FACULTAT DE QUÍMICA

Strategies to find optimal resolution in liquid chromatography

Estrategias para encontrar la resolución óptima en cromatografía líquida

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

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CERTIFICAN

Que la presente Memoria, "Estrategias para encontrar la resolución óptima en cromatografía líquida", constituye la Tesis Doctoral de

Dña. TAMARA ÁLVAREZ SEGURA

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 veinte de junio de dos mil dieciocho.

María Celia García Álvarez-Coque

José Ramón Torres Lapasió

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RESUMEN

La cromatografía líquida de alta resolución o alto rendimiento es actualmente la técnica de separación analítica más utilizada, debido a su versatilidad, fiabilidad, robustez y sensibilidad. Desafortunadamente, en cromatografía líquida, la eficacia suele ser inferior a la ofrecida por la cromatografía de gases, electroforesis capilar y otras técnicas de electromigración. Con una menor eficacia, no es posible el análisis de muestras de muy elevada complejidad. Así, se ha dedicado un considerable esfuerzo en investigación, tanto básica como aplicada, al incremento de la eficacia y selectividad, produciéndose avances muy significativos desde los orígenes de la técnica.

A pesar de los progresos conseguidos en las últimas décadas, con el desarrollo de una instrumentación cada vez más sofisticada (incluida la introducción de bombas de presión ultra-alta) y los avances en la tecnología utilizada para preparar columnas (con la síntesis de nuevos soportes y fases estacionarias), quedan desafíos por resolver, puesto que la necesidad de analizar muestras cada vez más complejas, en tiempos más cortos, es permanente.

Las investigaciones realizadas a lo largo del desarrollo de la Tesis Doctoral, y recogidas en la presente Memoria, contribuyen a la mejora del rendimiento de la cromatografía líquida, particularmente en su variante de fase inversa. Concretamente, se realizan propuestas relacionadas con la optimización de sistemas multi-columna en cromatografía líquida monodimensional y el desarrollo de nuevas funciones objetivo para medir la capacidad separativa de los sistemas cromatográficos, con motivos de optimización. La Memoria también incluye un desarrollo fundamental que mejora la medida de la dispersión producida en las columnas cromatográficas. A continuación, se resumen las investigaciones realizadas.

1. Optimización de sistemas multi-columna en cromatografía líquida monodimensional

El nivel máximo de resolución que es capaz de proporcionar una sola columna resulta a menudo insuficiente. Una solución relativamente sencilla es el acoplamiento de dos o más columnas convencionales, mediante acopladores de volumen muerto cero. Esta estrategia permite modular la naturaleza de la fase estacionaria para adaptarla a las necesidades de resolución y tiempo de análisis de una muestra determinada. Una segunda ventaja es que se puede implementar en instrumentos cromatográficos convencionales (equipados con una única bomba), y utilizar cualquier tipo de columna, siempre y cuando las condiciones de elución sean compatibles. En la actualidad se comercializan columnas cortas con fases estacionarias muy distintas y múltiples longitudes, lo que multiplica las posibilidades de éxito de los acoplamientos en serie.

La Memoria de Tesis incluye una revisión bibliográfica que analiza los desarrollos propuestos hasta la fecha, basados en la aplicación de la estrategia de combinación de columnas en serie. En los artículos publicados, los autores indicaron que no fueron capaces de resolver sus muestras con columnas individuales, mientras que encontraron una sustancial mejora cuando combinaban dos o más columnas. El acoplamiento de columnas en serie resulta, además, particularmente interesante para resolver el denominado "problema general de la resolución cromatográfica": la obtención de tiempos de retención que aumentan exponencialmente con la hidrofobicidad de los solutos, y cuya corrección conduce inevitablemente a la coelución de los solutos menos hidrofóbicos. La incompatibilidad entre la mejora del nivel de resolución y la disminución del tiempo de análisis constituye probablemente la mayor limitación de las separaciones isocráticas. Hasta ahora, la solución más usual ha

sido la aplicación de elución en gradiente. El acoplamiento de columnas en serie puede constituir una solución alternativa a los gradientes, con importantes mejoras en la selectividad. Esto representa un estímulo para el desarrollo de nuevos procedimientos isocráticos, especialmente en combinación con detección mediante espectrometría de masas.

En la Memoria de Tesis se investigan algunas estrategias para que la metodología de acoplamiento de columnas resulte de utilidad para los laboratorios de rutina e investigación, sobre todo desarrollando métodos de optimización de las condiciones de elución (contenido de disolvente orgánico, flujo o temperatura), y de selección de la combinación de columnas más apropiada, tanto en modo isocrático como en gradiente. Aunque todavía no es posible proporcionar recomendaciones específicas sobre las columnas que resultarán a priori más adecuadas para una aplicación particular, se puede afirmar que basta considerar una pequeña cantidad de fases estacionarias convencionales (por ejemplo, C18, ciano, amino y fenilo), para mejorar muy sustancialmente la resolución en separaciones donde aisladamente fallan. Una correcta selección de columnas (naturaleza y longitud), y de eluyente (fase móvil isocrática o programa de gradiente), multiplica de manera impresionante las probabilidades de éxito, especialmente a través de la aplicación de estrategias de optimización interpretativa y simulaciones por ordenador. El uso de columnas acopladas en serie tiene la ventaja de ser accesible a cualquier laboratorio, frente a las separaciones bidimensionales, que requieren una inversión y capacitación muy importantes, por lo que son mucho más restrictivas.

Con la conexión en serie de dos o más fases estacionarias mediante columnas de diferente longitud se produce una transición casi continua de selectividad, que permite adaptar el sistema separador a los requisitos de una

muestra en particular. Las consecuencias de combinar columnas que contienen diferentes fases estacionarias en serie equivalen, a nivel de resultados, a lo que en el laboratorio conseguiríamos si dispusiéramos de cientos, miles o incluso millones de columnas. Puesto que cada combinación de columnas se comporta como una nueva columna, el hecho de combinarlas en serie equivale a multiplicar la cantidad de columnas disponibles en el laboratorio. Con ello, la probabilidad de disponer de la "columna ideal" aumenta enormemente. Sin embargo, para conseguir convertir a la estrategia de combinación de columnas en una herramienta general, verdaderamente poderosa para la resolución de muestras complicadas, se han de resolver antes algunos problemas técnicos.

Primero, las columnas cromatográficas se deben poder conectar sin afectar a la naturaleza de la fase estacionaria en la proximidad de las conexiones entre columnas, y sin introducir volúmenes muertos. Estos problemas se han resuelto mediante el uso de conectores de volumen muerto cero, que disponen de roscas universales y permiten la conexión directa de columnas de distintos fabricantes, de un modo fiable y reproducible. En segundo lugar, se necesita un sistema que anticipe la combinación más correcta, y optimice su rendimiento. Para ello, se ha perfeccionado, dotándolas de nuevas características, algunas herramientas anteriormente desarrolladas en el laboratorio de investigación para la optimización de columnas individuales, tanto en separaciones isocráticas, como en gradiente. Con todo ello se construyó un software que permite explotar en todo su potencial las ventajas que ofrece la combinación de columnas en serie.

Seguidamente, se presentan algunos estudios sobre las posibilidades que ofrecen los métodos que utilizan columnas acopladas en serie para cromatografía líquida y se detallan las herramientas matemáticas necesarias para realizar la optimización de las condiciones de separación. Se estudia la separación de una mezcla de 15 sulfonamidas, considerando cuatro tipos de

fase estacionaria de diferente naturaleza: dos columnas C18 (una convencional y otra que incluye grupos polares), y otras dos que contienen una fase fenilo o una fase ciano. Todas estas columnas mostraron, cuando se utilizaron individualmente, un rendimiento insuficiente para la separación de las 15 sulfonamidas. Sin embargo, al usar una combinación optimizada de columnas, se obtuvo una excelente separación, tanto en términos de resolución como de tiempo de análisis. Estos resultados sugieren que se conseguirán igualmente buenas resoluciones en otras muestras complejas.

En realidad existen dos formas de conseguir que dos o más columnas cooperen en separaciones monodimensionales: su combinación en serie o en paralelo. En el segundo caso, se deben realizar dos (o más) inyecciones, cada una de ellas con una columna distinta, o bien dividir el flujo de fase móvil en dos corrientes idénticas, haciendo pasar cada una de ellas por una columna y detector distintos, ejecutando una única inyección. En la Memoria de Tesis se estudian las prestaciones de cada una de estas formas de acoplamiento, utilizando de nuevo la mezcla de 15 sulfonamidas y fases móviles en las que se modifica solamente un factor experimental (el contenido de disolvente orgánico), realizando la elución en régimen isocrático.

La optimización de columnas en paralelo de diferente naturaleza se llevó a cabo aplicando el principio de complementariedad al caso de dos inyecciones independientes (nótese que igualmente se puede extender a más de dos). Este principio se basa en dedicar una condición de separación a la resolución óptima de sólo algunos compuestos en la muestra, mientras que los otros -que permanecen sin resolver-, son resueltos de manera óptima en una segunda condición de separación. El rendimiento de las diferentes configuraciones se evaluó mediante el cálculo de las resoluciones limitantes (máximo nivel de resolución ideal que se puede conseguir considerando todas las condiciones

posibles de separación) y el porcentaje de capacidad de separación alcanzado en las condiciones óptimas, con cada sistema cromatográfico (refiriendo las resoluciones óptimas alcanzadas a los valores limitantes).

En el caso de estudio, la separación en serie superó ampliamente en prestaciones a las columnas en paralelo. En un estudio anterior, se había demostrado que si se introducía una mayor diversidad en la composición de la fase móvil (por ejemplo, uso de dos modificadores, o contenido de disolvente orgánico junto al pH), se obtenían buenas separaciones para muestras altamente complejas, utilizando una única columna e inyecciones paralelas. En el ejemplo estudiado, sin embargo, se utilizó sólo un factor en la fase móvil (la concentración de un único disolvente orgánico), por lo que las variaciones que se consiguen en la selectividad al cambiar la composición de la fase móvil son mucho más pequeñas, y por lo tanto, también las posibilidades de éxito.

Las consecuencias de las variaciones en longitud de las columnas son muy diferentes dependiendo de que el acoplamiento se realice en serie o en paralelo. En las separaciones en paralelo, no se producen cambios en la selectividad, mientras que la longitud de las columnas afecta muy notablemente a la selectividad en las separaciones en serie. Por lo tanto, aunque se intente realizar la comparación entre ambos métodos en idénticas condiciones, para evitar favorecer a una de las opciones, es imposible evitar la ventaja intrínseca que poseen las columnas acopladas en serie: la existencia de un factor de separación adicional (la longitud de la columna), con dramáticos efectos sobre la selectividad.

En la Memoria de Tesis se demuestra que, incluso aquellas columnas que ofrecen un rendimiento separador muy bajo cuando se usan de forma aislada, pueden dar lugar a una separación completa cuando se acoplan en serie. En nuestro estudio demostramos que, si bien las combinaciones que ofrecían una

mejor resolución implicaban cuatro columnas, era posible encontrar soluciones alternativas excelentes acoplando sólo dos columnas de polaridad diferente (como fenilo y ciano). Curiosamente, la mejor combinación tenía una mayor contribución de la columna más rápida colocada en primera posición. Esta misma conclusión se ha observado sistemáticamente en otras separaciones, por lo que constituye una recomendación general.

Por otro lado, es necesario comentar que con el uso rutinario, las columnas cromatográficas inevitablemente sufren un deterioro gradual y, como resultado, el nivel de resolución en los cromatogramas reales difiere progresivamente de los resultados esperados. Cuando el nivel de deterioro es ya considerable, es necesario pronosticar unas nuevas condiciones de separación óptimas, puesto que los modelos descriptores de la retención y forma de los picos, desarrollados inicialmente, ya no son válidos. El cromatografista se ve entonces en la coyuntura de decidir si repite todo el proceso experimental de modelización (lo que resulta poco deseable, ya que supondría el consumo de tiempo y reactivos, particularmente cuando se utiliza una metodología en la que aparecen involucradas varias columnas acopladas), o bien corrige los pronósticos, a partir de un número reducido de experiencias que cuantifiquen el cambio en el rendimiento separador.

En la Memoria de Tesis, se desarrolla un método práctico para adaptar las predicciones de tiempo y perfil de pico a las nuevas condiciones, en las que la(s) columna(s) ha(n) perdido capacidad separadora por envejecimiento. Así, los modelos originales se corrigen mediante la introducción de parámetros que cuantifican el nivel de deterioro, que se obtienen a partir de la elución de un pequeño subconjunto de compuestos. Dichos compuestos se seleccionan de entre aquéllos medidos en el conjunto de entrenamiento, con una elución representativa, comparando las medidas antes y después de haber sufrido las

columnas pérdidas en su rendimiento. Los parámetros de envejecimiento son comunes para el conjunto de solutos analizados, y se obtienen por regresión, de tal manera que se minimizan las discrepancias entre los datos predichos con los modelos de retención originales para la columna completamente nueva, y los datos experimentales medidos para la columna envejecida.

El método de corrección fue desarrollado y validado mediante la predicción del comportamiento cromatográfico del conjunto de 15 sulfonamidas anteriormente utilizado, que se analizó con las columnas individuales y combinadas en serie, utilizando tanto elución isocrática como de gradiente. A partir de la cuantificación del envejecimiento, se pronosticó el comportamiento separador esperado para el conjunto de compuestos con una columna o un conjunto de columnas acopladas. Se observó una concordancia excelente entre los cromatogramas previstos y experimentales en las columnas envejecidas.

Los resultados sugieren que, siempre que las columnas mantengan un rendimiento suficiente, es posible aprovechar el extenso trabajo experimental realizado cuando el sistema se modelizó para las columnas recién estrenadas. Con esta información, y un esfuerzo experimental adicional mínimo, es posible obtener predicciones exactas en condiciones de degradación moderada. Por lo tanto, la modelización completa del comportamiento cromatográfico sólo se lleva a cabo cuando las columnas son nuevas o los solutos se analizan por primera vez.

Quizá la principal limitación del método propuesto es que el tratamiento no considera efectos específicos (dependientes del soluto), que se ven amplificados cuando el nivel de deterioro es grave. Además, si los cambios en la química de la columna afectan a la cinética de adsorción-desorción, las predicciones de semi-anchura también serán dependientes del soluto. La

corrección de estos efectos implica un mayor esfuerzo experimental, por lo que se pierde el interés práctico de la metodología propuesta. Por supuesto, desde una perspectiva de calidad, las columnas antiguas deben regenerarse o reemplazarse si sus parámetros de separación (es decir, eficacias, factores de retención o selectividades) no cumplen con los criterios aceptables. Pero para un laboratorio de rutina, sacrificar una columna que todavía puede ofrecer buenas separaciones es antieconómico e irracional.

La última investigación incluida en este apartado de la Memoria de Tesis desarrolla un nuevo método de separación, utilizando columnas en serie, para el análisis de aminoácidos. A lo largo de los años, se ha invertido un gran esfuerzo en el desarrollo de métodos para analizar estos compuestos. Algunos métodos publicados tratan sobre la separación de los derivados (isoindoles) del *o*-ftalaldehído/*N*-acetil-L-cisteína de los 19 aminoácidos proteicos primarios, utilizando elución en gradiente. Sin embargo, con frecuencia los derivados sólo pueden ser resueltos en tiempos de análisis excesivamente largos, incluso cuando se utilizan gradientes multi-lineales o multi-isocráticos. Si se intenta reducir el tiempo de análisis, se produce la coelución parcial de varios compuestos. El uso de columnas individuales es, por lo tanto, insuficiente para resolver separaciones complejas de estos analitos, debido a su funcionalidad limitada y elevados tiempos de análisis.

Considerando los pronósticos de tiempos de retención, la ecuación de Neue-Kuss dio lugar a las mejores predicciones, para todas las columnas ensayadas. Sin embargo, la elevada diferencia de polaridad entre los derivados de los aminoácidos originó retenciones fuera de escala (demasiado cortas o demasiado elevadas), de modo que las medidas eran sólo viables en dominios estrechos de disolvente orgánico, específicos de cada aminoácido. Fuera de estos dominios, las predicciones de retención deben ser extrapoladas, y su nivel de

incertidumbre puede crecer hasta niveles inaceptables. Las consecuencias indeseables de operar fuera de márgenes y la obtención de separaciones exponenciales se atenuaron cuando las columnas se acoplaron en serie y la elución se llevó a cabo en gradiente, lo que disminuyó el tiempo de análisis de la mezcla de aminoácidos y repartió los solutos a lo largo de la ventana de elución. Las predicciones obtenidas fueron muy satisfactorias.

La complementariedad de interacciones entre las columnas PFP-C18 y C4 dio como resultado una resolución de los aminoácidos muy superior a la encontrada con cada columna por separado. No fue, sin embargo, posible disminuir el tiempo de análisis por debajo de los 50 min con las columnas utilizadas, debido a la coelución parcial de los isoindoles de los aminoácidos más retenidos (triptófano, fenilalanina, leucina y lisina), que muestran una hidrofobicidad demasiado similar, y picos muy amplios.

2. Desarrollo de nuevas funciones objetivo cromatográficas

La obtención de información química útil, en muestras que contienen múltiples compuestos, sigue constituyendo un desafío importante para un analista. La mayor complejidad corresponde a aquellas muestras en las que no se dispone de conocimiento previo sobre su composición química, o cuando se carece de estándares para todos o algunos constituyentes. La mayoría de las funciones para medir el rendimiento de la separación (i.e., funciones objetivo cromatográficas, COFs del inglés "*chromatographic objective functions*), descritas en la bibliografía, no son aplicables en estas circunstancias. En la Memoria de Tesis, se propone y valida una nueva COF que viene a suplir las carencias de las COFs convencionales, para tales situaciones. El objetivo último

de esta investigación es la mejora de los análisis de hierbas medicinales mediante la obtención de huellas dactilares cromatográficas.

Las huellas dactilares cromatográficas son cromatogramas de muestras de alta complejidad, como las que se encuentran en materiales biológicos y ambientales, con múltiples picos de diferentes alturas. Los picos de mayor tamaño pertenecen a componentes en concentraciones relativamente altas, mientras que los picos más pequeños pueden corresponder a compuestos a nivel traza, productos de degradación a diferentes concentraciones, impurezas introducidas en muestras particulares o ruido instrumental. Los cromatogramas obtenidos son útiles para determinar la identidad de las muestras, comparando las huellas dactilares de diferentes muestras, y llevando a cabo análisis de similitud y diversos métodos de clasificación.

La cuantificación de cromatogramas exige señales en las que, idealmente, cada componente debe estar completamente aislado del resto. Las condiciones que permiten tal nivel de separación se determinan de manera rutinaria a partir de la información proporcionada a partir de la inyección de estándares en un pequeño número de condiciones de elución, desarrolladas de acuerdo a un determinado diseño experimental. Sin embargo, cuando se analizan muestras en las que la identidad de los constituyentes es parcial o totalmente desconocida, y en consecuencia, se carece del soporte que puede aportar el uso de estándares para el desarrollo de métodos de separación, el cromatografista no puede aplicar las metodologías habituales de selección de las condiciones experimentales más adecuadas.

En la Memoria de Tesis, se presenta el desarrollo de un algoritmo no supervisado (COF), que mide el nivel de información en los cromatogramas a través del concepto de prominencia de pico, que mide la resolución de los picos de un cromatograma, sin la necesidad de disponer de estándares. La

prominencia de un pico se define como la fracción de área de un pico que sobresale respecto de los valles que lo delimitan (o la línea base). La combinación de valores de prominencia permite caracterizar la calidad de la separación de cromatogramas con diversos niveles de solapamiento y cantidades relativas de los solutos.

Las prestaciones de la combinación de prominencias fue evaluada utilizando una mezcla de seis compuestos de prueba, cuyo comportamiento cromatográfico (tiempos de retención y semi-anchuras de pico) se caracterizó cuidadosamente. A partir de los modelos construidos, se realizaron pronósticos de separación en condiciones diversas. Finalmente, se dibujó un diagrama que representaba la resolución frente a la composición de la fase móvil, de acuerdo con los criterios de prominencia de pico y pureza de pico (que mide el área superpuesta de un pico con otros picos en un cromatograma). La comparación realizada con el criterio de pureza de pico se debe a que este criterio es considerado como el mejor para evaluar la resolución, aunque posee el inconveniente de requerir de estándares de los solutos eluidos.

La prominencia de pico ofreció una gran sensibilidad para detectar cambios en la configuración de los cromatogramas, respondiendo sólo a los picos perceptibles. Las ventajas de este criterio son las siguientes: (i) no requiere de estándares, (ii) proporciona medidas asociadas a cada pico individual, lo que facilita la combinación ordenada de las resoluciones elementales y el recuento de picos, (iii) es sensible al perfil y tamaño de las señales, (iv) es una medida normalizada de fácil interpretación, (v) se puede medir a partir de la señal global bruta, y (vi) atiende a todos los picos del cromatograma, independientemente de que correspondan a uno o más compuestos.

Debe tenerse en cuenta que incluso con mezclas simples (como las formadas por los seis compuestos de prueba), se pueden observar más picos de los esperados en los cromatogramas. El estudio sugirió que el parámetro más significativo para caracterizar las huellas dactilares cromatográficas con picos múltiples, de tamaño muy distinto, es el número de picos perceptibles (recuento de picos significativos). Por otro lado, la resolución global sólo debe calcularse para los picos significativos. Dado que se desconoce el número real y la naturaleza de los componentes en estas muestras, es necesario establecer un umbral para discriminar entre los picos significativos y aquéllos que no son reproducibles en el procesamiento de datos (artefactos, picos de impurezas o ruido instrumental). En el estudio realizado, se consideraron significativos los picos que excedían un umbral preseleccionado de 0.05% del área de prominencia de pico relativa.

Otro de los capítulos de la Memoria de Tesis describe los factores necesarios para optimizar las condiciones de extracción y conservación de las hierbas medicinales, utilizando diseños experimentales de Plackett-Burman. El método descrito fue aplicado a muestras de té verde. Para cada factor experimental, se establecieron niveles de monitorización extremos. Debido a la incompatibilidad entre los factores experimentales, fue necesario distribuirlos en dos grupos. En una primera etapa, sólo se estudió la concentración de disolvente orgánico, el tiempo de sonicación (ultrasonidos) y la temperatura de la extracción. Se llevó a cabo una investigación independiente, utilizando acetonitrilo, etanol y metanol como disolventes de extracción. En una segunda etapa, se investigó la influencia del peso de la muestra, y del tiempo y la temperatura de conservación.

Para optimizar las condiciones de extracción, los picos en las huellas dactilares se clasificaron de acuerdo a las áreas de prominencia de pico. El

análisis de los resultados demostró que el metanol era el mejor disolvente, y que debería utilizarse preferiblemente en mezclas con agua al 30%, utilizando un tiempo de sonicación de 60 min y una temperatura de 80 ºC. El peso de la muestra contribuyó muy significativamente al número de picos observados, valor que se incrementaba cuando la temperatura de conservación era más elevada (4 ºC frente a −10 ºC), probablemente debido a la degradación de algunos compuestos a mayor temperatura. Por ello, el análisis debería realizarse inmediatamente después de realizar la extracción.

Aunque el método fue aplicado a muestras de té verde, la metodología es adecuada para encontrar las mejores condiciones de extracción y conservación de otros tipos de hierbas medicinales. Se espera que la prominencia de pico, junto con una estrategia adaptada a este tipo de muestras, sea útil para optimizar el programa de gradiente para análisis cromatográfico, a fin de obtener huellas dactilares de la más alta calidad.

La Memoria de Tesis propone y valida también una función de resolución global adecuada para situaciones de separación generales (en presencia o ausencia de estándares). La función, basada en la medida no supervisada de prominencias de pico, fue implementada en MATLAB y automatiza el análisis de los cromatogramas con substracción previa de la línea base, detección completa de todos los picos y cálculo de sus propiedades. El rendimiento proporcionado por esta función fue comparado con el ofrecido por la pureza de pico. Para la validación se tomó como caso de estudio la separación de una mezcla de los derivados de OPA/NAC de los 19 aminoácidos proteicos primarios, bajo elución en gradiente. Como se ha comentado anteriormente, cuando se analizan con una columna aislada, estos compuestos pueden resolverse completamente sólo en tiempos de análisis excesivamente largos, y ni siquiera el uso de gradientes multi-lineales o multi-isocráticos consigue

reducir los tiempos. Cualquier intento de reducción conduce a una superposición inaceptable de dos o más compuestos. Sin embargo, estas dificultades hacen muy interesante esta separación para evaluar funciones de resolución.

Se predijo la separación de los derivados de los aminoácidos, simulando alrededor de 1100 gradientes que barrían concentraciones en el intervalo 5–27.5% de acetonitrilo. Para realizar las simulaciones, se utilizó la información (valores de retención y anchura de pico) suministrada por los estándares de los aminoácidos, cuando se eluyeron los derivados en 10 condiciones isocráticas. Se consideraron distintos niveles de dificultad: picos cromatográficos con áreas similares y con diferencias sustanciales en magnitud, en presencia y ausencia de compuestos desconocidos, con diferentes niveles de ruido, e incluyendo líneas base muy irregulares.

Se compararon varias funciones de resolución global, en base a la coincidencia de los gradientes elegidos como óptimos de Pareto resolución-tiempo de análisis, utilizando dos criterios de resolución: prominencia y pureza de pico. La evaluación de la pureza de pico exige un conocimiento exhaustivo de cómo son las señales individuales de cada compuesto, en cada condición experimental, de modo que sólo es determinable mediante simulaciones. La prominencia de pico, en cambio, hace uso de la señal combinada, sin requerir ninguna información previa sobre el número de compuestos que la muestra contiene, de modo que es posible medir la resolución global cuando la única información disponible es un cromatograma experimental, sin necesidad de la información de estándares.

La aplicación práctica a muestras reales de huellas dactilares exige solucionar el problema de las diferencias de magnitud entre constituyentes (e.g., componentes mayoritarios y traza) para comparar la resolución en los

cromatogramas. Una buena función de resolución global debe ser insensible a estas diferencias, y a la vez conseguir una buena equivalencia entre las condiciones seleccionadas como óptimas por la prominencia y pureza de pico. Es decir, la definición de función global de resolución ensayada para la prominencia de pico ha de seleccionar los mismos gradientes óptimos que los que se habrían elegido en base a la pureza de pico. Ello permitiría comparar condiciones experimentales a la hora de realizar una optimización. De entre las funciones ensayadas, se seleccionó una basada en el uso de valores normalizados referidos a cada pico individual, de modo que si todos los picos quedan completamente resueltos, coincide la medida con el número de compuestos eluidos. En efecto, la coincidencia de condiciones óptimas entre los gráficos de Pareto de prominencia y pureza de pico, para la función seleccionada, indicó un rendimiento similar para ambos criterios, incluso aunque una de ellas (la prominencia) disponga de un nivel de información sobre la muestra mucho más pobre.

La Memoria de Tesis incluye también estudios sobre el uso de la selectividad multivariante de segundo orden (que incorpora información temporal y espectral proporcionada por un detector de fila de diodos) como función de resolución, con motivos de optimización. La optimización de la resolución en cromatografía líquida se realiza tradicionalmente atendiendo sólo a la información temporal. Sin embargo, algunos compuestos pueden permanecer solapados, incluso bajo las condiciones de elución seleccionadas como óptimas. Esta insuficiente resolución se puede complementar por medios matemáticos deconvolucionando los picos solapados. La calidad del resultado se incrementa considerablemente si se incluye información espectral en el proceso de deconvolución.

La selectividad multivariante se relaciona con el seno del ángulo que forma en el espacio multidimensional la señal del analito, respecto de la señal de los interferentes. Esta COF es sensible a las diferencias temporales y espectrales, cuando dos o más componentes coeluyen en una mezcla. En un estudio anterior se habían probado las prestaciones de esta COF con una mezcla de fenoles, que poseían diferencias espectrales apreciables. En el estudio incluido en esta Memoria, la selectividad multivariante de segundo orden evalúa las posibilidades de resolución cuando existen compuestos con espectros similares, que coeluyen parcial o completamente. Éste es el caso de una muestra que contiene los derivados de OPA/NAC de los aminoácidos proteicos, donde algunos compuestos presentan espectros idénticos y otros son sólo ligeramente diferentes.

La aplicación de la selectividad multivariante permite seleccionar las condiciones experimentales que darán lugar a cromatogramas que muestran picos solapados, pero que pueden recuperarse mediante deconvolución con garantías de éxito, en casos donde la separación completa es imposible. Para realizar la deconvolución, se utilizó la aproximación de proyecciones ortogonales y mínimos cuadrados alternantes. La aproximación de proyecciones ortogonales es una técnica de análisis multivariante, que permite localizar los espectros más puros en un espectrocromatograma. Los mínimos cuadrados alternantes reflejan los perfiles de concentración en un espectrocromatograma, para encontrar espectros normalizados, y viceversa, tras aplicar una serie de restricciones a los resultados.

Era posible resolver satisfactoriamente la mezcla de derivados de los aminoácidos, utilizando una columna C18 y un gradiente de acetonitrilo-agua, aunque en tiempos de análisis demasiado elevados. Al aumentar la fuerza eluyente, los tiempos de retención disminuían, produciéndose solapamientos

importantes entre picos. La optimización basada en la selectividad multivariante permitió encontrar condiciones experimentales con tiempos de análisis más bajos, mediante la recuperación (con errores bajos) de los picos solapados mediante deconvolución, aprovechando las pequeñas diferencias espectrales existentes.

Los tiempos de análisis más cortos se obtuvieron utilizando gradientes multi-isocráticos, optimizados considerando tanto el tiempo de análisis como la resolución espectral. El gráfico de Pareto indicó que este tipo de gradientes era capaz de proporcionar una resolución espectral aceptable con un tiempo de análisis tan reducido como 20 min. Sin tener en cuenta la resolución espectral, el tiempo de análisis más corto que ofreció una resolución aceptable fue de 60 min.

3. Estudios fundamentales sobre el rendimiento de las columnas

El estudio del rendimiento de una columna es de gran interés para caracterizar fases estacionarias, desarrollar nuevos materiales y aumentar la resolución de muestras complejas. Tradicionalmente, este estudio se ha realizado mediante la elución de un compuesto de prueba a diferentes flujos y la representación de los denominados gráficos de Van Deemter, que relacionan la altura de plato teórico de la columna con la velocidad lineal de fase móvil. El interés de estos gráficos reside en que pueden revelar las diferentes contribuciones al ensanchamiento del pico. Sin embargo, el método posee algunas desventajas, como son la necesidad de medir con exactitud la varianza extra-columnar (lo que puede ser complicado), la utilización de un único compuesto para conocer la relación entre la altura de plato teórico y la

velocidad lineal (lo que limita las conclusiones del método), y el aumento de la incertidumbre debido a la manipulación de dos parámetros de pico (la posición y la varianza) para obtener los valores de altura de plato teórico.

Cuando la contribución extra-columnar no es despreciable, la altura de plato teórico depende de la retención del compuesto ensayado. Los compuestos que eluyen a bajos tiempos de retención proporcionan valores de altura de plato teórico mayores. Por lo tanto, las conclusiones obtenidas de un gráfico de Van Deemter pueden variar según el compuesto seleccionado para trazar el gráfico.

En el último capítulo incluido en la Memoria de Tesis, se propusieron y compararon tres métodos complementarios que evitan las desventajas anteriores. El estudio se realizó con los datos provenientes de la elución de cinco sulfonamidas, analizadas con columnas C18 de micropartículas y monolítica, utilizando mezclas de acetonitrilo y agua. Los resultados mostraron que es posible caracterizar las columnas con mayor fiabilidad respecto al método clásico, utilizando los métodos propuestos.

El primer método se basa en el tratamiento directo de las varianzas de pico de varios compuestos eluidos con una columna, utilizando una o varias condiciones de elución isocrática. A partir de la función ajustada a una relación lineal, de la representación de las varianzas frente al tiempo de retención al cuadrado, se obtiene la altura de plato teórico, cuyo valor se obtiene a varios valores de flujo. Es interesante comentar que la ecuación ajustada incluye la variación extra-columnar como parámetro de ajuste. En un segundo método, la varianza de pico para cada sustancia (expresada en unidades de volumen) se ajusta respecto del flujo, y los parámetros del modelo se relacionan con los factores de retención. El tercer método se basa en una combinación del primer y segundo métodos.

Así, los tres métodos propuestos permiten un estudio gráfico completo del rendimiento de una columna, basado en el comportamiento de un conjunto de compuestos de prueba, sin la necesidad de obtener previamente la varianza extra-columnar. Los métodos proporcionan información sobre cómo el factor de retención afecta a los parámetros *A*, *B* y *C* de la ecuación de Van Deemter, que describen la importancia de la difusión y la transferencia lenta de masa en la fase estacionaria.

El estudio se complementó examinando los factores que afectan a la incertidumbre de los datos cromatográficos, utilizados para construir los gráficos de Van Deemter: el tiempo de respuesta (relacionado con la frecuencia de muestreo), los límites de los picos, el ruido de los datos, el método utilizado para restar la línea base antes de proceder a evaluar los parámetros de dispersión de los picos, el procedimiento y la altura del pico para obtener la mejor evaluación de la varianza, y la metodología para medir la altura de plato teórico.

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OBJECTIVES AND DEVELOPMENT OF THE RESEARCH

High performance liquid chromatography (HPLC) is currently the most widely used analytical separation technique, due to its applicability, reliability, robustness and sensitivity. Unfortunately, the efficiency in HPLC is usually smaller than that achieved in gas chromatography, capillary electrophoresis and other electromigration techniques. This constrains the analysis of complex samples. Hence, the high effort dedicated to increase the efficiency and selectivity in HPLC, with significant improvements since the beginning of the technique. Despite the progress in the last decades with the development of increasingly more sophisticated instrumentation (including the introduction of ultra-high pressure pumps), and the advances in the column technology (with the synthesis of new supports and stationary phases), there are still challenges to resolve.

The PhD. work collected in this Project gathers some proposals to increase the performance of HPLC. It was started according to the following objectives, whose main results are also briefly summarised:

Objective 1. *Optimisation of multi-column systems in mono-dimensional liquid chromatography.*

In spite of the variety of columns available in the market, their chemistry is still limited. When the separation fails with all assayed columns, an alternative solution is taking advantage of the different selectivity of two or more columns. The research group has proposed a robust, reliable and universal connection system, which allows building multi-column systems by serially coupling columns marketed by different manufacturers. The theoretical basis for making reliable predictions in isocratic and gradient elution was also developed. Throughout the development of this PhD. work, our purpose was:

Objective 1.1: To study in detail the scope and extend the possibilities of the serial combination of columns. We were particularly interested in appraising the real value of this approach by comparing its performance with that achieved with another separation strategy developed by the research group, which was extended to the use of parallel complementary columns.

Objective 1.2: To develop new applications for the analysis of new groups of compounds using serially-coupled columns, in order to extend the applicability of the approach and increase its knowledge. For this purpose, columns with diverse retention mechanisms were used using linear, multi-isocratic and multi-linear gradients.

