDS / 1.42 % (*v*/*v*) octane / 8.15 % (*v*/*v*) 1-butanol (XTerra C18 column).

The high retention times in MLC with SDS using a C18 column were decreased to adequate values (preserving the peak profiles), by using a C8 column (Eclipse XDB from Agilent, 150 mm × 4.6 mm i.d, 5 µm particle size) and a small amount of an organic solvent with strong elution strength (6 % (*v*/*v*) 1-pentanol) in the mobile phase (Figure 5.2b). In contrast, the neutral surfactant Brij-35 yielded short analysis times with a C18 column (Zorbax from Agilent, 150 mm × 4.6 mm i.d, 5 µm particle size). However, as shown in Figure 5.2c, Brij-35 without organic solvent yields poor peak profiles.

As can be observed in Figure 5.2d, the peaks obtained in MELC with a C18 column are symmetric, which confirms that the silanol suppressing effect of this surfactant in MLC is kept. Also, the addition of 1.42 % (*v*/*v*) octane was translated into a significant decrease in retention with regard to MLC with SDS (compare with Figure 5.2b), keeping narrow and symmetrical peaks. This indicates that MELC is a good solution to the high demand of organic solvent needed in MLC with SDS, or the need of a column with shorter alkyl chain length with this surfactant.

5.4.4. Method validation

The selected mobile phase in MELC contained 0.173 M SDS, 1.42 % (*v*/*v*) octane and 8.15 % (*v*/*v*) 1-butanol. Method validation with this mobile phase was carried out following the recommendations of the ICH Guideline [22]. The validation parameters evaluated were the linearity of calibration curves, accuracy, precision, robustness, and limits of detection (LOD) and quantification (LOQ).

Calibration curves were built by plotting the chromatographic peak areas for each TCA versus itsconcentration, obtained from duplicate injections of standard solutions at five concentrations in the 50–80 μg/mL range, uniformly distributed. The working solutions were obtained from the stock aqueous solutions by dilution with water, and renewed weekly. The calibration parameters (slope and intercept of the fitted straight-lines) were obtained for runs carried out during three non-consecutive days along three different weeks. The parameters of the calibration straight-lines are given in Table 5.2. As observed, all calibration curves met the linearity requirements, with determination coefficients usually $R^2 > 0.990$. The slopes and intercepts were stable throughout the validation process, which indicates a high prediction capability of the concentrations of the analytes from the fitted regression straight-lines, and the fact that the chromatographic column and mobile phase performance were maintained.

The intra- and inter-day reproducibilities were studied by measuring the signals of the chromatographic peaks of each TCA from solutions at three concentrations, inside the linear range of the calibration curves (50, 60 and 80 μg/mL). The measurements were made along three non-consecutive days, in the same week (inter-day reproducibility), making six replicates each day (intra-day reproducibility). Table 5.3 summarises the precision of the method expressed as the relative standard deviation (RSD), and its accuracy expressed as the relative error (relative difference between the values found from the calibration straight-line and the concentration of the standards). The intra- and inter-day precisions were always below 2.5 %, and the intra- and inter-day accuracies ranged from -1.7% (amitryptiline) to 1.2 % (clomipramine).

Table 5.2. Day-to-day calibration parameters obtained for the TCAs.

Compound		SDS / octane / 1-butanol		
		Slope	Intercept	R^2
Amitryptiline	[a]	0.536 ± 0.011	-2.12 ± 0.15	0.9676
	[b]	0.520 ± 0.007	-1.3 ± 0.7	0.9845
Clomipramine	[a]	0.41 ± 0.03	-0.2 ± 1.6	0.9992
	[b]	0.438 ± 0.009	-1.9 ± 0.7	0.9921
Imipramine	[a]	0.4994 ± 0.0011	-0.6 ± 0.3	0.9997
	[b]	0.502 ± 0.008	-0.6 ± 0.5	0.9995
Maprotiline	[a]	0.0344 ± 0.0007	-0.08 ± 0.04	0.9991
	[b]	0.0357 ± 0.0009	-0.16 ± 0.05	0.9911
Nortryptiline	[a]	0.52 ± 0.03	-1.1 ± 2.1	0.9997
	[b]	0.510 ± 0.011	-0.5 ± 1.1	0.9777

[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.

Table 5.3. Intra- and inter-day precision and accuracy for the MELC mode.

Compound	Added (µg/ml)	Intra-day [a]			Inter-day [b]		
		Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)	Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)
Amitryptiline	50.10	50.5 ± 0.6	0.4	0.8	50.57 ± 0.23	0.5	0.5
	65.13	65.0 ± 0.4	0.6	-0.6	64.3 ± 1.0	1.6	-1.7
	80.16	81.0 ± 0.3	0.4	0.6	80.0 ± 2.0	2.5	-0.6
Clomipramine	50.30	50.2 ± 0.8	1.6	0.2	50.3 ± 0.4	0.8	0.4
	65.39	64.4 ± 0.5	0.5	-0.8	64.2 ± 0.4	0.6	-1.4
	80.48	81.1 ± 0.9	1.1	1.2	80.0 ± 1.0	1.3	-0.2
Imipramine	50.10	50.4 ± 0.23	0.5	0.6	50.3 ± 0.4	0.8	0.4
	65.13	64.65 ± 0.24	0.4	-0.7	64.9 ± 0.4	0.6	-0.4
	80.16	80.0 ± 0.3	0.4	-0.2	80.1 ± 0.9	1.1	-0.1

[a] Measurements of six replicates made along the same day.

[b] Measurements made along three non-consecutive days, in the same week, making six replicates each day.

Table 5.3 (continued).

Compound	Added (µg/ml)	Intra-day [a]			Inter-day [b]		
		Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)	Found (µg/ml) (mean ± SD)	RSD (%)	Accuracy (%)
Maprotiline	50.60	50.4 ± 0.7	1.4	-0.4	50.5 ± 0.4	0.8	-0.2
	65.78	65.2 ± 1.5	2.3	-0.9	65.7 ± 0.7	1.1	-0.1
	80.96	80.9 ± 0.7	0.9	-0.1	81.1 ± 0.6	0.7	0.2
Nortryptiline	50.10	50.7 ± 0.3	0.6	0.4	50.6 ± 0.15	0.3	0.2
	65.13	65.3 ± 0.6	0.9	-0.5	65.5 ± 0.2	0.3	-0.2
	80.16	81.2 ± 0.3	0.4	0.5	80.6 ± 0.6	0.7	-0.2

[a] Measurements of six replicates made along the same day.

[b] Measurements made along three non-consecutive days, in the same week, making six replicates each day.

LODs and LOQs were determined using the 3s and 10s criteria, respectively. The standard deviation was calculated from ten-fold injections of solutions containing 0.25 μg/mL amitryptiline, imipramine, and nortryptiline, 0.50 μg/mL clomipramine, and 4 μg/mL maprotiline. The obtained values (LOD and LOQ, expressed as μg/mL) were: amitryptiline (0.05, 0.16), clomipramine (0.09, 0.31), imipramine (0.05, 0.17), maprotiline (1.15, 3.85), and nortryptiline (0.06, 0.021).

The robustness of the method was also evaluated by the mean value and absolute and relative standard deviations (RSD) of the chromatographic peak areas and retention times, for 65 μg/mL of each TCA. The experimental parameters were the flow-rate, and the concentrations of SDS, octane and 1-butanol in the mobile phase. Each of these parameters were varied within a range around the value used to develop the analytical 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. As seen in Table 5.4, the RSD values for the retention times were usually below 2 %. The highest values corresponded to the concentration of octane, which confirms the important role of the oil in the formation of the ME. For the peak areas, a higher variability was obtained, especially for clomipramine.

Table 5.4. Robustness of the proposed method for tricyclic antidepressants.

Compound	Parameter	Level	SDS / octane / 1-butanol	
			Retention time (min) (RSD, %)	Area (arbitrary units) (RSD, %)
Amitryptiline	Flow rate (ml/min)	0.99 – 1.01	5.37 ± 0.04 (0.7)	32.5 ± 0.3 (1.0)
	SDS (M)	0.170 – 0.177	5.39 ± 0.04 (0.7)	32.5 ± 0.13 (0.4)
	Octane (%)	1.35 – 1.49	5.39 ± 0.10 (1.9)	32.4 ± 0.14 (0.4)
	1-Butanol (%)	8.02 – 8.27	5.39 ± 0.02 (0.4)	32.41 ± 0.14 (0.4)
Clomipramine	Flow rate (ml/min)	0.99 – 1.01	6.00 ± 0.04 (0.7)	26.2 ± 0.8 (3.1)
	SDS (M)	0.170 – 0.177	6.02 ± 0.05 (0.8)	26.2 ± 0.6 (2.3)
	Octane (%)	1.35 – 1.49	6.12 ± 0.12 (0.1)	17.97 ± 6.06 (33.7)
	1-Butanol (%)	8.02 – 8.27	6.02 ± 0.02 (0.3)	22.64 ± 2.6 (11.5)
Imipramine	Flow rate (ml/min)	0.99 – 1.01	5.27 ± 0.04 (0.8)	31.88 ± 0.23 (0.7)
	SDS (M)	0.170 – 0.177	5.28 ± 0.04 (0.8)	31.74 ± 0.04 (0.1)
	Octane (%)	1.35 – 1.49	5.27 ± 0.10 (1.9)	31.92 ± 0.6 (1.8)
	1-Butanol (%)	8.02 – 8.27	5.279 ± 0.007 (0.1)	31.7 ± 0.2 (0.6)

Table 5.4 (continued).

Compound	Parameter	Level	SDS / octane / 1-butanol	
			Retention time (min) (RSD, %)	Area (arbitrary units) (RSD, %)
Maprotiline	Flow rate (ml/min)	0.99 – 1.01	6.24 ± 0.05 (0.8)	2.21 ± 0.03(1.4)
	SDS (M)	0.170 – 0.177	6.27 ± 0.05 (0.8)	2.17 ± 0.1 (4.6)
	Octane (%)	1.35 – 1.49	6.26 ± 0.13 (2.1)	2.06 ± 0.03 (1.5)
	1-Butanol (%)	8.02 – 8.27	6.27 ± 0.02 (0.3)	2.14 ± 0.06 (2.8)
Nortryptiline	Flow rate (ml/min)	0.99 – 1.01	5.83 ± 0.05 (0.9)	32.8 ± 0.4 (1.2)
	SDS (M)	0.170 – 0.177	5.83 ± 0.03 (0.5)	32.9 ± 0.3 (1.0)
	Octane (%)	1.35 – 1.49	5.87 ± 0.10 (1.7)	31.27 ± 0.9 (2.9)
	1-Butanol (%)	8.02 – 8.27	5.38 ± 0.01 (0.2)	32.7 ± 0.3 (1.0)

The results obtained for the TCAs assayed in this work, with the proposed MELC procedure, were also compared with other procedures published in previous reports, using hydro-organic mixtures with acetonitrile [5], micellar mobiles phases containing SDS and 1-pentanol [5], and pure micellar mobile phases with Brij-35 [6]. The calibration parameters (Table 5.5), intra- and inter-day precision and accuracy (Table 5.6), and LODs and LOQs (Table 5.7), are provided for the hydro-organic and both micellar modes with SDS and Brij-35.

As can be seen, all calibration curves of the three methods met the linearity requirements (Table 5.5). However, method precision was better for the MELC procedure described in this work, and the micellar mode with SDS and 1-pentanol (compare Tables 5.2 and 5.6), with RSD values usually below 2 %. Meanwhile, for the hydro-organic and Brij-35 pure micellar modes, the inter-day precision expressed as RSDs ranged from 0.65 % to 3.1 % (Tables 5.2 and 5.6).

In general, LODs and LOQs were smaller with the MELC procedure proposed in this work, except for amitryptiline and maprotiline, which yielded lower values with the hydro-organic procedure.

Table 5.5. Day-to-day calibration parameters obtained for the tricyclic antidepressants.

Compound		Acetonitrile / water			SDS / pentanol		
		Slope	Intercept	R^2	Slope	Intercept	R^2
Amitryptiline	[a]	0.137 ± 0.004	0.11 ± 0.11	0.9990	0.0391 ± 0.0010	0.04 ± 0.04	0.9997
	[b]	0.143 ± 0.004	-0.34 ± 0.20	0.9990	0.0397 ± 0.00006	0.019 ± 0.003	0.9999
Clomipramine	[a]	0.204 ± 0.005	-0.40 ± 0.35	0.9998	0.0519 ± 0.0004	0.005 ± 0.013	0.9998
	[b]	0.205 ± 0.004	-0.72 ± 0.21	0.9994	0.0519 ± 0.0004	0.02 ± 0.02	0.9999
Maprotiline	[a]	0.0250 ± 0.0007	-0.033 ± 0.015	0.9995	0.0057 ± 0.0012	-0.001 ± 0.005	0.9995
	[b]	0.0260 ± 0.0009	-0.03 ± 0.05	0.9995	0.00560 ± 0.00011	0.003 ± 0.006	0.9999
Nortryptiline	[a]	0.1460 ± 0.0017	-0.17 ± 0.13	0.9998	0.0397 ± 0.0008	0.01 ± 0.02	0.9998
	[b]	0.136 ± 0.005	0.11 ± 0.27	0.9997	0.0403 ± 0.00008	0.002 ± 0.004	0.9999

[a] Mean value of the parameters of three calibration straight-lines obtained in three consecutive days.

[b] Values from calibration straight-lines obtained 1–3 months later.

Table 5.6. Intra- and inter-day precision and accuracy for the hydro-organic and micellar mode with SDS and Brij-35.

Mobile phase		Acetonitrile / water		SDS / pentanol			Brij-35	
Compound	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	30	0.20	2.6	0.27	0.94	30	0.4	0.9
	50	0.14	2.3	0.17	1.10	40	0.16	0.9
	70	0.13	0.98	0.13	0.44	50	0.3	1.6
Clomipramine	30	0.29	0.99	0.40	1.10	30	2.2	1.7
	50	0.50	0.93	0.25	1.30	40	0.4	2.1
	70	0.16	1.4	0.63	0.46	50	0.3	1.1
Imipramine	–	–	–	–	–	30	0.6	1.9
	–	–	–	–	–	40	1.1	2.3
	–	–	–	–	–	50	0.17	2.2
Maprotiline	30	0.33	2.1	0.19	1.80	30	0.8	2.9
	50	0.15	1.0	0.16	1.90	40	1.4	1.8
	70	0.15	1.3	0.29	1.30	50	0.6	0.9
Nortryptiline	30	0.30	1.5	0.30	0.46	30	0.6	1.2
	50	0.33	3.1	0.20	1.20	40	0.8	1.8
	70	0.24	0.65	0.14	0.23	50	0.7	1.7

Table 5.7. Comparison of limits of detection and quantification (μg mL^{-1}) using mobile phases of different nature.

Compound	Acetonitrile / water [a]		SDS / pentanol [b]		Brij-35 [c]		SDS / octane / 1-butanol [d]	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
Amitryptiline	0.02	0.07	0.54	1.80	0.16	0.53	0.05	0.16
Clomipramine	0.19	0.63	0.18	0.60	0.38	1.27	0.09	0.31
Imipramine	–	–	–	–	0.25	0.83	0.05	0.17
Maprotiline	0.21	0.70	1.7	5.67	1.53	5.1	1.15	3.85
Nortryptiline	0.22	0.73	0.40	1.33	0.29	0.97	0.06	0.21

[a] 35 % (*v*/*v*) acetonitrile (XTerra C18) (Ref. (5)).

[b] 0.075 M SDS / 6% (*v*/*v*) 1-pentanol (C8) (Ref. (5)).

[c] 0.02 M Brij-35 (C18) (Ref. (6)).

[d] 0.173 M SDS / 1.4% (*v*/*v*) octane / 8.15% (*v*/*v*) 1-butanol (XTerra C18).

5.4.5. Analysis of pharmaceutical formulations

The validated method was applied to determine the TCAs amitryptiline, clomipramine, imipramine, maprotiline and nortryptiline in several pharmaceutical formulations prescribed in Europe (Table 5.8). The analyses were carried out taking five portions of powder, for each formulation, previously homogenised in a mortar. The injected solutions of each sample were prepared by weighting the adequate amount of the homogenous powder to obtain solutions of ca. 65 μg/mL.