Objective 2. *Development of new chromatographic objective functions.*

In order to find out the best separation conditions, the information contained in simulated chromatograms should be transformed to a numerical value that describes the achieved resolution, which is monitored against changes in the experimental factors, with the aim of maximizing the separation. For this purpose, an appropriate descriptor called "chromatographic objective function" (COF) has to be evaluated. COFs allow the detailed *in silico* inspection of the resolution behaviour in a wide range of conditions, without the requirement of more experimental effort. However, available COFs are not useful for situations of extremely low resolution. Our purpose was:

Objective 2.1: To develop a COF that could work when there are no standards available, as is the case of the separation of many biological and environmental samples that give rise to the so-called "chromatographic fingerprints". Generally, samples with similar fingerprints also have similar properties. Therefore, this type of chromatogram has a potential interest to determine the

identity, authenticity and consistency between batches of natural products of diverse nature. To maximise the informative content of such chromatograms, new COFs should be developed to grade the quality of a set of chromatograms in a rigorous way. This will improve the classification and recognition of these samples.

Objective 2.2: To solve the incomplete resolution in complex chromatograms, using mathematical techniques to deconvolve overlapped peaks. Particularly, our purpose was to inspect the possibilities of success of a COF that considered both time and spectral information in cases of small spectral differences.

It should be commented that as the work was developing, it was found interesting to carry out a fundamental study on the measurement of the efficiency of chromatographic columns. Therefore, the PhD. Project is divided in three parts:

Part 1. *Optimisation of multi-column systems in mono-dimensional liquid chromatography.*

Part 2. *Development of new chromatographic objective functions.*

Part 3. *Fundamental studies on column performance.*

The research work leading to the PhD. degree in Chemistry was started in September 2014, once the Master degree on "Experimental techniques in Chemistry" taught by the Departments of Analytical Chemistry and Inorganic Chemistry of the University of Valencia was finished. The experimental work reflected in this Project was developed in the Department of Analytical Chemistry (University of Valencia), under the supervision of Profs. María Celia García Álvarez-Coque and José Ramón Torres Lapasió. Acknowledge should

be also given to the collaboration with Prof. Juan José Baeza Baeza in some fundamental studies.

The PhD. period has included two three-month stays:

September to November 2017: under the supervision of Prof. Martí Rosés in the Department of Analytical Chemistry of the University of Barcelona (Spain), working in the field of hydrophilic liquid chromatography (HILIC) for the characterization of liquid chromatography systems. This work was presented to the 32nd International Symposium on Chromatography held in Cannes (France) in September 2018 and sent for publication.

September to November 2018: under the supervision of Prof. Karine Faure in the Institute of Analytical Sciences (CNRS at the University of Lyon, France), working in the field of comprehensive two-dimensional chromatographic systems to analyse fingerprints with a preparative perspective.

The work here presented has implied a large experimental effort, designed to explore and extract information on the chromatographic behaviour of compounds of different nature (sulphonamides, β-blockers, diuretics, amino acids, medicinal herbs, and a variety of acidic and basic compounds). A great diversity of columns and experimental conditions with acetonitrile-water mobile phases has been used along the studies. The work has also involved an extensive data treatment, mainly related with the construction of models to predict the chromatographic retention, peak shape and resolution of mixtures of compounds, with optimisation purposes.

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

Published developments in several scientific journals, carried out in the Department of Analytical Chemistry of the University of Valencia, are collected along 10 chapters. Specifically, the published articles directly related to the chapters in this PhD. Project (in order of publication) are:

1. Tamara Álvarez Segura, Casandra Ortiz Bolsico, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Serial versus parallel columns using isocratic elution: A comparison of multi-column approaches in mono-dimensional liquid chromatography.
   Journal of Chromatography A 1390 (2015) 95–102 (Chapter 4).
2. Tamara Álvarez Segura, María Celia García Álvarez-Coque, Casandra Ortiz Bolsico, José Ramón Torres Lapasió,
   Interpretive approaches to optimize serially-coupled columns in reversed-phase liquid chromatography
   Current Chromatography 2 (2015) 110–121 (Chapter 3).
3. Tamara Álvarez-Segura, Antonio Gómez Díaz, Casandra Ortiz Bolsico, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   A chromatographic objective function to characterize chromatograms with unknown compounds or without standards available
   Journal of Chromatography A 1409 (2015) 79–88 (Chapter 7).

4. Tamara Álvarez Segura, Elsa Cabo Calvet, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   An approach to evaluate the information in chromatographic fingerprints: Application to the optimisation of the extraction and conservation conditions of medicinal herbs
   Journal of Chromatography A 1422 (2015) 178–185 (Chapter 8).

5. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Optimisation of chromatographic resolution using objective functions including both time and spectral information. Part 2: Compounds exhibiting small spectral differences
   Current Chromatography 3 (2016) 34–43 (Chapter 10).

6. Tamara Álvarez Segura, José Ramón Torres Lapasió, C. Ortiz Bolsico, María Celia García Álvarez-Coque
   Stationary phase modulation in liquid chromatography through the serial coupling of columns: A review
   Analytica Chimica Acta 923 (2016) 1–23 (Chapter 2).

7. Tamara Álvarez Segura, Carolina Camacho Molinero, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Analysis of amino acids using serially coupled columns
   Journal of Separation Science 40 (2017) 2741–2751 (Chapter 6).

8. Tamara Álvarez-Segura, Manuel David Peris Díaz, María Celia García Álvarez-Coque, Juan José Baeza Baeza
   New approaches to evaluate the dispersion parameters in liquid chromatography based on the information obtained from a set of compounds
   Current Chromatography 4 (2017) 156–166 (Chapter 11).

9. José Antonio Navarro Huerta, Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Study of the performance of a resolution criterion to characterise complex chromatograms with unknowns or without standards
   Analytical Methods 9 (2017) 4293–4303 (Chapter 9).

10. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Alvarez-Coque
    Updating chromatographic predictions by accounting ageing for single and tandem columns
    Journal of Separation Science DOI: 10.1002/jssc.201800264 (2018) (Chapter 5).

The work not included in this PhD. Project is:

11. Tamara Álvarez Segura, Xavier Subirats, Martí Rosés
    Retention-pH profiles of acids and bases in HILIC
    Sent article (2018)

12. Tamara Álvarez Segura, Elsa Cabo Calvet, Juan José Baeza Baeza, María Celia García Álvarez-Coque
    Study of the efficiency of chromatographic columns in gradient elution
    Sent article (2018)

The developed research has been also presented in 15 scientific conferences in the period October 2014 to October 2018 (17 communications in international conferences and 12 communications in national conferences). The PhD. candidate has attended to several of these conferences:

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

1. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, Casandra Ortiz Bolsico, Tamara Álvarez Segura
   Selectivity modulation with serially-coupled columns in RPLC for the analysis of complex samples
   (Poster CTQ-P01)

First Virtual Symposium on Applied Separation Science (VSASS'2015)
Sydney (Australia), May 2015 (international)

2. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, Antonio Gómez Díaz, Tamara Álvarez Segura
   Peak prominence: A new chromatographic function to evaluate chromatographic performance for complex samples without standards
   (Poster 239, awarded with Notable mention to best Posters)
3. María Celia García Álvarez-Coque, José Ramón Torres-Lapasió, Tamara Álvarez Segura
   Serially-coupling of columns: An approach to expand the separation capability in HPLC
   (Poster 240)

42th International Symposium on High-Performance Liquid Phase Separations and Related Techniques (HPLC'2015)
Geneva (Switzerland), June 2015 (international)

4. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, Casandra Ortiz Bolsico, Tamara Álvarez Segura
   Optimisation of serially-coupled columns: Isocratic and gradient elution
   (Oral communication: FUN-TH-O-12:10)

5. José Ramón Torres Lapasió, María Celia García Álvarez-Coque, Antonio Gómez Díaz, Casandra Ortiz Bolsico, Tamara Álvarez Segura
   Measurement of resolution in fingerprinting analysis of herbal medicines
   (Poster PSA-DATA-02)

6. José Ramón Torres Lapasió, María Celia García Álvarez-Coque, Tamara Álvarez Segura
   Transference of optimal linear and multi-linear gradients to aged single and coupled columns
   (Poster PSB-COL-04)

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

7. Tamara Álvarez Segura, Elsa Cabo Calvet, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Maximization of the chromatographic information in green tea fingerprinting
   (Poster AA30)

VI Chemometrics Workshop for Young Researchers
Valencia (Spain), October 2015 (international)

8. Tamara Álvarez Segura, Elsa Cabo Calvet, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Optimization of the extraction and conservation conditions of a green tea sample maximizing the information obtained in fingerprints
   (Oral communication: Proceedings pp 103–107)

9. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
   Optimization of the chromatographic selectivity of o-phthaldehyde amino-acids derivatives using diode array detection
   (Oral communication: Proceedings pp 113–117)

XV Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECyTA'2015)
Castellón (Spain), October 2015 (national)

10. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, Tamara Álvarez Segura
    Implementation of methods in high performance liquid chromatography using serially-coupled columns
    (Oral communication: O-15)

11. José Ramón Torres Lapasió, Tamara Álvarez Segura, María Celia García Álvarez-Coque
    Optimization of the information in chromatographic fingerprints of herbal medicines
    (Poster 83)

12. José Ramón Torres Lapasió, Tamara Álvarez Segura, María Celia García Álvarez-Coque
    Optimization of resolution in high-performance liquid chromatography of proteic amino acids considering both time and spectral information
    (Poster 84)

XVI Chemometrics in Analytical Chemistry (CAC'2016)
Barcelona (Spain), June 2016 (international)

13. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, Tamara Álvarez Segura
    Optimisation of multilinear gradients in serially coupled columns assisted by Pareto plots and genetic algorithms
    (Poster P-001)

14. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, José Antonio Navarro Huerta, Tamara Álvarez Segura
    Measurement of resolution for highly complex multi-analyte samples
    (Poster P-002)

15. José Ramón Torres Lapasió, José Antonio Navarro Huerta, María Celia García Álvarez-Coque, Tamara Álvarez Segura
    Maximisation of the information in chromatographic fingerprints
    (Poster: P-003)

31st International Symposium on Chromatography (ISC'2016)
Cork (Irland), August-September 2016 (international)

16. María Celia García Álvarez-Coque, José Ramón Torres Lapasió, Tamara Álvarez Segura, José Antonio Navarro Huerta

Measurement of resolution and optimization of chromatographic conditions in the absence of standards
(Poster P-01-001-017)

17. José Ramón Torres Lapasió, Tamara Álvarez Segura, Carolina Camacho Molinero, María Celia García Álvarez-Coque
Analysis of primary proteic amino acids using serially-coupled RPLC columns
(Poster P-01-001-069)

18. María Celia García Álvarez-Coque, Manuel David Peris Díaz, Tamara Álvarez Segura, Juan José Baeza Baeza
Approach to evaluate the dispersion parameters in HPLC based on the information obtained from a set of compounds
(Poster P-02-002-057)

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

19. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
Increased resolution of complex mixtures of diuretics using a serial combination of C18 and cyano columns
(Poster P FCH-10)

20. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
    Optimal multi-step gradients using single and tandem columns: Chromatographic separation of proteic amino acids derivatised with o-phthalaldehyde and N-acetyl-L-cyteine
    (Poster P FCH-12)

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

21. José Antonio Navarro Huerta, Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
    A resolution criterion to characterize complex chromatograms with unknown compounds or without standards available: A validation study
    (Oral communication)

22. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
    Serially coupled-columns: An interesting solution for the RPLC analysis of amino acids
    (Oral communication)

45th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2017)
Prague (Czech Republic), June 2017 (international)

23. José Antonio Navarro Huerta, T. Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
    Measurement of resolution in complex chromatograms aimed to optimised the separation
    (Oral communication: FUN13-O2-Th)

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

24. José Antonio Navarro Huerta, Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
Benefits of serially-coupling chromatographic columns
(Poster 018 Tu-SEP)

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

25. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
Transference of optimal separations in multi-linear gradient elution to aged serially-coupled columns
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26. Juan José Baeza Baeza, Elsa Cabo Calvet, Tamara Álvarez Segura, María Celia García Álvarez-Coque
Study of the efficiency of chromatographic columns in gradient elution
(Poster PO-TS-01)

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

27. José Antonio Navarro Huerta, Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
Comparison of search strategies for finding optimal multi-linear gradients
(Poster)

6th International Conference and Exhibition on Advances in Chromatography & HPLC Techniques
Barcelona (Spain), August 2018 (international)

28. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
    Updating chromatographic predictions by accounting ageing for single and tandem columns
    (Poster)

29. Tamara Álvarez Segura, José Ramón Torres Lapasió, María Celia García Álvarez-Coque
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CHAPTER 1

GENERAL INTRODUCTION: REVERSED PHASE LIQUID CHROMATOGRAPHY, MODELS AND CHROMATOGRAPHIC OBJECTIVE FUNCTIONS

1.1. Reversed phase liquid chromatography

1.1.1. Introduction

Reversed phase liquid chromatography (RPLC) is a mode of high-performance liquid chromatography (HPLC) that employs a nonpolar stationary phase and a polar mobile phase constituted by water and at least a water-miscible organic solvent, which performs as a modifier [1–4]. In this technique, retention is fundamentally determined by the distribution of solutes between mobile phase and stationary phase, as they progress along the chromatographic column. One of the main factors responsible for the development of RPLC was the need of separating mixtures containing compounds that were not sufficiently volatile or that lacked the necessary thermal stability to be analysed by gas chromatography, such as many polar and ionic organic and inorganic compounds, drugs, and biomolecules.

The HPLC mode known as normal-phase liquid chromatography (NPLC), which uses silica and alumina polar porous microparticles and nonpolar mobile phases, was first developed. Polar microparticles were common in the early development of HPLC [2]. However, silica is unsuitable to retain many highly hydrophobic organic molecules and yields poor efficiency and extreme peak tailing for basic compounds, due to the interaction with acidic silanol groups (silanols) on the silica surface. Moreover, polar compounds are not soluble in the hydrophobic mobile phases or exhibit very long retention times. Furthermore, retention in NPLC significantly depends on the presence of traces of water and impurities of other polar solvents in the nonpolar or weakly polar mobile phases. In order to overcome these limitations, a modification of the silica surface was required to provide less polar or nonpolar (hydrophobic) materials to be used as stationary phases with polar solvents. This new

chromatographic mode utilised the opposite approach to, at that time, the normal mode of operation (NPLC), namely, a nonpolar stationary phase and a polar mobile phase. For this reason, it was considered a “reversed” mode [5,6].

The first reports in HPLC using covalently modified silica supports, stable in most common water-miscible solvents, appeared in the mid-1960s, and the first commercial RPLC column (Bondapak) was introduced in 1973. RPLC with alkyl-bonded stationary phases is an extraordinary separation technique that has become the most popular HPLC mode, representing the vast majority of all HPLC separations. It is suitable for the analysis of compounds of a wide range of polarities and structures. Although most applications correspond to small organic molecules (with a molecular mass of $M_r < 500$), the analysis of proteins and other large biomolecules is also possible. In RPLC, once a suitable column has been selected, the absolute retention and selectivity (retention relative to other solutes) can be easily manipulated using several experimental factors, such as the percentage of organic modifier and the concentration of a wide range of additives (such as buffers, weak acids, neutral salts, ion pairing reagents, surfactants, ionic liquids, and chiral selectors) [7], pH [8], and temperature [9]. Other advantages of this popular technique are:

(i) the easy implementation of gradient elution
(ii) the compatibility with aqueous samples
(iii) the possibility of separating nonpolar, polar, and ionic compounds in the same run
(iv) the wide variety of available commercial stationary phases
(v) the large number of published applications

RPLC is a mature technique, employed in science and technology by chemists, biochemists, and pharmacists for analysis and purification. The applications cover environmental control, food, clinical, pharmaceutical, and

industrial analyses, drug and chemical manufacturing (at both quality control and preparative scales), biomedical studies, and measurement of physico-chemical properties.

In this chapter, the different factors that contribute to the separation in RPLC are described, together with the main approaches proposed to understand the observed behaviours.

1.1.2. The stationary phase

The most common column packings used in RPLC contain a monomolecular layer of a hydrophobic material, which is covalently bound to porous amorphous silica microparticles. The availability of sufficiently stable and reproducible bonded stationary phases, with a broad selectivity range, has been a key factor in the success of RPLC as a rapid, efficient, and reliable analytical and preparative technique [10–14]. Today, a very large number of RPLC columns are commercially available, and new brands are introduced every year.

In RPLC, the stationary phase should be mechanically stable and possess the desired nonpolar, moderately polar, or polarisable properties. The development of a technology for immobilizing the chromatographic ligand on the solid support through chemical bonding (grafting) explains the success of RPLC. Bonded phases are capable of withstanding without appreciable deformation the high pressures (a few tens of MPa) that are generated when the mobile phase is forced through the packed columns at reasonable flow rates. Bonding to the silica support makes the design of a wide variety of stationary phases possible, including nonpolar, weakly polar, rather polar, and even charged (anionic, cationic, and zwitterionic), thus broadening the selectivity and

application range of HPLC. Also, a wide variety of aqueous-organic mixtures and even pure organic solvents can be used without risk of losing the nonpolar or weakly polar stationary phase.

Hard inorganic materials are by far the most frequent supports of the bonded stationary phases. For this purpose, mainly silica, and also hydroxyapatite, graphite, and metal oxides (such as titanium and zirconium oxides) are used [13]. Among these, silica is an almost ideal support in view of a number of properties, including a rich and easily manipulable surface chemistry, high mechanical resistance under high pressures, thermal stability, easily controllable particle size, adequate porosity, and large surface area. The properties of the silica support (composition, impurities, particle size and shape, size distribution, pore size, and surface area) and the bonded phase parameters (bonding chemistry, functionality, bonding density, also known as grafting density or surface coverage, and deactivation of the uncovered surface) assure rapid mass transfer, reduced eddy dispersion, large solute loadability, high retention, and peak profile reproducibility.

Although bonded silica stationary phases have many advantages, their application is limited to the relatively narrow pH range over which they are stable. Most chemically modified silicas are useful only from pH 2 to pH 8, although this range and the degradation rates in acidic and basic media depend on the type of silica, bonded reagent, bonding method, and bonding density [3]. Even the most stable improved alkylsilane surfaces present serious stability problems at both very high (> 10) and low (< 2) pH values.

The general and most widespread method for silica surface functionalisation has been organosilanisation, in which silanols located on the silica surface react with an organosilane or silanizing reagent [14,15]. Before the development of RPLC, chemical bonding by reaction with silanes was already known in the

field of industrial silica (e.g., to provide better properties as a thickener in paints) [14]. Silica modification through silanisation results in a hydrophobic surface that preferentially retains the nonpolar regions of the solute molecules. Porous silica has a high surface area (typically, 100–300 m^2/g). Therefore, the amount of hydrophobic chains bonded to the support can be very large, providing high solute loading capabilities.

Many available commercial stationary phases are monomeric, where the silica support has been functionalised with a silanizing reagent containing a single hydrolysable group. In the resulting monomeric phases, a Si atom is attached to the surface by means of a single siloxane bridge, Si-O-Si. Meanwhile, polymeric stationary phases are constructed with silanizing reagents with two or three hydrolysable leaving groups on the same Si atom. Then, the Si atom becomes either attached to the surface through two or three bonds or linked both to the surface and to one or two neighboring ligands. Cross-linked polymeric structures having higher bonding densities than monomeric phases (3–6 $\mu mol/m^2$ versus 2–3.5 $\mu mol/m^2$, respectively) may result. Polymeric phases show improved shape selectivity, that is, enhanced capability of separating positional isomers.

1.1.3. The mobile phase

The second major contributor to the RPLC environment is the mobile phase, which has a considerable influence on the separation of solutes. Usually, binary mixtures of water and an organic solvent are used. The selection of the mobile-phase composition, including solvents, buffer, and other additives, will determine the degree of interaction between solutes and stationary phase.

In Section 1.2, an overview of the approaches followed to optimise all the involved factors that affect retention, selectivity and resolution in HPLC are discussed. In principle, there is a wide range of water-miscible organic solvents that can be used as modifiers; however, only a few of them are used in RPLC, namely, acetonitrile, methanol, ethanol, tetrahydrofuran, and isopropanol [16,17]. Among them, acetonitrile followed by methanol are by far the most frequently used. The reasons that make acetonitrile the most popular choice as a modifier are its low viscosity that helps in reducing the back pressure, low cutoff wavelength for UV detection, sufficiently large elution strength, reduced reactivity, and ability to dissolve a wide range of solutes. The viscosity of methanol-water mixtures is particularly large, having a maximum at about 50% methanol, which does not occur with acetonitrile. In turn, methanol is less expensive and less toxic, and its higher polarity reduces the risk of buffer precipitation.

There may be other reasons to switch to another solvent, including unavailability or legal restrictions. For instance, from late 2008 to early 2009, the production of acetonitrile came down giving rise to a substantial increase in its price [18]. As alternative, solvent consumption was recommended to be reduced by using columns with narrower diameters. Ethanol, which is less toxic than acetonitrile and methanol, was also suggested, but it is subjected to restrictions in some countries to avoid illegal diversion to human consumption. Finally, solvent recycling technologies were also given as a solution to reduce organic solvent consumption and its environmental impact.

1.1.4. Chromatographic elution modes

RPLC is the technique of choice for the separation of complex mixtures of solutes within a wide range of polarities. Retention in RPLC is mainly related to solute hydrophobicity. Therefore, the 1-octanol/water partition coefficient (log $P_{o/w}$) for solutes can be used to assess retention. If the solute is ionised at the mobile-phase pH, the apparent partition coefficient (log P_{app}), which is smaller than log $P_{o/w}$) will take into account the ionisation degree [19]. Separations can be carried out in either the isocratic or gradient elution modes. In isocratic elution, the mobile-phase composition is held constant during the separation, while in gradient elution, it is varied.

1.1.4.1. Isocratic elution

Due to its inherent simplicity, lower cost, and higher robustness, many RPLC separations are carried out in the isocratic mode. This approach is suitable when the chromatogram contains a few peaks for solutes within a small or moderate polarity range. In such cases, all solutes in the sample can be separated over a reasonable time period. However, isocratic elution is not recommended if the polarity of the solutes in the sample spreads over a wide range. In this case, if the most retained solutes are eluted within the optimal range (at high φ_B), the early eluted peaks will be poorly resolved and even lost in the solvent front. On the contrary, if the less retained solutes are well resolved (at low φ_B), then the most retained solutes will be eluted with excessively long run times and broadened peaks, giving rise to a reduced detection sensitivity. Some highly retained solutes may even be buried into the baseline noise or overlap the following sample. Therefore, it is not possible to

improve both extremes of the chromatogram at the same time by using isocratic elution. This situation has been called the "general elution problem of RPLC".

1.1.4.2. Gradient elution

As described, a mobile phase with fixed composition is often not suitable to satisfactorily resolve complex samples. The usual solution to the general elution problem is the application of gradient elution with programmed changes of organic modifier [20–23]. The main objective of gradient elution is to achieve adequate resolution, and acceptably short times during a single run, by increasing the retention of the poorly retained solutes and speeding up the elution of those strongly retained. For this purpose, the elution strength of the mobile phase should be initially low and become steadily stronger by increasing the concentration of the modifier as the separation progresses.

As the concentration of the organic modifier is increased, the polarity of the mobile phase decreases. In mobile phases with low elution strength, the strongly retained solutes are stuck in a narrow band near the head of the column, migrating only very slowly, so that this range of mobile phase compositions does not contribute significantly to their elution. As the elution strength of the mobile phase increases, the rate of partitioning into the mobile phase also increases and the solutes are "accelerated" through the column. At some point within the column, a given solute may partition mostly into the mobile phase, and will move almost with the same linear velocity as the mobile phase [24]. Solutes in gradient RPLC seldom experience the whole range of mobile-phase compositions. The fraction of the solvent composition range that actually affects solute migration has been called "significant solvent concentration range" [25].

Gradient experiments are often used in a scouting technique to obtain information for selecting isocratic separation conditions. From the above discussion, it can be concluded that gradient data can be transformed into accurate isocratic retention factors only in a narrow concentration range. The concentration dependence of the extrapolated values in a broader concentration range may be subjected to significant errors. Isocratic measurements are more laborious but generally provide more reliable information for selecting optimal isocratic separation conditions.

1.2. Models and chromatographic objective functions for the optimisation of selectivity in RPLC

1.2.1. Introduction

As commented above, HPLC separations can be approached from two perspectives: isocratic and gradient elution. The number of mobile phase compositions, and especially, of gradient programs that can be implemented is virtually unlimited, and the success of the separation depends critically on the profile selected. In practice, gradients consist usually of simple linear increases of an experimental factor, but several isocratic steps (i.e., multi-isocratic gradients), or several segments of different slope, which can be linear (i.e., multi-linear gradients) or curvilinear, can be implemented.

When the chromatographer tackles a new problem, some decisions concerning non-adjustable factors (e.g., column, solvent and buffer nature) should be taken, often through screening or based on prior knowledge. Then, readily adjustable properties are examined, such as the concentration of organic solvent, pH, temperature, or the gradient program where one or more of these factors are varied. When the results are not satisfactory, a major

change in the chromatographic system (i.e., the column and/or the mobile phase components) is required. This means more experimental work, more cost and an undesirable delay in method development. In the new system, some previously overlapped compounds may be now better resolved, but others, which were formerly well resolved, may co-elute.

This panorama suggests that finding the best chromatographic separation conditions is not easy. Several optimisation strategies have been proposed to assist the resolution of complex problems. In spite of being particularly slow and inefficient, trial-and-error strategies are still frequent. Many mixtures are, however, so complex that they cannot be solved by these strategies. Fortunately, method development can be notably expedited with more reliable results by applying computer-assisted interpretive optimisations. A number of references that explain and apply these tools to different samples have been published in the last two decades [26–34].

Experiment-based optimisations include two steps: modelling of the system and prediction of the resolution through computer simulation. In the first step, the chromatographer develops a number of experiments as reduced and informative as possible, in order to fit equations or train algorithms that will allow the prediction of retention, and incidentally, other properties that summarise a chromatogram. The aim is to develop systems capable of predicting the separation at any new arbitrary condition. In the second step, the separation quality is scanned for a large number of separation conditions, and the one that is expected to give maximal resolution is selected. In practice, this is done by simulating the chromatograms for a pre-fixed distribution (regular or chosen on a random basis) of the experimental factors being optimised. For this purpose, synthetic chromatograms are built by adding the predicted signals for the compounds in the mixture analysed.

In order to find the best conditions, the information contained in the simulated chromatograms should be transformed to a numerical value, which is monitored throughout the optimisation. Ideally, this value should correlate with the analyst's appraisal of resolution. The mathematical expression that allows the evaluation of the separation quality is called "chromatographic objective function" (COF), which is maximised throughout the optimisation process. Usually, the COF only considers the resolution, but it may gather additional aims, more or less subjective, such as shorter analysis time or desirable peak profiles (i.e., high efficiencies and low asymmetries).

Very often just one factor (e.g., the concentration of organic solvent in isocratic elution) is enough to succeed in the separation. The optimisation of two or more factors is less frequent (e.g., the concentration of organic solvent and pH or temperature, or the concentrations of two modifiers and pH), due to the increased experimental effort needed to achieve accurate predictions [35,36]. Gradient elution implies finding the suitable gradient program, which is usually, numerically speaking, more complex. This can be carried out in many ways, depending on the selected factors that determine the gradient profile: gradient time, gradient shape and initial value of the experimental factor(s).

Several software packages have been marketed to facilitate the implementation of interpretive methodologies, especially for RPLC, which is nowadays the prevalent chromatographic mode. The development of analytical methods can be greatly expedited with these packages, which in addition can assist untrained users to set up separations. Some examples are DRYLAB [37], PREOPT-W [38], OSIRIS [39], MICHROM [40,41], and CHROMSWORD [42].

1.2.2. Prediction of chromatographic retention

As mentioned, the first step in the experiment-based optimisation of resolution is gathering information about the chromatographic behaviour of the compounds in the sample, covering a reasonably wide factor region usually attending only to retention. This will allow, among other aims, the inference of a relationship to describe the retention as a function of the experimental factors, for each solute. The accuracy of these predictions is decisive for the reliability of the optimisation.

Retention models can be fitted from either isocratic or gradient experiments [43–45], which means finding the best values of the model parameters for each solute under a least-squares basis. For this purpose, a convenient algebraic expression (an empirical or mechanistic model), or a black-box algorithm, able to predict the isocratic or gradient retention times, should be available for each solute. The assignation of each peak to each solute can be facilitated by the injection of standards of the individual compounds, although solutions containing two or more compounds with separate retention can be used. If needed, peak tracking can be made by varying the concentration of the injected standards, or with the aid of a selective detection technique [46–50].

The quality of the predictions is compromised by the equation selected to fit the training data and the fitting procedure. However, the ranges of the experimental factors, the distribution of the experiments within each factor domain, and the elution mode (isocratic or gradient) where the retention and other peak properties of the training set were measured can also be important.

1.2.2.1. Isocratic elution at fixed pH

The concentration of organic solvent in the mobile phase is the factor most frequently optimised in RPLC. Several models allowing accurate predictions at varying concentrations of organic solvent have been exhaustively tested [51–61]. The retention behaviour can be modelled accurately by establishing a quadratic relationship between the logarithm of the retention factor, k, and the volume fraction of organic solvent in the aqueous-organic mobile phase, φ:

$$\log k = \log \frac{t_R - t_0}{t_0} = c_0 + c_1 \varphi + c_2 \varphi^2 \qquad (1.1)$$

where t_R and t_0 are the retention time and dead time, respectively, and c_i are regression coefficients with characteristic values for a given solute and column/solvent system. This model can be simplified for narrow ranges of organic solvent to a linear relationship:

$$\log k = c_0 + c_1 \varphi = \log k_w - S \varphi \qquad (1.2)$$

The intercept of the fitted straight-line refers to the extrapolated log k value for water as the mobile phase, and the slope indicates the sensitivity of retention to changes in the organic solvent content (i.e., elution strength). The deviations from linearity are especially important at the highest and lowest concentrations.

Although it is generally accepted that Eq. (1.2) accurately describes the retention in RPLC only in narrow or moderate domains of organic solvent, and Eq. (1.1) is preferable for wider ranges, the linear model is usually arbitrarily selected (i.e., without statistical support). This means that a certain level of subjectivity is always present, so that predictions can result more or less biased. Statistical tests that guarantee the absence of subjectivity in the

selection of the model have been used [62–64]. These tests reduce the risk of overfitting (i.e., obtaining a wrong inference of the system behaviour, owing to the excessive importance given to small scale details instead of fitting the general trend). This is particularly important when extrapolations are involved, such as the prediction of isocratic data from gradient training sets.

1.2.2.2. Gradient elution

Retention times for a solute eluted under a given gradient program in RPLC can be described by resolving the fundamental equation for gradient elution:

$$t_0 = \int_0^{t_g - t_0} \frac{dt}{k(\varphi(t))} \tag{1.3}$$

where t_0 is again the dead time, t_g is the solute retention time under gradient conditions, and $k(\varphi(t))$ the equation that describes the solute retention at the column inlet as a function of time. The retention time can be calculated for any gradient, provided the composite function $k(\varphi(t))$ is known. This dependence implies two nested equations: the variation of the concentration of organic solvent with time (i.e., the gradient program), and the dependence of k on φ (i.e., the retention model). For mathematical convenience, gradients can be outlined as small isocratic steps. This allows the use of models that cannot be integrated algebraically, even for linear gradients, or the application to any type of gradient of models that can be integrated only for linear gradients.

If the gradient involves only variations in φ, and both the dependence between log k at the column inlet and time, and the gradient program are linear, Eq. (1.3) has the following algebraic solution [61]:

$$t_g = \frac{t_0}{b} \log (2.3\, k_0\, b + 1) + t_0 + t_D \quad (1.4)$$

where b is a parameter related to the solvent strength (i.e., the slope of the gradient program, φ') and t_0 through:

$$b = S\, \varphi'\, t_0 \quad (1.5)$$

being

$$\varphi' = \frac{\Delta\varphi}{t_G} \quad (1.6)$$

where $\Delta\varphi$ is the difference between the final and starting organic solvent concentrations, and t_G the gradient time (i.e., the time at which the gradient program reaches the final composition). In Eq. (1.4), t_D is the dwell time (i.e., the time required for the gradient front to reach the column inlet), and k_0 the solute retention factor at the gradient starting concentration. The dwell time is usually obtained by running a blank gradient, where the content in acetone is increased from 0 to a small percentage (e.g., 1%) in an arbitrary time (e.g., 20 min). In retention modelling, the dwell time is implemented as a small isocratic step at the beginning of any gradient program. This means that actual gradient profiles should be obtained by adding t_D to the programmed time values.

In other cases, the integral equation (Eq. (1.3)) lacks an algebraic solution, and the gradient retention time (t_g) should be obtained through numerical integration [63,65].

Gradient retention times (t_g) can be calculated by splitting the integral in small isocratic steps, and finding the last upper limit:

$$t_0 = \int_0^{t_g - t_0} \frac{dt}{k(t)} = \int_0^{t_1} \frac{dt}{k(t)} + \int_{t_1}^{t_2} \frac{dt}{k(t)} + \ldots + \int_{t_{i-1}}^{t_i} \frac{dt}{k(t)} + \int_{t_i}^{t_{i+1}} \frac{dt}{k(t)} \quad (1.7)$$

Since $k(t)$ can be assumed to be constant in each of these steps, t_0 can be approximated to:

$$t_0 \approx \frac{t_1}{k_{0,1}} + \frac{t_2 - t_1}{k_{1,2}} + ... + \frac{t_i - t_{i-1}}{k_{i-1,i}} + \frac{t_{i+1} - t_i}{k_{i,i+1}} \quad (1.8)$$

with:

$$k_{i,i+1} = \frac{k(t_i) + k(t_{i+1})}{2} \quad (1.9)$$

The precision in t_g can be increased up to any desired level, by just narrowing the time intervals ($t_{i+1} - t_i$). However, the higher the precision, the longer the computation time. Moreover, there is a limitation associated with the pumping system, since gradients are generated by the instrument by approximating changes in composition in small steps, which makes investing effort in obtaining a precision level in t_g beyond the capability of the instrument, useless. The time increments in the numerical integration should, thus, be fixed by taking into account the instrument specifications. Typically, a minimal variation in the organic solvent content could be ca. 0.1%.

The prediction of retention under gradient conditions is usually made from training sets involving gradient experiments [67]. Crossing the information between both elution modes is also possible: data coming from isocratic experiments can be used to anticipate the retention in gradient runs, and *vice versa* [45]. When gradient elution is predicted from isocratic data, the model parameters are straightforwardly obtained, due to the explicit relationship between retention data and parameters. Gradient training experiments are maximally efficient when a solution containing all standards can be injected in a single run and the identity of each peak can be unambiguously known. Otherwise, modelling gradient retention from wisely planned isocratic experiments is often not only safer but also faster,

since no re-equilibration step is needed. Isocratic data may also be available from the literature or from previous user data sets, and the chromatographer may wish to explore whether a gradient separation would give satisfactory results before carrying out any gradient experiment.

1.2.3. pH and temperature as experimental factors

For many solutes, pH is an additional experimental factor with an extreme influence on selectivity. When the analysed mixture contains one or more compounds with acid-base behaviour, pH tuning can offer unique opportunities to reach resolution. However, variations in retention and selectivity with pH are particularly hard to model [68,69]. Not surprisingly, a widely extended practice consists of fixing the pH at a convenient value, which means that the benefits of this experimental factor on selectivity are mostly lost.