Figure 5.3 shows the chromatograms of the analysed pharmaceutical formulations containing one of the five TCAs, using a mobile phase with 0.173 M SDS, 1.42 % (*v*/*v*) octane and 8.15 % (*v*/*v*) 1-butanol. The excipients were eluted at the dead time or did not absorb at the wavelength of detection. Table 5.8 gives the found contents, together with the label claim percentages. Tryptizol analysed with the acetonitrile/water and SDS/pentanol methods contained 50 mg amitryptiline chlorhydrate per tablet, and with the Brij-35 and SDS/octane/1-butanol methods contained 25 mg amitryptiline chlorhydrate per tablet.

The results are compared with those obtained with procedures using mobile phases containing either 35 % (*v*/*v*) of acetonitrile, 0.075 M SDS / 6 % (*v*/*v*) 1-pentanol, or 0.02 M Brij-35. The recoveries for all the formulations analysed were in the range from 80 to 120 % of the drug content (except nortryptiline analysed by MLC with Brij-35). These values are considered acceptable by the ICH guideline for the assay of finished pharmaceutical products.

Table 5.8. Analysis of several formulations containing tricyclic antidepressants.

Formulation (laboratory)	Composition (mg)	Acetonitrile / water		SDS / pentanol	
		Found (mg)	Label claim (%)	Found (mg)	Label claim (%)
Tryptizol (Merck, Sharp and Dohme)	Per tablet: amitryptiline hydrochloride (50), lactose, corn starch, and other excipients	50.1	100.2	49.0	98.0
Anafranil (Novartis)	Per tablet: clomipramine hydrochloride (25), saccharose, lactose, and other excipients	24.5	98.0	24.8	99.2
Tofranil (Amdipharm)	Per tablet: imipramine hydrochloride (25), colloidal silica, glycerol, and other excipients	–	–	–	–
Ludiomil (Amdipharm, Novartis)	Per tablet: maprotiline hydrochloride (25), magnesium stearate, and other excipients	26.4	105.6	25.4	101.6
Paxtibi / Norfenazin (BIOMED, Reig Jofré)	Per tablet: nortryptiline hydrochloride (25), lactose, and other excipients	21.9	87.6	24.4	97.6

Table 5.8 (continued).

Formulation (laboratory)	Composition (mg)	Brij-35		SDS /octane / 1-butanol	
		Found (mg)	Label claim (%)	Found (mg)	Label claim (%)
Tryptizol (Merck, Sharp and Dohme)	Per tablet: amitryptiline hydrochloride (25), lactose, corn starch, and other excipients	22.5 ± 0.7	90.0	24.95 ± 0.21	99.8
Anafranil (Novartis)	Per tablet: clomipramine hydrochloride (25), saccharose, lactose, and other excipients	23.76 ± 0.16	95.0	28.6 ± 1.8	114.4
Tofranil (Amdipharm)	Per tablet: imipramine hydrochloride (25), colloidal silica, glycerol, and other excipients	23.7 ± 0.6	94.8	28.2 ± 2.3	112.8
Ludiomil (Amdipharm, Novartis)	Per tablet: maprotiline hydrochloride (25), magnesium stearate, and other excipients	24.5 ± 0.5	98.0	26.6 ± 0.9	106.4
Paxtibi / Norfenazin (BIOMED, Reig Jofré)	Per tablet: nortryptiline hydrochloride (25), lactose, and other excipients	18.8 ± 0.5	75.2	26.55 ± 0.17	106.2

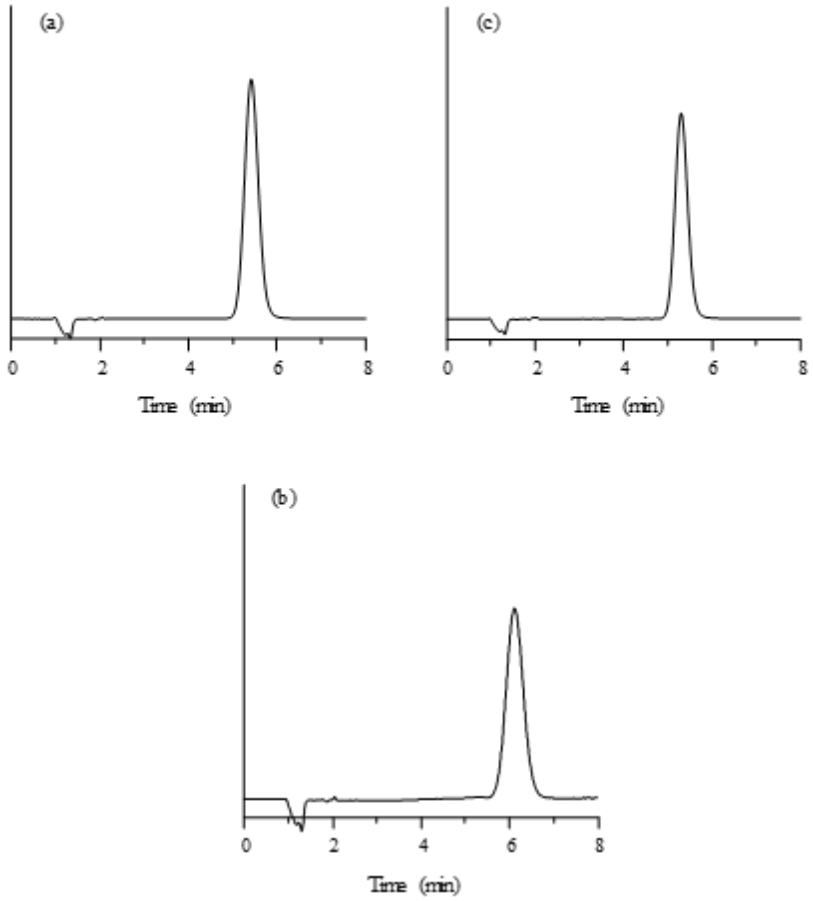

Figure 5.3. Chromatograms of the formulations containing TCAs, eluted with a mobile phase of 0.173 M SDS, 1.42 % (*v*/*v*) octane, and 8.15 % (*v*/*v*) 1-butanol from an XTerra C18 column: (a) Tryptizol, (b) Anafranil, and (c) Tofranil (see Table 5.8).

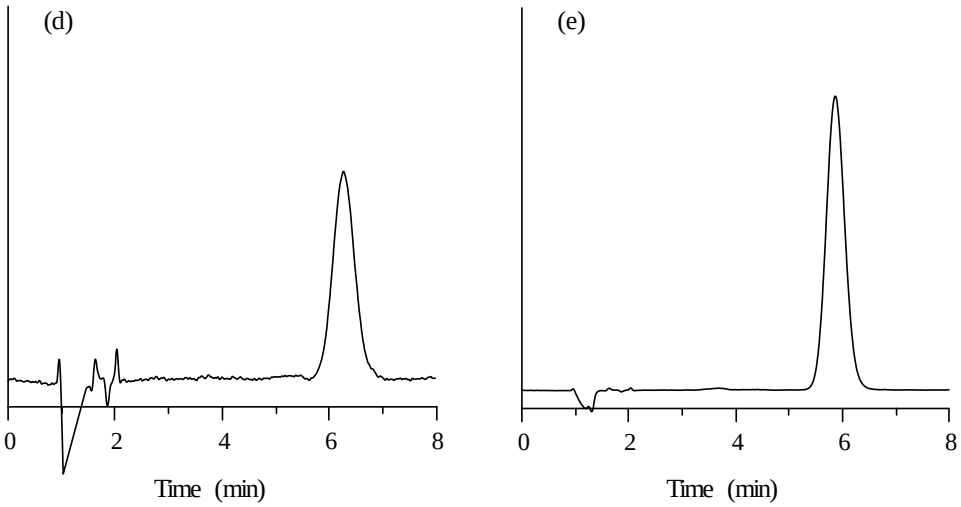

Figure 5.3 (continued). Chromatograms of the formulations containing TCAs, eluted with a mobile phase of 0.173 M SDS, 1.42 % (v/v) octane, and 8.15 % (v/v) 1-butanol from an XTerra C18 column: (d) Ludiomil, and (e) Paxtibi (see Table 5.8).

5.5. Conclusions

The suitability of the use of a ME containing SDS, octane and 1-butanol, as mobile phase, was studied for the analysis of five TCAs in commercialised pharmaceutical formulations. The optimised procedure using a mobile phase formed by 0.173 M SDS, 1.42 % (*v*/*v*) octane, and 8.15 % (*v*/*v*) 1-butanol, was validated, and compared with the results obtained with previous procedures that employed a hydro-organic mixture, and micellar media containing SDS and 1-pentanol or Brij-35 without organic solvent, as mobile phases.

Method validation indicated good linearity and intra- and inter-day precision and accuracy for the proposed MELC procedure. Moreover, it was found robust, but requiring the control of octane concentration. The MELC procedure showed better precision and lower LODs and LOQs than the micellar approaches, and similar to the hydro-organic, but without the need of adding high amounts of organic solvent (35 % (*v*/*v*) acetonitrile).

An advantage of the MELC procedure is the reduction in the retention times, compared with conventional RPLC and MLC with SDS at the same concentration, even when 1-butanol is added as co-surfactant. The MELC procedure maintains the good peak profiles achieved in MLC. When applied to commercialised formulations, satisfactory results are obtained without the need of any pre-treatment of the sample (only solubilisation and filtration).

The method can be run at pH 3, which is appropriate for more conventional columns. It should be also noted that the mobile phase composition would need a particular optimisation to develop a screening method for TCAs in other type of samples, as physiological fluids.

5.6. References

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CHAPTER 6

SOLUTE INTERACTIONS IN CHROMATOGRAPHIC MODES WITH SURFACTANT AND IONIC LIQUID

6.1. Abstract

Aqueous microemulsions (MEs), where an oil co-exist with water in the presence of the anionic surfactant sodium dodecyl sulphate (SDS), have been proposed as a solution to decrease the amount of organic solvent in the mobile phase needed in Reversed-Phase Liquid Chromatography (RPLC). However, the oil phase of typical MEs is volatile, toxic and flammable, and although it is added in a small amount, it would be convenient to avoid it from an environmental perspective. This is the reason of the proposal of Peng *et al.* (J. Chromatogr. A 1499 (2017) 132–139) of replacing the oil in Microemulsion Liquid Chromatography (MELC) by the non-polar ionic liquid hexyl-methylimidazolium hexafluorophosphate ($[C_6C_1IM][PF_6]$), for the analysis of phenolic acids at pH 2.5, where these compounds are not ionised. Based on this report, a procedure is here proposed to analyse basic compounds (β-adrenoceptor antagonists) at pH 1.35 (where they exist as cations). In order to check the possible formation of MEs and elucidate the interactions between the cationic basic compounds and the cations and anions in the additives (the SDS anion, and the IL cation and anion), an extensive study was made with several methylimidazolium ILs with either the cations $[C_2C_1IM]^+$, $[C_4C_1IM]^+$, or $[C_6C_1IM]^+$, combined with the anions Cl^-, BF_4^-, or PF_6^-, using 1-butanol as co-surfactant. The study was performed in comparison with the behaviour observed in classical MELC with octane, Micellar Liquid Chromatography with SDS and 1-propanol, and RPLC with mobile phases containing the ILs and acetonitrile. A mixture of SDS and a soluble IL ($[C_6C_1IM][Cl]$), without the addition of alcohol, was also considered as a greener mobile phase in RPLC.

6.2. Introduction

Common reagents in Microemulsion Liquid Chromatography (MELC) are the surfactant sodium dodecyl sulphate (SDS, anionic) and polyoxyethylene(23) lauryl ether (Brij-35, non-ionic), the oils heptane, octane, cyclohexane, diisopropylether and ethyl acetate, and the alcohols 1-propanol, 1-butanol and 1-pentanol, which are added as co-surfactants to stabilise the micelles [1,2]. MELC systems require smaller concentration of organic solvent in the mobile phase than conventional Reversed-Phase Liquid Chromatography (RPLC), below 1 % and 10 % for the oil phase and co-surfactant, respectively. Since any change in the nature and concentration ranges of the reagents (surfactant, oil and co-surfactant), in the MELC mobile phase, may affect significantly the chromatographic behaviour of solutes, a detailed systematic investigation is usually required to obtain successful separations. Therefore, finally, the large amount of experimental work needed may generate a toxic waste with a negative impact on both environment and health of the analyst.

In general, the replacement of harmful and volatile solvents, traditionally used in many processes, has generated major interest in recent years. Ideally, the best solvent would be no solvent (i.e. a solvent-free process), considering health hazards, waste generation and treatment, and economy [3]. Since the absence of solvent is not always possible, several greener solvents have been proposed to substitute the organic solvents conventionally employed, in order to decrease the environmental impact and overall risk of chemical exposure. Among the proposed alternatives are ionic liquids (ILs) [4], which are salts with low melting points (usually below 100 ºC), formed by a bulky organic cation associated with a smaller inorganic/organic anion to get electrical neutrality [5–7].

The interest in ILs can be attributed to the wide range of intermolecular interactions with solutes (strong and weak ionic interactions, hydrogen bonding, and van der Waals, dispersive, n-π and π-π interactions). The possibility of all these interactions gives rise to interesting solvation properties, compared to conventional organic solvents [8]. Other interesting features of ILs, such as their low volatility and flammability, and high thermal stability, have led to the replacement of pollutant conventional solvents by ILs, which have gained the label of benign or green solvents. However, some recent reports have shown that some ILs are not so safe and non-toxic [9,10], although it should be noted that the physico-chemical properties of ILs, including their toxicity, can be tuned and modulated by appropriate selection of the IL cation and anion.

In the analytical field, ILs have been widespread applied in sample preparation [11,12] and chromatographic analysis [13,14]. They have also been used immobilised on stationary phases in gas chromatography [15,16] and liquid chromatography [17,18], and as mobile phase additives in the hydro-organic mobile phases used in RPLC [19]. In these applications, ILs lose their characteristic physical features as solvents, being just salts that are dissociated in aqueous medium [20]. It should be noted that the addition of ILs to the mobile phase, in RPLC, minimises ion-exchange interactions of cationic solutes with residual anionic silanols, which are present in conventional silica stationary phases. This enhances peak performance, which has been explained by the adsorption of both cation and anion on the stationary phase, creating an asymmetrical bilayer, positively or negatively charged that mask the silanols. The effect is stronger with ILs with a cation of larger size [21].

Recently, alkyl-methylimidazolium ILs, associated to the anions tetrafluoroborate (BF_4^-) and hexafluorophosphate (PF_6^-), were proposed to prepare ionic liquid-in-water (IL/w) microemulsions (MEs) (also called aqueous

IL-based MEs), for the MELC analysis of hydrophilic phenolic compounds (danshensu, caffeic acid, protocatechualdehyde, rosmarinic acid and salvianolic acid B) in Danshen samples (a traditional Chinese herbal medicine), in acidic medium (pH = 2.5) [22] (see Figure 1.6 in Chapter 1). The procedure yielded excellent selectivity and appropriate resolution. Since the non-polar organic solvent (octane) was substituted in the ME by an IL, the authors claimed the smaller toxicity and low consumption of organic solvent in the proposed procedure as a remarkable advantage.

In this work, the application of IL/w MEs, using alkyl-methylimidazolium ILs with alkyl chains of diverse length, associated to anions of diverse nature (Cl^-, BF_4^-, and PF_6^-), which are the most common ILs added to the mobile phase in RPLC [19], is investigated for the analysis of cationic basic solutes (β-adrenoceptor antagonists) in acidic medium. The results were compared with those found with MELC mobile phases containing octane as oil (see Chapter 3), and with RPLC mobile phases containing SDS and 1-propanol, or ILs and acetonitrile.

6.3. Experimental

6.3.1. Reagents

Seven β-adrenoceptor antagonists (atenolol, acebutolol, carteolol, metoprolol, timolol, oxprenolol, and propranolol), all from Sigma (St. Louis, MA, USA) were used as probe compounds. Their structures, acidity constants and polarities, measured as the logarithm of the partition coefficient between octanol and water, are given in Chapter 4 (Table 4.2). The drugs were dissolved in 1 mL of methanol from VWR International (France) with the aid of an Elmasonic S15 H ultrasonic

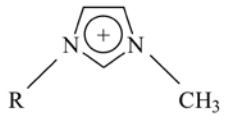
bath from Elma (Singen, Germany), and diluted with water. The concentration of the stock solutions, which remained stable during at least two months at 4 ºC, was approximately 100 μg/mL. These solutions were diluted with water to a final concentration of 20 μg/mL, prior to injection into the chromatograph. Uracil from Acros Organics (Geel, Belgium) was used as dead time marker.