Retention also depends on temperature through the Van't Hoff equation [70,71]:

$$\log k = \frac{\Delta S}{2.3\,R} - \frac{\Delta H}{2.3\,RT} + \log \Phi \qquad (1.10)$$

where ΔS and ΔH are the system entropy and enthalpy variations, T is the absolute temperature, R the universal gas constant, and Φ the system phase ratio.

1.2.4. Prediction of peak profiles

Very often, the optimisation of resolution is carried out using information from simplified chromatograms, where only retention times are considered. This treatment is supported by the idea that the most relevant peak property is retention. Realistic simulations of chromatograms require, however, more elaborated treatments, where bandwidths or even, full peak profiles, are considered. In some instances, the areas of individual peaks are also needed to get a reliable optimisation (e.g., to take into account a minor component), although in most situations, using normalised areas are a valid choice, since this strategy provides a general solution.

For the calculation of resolution (see Section 1.2.5), the most comprehensive criteria require full simulation of chromatograms, which are built by adding the individual peaks. There are other criteria where this is not essential, but they offer less informative evaluations of resolution. Furthermore, the possibility of simulation is of interest to appraise the quality of predictions by comparison with the corresponding experimental chromatograms.

A number of theoretical and empirical mathematical functions have been reported for the description of chromatographic peak profiles, which solve the problem with different success [72–76]. The elution profiles of symmetrical and non-overloaded chromatographic peaks are well described by the Gaussian model. Non-ideal peaks (either tailing or fronting) are, however, quite common in practice. In addition, the description of asymmetrical peaks should consider incidental variations in peak profile with mobile phase composition and other factors [77]. Hence, an adequate and handy mathematical peak model is needed to describe each peak in a chromatogram. The model should take

advantage of the properties usually monitored (i.e., retention time, efficiency, asymmetry, and area or height).

One of these handy equations consists of a modification of the Gaussian equation, where the standard deviation varies with the distance to the peak maximum, which has been called polynomially modified Gaussian (PMG) model [73,74]. In its initial formulation, this model was expressed as:

$$h(t) = h_0 \exp\left[-\frac{1}{2} \left(\frac{t - t_R}{s_0 + s_1 (t - t_R) + s_2 (t - t_R)^2 + \ldots} \right)^2 \right] \qquad (1.11)$$

where $h(t)$ and h_0 are the height at time t and the maximal peak height, respectively, t_R is the solute retention time, s_0 a measurement of the peak width established on a Gaussian basis (i.e., a Gaussian peak that behaves as the asymmetrical one in the maximum neighbourhood of the latter), and s_1 and higher order terms account for peak distortion. The equation actually represents a family of models, since changes in the polynomial degree within the standard deviation term give rise to several functions. The higher the degree, the more flexible the model and the more the chances to fit the experimental data. Theoretically, there is no limit to the polynomial degree, but in practice, parabolic or cubic functions are enough to account for most chromatographic signals without significant under- or over-fitting.

The chromatogram of a sample may contain a large number of peaks that should be modelled or predicted individually. For optimisation purposes, a PMG model with a linear standard deviation term (i.e., including only the s_0 and s_1 terms) is most convenient. The advantage of this model, besides its simplicity, is the easiness to relate its four parameters to four convenient peak properties: retention time, efficiency, asymmetry, and area or height. If the

efficiency (N) is calculated according to the equation proposed by Foley and Dorsey [78], s_0 and s_1 can be calculated as:

$$s_0 = 0.466 \frac{\sqrt{\frac{41.7\, t_R^2}{N\left(1.25 + f_{B/A}\right)}}}{1 + \frac{1}{f_{B/A}}} \left(1 - \frac{f_{B/A} - 1}{f_{B/A} + 1} \right) \tag{1.12}$$

$$s_1 = 0.466 \frac{f_{B/A} - 1}{f_{B/A} + 1} \tag{1.13}$$

where $f_{B/A}$ is the asymmetry factor measured at 10% of peak height, which is calculated by dividing the right (B) and left (A) peak half-widths.

1.2.5. Measurement of resolution

The goal of experiment-based interpretive optimisation is to establish the conditions to achieve maximal resolution, using the data from a set of experiments as reduced and informative as possible. The process implies measuring the performance of hundreds of computer-generated chromatograms. These chromatograms can range from only the location of the peaks for the most simple resolution assessments, up to simulations of comprehensive signals versus time. Each of these chromatograms corresponds to a particular set of experimental factors, which are wisely-tuned throughout the optimisation process. Such an exploration may be carried out visually, but this is time consuming and inefficient. The examination can be more exhaustive, rigorous and practical with the assistance of a COF, which is a mathematical expression that qualifies the resolution level associated with any peak arrangement [79–83]. The COF may include additional quality criteria, such as the analysis cost, the retention of the first and last peak, and the number of resolved peaks. However, owing to the

prevalence of resolution as a qualifier of separation, frequently the COF only considers this property.

The maximal COF value denotes the best separation conditions. The validity of this approach is, however, bound to the premise that the applied function is able to appraise chromatograms in the same way as an expert chromatographer would do. Actually, the reliability of an optimisation process depends on two factors. First, the simulated chromatograms should be realistic. As already indicated, simulations have been benefited by the advances in modelling of retention time and peak shape. Second, the selected resolution criterion should quantify the peak resolution properly. This is particularly important for asymmetrical peaks or when the peak areas from the individual peaks differ significantly. Unfortunately, the validity of the optimal conditions provided by an interpretive procedure is affected by the COF choice.

The analyst estimates the quality of a chromatogram attending first to the individual separation achieved for each peak. In the next step, the global peak distribution is examined. This process can be mimicked numerically by calculating a set of elementary resolution values for each particular solute or solute-pair, which are next combined through a reduction function to obtain a single numerical value. The outlined process requires the calculation of intermediate functions denoting the retention, efficiency or width, and asymmetry, to evaluate the resolution.

1.2.5.1. Elementary resolution criteria

Elementary resolutions score the particular separation of each solute from the others, or between each pair of consecutive peaks. Different measurements

of diverse complexity have been proposed for this purpose. The simplest one, which considers only peak position, is the selectivity:

$$\alpha_{i,i+1} = \frac{k_{i,i+1}}{k_i} \tag{1.14}$$

where k_i and k_{i+1} (with $k_{i+1} > k_i$) are the retention factors of two consecutive peaks. Accordingly, this criterion provides one value for each peak pair. However, because peak width is not considered, the selectivity is only suited for comparing chromatograms where the peaks are relatively narrow with regard to the peak distance.

Other elementary resolution criteria consider individual peak widths or even peak profiles and sizes. Such more comprehensive assessments are not only interesting when asymmetrical or low-efficiency peaks are involved, but also in situations where the separation space is narrow, or when an impurity is concerned. The so-called chromatographic resolution, R_S, is certainly the most popular criterion:

$$R_S = \frac{t_{R,i+1} - t_{R,i}}{B_i + A_{i+1}} \tag{1.15}$$

where $t_{R,i+1}$ and $t_{R,i}$ are the retention times of two consecutive peaks, and B_i and A_{i+1}, the tailing and front peak half-widths. The threshold of baseline separation for R_S is usually adopted as 2.5, although this value is actually only appropriate for Gaussian peaks. Modifications of the R_S parameter that consider peak widths, asymmetries and height ratios have been proposed [77].

A measurement with a particularly good behaviour is the peak purity, which is the complement of the overlapped fraction [79,82]. This measurement quantifies the peak area percentage free of interference:

$$p_i = 1 - \frac{o_i'}{o_i} \tag{1.16}$$

where o_i' is the area under the peak overlapped by a hypothetical chromatogram built with the peaks of the accompanying compounds in the sample studied, and o_i the total area of the peak of interest. This measurement ranges from zero for full overlapping to one for full resolution, and depends on the relative peak areas. The calculation of peak purity is laborious, only being feasible through numerical computation. It offers, however, unique advantages. Fortunately, the state-of-the-art of computers and the proposal of more practical peak models have revived its interest. Other criteria bearing the same problem although with similar advantages, such as the vector–based representation of chromatograms, are nowadays fully feasible with the development of faster computers [84].

Chromatograms selected as optimal by different COFs can differ significantly, and the chromatographer may find some of them considerably better than others. This fact denotes the diverse ability of the functions to address the search, even when the factor space explored is the same [79,80]. The reason is that COFs are too simple to take into account all the features the chromatographer's mind considers in the quality evaluation. Among the reported functions, peak purity correlates particularly well with the appraisal of resolution of expert analysts, even for peaks remarkably skewed and overlapped.

1.2.5.2. Global resolution criteria

As mentioned, the global separation in a chromatogram is evaluated by reducing the elementary resolutions (r_i) to a single value that describes the overall peak distribution (R). The most widely used reduction function, owing to its simplicity, consists of finding the worst elementary resolution, that is, the least-resolved peak pair (i.e., critical pair):

$$R = \min (r_i) \quad 1 \leq i \leq n \tag{1.17}$$

In other cases, the scope of the optimisation is extended to more peaks than the critical one by combining the elementary resolutions in a more or less complex fashion. Two common criteria are the normalised-by-the-mean resolution product:

$$R = \frac{\prod_{i=1}^{n} r_i}{\left[\left(\sum_{i=1}^{n} r_i\right) / n\right]^n} \tag{1.18}$$

and the unnormalised resolution product:

$$R = \prod_{i=1}^{n} r_i \tag{1.19}$$

where n represents, depending on the elementary criterion, the number of peak pairs (case of the selectivity factor and R_S) or number of peaks (case of peak purity). With normalised elementary values, the three global functions (Eqs. (1.17)–(1.19)) vary between 0 (when at least one peak is fully overlapped) and 1 (when all peaks are baseline resolved); the closer to 1, the better the separation. Products of elementary resolutions are dominated by poorly resolved peaks, meaning that an optimisation based on these products tends to

discard rapidly conditions where two or more peaks remain unresolved. The situation is even worse with unnormalised measurements, particularly when the results have to be interpreted.

An optimisation based on the separation of the poorest-resolved peak is reasonable, but it only attends the results for the critical peak or peak pair. It is, therefore, insensitive to the separation attained for the remaining peaks. Thus, chromatograms scoring the same resolution may show important differences in the separation of non-critical peaks. For this reason, combined criteria that attend to all peaks are preferable.

1.2.5.3. Peak purity and limiting peak purity

Peak purity grants a particularly good performance in optimisation, with interesting advantages in comparison with the classical R_S [79] First, its meaning is very intuitive: a peak purity value of 0.95 just means that 95% of the peak is free of interferences (or 5% interfered). Accordingly, it is related to what the chromatographer actually wishes: peaks free of interference. Also, this criterion considers not only the position of the peak but also its profile and size. Therefore, the corresponding resolution diagrams provide a more realistic picture of the system separation performance. Finally, peak purity is an intrinsically normalised measurement, which facilitates the combination of elementary resolutions into a single global value and, eventually, the further combination with other quality criteria.

However, the feature of the peak purity concept with perhaps more consequences, at least at a calculation level, is that it provides the separation quality for each individual peak, instead of peak pair. There is, thus, an unambiguous relationship between compound identities and numerical values,

and therefore, knowledge of the identity of neighbouring peaks is not as important as for other criteria related to peak pairs (e.g., R_S). Furthermore, certain operations, such as weighting or exclusion of peaks are easier, and problems related to peak crossing are avoided: any other remaining peak is considered as interference.

Perhaps, the most useful measurement derived from the peak purity concept is the so-called "limiting peak purity": the maximal elementary value found for each compound. The limiting purities measure the maximal expectancies of resolution for each compound. The meaning of limiting purities is the same as for conventional purities: if a solute reaches full separation, the limiting purity will be 1. When a certain overlap remains with another or more solutes, it will be <1. If the limiting value is small, no mobile phase will resolve the solute. This means that the capability of the system will have been fully exploited, and a further enhancement will need a drastic change in the separation system.

The degree of completion of the separation capability reached for a particular compound, *j*, in a given chromatographic system, at the i^{th} separation condition, is given by [85]:

$$p_{\mathrm{rel}}(i, j) = \frac{p(i, j)}{p_{\mathrm{lim}}(j)} \times 100 \tag{1.20}$$

The combined limiting peak purity prospects the capability of the chromatographic system. The meaning is similar to the global resolution: it indicates the maximal separation capability that can be reached for a set of compounds. The percentage of global resolution informs about the degree of exploitation of the separation system:

$$P_{\mathrm{rel}}(i) = \frac{P(i)}{P_{\mathrm{lim}}} \times 100 \tag{1.21}$$

When P_{rel} is close to 100%, no significant improvement can be expected by changing the separation conditions. However, the interpretation of the percentage of global resolution is less evident than for the elementary values, and should be carried out with caution, since it includes other effects that influence the numerical values, such as the number of peaks and the distribution of the individual values.

The peak purity concept has allowed the development of new optimisation strategies [34]. On the one hand, the fact that it is able to anticipate the maximal resolution capability of the separation system, is particularly useful for tackling low resolution situations, where conventional resolution criteria fail [8].

Isocratic elution allows the maximal capability of the system, since it maximally expands the separation among peaks. However, isocratic elution can imply absolutely impractical analysis times for complex mixtures, which obliges the use of gradient elution. As the complexity of the gradient increases, the limiting purities tend to that for the isocratic elution. Furthermore, if the isocratic limiting purities indicate that one or more pairs cannot be separated up to the baseline, no gradient will be able to succeed.

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PART 1

OPTIMISATION OF MULTI-COLUM SYSTEMS IN MONO-DIMENSIONAL LIQUID CHROMATOGRAPHY

CHAPTER 2

STATIONARY PHASE MODULATION IN LIQUID CHROMATOGRAPHY THROUGH THE SERIAL COUPLING OF COLUMNS

2.1. Abstract

Liquid chromatography with single columns often does not succeed in the analysis of complex samples, in terms of resolution and analysis time. A relatively simple solution to enhance chromatographic resolution is the modulation of the stationary phase through the serial coupling of columns. This can be implemented with any type of column using compatible elution conditions and conventional instruments. The key features of column coupling and published procedures, where two or more columns were coupled in series to solve separation problems are here described. In all reports, the authors could not resolve their samples with single columns, whereas significant enhancement in chromatographic performance was obtained when the columns were combined. Particularly interesting is the reduction in the analysis time in the isocratic mode, which alleviates the "general elution problem" of liquid chromatography, and may represent a stimulus for the proposal of new procedures, especially in combination with mass spectrometric, electrochemical and refractometric detection. Developments proposed to make the serial coupling of columns useful in routine and research laboratories are outlined, including optimisation strategies that facilitate the selection of the appropriate column combination and elution conditions (solvent content, flow rate or temperature) in both isocratic and gradient modes. The availability of zero dead volume couplers, able to connect standard columns, and the commercialisation of short columns with multiple lengths, have expanded the possibilities of success.

2.2. Introduction

Since the 1970s, liquid chromatography (LC) has expanded in the analytical laboratories, due to its versatility, ease of use, robustness, sensitivity and applicability to multiple problems in environmental, pharmaceutical, clinical and food analysis [1,2]. Reversed-phase liquid chromatography (RPLC) is the most frequent LC mode for the separation of non-volatile compounds, from small molecules to large biological macromolecules in a wide range of polarities [3]. However, LC has the important drawback that, in comparison to gas chromatography (GC), the analysis of complex samples requires a relatively higher selectivity to get resolution, owing to the substantially lower efficiency [4]. Moreover, selectivity and analysis time depend in a complex way on several experimental factors that interact with each other (e.g., organic solvent content, pH and temperature), which are difficult to adjust in practice to the optimal levels because they affect the analytes through a series of complex interactions.

In LC, the very first important step in method development is the selection of an adequate column (i.e., stationary phase, particle size and length). The column may be chosen from those available in the laboratory, attending to previous experience, from literature, or from recommendations obtained from manufacturers. The performance of the LC column is investigated under different elution conditions (using one or more organic solvents, pH values or temperatures, under isocratic or gradient elution, and perhaps with one or more types of buffers or additives). The choice of solvent is particularly limited (usually acetonitrile or methanol and water in RPLC), but its concentration is easy to adjust giving rise to dramatic consequences. Since initial runs seldom provide adequate separation, an optimisation protocol is required to establish an appropriate isocratic mobile phase composition or gradient program [5–7]. This

step is rather complex due to the type of dependence between the retention factor and eluent composition. Therefore, a large number of experimental runs may be needed. Often, the final decision is based on an incomplete exploration of the experimental domain, which results in the selection of conditions that do not necessarily yield the best separation.

The aim of chromatographic optimisation is the achievement of maximal resolution with an acceptable analysis time. If the search does not succeed, other conditions (such as another solvent, pH or temperature) may be then selected, and the optimisation process is repeated. Only when all alternative solutions fail, the analyst decides to change the column to get different selectivity. This trial-and-error method can be extremely time-consuming, and its success depends strongly on the analyst experience and intuition. It is not surprising that usually only one column is tried, even when the final separation is not fully acceptable. The frequent situation with complex samples is that after probing several columns, the sample remains unresolved.

Therefore, in spite of the extraordinary advances in the development of new supports and stationary phases, the traditional strategy of using a single chromatographic column in LC is still insufficient, due to the limited chemistry of conventional stationary phases. An interesting solution, which may contribute to expand the applicability of LC, is the modulation of the stationary phase through the serial connection of columns. The term "modulation" is associated to transitions or combinations produced deliberately. However, the implementation of the modulation concept in LC has not been simple, due to a number of technical problems.

This review describes the developments proposed by several authors to make the serial connection of columns useful in laboratories. The limitations of single columns are first commented. Serially coupling of columns containing

different stationary phases is next framed among a group of approaches that take advantage of the combination of stationary phases. A literature survey offers information on the type of columns that can be combined and the range of applications. The main problems and limitations related to column coupling, and the development of optimisation strategies are discussed. Finally, different modalities in method development with coupled columns that involve the search of the best column combination, the optimal elution conditions, and the benefits of controlling the temperature and flow rate, are described.

2.3. Selection of single columns

LC analysis is usually implemented using a single column. In the last decades, increasingly sophisticated and selective LC columns have been marketed, especially in the field of RPLC [8–11]. The advances in the knowledge of the surface properties of silica, and the influence of residual silanols and metal impurities on adsorption-desorption processes, are partially responsible for this progress. This has given rise to successive generations of supports and bonded phases. Most research in column technology responds to the need of better reproducibility and stability of the columns at extreme pH values, more selective separations, smaller analysis times for diverse groups of compounds, and the elimination of free silanols to avoid skewed peaks. More recently, hydrophilic interaction chromatography (HILIC) has attracted great attention to analyse highly polar compounds [12–14].

Compared to the narrow selection of organic solvents in RPLC, the number of stationary phases is overwhelming, with several hundreds of columns in the market. This number increases continuously, without often offering alternative or enough selectivity. Most commercially available stationary phases are based on octadecyl-silica. Other usual types of silica-based materials are octyl-,

cyano-, amino-, phenyl-, and polar embedded. New materials have been introduced to overcome the poor chemical and thermal stability of silica, and its surface chemical heterogeneity, including polymer-, alumina-, and zirconia-based phases [10,11]. Another solution to expand column selectivity is to alter the nature of conventional stationary phases by a temporary or permanent modification, through the addition of additives [15].

The choice of stationary phase is crucial. Many attempts have been made to develop strategies to facilitate this selection, based on classification models [16–21]. However, these models are not commonly applied or even useful. In addition, manufacturers do not use harmonised approaches to describe column selectivity. Therefore, there is no reliable guidelines that guarantee the selection of the right column, and this is often found by chance (or not found), being like a "hit or miss proposition" [22]. To succeed in the analyses, it is common to check the performance of columns of different types (or even columns of the same type, from different suppliers), to achieve the desired separation. Owing to economic reasons, the number of columns available in a laboratory and the number of assays are small. Consequently, the ideal column for a given separation may not be available or may be missed.

Another problem related to the selection of single columns is that their selectivity cannot be modulated continuously (unlike the mobile phase composition), to finely tune the separation. Also, by using a new column type, most of the effort expended on a previous column for method development is wasted, and the possible benefits of that column totally discarded.

2.4. Approaches that combine stationary phases to improve the chromatographic performance

2.4.1. Use of parallel columns

When a single column fails in the separation of the whole set of compounds in a sample, within a single run, the most immediate solution is to perform the analysis in two runs. The second run has the aim to resolve at least the overlapped compounds in the first run. The same or a different column can be used in each run. The approach is apparently simple, but with complex samples a rather sophisticated software is needed to find true complementary situations that give adequate resolution of all analytes [23,24].

2.4.2. Mixed-mode columns

Stationary phases exhibiting intermediate selectivity can be prepared by mixing different separation mechanisms, such as reversed phase or size exclusion, and cation or anion exchange [25–30]. The development of mixed-mode columns is currently an active field. In a mixed-mode column, the separation mechanisms can be implemented by either chemically bonding the different stationary phases on the same particles, or by mixing particles that incorporate different stationary phases within a single column. Mixed-mode columns exhibit the properties of each constituent stationary phase. By varying their ratio, the selectivity can be modulated.

The mixed-mode column approach presents, however, several drawbacks. First, dedicated columns are needed. Second, the interaction between the different mixed stationary phases can give rise to complex non-linear retention behaviours [25,27]. Finally, analysts cannot usually pack columns by their own (or even synthesize their own stationary phases), due to the lack of equipment

and expertise. Mixed-mode columns should be purchased from catalogue (if available), or provided by manufacturers in a custom-made request. This is often very expensive (when possible). Therefore, more versatile solutions are needed.

2.4.3. Tandem columns in a multi-dimensional configuration

An alternative to mixed-mode columns is the elution of the sample through two or more tandem (i.e., subsequent) columns, each one filled with a specific stationary phase. Several modes of operation have been developed. An approach that has gained high popularity in recent years is "multi-dimensional LC" (for two columns, "two-dimensional" or "dual column LC"). In two-dimensional LC (2DLC), fractions of the eluent from the outlet of the first (primary) column are transferred to the inlet of the second column. Each transferred volume can correspond to a group of peaks, a single peak, a fraction of a peak, or baseline region. The transference can be carried out either off-line or on-line. The separation in the second dimension can involve all the fractions collected in the first dimension (comprehensive approach) [31–34], or only selected fractions (heart-cutting approaches) [35–38].

Together with the advantage of exploiting different retention mechanisms, two-dimensional LC offers the freedom to manipulate independently the eluent composition, gradient program, flow rate and temperature, for each column. For this purpose, dual pumping systems and multiport valves with intermediate sample storage mechanisms are used between the coupled columns (Fig. 2.1). This gives rise to a considerable increase in peak capacity (i.e., maximal number of compounds that ideally can be separated in a single run) [39]. Columns of the same type (with the same or different lengths), or different

type, can be combined. In principle, all established interaction mechanisms (e.g., reversed phase, normal phase, ion exchange, size exclusion and hydrophilic interaction) can be combined. However, the best performance for samples containing analytes in a wide range of polarities is reached by coupling orthogonal (i.e., independent) mechanisms [40,41].

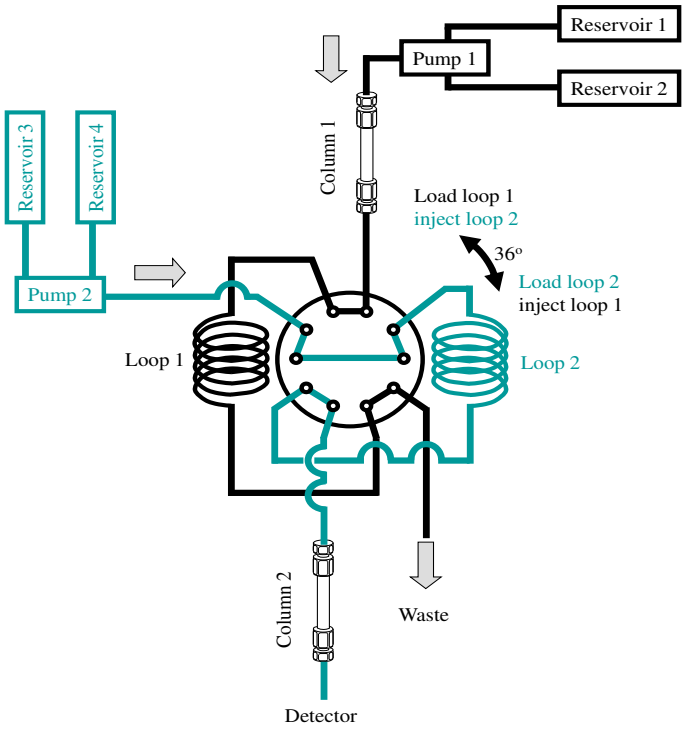

Figure 2.1. Tandem columns in a comprehensive two-dimensional configuration with a 10-port valve.

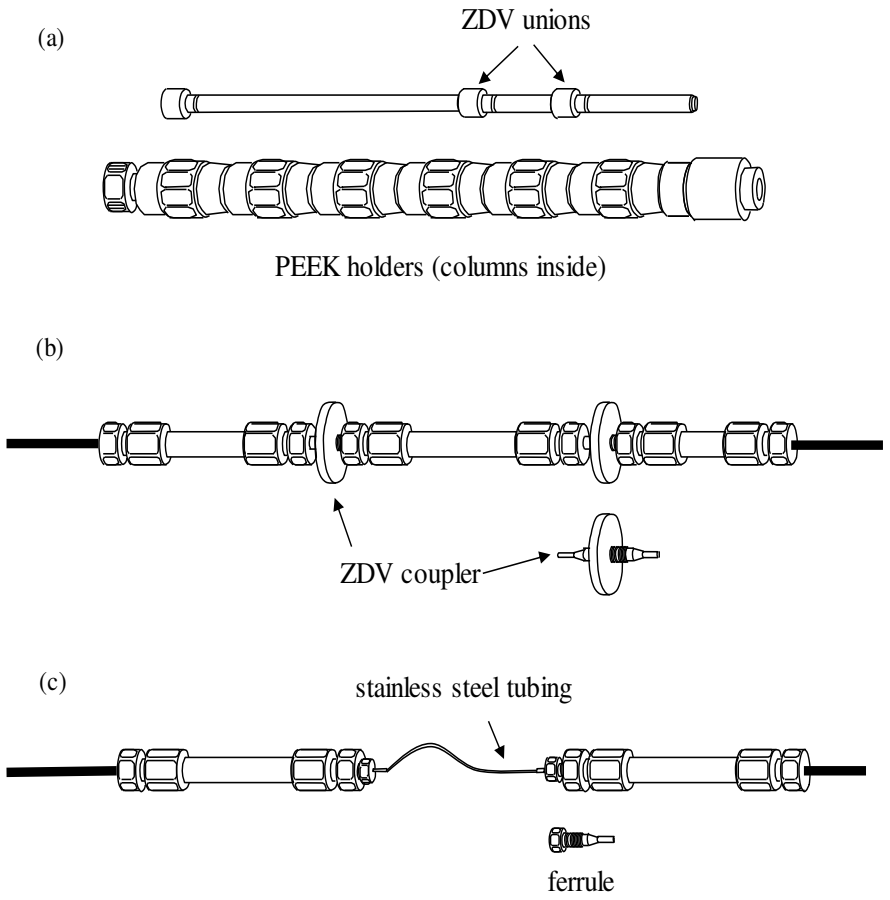

Figure 2.2. Tandem columns in serial configurations, using different types of connectors: (a) ZDV unions and PEEK holders (POPLC system), (b) universal ZDV fingertight couplers, and (c) stainless steel tubing.

In spite of the attractive peak capacity of two-dimensional LC, the expensive equipment needed is not common in most laboratories. It is also beyond the capabilities of less experienced analysts. Other disadvantages are the considerable sample dilution that may occur and the longer analysis times.

Chromatographic optimisation in two-dimensional LC is also not trivial, due to the large number of mutually dependent factors, and the uncertainties associated to the modulator (i.e., the transfer system that cuts and re-injects portions of the primary column effluent into the second column throughout the analysis). The modulator must be capable of operating repeatedly at high pressure with very low dead volume, so that the transferred peaks do not result significantly broadened.

2.4.4. Tandem columns in a serial configuration

A more modest design using tandem columns, at least from a technical point of view, consists of connecting directly two or more columns in series, and flowing the same mobile phase (or gradient) through all columns (Fig. 2.2, see also Section 2.6.1). Similarly to comprehensive multi-dimensional LC, all the molecules contained in the sample injected into the first column migrate through all tandem columns. This approach, which has been called "serially-coupled columns", "multi-segment LC", "columns in-line" and "stationary phase gradient LC", is the topic of this review. LC with serially-coupled columns (SCC) only requires a conventional instrument equipped with a single pump, without the need of any device between the columns to store fractions of the eluent. The approach is thus much easier to implement in a standard laboratory, compared to comprehensive or heart-cutting multi-dimensional LC, not requiring any special training. It is also faster and more robust. However, since serial columns are operated with the same eluent and experimental conditions, the elution conditions (pH, temperature, solvent composition, gradient program, pressure, etc.) of the coupled stationary phases should be compatible, a problem that is less important in multi-dimensional LC.

Selectivity changes can be achieved in some cases by coupling columns differing in their end capping or alkyl chain length, but often a stationary phase based on a different mechanism is needed. The selectivity of the column combination will be only strikingly different if the selectivity of each individual stationary phase differs from each other to a great extent. Thus, unless the behaviour of the critical pairs for each individual column is different, the selectivity will not change with the combined columns. However, interesting behaviours are observed even if this is not the case.

The direct connection of columns with different stationary phases and lengths significantly improves the resolution expectancies, with regard to the separation offered by each single column (without reaching the peak capacity of multi-dimensional LC). Even when all assayed columns offered a deficient separation when used isolatedly, their wise combination may yield baseline separation (Figs. 2.3 to 2.6). In addition, the analysis time can be reduced with regard to the single columns, making the approach competitive against gradient elution. Finally, the columns can be disassembled and re-used for other separation problems. A given serial column combination behaves like a totally new column, in some instances with extraordinarily better performance [24]. This increases the probability of having the ideal column for a particular separation.

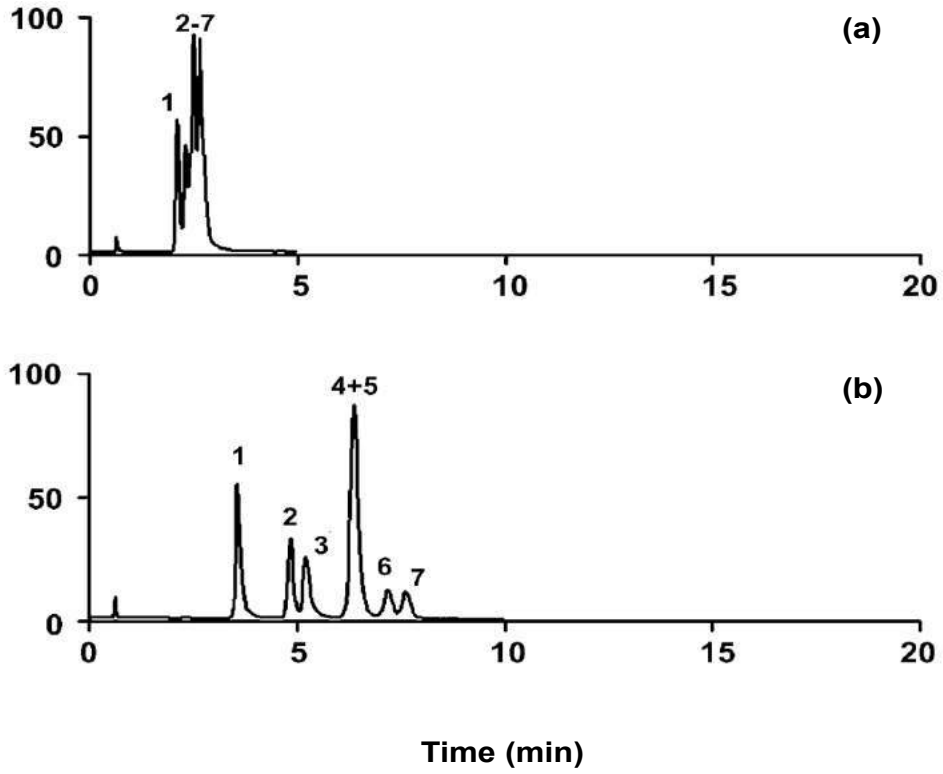

Figure 2.3. Optimised chromatograms of probe compounds using POPLC Prontosil columns and the SOSLC software from Bischoff Chromatography (Leonberg, Germany). Columns: (a) Cyano and (b) phenyl. The dimensions of the single columns were 10 cm × 3.0 mm I.D. Other conditions: Column temperature, 30 ºC; mobile phase, 30% (v/v) acetonitrile/water; flow-rate, 0.6 mL/min; UV detection at 220 nm. Compounds: (1) Methylparaben, (2) acetophenone, (3) ethylparaben, (4) dimethylphtalate, (5) 2,3-dimethyl-phenol, (6) methylbenzoate, and (7) anisol. Reproduced with permission of Elsevier from Ref. [61].

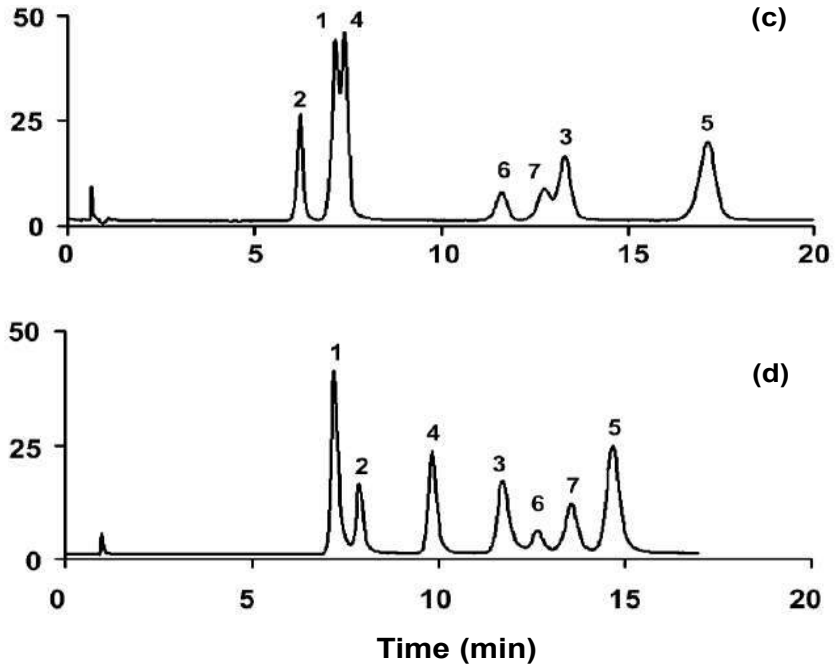

Figure 2.3 (continued). Columns: (c) C18-EPS, and (d) serial coupling of the three columns (a), (b) and (c). The assembled column in (d) was 4 cm cyano + 10 cm phenyl + 6 cm C18-EPS. See previous page for more details.