The reagents used to prepare the mobile phases were sodium dodecyl sulphate from Merck (99% purity, Darmstadt, Germany), acetonitrile and 1-butanol from Scharlab (Barcelona), octane from Alfa Aesar (Kandel, Germany), and the ILs indicated in Table 6.1 from Sigma (St. Louis, MA, USA). Molar concentrations were used for the surfactant, and volumetric fraction (expressed as percentage) for acetonitrile, 1-butanol and octane.

The mobile phases contained: (i) SDS, 1-butanol and IL, (ii) SDS, 1-butanol and octane, (iii) SDS and 1-propanol, or (iv) IL and acetonitrile. The pH was fixed at 1.35 with 0.05 % trifluoroacetic acid for the MELC mobile phases with IL and octane, and at 3.0 with 0.01 M citric acid monohydrate and sodium hydroxide (Panreac, Barcelona), for the other mobile phases. The pH meter was calibrated with aqueous buffers, while the pH of the mobile phases was always fixed in the presence of the organic solvent. β-Adrenoceptor antagonists have a strong basic character ($pK_a \geq 9$), which means that at the acidic pH of the mobile phases the cationic species was dominant.

The solutions of the β-adrenoceptor antagonists and mobile phases were filtered through 0.45 μm Nylon membranes from Micron Separations (Westboro, MA, USA). Nanopure water from Adrona (Riga, Latvia) was used throughout.

Table 6.1. Structure and properties of the studied ionic liquids[a].

Ionic liquid	1-R-3-Methylimidazolium cation 	Anion	Melting point (°C)	Density (g/ml)	Water solubility
$[C_2C_1IM][PF_6]$	Ethyl–	$[PF_6]^-$	59	1.48	Partially soluble
$[C_4C_1IM][PF_6]$	Butyl–	$[PF_6]^-$	16	1.38	Non soluble
$[C_6C_1IM][PF_6]$	Hexyl–	$[PF_6]^-$	–61	1.30	Non soluble
$[C_4C_1IM][BF_4]$	Butyl–	$[BF_4]^-$	–82	1.17	Soluble
$[C_6C_1IM][BF_4]$	Hexyl–	$[BF_4]^-$	–81	1.21	Non soluble
$[C_6C_1IM][Cl]$	Hexyl–	$[Cl]^-$	–70	1.03	Soluble

[a] From Ref. [23].

6.3.2. Apparatus and columns

The chromatograph, from Agilent (Waldbronn, Germany) was equipped with quaternary pump (Series 1200), autosampler (Series 1260 Infinity II), thermostated column compartment (Series 1290 Infinity II), and diode array detector (Series 1100). The β-adrenoceptor antagonists were monitored at 225 nm, except timolol, which was detected at 300 nm. Uracil was detected at 254 nm. The retention data were obtained at 25 ºC, using isocratic conditions with a flow rate of 1 mL/min. Duplicate injections of 20 µL were made.

The system was controlled with an OpenLAB CDS LC Chemstation (Agilent B.04.03). The mathematical treatment was performed with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were processed with the MICHROM software to obtain the peak parameters (retention times and peak half-widths) [24].

An XTerra-MS C18 column from Waters (MA, USA) was used with the MELC mobile phases of SDS, 1-butanol and IL or octane, mixtures of IL and acetonitrile, and SDS and IL. The column has the following characteristics: 150 mm × 4.6 mm i.d., 5 µm particle size, 120 Å average pore diameter, 175 m^2/g surface area, and 12 weight % total carbon. XTerra MS C18 replaces one out of every three silanols with a methyl group during particle synthesis. The analytical column was preceded by similar 30-mm guard columns to protect them from the mobile phase.

A Kromasil C18 column (Análisis Vínicos, Ciudad Real, Spain) with the following characteristics 150 mm × 4.6 mmi.d., 5 µm particle size, 110 Å average pore diameter, 320 m^2/g surface area, and 19% carbon load, was used for micellar mobile phases and mobile phases containing ILs and acetonitrile or 1-propanol.

The mobile phases were recycled between runs and also during the analysis to reduce reagent waste. The chromatographic system was periodically rinsed with pure water and methanol or 2-propanol (around 30 mL), to remove the surfactant and the IL from the stationary phase. During weekend, the column was kept with 2-propanol.

6.4. Results and discussion

6.4.1. Solubility of [C_6C_1IM][PF_6] in mixtures of SDS and 1-butanol

In a recent study [22], alkyl-methylimidazolium ILs formed with $[C_4C_1IM]^+$, $[C_6C_1IM]^+$ and $[C_8C_1IM]^+$, associated to BF_4^-, PF_6^- and bis[(trifluoromethyl) sulfonyl] imide (TF_2N^-), were assayed in MELC. As indicated in Table 6.1, the solubility of [C_6C_1IM][PF_6] and [C_6C_1IM][BF_4] in water is low, making them alternative "greener" oils to form IL/w MEs composed by SDS, IL and 1-butanol. [C_6C_1IM][PF_6] was selected as the optimal to form the oil phase, based on the analysis time and separation selectivity obtained for the group of phenolic compounds. We took this ME as starting point for the analysis of the β-adrenoceptor antagonists.

In order to check the conditions of formation of a clear (transparent) medium to be used as mobile phase, or the possible appearance of two well differentiated phases, several mixtures containing different amounts of SDS, [C_6C_1IM][PF_6] and 1-butanol were first prepared. The effect of [C_6C_1IM][PF_6] was checked by increasing its concentration in the 0.01–0.10 M range, in solutions containing 0.10 M SDS and 0.02–0.14 M 1-butanol, or 0.02–0.25 M SDS and 0.09 M 1-butanol. Once the reagents were mixed, each mixture was allowed to stand for at least 12 hours. When the mixture did not initially give rise to two well-differentiated phases, it was left several weeks at rest to check its stability.

The formation of transparent and stable mixtures within two weeks was visually verified at room temperature.

In Chapter 4, the formation of an emulsion at increasing octane or decreasing 1-butanol concentrations, in mixtures with SDS, gave rise to an upper phase that increased in thickness and turned whitish, effect which was larger at the smallest concentration assayed for the surfactant. By substituting the oil by $[C_6C_1IM][PF_6]$, phase separation was not so clear, being only evidenced by the observation of a yellowish drop of the IL solution falling through the solution. However, in most assayed mixtures a transparent mixture was obtained.

The composition of the tested transparent mixtures and those showing phase separation (i.e., formation of emulsions), is represented in Figures 6.1a and 6.1b. At fixed concentration of 0.10 M SDS (Figure 6.1a), stable mixtures were always formed with a maximal concentration of $[C_6C_1IM][PF_6]$ close to 0.08 M, at both lower (0.02 M) and upper (0.14 M) extreme concentrations of 1-butanol (i.e., 1.81 % and 12.7 *v*/*v*). This means that the surfactant was capable of solubilising the IL without the need of a high amount of co-surfactant. When the concentration of 1-butanol was fixed at 0.09 M (8.15 % *v*/*v*) (Figure 6.1b), increasing amounts of $[C_6C_1IM][PF_6]$ required a larger concentration of SDS to get stable mixtures.

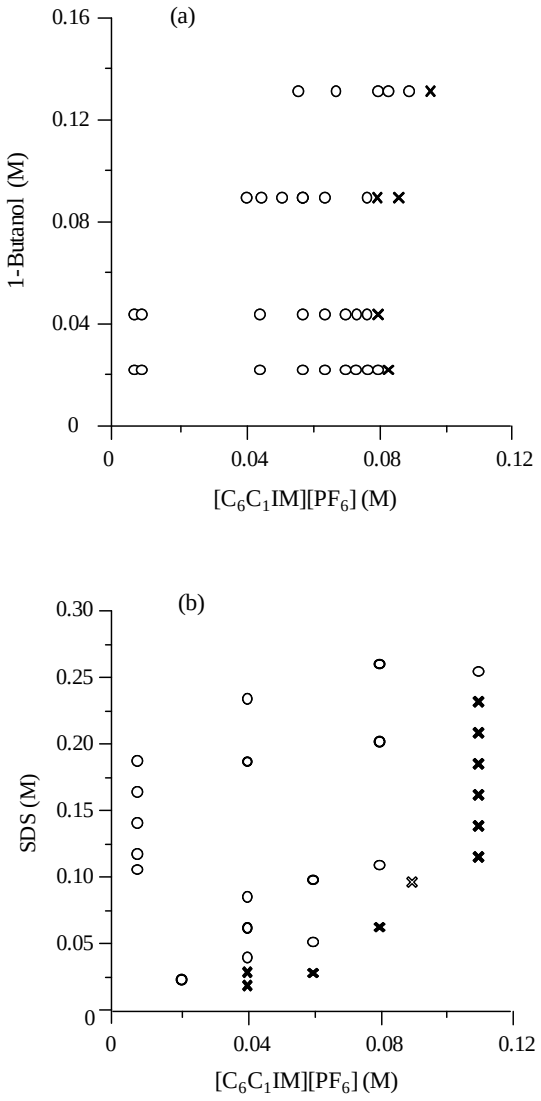


Figure 6.1. Concentration range for: (a) 1-butanol and $[C_6C_1IM][PF_6]$ in the presence of 0.10 M SDS, and (b) SDS and $[C_6C_1IM][PF_6]$ in the presence of 0.09 M 1-butanol. The circles correspond to the compositions that led to the formation of transparent mixtures, whereas the crosses correspond to the compositions that yielded phase separation.

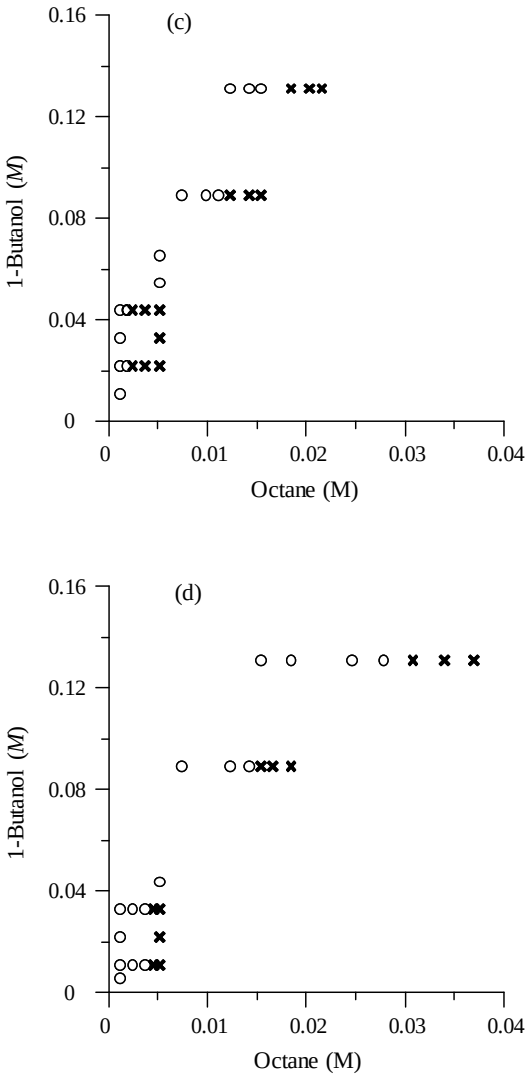


Figure 6.1 (continued). Concentration range for: 1-butanol and octane in the presence of 0.10 M (c) and 0.18 M (d) SDS. The circles correspond to the compositions that led to the formation of transparent mixtures, whereas the crosses correspond to the compositions that yielded phase separation.

Maximal concentrations of 0.10 M and 0.25 M were assayed for $[C_6C_1IM][PF_6]$ and SDS, respectively, which gave rise to stable transparent mixtures of these two reagents. It should be noted that, in RPLC, the range of concentrations used for this IL in the mobile phase is usually narrow, with an upper value below 0.04 M to avoid high viscosity. The capability of SDS to solubilise this IL could be explained by the formation of a stable ME, where the IL would act as oil (IL/w ME). However, the formation of a neutral ion pair or any other structure between the anionic SDS micelles and the alkyl-methylimidazolium cation should be also considered. This could explain the side role of 1-butanol in the solubilisation process.

The results in Figures 6.1a and 6.1b should be compared with those in Figures 6.1c and 6.1d, which correspond to the SDS / octane / 1-butanol system, where the role of the organic solvent is relevant for octane solubilisation. At both SDS concentrations (0.10 M, Figure 6.1c) and (0.18 M, Figure 6.1d), a high concentration of 1-butanol solubilises higher amounts of octane.

The chromatographic studies shown below try to gain more insight in the formation of organised structures in the SDS / $[C_6C_1IM][PF_6]$ / 1-butanol mixtures.

6.4.2. Retention behaviour of basic compounds with mobile phases containing SDS, $[C_6C_1IM][PF_6]$ and 1-butanol

In a chromatographic system with mobile phases containing SDS and IL, the stationary phase is probably coated by layers of surfactant monomers, IL cation, and to a lesser extent, IL anion. Alkyl-methylimidazolium cations with sufficiently long alkyl chains (such as $[C_6C_1IM]^+$), and chaotropic anions (such as PF_6^-), have been reported to be significantly adsorbed on the stationary phase

(~32 µmol) [23]. The adsorbed ionic reagents change the stationary phase from a non-polar (hydrophobic) to a polar (hydrophilic) charged surface. The charge sites on the stationary phase produced by this adsorption serve as ion-exchangers for the cationic solutes. The extension of the interactions of the anionic surfactant and IL cation and anion with the stationary phase, and the interactions of the cationic solutes with the surfactant and IL ions in the mobile phase and adsorbed on the stationary phase, makes the interpretation of the chromatographic behaviour (i.e., the retention mechanism) difficult.

The retention factors of the β-adrenoceptor antagonists obtained with mobile phases containing SDS in the range 0.05–0.25 M, 0.01 M [C_6C_1IM][PF_6], and 8.15 % (*v*/*v*) 1-butanol, are shown in Figure 6.2a. As observed, the addition of surfactant at increasing concentration yielded the expected decrease in retention. This is explained because there is a maximal amount of adsorbed surfactant on the C18 column which attracts the cationic solutes, while the concentration of SDS micelles in the mobile phase (which also interact with solutes) increases [25]. Therefore, the cationic solutes suffer a progressive distribution into an increased volume of microemulsion droplets (micelles containing IL in its core or surface), which increases the elution strength.

The observed behaviour should be compared with the changes in retention observed for the β-adrenoceptor antagonists with MEs formed by SDS, 1.14 % octane and 8.15 % 1-butanol (Figure 6.2b). The trend for SDS in the microemulsion mobile phase is similar, but with lower retention when octane is used instead of the IL [C_6C_1IM][PF_6]. Note that, with octane, a small increase in retention is observed for the upper SDS concentration.

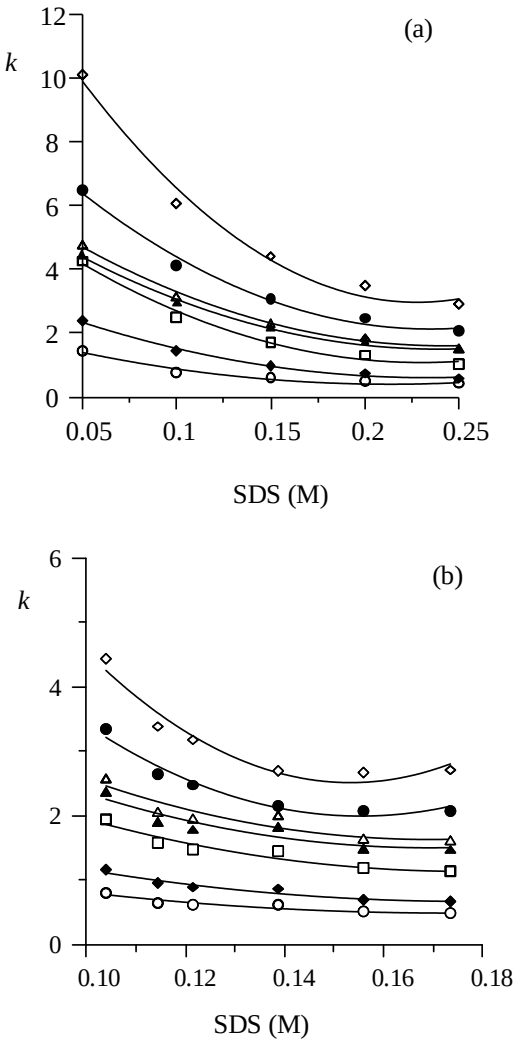


Figure 6.2. Variation of retention at increasing concentration of SDS in the presence of 8.15 % (*v*/*v*) 1-butanol and: (a) 0.01 M [C_6C_1IM][PF_6], or (b) 1.14 % octane. Solute identity: (□) acebutolol, (○) atenolol, (♦) carteolol, (Δ) metoprolol, (●) oxprenolol, (◇) propranolol, and (▲) timolol.