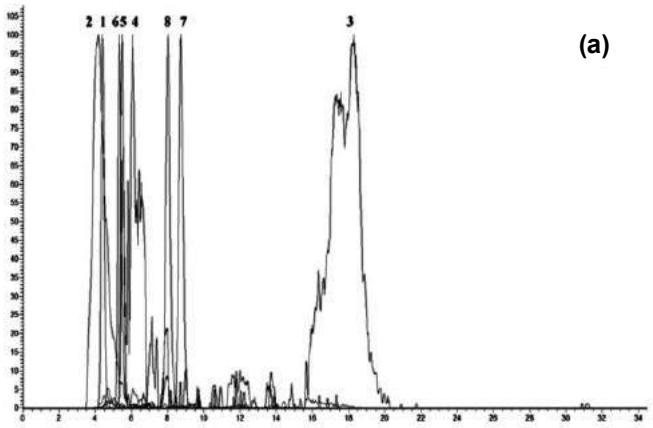

Figure 2.4. Optimised chromatograms of polar and non-polar metabolites in beer. Column: (a) SeQuant ZIC-pHILIC, 15 cm × 4.6 mm I.D. (Merck, Darmstadt, Germany). Gradient conditions: (a) solvent A, water + 20 mM ammonium carbonate, pH 9; solvent B, acetonitrile; flow-rate, 350 μL/min. Column temperature was 25 ºC for all separations. Detection was carried out with a Thermo Scientific Exactive Orbitrap system equipped with a HESI II interface. Compounds: (1) ad-Humulone, (2) iso-xantholhumol, (3) cohumulone, (4) syringaldehyde, (5) tyrosine, (6) ethylvanillin, (7) succinic acid, and (8) phenylalanine. Reproduced with permission of Springer from Ref. [89].

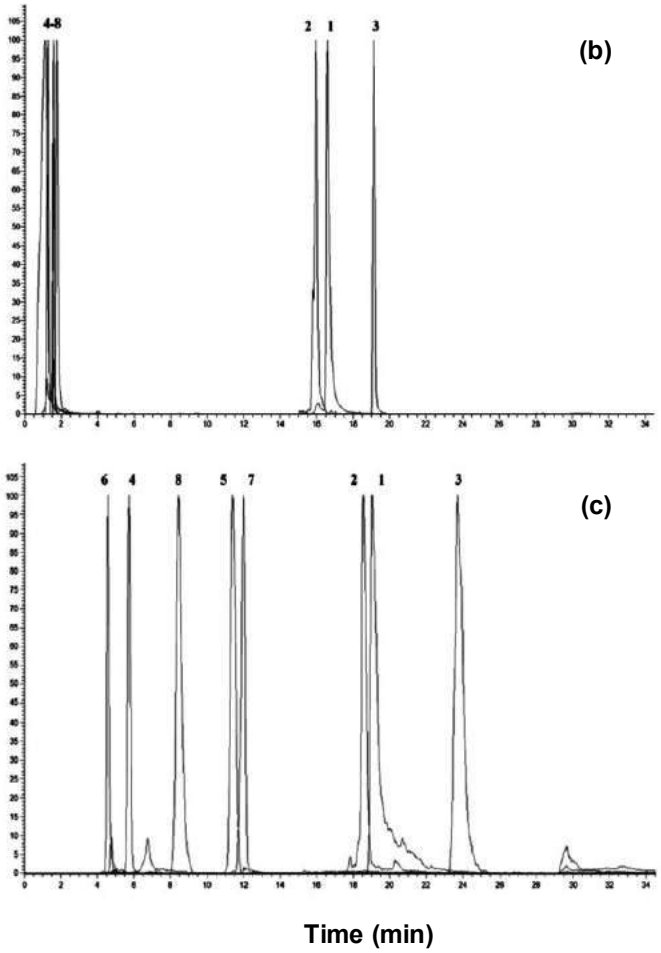

Figure 2.4 (continued). Optimised chromatograms of polar and non-polar metabolites in beer. Columns: (b) Hypersil GOLD 10 cm × 1.0 mm I.D. (Thermo Scientific, Hemel Hempstead, UK), and (c) serial coupling of the columns in (a) and (b), where the HILIC column was placed after the C18 column (see Fig. 2.9b). Gradient conditions: (b) solvent A, water; solvent B, acetonitrile; flow-rate, 65 µL/min. The same conditions were applied for (c). See previous page for more details.

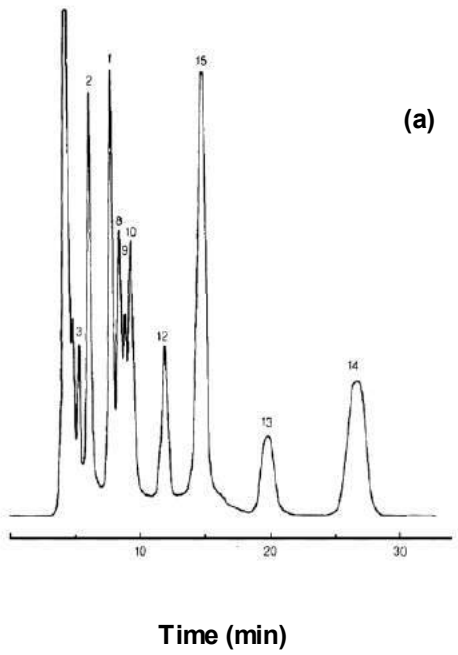

Figure 2.5. Optimised chromatograms of oligosaccharides, monosaccharides and related products. Column: (a) Ca-loaded sulphonated polystyrene-divinylbenzene resin, 30 cm × 7.8 mm I.D. Other conditions: Column temperature, 85 ºC; mobile phase, water; flow-rate, 0.6 mL/min; refractometric detection. The oligosaccharides were obtained from a starch syrup and are identified by their degree of polimerisation, DP. Compounds: (1) D-Glucose, (2) DP2, (3) DP3, (4) DP4, (5) DP5, (6) DP6, (7) DP7, (8) D-xylose, (9) D-fructose, (10) D-arabinose, (11) dihydroxyacetone, (12) 1,6-anhydro-β-glucose, (13) hydroxymethylfurfural, (14) furfural, and (15) ethanol. Reproduced with permission of Elsevier from Ref. [44].

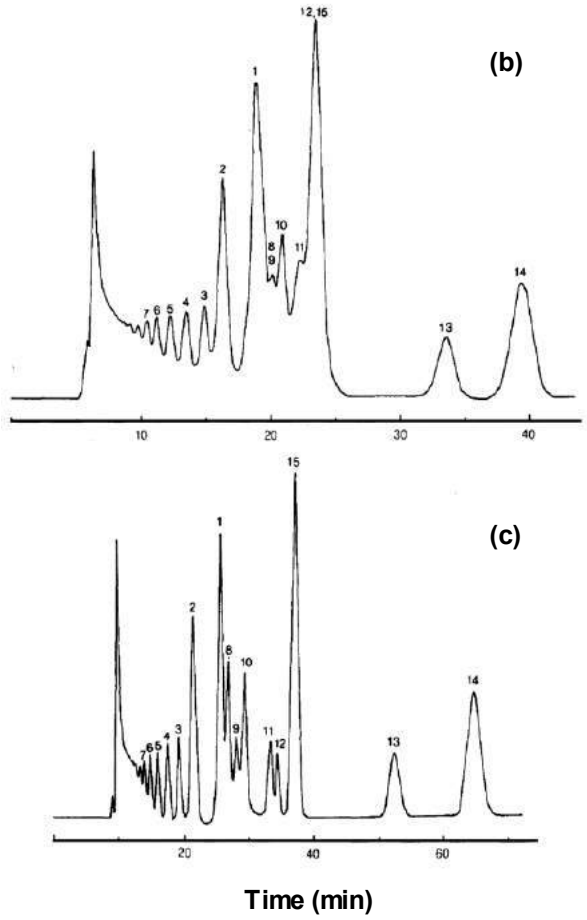

Figure 2.5 (continued). Optimised chromatograms of oligosaccharides, monosaccharides and related products. Columns: (b) Ag-loaded cation-exchange stationary phase, 10 cm × 7.8 mm I.D., and (c) serial coupling of the columns in (a) and (b). See previous page for more details.

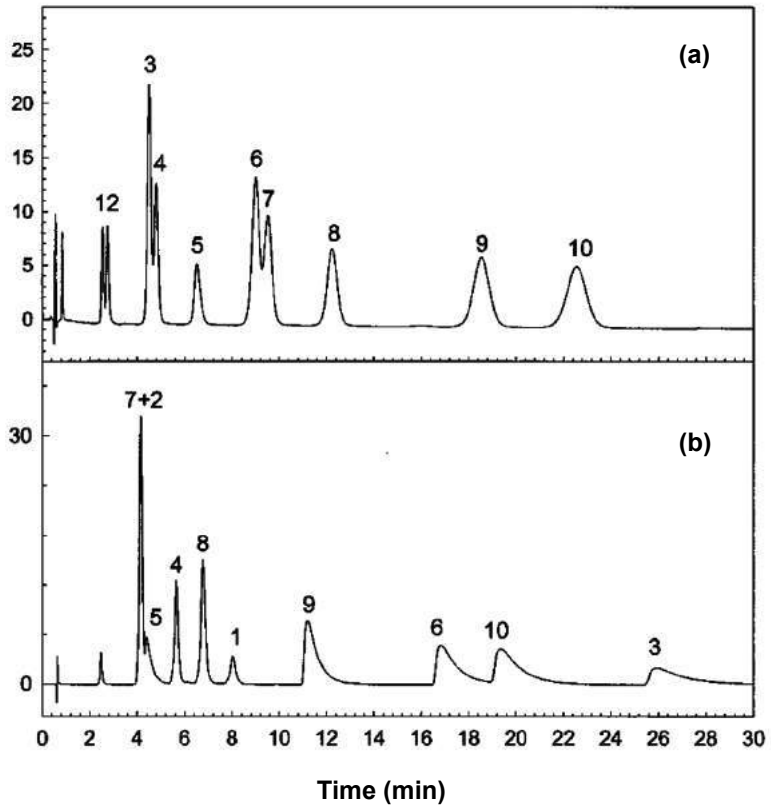

Figure 2.6. Optimised separations of triazine herbicides. Columns: (a) Zorbax SB-C18 (Hewlett-Packard, Wilmington, DE, USA), (b) carbon-coated ZrO_2 (ZirChrom Separation, Anoka, MN, USA) The dimensions of the single columns in (a) and (b) were 5 cm × 4.6 mm I.D. Column temperature: (a) 30 ºC, (b) 60ºC. Flow rate: (a,b) 1 mL/min. Other conditions: Isocratic elution with 30% (v/v) acetonitrile; UV detection at 254 nm. Compounds: (1) Simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) prometon, (6) ametryn, (7) propazine, (8) turbulazine, (9) prometryn, and (10) terbutryn. Reproduced with permission of American Chemical Society from Ref. [81].

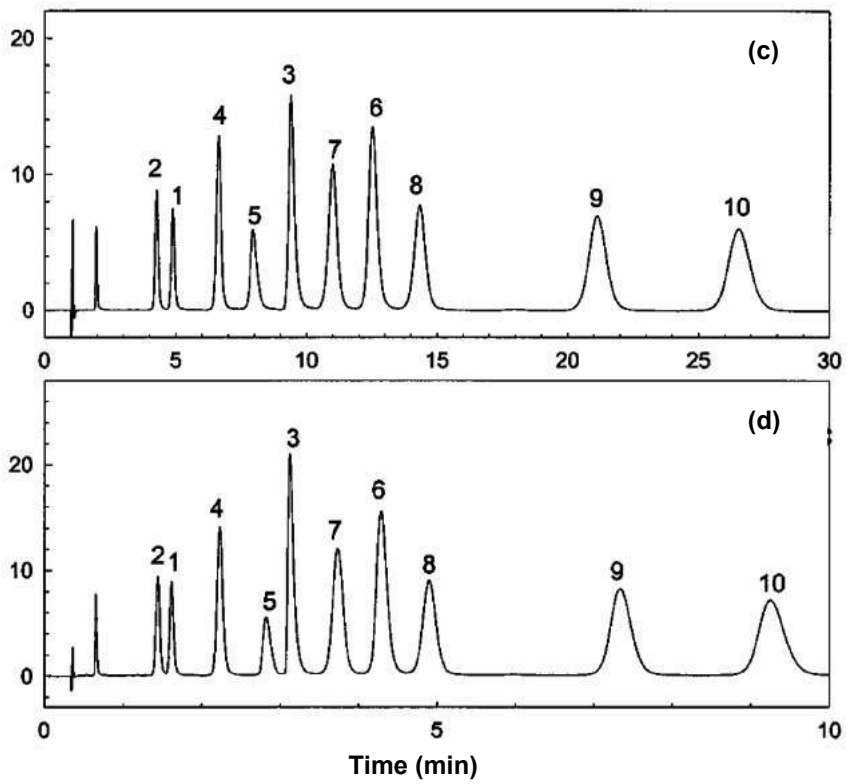

Figure 2.6 (continued). Optimised separations of triazine herbicides. Columns: (c,d) serial coupling of columns in (a) and (b). Column temperature: (c, d) 30 ºC for SB-C18 and 125 ºC for C-ZrO_2. Flow rate: (c) 1 mL/min, and (d) 3 mL/min. See previous page for more details.

The contour map in Fig. 2.7 illustrates the resolution in the separation of 15 sulphonamides (see compounds in Fig. 2.10) by coupling cyano and phenyl columns. The global resolution was measured as the product of the peak purity (P) for all compounds (peak area fraction for each peak, free of interference [5]; $P = 1$ denotes full resolution, and $P = 0$, full overlapping). The bottom border depicts the resolution for a 10 cm phenyl column and the top border the resolution for a 10 cm cyano column. Intermediate values correspond to hybrid combinations of both stationary phases with a total length of 10 cm. As observed, neither the cyano nor the phenyl pure columns were able to resolve the sample at any acetonitrile content, while the resolution was enhanced with the combination of both stationary phases. The highest performance with almost baseline resolution for all sulphonamides was obtained with SCC built with cyano columns in the range 6–8 cm and phenyl columns in the range 2–4 cm, and isocratic mobile phases with acetonitrile below 12%. The white region in the diagram corresponds to combinations that were considered not appropriate due to excessive retention.

2.5. Reported procedures where two or more columns were serially connected

The idea of serially coupling columns containing different stationary phases to analyse complex samples appeared early in the development of LC [42–46], inspired by a similar approach in GC [47–50]. Reported procedures where two or more columns were coupled in series are listed in Table 2.1. In all cases, the authors indicated that the separation using one or several single columns was unsuccessful, in terms of resolution (most often) and analysis time. Meanwhile, by serially coupling two or more columns of different selectivity, the resolution was highly satisfactory and often with smaller

analysis time. Refs. [68,72] also highlighted the increased number of drugs that could be monitored using coupled columns.

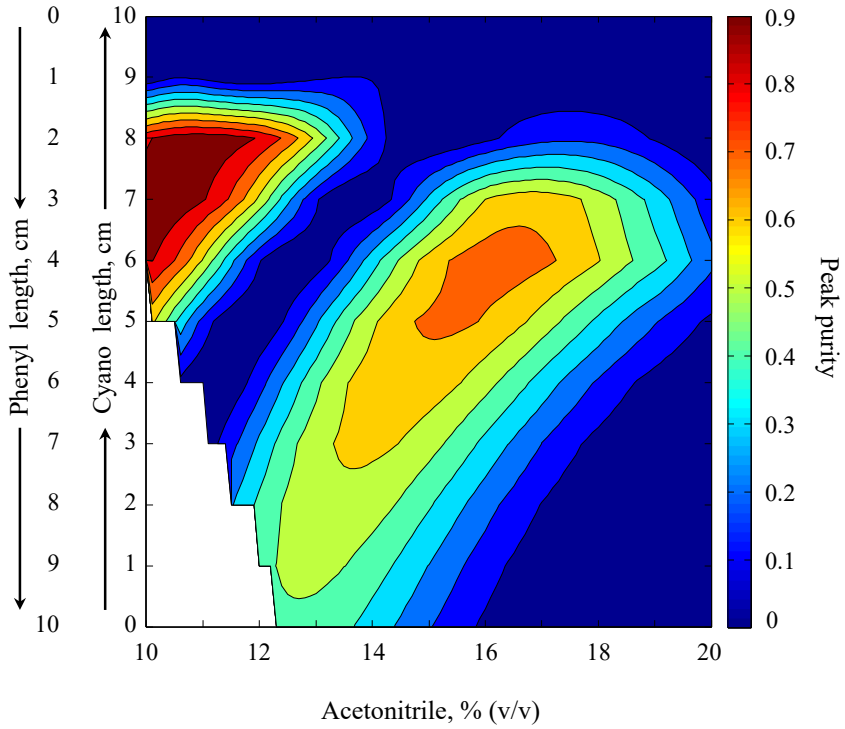

Figure 2.7. Contour map depicting the global resolution in the separation of 15 sulphonamides with serial combinations of cyano and phenyl columns of variable length, versus the mobile phase composition in isocratic elution. The length of the combined columns was fixed at 10 cm, so that when the length of one column in the combination increased (x in increments of 1 cm), the length of the other column decreased as $(10 - x)$ cm. The global resolution was measured as the product of the peak purity (P) for all compounds (see text for meaning). The optimal values of column length and mobile phase composition were: 3 cm phenyl + 7 cm cyano and 10.0% acetonitrile (P = 0.990). Reproduced with permission of Elsevier from Ref. [24].

Table 2.1. Some reported analytical procedures using serially coupled columns.

Columns [a]	Compounds and samples	Elution conditions and detection	Ref.
Shodex RSpak RP18-413 and Puresil C18	Rat liver metallothionein I and II, rabbit liver MT-1 and MT-2, mouse liver MT-1 and MT-2	20 mM Potassium phosphate buffer (pH 7.5)--acetonitrile-methanol (80:18:2) Fluorimetric detection	51
Zorbax SB-C18 and Betasil C18	Antihistamines, barbiturates, triazines and phenylthiohydantoin amino acid derivatives	Isocratic elution with THF-, acetonitrile- and methanol-water UV detection	52
UltraSep ES PHARM RP18, AMID RP18 P, RP18 M500 and CHAIR	Mixture of 5 probe compounds	Isocratic elution with acetonitrile-water UV detection	53
Nucleosil C18, Supelcosil LC-18-DB and Nucleosil C18 AB	Octabromo isomers in technical brominated diphenyl ethers	Isocratic elution with acetonitrile-water Diode array UV detection	54
50 mμ C18 and 100–200 mμ C18 + C1	Alkylbenzenes	Gradient elution with acetonitrile-water UV detection	55
Varian Pursuit C18 and Waters Atlantis dC18	Cortisol and cortisone in human urine	Isocratic elution with acetonitrile-water Diode array UV detection	56
C18 and C8	Dihydroxyphenylalanine in urine and plasma	Isocratic elution with 50 mM citric acid-sodium acetate buffer (pH 2.35) Electrochemical detection	43
C18 and phenyl	Mixture of 6 probe compounds	Isocratic and gradient elution with methanol-water Diode array UV detection	45
LiChrospher RP-18, cyano, NH_2, and modified ES-Gel with nitro, C7, diol and safrol groups	Chlorophenols and nitroaromatic compounds	Isocratic elution with *n*-heptane-ethanol, and acetonitrile- and methanol-water UV detection	57
Purospher STAR RP-18e, Discovery HS F5 and Discovery HS PEG	Phenolic antioxidants in beer and hop extracts	Isocratic and gradient elution with acetonitrile-ammonium acetate Diode array UV detection	58

Table 2.1 (continued).

Columns [a]	Compounds and samples	Elution conditions and detection	Ref.
Zorbax SB-C18 and Supelcosil LC-NH_2	Ibuprofen, arginine and related impurities in pharmaceuticals	Isocratic elution with acetonitrile-water UV detection	59
Zorbax-C8, cyano, benzyl and phenethyl	Phenylthiohydantoin amino acid derivatives	Isocratic elution with THF-, methanol- and acetonitrile-water UV detection	42
POPLC system with Prontosil C18-EPS, cyano and phenyl	Flavonoids and pesticides	Isocratic elution with acetonitrile-water Diode array UV detection	60
POPLC system with Prontosil C18-EPS, cyano and phenyl	Mixture of 7 probe compounds	Isocratic elution with acetonitrile-water Diode array UV detection	61
POPLC system with Prontosil C18-EPS, C18-SH, phenyl and C30	Steroids	Isocratic elution with methanol-water Diode array UV detection	62
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Synthetic thyroid hormones	Isocratic and gradient elution with acetonitrile-water UV detection	63
POPLC system with Prontosil C18-SH, phenyl and C30	Nitroaromatic explosives in raw water from an ammunition plant	Isocratic elution with methanol-water Diode array UV and electrochemical detection	64
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Steroids	Isocratic and gradient elution with acetonitrile-water Diode array UV detection	65
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Polymer electrolyte membrane degradation products	Isocratic and gradient elution with acetonitrile-water Diode array UV and MS detection	66
POPLC system with Prontosil C18, C18-EPS, cyano, phenyl and C30	Mixtures of probe compounds and mixtures of 13 steroids	Isocratic and gradient elution with acetonitrile-water Diode array UV detection	67

Table 2.1 (continued).

Columns [a]	Compounds and samples	Elution conditions and detection	Ref.
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Mixture of 14 probe compounds	Isocratic and gradient elution with ethanol-water UV detection	68
POPLC system with Prontosil C18, C18-EPS, cyano and phenyl	Mixtures of 13 oligopeptides	Isocratic and gradient elution with acetonitrile-water Diode array UV detection	69
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Screening of drugs in human autopsy material	Isocratic and gradient elution with acetonitrile-2 mM ammonium acetate-formic acid (30:70:0.2) MS detection	70
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Sulphonamides	Isocratic elution with acetonitrile-water UV detection	71
POPLC system with Prontosil C18-EPS, C18-SH, cyano, phenyl and C30	Pharmaceutical compounds in food supplements	Isocratic and gradient elution with methanol-0.025 M ammonium acetate Diode array UV detection	72
Alltima C18, C18-EPS, cyano and phenyl	Phenones, benzoic acids and hydroxybenzoates	Isocratic and gradient elution with acetonitrile-water Diode array UV detection	73
ACE C18, C18-HL, C18-AQ, cyano and phenyl	Sulphonamides	Isocratic elution with acetonitrile-water UV detection	71,74
ACE C18, C18-HL, C18-AQ, cyano and phenyl	Sulphonamides	Isocratic and gradient elution with acetonitrile-water UV detection	75,76
ACE C18-HL, C18-AQ, cyano and phenyl	Sulphonamides	Isocratic elution with acetonitrile-water UV detection	24

Table 2.1 (continued).

Columns [a]	Compounds and samples	Elution conditions and detection	Ref.
ACE C18-HL, C18-AQ, cyano and phenyl	Sulphonamides	Isocratic and gradient elution with acetonitrile-water UV detection	77
Spherisorb Reversible phenyl and nitrile	Dextromethorphan and its main metabolites in urine and microsomal preparations	Isocratic elution with acetonitrile-water Fluorimetric detection	78
Cyano and PEG silica	Mixture of 17 probe compounds	Isocratic and gradient elution with hexane-dioxane UV detection	79
Microcolumns packed with C18 and β-cyclodextrin silica	Isomeric polynuclear aromatic hydrocarbons	Isocratic and gradient elution with methanol- and acetonitrile-water Laser-induced fluorimetric and UV detection	80
Zorbax SB-C18 and C-ZrO_2	Triazine herbicides and carbamate pesticides	Isocratic elution with THF-, acetonitrile- and methanol-water UV detection	81
Zorbax SB-C18 and C-ZrO_2	Barbiturates and phenylthiohydantoin amino acid derivatives	Isocratic elution with acetonitrile-water UV detection	82
Zorbax Extended C18 and PDB-ZrO_2	Antihistamines	Isocratic elution with acetonitrile-water Diode array UV detection	83
PBD-ZrO_2 and C-ZrO_2	Benzonitrile, anisole, methylbenzoate, ethylbenzene, p-xylene and *n*-propylbenzene	Isocratic elution with acetonitrile-water UV detection	22
Ca-loaded sulphonated polystyrene-divinylbenzene resin, Ca-loaded cation-exchange, H-loaded ion-exchange and Ag-loaded cation-exchange phases	Oligosaccharides, monosaccharides and sugar degradation products	Isocratic elution with water Refractometric detection	44

Table 2.1 (continued).

Columns [a]	Compounds and samples	Elution conditions and detection[b]	Ref.
Zorbax Bio Series WCX-300, SCX-300, WAX-300, SAX-300 and phenyl Zorbax-300	Mixture of 10 proteins	Isocratic and gradient elution with sodium chloride in 20 mM Tris-HCI (pH 7) UV detection	46
AFPak heparin (affinity column) and Asahipak GS-520HQ (size exclusion)	Selenium-containing proteins in human and mouse plasma	Gradient elution ICP-MS detection	84
Phenomenex Luna C18 and Supelco HILIC Ascentis Si	Sugars and sulphonamides	Isocratic and gradient elution with acetonitrile-formic acid and acetonitrile-10 mM ammonium formate Diode array UV, CAD and TOF-MS detection	85
C18 and HILIC polyhydroxyethyl aspartamide	Quaternary ammonium compounds in brain tissue	Isocratic and gradient elution with formic acid-water or acetonitrile-formic acid and acetonitrile-10 mM ammonium hydroxide or 10 mM ammonium hydroxide-water FT-MS detection	86
Poroshell 120 EC-C18 and ZIC®-HILIC	Polar and non-polar phenols in wine	Gradient elution with acetonitrile-10 mM ammonium acetate and acetonitrile-water ESI-TOF-MS detection	87
Kinetex XB-C18, Luna aminopropyl, Kinetex HILIC, ZIC HILIC sulfonylbetaine, Kinetex PFP pentafluorophenyl and Halo RP-amide	Polar and non-polar compounds in mouse serum	Gradient elution with acetonitrile-5 mM ammonium acetate-formic acid (pH 3) ESI-TOF-MS detection	88
Hypersil GOLD C18 and SeQuant -ZIC-pHILIC	Bile acids and metabolites in beer	Gradient elution with LC–MS grade water (solvent A) and acetonitrile (solvent B) ESI-FTMS detection	89

[a] Columns used to build serially coupled columns. [b] CAD = corona charged aerosol, ESI = electrospray ionisation, FT = Fourier transform, ICP = inductively coupled plasm, MS = mass spectrometry, PBD = polybutadiene, PEG = polyethylene glycol, THF = tetrahydrofuran, TOF = time-of-flight, Tris = tris(hidroximetil)aminometano, UV = ultraviolet.

Figs. 2.3 to 2.6 depict some chromatograms illustrative of the observed behaviour. In the figures, the performance with optimised SCC is compared with that obtained with each single column. In most reports, acetonitrile was used as the organic modifier, due to its low viscosity that decreases the impact on the backpressure by the increased column length with coupled columns. The columns to be combined were selected attending to the dissimilarity in their retention mechanisms and mobile phase compatibility. As can be observed in Table 2.1, a large variety of stationary phases and combinations were used: from combinations of C18 columns of different manufacturers or C18 columns with cyano, phenyl and amino columns (Fig. 2.3) or HILIC columns (Fig. 2.4), to combinations of polystyrene-divinylbenzene resins and ion-exchange columns (Fig. 2.5) or silica-based C18 columns and zirconia columns (Fig. 2.6). The variety of analytes and samples in the different reports does not allow inferring a specific guideline about the most suitable column combinations to succeed in the analyses. In most cases, these were selected by trial-and-error, or simply, among those found in the laboratory. Only for Refs. [24,60–77], the selection was made based on interpretive (i.e., based on models) optimisation protocols, which are described in Section 2.7. Mainly UV detection was used, but in some procedures fluorimetric, electrochemical, refractometric, ICP and mass spectrometric detection was applied.

Besides the development of analytical procedures, an attempt was made to use SCC in preparative chromatography for separating multi-component mixtures [90]. Unlike analytical LC with SCC, which is aimed to the rapid analysis of complex mixtures with high resolution, preparative LC focuses on isolating a target component with the objective of maximising the production rate and/or recovery. The authors reported an enhanced production by combining two columns in series.

It should be finally commented that the analysis of samples containing mixtures of chiral and achiral compounds, by coupling chiral and achiral stationary phases, is another active field, which would need a specific report [91–95].

2.6. Implementation of serial columns

2.6.1. Devices to connect the columns

It should be first indicated that the connection of columns is not needed if the stationary phases are packed inside the same cartridge in separated zones, or the particle composition is gradually varied along the column, giving rise to a real stationary phase gradient. However, such columns can only be used for specific applications, and the stationary phases cannot be dismantled and recombined again. In this review, we will focus only on the coupling of conventional micro-particulate columns, at reach of common LC laboratories. This approach is also more flexible because the different stationary phases are packed in independent cartridges that can be disassembled and re-used to build other combinations.

Several devices have been developed to connect two columns in series. Some are here commented but others are available from column manufacturers, or can be built in the laboratory. Coupling elements are usually very simple. Some are shown in Fig. 2.2: zero dead volume (ZDV) unions (Fig. 2.2a) [61,96], ZDV fingertight couplers screwed directly to the columns (Fig. 2.2b) [71,74–76], and stainless steel (Fig. 2.2c) or PEEK (polyether ether ketone) tubing [55,73]. The use of T connectors (Fig. 2.8a and 2.9 is another option [79,87,89]. Valve devices used for switching columns in 2DLC are not needed. However, these are useful to re-direct the flow either to each individual column or to the combination of columns (Fig. 2.8b) [44].

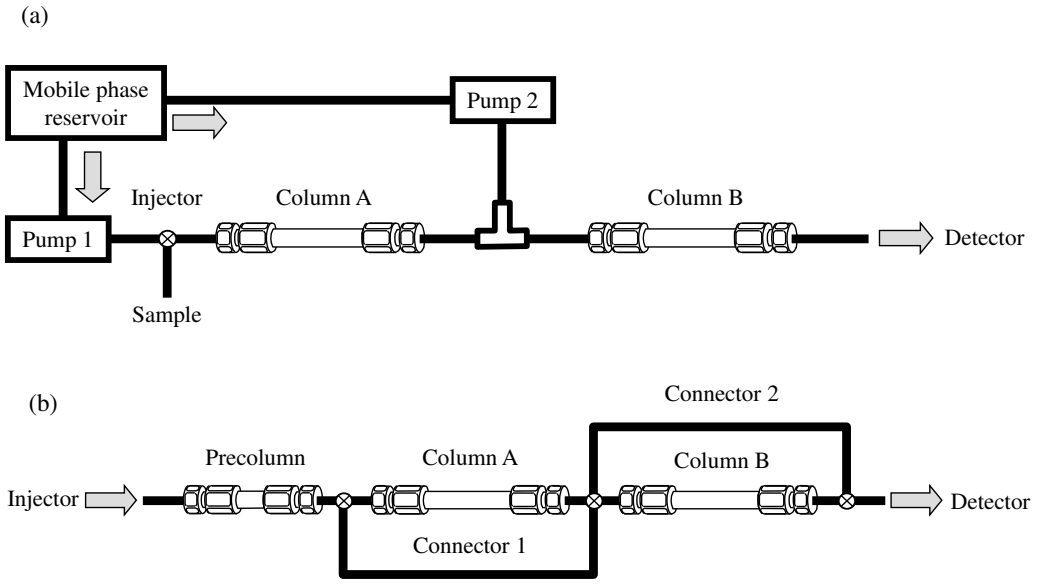

Figure 2.8. Configurations of SCC, using: (a) T connectors, which allow a second stream of the same eluent being incorporated through Pump 2 to increase the flow-rate [79], and (b) switching valves, which allow the flow being re-directed to each individual column or to their combination [44].

2.6.2. Availability of columns of different lengths to facilitate selectivity tuning

Coupling of columns available in the laboratory with a specific length can give interesting performance, but the possibility of making multiple combinations of columns of different lengths will increase the selectivity tuning, and hence, the possibility of reaching high resolution. The basis of this approach is rather simple, but its implementation has been troublesome. The first problem has been getting a correct connection of the serial columns, which ideally should not affect the sum of column behaviours. For this purpose, a

ZDV union and a system to link tightly the columns to each other are needed. The second problem has been the development of appropriate and specific tools and software to assist in the selection of the column combination, and make accurate predictions of the chromatographic behaviour using isocratic and gradient elution. Once these problems are solved, the routine application of SCC is straightforward. In this section, the alternatives that have been proposed to link columns of several lengths are discussed. Section 2.7 describes the optimisation tools and strategies.

2.6.2.1. The POPLC system

The idea of serially coupling two or more columns is highly attractive, especially if not only the stationary phase nature but also the column length is optimised, giving rise to tailor-made columns. In 2006, Nyiredy *et al.* breathed new life to the idea by commercialising a patent with Bischoff Chromatography, under the trademark PopLink® or POPLC® (the acronym POPLC comes from "Phase OPtimised Liquid Chromatography") [96]. The system attracted some attention and several authors have reported applications (Table 2.1).

The POPLC system consists of a kit of five sets of stainless steel cartridges (column segments) of different lengths (1, 2, 4 and 8 cm) and 3.0 mm internal diameter, filled with 5 μm particles with bonded stationary phases of different nature [61,96]. The availability of single columns with different lengths allows building combined columns of variable lengths, in 1 cm increments. The stationary phases are ProntoSIL C18-SH2 (with maximal carbon load aimed to separate by purely hydrophobic interactions), polar embedded C18-EPS and cyano (which offer polar selectivity), phenyl (based on π–π interactions), and C30 (which includes molecular shape recognition). The ends of the column

segments are covered with a metal grid and are connected through ZDV unions (Fig. 2.2a). When the columns are assembled, the grids on the column ends contact each other. However, the ZDV unions in these columns are not able to tightly fit the column segments at high pressures. Therefore, these are inserted inside a series of PEEK holders with lengths of 1 and 2 cm, which are screwed to each other to keep the cartridges firmly pressed. When the holders are properly fitted, the pressure is stabilised as quickly as for conventional columns.

In order to get good performance with the POPLC columns, careful handling is needed [71]: the column segments should be tightly pressed after proper alignment to prevent mobile phase leakages due to the high pressure, correct alignment of the PEEK holders is needed to screw them properly, and the column ends should not be too tightly pressed to avoid their damage. Even with careful handling, after routine use, the holders may become strongly grasped to each other and can get broken. It has been suggested that this problem can be solved by replacing the PEEK material in the holders by stainless steel [71].

2.6.2.2. Connection of conventional columns from different manufacturers

The POPLC system is a very interesting solution exclusively designed to build serial columns. However, it has the disadvantage of needing dedicated columns, which makes the user depend on only one manufacturer. Therefore, other options have been explored to get a more robust and versatile system. Recently, a solution was proposed that consists in connecting the analytical columns with ZDV fingertight couplers made of PEEK material (Fig. 2.2b) [71]. These couplers are available from several manufacturers to connect analytical and guard columns, and can be easily and reproducibly screwed to the column ends, maintaining them tightly attached, without the need of column holders. The same couplers can be used for a long time (months) without perceptible deterioration. The PEEK material in the couplers is inert and biocompatible with a broad range of applications. Another good option to connect conventional short columns is the use of standard stainless steel or PEEK tubing (Fig. 2.2c) [73].

With these connectors, any conventional column available in the market can be coupled. Also, new stationary phases can be used to increase the realm of possibilities. In turn, when using conventional columns to build SCC, an eventual problem is the lack of enough variety of lengths (which is the big advantage of the POPLC system). Short columns from Advanced Chromatography Technologies (ACE, Aberdeen, Scotland, UK), which are commercialised with different lengths (2, 3, 3.5, 5 and 7.5 cm) for mass spectrometric (MS) applications, have been found especially adequate to build SCC [71]. Such variability in lengths is hardly found for most manufacturers.

2.6.2.3. The dead volume problem associated to the connection of serial columns

The success of SCC depends critically on the availability of connectors with low dead volumes. This requirement is mandatory to make reliable predictions of the retention behaviour and avoid an extra band broadening. In this regard, PEEK fingertight couplers seem to be superior to the system used to connect the POPLC columns, which has been reported to have a number of problems: retention times above the theoretical values [68,90], dependence of the retention times and selectivity on the column order [61,71], and formation of fronting peaks [71]. An empirical calibration was proposed to correct the shifts in the predicted retention times [68], but other problems remain. It has been suggested that the non-negligible dead volumes in the column connections, produced by an imperfect screwing of the PEEK holders, may create a head space in the columns that could explain all these effects [68,71].

The use of generic tubing to connect the columns does not seem to be either a good solution [73]. Tubing of 10.5 cm × 0.12 mm I.D. was found to produce an appreciable dead volume (larger compared to the POPLC system), which yielded significant band broadening and deviations in the predicted retention times. The authors suggested the use of shorter tubing (7 cm), or one-piece couplers to improve the performance.

The dead volume problem is reduced by using ZDV fingertight couplers screwed to conventional columns (Fig. 2.2b) [71]. Since the dead volume in these couplers is negligible, and no other element is required to hold the columns together, the accuracy in the prediction of retention times is improved and there is no loss in column efficiency. The column order does not affect the retention times and peak shape, either.

2.6.3. Coupling of reversed-phase and hydrophilic interaction columns

RPLC is very successful in the separation of a wide range of non-polar analytes. However, polar and ionic compounds are not sufficiently retained on these columns unless ion pairing reagents are added [15]. Common approaches to analyse samples comprising compounds in a wide range of polarities make use of two or more columns in parallel, involving different separation modes (e.g., RPLC, HILIC and/or ion exchange), or columns using the same separation mode but rather different selectivity. While performing parallel runs with different columns can be successful, it is unpractical for high throughput analysis.

A great challenge in LC has been the expansion of the elution window to determine simultaneously groups of compounds with different polarities. RPLC and HILIC are highly orthogonal. Therefore, their serial coupling is especially attractive for analysing such samples. However, despite RPLC and HILIC use the same solvent components, the ratio of the components is diametrically opposed (HILIC needs a high organic solvent content, while RPLC needs a high amount of water). Considerable effort has been invested to overcome the solvent strength incompatibility of RPLC and HILIC. For this purpose, several on-line combined systems based on valve switching have been developed [97–99]. However, these systems are expensive and at present not suitable for routine analysis in most laboratories. Also, analysis times may be too long and the results may lack the required robustness.

Therefore, the development of approaches capable of simultaneously separating polar and non-polar analytes within a single chromatographic run, using a direct connection of RPLC and HILIC [85–87,89], is highly attractive. In the proposed configurations, the RPLC column, aimed to the separation of low polarity solutes, was placed first, while the HILIC column worked as the

second column to separate highly polar solutes. In Refs. [85–87], the solvent strength incompatibility between RPLC and HILIC was solved by increasing the acetonitrile content in the eluate from the RPLC column by the on-line mixing with an acetonitrile-rich solvent (≥80%), via a T-connector (Fig. 2.9a). To make the system work in the appropriate pressure range, the RPLC column had a smaller diameter than the HILIC column (e.g., 25 cm × 2 mm I.D. with a flow rate of 0.1–0.2 mL/min versus 5 cm × 4.6 mm I.D. with a flow rate of 1.5–2.0 mL/min). In a more sophisticated configuration, independent gradient control was applied to the two columns (Figs. 2.4 and 2.9b) [89].

The high acetonitrile content in the eluent coming from the HILIC column facilitates compound desolvation and ionisation in the electrospray process in MS detection. Also, the reduction of the total number of analytes entering the electrospray source at a given time contributes to reduce the degree of ion suppression, especially in the region near the dead volume [85–87]. Consequently, the serial coupling of RPLC and HILIC offers the possibility of increasing the number of analytes identified in MS, and is a valid approach for non-target analysis of complex samples making a single injection. A disadvantage is, however, the chromatographic dilution, which reduces the sensitivity for UV detection. The dilution effect has less impact in MS detection, but the higher flow rates could be detrimental for the sensitivity.

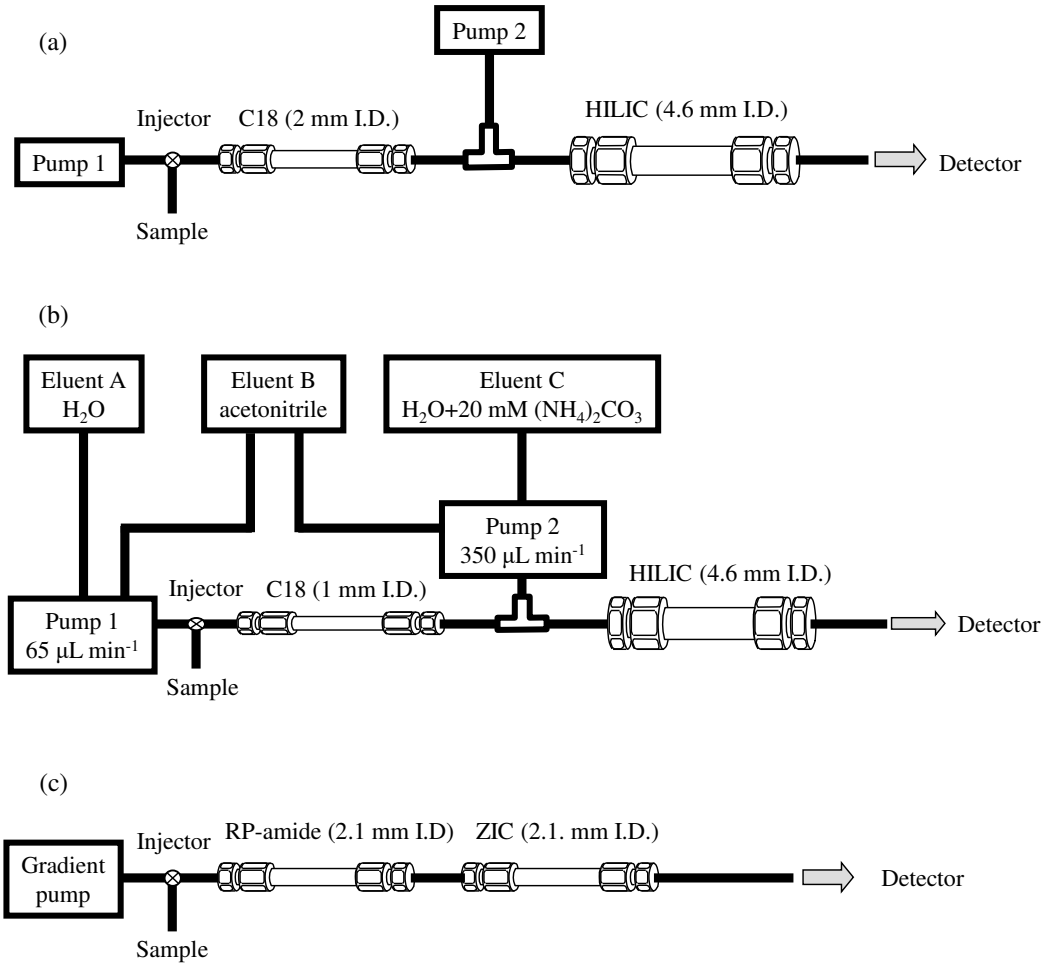

Figure 2.9. Serial coupling of RPLC and HILIC columns: (a) The acetonitrile content in the mobile phase is increased by incorporating a secondary stream of organic solvent through Pump 2 [85], (b) independent gradient control for each coupled column, and (c) direct connection of the RPLC and HILIC columns. The column order in (c) should not have a significant impact on the resolution.

The direct connection of RPLC and HILIC, using a single gradient that fulfils the solvent requirements for both columns, has been also reported (Fig.

2.9c) [88]. The gradient program started at sufficiently high organic composition, compatible with RPLC and HILIC. The advantage of such gradient is again the enhanced ionisation efficiency in MS in the presence of high content of organic solvent, due to its higher volatility compared to aqueous buffers. The approach did not intend providing maximal peak capacity, but optimal MS conditions by reducing the total number of analytes entering the detector at a given time. Therefore, the tandem columns allowed the detection of more mass spectral features than a single column under either the HILIC or RPLC gradient elution conditions.

2.7. Interpretive optimisation of serial columns

2.7.1. Is stationary phase optimisation possible?

Ideally, a parameter to be adjusted during method development should have a great impact on the selectivity. The parameter should also be easy to modulate and robust. Several parameters associated with the mobile phase (e.g., organic solvent content, pH, temperature and flow rate) can be modified in a continuous and simple fashion to generate intermediate conditions yielding appropriate selectivity. In contrast, the stationary phase is a discrete factor difficult to vary. As a consequence, most analyses are performed with a single stationary phase of pre-established length, selected by trial-and-error, or based on expected interactions or previous experience.

The work of several authors has, however, demonstrated that serial coupling of columns with different stationary phases introduce new degrees of freedom, such as the type, number, relative lengths and order of the individual columns, with outstanding consequences on selectivity and retention in LC. It is possible to achieve almost continuous transitions between the selectivity of two or more

stationary phases by combining columns of different lengths. The optimisation of column coupling may yield separations that are not possible with single columns, based on their enhanced selectivity. The proposed approaches are suitable for complex separation problems that cannot be solved satisfactorily by optimising only the eluent composition.

Using trial-and-error strategies, some authors have shown that column combinations (built in most cases with columns already available in their laboratories) outperform the results offered by each single column [51,56,59]. However, full benefit of such coupling is only achieved through the interpretive optimisation of both the column nature and length, together with the elution conditions. In this sense, the pioneer work of Issaq and Gutiérrez [25], Glajch *et al.* [42], and Lukulay and McGuffin [80], who developed approaches for multi-column systems, should be mentioned. However, the most significant advances, which boosted the interest of a number of authors, started with the commercial release of the POPLC system associated to a software package that makes the optimisation of SCC accessible to any user. The software assists in the selection of the optimal combination of column nature and length for a pre-selected mobile phase [61,96].

Before going further, it should be noted that in the implementation of optimisation protocols, the column length is limited by the available column segments. Also, there are pressure constraints that prevent long coupled columns. The analysis time, which increases with column length, is another restriction. All these constraints should be considered to reduce the bulk calculation during the optimisation process.

2.7.2. Modelling of reference columns and prediction of the best conditions

The interpretive optimisation of the column nature and length (and eventually, of the elution conditions) involves several steps: (i) Gathering basic information on the retention time and peak shape (width and asymmetry) from a limited number of runs for all analytes in each stationary phase; (ii) measurement of the extra-column volume, and dead volume associated to each column, and the corresponding peak shape properties; (iii) modelling of the chromatographic behaviour of analytes for each stationary phase; (iv) prediction of chromatograms for a number of column combinations at fixed or variable eluent composition; (v) search of the optimal column combination based on the simulated chromatograms; and finally, (vi) preparation and testing of the selected column combination. The methodologies used for predicting the resolution and peak shape, and the search of the best separation conditions, are the same as those applied for the optimisation of the mobile phase composition and gradients for a single column. Detailed algorithms have been described in several reports [61,67,68,71,74–77].

Key factors that determine the reliability of the approaches with SCC are the accurate modelling of the retention time and peak shape parameters, and the availability of a peak shape model to achieve accurate simulation of chromatograms. For this purpose, a relatively small set of experimental data is obtained for each analyte eluted from the single columns, at one or more mobile phase compositions [71,74]. In spite of being more laborious, the most informative data come from isocratic experiments [100], but for practical reasons, gradient experiments can be run instead. Achievement of the information from several columns can be expedited with the assistance of an automatic column selector.

For a given mobile phase composition and in the absence of additional volumes between the coupled columns, the retention factors of analytes eluted from columns of the same nature and diameter should be the same, independently of the column length. On the other hand, once the extra-column contributions are subtracted, the intra-column dead volume will increase linearly with the column length. Therefore, the chromatographic data and dead volume do not need to be measured for columns of different lengths. The required information can be obtained from experiments carried out with columns of an arbitrary length (i.e., reference columns) [71,74]. To get maximal accuracy, the length of these columns should be the longest available in the laboratory for each type of stationary phase (provided the retention is not excessive). Single columns can be used as reference columns, or these can be built by combining several short column segments containing the same stationary phase. The achieved information will allow the calculation of the retention times and peak shapes for serial combinations of columns of different lengths.

Since the stationary phases giving rise to different separation environments are packed in separate cartridges, the solutes will interact independently within each one. The retention time for a solute *j* eluted isocratically at constant flow rate using SCC, $t_{Rc,j}$, is thus a linear combination of the extra-column contribution, t_{ext}, and the retention times for each column, $t_{R,ij}$ [71,74,77]:

$$t_{Rc,ij} = t_{ext} + \sum_{i=1}^{nc} t_{R,ij} = t_{ext} + \sum_{i=1}^{nc} t_{0int,i}(1+k_{ij}) = t_0 + \sum_{i=1}^{nc} t_{0int,i}\, k_{ij} \qquad (2.1)$$

where *i* is the column index, *nc* the total number of combined columns, $t_{0int,i}$ the intra-column dead times, t_0 the dead time for the whole column combination, and k_{ij} the retention factor for solute *j* in column segment *i*, expressed as:

$$k_{ij} = \frac{t_{\mathrm{R},ij} - t_{0\mathrm{exp},\,i}}{t_{0\mathrm{exp},\,i} - t_{\mathrm{ext}}} \tag{2.2}$$

The experimental dead time for a column i, $t_{0\mathrm{exp},i}$, is given by:

$$t_{0\mathrm{exp},\,i} = t_{\mathrm{ext}} + t_{0\,\mathrm{int},i} \tag{2.3}$$

The gradient retention time for the column combination, $t_{\mathrm{g},j}$, is also a linear combination of the retention times associated to each column, to which the extra-column contribution should be added:

$$t_{\mathrm{g},\,j} = t_{\mathrm{ext}} + \sum_{i=1}^{nc} t_{\mathrm{g},ij} \tag{2.4}$$

Gradient elution for each column can be outlined based on the following integral equation:

$$t_{0,i} = \int_0^{t_{\mathrm{g},ij} - t_{0,i}} \frac{dt}{k_{ij}(\varphi(t))} \tag{2.5}$$

where $\varphi(t)$ is the gradient program, and t the time measured at the column inlet. With SCC, Eq. (2.5) should be solved numerically. When the cumulative sum in the numerical integration equals the column dead time ($t_{0,i}$), the solute has reached the outlet of that column. If the solute enters a new column, the $k_{ij}(\varphi)$ function for the new column should be adopted, and a new integral equation should be solved, using as dwell time the instrument dwell time plus the sum of the dead times for the previous columns. Owing to the different speeds of analytes, column transitions may happen at different times for each solute, and consequently, will be associated to different stationary phases.

In isocratic elution, if the columns are ideally connected (i.e., the volume associated to the column connections is negligible, see Section 2.6.2.3),

according to Eq. (2.1) the change in column order should not introduce significant differences in the chromatographic behaviour. Therefore, segments containing the same stationary phase can be consecutively coupled or not. The situation is different for gradient elution, as will be detailed in Section 2.7.6. In contrast, the prediction of peak width and asymmetry is similar for both isocratic and gradient elution. The prediction of half-widths can be done assuming the additivity of variances, which is only strictly valid for Gaussian peaks and independent separation mechanisms [71,74,75]. Nevertheless, the predictions have been found highly satisfactory. The longer length of some combined columns benefits larger efficiencies, and therefore, better separations.

Owing to the multi-dimensional nature of the optimisation factors, Pareto plots (which balance good resolution and short analysis time) have been proposed to assist in the selection of the most suitable column combination and elution conditions (Fig. 2.10a and b). In spite of the apparent complexity of the optimisation methodology compared to single columns, an outstanding agreement has been reported between predicted and experimental chromatograms [74–76].

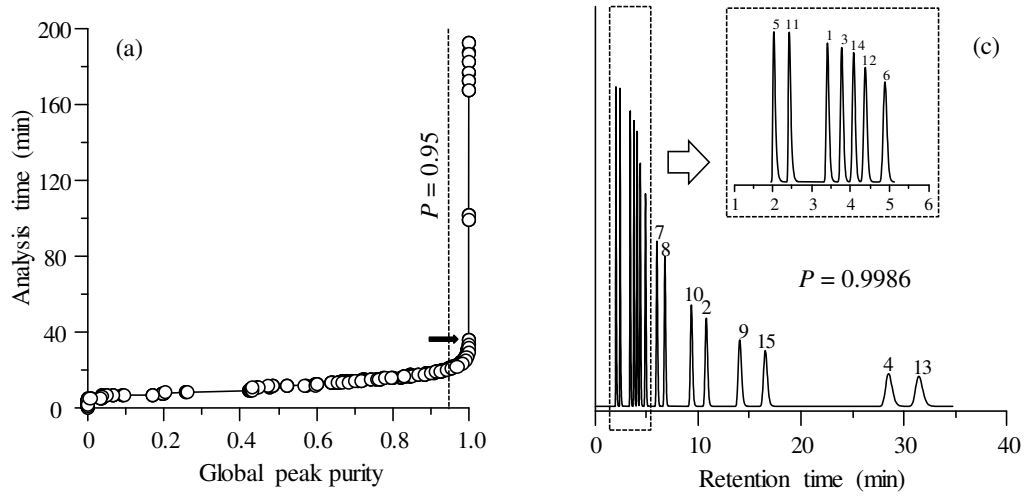

Figure 2.10. Pareto plot for: (a) simultaneous optimisation of column nature and length, and mobile phase composition in isocratic elution. The arrow in (a) point out the resolution (measured as global peak purity, *P*) and analysis time for the experimental conditions used to obtain the chromatogram in (c). Selected column combination for chromatogram (c): 5 cm C18 + 2 cm phenyl + 3 cm C18-HL + 2 cm C18-AQ (columns from Advanced Chromatography Technologies). Experimental conditions in (c): Isocratic elution with 16.6% acetonitrile. Compounds: (1) Sulphacetamide, (2) sulphachloropyridazine, (3) sulphadiazine, (4) sulphadimethoxine, (5) sulphaguanidine, (6) sulphamerazine, (7) sulphamethazine, (8) sulphamethizole, (9) sulphamethoxazole, (10) sulphamonomethoxine, (11) sulphanilamide, (12) sulphapyridine, (13) sulphaquinoxaline, (14) sulphathiazole, and (15) sulphisoxazole. Reproduced with permission of Elsevier from Ref. [76].

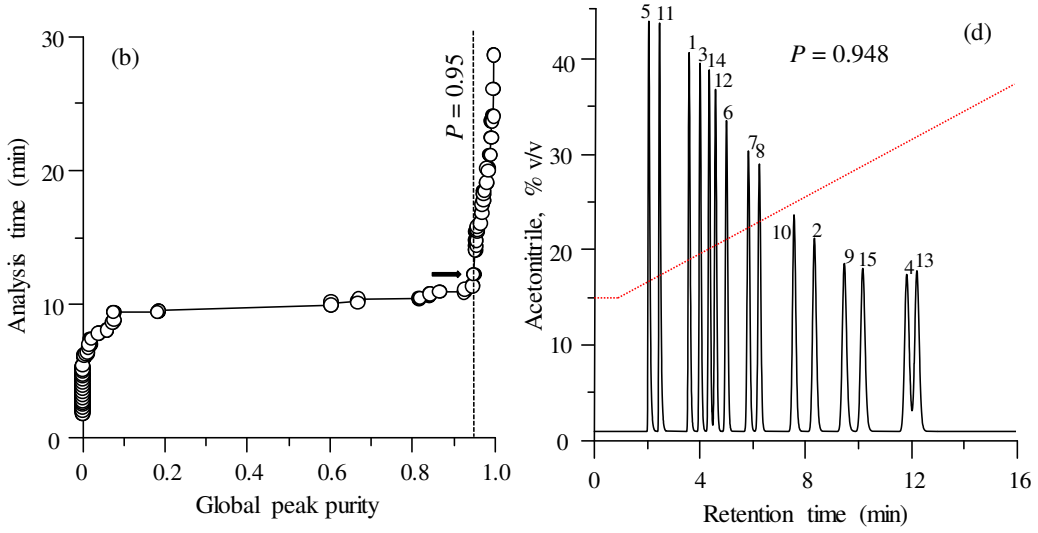

Figure 2.10 (continued). Pareto plot for: (b) optimisation of the gradient profile using the column combination marked in (a). The arrow in (b) point out the resolution and analysis time for the experimental conditions used to obtain the chromatogram in (d). Experimental conditions in (d): gradient elution with 15.0–43.0% acetonitrile in 20 min. See previous page for more details.

2.7.3. Search of the best column combination in isocratic elution: The SOSLC approach

Since commercial columns are only available at a few lengths, combined columns cannot be built at any arbitrary length. This limitation contrasts with the selection of the organic solvent content in an isocratic mobile phase or gradient program, which can be prepared with high precision within a range of values. Regarding this panorama, the POPLC column kit manufactured by

Bischoff Chromatography (see Section 2.6.2.1) [96] represents an interesting development to broaden the selectivity in LC. The kit is marketed with a software package to optimise the column combinations, although restricted to pre-selected isocratic conditions. The basic idea is that the selectivity can be adapted by changing only the column nature and length, which is highly attractive (Fig. 2.3). The stationary phase becomes thus a tuneable parameter, whereas the mobile phase composition, flow rate and column temperature are fixed [60,61]. The authors named this approach "Stationary phase Optimised Selectivity Liquid Chromatography" (SOSLC).

In the SOSLC approach, the mathematics for the predictions of optimal conditions is rather simple, since the retention time for each column segment contributes linearly to the overall retention (Eq. (2.1)). According to the authors, the SOSLC software is based on the PRISMA model, previously developed for solvent optimisation [101], and inspired in the Snyder solvent classification [102] and Kirkland-Glajch solvent strength prism for multi-solvent elution [103]. Fig. 2.11 shows all possible combinations built with columns containing three different stationary phases and assuming the availability of 10 column segments of equal length. The number of possible combinations is 66, from which 3 are homogeneous columns (located at the triangle vertices), and 63 are heterogeneous: 27 are combinations of two types of phases (located along the edges of the triangle), and 36 are combinations of three phases (located inside the triangle).

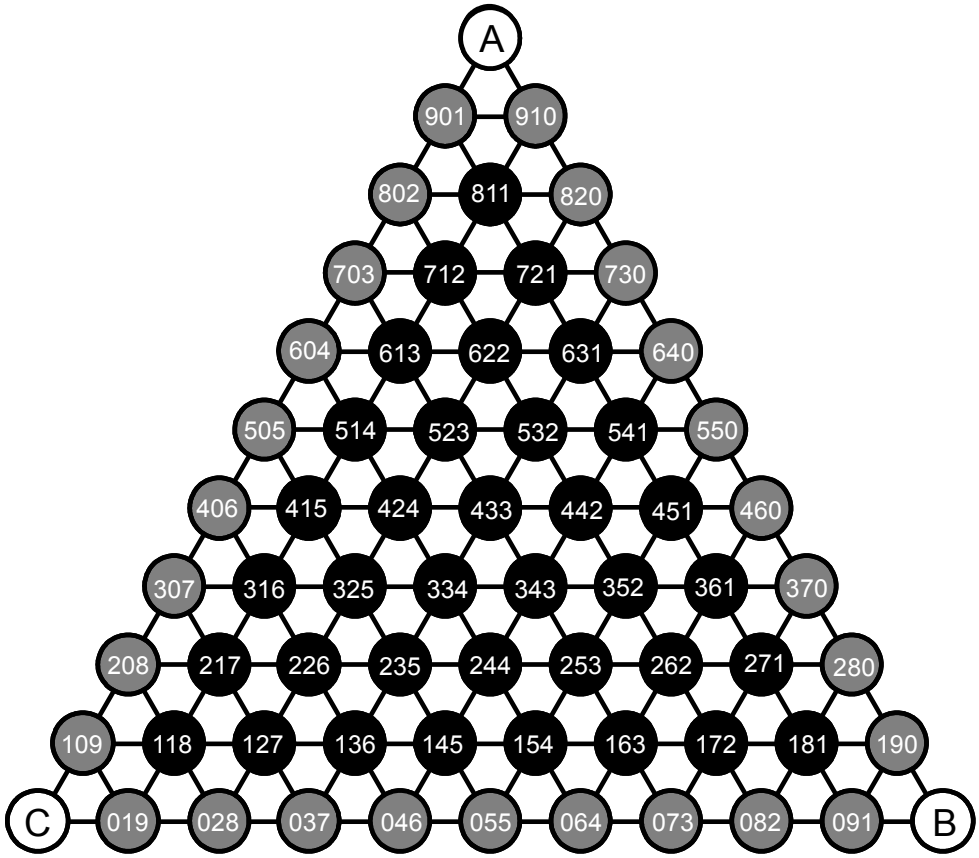

Figure 2.11. Ternary diagram showing all possible combinations of 10 column segments of equal length for three stationary phases (A, B and C). The number of segments for A, B and C are given inside each circle (the left, central and right number, respectively). The length of the segments for each stationary phase is not necessarily the same.

In a rough approximation and ideally, if the length of the elementary column segments were adapted so that they had the same polarity for all columns, all combinations would yield the same analysis time, provided that the total number of segments is the same for all combinations. Since this is not usually the case, the analysis time will change for different combinations. Thus, for the POPLC system, the length of the elementary segments is 1 cm for all stationary phases. The approach can be generalised to more than three stationary phases [60,61]. Interestingly, in an earlier report, Eppert and Heitmann considered the geometrical domain of combinations with three and four stationary phases, represented by a triangle and a tetrahedron, respectively [53]. With five stationary phases, there is no three-dimensional equivalent figure [61,71].

The SOSLC software explores all possible segment combinations within the restriction of maximal predicted column length and analysis time, calculates the resolution of the critical peak pair for each column combination, and ranks the combinations according to their decreasing resolution. The segment combination yielding the maximal value is finally selected. The POPLC system, assisted by the SOSLC software, has been used by several authors to develop applications for the determination of diverse groups of compounds in isocratic conditions (Table 2.1).

2.7.4. Simultaneous search of the best column combination and mobile phase composition in isocratic elution

The mobile phase composition in isocratic elution has a decisive influence on the performance of an optimisation focused exclusively on the nature and length of the coupled columns [74]. Therefore, it is possible that the optimisation with a pre-selected mobile phase (Section 2.7.3) would offer an interesting solution (i.e., column combination) but this will not be the best in

terms of resolution and analysis time. The same could be said of an optimisation restricted to the mobile phase composition applied to a column combination arbitrarily selected.

Therefore, although the exclusive optimisation of column nature and length can yield satisfactory performance, the addition of a second factor will increase the possibilities of success. Thus, the simultaneous optimisation of mobile phase composition, column nature and length, has been suggested as the most powerful approach to finely tune the selectivity with SCC in isocratic elution [42,61,62,80]. Recently, an approach has been developed to implement this strategy [74]. The optimisation process is more complex with regard to that carried out with a pre-selected mobile phase, since the dependence of the retention behaviour with the mobile phase composition for each stationary phase should be considered, and the number of possible solutions quickly outperforms the computation capabilities. The most economical experimental design requires two experiments for each stationary phase at different mobile phase compositions, but some extra experiments are convenient to allow degrees of freedom. The benefits of the simultaneous optimisation of mobile phase composition, column nature and length, however, compensate the increased complexity. The main advantage is the better performance in terms of resolution and analysis time (compare Fig. 2.12d and e). Other advantages are next commented.

A drawback of the optimisation approach run at fixed mobile phase composition is the need of multiple column lengths for each stationary phase, as is the case of the POPLC system, which allows building columns differing in 1 cm up to at least 15 cm. This guarantees enough precision in the search of the ideal column to resolve a particular sample. However, the inclusion of the mobile phase composition as a second factor in the optimisation process yields

equivalent results in terms of resolution and analysis time, using a smaller number of column lengths [74]. This reduces drastically the implementation cost. The inclusion of the mobile phase composition also allows using columns with shorter lengths. Thus, short columns at two lengths for each stationary phase (e.g., 2 and 4 cm) may be satisfactory, provided the quality of the retention data allows an accurate modelling.

A side advantage of the reduction in the number of column segments is the decrease in the system dead volume associated to the connections. Note that in the POPLC approach several short segments (sometimes 1 cm long) are connected to get the required column length, which may represent a significant increase in dead volume.

2.7.5. Advantages of the optimised isocratic approaches with coupled columns

Besides the significant enhancement in the separation performance, an attractive advantage of the optimisation of column combinations, using the approaches described in Sections 2.7.3 and 2.7.4, is the fact that the expensive testing of multiple columns to solve complex separation problems is not more needed. The probability of success increases enormously by combining two to four columns with different behaviour. Fig. 2.10c and d and Fig. 2.12a to e compare the performance of the isocratic separation of a mixture of 15 sulphonamides using columns that individually yielded poor resolution, with that obtained with optimal combinations of two and three columns.

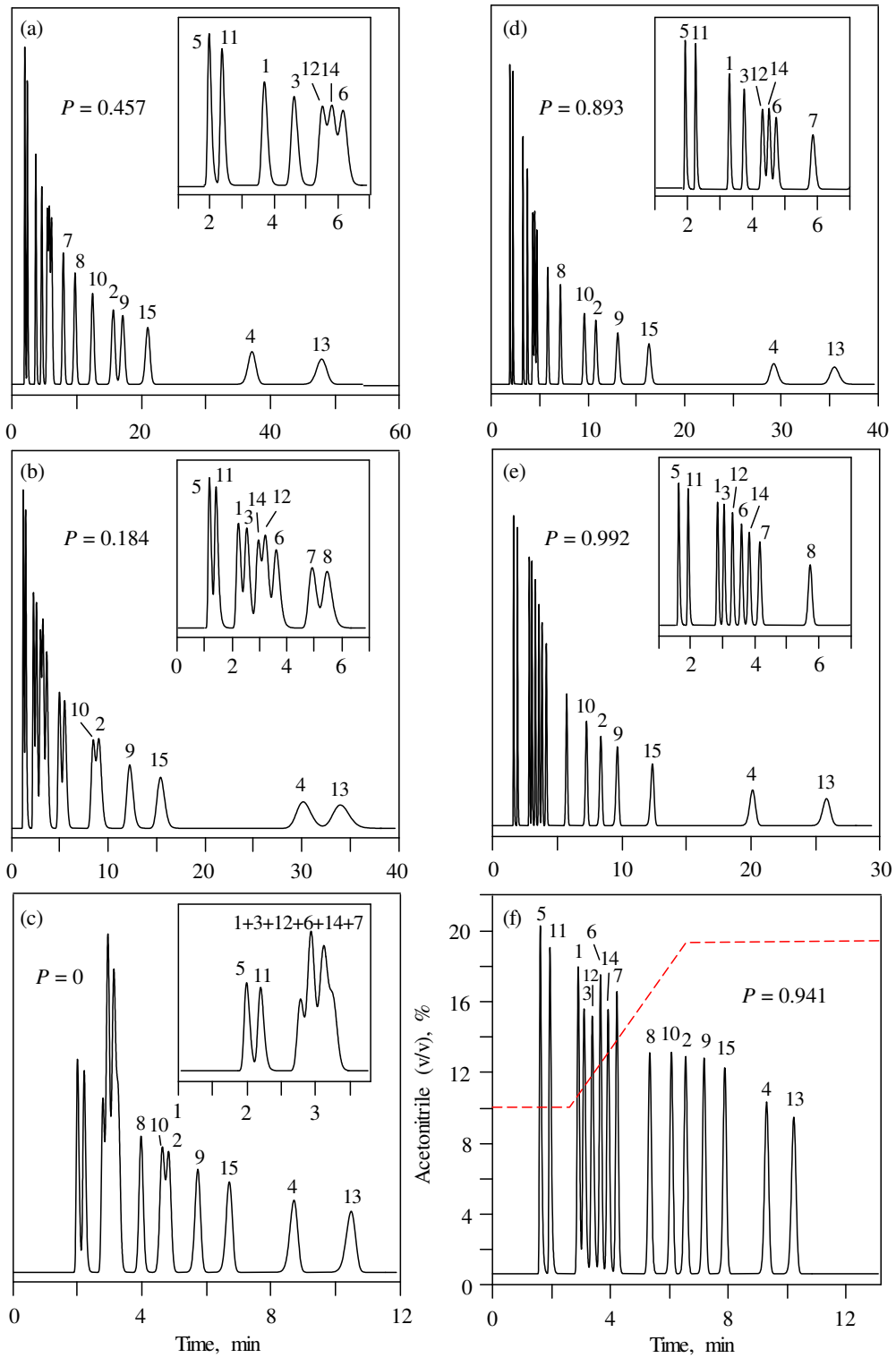
(a)
P = 0.457
(b)
P = 0.184
(c)
P = 0
1+3+12+6+14+7
(d)
P = 0.893
(e)
P = 0.992
(f)
P = 0.941
Acetonitrile (v/v), %
Time, min
Time, min

Figure 2.12. Chromatograms balancing resolution and analysis time in different situations (optimised column and elution conditions are indicated in parenthesis) [24,77]. Optimal isocratic separation with single columns: (a) 10 cm phenyl (12.5% acetonitrile), (b) 6 cm C18-HL (13.9%), (c) 11 cm cyano (14.1%). Optimal separations with combined columns: (d) attending only to the column combination at a pre-selected isocratic eluent composition of 12.5% acetonitrile (3 cm phenyl + 2 cm C18-HL + 5 cm cyano), (e) attending to both column combination and isocratic eluent composition (2 cm phenyl + 6 cm cyano and 10.3% acetonitrile), (f) using an optimised gradient with three nodes for 2 cm phenyl + 6 cm cyano (the gradient is overlaid on the chromatogram). The resolution, measured as peak purity (*P*), is indicated in the chromatograms. In all instances, the temperature was 25 ^{o}C and the flow rate, 1 mL/min. See Fig. 2.10 for compound identity and column manufacturer.

Fig. 2.12 illustrates the performance of the combination of phenyl, C18 and cyano columns. The chromatogram in Fig. 2.12d was obtained by optimising the column nature and length at the optimal mobile phase composition for the best single column (i.e., the phenyl column). The required measurements for such optimisation (i.e., the chromatographic behaviour for the analytes in each stationary phase at the pre-selected mobile phase) took only a few hours. However, the performance of the optimal column combination depends on the mobile phase composition, as can be observed in Fig. 2.13, where the global resolution (measured as peak purity) and analysis time for the optimal combinations of the three stationary phases (phenyl, C18 and cyano) are plotted against the mobile phase composition in isocratic elution. The points in the graph correspond to different column combinations or even single columns at

several lengths). As observed, maximal global resolution and minimal analysis time are obtained with mobile phases below 12% acetonitrile. The simultaneous optimisation of the column nature and length together with the mobile phase composition would point out directly to these solutions. Fig. 2.12e shows a chromatogram obtained at the optimal conditions.