6.4.3. Effect of IL cation and anion on retention

In order to gain more insight on the effect of hybrid systems of SDS and ILs on the retention of the group of β-adrenoceptor antagonists, several mobile phases were assayed containing 0.05 M SDS, 8.15 % (*v*/*v*) 1-butanol and alkylimidazolium ILs with different cations and anions, and consequently, different water solubility. Two series were considered: the effect of anions using hexyl-methylimidazolium with different anions ([C_6C_1IM][Cl], [C_6C_1IM][BF_4] and [C_6C_1IM][PF_6]), and the effect of alkyl-methyl-imidazolium cations with different alkyl length using hexafluorophosphate as anion ([C_6C_1IM][PF_6], [C_4C_1IM][PF_6] and [C_2C_1IM][PF_6]), all at concentrations 0.01 and 0.03 M. Among the studied anions, Cl^- has low affinity for the stationary phase (~2.5 μmol), whereas BF_4^- and PF_6^- show moderate (~15 μmol) and strong adsorption (~32 μmol) on C18 stationary phases, respectively [23]. Note that these values were obtained with a Kromasil C18 column, and mobile phases containing 30 % acetonitrile and 0.05 M NaCl, $NaBF_4$ or $NaPF_6$.

Figure 6.3 depicts the behaviour for metoprolol, with an intermediate retention among the studied β-adrenoceptor antagonists (similar trends were observed for the other probe compounds). The retention decreased at increasing concentration of the ILs, being the effect stronger as the alkyl chain in the IL increased: $[C_2C_1IM]^+$ < ($[C_4C_1IM]^+$ < $C_6C_1IM]^+$ (i.e., the retention with the ILs with shorter side chains was significantly longer). This decreasing trend was also observed in mobile phases containing ILs without SDS (in combination with a weakly adsorbed anion as BF_4^- and Cl^-, see Figure 6.4). In Ref. [26], this was explained by considering the stronger adsorption of the more hydrophobic IL cation with longer alkyl chain, which repels the cationic solutes significantly.

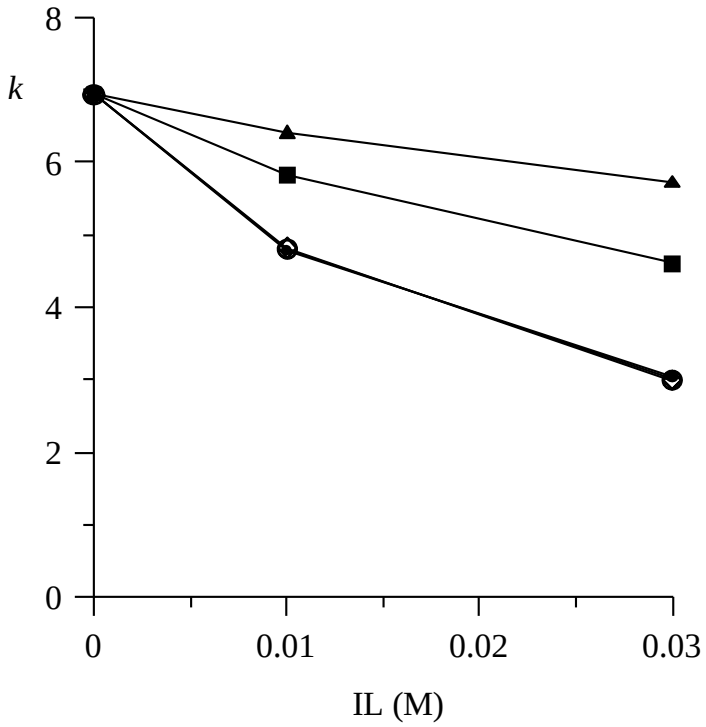


Figure 6.3. Retention behavior of metoprolol in different RPLC systems with 0.05 M SDS and 8.15 % (*v/v*) 1-butanol, and increasing concentration of IL. Assayed ionic liquids: (▲) $C_2C_1IM][PF_6]$, (■) $[C_4C_1IM][PF_6]$, (●) $[C_6C_1IM][PF_6]$, (◇) $[C_6C_1IM][BF_4]$, and (○) $[C_6C_1IM][Cl]$. The retention times agreed for $[C_6C_1IM][PF_6]$, $[C_6C_1IM][BF_4]$, and $[C_6C_1IM][Cl]$.

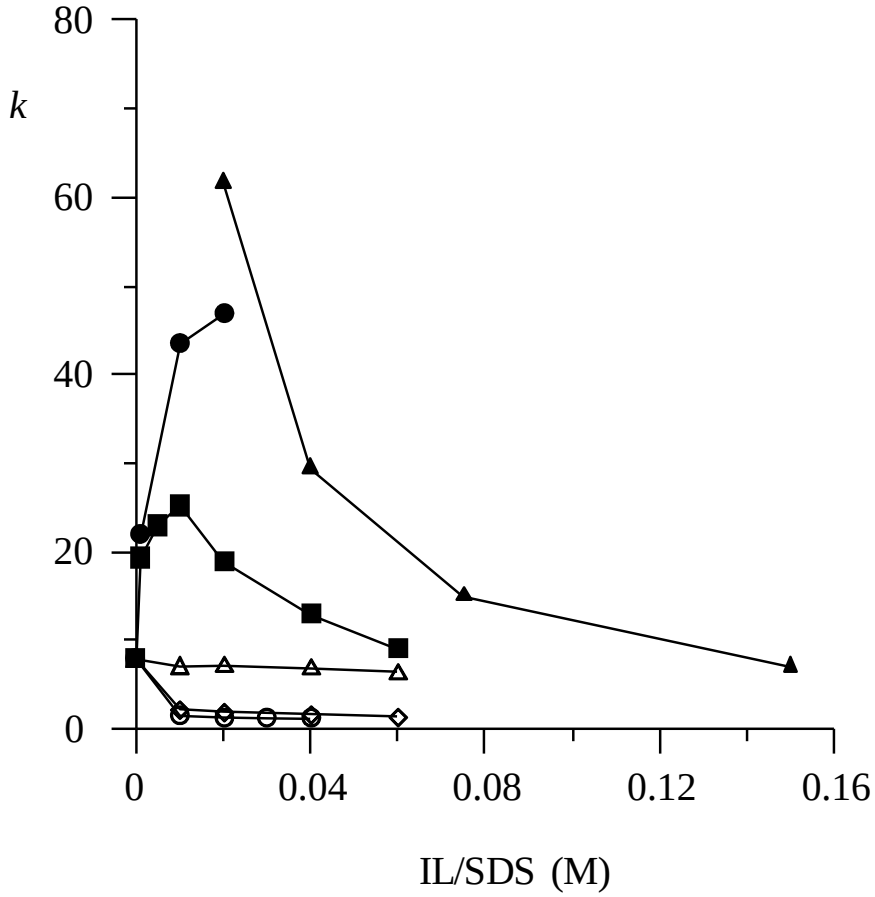


Figure 6.4. Retention behavior of metoprolol in different RPLC systems with mobile phases containing: (▲) SDS in the presence of 15 % 1-propanol (*v/v*), or either (●) [C_2C_1IM][PF_6], (■) [C_4C_1IM][PF_6], (Δ) [C_4C_1IM][BF_4], (◇) [C_6C_1IM][BF_4], or (○) [C_6C_1IM][Cl], in the presence of 15 % (*v/v*) acetonitrile [26].

Note that the IL cation dissolved in the mobile phase will also repel the cationic solutes, but this would be shifted towards the stationary phase, increasing the retention (i.e., the opposite effect). Also, a stronger adsorbed IL anion will attract the cationic solutes (increasing also the retention).

The decreased retention of the basic solutes at increased IL concentration, in the 0 to 0.03 M assayed range, suggested that the interaction of the cationic basic compounds with the imidazolium cations (electrostatic repulsion with the adsorbed IL cation) should prevail over the association with the adsorbed IL anions on the stationary phase, whose concentration is also changed by addition of IL to the mobile phase. This can be interpreted by considering that the strongly adsorbed SDS hinders the adsorption of the IL anion (even for the ILs associated to PF_6^-). In Figure 6.3, note that in the presence of SDS, the retention times for $[C_6C_1IM][PF_6]$, $[C_6C_1IM][BF_4]$, and $[C_6C_1IM][Cl]$ agreed. Seemingly, in the presence of SDS, the decreasing behavior for $[C_4C_1IM][BF_4]$ and $[C_4C_1IM][PF_6]$ will be probably similar, as is the case for $[C_6C_1IM][BF_4]$ and $[C_6C_1IM][PF_6]$.

Figure 6.4 (right curve) shows the retention of the cationic solutes with a mobile phase of SDS in the range 0.02–0.15 M and 15 % (*v*/*v*) 1-propanol. The high retention at low concentration of the surfactant reveals the attraction of the cationic solutes towards the SDS adsorbed on the stationary phase. Once the stationary phase is saturated with SDS, the amount of surfactant in the mobile phase (forming micelles) is increased, which increases the elution strength by attraction of the cationic solutes to the anionic micelles (which decreases the retention). A similar behaviour is observed with mobile phases that contain an increased concentration of SDS, and fixed amounts of IL and 1-butanol (Figure 6.2a), or octane and 1-butanol (Figure 6.2b), although the retention is globally smaller due to the presence of the organic solvents and IL.

The comparison of the trends in retention at increasing concentration of IL, in the presence of SDS (Figure 6.3) (MELC with IL), and without SDS (Figure 6.4) (RPLC with IL), can help to interpret the possible interactions. Only the behaviour in the presence of $[C_2C_1IM][PF_6]$, $[C_4C_1IM][PF_6]$, $[C_4C_1IM][BF_4]$, $[C_6C_1IM][BF_4]$, and $[C_6C_1IM][Cl]$, could be studied, since the solubility of $[C_6C_1IM][PF_6]$ in the absence of SDS was too low.

In the absence of surfactant (Figure 6.4), the retention was significantly affected by the presence of specific IL cations and anions, which should be explained by their particular adsorption capability on the C18 stationary phase. The adsorption of some cations and anions is stronger and also the saturation of the stationary phase towards the adsorption of these ions. As commented above, the adsorption of the IL cation on the stationary phase increases at increasing length of its alkyl chain, whereas the adsorption of PF_6^- is significantly stronger compared to BF_4^- and Cl^-. In fact, it is observed that the decreasing trend in the retention with mobile phases containing $[C_6C_1IM][BF_4]$ and $[C_6C_1IM][Cl]$, at increasing IL concentration, was similar. Meanwhile, in the absence of SDS, the combined effect of BF_4^- with an IL with shorter length ($[C_4C_1IM][BF_4]$), gave rise to an almost constant retention at increasing amount of the IL, which can be explained by the smaller adsorption of $[C_4C_1IM]^+$, compared to $[C_6C_1IM]^+$ (both with a decreasing effect on the retention), which makes the adsorption of BF_4^- (which would increase the retention) more competitive.

On the other hand, for $[C_4C_1IM][PF_6]$, in the mobile phases without surfactant, the combined effect of cation and anion gave rise to an increased retention trend at low concentration of the IL with decreased retention at higher concentration (Figure 6.4). The interpretation of this behavior is not easy, due to the significant amount for both cation ($[C_4C_1IM]^+$) and anion (PF_6^-), adsorbed on the stationary phase and dissolved in the mobile phase, giving rise to repulsion

and attraction of the cationic solutes, respectively. In this regard, the trend observed for $[C_2C_1IM]^+$ associated to the PF_6^- anion is interesting, since the smaller adsorption of an IL cation with smaller alkyl length ($[C_2C_1IM]^+$) is combined with an anion showing strong adsorption (PF_6^-). In this case, the retention increased up to reach the maximal assayed concentration (note that this IL is partially soluble, Table 6.1), indicating clearly that the adsorption of the anion (which attracts the cationic solutes to the stationary phase) is predominant. Note that for $[C_4C_1IM][PF_6]$ and $[C_2C_1IM][PF_6]$, in the presence of SDS, the retention always decreases with added IL.

6.4.4. Effect of IL cation and anion on the peak profiles

Peak profiles in chromatography are characterised by their height, position, width and asymmetry; the two latter depend on the values of the left and right peak half-widths. The observation of the trend of peak half-widths is also useful to evaluate the interactions of solutes with the stationary phase (which affect the kinetics), and obtain equations that allow the prediction of peak profiles with optimisation purposes. Fortunately, simple correlations can be built between the peak half-widths and the retention times, which in isocratic elution can be approximated to straight-lines. These plots can be obtained with the half-widths/retention time data for a set of solutes experiencing the same kinetics, eluted with a mobile phase at fixed or varying composition [27]. When the analysed solutes in a mixture experience different resistance to mass transfer, the plots will be solute dependent, and will show significant scattering.

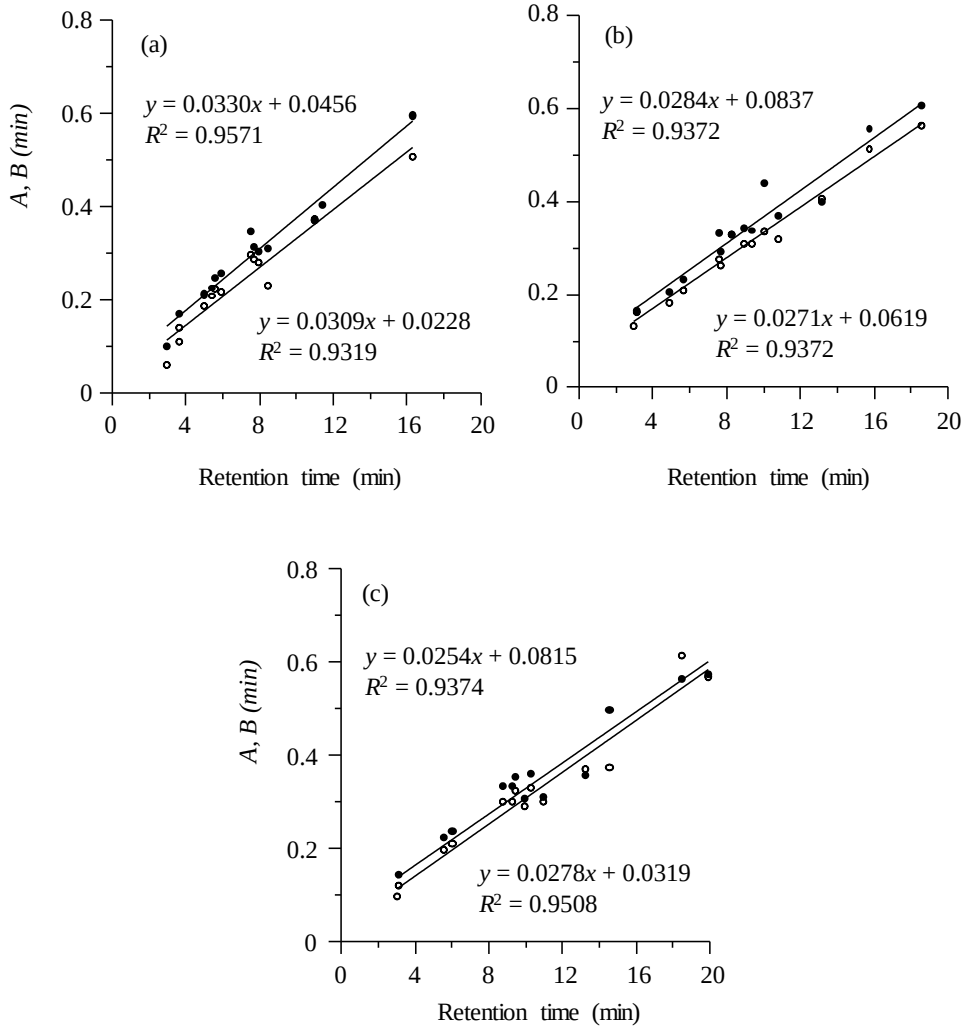


Figure 6.5. Half-width plots (left, *A* (○) and right, *B* (●)), built with the data obtained for the seven β-adrenoceptor antagonists, analysed with mobile phases containing 0.05 M SDS, 8.15 % 1-butanol, and 0.01 and 0.03 M ILs: (a) [C_6C_1IM][PF_6], (b) [C_4C_1IM][PF_6], and (c) [C_2C_1IM][PF_6].

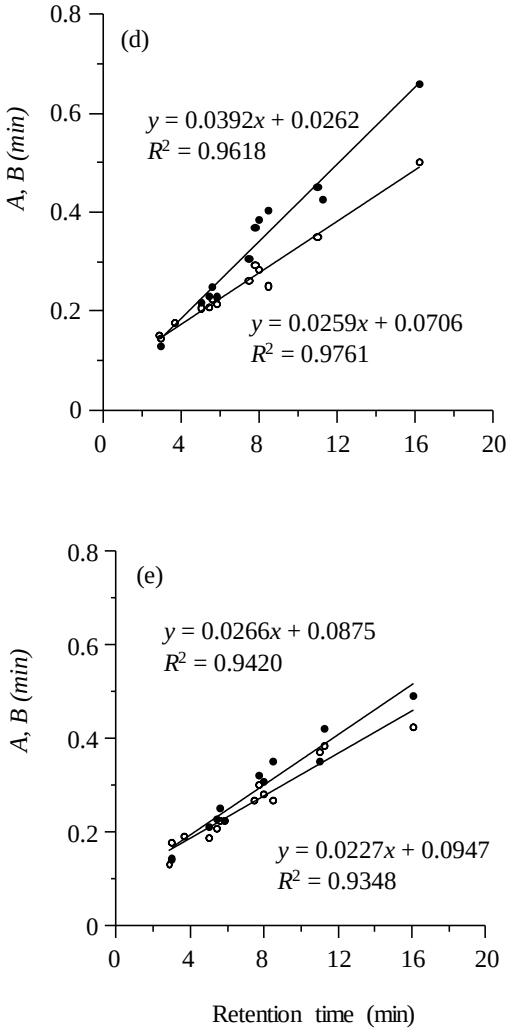


Figure 6.5 (continued). Half-width plots (left, *A* (○) and right, *B* (●)), built with the data obtained for the seven β-adrenoceptor antagonists, analysed with mobile phases containing 0.05 M SDS, 8.15 % 1-butanol, and 0.01 and 0.03 M ILs: (d) $[C_4C_1IM][BF_4]$, or (e) $[C_4C_1IM][Cl]$.

The half-width plots for the set of β-adrenoceptor antagonists are depicted in Figure 6.5 for four ILs with different cation and anion. The plots were obtained using the information for the set of solutes eluted at varying mobile phase composition. Table 6.2 gathers the features of the plots: the slopes of the left (m_A) and right (m_B) half-widths, and the sum of slopes and their ratio, where they are compared with the peaks obtained with acetonitrile-water mixtures, and mobile phases of SDS/1-propanol and IL/acetonitrile [21,26]. The presence of additive in all cases yielded a significant improvement in the peak profiles with respect to classical hydro-organic RPLC, which can be explained by a masking effect of the free anionic silanols in the silica-based stationary phases.

In the presence of IL, the peaks are significantly more symmetric compared to acetonitrile-water mixtures, especially for $[C_2C_1IM][PF_6]$, $[C_4C_1IM][PF_6]$ and $[C_6C_1IM][PF_6]$, in the presence of SDS, and $[C_4C_1IM][BF_4]$ and $[C_4C_1IM][Cl]$, without SDS (B/A = 0.9–1.1).

The half-width plots in Figure 6.5 should be compared with those obtained for an MELC mobile phase with octane. In Figure 3.6 in Chapter 3, the plots for several mobile phase compositions in the 0.104–0.173 M SDS, 0.25–1.28 % octane, and 8.15–17.3 % 1-butanol ranges are shown. As indicated in Table 6.2, the mean asymmetry when all assayed mobile phases were considered was B/A = 1.0 . Figure 6.6 depicts the plots for particular mobile phases: 0.114 M SDS / 0.28 % octane / 8.15 % 1 butanol, and 0.156 M SDS / 1.14 % octane / 8.15 % 1-butanol. The B/A values were 1.1 and 0.9, respectively.

Table 6.2. Half-width plots parameters for several chromatographic systems: slopes of the left (m_A) and right (m_B) half-widths, sum of slopes and slope ratio.

IL	m_A	m_B	$m_A + m_B$	m_B/m_A
Without additive[a]	0.021	0.047	0.068	2.3
SDS / 1-butanol / IL				
$[C_2C_1IM][PF_6]$	0.028	0.025	0.053	0.9
$[C_4C_1IM][PF_6]$	0.027	0.028	0.055	1.0
$[C_6C_1IM][PF_6]$	0.031	0.033	0.064	1.0
$[C_6C_1IM][BF_4]$	0.026	0.039	0.065	1.5
$[C_6C_1IM][Cl]$	0.023	0.027	0.049	1.2
Classical MELC with non-polar solvent				
SDS/1-butanol/octane	0.043	0.044	0.087	1.0

[a] Acetonitrile-water, from Ref. [28].

Table 6.2 (continued).

IL / acetonitrile without SDS[b]				
$[C_2C_1IM][PF_6]$	0.026	0.038	0.064	1.5
$[C_4C_1IM][PF_6]$	0.026	0.040	0.066	1.5
$[C_2C_1IM][BF_4]$	0.018	0.022	0.040	1.2
$[C_4C_1IM][BF_4]$	0.020	0.022	0.042	1.1
$[C_6C_1IM][BF_4]$	0.022	0.17	0.039	0.8
$[C_2C_1IM][Cl]$	0.017	0.023	0.041	1.3
$[C_4C_1IM][Cl]$	0.019	0.019	0.039	1.0
$[C_6C_1IM][Cl]$	0.020	0.016	0.036	0.8

[b] From Refs. [21,26].

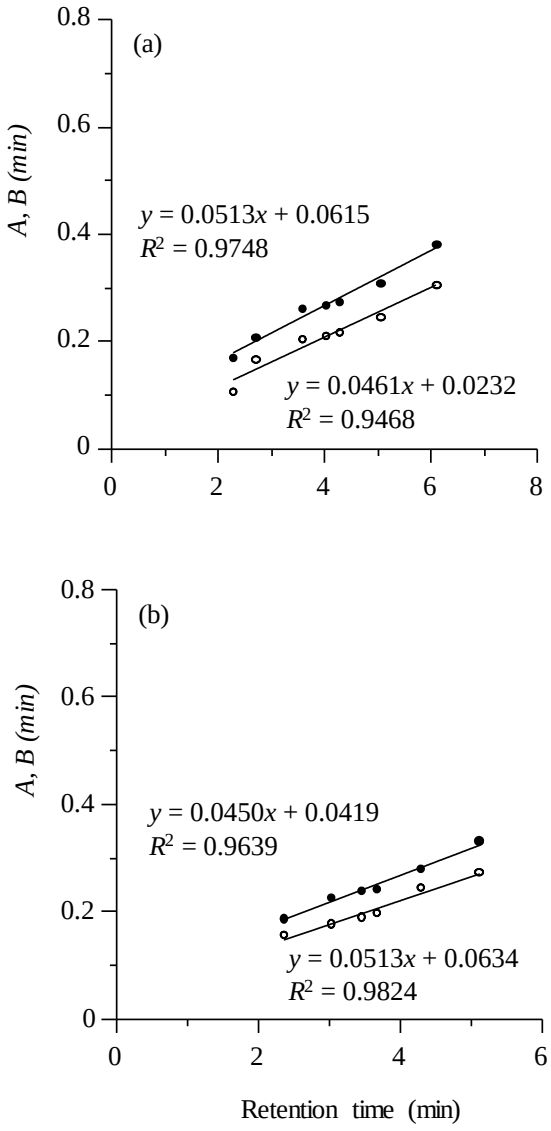


Figure 6.6. Half-width plots (left, *A* (○) and right, *B* (●)), built with the data obtained for the β-adrenoceptor antagonists with mobile phases containing: (a) 0.114 M SDS / 0.28 % octane / 8.15 % 1-butanol, and (b) 0.156 M SDS / 1.14 % octane / 8.15 % 1-butanol.

6.4.5. Is a microemulsion being formed in the mobile phase?

Up to this point, we raised the question if a microemulsion was really being formed in the mixture of SDS, 1-butanol and IL (even with the most non-polar IL [C_6C_1IM][PF_6]). To answer this question, we made a literature survey on the formation of MEs with ILs to get more information.

The development of chemical systems free of organic solvents is becoming increasingly important. The best solvent would be pure water, but it has the drawback of the low solubility of most organic compounds. Aqueous MEs, where an oil co-exist with water has been found as a solution, but the oil phase of typical MEs is volatile, toxic and flammable, which is deleterious under an environmental perspective. This is the reason of the proposal, for diverse purposes, of water-immiscible ILs as ideal replacements of typical oils in MEs, considering their attractive physico-chemical properties. Also, despite the variety and wide application range of typical surfactants, these have some drawbacks in ME formation, such as the relatively high concentration required of surfactant and the need of adding a co-surfactant (an organic solvent). In this regard, amphiphilic (i.e., with both hydrophobic and hydrophilic regions) imidazolium-based ILs with long alkyl chains which form micelles (the so-called surface active ionic liquids, SAILs), typically with eight or more carbon atoms, constitute a good alternative.

In the last decade, an increasing number of studies dealing with MEs have been published, where either the aqueous phase, oil phase, surfactant, or two of these components are replaced with ILs [29,30]. Due to the tunable properties of MEs containing ILs, these have been revealed as much more versatile than conventional MEs, or even compared to solutions with only ILs, being useful for

a wide range of fields, such as synthesis, bio-catalysis, polymerisation, preparation of nano-materials, drug delivery and separations.

Formation of stable MEs between two inherently immiscible liquids (oil and water), requires the presence of surfactants to reduce the interfacial tension between the two phases. The ME structure depends on the mass fraction of water, oil, and amphiphile, as well as on the nature of the interfacial film. Depending on their cation and anion properties, ILs can be used as polar or non-polar solvents in the formation of MEs [30–34]. This gives rise to different types of ME systems [29]:

(i) Non-aqueous IL-based MEs, usually formed by the combination of a neutral surfactant, such as Triton-X100 or Tween-80, with cyclohexane, benzene or toluene as oil, and a water-soluble IL, such as $[C_4C_1IM][BF_4]$ or $[C_2C_1IM][NTf_2]$ (NTf_2 being bis(trifluoromethylsulfonyl)imide). Another proposed combination is the mixture of the cationic surfactant cetyltrimethylammonium bromide (CTAB), using pentanol as co-surfactant, toluene as oil, and the water-soluble IL $[C_2C_1IM][C_6SO_4]$.

(ii) Aqueous IL-based MEs, formed by the combination of the neutral surfactants Triton-X100, Tween-20 or Brij-35, and the anionic bis(2-ethylhexyl) or dioctyl sulfosuccinate sodium (AOT), or the zwitterionic N-dodecyl-N,N-dimethyl-3-ammonium-1-propanesulphonate (SB-12), with or without 1-butanol, 1-hexanol or ethanol as co-surfactant, and the ILs $[C_4C_1IM][PF_6]$ and $[C_8C_8IM][NTf_2]$.

(iii) IL / oil/water MEs, where the IL acts as a self-assembly and structural organisation of amphiphilic molecule. Some examples of this type are formed by the SAILs $[C_{16}C_1IM][Br]$ or $[C_{14}C_1IM][Cl]$, and the oils p-xylene, 1-decanol or n-heptane. A conventional surfactant may be also

added to form a stable ME. Such is the case of [C_2C_1IM][Cl] and AOT, with isooctane as oil, or [C_4C_1IM][BF_4] and dioctadecyldimethyl-ammonium chloride or SDS, using 1-butanol as co-surfactant and heptane as oil.

The ME proposed by Peng *et al.* [22] to analyse phenolic acids was formed by a mixture of SDS, [C_6C_1IM][PF_6] and 1-butanol. Therefore, it should belong to the class of aqueous IL-based MEs. However, in such type of ME, non-ionic surfactants are the most usual. The most similar aqueous IL-based ME included in the review by Hejazifar *et al.*, published in 2020 [29], was prepared with [C_8C_8IM][NTf_2], the anionic AOT and 1-hexanol as co-surfactant. The solubility of [C_6C_1IM][PF_6] is low and can be associated to the core of the SDS micelle, but the solubility of other ILs assayed in Section 6.4.3 ([C_6C_1IM][Cl], [C_6C_1IM][BF_4], [C_4C_1IM][PF_6] and [C_2C_1IM][PF_6]) is appreciably higher and [C_6C_1IM][Cl] is soluble in water (see Table 6.1). However, as commented above, the maximal concentration of all these ILs was increased in the presence of SDS, which indicates an association between IL and surfactant. However, the role of 1-butanol to form stable mixtures is not sufficiently clear.

6.4.6. Retention of basic compounds with SDS / ionic liquid mobile phases without organic solvent

We should remind that the purpose of the addition of 1-butanol is the stabilisation of MEs, but when an IL is used instead of a non-polar organic solvent (e.g., octane), the presence of 1-butanol does not seem so relevant to get transparent mixtures (see Section 6.4.1). On the other hand, the retention of β-adrenoceptor antagonists with mobile phases containing SDS, [C_6C_1IM][PF_6] and 1-butanol, was too short (usually below 10 min), and significant overlapping of the basic compounds was observed (Figure 6.7a).

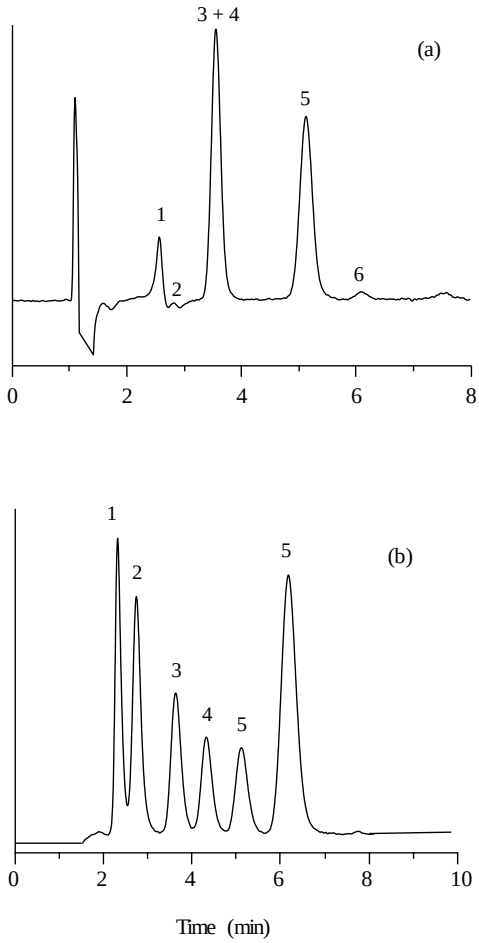


Figure 6.7. Experimental chromatograms obtained for mixtures of β-adrenoceptor antagonists with: (a) 0.1 M SDS / 0.01 M [C_6C_1IM][PF_6] / 8.15 % 1-butanol, and (b) 0.114 M SDS / 1.14 % octane / 8.15 % 1-butanol. Solute identity: (1) atenolol, (2) carteolol, (3) acebutolol, (4) metoprolol, (5) oxprenolol, and (6) propranolol.