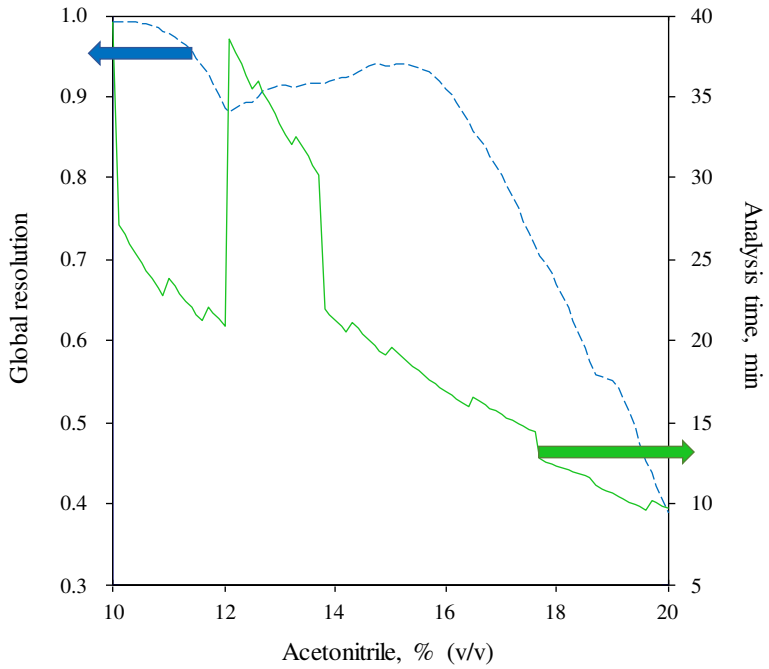

Figure 2.13. Optimisation of the column nature and length at several mobile phase compositions, for the separation of 15 sulphonamides using SCC with phenyl, C18-HL and cyano columns, up to a maximal combined length of 20 cm. Global resolution (measured as peak purity) of different optimised column combinations of one to three columns containing different stationary phases and corresponding analysis time are plotted versus the isocratic mobile phase composition. The graph represents the performance of several column combinations or single columns. See text for other details.

Besides the better resolution, the "general elution problem" of RPLC (i.e., the long analysis times for samples having components in a wide range of polarities) is alleviated with the use of an appropriate column combination. This makes gradient elution less necessary. Method transference between different chromatographic equipment should be also easier. The optimisation of the column combination in isocratic elution is also more MS-friendly, due to an easier interfacing. During isocratic elution, there is no need of compromises for ion-source parameters, such as electrospray voltage, spray gas flow, or dry gas temperature, which are significantly dependent on the organic solvent content. Isocratic elution with coupled columns also benefits other detection techniques. The chemical shifts in nuclear magnetic resonance detection due to the change in solvent polarity are minimised. In general, signal instability and baseline drifts are reduced in electrochemical and refractometric detection, which is a pre-requisite for a reliable quantification [43,44,64].

2.7.6. Gradient elution with serially-coupled columns

SCC in the isocratic mode often yields shorter analysis times compared to the single columns. However, when complex mixtures containing compounds in a wide range of polarities are analysed, it may be convenient to apply a gradient to decrease the analysis times even more. Gradients yield more homogeneous peak distributions along the separation space. Owing to the peak width compression effect, they also avoid sensitivity losses in late eluting compounds. With regard to isocratic elution, the use of gradients with SCC implies an additional factor: the column order. Depending on the location of each column segment inside the combination, this will be affected by a different organic solvent domain along the gradient. The number of possible

permutations of the coupled columns may represent an important increment in the computation time. For example, for four stationary phases, the calculation volume is multiplied by 24 when all column permutations are examined. In spite of the remarkable complexity of optimising gradients with SCC, several authors have been interested in their development.

2.7.6.1. Search of the best gradient by trial-and-error

Some authors have extended the application of the POPLC columns to gradient elution. In several reports, the best gradient was searched by trial-and-error, for column combinations previously optimised using the SOSLC approach. First, a multi-isocratic (i.e., stepwise) gradient was developed, which consisted of consecutive isocratic steps with compositions appropriate for MS detection [63]. In another report, the analyses were carried out isocratically, and a final gradient was applied to elute unknown late eluting compounds [66]. Later, a gradient was designed attending to the isocratic behaviour of the analytes with the combined columns, using a long gradient time to assure the separation and the detection of unknown compounds [72].

2.7.6.2. Search of the best column combination for a pre-selected gradient

Other authors working with SCC outlined the search of the best gradient conditions performing the exploration in an interpretive fashion. De Beer *et al.* reported two approaches. A stepwise gradient was first designed, inspired in the SOSLC optimisation [65]. The approach consisted of six steps: (i) Analysis of the sample with a C18 column using a linear gradient; (ii) classification of the analytes into a few groups (3 or 4), according to their elution order; (iii) for each group, selection of an isocratic mobile phase composition from those

inside their elution window along the gradient; (iv) independent SOSLC optimisation (i.e., search of the best column combination) for each group at their respective selected mobile phase composition; (v) selection of a common column combination for all groups to yield sufficient resolution in a convenient analysis time; (vi) development of a stepwise gradient, using the common column combination, consisting of the sequence of selected isocratic levels for each group. With this approach, most probably, the best column combination for each group of analytes will be different. In such a case, the next best common combination for each group should be selected instead (although the resolution will not be optimal). This group comparison is not possible with the original SOSLC software, and required the preparation of new software [65].

Later, the same research group developed a prediction algorithm based on numerical integration, which allowed the analysis with coupled columns using different types of pre-selected gradients (stepwise, linear and multi-linear), all of them outlined as a sequence of infinitesimal isocratic steps [67,68]. The software considered the substitution of the retention model when an analyte band is transferred to a new column, and the increase in the dwell time along the columns. The optimisation was again restricted to the selection of the best column combination (nature and length). The software was written using the Visual Basic platform of Microsoft Excel, and is available free of charge from the Pfizer Analytical Research Centre (Ghent University, Belgium), and from Bischoff Chromatography [67]. The authors applied their approach to the POPLC columns [67,68], and to conventional columns from other manufacturers, coupled using stainless steel tubing [73]. A linear relationship between the peak standard deviation and the retention time was applied after the optimisation to make the correction of peak widths. Although such

relationship is not strictly valid for gradient elution and skewed peaks, it improved the prediction of peak profiles [73].

More recently, a different software application has been developed to optimise column combinations using a pre-selected gradient, which incorporated the prediction of peak shapes throughout the optimisation [75]. It should be noted that the number of column combinations to be investigated in gradient elution is significantly larger with respect to isocratic elution and the column order affects the separation. Obviously, with gradient elution, all segments containing the same stationary phase should be located sequentially to decrease the complexity of the approach. However, non-consecutive location of these column segments may offer benefits in some instances.

2.7.6.3. Search of the best gradient program for a pre-selected column combination

The optimisation of SCC using a pre-selected gradient program has the same drawback as the optimisation of a pre-selected mobile phase composition in isocratic elution: the optimal combination will depend critically on the pre-selected gradient, and usually, the best separation will be lost. However, with gradient elution, the simultaneous optimisation of the column combination and gradient program becomes unpractical, due to the massive computation. Instead, a good alternative is focussing the optimisation on the gradient program, using a column combination previously chosen in the isocratic mode (Fig. 2.10 and Fig. 2.12e and f), as reported previously for trial-and error approaches [63,66,72]. The optimisation of both linear and multi-linear gradients has been addressed [75,76].

The use of a pre-selected column is supported by the fact that isocratic elution yields the maximal global peak resolution (although often with

unpractical analysis times). Also, multi-linear gradients tend to the resolution reached in isocratic elution, as their complexity (i.e., number of nodes) increases [104]. The isocratic optimisation of the column combination tends to discard redundant stationary phases (i.e., those offering separations similar to others), or those that do not contribute to the separation. As observed in Fig. 2.10a, the selection of the column combination to carry out a further optimisation of the gradient program is not critical: several options may offer satisfactory results (Fig. 2.10).

The gradient optimisation focused on a pre-selected column combination offers more solutions than the optimisation of the column combination restricted to a pre-selected gradient, usually with better performance. Several refinements concerning the gradient delays along the solute migration have been suggested to increase the accuracy of predictions [75,76]. For a single gradient, the parameters to be optimised are the initial and final eluent composition and the gradient slope (or time). For multi-linear gradients, at least the location of the coordinates for one or more intermediate nodes should be added. In this case, even if only the nodes are optimised, the computation time will be much longer when a systematic search of all conditions is carried out. Genetic algorithms (GAs) have been proposed to assist in the search of the best gradient program and reduce the computation time to minutes [75,76]. GAs are constrained global optimisation methods, where the exploration focuses progressively on the most promising regions in the search space. This gives response to large scale problems with a high number of local solutions.

An early report containing a theoretical study should be finally commented, where numerical integration was applied to calculate the retention under gradient elution with a previously selected combination of columns. The

software was designed to tackle up to six coupled columns in any order, from isocratic elution to complex gradients [45].

2.8. Other approaches to control the selectivity using combined columns

As commented, the combination of serial columns was first proposed for GC, where the selectivity is often optimised by fixing the nature and length of the coupled columns. Only the carrier gas flow, the temperature of the individual columns, or both is adjusted [47–50,105]. Leaving unchanged the column lengths and getting the desired resolution by independent modification of the flow rate and/or temperature, has been also the subject of research in LC, as described next. These approaches can be considered "on-line" modes, since a continuous tuning of the system polarity is carried out without disassembling the coupled columns. The effect is similar to the change in the relative lengths of the combined columns.

2.8.1. Use of different flow rates for each combined column

The potential of tuning the selectivity in LC with SCC, by varying the flow rate for each column, has been explored by several authors [55,57,79]. In order to change the flow rate in the second column, a second stream of eluent is added by inserting a T-connector after the first column (Fig. 2.8a). The needed flow rate ratio of both streams for getting optimal resolution is easily calculated on the basis of the retention factors for the individual columns. However, it should be noted that the column order will affect the separation.

The approach requires coupling two columns with sufficiently different polarity but with a compatible eluent. In an early report, several RPLC packings were synthesised from commercially available silica gel to get different

functionalities and enhance selectivity differences [57]. More recently, the approach has been implemented by coupling a crosslinked commercial polyethylene glycol phase and a cyano phase. The system was operated in the normal phase mode using a mobile phase composed of 90/10 (v/v) hexane/dioxane [79]. The authors in these reports found dramatic changes in selectivity when the flow through the individual columns was altered.

A different approach was developed using capillary columns of different diameter, packed in the laboratory and connected with stainless steel tubing [55]. A first 50 μm I.D. column containing a C18 phase was serially-coupled to a 100–200 μm I.D. column packed with a mixture of C18 and C1 particles. The inner diameter of the capillary columns determined the flow rate, which was appreciably different in each column. Both the chemical nature of the stationary phase and the flow rate were changed along the column system. This dual effect yielded a gradient-like elution of the analytes using isocratic conditions.

2.8.2. Independent temperature control for each coupled column

The introduction of LC stationary phases designed to withstand high temperatures has provided the analysts with a new parameter to modulate the selectivity and analysis time. In a series of reports, Carr *et al.* described the enhancement of selectivity with SCC by controlling individually the temperature of each column (see Table 2.1) [22,52,81–83]. For this purpose, the columns were placed in two independent compartments at different temperature. The authors named the approach "thermally tuned tandem column" (T^3C) optimisation.

The approach solves the discontinuity problem intrinsic to the coupled columns, since the stationary phase selectivity can be tuned in a continuous

fashion without the need of changing the column lengths. By changing the temperature of each column, their contribution to the overall retention time changes. Even small variations in the temperature may have substantial effects on the overall selectivity, outperforming those found with the single columns (Fig. 2.6a and c). The approach was qualified as more powerful than manipulating the eluent type and composition, with baseline separations even when the sample could not be separated in either individual column. The high selectivity with the T^3C approach allows also shortening significantly the analysis time by using a high flow rate (compare Fig. 2.6c and d).

The retention behaviour in T^3C is additive, similarly to the conventional approaches at fixed or variable isocratic conditions (Sections 2.7.3 and 2.7.4). If the temperature in the first column is much higher, its contribution to the retention will be negligible, and consequently, the overall selectivity will be the same as that on the second column. At intermediate temperatures, the selectivity will be in between both columns. The effect of temperature on retention for a single column in moderate ranges can be accurately modelled by a simple two-parameter van't Hoff equation. The fitting of this relationship requires at least two initial runs for each column for method development, which makes T^3C very practical and convenient.

Similarly to the approach based on the flow rate tuning, the coupled columns used in the T^3C approach must exhibit quite different selectivity, so that the unresolved peaks in one column are separated by the other. The developers of the approach used combinations of C18 silica and coated polybutadiene- or carbon-type zirconia. Zirconia columns differ more in selectivity with respect to the C18 phase than any other type of silica-bonded phase (such as C8, phenyl, or cyano). One of the coupled columns may be set at room temperature, but the other should preferably be operated at high temperature. In this regard,

zirconia-based phases are appropriate due to their high stability up to 200°C. This allows tuning the overall selectivity over a very substantial range, with dramatic changes in the retention factors, elution sequences and band spacing.

For closely related non-ionic compounds, the changes in selectivity in RPLC with temperature using a single column are small, in accordance with the small differences in the retention enthalpies. In contrast, when both the charged and neutral species of ionisable analytes are present in the eluent, temperature will significantly affect the retention if the ionisation degree (related to the dissociation constant, pK_a) is altered. Thus, the aqueous pK_a of basic analytes decreases with increasing temperature, while the pK_a of phosphate buffer in water remains relatively constant. The net effect is that, at higher temperature, the concentration of the neutral species increases and for the protonated cationic species is reduced. The neutral species interacts with the C18 phase much stronger than the charged species does. Therefore, retention should increase at increasing temperature in this column. In contrast, cation exchange is dominant in a polybutadiene-coated zirconia column, making the charged analyte interact more strongly than the neutral species. Therefore, the retention decreases with increasing temperature. This behaviour has given rise to a very convenient method to analyse mixtures of basic compounds using the T^3C approach (Fig. 2.6) [81].

2.9. Conclusions

According to Mao and Carr, “one of the key reasons for the use of a single stationary phase is the long-held belief that the retention and selectivity in LC are dominated by the mobile phase” [22]. Meanwhile, the work of several authors has shown the potential of serially coupling of two or more columns

containing different stationary phases to analyse complex mixtures. SCC behave like totally new columns, in some instances with extraordinarily better performance. The approach can become sufficiently powerful to succeed in the separation of many complex samples that cannot be analysed using single columns. The probability of having the ideal column for a particular separation is thus increased. However, column combination would not have been so appealing without the concomitant advances in column technology. The stationary phases currently available, together with the availability of ZDV couplers, columns of multiple lengths, some of them capable of withstanding high temperatures or flow rates, have expanded the probabilities of success. Obviously, when the separation is good enough with a single column there is no need to use SCC.

A detailed study on the peak capacity has not been reported for SCC yet. However, it can be expected to be similar to that for the individual columns of similar length [71]. Therefore, SCC cannot compete with 2DLC in terms of peak capacity. Peak capacity does not either explain the success of SCC compared to single columns in improving the chromatographic resolution. The key point is the change in selectivity achieved by coupling two or more columns, which depends on the orthogonality of the separation mechanisms in each column. Besides the lower peak capacity with regard to 2DLC, there is an additional limitation for SCC related to the continuity of the mobile phase that emerging from one column should flush through the next. In 2DLC there is more freedom in this regard: even if the same stationary phase is placed in both dimensions, two widely different mobile phases or gradients can be used. Finding a solution for the combination of fully orthogonal columns (e.g., RPLC and HILIC) is also more difficult with serial coupling. Therefore, this approach should be considered as an intermediate solution between the use of single

columns and tandem columns in 2DLC that can fulfil the resolution requirements of many complex samples. It can be also considered as a solution to enhance the separation capability in each dimension in 2DLC.

A specific guideline about the most suitable columns that should be combined for a particular application cannot be given yet, based on the gathered experience. It can be instead affirmed that the combination of a small number of common columns (e.g., C18, cyano, amino and phenyl) may succeed in most separations where isolatedly they fail [24]. A wise selection of columns (nature and length) and solvent (isocratic mobile phase or gradient program), through the application of interpretive optimisation approaches, multiplies impressively the probabilities of success. This is also true for 2DLC. However, SCC has still the advantage of being accessible to any laboratory, whereas 2DLC requires a very considerable investment and training, and in this sense is more restrictive. When SCC can solve the problem, the analysis time is also smaller. In a comprehensive study to analyse phenolic antioxidants in beer and hop-extract samples, comprehensive 2DLC with a short PEG column in the first dimension and a C18 column in the second dimension resulted in good separation, but a serial column setup offered significantly shorter analysis times [58].

An additional advantage of SCC, compared to the use of single columns, is that the enhanced selectivity gives rise to a certain gradient effect that reduces the analysis time. The usefulness of isocratic elution is thus powered. This may represent a stimulus for new developments, especially in combination with MS, electrochemical and refractometric detection. However, a limitation has been suggested related to the non-idealities that may appear at ultrahigh pressure using sub-2 μm particles [67]: the increase in temperature and drop in retention

produced by the frictional heating can be expected to make method development with coupled columns more challenging.

So far, the work with SCC has focused on the achievement of enhanced selectivity. Although some authors report limits of detection, there is no formal comparison study with the values obtained with the single columns. The topic is not yet in a routine analysis step, but a few authors have applied SCC to improve screening and quantification studies. There is no reason to think any kind of quantitative problem. The results will have basically the same features than those obtained for resolved peaks with single columns, because the column combinations behave as new single columns with enhanced performance. It should be noted that with regard to 2DLC there is no dilution of the injected sample.

When a given separation fails with single columns, and each column gives rise to a different critical pair, several reports have shown that SCC will enhance the resolution. The gathered information obtained with the single columns can be re-used to find out the best column combination and separation conditions. If the analysis time with the combined columns under isocratic conditions is not convenient, the information can now be re-used to predict the best gradient separation. The only additional data to carry out the interpretive optimisation is the dwell time.

The combination of several columns of multiple lengths containing different stationary phases has the same effect as having a stock of different single columns (tens, hundreds, thousands, or even more) in the laboratory, each of them behaving as a new column with new selectivity expectancies and limitations. However, to exploit it, some problems should be still addressed. First, although dedicated columns are available in the market (the POPLC kit), coupling columns from different manufacturers is more attractive. However, up

to date, the availability of columns of different lengths bought from catalogue is rather poor and still expensive. If the demand persuades manufacturers for producing short columns at a reasonable cost, SCC will have a promising future. A full exploitation of all the potential also requires commercial software oriented to find the proper column combination and the separation conditions. Ideally, both isocratic and gradient optimisations with single and SCC should be carried out with the same software application. It is expected that these developments will produce significant advances in both routine analysis and research laboratories in several fields, in the next future.

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CHAPTER 3

INTERPRETATIVE APPROACHES TO OPTIMISE SERIALLY-COUPLED COLUMNS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

3.1. Abstract

The serial coupling of chromatographic columns of controlled lengths containing different stationary phases multiplies the available selectivity in reversed-phase liquid chromatography, which expands the probabilities of success in the separation of complex samples. However, the full exploitation of the separation capability of coupled columns requires a reliable predictor system of solute retention, peak profile and resolution, for both isocratic and gradient elution. This work summarises the modelling and optimisation strategies needed to implement a separation with serially-coupled columns, offering as example the analysis of a mixture of 15 sulphonamides with four columns of different nature (two C18 columns, a phenyl phase and a cyano phase). None of these columns was able to reach good resolution, but when properly combined, all compounds were resolved in an acceptable analysis time.

3.2. Introduction

The traditional strategy of using a single column in reversed-phase liquid chromatography (RPLC) is often insufficient to solve complex separations, due to the limited functionality of conventional stationary phases, in spite of the enormous advances in column technology [1,2]. A relatively simple solution to expand the possibilities of RPLC is to serially connect two or more columns containing different stationary phases. The effect was described by Garay, who indicated that "it is similar to the technique of a painter preparing green by mixing cyan and yellow paints, including the modification of the hue by altering the ratio of the two paints" [3]. In the context of chromatography, we would refer to selectivity instead of color: a nearly continuous transition is possible by serially connecting RPLC columns, which allows fine-tuning the selectivity for each particular separation. Additionally, the analysis time is decreased making gradient elution less necessary. These features are convenient to yield significant advances in both routine analysis and research laboratories in several fields.

The practical consequences of serially combining columns containing different stationary phases are the same as multiplying the number of columns in the laboratory to thousands or even millions, each combination behaving as a new column. The probability of having the ideal column for a particular separation is highly increased. In fact, the idea of serially connecting columns appeared early in the development of chromatography (especially in the field of gas chromatography) [4–6]. Along the years, some authors have proposed the serial connection of two or more columns in HPLC to solve their particular problem. Some examples are given in Table 3.1. However, being the combination of different columns a highly powerful tool to resolve complex

samples, its successful development and widespread application required solving some technical problems.

Firstly, the chromatographic columns should be coupled without adding extra dead volumes, and without affecting the packing close to the column connections. These problems are solved by connecting conventional chromatographic columns through zero dead volume fingertight couplers, which are screwed directly to the columns [20]. This is also a universal and reliable system that has the advantage of coupling columns from different manufacturers. Secondly, an approach is needed to find the correct combination and optimise its performance. For this purpose, some Chemometrics tools used for the optimisation of single columns should be adapted [20–24]. Finally, adequate software to properly exploit all the potential that the separation system offers should be developed.

This work reports models and other tools useful to describe the retention and peak profile in RPLC needed to implement the optimisation of serially-coupled columns. The good performance of coupled systems is demonstrated considering a sample consisting of 15 sulphonamides to be resolved with four stationary phases (two C18 columns with different retention behaviour, a phenyl phase and a cyano phase), each one giving rise to poor performance when used as single columns. However, using an optimised combination of these columns, an excellent separation was obtained in terms of resolution and analysis time.

Table 3.1. Some reported procedures using serially-coupled columns.[a]

Compounds and samples	Experimental conditions	Ref.
Dihydroxyphenylalanine in urine and plasma	C8 and C18 columns Isocratic elution with citric acid-sodium acetate buffer Electrochemical detection	[7]
Phenylthiohydantoin amino acid derivatives	Cyano and benzyl columns Isocratic elution with methanol-acetonitrile-tetrahydrofuran-water UV absorbance detection	[8]
Aromatic nitro compounds	Modified ES-Gel with nitro, C7, diol and safrol groups and LiChrospher 100 NH2, 100 CN and RP-18 columns Isocratic elution with *n*-heptane-ethanol, and acetonitrile- and methanol-water UV absorbance detection	[9]
Polynuclear aromatic hydrocarbons	Microcolumns packed with octadecylsilica and β-cyclodextrin silica materials Isocratic and gradient elution with methanol- and acetonitrile-water Laser-induced fluorescence and UV absorbance detection	[10]
Triazine herbicides, and urea and carbamate pesticides	C-ZrO_2 and Zorbax SB-C18 columns Isocratic elution with tetrahydrofuran-, acetonitrile- and methanol-water UV absorbance detection	[11]
Model mixture of 17 analytes	Cyano and PEG silica columns Isocratic and gradient elution with hexane-dioxane UV absorbance detection	[12]
Phenolic antioxidants in beer samples	Purospher STAR RP-18e, Discovery HS F5 and Discovery HS PEG columns Isocratic and gradient elution with acetonitrile-water Diode array UV detection	[13]
Arginine, ibuprofen and ibuprofen impurities	Zorbax SB-C18 and Supelcosil LC-NH2 columns Isocratic elution with acetonitrile-water UV absorbance detection	[14]
Model mixture of 7 analytes	POPLC system with phenyl, cyano and C18 EPS columns Isocratic elution with acetonitrile-water Diode array UV detection	[15]
Cortisol and cortisone in urine samples	Varian Pursuit C18 and Waters Atlantis dC18 columns Isocratic elution with acetonitrile-water Diode array UV detection	[16]
Polymer electrolyte membrane degradation products	POPLC system with C18, phenyl and C18 EPS columns Isocratic and gradient elution with acetonitrile-water Diode array UV and mass spectrometric detection	[17]
Mixture of 13 peptides	POPLC system with C18, phenyl, cyano and C18EPS columns Isocratic and gradient elution with acetonitrile-water Diode array UV detection	[18]
Phenones, benzoic acids and hydroxybenzoates	POPLC system with C18, phenyl, cyano and C18EPS columns Isocratic and gradient elution with acetonitrile-water Diode array UV detection	[19]

[a] The serially-coupled columns were built with the indicated columns containing different stationary phases.

3.3. Experimental

The following sulphonamides were considered: (1) sulphacetamide, (2) sulphachloropyridazine, (3) sulphadiazine, (4) sulphadimethoxine, (5) sulphaguanidine, (6) sulphamerazine, (7) sulphamethazine, (8) sulphamethizole, (9) sulphamethoxazole, (10) sulphamonomethoxine, (11) sulphanilamide, (12) sulphapyridine, (13) sulphaquinoxaline, (14) sulphathiazole, and (15) sulphisoxazole. Their chromatographic behaviour was examined with columns of different lengths containing four stationary phases, supplied by Advanced Chromatography Technologies Ltd. (ACE, Aberdeen, Scotland, UK): C18-HL (with a high surface area and carbon load, 2 cm and 3 cm), C18 AQ (with an embedded polar group, 2 cm and 5 cm), phenyl (2 cm and 5 cm) and cyano (3.5 cm and 7.5 cm). In order to model the chromatographic systems (columns and mobile phase compositions), isocratic runs were carried out with acetonitrile-water mobile phases in the 10–20% (v/v) range, buffered at pH 3.5.

The HPLC system (Agilent, Waldbronn, Germany) was equipped with a quaternary pump (Series 1200) and a UV-visible detector (Series 1200), a temperature controller (Series 1100) and an automatic sampler (Series 1100) with 2 mL vials. The detector and column temperature were set at 254 nm and 25 ºC, respectively. The injection volume was 20 µL and the flow-rate, 1 mL/min. The system was controlled by an OpenLAB CDSLC ChemStation (Agilent B.04.03). Other details can be found elsewhere [20,22].

The retention and peak shape behaviours needed to simulate the expected chromatograms, for single or serially-coupled columns, were computed assisted by MATLAB 2014b (The MathWorks Inc., Natick, MA) routines.

3.4. Optimisation outline and results

3.4.1 Models for single and coupled columns

To carry out the optimisation of the chromatographic performance in either isocratic or gradient elution, the behaviour of each stationary phase should be modelled. Since the analyst is usually interested in achieving good resolution and short analysis time, a good tool for the selection of the best separation conditions is the construction of Pareto plots. In order to increase the reliability of the optimisation, the prediction system should consider not only the retention, but also the peak profiles summarised by the width and asymmetry of chromatographic peaks.

In principle, for single columns, the needed information may be obtained from isocratic or gradient runs. Isocratic measurements are more laborious, but generally provide more reliable information for selecting optimal isocratic separation conditions, since gradient data can be only transformed in accurate isocratic predictions in narrow ranges of organic solvent [25]. To predict the separation with combined columns, the behaviour with each stationary phase should be modelled. In this case, the data needed to predict either isocratic or gradient elution should be isocratic. Since in this approach the column length is also optimised, an arbitrary length should be set as reference for modelling the system, which should not be necessarily the same for all stationary phases. However, in order to obtain the maximal accuracy, the use of the maximal available column length is convenient.

The same protocol may be applied to model columns that will be used in a single fashion or in combinations. For this purpose, several runs should be developed in a selected range of mobile phase compositions. In this work, several acetonitrile-water mixtures in the 10–20% (v/v) range were used. At

each mobile phase composition, the retention times and peak half widths were measured for each probe compound. The dead time for each column was obtained using uracil as marker.

3.4.1.1. Models for single and coupled columns

The retention time for a solute *j* eluted through a single column *i* ($t_{R,ij}$) consists of two contributions: the extra-column effect on retention (t_{ext}, produced by the external tubing and the connections with the pump through the injector), and the retention inside the column ($t_{R,int,ij}$):

$$t_{R,ij} = t_{ext} + t_{R,int,ij} \tag{3.1}$$

Since the retention time depends on the column length and flow-rate, the retention factor, which is a normalised parameter, is used instead:

$$k_{ij} = \frac{t_{R,ij} - t_{0exp,i}}{t_{0exp,i} - t_{ext}} \tag{3.2}$$

$t_{0exp,i}$ being the experimental dead time, which includes both the extra- and intra-column contributions:

$$t_{0exp,i} = t_{ext} + t_{0int,i} \tag{3.3}$$

where $t_{0int,i}$ represents the fraction of dead time associated exclusively to the column.

The most common and reliable model to describe the retention behaviour in RPLC is the quadratic relationship between the logarithm of the retention factor (usually expressed as decimal logarithm) and the organic solvent content (usually expressed as volume fraction, φ) [26]:

$$\log k_{ij} = c_{0,ij} + c_{1,ij}\,\varphi + c_{2,ij}\,\varphi^2 \tag{3.4}$$

In narrow ranges of organic solvent, the quadratic term can be dropped. Another model that has demonstrated good performance is based on polarity parameters [27]:

$$\log k_{ij} = c_{0,ij} + c_{1,ij}\left(1 - \frac{a\,\varphi}{1 + b\,\varphi}\right) \tag{3.5}$$

The fitting parameters $c_{0,ij}$, $c_{1,ij}$ and $c_{2,ij}$ in Eqs. (3.4) and (3.5) have particular values for a given solute and chromatographic system (column/modifier), and a and b are specific parameters for each organic solvent (a = 2.13 and b = 1.42 for acetonitrile-water mixtures, and a = 1.33 and b = 0.47 for methanol-water mixtures).

Eqs. (3.4) and (3.5) also describe the retention in the gradient mode. However, the prediction of the retention is more complex, since the organic solvent content changes along the gradient. Therefore, an integral equation should be solved for both single and combined columns. In the most usual approach, the integral is expressed as [28]:

$$t_{0,i} = \int_0^{t_{g,ij} - t_{0,i}} \frac{dt}{k_{ij}(\varphi(t))} \tag{3.6}$$

where $t_{g,ij}$ is the retention time of solute j under gradient elution with column i, and k_{ij} the retention factor, which varies along the column as the gradient program $\varphi(t)$ changes. Eq. (3.6) can be solved analytically only in simple situations, as is the case of single columns for which the retention is described by a linear logarithmic relationship with the mobile phase composition and a linear gradient program is applied. When this is not the case, the integration should be numerical.

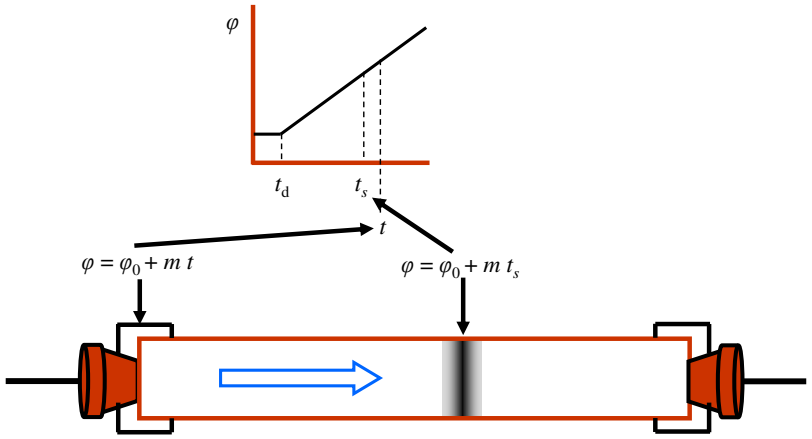

Figure 3.1. Chromatographic column illustrating the solute elution band and change in eluent composition (φ) at the column inlet along the gradient. The plot indicates the dwell time (t_d), the time measured at the column inlet (t), and the time at the column inlet corresponding to the eluent composition affecting the solute (t_s).

It should be noted that the analyst has only direct information about the gradient front, which does not reach the column instantaneously (Fig. 3.1). Therefore, initially, the solutes are eluted isocratically during the so-called dwell time (t_d), and strictly, only when the gradient front reaches the solute its retention will depend on the changes in organic solvent along the gradient program. Therefore, the mobile phase composition at the solute location is needed to predict the retention. Note that the modifier content at the solute location is different (smaller for positive gradients) to that at the column inlet, which is the available information. As the solute migrates along the column, a progressively longer time is needed for the gradient to reach the solute. Thus, this internal (intra-column) delay should be included in the calculation [29], which is taken into account through the difference $t_{g,ij} - t_{0,i}$ in the upper integral

limit, and should also be considered in the numerical integration, which splits Eq. (3.6) in a series of integrals:

$$t_{0,i} = \int_{0}^{t_d} \frac{dt}{k_{ij}(\varphi_0)} + \int_{t_d}^{t_1 - t_{01}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_1 - t_{01}}^{t_2 - t_{02}} \frac{dt}{k_{ij}(\varphi(t))} + \ldots + \int_{t_{n-1} - t_{0n-1}}^{t_n - t_{0n}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_n - t_{0n}}^{t_{g,ij} - t_{0,i}} \frac{dt}{k_{ij}(\varphi(t))} \quad (3.7)$$

Each term in the summation is associated to an isocratic step along the gradient program, and corresponds to the migrated column fraction during a given time interval (defined by the integration limits). The times t_1, t_2, ..., t_n correspond to the intervals in which the gradient time has been split (in this work, Δt = 0.01 min intervals were adopted), and t_{01}, t_{02}, …, t_{0n} are the times (measured from the column inlet) for the gradient front to reach the solute in each column section. Since $k_{ij}(\varphi)$ can be considered constant in small enough time steps (usually adopting the mean value in each interval) and $t_{0n} \approx t_{0n-1}$, the integration can be approximated as follows:

$$t_{0,i} \approx \frac{t_d}{k_{ij}(\varphi_0)} + \frac{t_1 - t_d}{k_{ij}(\varphi_1)} + \frac{t_2 - t_1}{k_{ij}(\varphi_2)} + \ldots + \frac{t_{g,ij} - t_n}{k_{ij}(\varphi_n)} = \frac{t_d}{k_{ij}(\varphi_0)} + \frac{\Delta t}{k_{ij}(\varphi_1)} + \frac{\Delta t}{k_{ij}(\varphi_2)} + \ldots + \frac{\Delta t}{k_{ij}(\varphi_n)} \quad (3.8)$$

When dealing with multi-linear gradients with isocratic steps, or steps with small slopes, the internal delay should be preferably implemented in the solvent content direction (i.e., $\Delta\varphi$ instead of Δt) [23]. Note that the required information is the solvent content at the solute location.

3.4.1.2. Modeling the peak profiles

A chromatographic peak can be described based on four parameters (Fig. 3.2): the retention time (t_R, whose prediction has been commented in Section 3.4.1.1), the maximal peak height (h_0, which depends on the solute nature and concentration, and the detector sensitivity), the peak width (w) and the asymmetry (f). For a solute j eluted with a column i:

$$w_{ij} = A_{ij} + B_{ij} \tag{3.9}$$

$$f_{ij} = \frac{B_{ij}}{A_{ij}} \tag{3.10}$$

which depend on the left (A) and right (B) half-widths, and should be more conveniently measured close to the peak base. Measurements at 10% peak height are recommended to avoid biases produced by the baseline noise.

In isocratic elution, the half-widths can be predicted with good accuracy, using the following global models [30]:

$$A_{ij} = a_{0,i} + a_{1,i}\, t_{R,ij} + a_{2,i}\, t_{R,ij}^2 \tag{3.11}$$

$$B_{ij} = b_{0,i} + b_{1,i}\, t_{R,ij} + b_{2,i}\, t_{R,ij}^2 \tag{3.12}$$

where $a_{0–2,i}$ and $b_{0\text{-}2,i}$ are fitting parameters that depend on the column nature and length.