In any case, the separation was poorer compared to that achieved with the mixture of SDS, octane and 1-butanol (Figure 6.7b). It was, thus, evident that the organic solvent (1-butanol) did not help to achieve chromatographic resolution for these analytes with added IL. On the other hand, the studies in Section 6.4.3 indicated that the separation is dominated by the association of the cationic solutes with the adsorbed SDS monomers and their repulsion from IL cation adsorbed on the stationary phase. Therefore, the possibility of eliminating the alcohol from the mobile phase was considered. Also, we thought that the combined effect of both reagents (attraction of the cationic analytes to the anionic SDS and repulsion from the IL cation) should be able to modulate the separation of the analytes, and get appropriate separation without the need of the alcohol. Therefore, a mixture containing only SDS and [C_6C_1IM][Cl] in aqueous solution was prepared to be used as mobile phase. Here, we should also think that [C_6C_1IM][Cl] is too soluble to form a ME with SDS (i.e., be included in the micelle core). Anyway, a transparent mixture was obtained that could be used with RPLC column.

It should be noted that the retention times for the β-adrenoceptor antagonists are excessive with both aqueous micellar mobile phases containing either SDS or [C_6C_1IM][Cl] as unique reagents, in the absence of organic solvent: the retention times for atenolol, carteolol, acebutolol, metoprolol, oxprenolol and propranol eluted with 0.1 M SDS from the XTerra column, were 9.9, 14.3, 16.8, 34.8, 57.1 and 83.5 min, respectively, whereas the retention times with 0.02 M [C_6C_1IM][Cl] were 3.5 and 11.6 min for atenolol and carteolol, respectively and > 60 min for metoprolol, oxprenolol and propranolol.

Figure 6.8 depicts the chromatogram obtained for a mixture of six β-adrenoceptor antagonists, using an isocratic mobile phase containing 0.10 M SDS and 0.02 M [C_6C_1IM][Cl], without organic solvent, buffered at pH 3. The

achieved separation suggests that the aqueous mixture of SDS and [C_6C_1IM][Cl] is promising to succeed in the separation of mixtures of β-adrenoceptor antagonists, with a favourable effect on retention (analysis time below 30 min) and good resolution. However, the most remarkable is that the separation was achieved without the need of an organic solvent in the mobile phase.

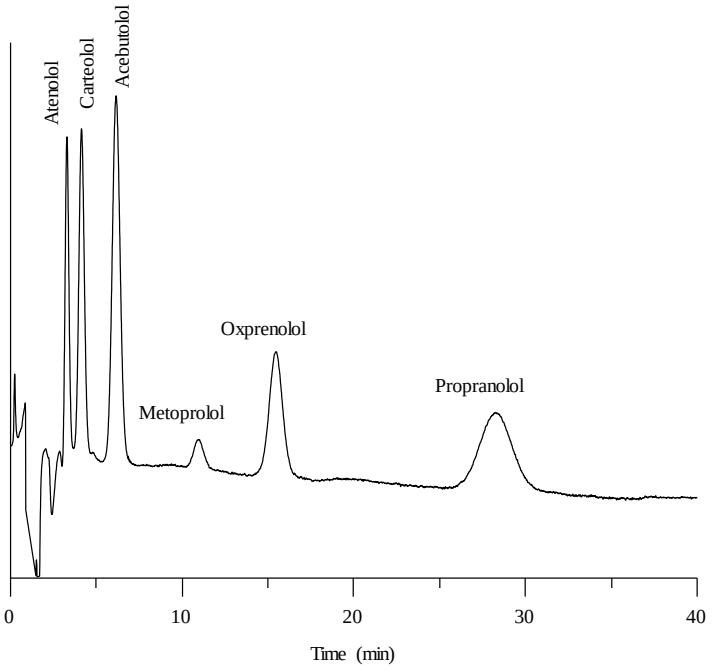


Figure 6.8. Chromatogram for a set of six β-adrenoceptor antagonists, eluted with mobile phases containing 0.10 M SDS and 0.02 M [C_6C_1IM][Cl], without organic solvent.

6.4.7. Some information on surface active ionic liquids (SAILs) that can help to understand the observed behaviour

In Section 6.4.5, the use of IL / oil / water MEs containing ILs with combined properties of ILs and surfactants (SAILs), was commented. With this type of IL, the aggregation behaviour in water (i.e., the capability of forming structures as micelles) can be tuned not only through the addition of salts or alcohols, or other (conventional) surfactants, which is usual for conventional surfactants, but also by chemical modifications in the ILs that alter the interactions. Even more, often SAILs do not require a co-surfactant or salt to form organised structures.

In view of the results included in Section 6.4.6, we thought that an extensive literature survey was also needed for this type of ILs, in order to understand the observed behaviour in the assayed mixture of SDS and [C_6C_1IM][Cl]. This is summarised next.

Practically unlimited combinations of cations and anions with substituted head-groups can be envisaged with ILs [7]. When one of the components of the IL (either the anion or cation) has amphiphilic properties, ILs-like surfactants (SAILs) are obtained [35,36]. Such molecules possess both a hydrophilic polar moiety with affinity for polar solvents and a hydrophobic (lipophilic) non-polar moiety (usually a hydrocarbon chain) with affinity for non-polar solvents. However, amphiphiles do not have surface activity if their interactions with solvents are dominated by either hydrophilic or lipophilic moiety.

In recent years, there has been an increasing interest in the synthesis of ILs with cations containing long alkyl chains to obtain SAILs. An important advantage of SAILs is that their hydrophobic and hydrophilic character can be fine-tuned through structural / functional alterations in the substituent groups of both cation and anion. SAILs, as conventional ionic surfactants, can be classified

as anionic, cationic, catanionic or zwitterionic (with positive and negative charge in the head group) [37].

Initial studies of SAILs were limited in scope, since only the cation was amphiphilic and the anion was not (called cationic SAILs). New proposals followed, and the field is still increasing. SAILs with diverse cations, such as imidazolium, pyridinium, and piperidinium, have been widely studied, although those based on the imidazolium cation with minor chemical variations are still the most usual. However, it should be noted that the associated IL anions has a significant effect on the surface properties and potential applications of SAILs. Thus, for example, in a study on the effect of different common counterions on the aggregation behaviour of $[C_{12}C_1IM]^+$ ILs in water, formation of aggregates in aqueous solution was observed only with Cl^-, whereas two-phase separation was obtained with PF_6^- and NTf_2^- [38].

However, increasing attention is being paid to SAILs with amphiphilic anions, including catanionic SAILs in which both the anion and the cation are amphiphiles. Although most known SAILs are formed by cations with a long alkyl chain, the combination of cations with shorter chain and anions of long chain has been checked to yield also ILs with amphiphilic properties (anionic SAILs). Thus, for example, $[C_6C_1IM][Cl]$ can be transformed to a SAIL by increasing the hydrophobicity via longer alkyl chain length, such as the case of $[C_nC_1IM][Cl]$ with n = 10, 12, 14 and 16 carbons, but an alternative is maintaining the alkyl length and substitute Cl^- by an alkyl sulphate anion ($[C_6C_1IM][C_8H_{17}SO_4]$) [39,40]. Blesic *et al.* reported that while an alkyl-imidazolium methylsulphate ($[C_nH_{2n+1}C_1IM][CH_3SO_3]$) behaves as cationic surfactant for $n > 8$, alkylimidazolium alkylsulphate SAILs ($[C_nH_{2n+1}C_1IM][C_mH_{2m+1}SO_3]$) with n = 4, and m = 8 are catanionic and have greater surface activity [41]. Two other classes of SAILs that must be mentioned

are the gemini (with two linked replicates of an amphiphilic moiety), and polymeric SAILs [36].

Anionic and catanionic SAILs, obtained by combining the properties of imidazolium-based ILs and surfactants, have been found more tailorable than the chemically limited sub-set of cationic SAILs of imidazolium, commonly employed, giving rise to changes in critical micelle concentration (CMC), mesophase behaviour, and bulk physico-chemical properties, such as melting point and solvent miscibility [42]. These compounds are also cheaper and more environmentally friendly compared to standard cationic SAILs.

A surprising aspect is that the physico-chemical properties of SAILs are dominated by the nature of the surfactant anion and that the chemical structure of the added cation plays only a secondary role [42,43]. Nonetheless, development of these SAILs is advantageous as they offer interesting opportunities to combine the properties of surfactants with those of imidazolium-based ILs, and this dual nature may be beneficial in applications such as separation and extraction [44].

It should be also considered that such halogen-free ILs are more environmentally friendly than traditional imidazolium-based ILs, such as those with the Cl^-, Br^-, BF_4^- and PF_6^- anions. Fluorinated anions such as BF_4^- and PF_6^- are frequently used, which might be attributed to their relatively simple preparation and reasonable price. However, despite their popularity, care should be taken with these ILs in aqueous MEs, since ILs associated to BF_4^-, and especially PF_6^-, are unstable in the presence of water due to slow hydrolysis that releases highly toxic and corrosive hydrofluoric acid [23,45–47].

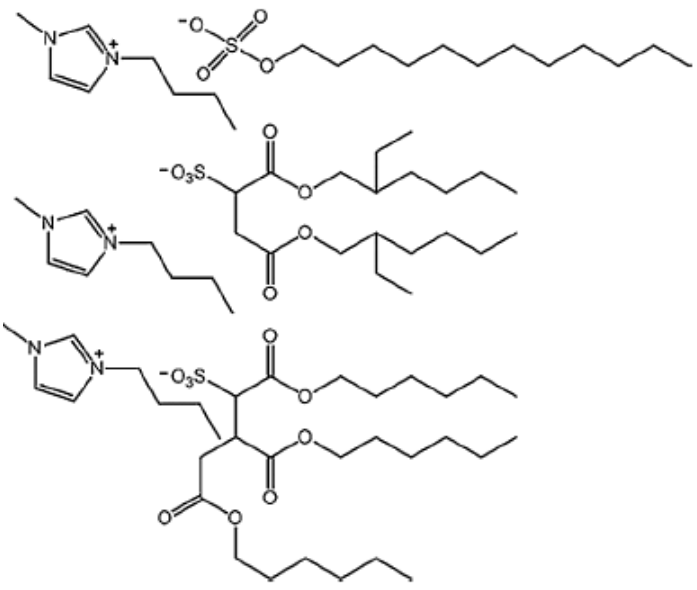


Figure 6.9. SAILS of the cationic IL 1-butyl-3-methyl-imidazolium combined with anionic surfactants of increasing complexity: SDS (single tail), AOT (double tail) and tris(hexyl) sulfosuccinate (triple tail) [43].

We should here comment that the anionic surfactant sodium dodecyl sulphate (SDS, $C_{12}H_{25}SO_4^-$) has been also reported as an option to form SAILs combined with an imidazolium cation with short chain, such as $[C_2C_1IM][C_{12}H_{25}SO_4]$, $[C_4C_1IM][C_{12}H_{25}SO_4]$ and $[C_5C_1IM][C_{12}H_{25}SO_4]$ [48]. In another interesting report, $[C_6C_1IM]^+$ was combined with three surfactant anions that bear bulky hydrophobic chains of increasing complexity: single tailed (dodecyl sulphate), double tailed (bis(2-ethylhexyl) sulfosuccinate, AOT) and triple tailed (tris(hexyl) sulfosuccinate) [43] (Figure 6.9).

As an example of a procedure used for the synthesis of a dodecyl sulphate-based anionic SAIL is the following [48]: Sodium dodecyl sulphate (0.035 mol) and $[C_2C_1IM]Cl$ (0.03 mol) are dissolved together in deionised water (50 mL) at 40 °C and the solution is stirred for 12 hours at 60 °C. The mixture is isolated by solvent extraction using dichloromethane (3×40 mL), and the solvent removed under vacuum. The crude product ($[C_2C_1IM][C_{12}H_{25}SO_4]$) is recrystallised with petroleum ether (3×40 mL), and dried in vacuo for 24 hours at 60 °C.

In this context, Pino *et al.* [49] examined the effect of several organic modifiers (methanol, 1-propanol, 1-butanol, 1-pentanol, and acetonitrile) on the micellisation behaviour of $[C_{16}C_4IM][Br]$ and 1,3-didodecylimidazolium bromide in water. In another study, the modulation in the aggregation behaviour of $[C_4C_1IM][C_8OSO_3]$ in aqueous solutions of the alcohols 1,2-propanediol, 1-propanol, and 2-propanol, at low concentration (10, 15 and 20 % *v*/*v*), was studied finding CMC increases in the aqueous-alcohol media [50].

6.5. Conclusions

In the literature, ILs appear to be an ideal replacement of the organic solvents used as oil phase in MEs, due to their attractive physico-chemical properties and low toxicity. However, in reported work, aqueous IL-based MEs consisting of IL, water and surfactant (and in some cases, an alcohol as co-surfactant) are usually formed by non-ionic surfactants, such as Brij-35 and Triton X-100, instead of the anionic SDS. The work by Peng *et al.*, reported in 2017 [22], was pioneer in the use of MEs in RPLC, where the oil was replaced with a non-soluble IL ($[C_6C_1IM][PF_6]$), and the anionic surfactant SDS (which is rather unusual for the preparation of aqueous IL-based MEs) was used in combination with

1-butanol as co-surfactant. The authors developed an analytical procedure for neutral phenolic acids.

In this work, the feasibility of using the aqueous IL-based ME recommended by Peng *et al.* as mobile phase, for the analysis of a group of basic compounds (β-adrenoceptor antagonists), which are positively changed, was investigated. The research was centred on the effect on retention and peak profiles produced by imidazolium ILs with alkyl chains of increasing length (with n = 2, 4 and 6) and Cl^-, BF_4^-, or PF_6^- as anions. The research group had previously developed a detailed work on the interactions of cationic solutes, in RPLC procedures that used C18 columns and mobile phases containing aqueous solutions of these imidazolium ILs in the presence of acetonitrile (without surfactant). A comparison of the effect of the cation and anion in different ILs, in the presence of SDS and 1-butanol, is made here with regard to previous work with mobile phases containing ILs in the absence of SDS (and acetonitrile instead of 1-butanol). The study gives some insight on the retention mechanisms.

It was found that the anionic surfactant SDS competes with the IL anions for adsorption, with a similar behaviour to that found without SDS when an IL cation showing sufficiently strong adsorption is associated to a weakly adsorbed anion. In these situations (i.e., IL mixed with the sodium salt of SDS or with the ILs $[C_6C_1IM][BF_4]$ or $[C_6C_1IM][Cl]$), the retention decreases upon addition of increasing concentration of IL. Meanwhile, in the absence of SDS, for an IL containing a less adsorbed cation or more strongly adsorbed anion, the retention is kept constant or increases with a maximum at a particular concentration of IL. On the other hand, in the presence of all assayed ILs, the peak profiles of the basic probe compounds are enhanced, but the effect is stronger in the presence of SDS. Peaks were completely symmetrical (B/A = 1.0) for $[C_4C_1IM][BF_4]$ and $[C_4C_1IM][Cl]$, indicating an efficient masking of the silanol effect.

The formation of transparent and stable MEs with surfactant (SDS), co-surfactant (1-butanol) and non-polar solvent (IL or octane), useful for RPLC, was found less dependent on the concentration of co-surfactant, when octane was replaced with an IL. Also, SDS allowed more concentrated solutions of the ILs, which indicated the formation of stable structures. In view of this behaviour, and considering that the addition of 1-butanol produced too short retention times and low resolution, in the separation of the group of β-adrenoceptor antagonists with the aqueous IL-based ME, the elimination of 1-butanol from the mobile phase was considered. In the literature, there has been big interest in the synthesis of surface active ionic liquids (SAILs) for diverse purposes, where an IL cation is associated to a surfactant anion, but there is no previous report on the use of a mixture of an imidazolium IL with a surfactant (such as SDS), in RPLC. The micelles formed by these SAILS should comprise alternate palisades of the IL cation and surfactant anion. Considering that, an aqueous solution of a soluble IL contains its dissociated cation and anion, the same effect would be obtained by dissolving a SAIL composed of IL cation and SDS anion, or a mixture of the chloride salt of the IL and the sodium salt of dodecyl sulphate (SDS).

The effect of a mobile phase composed of a mixture of the IL $[C_6C_1IM][Cl]$ and SDS, on the separation of the group of β-adrenoceptor antagonists, was thus investigated. The resulting chromatogram showed that the attraction of the cationic basic compounds by the SDS anion and repulsion by the IL cation should be useful to modulate the retention of the basic compounds to practical values, by modifying the concentrations of SDS and IL in the mobile phase, without the requirement of adding an organic solvent. This may give rise to an interesting “green mobile phase”. Here, we should remind that a mobile phase with only IL requires an amount of organic solvent to get appropriate analysis times and the retention with aqueous solutions of SDS may be extremely high. The observed

behaviour is expected to depend on the nature of the added IL in the mobile phase composition, and the concentration ratio of IL and SDS. This study will be the purpose of future work.

6.6. References

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SUMMARY AND CONCLUSIONS

Microemulsion Liquid Chromatography (MELC) is a Reversed-Phase Liquid Chromatographic (RPLC) mode, where mobile phases contain oil-in-water microemulsions (MEs). These allow new interactions of solutes with the chromatographic system. MEs are obtained spontaneously by mixing two immiscible liquids (water and oil), in the presence of a surfactant. A co-surfactant is also often needed to stabilise them.

A water/oil mixture gives rise to two immiscible phases, due to the large surface tension that exists between the two liquids. However, in the presence of surfactant, an organised, macroscopically homogeneous and thermo-dynamically stable liquid system is obtained. Surfactant molecules are made up of a bulky non-polar tail and a polar head group. By incorporating the surfactant into the water / oil mixture, a micro-structure is formed with a defined boundary between the oil and water phases: the oil penetrates the surfactant micelle and is stabilised at its core in the form of tiny droplets. The complex nature of the mobile phases in MELC allows numerous composition options (type and concentration of surfactant, oil, and co-surfactant), which can yield good separation performance compared to other chromatographic modes.

One of the main attractions of the oil-in-water MEs is the ability to solubilise compounds in a wide range of polarities, from polar to highly hydrophobic, which is of big interest in many fields. The solubilising effect on the non-polar matrices of some samples is also important. This makes it possible to analyse mixtures of compounds of different polarities and carry out the direct injection of samples, which in conventional RPLC require long previous treatments. Most applications in MELC refer to the analysis of samples containing hydrophobic compounds, in non-polar pharmaceutical products, such as creams, ointments and suppositories, and in physiological fluids and other biological matrices.

However, there are also some issues, which should be taken into account: the adsorption of surfactant on the silica-based stationary phases, the higher viscosity of the ME mobile phase which may generate higher high back-pressure, the need of more column cares, and the correct selection of the non-polar organic solvent and its concentration to form the ME droplets.

This PhD. Project includes fundamental studies to increase the knowledge in MELC. The successful application of the technique to the analysis of pharmaceutical formulations is also shown. For this purpose, mobile phases containing the anionic surfactant sodium dodecyl sulphate (SDS), octane as non-polar solvent (oil), and 1-butanol as co-surfactant, are used. The use of ionic liquids in the MEs, instead of octane, is also explored. The studies were carried out with several compounds: the parabens butylparaben, ethylparaben, methylparaben and propylparaben, and two groups of basic compounds, the β-adrenoceptor antagonists acebutolol, atenolol, celiprolol, carteolol, esmolol, labetalol, metoprolol, oxprenolol, pindolol, propranolol and timolol, and the tricyclic antidepressants (TCAs) amitryptiline, clomipramine, imipramine, maprotiline and nortryptiline. The studies on the chromatographic behaviour of these analytes considered both peak retention and profile. In the next pages, the general conclusions from each chapter in the PhD Project are outlined.

C.1 and C.2. Oil-in-water Microemulsion Liquid Chromatography

The investigations included in the PhD Project belong to the field of oil-in-water MELC, in which MEs are used as mobile phases in liquid chromatography. They represent the beginning of a new line for the research group to which the supervisors of the work belong. They became interested in 1988 in the use of organised media, performing a series of studies where

surfactant micelles were used to increase the absorptivity and fluorescence of several analytes to be used in spectrometric analysis. Following contacts with Prof. Alain Berthod from the University Claude Bernard of Lyon (France), who made pioneering research in Micellar Liquid Chromatography (MLC), the group began research in this chromatographic mode. Later, starting in 2008, the PhD. supervisors also became interested in the use of mobile phases where a relatively large amount of organic solvent is added to a surfactant solution to prevent the formation of micelles (the so-called High Submicellar Liquid Chromatography, HSLC). In recent years, the use of MEs as mobile phases in liquid chromatography had called their interest, since MEs, as submicellar media, can allow the RPLC analysis of compounds that appear excessively retained in MLC.

The number of published reports in MELC is still limited, but several researchers have offered valuable information. In any case, it is evident that this chromatographic mode is receiving growing attention. Nevertheless, despite the publication of some review articles on MELC in previous years, the given information was found contradictory and confusing. It should be considered that mobile phases prepared with MEs are complex, being formed of mixtures, in aqueous medium, of at least three reagents with a wide variety of natures (especially for the non-polar solvent and co-surfactant). Therefore, before starting the work in MELC, it was considered necessary to know in depth the information known to date. Thus, the PhD work began by carrying out a detailed study of the existing reports, which gave rise to two review articles that critically expose the fundamental aspects and selection of the experimental conditions in this chromatographic technique.

These review articles gather information on the different factors involved in MELC, updating the knowledge on the technique, and trying to understand the

conditions needed to obtain a successful separation. The large amount of information found in the published articles was organised and analysed, giving rise to an introductory guide, which can be useful for researchers interested in this chromatographic mode. The reviews were intended to encourage the development of analytical procedures in MELC, which appears as a competitive technique compared to other RPLC modes for the determination of hydrophobic compounds. Some practical tips are given to prepare stable ME mobile phases that yield reproducible results.

The most interesting aspects provided in the literature, for the development of the experimental work in MELC, are related to the information on the nature and concentration of the reagents used to prepare mobile phases containing MEs. Other relevant studies refer to the retention mechanisms, selectivity and experimental practice used in the determination of drugs in clinical and pharmaceutical samples. Oil-in-water MEs offer unique selectivity and reduced retention times, with equivalent or higher efficiency compared to conventional RPLC, resulting in satisfactory isocratic separations. The problem found in conventional RPLC, related to the exponential increase in the retention of compounds of decreased polarity (the so-called general problem of chromatographic elution) is decreased, making the elution with gradients of organic solvent less necessary.

Researchers working in MELC show MEs are competitive for some analytes, compared to mobile phases prepared with or without surfactant. In addition, these mixtures are considered more environmentally friendly, since pollution and wastes are significantly reduced. Some relevant experimental information is summarised below:

(a) *Surfactant*: It is needed to stabilise the MEs. The size of the droplets and the charge of the stationary phase and micelles depend on the type of surfactant. Therefore, the separation is highly affected by changing the surfactant molecule. The most usual surfactants in reported MELC methods are the anionic SDS and the non-ionic Brij-35. The concentration of surfactant used in the preparation of a mobile phase in MELC should be, in any case, above its critical micelle concentration (CMC), since micelles are needed to form the ME droplets.

(b) *Oil*: The choice of non-polar solvent is also important to form the ME droplets. Diverse alkanes and alcohols of varying chain length are usually employed as oils in MELC. One of the most usual oils is octane. The studied concentration range for this solvent is usually between 0 and 1.2 % *w*/*w*, but in order to elute insoluble compounds more quickly, more hydrophobic MEs are prepared by increasing the concentrations of surfactant and co-surfactant, and raising the oil content up to 2 % *w*/*w*. Recently, in order to decrease the consumption of organic solvent, reducing the wastes and environmental impact, ionic liquids (ILs) have been recommended as oil phase in MELC.

(c) *Co-surfactant*: A medium chain-length alcohol is usually added as co-surfactant to decrease the interfacial tension to nearly zero, and form stable MEs. To form successful MEs for liquid chromatography, the choice of co-surfactant appears to be more important than the choice of oil. By changing the type of co-surfactant, the polarity of the mobile phase is modified, which affects solute retention. This is decreased by increasing the concentration of co-surfactant in the ME mobile phase, since the amount of organic solvent in the aqueous component increases. The most usual co-surfactants in MELC are 1-propanol and 1-butanol. The concentration

ranges are usually in the 5–15 % *v*/*v* range for 1-propanol, and 6.6–16.5 % *v*/*v* for 1-butanol. Out of these ranges, ME are not formed, and above 8.6 % *v*/*v*, the column back-pressure increases excessively.

(d) *Other reagents*: The water continuous phase in MEs usually contains other additives, such as buffer reagents to control the pH of the mixture. As for other RPLC modes, the retention of ionisable compounds is strongly affected by the pH of the mobile phase.

(e) *Columns*: Most common columns used in RPLC, packed with chemically bonded C18 or C8 stationary phases, with 3–5 μm particle size, are also usual in MELC. Since MEs can yield high viscosity mobile phases, excessive back-pressure can affect the column. To solve this limitation, the use of shorter columns has been suggested. Monolithic columns can give rise to shorter analysis times, especially at higher flow-rates.

The complexity of the composition in a successful ME, and the fact that the different factors affecting the retention interact each other, may require many manipulations during method development to achieve an acceptable separation for complex multi-analyte samples. This is the reason of the proposal from several authors of a standard ME, as starting point, when developing a method for a new separation with no previous reports. Based on these initial conditions, several authors have proposed computer-assisted approaches to optimise the composition of ME mobile phases, reducing significantly the time and reagent consumption for method development.

Apparently, the preparation of mobile phases in MELC and their management can be rather difficult, if no information nor previous experience is available. Before beginning our research, several weeks were devoted to reproduce the work carried out in the report by Altria, Marsh and Clark, High-

performance liquid chromatographic analysis of pharmaceuticals using oil-in-water microemulsion eluent and monolithic column (Chromatographia 63 (2006) 309–314), using the same conditions and sequence of runs, in order to learn the technique. We were interested in verifying that the experimental work carried out to be useful in liquid chromatography was correct. These authors used a standard mobile phase prepared with SDS, octane and 1-butanol, to find the optimal conditions in an MELC procedure for parabens. We also adopted this composition, and other compositions in our work were obtained by modifying the concentration of only one reagent (surfactant, oil or co-surfactant), at a time. Throughout the PhD. work, the preparation of useful MEs for liquid chromatography was mastered, which gave rise to new recommendations to implement successful procedures.

C.3. Comparison of surfactant-mediated liquid chromatographic modes with sodium dodecyl sulphate for the analysis of basic drugs

The research group has long experience in the development of methods for the analysis of basic compounds, starting in 1996. Basic compounds are of big interest in pharmacology, because many active principles have this character, whose control is carried out mainly by liquid chromatography. However, the analysis of basic compounds using conventional RPLC has been a challenge since the first reports, since in the usual mobile phase pH range (below 7), basic compounds form cationic protonated species that interact with residual anionic silanols on silica-based stationary phases (the most usual in the experimental practice). This produces poor performance (increased retention and formation of broad and tailed peaks). Peak profile can be significantly improved by adding diverse reagents (additives) that are adsorbed on the stationary phase, such as

some surfactants or ionic liquids, which coat the column, preventing the access of solutes to free anionic silanols.

Besides amines, which are classically used as additives in RPLC, SDS used in the mobile phases in MLC is capable of suppressing the deleterious silanol effect. The long hydrophobic chains of SDS monomers are associated to the chains in the alkyl-bonded phases (usually C18), with the sulphate group oriented outside from the surface. This creates a stationary phase with a negative charge that masks silanols, but it is also able to attract the cationic solutes, which is a drawback since long retention times are yielded forcing the addition of a large amount of organic solvent to the mobile phase to increase the elution strength. In studies carried out by the group starting in 2008, it was found that when SDS is added at moderate concentration in the presence of a high content of organic solvent, such as 1-propanol (HSLC), advantageous procedures are obtained, in spite that micelles are not formed. At the beginning of this PhD work, it was thought that MELC could be even more advantageous, since it would make possible the further reduction of the amount of organic solvent, giving rise to a greener method.

There was no previous reference describing the application of MELC to basic compounds. It was thus found interesting to carry out a detailed study to examine this possibility, comparing exhaustively MELC with the results obtained in conventional RPLC, MLC and HSLC, the two latter techniques using also the anionic surfactant SDS. A comprehensive study of the change of behaviour, with regard to peak retention and profile, was thus made, as the environment inside the column (presence of surfactant micelles or monomers in the mobile phase, and nature of the stationary phase) was changed, using the different chromatographic modes. The modifications in column performance were discussed, when the concentration of SDS (in the surfactant-mediated liquid

chromatographic modes), octane (in MELC), and the organic solvents acetonitrile, 1-propanol and 1-butanol (in the different modes) were varied.

A group of eleven β-adrenoceptor antagonists (acebutolol, atenolol, carteolol, celiprolol, esmolol, labetalol, metoprolol, oxprenolol, pindolol, propranolol, and timolol) were used as probe compounds. This group of compounds are ideal for studying the behaviour of basic compounds, since there is a large number of commercialised compounds with diverse polarities, and the kinetics of the interactions with the stationary phase is similar for different compounds. The effect produced at varying concentrations of the experimental factors was examined, by observing variations in the retention of solutes and peak profiles, which affected the resolution. Another factor examined in the study was the consumption of organic solvent.

The study showed the possibilities of MLC, HSLC and MELC. The retention times of basic compounds, which were high with mobile phases containing only surfactant, could be modulated to practical values by the addition of different amounts of one or two organic solvents, giving rise to competitive procedures. Submicellar media (HSLC) reduce the retention and improved the chromatographic efficiency, in the separation of the β-adrenoceptor antagonists, compared to conventional RPLC and MLC. MELC also allowed very short analysis times, but with a smaller amount of organic solvent to get the elution of the most retained solutes, compared to the submicellar mode. Obtaining narrower and symmetric peaks in MLC, HSLC and MELC, compared to conventional RPLC, reveals that the silanols are effectively masked.

The concentration ranges explored in MELC were 0.104 to 0.173 M for SDS (which guaranteed the formation of micelles), 0.28 to 1.28 % for octane, and 8.2 to 17.3 % for 1-butanol. Outside these ranges, MEs were not stable or could not

be formed. The concentration of 1-butanol could not be increased above 17.3 %, due to excessive back-pressure, which could damage the column.

HSLC with acetonitrile offers the best peak profiles. However, the high volume of organic solvent that this mode requires to achieve sufficiently small retention times, for most hydrophobic solutes, makes this technique less attractive. In contrast, MELC yields a significant reduction of the retention times using very small amounts of the organic solvents (1-butanol and octane). With an adequate optimisation, it is possible to get satisfactory resolution in the separation of mixtures of the β-adrenoceptor antagonists in just a few minutes.

In general, chromatographic peaks are characterised by their height, position, width, and skewness, the last two parameters depending on the values of the left and right peak half-widths. A decade ago, the research group reported simple correlations between the values of the peak half-widths and their retention times, which were called half-width plots. For isocratic elution, the plots are actually parabolic, although often the parabolas can be approximated to straight-lines. The plots are obtained with the data of peak half-widths and retention times, for a set of solutes that experience the same kinetics of interaction, which are eluted with a mobile phase with a fixed or variable composition. When the resistance of solutes to mass transfer is different, the plots show some scattering. This PhD Project shows that the construction of half-width plots is very useful for the prediction of the peak profiles in the chromatograms for optimisation purposes. In addition, the plots reveal the similarity in the kinetics of interaction of solutes, when they are analysed in different conditions.

The experimental data obtained along this work allowed a global comparison of the performance of four chromatographic modes (conventional RPLC, MLC, HSLC and MELC). The reduction in the silanol effect using the surfactant-mediated modes was significant. The most remarkable characteristic is that the

slope of the right half-width was very similar to the slope of the left half-width, due to the formation of almost symmetrical peaks at diverse retention times. In conventional RPLC, the peaks were significantly tailing, while in HSLC with 1-propanol they changed from tailing to fronting when the concentration of SDS was low (0.02–0.04 M) and the concentration of 1-propanol, high (25 %).

The high efficiencies obtained in HSLC and MELC guaranteed high resolution. The elution order of the β-adrenoceptor antagonists in MLC and HSLC changed with respect to conventional RPLC, and was also different between HSLC and MELC. Peak resolution was maximal with the mobile phase containing SDS and 35 % acetonitrile (HSLC), because of the wider peaks in MELC, albeit at the cost of a higher consumption of organic solvent.