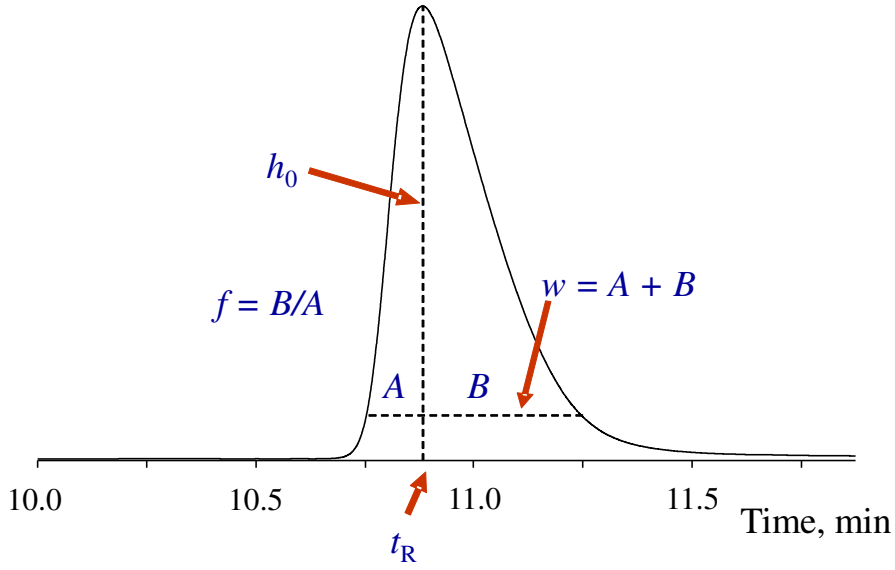

Figure 3.2. Chromatographic peak illustrating its relevant parameters (retention time, maximal peak height, left and right half-widths, width and asymmetry).

Eqs. (3.11) and (3.12) can be fitted using a set of isocratic experimental data ($t_{R,ij}$, A_{ij}, B_{ij}), for each target solute eluted at several mobile phase compositions or, when the same partitioning kinetics holds, for several solutes eluted using one or more compositions. The fitted models may be useful to predict the peak half-widths at mobile phase compositions different from those in the training experimental design. The half-width plots can often be simplified to straight-lines. For Gaussian peaks ($A_{ij} = B_{ij}$), a unique equation is used. Eqs. (3.11) and (3.12), fitted with isocratic data, allow also reliable predictions in gradient elution by substituting the equivalent time for isocratic elution at the instant composition when the solute leaves the column.

In all situations, the peaks in the chromatograms for both single and combined columns can be simulated based on the ($t_{R,ij}$, A_{ij}, B_{ij}) data set for

each solute. Realistic simulations can be built by reformulating the Gaussian model, introducing a linearly time-dependent standard deviation [31]:

$$h_{ij}(t) = h_{0,ij} \exp\left[-\frac{1}{2} \left(\frac{t - t_{R,ij}}{s_{0,ij} + s_{1,ij}(t - t_{R,ij})} \right)^2 \right] \tag{3.13}$$

where $h_{ij}(t)$ and $h_{0,ij}$ are the height at time t and the maximal peak height for the peak of solute i, respectively; $s_{0,ij}$ is a parameter associated to the width at the peak maximum; and $s_{1,ij}$ considers the peak asymmetry:

$$s_{1,ij} = 0.466 \frac{f_{ij} - 1}{f_{ij} + 1} \tag{3.14}$$

$$s_{0,ij} = B_{ij} (f_{ij} - s_{1,ij}) \tag{3.15}$$

To avoid baseline increments outside the peak region, exponential decays should substitute the signal at both peak sides below 10% peak height [32]:

$$h_{ij} = k_{1,ij} \exp[k_{2,ij} (t - t_{R,ij})] \tag{3.16}$$

For $t < t_{R,ij} - A_{ij}$:

$$k_{2,ij} = \frac{s_{0,ij} A_{ij}}{(s_{0,ij} - s_{1,ij} A_{ij})^3} \tag{3.17}$$

$$k_{1,ij} = 0.1 h_{0,ij} \exp(k_{2,ij} A_{ij}) \tag{3.18}$$

and for $t > t_{R,ij} + B_{ij}$:

$$k_{2,ij} = -\frac{s_{0,ij} B_{ij}}{(s_{0,ij} + s_{1,ij} B_{ij})^3} \tag{3.19}$$

$$k_{1,ij} = 0.1 h_{0,ij} \exp(k_{2,ij} B_{ij}) \tag{3.20}$$

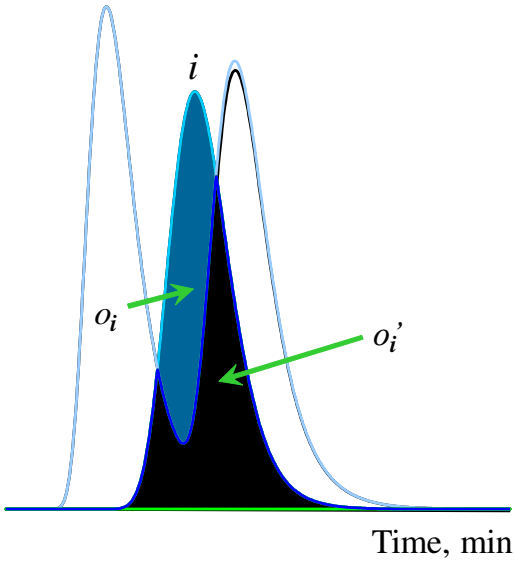

Figure 3.3. Peak areas considered in the calculation of the peak purity (Eq. (3.21)), for compound *i* overlapped by the peaks of two interferences.

3.4.1.3. Chromatographic resolution

The resolution can be reliably evaluated using the concept of peak purity (i.e., the non-overlapped area fraction) [33], which associates a value to each peak in the chromatogram calculated as follows:

$$p_{ij} = 1 - \frac{o'_{ij}}{o_{ij}} \tag{3.21}$$

where o_{ij} is the total peak area, and o'_{ij} the area under the peak overlapped by a hypothetical chromatogram built with the peaks of the other compounds in the sample (Fig. 3.3). The peak purity is a normalised measurement, with an easily interpretable meaning: $p_{ij} = 0$ is associated to a fully overlapped peak and $p = 1$

corresponds to a peak resolved up to the baseline. The overall resolution (P_i) is most conveniently calculated as:

$$P_i = \prod p_{ij} \tag{3.22}$$

and consequently, it is also constrained in the $0 \leq P_i \leq 1$ range.

3.4.2. Optimisation of the single columns

Section 3.4.1 describes several models that offer accurate prediction of the RPLC retention, peak profile and resolution, in isocratic and gradient elution. These models are useful to search reliable optimal separation conditions for single and combined columns. The expected performance of the assayed single columns (12 cm column lengths in all cases) in isocratic elution is shown in Fig. 3.4 as Pareto plots. Note that none of the available columns (neither the phenyl, C18AQ, C18HL nor the cyano column) was able to resolve completely the mixture of 15 sulphonamides. The phenyl column was the best, with maximal peak purity of $P = 0.434$, which means significant overlapping. Especially poor is the separation with the cyano column ($P < 0.01$), which seems to point out that it will be useless for the separation of the mixture of the probe compounds in the assayed range.

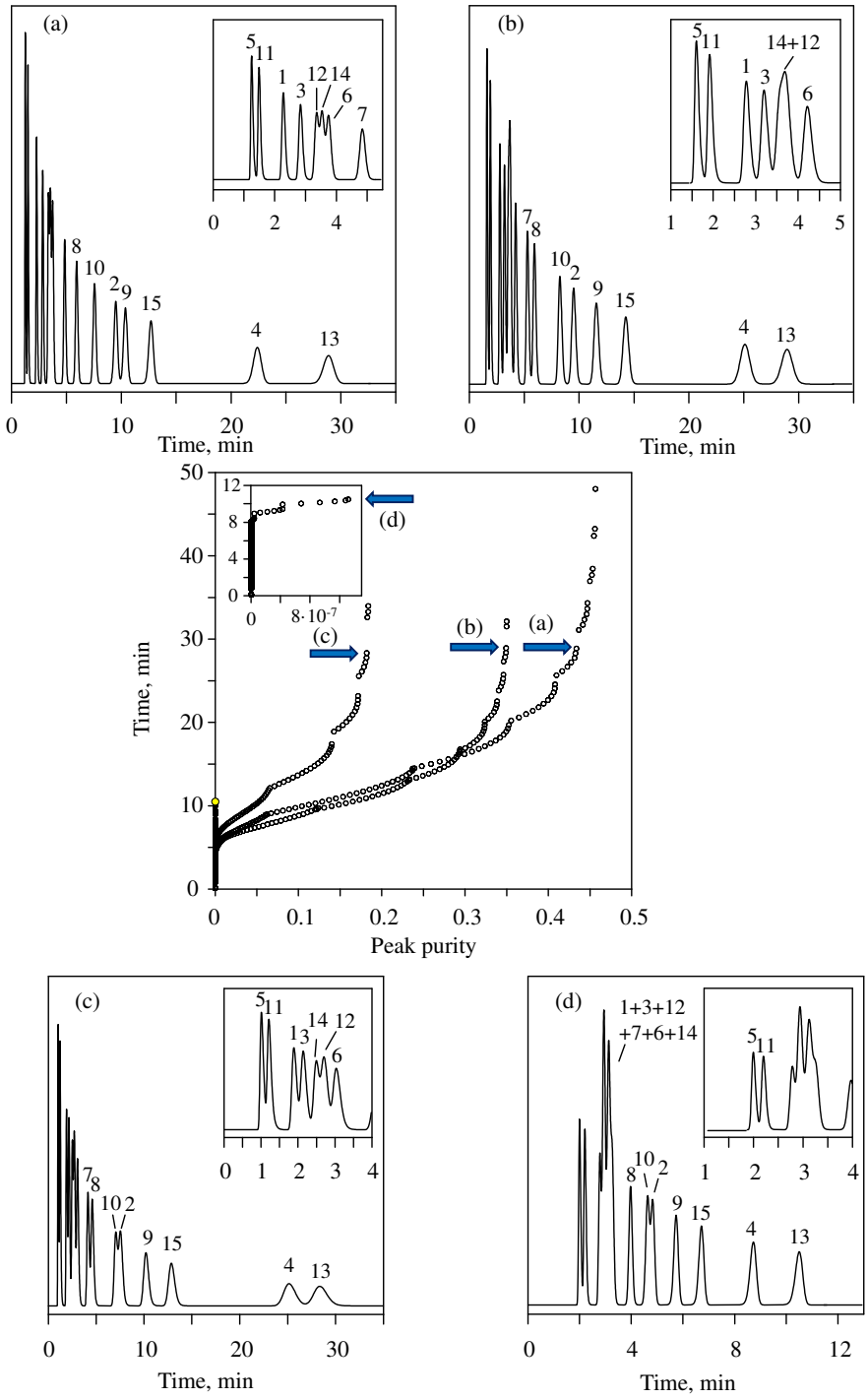
(a)
5
11
1
3
12
14
6
7
8
10
2
9
15
4
13
0
10
20
30
Time, min
(b)
5
11
14+12
1
3
6
7
8
10
2
9
15
4
13
0
10
20
30
Time, min
50
40
30
20
10
0
Time, min
12
8
4
0
0
8·10-7
(d)
(c)
(b)
(a)
0
0.1
0.2
0.3
0.4
0.5
Peak purity
(c)
5
11
13
14
12
6
7
8
10
2
9
15
4
13
0
1
2
3
4
0
10
20
30
Time, min
(d)
1+3+12
+7+6+14
5
11
8
10
2
9
15
4
13
1
2
3
4
0
4
8
12
Time, min

Figure 3.4. Optimisation of 12-cm single columns using isocratic elution. Pareto fronts are shown for the four assayed columns (phenyl, C18AQ, C18HL and cyano). The chromatograms correspond to the experimental conditions marked with an arrow on the Pareto fronts (the global peak purity is given): (a) Phenyl column and 12.5% acetonitrile (P = 0.434), (b) C18AQ and 15.8% acetonitrile (P = 0.349), (c) C18HL and 13.9% acetonitrile (P = 0.182), and (d) cyano and 14.1% acetonitrile (P = 0.000).

Four selected chromatograms are depicted in Fig. 3.4a to d. In all cases, the peaks for sulphapyridine and sulphathiazole (peaks #12 and #14) are almost completely overlapped, while only 5 sulphonamides (sulphadimethoxine, sulphamethizole, sulphamethoxazole, sulphaquinoxaline and sulphisoxazole) can be resolved with the cyano column. Note that the elution order is the same with all columns, except for sulphapyridine and sulphathiazole with phenyl and cyano. The separation did not improve using gradient elution due to the high overlapping. However, we will see below that the serial combination of the assayed columns offers remarkably better performance.

3.4.3. Optimisation of serially-coupled columns in isocratic elution

The models in Section 3.4.1 are also useful to predict the resolution with the combined columns, but some adaptations are needed, which are next described. First, it should be commented that the column system can be built with columns formed by only one segment, or by several segments containing the same stationary phase, in combination with other segments containing a stationary phase with different selectivity.

3.4.3.1. Modeling the retention behaviour

The retention time for a solute *j* eluted through serially-coupled columns ($t_{\mathrm{Rc},j}$) is a combination of the extra-column contribution (t_{ext}) and the retention inside each column ($t_{\mathrm{R,int},ij}$).

$$t_{\mathrm{Rc},j} = t_{\mathrm{ext}} + \sum_{i=1}^{n} t_{\mathrm{R,int},ij} \tag{3.23}$$

When several columns are serially coupled, the extra-column time includes, besides the contribution of the external tubing and the connections with the pump, the time associated to the connections between the serially-coupled columns. The retention time can be expressed in terms of retention factors, as follows [20]:

$$t_{\mathrm{Rc},j} = t_{\mathrm{ext}} + \sum_{i=1}^{n} t_{0\mathrm{int},i}(1+k_{ij}) = t_{\mathrm{ext}} + \sum_{i=1}^{n} t_{0\mathrm{int},i} + \sum_{i=1}^{n} t_{0\mathrm{int},i}\,k_{ij} = t_{0\mathrm{exp}} + \sum_{i=1}^{n} t_{0\mathrm{int},i}\,k_{ij} \tag{3.24}$$

where *n* is the number of combined columns, $t_{0\mathrm{int},i}$ is the intra-column dead time for each column, and $t_{0\mathrm{exp}}$ is the overall dead time associated to the column combination including the external contribution. The term $\sum_{i=1}^{n} t_{0\mathrm{int},i}$ is the dead time associated to the transit through the coupled columns.

Eq. (3.24) isolates the contribution to the retention of each column through each $t_{0\mathrm{int},i}\,k_{ij}$ term. This equation has the advantage of assigning a retention value to each column (or column segment), which is an interesting feature for tracking the changes in the peak profiles, and to predict gradient elution. In isocratic elution using column combinations with negligible dead volumes associated to the connections between columns, the change in column order does not introduce significant differences in the retention (see Eq. (3.23)).

Consequently, the column order is not important and segments containing the same stationary phase can be coupled or not consecutively.

Also, in the absence of extra-column volumes between the coupled columns, the retention factor of columns containing the same stationary phase and support operated at the same mobile phase composition would be expected to be identical, regardless of their column length. In contrast, the intra-column dead volume (and time, $t_{0\mathrm{int},i}$) should increase linearly with the column length, provided the column diameter is the same. This linear relationship allows the calculation of the dead time for columns of any length (l), based on the experimental dead time for a reference column ($t_{0\mathrm{exp,r}}$) with length l_r:

$$t_{0\mathrm{exp},i} = t_{\mathrm{ext}} + t_{0\mathrm{int},i} = t_{\mathrm{ext}} + (t_{0\mathrm{exp,r}} - t_{\mathrm{ext}})\,\frac{l}{l_\mathrm{r}} \tag{3.25}$$

Strictly, the dead time does not only depend on the column length, but also slightly on the mobile phase composition, due to the residual interaction of the dead time marker with the stationary phase and the change in the mobile phase viscosity, among other reasons:

$$t_{0\mathrm{int},i}(l,\varphi) = (d_{0,i} + d_{1,i}\,\varphi + d_{2,i}\,\varphi^2)\,\frac{l}{l_\mathrm{r}} \tag{3.26}$$

where $d_{0,i}$, $d_{1,i}$ and $d_{2,i}$ are fitting parameters for the model that describes the retention of the dead time marker in the reference column.

3.4.3.2. Modeling the peak profiles

Along the elution through the serially-coupled columns, solutes experience band broadening. The peak profiles depend on the peak widths when the solutes enter inside each coupled column. Since each column generates an independent peak broadening, for Gaussian peaks, the total width can be calculated by applying the principle of additivity of variances. For non-Gaussian peaks, this property is not strictly valid. Nevertheless, the additivity principle offers a simple and sufficiently accurate estimation of the peak profiles. The following approximations yield acceptable predictions:

$$w_{c,j}^2 = (A_{c,j} + B_{c,j})^2 = (A_{ext} + B_{ext})^2 + \sum_{i=1}^{n} (A_{int,ij} + B_{int,ij})^2 \tag{3.27}$$

$$f_{c,j} = \left(\frac{B_{c,j}^2}{A_{c,j}^2} \right)^{1/2} = \left(\frac{B_{ext}^2 + \sum_{i=1}^{n} B_{int,ij}^{\ 2}}{A_{ext}^2 + \sum_{i=1}^{n} A_{int,ij}^{\ 2}} \right)^{1/2} \tag{3.28}$$

where (A_{ext}, B_{ext}), and ($A_{int,ij}$, $B_{int,ij}$) are the extra- and intra-column contributions to the peak half-widths, respectively.

The half-widths for each column in the serial combination (A_{ij}, B_{ij}) can be predicted from Eqs. (3.11) and (3.12). Specific models for each stationary phase are required, but there is no need to develop models for each column length, since these can be derived from the models obtained for the reference columns of length l_r . Since this correction only affects the intra-column contribution, Eqs. (3.11) and (3.12) for the reference column may be referred directly to $A_{int,ij}$ and $B_{int,ij}$. This simplifies the prediction process:

$$A_{\text{int},ij}(l_\text{r}) = a_{0,i} + a_{1,i}\, t_{\text{R},ij} + a_{2,i}\, t_{\text{R},ij}^2 \tag{3.29}$$

$$B_{\text{int},ij}(l_\text{r}) = b_{0,i} + b_{1,i}\, t_{\text{R},ij} + b_{2,i}\, t_{\text{R},ij}^2 \tag{3.30}$$

The intra-column half-widths for column length l will be obtained as:

$$A_{\text{int},ij}(l) = A_{\text{int},ij}(l_\text{r})\left(\frac{l}{l_\text{r}}\right)^{1/2} = \left((A_{ij}^2(l_\text{r}) - A_{\text{ext}}^2)\frac{l}{l_\text{r}}\right)^{1/2} \tag{3.31}$$

$$B_{\text{int},ij}(l) = B_{\text{int},ij}(l_\text{r})\left(\frac{l}{l_\text{r}}\right)^{1/2} = \left((B_{ij}^2(l_\text{r}) - B_{\text{ext}}^2)\frac{l}{l_\text{r}}\right)^{1/2} \tag{3.32}$$

The chromatographic peaks are finally predicted using Eqs. (3.13) to (3.20).

3.4.3.3. Optimisation strategies

In isocratic elution at fixed temperature and flow-rate, two optimisation strategies are possible for serially-coupled columns: the search of the best column combination (nature and length of the coupled columns), or the search of the best column combination and mobile phase composition. The simplest option is to optimise only the column combination, pre-selecting the mobile phase composition. However, the performance of the serially-coupled columns depends on the selected mobile phase. Better results are obtained by simultaneously optimizing the mobile phase composition.

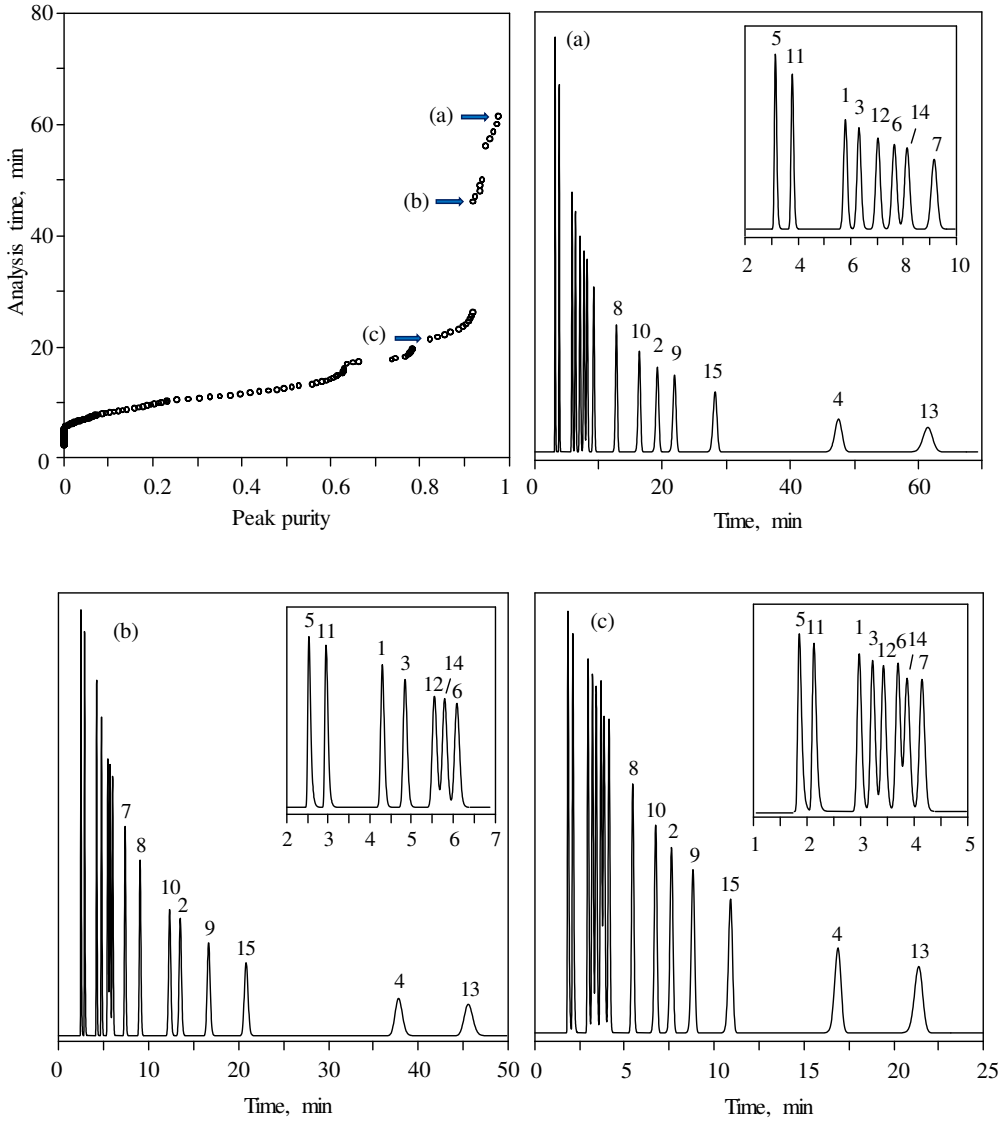

Figure 3.5. Optimisation of serially-coupled columns using isocratic elution. Pareto front (top left), and chromatograms for selected experimental conditions marked with an arrow (the global peak purity is given): (a) 5 cm phenyl + 11 cm cyano and 10% acetonitrile (P = 0.978), (b) 2 cm phenyl + 7.5 cm cyano + 2 cm C18AQ + 2 cm C18HL and 12.5% acetonitrile (P = 0.920), and (c) 2 cm phenyl + 7.5 cm cyano and 11.7% acetonitrile (P = 0.822).

Fig. 3.5 shows the Pareto front for the combination of the four columns. As observed, there are several experimental conditions with acceptable resolution, with $P > 0.8$. The three groups of experimental conditions with higher resolution correspond to three different column combinations: (i) 5 cm phenyl + 11 cm cyano, (ii) 2 cm phenyl + 7.5 cm cyano + 2 cm C18AQ + 2 cm C18HL, and (iii) 2 cm phenyl + 7.5 cm cyano. Note that the first and third group of solutions in the Pareto plot involve combinations of only two columns (phenyl and cyano), whereas the intermediate group involves the four columns.

Three representative chromatograms of the three groups of solutions are shown in Fig. 3.5, whose performance is marked with arrows in the Pareto front. Chromatogram (a), obtained with 5 cm phenyl + 11 cm cyano, exhibits nearly baseline resolution, but the analysis time exceeds one hour. By shortening the lengths of both phenyl and cyano columns (2 cm phenyl + 7.5 cm cyano), and increasing the acetonitrile content, still satisfactory resolution is kept with much shorter analysis time (Chromatogram (c)). Note that the elution order is the same as observed for the single phenyl and cyano columns (Fig. 3.4a and d), but the combination of these columns succeeded in resolving the critical pair (sulphapyridine/sulphathiazole, peaks #12 and #14) and reversed the elution order of sulphamerazine (peak #6), making sulphathiazole and sulphamerazine now the critical pair, but with enough resolution. The column combination corresponding to Chromatogram (b) involved the four columns by adding segments of 2 cm of C18AQ and C18HL to the column combination in (b). This is translated in better resolution, but again implies longer analysis time. Note that here sulphamerazine elutes after the critical sulphapyridine/sulphathiazole pair.

3.4.4. Optimisation of serially-coupled columns in gradient elution

The analysis time for the column combination will decrease using gradient elution. However, the complexity of the optimisation is remarkably larger with regard to isocratic elution. Single linear gradients of organic solvent require the optimisation of the initial and final organic solvent content, or alternatively, the initial concentration and the gradient slope, besides the gradient time. With multi-linear gradients, at least the location of the nodes (or equivalent parameters) should be optimised, and all this besides the nature and length of the combined columns.

3.4.4.1. Modeling the chromatographic behaviour

The prediction of the retention with gradient elution using serially-coupled columns is also more complex. Similarly to isocratic elution (Eq. (3.23)), the gradient time is a combination of the retention associated to each coupled column, to which the extra-column contributions should be added:

$$t_{\mathrm{gc},j} = t_{\mathrm{ext}} + \sum_{i=1}^{n} t_{\mathrm{g},ij} \tag{3.33}$$

where $t_{\mathrm{gc},j}$ and $t_{\mathrm{g},ij}$ are the retention times under gradient elution for the whole column combination and for each individual column, respectively. In contrast to isocratic elution, the performance depends on the column order, since the organic solvent range affecting a given column will change with the column order. Note also that with combined columns, the numerical integration can be the only solution to Eq. (3.6), since the retention time in a given column depends on the composition at which the solute left the previous column(s), and the retention model and time interval to which it holds are specific for each

solute and column. The situation is even more complex with multi-linear gradients.

Eq. (3.7) can be applied to predict the gradient time for the first column in the combination. For the subsequent coupled columns, it should be considered that the information on the eluent composition along the gradient corresponds to the first column inlet. Therefore, in addition to the extra- and intra-column time delays, the delay generated by the transit through previous columns (the sum of the dead times for each column) should also be included:

$$t_{0,i} = \int_{t_{\mathrm{p},ij}}^{t_1 - t_{01}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_1 - t_{01}}^{t_2 - t_{02}} \frac{dt}{k_{ij}(\varphi(t))} + \ldots + \int_{t_{n-1} - t_{0n-1}}^{t_n - t_{0n}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_n - t_{0n}}^{t_{\mathrm{g},ij} - t_0} \frac{dt}{k_{ij}(\varphi(t))} \qquad (3.34)$$

The times t_1, t_2, ... , t_n and t_{01}, t_{02}, ... , t_{0n} have the same meaning as for the first column, while $t_{\mathrm{p},ij}$ is the gradient time when the solute reaches the inlet of the column where the solute is migrating through, after crossing all previous columns (one or more). When the cumulative summation in Eqs. (3.7) and (3.34) matches the column dead time, the solute has reached the column outlet. From that point, if the solute entered a new column, the corresponding $k_{ij}(\varphi)$ function should be adopted and a new integral equation solved, using the sum of the extra-column time and the dead times for the previous columns as total delay time.

Owing to the different elution strengths for different solutes in the coupled columns, the transitions between them may occur at particular times for each solute, and thus, these will be associated to different organic solvent contents. Also, it should be noted that the extra-column contributions are a source of error in the prediction of both retention and peak profiles with serially-coupled columns. Therefore, all calculations must be carried out by excluding these contributions, which should be added at the end of the process, once the

retention times and peak profiles associated to the transit through the columns have been calculated.

3.4.4.2. Optimisation strategies

The optimisation of serially-coupled columns using a pre-selected gradient suffers from the same drawback as commented when the mobile phase composition is pre-selected in isocratic elution: the performance depends on the gradient used, and usually, the best separation is lost. However, the simultaneous optimisation of the column combination (nature and length) and gradient program is unfeasible, due to the massive number of candidate solutions. Therefore, the most reasonable strategy is to optimise the gradient program using a column combination previously selected. Such pre-selection can be carried out attending to the best combination obtained in isocratic elution. The selection is not critical, since several combinations may offer satisfactory results. However, the column order should be still optimised, together with the gradient.

When the optimisation is based on a systematic scan of all experimental conditions, even pre-selecting the column combination, the computation time is still significantly longer, compared to the isocratic elution. With single linear gradients, it may take several hours or days, and it is totally unfeasible with multi-linear gradients. The reduction in the computation time is possible by performing a probabilistic search of the optimal conditions, using for instance, genetic algorithms [23,34]. The populations generated along the genetic algorithm search can be represented as a Pareto plot. The best solution balancing acceptable resolution and analysis time will be one of the intermediate solutions of the probabilistic search (it will be rarely its optimum).

However, in this work, we used a computer-assisted stepwise selection of the gradient program setting as starting point the optimal conditions (not only the column combination, but also the mobile phase composition) found in the isocratic elution. The selection was carried out by observing the changes in the simulated chromatograms, inserting a linear gradient between two isocratic steps. Fig. 3.6 shows the chromatograms for the combinations of 5 cm phenyl + 11 cm cyano, and 2 cm phenyl + 7.5 cm cyano. The chromatograms for the two possible column orders in both cases are represented. The first isocratic step was fixed at the optimal mobile phase composition in isocratic elution (10% and 11.7% acetonitrile, respectively). The final isocratic step for all gradients was set at 20% acetonitrile, which was the maximal concentration in the studied range. To avoid decreases in the resolution, these gradient programs only affected the retention of the most retained peaks, reducing the distance between them. It should be noted that when the cyano column was the first column in the combinations, the analysis time was shorter. Also, some changes in the selectivity were observed.

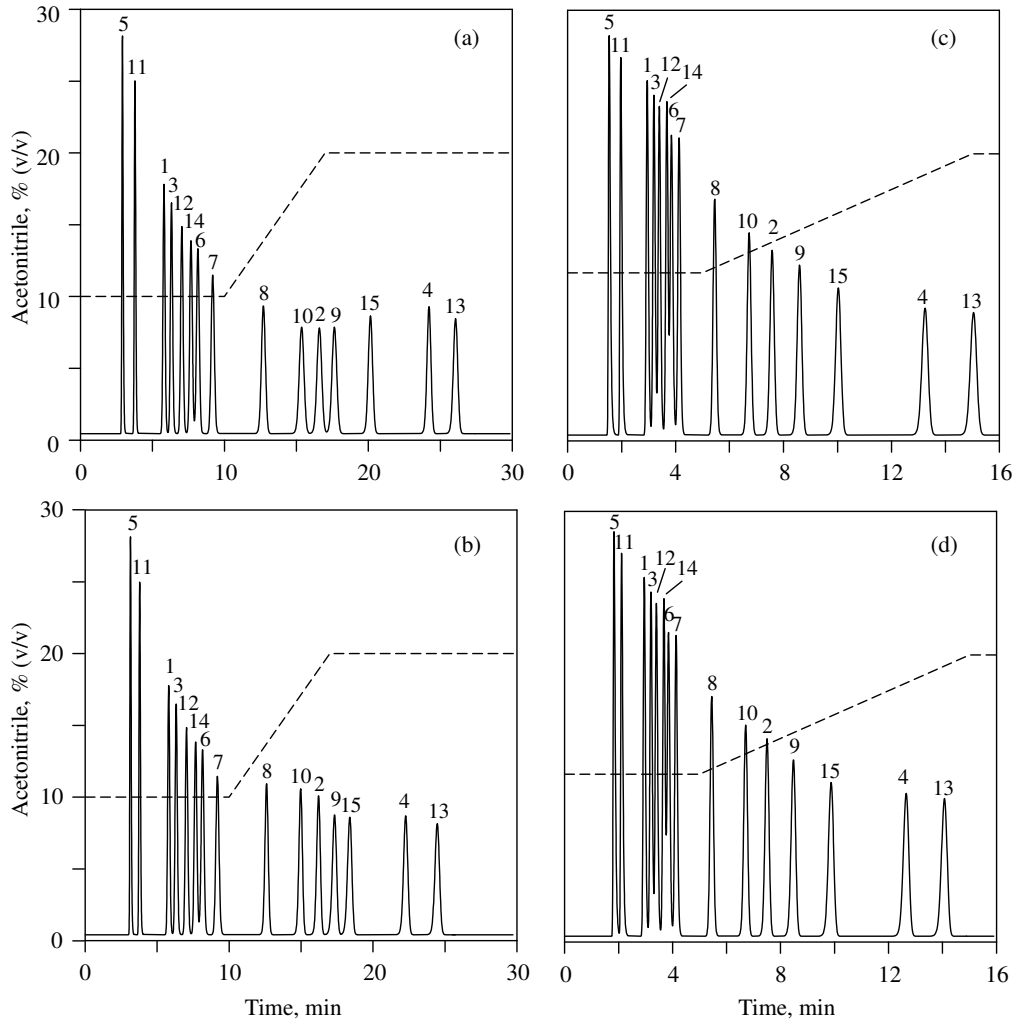

Figure 3.6. Optimisation of serially-coupled columns using gradient elution. Chromatograms obtained with different column combinations (the global peak purity is given): (a) 5 cm phenyl + 11 cm cyano (P = 0.970), (b) 11 cm phenyl + 5 cm cyano (P = 0.974), (c) 2 cm phenyl + 7.5 cm cyano (P = 0.826) and (d) 7.5 cm phenyl + 2 cm cyano (P = 0.823). The gradient program is depicted on the chromatograms.

3.5. Conclusions

The described interpretive optimisation approaches offer successful solutions to the separation of samples of diverse complexity in RPLC. The analyst should first assay one or more chromatographic columns. When the single columns fail, their serial combination may offer a valid solution. The optimisation of the experimental conditions can be carried out using the same information (i.e., retention times and peak profile models) for both single and serially-coupled columns. The need of gradient elution is less critical with serially-coupled columns with regard to single columns, since combined columns decrease significantly the analysis time. Also, in general, the separations are closer to those achieved in two-dimensional liquid chromatography, with the advantage of requiring only a mono-dimensional chromatographic system.