C.4. Modelling the retention in Microemulsion Liquid Chromatography

Retention modelling, depending on the composition of the mobile phase, is a common task in the chromatographic practice, of big importance in liquid chromatography in order to find the optimal separation conditions, and understand the retention mechanism of the eluted compounds. The research group had previous experience in the modelling of retention in conventional RPLC with hydro-organic mobile phases, as well as with mobile phases with additives, with excellent results. Although there was some background in the field of optimisation in MELC from other authors, the methods were not interpretive (i.e., based on models). Therefore, it was considered interesting to develop a mathematical equation that allowed the description of the effect, on the retention of compounds of different nature, of each of the main factors in MELC (concentrations of SDS, octane and 1-butanol), considering their mutual interaction.

The study was carried out with two groups of compounds with different nature (parabens and β-adrenoceptor antagonists). Pure micellar mobile phases (i.e., without organic solvent) provided extremely long retention times for both families of compounds, fully unpractical to be used in analysis. To obtain sufficiently short retention times for these compounds, it was necessary to add an organic solvent that offered high elution strength, such as 1-butanol. In MELC, the analysis times of mixtures of parabens and β-adrenoceptor antagonists was decreased further down to 4–5 min by adding octane. Thus, for example, the analysis time for parabens and β-adrenoceptor antagonists was 5 and 6.5 min, respectively, using 0.10 M SDS, 1 % octane and 7 % 1-butanol as mobile phase (MELC), and 4.4 and 5.7 min with 0.18 M SDS and 12 % 1-butanol (MLC in the presence of a high amount of surfactant).

No rigorous study on the concentration ranges of the ME reagents that must be mixed to form stable MEs was found in the published literature in MELC. It should be noted that, in order to guarantee the success in their formation, not only the choice of components is important, but also the concentration in the mobile phase of each component. Since we were interested in investigating the modelling performance of retention in wide concentration ranges, it was necessary to previously ensure the ranges that lead to the formation of MEs, instead of emulsions. Therefore, with the aim of verifying the formation of a transparent medium, suitable to be used in liquid chromatography, modelling of retention was preceded by a study based on the visual observation of a number of mixtures, in order to establish the concentration ranges of SDS, octane and 1-butanol that could be mixed to form a stable ME, avoiding the formation of an emulsion that could damage the equipment or column. The study also showed the time period MEs remained stable. After mixing the reagents, the mixtures were allowed to stand for at least 12 hours. After this, they were centrifuged,

and when did not initially give rise to two well-differentiated phases (i.e., an emulsion), the mixtures were left several weeks at rest to check the ME stability.

A plot depicting the limits of octane and 1-butanol where MEs were stable, at two concentrations of SDS (0.10 M and 0.18 M), was designed. The concentration plot indicated that, with a relatively low amount of 1-butanol and 0.10 M SDS, at increasing concentration of octane, MEs are unstable after a few weeks, but by increasing the amount of SDS to 0.18 M, phase separation was not observed.

Despite the benefit that the use of a higher concentration of 1-butanol could bring over the solubilisation of a higher amount of octane, the upper concentration limit of co-surfactant was limited to avoid high pump back-pressure, which could damage the chromatographic column and apparatus. On the other hand, when a low amount of octane (0.2 %) was added, phase separation was not visible at any assayed SDS concentration, even with very low concentration of 1-butanol. This indicated that the surfactant was capable of solubilising small amounts of octane without the need of co-surfactant. Finally, with the aim of preserving column performance, avoid damage to the apparatus and reduce the environmental impact, the upper limit of 1-butanol was set at 12 %, in the absence and presence of octane (for MLC and MELC, respectively).

The feasibility of modelling the retention in MELC, with satisfactory accuracy, considering altogether the three components in the mobile phase (surfactant, alcohol and oil), was demonstrated, with errors in the 1.1–2.5 % range. The equation derived was based on a previous model that the research group proposed in 1996 to describe the retention, using micellar mobile phases containing a co-surfactant (hybrid MLC). In general, MELC data yielded better fitting of the retention behaviour compared to MLC, with fitting errors in the 0.43–3.2 % range. The study was carried out at a mobile phase pH slightly

above 2, using trifluoroacetic acid to fix it, and with a column with high pH tolerance (XTerra). However, the results would also be satisfactory with conventional stationary phases buffered at pH 3–3.5.

When performing the fitting of the retention data to the model, assisted by Microsoft Excel Solver application, the convergence process was observed to be problematic, since it required initial values very close to the optimum to succeed. To solve this problem, the equation that described the retention was transformed moving the origin to the mobile phase that showed maximal retention (i.e., the phase with the smallest elution strength). The influence of each modifier on the elution strength was very similar for all probe compounds, with mean values of 0.072, 0.119, and 0.98 for surfactant, co-surfactant and oil, respectively. Therefore, octane offered the highest elution strength, appreciably above SDS and 1-butanol. The proposed model for MELC revealed that, when octane is inserted inside the micelle, this is modified. Therefore, the interaction between solute and micelle is changed, as indicated by the values of the model parameters in both MLC and MELC.

Despite the fact that the mobile phase with 0.18 M SDS, 1 % octane and 12 % 1-butanol showed the best results in terms of analysis time, for both parabens and β-adrenoceptor antagonists, peak resolution was only satisfactory for parabens, making this mobile phase the most suitable. For β-adrenoceptor antagonists, the peaks of atenolol and carteolol were overlapped; therefore, a mobile phase with smaller octane concentration (0.25 %) was more convenient.

C.5. Analysis of tricyclic antidepressants in pharmaceuticals

The work carried out with the β-adrenoceptor antagonists (with basic character and high or intermediate polarity) indicated the suitability of MELC for the analysis of basic compounds. As explained, the chromatographic performance of basic compounds is very poor with conventional RPLC (long analysis times, deformed peaks and high consumption of organic solvent), but in MLC with the anionic surfactant SDS the retention times are also rather high, requiring high amount of organic solvent to decrease the retention. This is especially problematic in the analysis of tricyclic antidepressants (TCAs), which are compounds very widely used, with basic character and very low polarity. The development of an analytical method to analyse TCAs in pharmaceutical preparations was thus considered that took advantage of the previous research. A mobile phase of 0.173 M SDS, 1.42 % (*v*/*v*) octane, 8.15 % (*v*/*v*) 1-butanol and UV detection was used. The method demonstrated advantages with regard to the analysis time and consumption of organic solvent.

In fact, the research group had been interested since 2003 in the control of these compounds in pharmaceutical formulations, for which conventional RPLC offers very poor results. Trying to improve these analyses, MLC with mobile phases containing SDS and 1-pentanol or aqueous solutions of Brij-35 without organic solvent were proposed, starting in 2012. Hence the interest in performing a comparative study of the chromatographic behaviour of TCAs, when hydro-organic mixtures, micellar media and MEs are used as mobile phases. Analytical performance was compared, in terms of intra- and inter-day linearity, accuracy and precision.

An extensive method validation was carried out, which included five TCAs (amitriptyline, clomipramine, imipramine, maprotiline and nortriptyline) and

five pharmaceutical preparations (each containing one TCA), commercialised in Spain. The results were very satisfactory, with good recoveries and very simple sample preparation without the need of any pre-treatment, requiring only solubilisation and filtration prior to injection. The recoveries were in the 80-120 % range, which is considered acceptable for finished pharmaceutical products. Therefore, the optimised MELC method is useful for the quality control of pharmaceuticals that contain TCAs. An advantage of the MELC procedure is the reduction in the retention times, compared to conventional RPLC and MLC with SDS at the same concentration, even using 1-butanol in MLC as co-surfactant. The MELC procedure maintains the good peak profiles achieved in MLC.

The validation of the method was made according to the ICH (International Conference of Harmonization) guidelines and offered good results for the tested drugs, with the following results:

(a) The calibration curves met the linearity requirements, with determination coefficients $R^2 > 0.990$. The slopes and intercepts of the fitted straight-lines were stable during three non-consecutive days and along three different weeks, indicating column performance was maintained with a good prediction capability of the concentrations of analytes from the fitted regression straight-lines.

(b) Intra and inter-day precisions were always below 2.5 %, and the intra- and inter-day accuracies were in the –0.9 % to +1.2 %, and –1.7 % to +0.5 %, ranges, respectively.

(c) Limits of detection and quantification for TCAs were usually below 0.09 µg/mL and 0.31 µg/mL, respectively, except for maprotiline, which were 1.15 µg/mL and 3.85 µg/mL.

(d) Robustness was evaluated by modifying the flow-rate and concentrations of SDS, octane and 1-butanol in the mobile phase. Each of these factors was varied within a range around the value used to develop the analytical procedure, following the one-variable-at-a-time (OVAT) method, where the variables are varied one by one, keeping all other constant at their original value. The values of relative standard deviation (RSD) for the retention times were usually below 2 %, corresponding the highest values to the concentration of octane, which confirms the important role of the oil in the formation of the ME. A higher variability was obtained for the peak areas.

The results were compared with those obtained with procedures using mobile phases containing either 35 % (*v*/*v*) acetonitrile, 0.075 M SDS / 6 % (*v*/*v*) 1-pentanol, or 0.02 M Brij-35 without organic solvent. Method precision was better for the MELC procedure, and the micellar mode with SDS and 1-pentanol, with RSD values usually below 2 %. Meanwhile, for the hydro-organic and Brij-35 pure micellar modes, the inter-day precision expressed as RSD ranged from 0.65 % to 3.1 %. The LODs and LOQs were smaller for the MELC procedure, except for amitryptiline and maprotiline, which yielded smaller values with the hydro-organic procedure.

C.6. Solute interactions in chromatographic modes with surfactant and ionic liquid

Ideally, the best organic solvent would be no solvent (only water), considering health hazards, waste generation and economy. However, the absence of organic solvent is not always possible. Therefore, greener solvents have been proposed to substitute the organic solvents conventionally employed, to decrease the environmental impact and risk of chemical exposure. In this regard, ionic liquids, which are salts frequently in liquid state at room temperature, formed by a bulky organic cation associated with a usually smaller inorganic / organic anion, have called high attention in several scientific and technological fields.

In the field of MELC, a report published by Peng *et al*. in 2017 for the analysis of neutral phenolic acids, using a water-immiscible IL (1-hexyl-3-methylimidazolium hexafluorophosphate, $[C_6C_1IM][PF_6]$) to replace octane as oil in the mobile phase with SDS and 1-butanol, is relevant. It should be noted that the class of MEs used by these authors is aqueous IL-based MEs, consisting of IL, water and surfactant (and in some cases, an alcohol as co-surfactant). However, in the literature, these MEs are usually formed by non-ionic surfactants, such as Brij-35 and Triton X-100, instead of the anionic SDS.

As commented, the analysis of a group of cationic basic drugs (β-adrenoceptor antagonists), using MEs of SDS, octane and 1-butanol, was previously investigated. In view of the results by Peng *et al.*, the feasibility of aqueous IL-based MEs with IL, instead of oil, was considered interesting to eliminate octane from these analyses. The use of $[C_6C_1IM][PF_6]$ was first considered, but later the research was extended to other imidazolium ILs with alkyl chains of diverse lengths (n = 2, 4 and 6), associated to the Cl^-, BF_4^-, or PF_6^- anions. These ILs offer diverse solubility and adsorption capability on C18 stationary phases,

and are the most common added to the mobile phase in RPLC. The study allowed to increase the knowledge on the effect of IL cation and anion on the chromatographic system in the presence of ionic additives, with consequences on solute retention and peak profile for the cationic drugs. The results were interpreted by comparison with mobile phases containing ILs without SDS, and acetonitrile instead of 1-butanol.

Plots of retention, versus the concentration of additive, showed that in the IL-based MEs the anionic surfactant SDS competes with the IL anions for adsorption on the chromatographic column. The observed behaviour (decreased retention at increasing concentration of IL) is similar to that found in the absence of SDS, for ILs formed by a cation with sufficiently strong adsorption associated to a weakly adsorbed anion (case of $[C_6C_1IM][BF_4]$ and $[C_6C_1IM][Cl]$). Meanwhile, in the absence of SDS, for an IL containing a less adsorbed cation or a more strongly adsorbed anion, the retention is kept constant or increases showing a maximum at a particular concentration of IL, depending on its nature. Nevertheless, in all mobile phases with ILs, the peak profiles of the basic compounds was improved, in comparison with hydro-organic mixtures in RPLC, giving rise to symmetrical (or almost symmetrical) peaks, with a stronger effect in the presence of SDS. The peak profile enhancement is explained by the masking effect of the silanol effect by the additives.

When octane was replaced by an IL, the role of 1-butanol was less important to form transparent and stable mixtures with SDS, useful for RPLC. Moreover, the surfactant allowed more concentrated solutions of the ILs, which suggested the formation of an organised SDS-IL structure in the mobile phase. Since 1-butanol yielded too short retention with low resolution for the basic compounds, its elimination was investigated. In effect, a mobile phase composed of only the surfactant sodium dodecyl sulphate (SDS) and the IL $[C_6C_1IM][Cl]$

gave rise to promising results: satisfactory peak profiles and good resolution, with still appropriate retention for the studied compounds. We should note that, in these mixtures, the retention of the basic compounds can be modulated to reach practical values, by modifying the concentrations of SDS and IL, based on the attraction of the cationic basic compounds to the SDS anion and repulsion from the IL cation, without the requirement of adding an organic solvent. This may give rise to an interesting "green mobile phase" (note that a mobile phase with only IL requires the addition of organic solvent to get appropriate analysis times, and the retention in mobile phases with only SDS is excessive). It is expected that the retention behaviour will depend on the concentration ratio of IL and surfactant in the mobile phase, and the nature of the added IL.

In the literature, there has been big interest in the synthesis of surface active ILs, where the IL cation is associated to the anion of a conventional surfactant. A similar environment in aqueous solution is obtained with the assayed mixture of alkyl-imidazolium IL chloride and the sodium salt of dodecyl sulphate (SDS), where the micelles are composed seemingly of alternate palisades of the IL cation and surfactant anion. It should be noted that there is no previous work on the use of such additive combination in RPLC.

CONTRIBUTION OF THE PhD WORK TO PUBLISHED ARTICLES

The chapters in this PhD. work correspond to the following publications, listed according to the publication date. The percentage of contribution of Nikitaben Pankajkumar Patel as PhD. student is indicated with each article.

- Nikitaben 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 (Chapter 2).
  Contribution: 100% Nikitaben Pankajkumar Patel

- Ester Peris García, Nikitaben Pankajkumar Patel, Samuel Carda Broch, María José Ruiz Ángel, María Celia García Álvarez-Coque
  Oil-in-water Microemulsion Liquid Chromatography.
  Separation & Purification Reviews. 49 (2020) 89–111 (Chapter 1).
  Contribution: 100% Nikitaben Pankajkumar Patel

- Nikitaben Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
  Comparison of surfactant-mediated liquid chromatography modes with sodium dodecyl sulphate for the analysis of basic drugs.
  Analytical Methods 12 (2020) 2443–2452 (Chapter 3).
  Contribution: 100% Nikitaben Pankajkumar Patel

- Nikitaben Pankajkumar Patel, Ester Peris García, Olga Schiopu, María José Ruiz Ángel, Juan José Baeza Baeza, María Celia García Álvarez-Coque
  Modelling the retention in Microemulsion Liquid Chromatography
  Journal of Chromatography A 1634 (2020) 461651 (Chapter 4).
  Contribution: 70% Nikitaben Pankajkumar Patel; 30% Olga Schiopu

- N. Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
  Analysis of tricyclic antidepressants in pharmaceuticals by Microemulsion Liquid Chromatography
  Microchemical Journal 160 (2020) 105659 (Chapter 5).
  Contribution: 100% Nikitaben Pankajkumar Patel

- Nikitaben Pankajkumar Patel, Ester Peris García, María José Ruiz Ángel, María Celia García Álvarez-Coque
  Solute interactions in Microemulsion Liquid Chromatography with ionic liquids

  In preparation (2021) (Chapter 6).
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The supervisors of this PhD. work (María Celia Álvarez-Coque, and María José Ruiz Ángel) appear as co-authors. Both of them belong to the FUSCHROM (FUndamental Studies in CHROMatography) group, whose main researcher is María Celia García Álvarez-Coque.

The articles in Chapters 1 and 2 were written in collaboration with Samuel Carda Broch, Professor at the University Jaume I in Castellón, who contributed in the beginning of the group's research in the field of Microemulsion Liquid Chromatography.

Ester Peris García participated in the whole 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). The research work in Chapter 4 contributed to the training of the Degree student Olga Schiopu.