However, the full potential of the serially-coupled columns approach can only be reached with the development of a powerful interpretive optimisation strategy, such as the one described in this work. For this purpose, a unique predictor system that implements the different strategies with single and serially-coupled columns in isocratic and gradient elution is highly useful. In spite of the apparent complexity of the methodology with regard to the single column optimisation, an outstanding agreement has been found between experimental and predicted chromatograms [20–22].

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CHAPTER 4

SERIAL VERSUS PARALLEL COLUMNS USING ISOCRATIC ELUTION: A COMPARISON OF MULTI-COLUMN APPROACHES IN MONO-DIMENSIONAL LIQUID CHROMATOGRAPHY

4.1. Abstract

When a new separation problem is faced with high performance liquid chromatography (HPLC), the analysis is addressed conventionally with a single column, trying to find out a single experimental condition aimed to resolve all compounds. However, in practice, the system selectivity may be insufficient to achieve full resolution. When a separation fails, the usual practice consists of introducing drastic changes in the chromatographic system (e.g. use of another column, solvent or pH). An alternative solution is taking benefit of the combined separation capability of two or more columns, which can be attained in multiple ways, such as diverse modalities of two-dimensional HPLC, or mono-dimensional HPLC with serial or parallel columns. In this work, the separation performance offered by the serial coupling of columns of different nature and length, operated at varying mobile phase composition in isocratic elution, is compared with the results offered by parallel columns. The resolution capability of both approaches is characterised through the limiting peak purities. It is demonstrated that serial columns of different lengths perform as new columns that increase enormously the probabilities of success. The potential of the approach is illustrated through the separation of 15 sulphonamides. In spite of the poor individual performance of the four selected columns (phenyl, cyano and two C18 columns, with nearly null resolution for the cyano column), it was found that the serial coupling of the phenyl and cyano columns of appropriate lengths succeeded in the full resolution of the 15 compounds in 20–25 min, and the serial coupling of the two C18 columns yielded acceptable resolution in less than 20 min.

4.2. Introduction

High performance liquid chromatography (HPLC) is currently the most widely used analytical separation technique, due to its applicability, robustness and sensitivity, responding to multiple problems in environmental, pharmaceutical, clinical and food control, as well as in genomics, proteomics, lipidomics and metabolomics. The advances in this field are the result of fundamental research in several directions, which have gradually addressed increasingly complex problems [1–3]. However, HPLC with a single column still presents challenges in terms of resolution, analysis time and baseline drift in gradient elution, due to the limited chemistry of conventional stationary phases. The combination of retention mechanisms by coupling two or more columns in mono- [4–12], or multi-dimensional [13–16] configurations, is currently the best solution to solve this drawback, which has opened enormously the range of resolutions. It is possible to achieve almost continuous transitions between the selectivity of two or more stationary phases by combining columns. However, being the combination of different columns in multi-column HPLC a highly powerful tool to resolve complex samples, its success requires the assistance of an interpretive optimisation of the separation conditions [7,9,12,17].

We will refer here to the combination of columns in mono-dimensional HPLC. The idea of serially coupling stationary phases (Fig. 4.1a) to analyse complex samples appeared early in the development of chromatography [4,18,19]. However, this approach has been only feasible when the connection between columns has been improved to avoid the undesirable presence of void volumes and changes in the packing material close to the junctions. We have recently proposed a reliable and flexible system, to

connect conventional columns from different manufacturers through zero dead volume fingertight couplers, screwed directly to the columns [8].

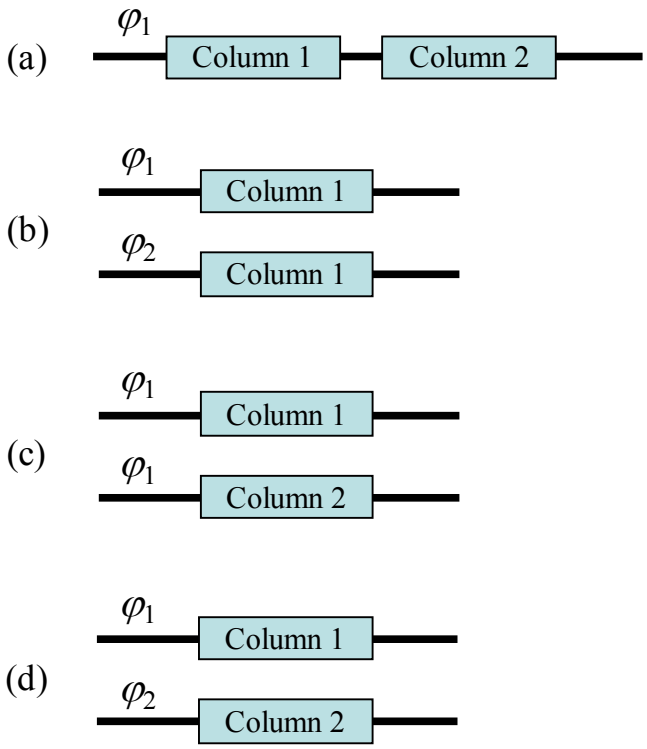

Figure 4.1. Column/solvent configurations studied in this work: (a) Serially-coupled columns, (b) two identical parallel columns with independent mobile phases, (c) two parallel columns of different nature with a common mobile phase, and (d) two parallel columns of different nature with independent mobile phases.

Also, along the last decade we have reported a different separation approach, based on the complementarity concept, which has succeeded in the resolution of highly complex samples [20]. In this approach, a separation condition focuses on the resolution of some compounds in the sample, while the other compounds remain unresolved, but are optimally resolved in a second (or subsequent) condition(s). Consequently, the separation space increases, and so the chances of success. The selection is done in such a way that when the results of the optimal complementary separation conditions are considered altogether, all compounds are maximally resolved. In previous work, we have checked the capability of complementary mobile phases (Fig. 4.1b), containing the same or two different organic solvents, to improve the resolution of a complex sample. In this work, we extend this development to the optimisation of two different parallel columns, sharing the same (Fig. 4.1c) or different (Fig. 4.1d) mobile phase compositions.

The capability of both mono-dimensional multi-column approaches (serial and parallel columns) to resolve a complex sample is compared, using pairs of combined columns, and considering both column nature and lengths together with the mobile phase composition. In order to find out the maximal performance of the systems in both approaches, a rigorous optimisation of the experimental factors based on the modelling of the retention and peak shape was carried out. It is shown how the combination of two serially-coupled columns, both offering very poor resolution when used as single columns, can yield outstanding full resolution, highly extending the separation power of the parallel columns.

4.3. Experimental

For evaluating the performance of the different approaches, a set of 15 sulphonamides studied in previous work [8,9,12] was considered: (1) sulphacetamide, (2) sulphachloropyridazine, (3) sulphadiazine, (4) sulphadimethoxine, (5) sulphaguanidine, (6) sulphamerazine, (7) sulphamethazine, (8) sulphamethizole, (9) sulphamethoxazole, (10) sulphamonomethoxine, (11) sulphanilamide, (12) sulphapyridine, (13) sulphaquinoxaline, (14) sulphathiazole, and (15) sulphisoxazole. The chromatographic behaviour of these compounds was examined with columns of different lengths containing four different stationary phases, supplied by Advanced Chromatography Technologies Ltd. (ACE, Aberdeen, Scotland, UK): phenyl, cyano, C18-HL and C18-AQ. Isocratic runs were carried out with acetonitrile/water mobile phases in the 10–20% (v/v) range, buffered at pH 3.5. The chromatographic system consisted of an isocratic pump operated at a flow rate of 1 mL/min, autosampler, thermostated column compartment set at 25 ^{o}C, and variable wavelength UV-visible detector set at 254 nm, all from Agilent (Waldbronn, Germany). Other details can be found elsewhere [8,9].

The retention and peak shape behaviours needed to simulate the expected chromatograms under any configuration (single, serial and parallel columns) were modelled assisted by MATLAB 2014b (The MathWorks Inc., Natick, MA) routines. This information was used to evaluate the limiting resolutions and the expected optimal separations.

4.4. Results and discussion

4.4.1 Columns and optimisation of the resolution

The below discussion intends to offer some light on the behaviour found with serial columns, where only the nature and length of the coupled columns or these together with the mobile phase composition are optimised. For this purpose, several configurations of serial and parallel columns were compared. In order to facilitate the comparisons, the studies were carried out using isocratic elution with mobile phases containing only one factor (the organic solvent content) and attending exclusively to the resolution (except in Section 4.4.5, where the analysis time was also considered). Four stationary phases, which showed very poor performance when used as single columns, were selected deliberately to demonstrate the capability of the multi-column approaches, and highlight their differences: phenyl, cyano, and two C18 columns (C18-HL and C18-AQ, the former with a high surface area and carbon load, and the latter with an integrated polar functionality). The retention and peak profile behaviours (required to predict the resolution in the single and combined columns of any length) were modelled with single columns of the following lengths (arbitrarily selected): 5 cm phenyl, 11 cm cyano, 3 cm C18-HL and 5 cm C18-AQ.

In serially-coupled columns, where the mobile phase elutes the solutes sequentially (Fig. 4.1a), the retention is the sum of the contributions of each column, being independent of their coupling order in isocratic elution [8,9]. Meanwhile, in the complementarity approaches, where two mobile phases are optimised for the same column (Fig. 4.1b), or two different parallel columns are optimised using the same or different mobile phases (Fig. 4.1c and d, respectively), the retention time for each solute is simply the retention time in

the column (or mobile phase) where it is best resolved. The effect of the mobile phase composition on retention was considered using a quadratic dependence of the retention factor with the organic solvent content. The peak profiles were predicted as explained in Ref. [9].

The optimisation of the resolution along this work was made based on an exhaustive examination of synthetic designs of separation conditions (e.g., column nature and length, and mobile phase composition). For these conditions, the corresponding chromatograms were predicted and the separation quality evaluated. Finally, the separation conditions offering the maximal separation quality were selected. The chromatographic resolution was measured using the peak purity concept, which is the peak area fraction free of overlap for a given compound, considering all other compounds as interferences [17]. For a fully overlapped peak and a peak resolved up to the baseline, the elementary peak purity for a solute j is $p_j = 0$ and 1, respectively. The global resolution (P) was calculated as the product of the elementary peak purities; therefore, the range of variation is also $0 \leq P \leq 1$. Limiting peak purities (i.e., maximal resolution thresholds that can be reached for each compound in a sample considering all possible separation conditions) were used to investigate the degree of exploitation of the capability of the different column configurations [17].

The optimisation of complementary separations deserves a more detailed explanation. The search of two complementary mobile phases (Fig. 4.1b and d) was carried out by considering all possible distributions of the solutes in the sample in two groups and establishing the condition that each group should be separated with a particular mobile phase using the same (Fig. 4.1b), or a different column (Fig. 4.1d). In contrast, when the same mobile phase was flushed in both parallel columns (Fig. 4.1c), the solutes were directly

assigned to the column where they are best resolved. In each case, the partial resolution for each complementary separation condition was calculated, and the global resolution obtained as the product of the partial resolutions. The combination of complementary conditions that offered the maximal global resolution was finally selected. For other details, see Ref. [20].

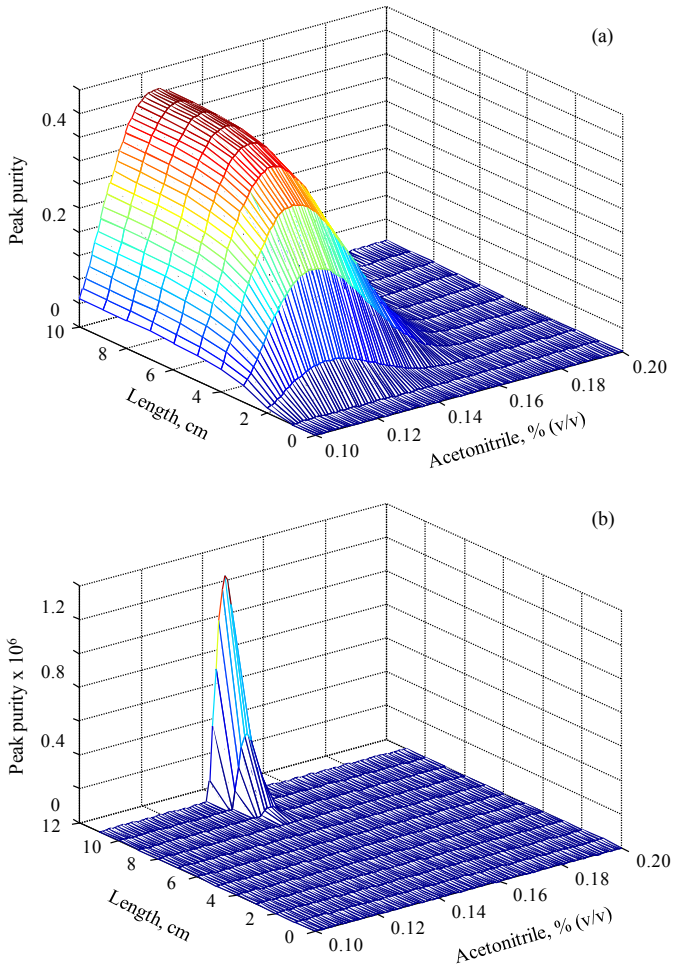

Figure 4.2. Resolution capability for single columns at variable column length and mobile phase composition: (a) phenyl, and (b) cyano.

4.4.2. Resolution capability of the single columns

The capability of single columns to resolve the probe compounds was first evaluated, considering the mobile phase composition and column length as factors. The increase in column length favours the efficiency, and thus it may have a positive effect on the separation capability. However, the improvements are only significant at short lengths, owing to the quick loss of weight of the extra-column contributions to the peak width. Also, it should be noted that the pressure limits the maximal possible length. Fig. 4.2 depicts the global resolution achieved at different column lengths and mobile phase compositions for the phenyl and cyano columns (the behaviour for C18-HL and C18-AQ is similar to the phenyl column). As observed, the resolution increases with the column length tending asymptotically to a maximal value, and that offered by the cyano column is extremely poor at all conditions.

The limiting peak purities in Table 4.1 show, for the four selected columns, that there is no condition (column length or mobile phase composition) able to yield full resolution for each single column, with global limiting resolutions (P_{lim}, calculated as the product of the limiting elementary values) ranging between 0.001 (for cyano) and 0.628 (for phenyl). These values represent the performance limits that cannot be exceeded with each column, unless new selectivity changes are introduced. The extremely low resolution for the cyano column is noteworthy and it would suggest that this column does not deserve more attention. However, we demonstrate below that this column is really useful.

Table 4.1. Limiting peak purities for each single column, considering both column length and mobile phase composition. [a]

Compound	Phenyl	Cyano	C18-HL	C18-AQ
Sulphacetamide	1.0000	**0.8423**	0.9875	1.0000
Sulphachloropyridazine	0.9989	0.9999	0.9909	0.9999
Sulphadiazine	1.0000	**0.0955**	0.9872	1.0000
Sulphadimethoxine	1.0000	1.0000	0.9980	1.0000
Sulphaguanidine	0.9979	1.0000	0.9654	0.9967
Sulphamerazine	0.9710	**0.1452**	0.9106	0.9932
Sulphamethazine	1.0000	**0.2246**	0.9975	1.0000
Sulphamethizole	1.0000	1.0000	0.9972	1.0000
Sulphamethoxazole	0.9986	1.0000	1.0000	1.0000
Sulphamonomethoxine	1.0000	0.9998	0.9910	1.0000
Sulphanilamide	0.9976	0.9999	0.9651	0.9964
Sulphapyridine	0.9664	**0.3334**	**0.7022**	**0.6901**
Sulphaquinoxaline	1.0000	1.0000	0.9977	1.0000
Sulphathiazole	**0.6737**	0.9997	**0.7647**	**0.6152**
Sulphisoxazole	1.0000	1.0000	1.0000	1.0000
Limiting global purity	0.628	0.001	0.432	0.419

[a] Critical compounds are marked in bold.

Table 4.2. Optimal peak purities for each single column, considering both column length and mobile phase composition.[a]

Compound	Phenyl	Cyano	C18-HL	C18-AQ
Sulphacetamide	1.0000	**0.8279**	0.9069	0.9903
Sulphachloropyridazine	0.9896	**0.8263**	**0.8119**	0.9993
Sulphadiazine	0.9999	**0.0115**	0.8693	0.9611
Sulphadimethoxine	1.0000	1.0000	0.9838	1.0000
Sulphaguanidine	0.9954	0.9778	0.9262	0.9887
Sulphamerazine	**0.8510**	**0.1396**	0.9085	0.9883
Sulphamethazine	1.0000	**0.0837**	0.9233	0.9904
Sulphamethizole	1.0000	1.0000	0.9232	0.9902
Sulphamethoxazole	0.9896	1.0000	1.0000	1.0000
Sulphamonomethoxine	1.0000	**0.8260**	**0.8122**	0.9995
Sulphanilamide	0.9951	0.9776	0.9262	0.9885
Sulphapyridine	**0.8210**	**0.0266**	**0.7008**	**0.6323**
Sulphaquinoxaline	1.0000	1.0000	0.9835	0.9998
Sulphathiazole	**0.6737**	**0.6826**	**0.7507**	**0.6146**
Sulphisoxazole	1.0000	1.0000	0.9999	1.0000
Optimal global purity	0.457	0.000	0.176	0.349
Acetonitrile (%, v/v)	12.5	14.1	13.9	15.8

[a] Critical compounds are marked in bold.

Limiting peak purities point out the best separation conditions (in this case, mobile phase compositions) that would resolve each compound from all others in a sample. It may happen that each compound requires a specific separation condition non-compatible with those needed by other compounds to be optimally resolved. However, in practice, compromise separation conditions able to separate several compounds maximally may be found. Analysts usually search only one separation condition, but more than one condition can be selected. This is the basis of the complementary optimisation approaches.

The optimal elementary and global resolutions for the mixture of 15 sulphonamides, when a single optimal mobile phase composition is selected for each column, are given in Table 4.2. It is observed that the optimal global purities (P_{opt}) are smaller than the limiting ones (compare with Table 4.1), ranging between 0.000 (for cyano) and 0.457 (for phenyl). In the table, the resolutions for the critical compounds are marked in bold. As observed, the cyano column has serious problems with eight of the 15 compounds in the sample, with extremely low best resolution for sulphadiazine, sulphamerazine, sulphamethazine and sulphapyridine (which also showed very low limiting resolutions with this column). In contrast, the phenyl and C18-AQ columns showed poor resolution only for sulphamerazine, sulphapyridine and sulphathiazole.

The complementarity principle allows getting closer to the limiting resolutions. However, for the case of study, the limiting purities for each column in Table 4.1 indicate that complementary mobile phases (Fig. 4.1b) where only the solvent content is optimised would be yet deficient: the limiting global value for the best column (phenyl) is $P_{lim} = 0.628$. Thus, when two optimal complementary mobile phases were searched for each column,

the global purities ($P_{c,opt}$) were: 0.558, 0.0006, 0.363 and 0.393 for the phenyl, cyano, C18-HL and C18-AQ columns, respectively (Fig. 4.3). Consequently, none of the four selected columns was selective enough to resolve the 15 probe compounds, even using complementary mobile phases.

4.4.3. Separation performance of parallel columns

4.4.3.1. Two columns and a common mobile phase composition

The use of approaches with combined columns to increase the separation space was next evaluated. We will first consider the use of two parallel columns of different nature, flushing the same mobile phase through both columns (Fig. 4.1c). For the case of study, where four columns were available, six combinations of two columns were possible: phenyl/cyano, phenyl/C18-HL, phenyl/C18-AQ, cyano/C18-HL, cyano/C18-AQ and C18-HL/C18-AQ. Fig. 4.4 illustrates the changes observed in the global complementary resolution for these combinations operated in parallel at varying mobile phase composition. As expected, the optimal resolution was achieved with the maximal available length for each column: 10 cm for phenyl, C18-HL and C18-AQ, and 11 cm for cyano.

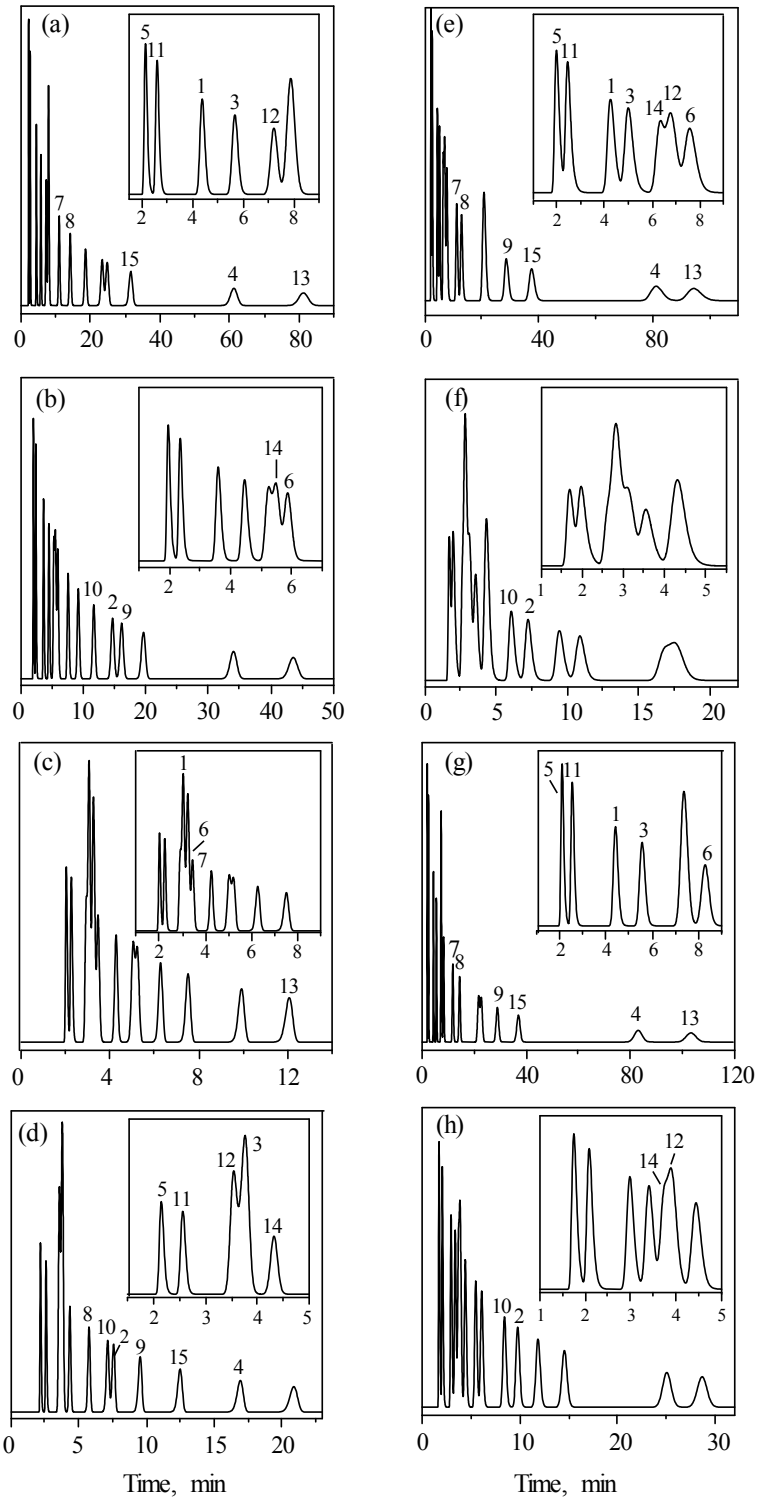
(a)
(e)
(b)
(f)
(c)
(g)
(d)
(h)
Time, min
Time, min

Figure 4.3. Optimal chromatograms for the four columns, using complementary mobile phases (mobile phase compositions and partial peak purities are given). Phenyl: (a) 10.0% (P = 0.962) and (b) 13.0% (0.580). Cyano: (c) 13.0% (0.021) and (d) 10.0% (0.029). C18-HL: (e) 11.5% (0.370) and (f) 20.0% (0.982). C18-AQ: (g) 10.2% (0.985) and (h) 16.4% (0.399). The global peak purity for phenyl, cyano, C18-HL and C18-AQ is: 0.558, 0.001, 0.363 and 0.393, respectively. The column length is 10 cm for phenyl, C18-HL and C18-AQ, and 11 cm for cyano. The compounds that are best resolved with each complementary mobile phase are indicated. See Section 4.3 for compound identity.

The maximal resolution and optimal mobile phase compositions were: phenyl/cyano (0.656 and 12.1%), phenyl/C18-HL (0.600 and 11.1%), phenyl/C18-AQ (0.558 and 11.9%), cyano/C18-HL (0.491 and 10.0%), cyano/C18-AQ (0.375 and 13.4%), and C18-HL/C18-AQ (0.512 and 13.3%). Interestingly, the best combination was formed by the columns with the best (phenyl) and worst (cyano) performance (see Tables 4.1 and 4.2). The global resolution of this pair of columns (P = 0.656) improves the results obtained with the best single column (P = 0.457 for phenyl), but does not still yield enough resolution (Fig. 4.5a and b).

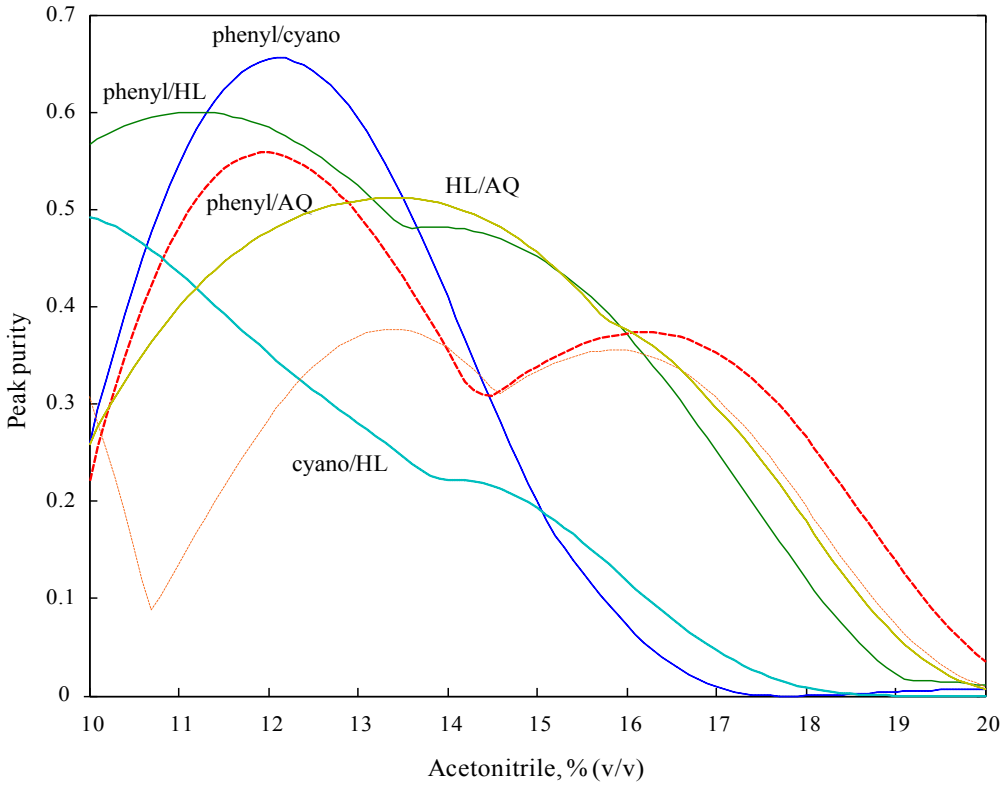

Figure 4.4. Resolution capability for parallel columns at varying mobile phase composition. The combined columns are indicated.

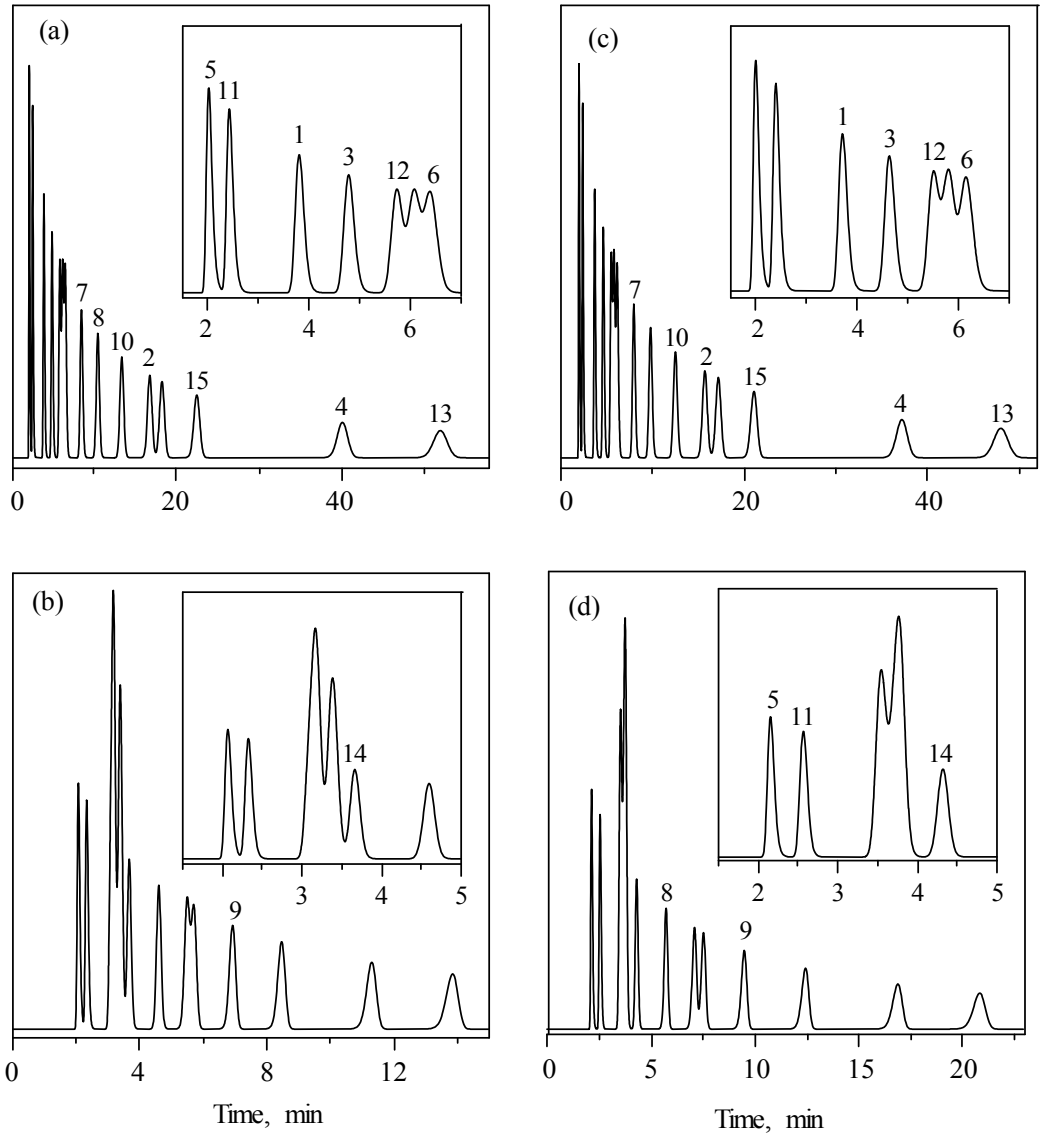

Figure 4.5. Chromatograms for the optimal separation conditions using two parallel columns (10 cm phenyl and 11 cm cyano) and: (a,b) the same mobile phase composition for both columns (P = 0.656), and (c,d) an optimised composition for each column (P = 0.691). Acetonitrile contents: (a,b) 12.1%, (c) 12.5% and (d) 10.0%. The compounds that are best resolved in each chromatogram are indicated. See Section 4.3 for compound identity.

4.4.3.2. Two columns and an optimised mobile phase for each column

Table 4.3 shows the limiting purities for the combination of two columns used in parallel. The possibilities of resolution of the approach increase remarkably with respect to the single columns, reaching for the best combination (phenyl/cyano) high resolution (P_{lim} = 0.938). As commented, in order to get such a value, each compound in the sample may require different conditions. In the previous section, we have seen that the optimal resolution using a common mobile phase for both columns is far from this value (P_{opt} = 0.656). This means that only 69.9% ((0.656/0.938)×100) of the system capability was reached.

In order to extend the possibilities of resolution, a different mobile phase was optimised for each column (Fig. 4.1d). The maximal global resolution and optimal mobile phase composition for each column were: phenyl/cyano (0.691, 12.5%/10%), phenyl/C18-HL (0.633, 10.4%/12.9%), phenyl/C18-AQ (0.585, 10.0%/15.9%), cyano/C18-HL (0.518, 10.0%/11.5%), cyano/C18-AQ (0.588, 10.0%/15.9%), and C18-HL/C18-AQ (0.512, 13.3%/13.5%) (see the optimal elementary, partial and global resolutions in Table 4.4, and the chromatograms for the best combination, phenyl/cyano, in Fig. 4.5c and d). The improvement with respect to the use of the same mobile phase for both columns is not significant, being the resolution values still far from the corresponding limiting ones (Table 4.3). Therefore, for the case of study, parallel columns with one experimental factor in the mobile phase were not powerful enough to get acceptable resolution for the studied sample.

Table 4.3. Limiting peak purities for the single columns and for the combination of two parallel columns at varying column length and mobile phase composition.

Compound	Phenyl	Cyano	Phe-CN	Phenyl	HL	Phe-HL	Phenyl	AQ	Phe-AQ
Sulphacetamide	1.0000	0.8423	1.0000	1.0000	0.9875	1.0000	1.0000	1.0000	1.0000
Sulphachloropyridazine	0.9989	0.9999	0.9999	0.9989	0.9909	0.9989	0.9989	0.9999	0.9999
Sulphadiazine	1.0000	0.0955	1.0000	1.0000	0.9872	1.0000	1.0000	1.0000	1.0000
Sulphadimethoxine	1.0000	1.0000	1.0000	1.0000	0.9980	1.0000	1.0000	1.0000	1.0000
Sulphaguanidine	0.9979	1.0000	1.0000	0.9979	0.9654	0.9979	0.9979	0.9967	0.9979
Sulphamerazine	0.9710	0.1452	0.9710	0.9710	0.9106	0.9710	0.9710	0.9932	0.9932
Sulphamethazine	1.0000	0.2246	1.0000	1.0000	0.9975	1.0000	1.0000	1.0000	1.0000
Sulphamethizole	1.0000	1.0000	1.0000	1.0000	0.9972	1.0000	1.0000	1.0000	1.0000
Sulphamethoxazole	0.9986	1.0000	1.0000	0.9986	1.0000	1.0000	0.9986	1.0000	1.0000
Sulphamonomethoxine	1.0000	0.9998	1.0000	1.0000	0.9910	1.0000	1.0000	1.0000	1.0000
Sulphanilamide	0.9976	0.9999	0.9999	0.9976	0.9651	0.9976	0.9976	0.9964	0.9976
Sulphapyridine	0.9664	0.3334	0.9664	0.9664	0.7022	0.9664	0.9664	0.6901	0.9664
Sulphaquinoxaline	1.0000	1.0000	1.0000	1.0000	0.9977	1.0000	1.0000	1.0000	1.0000
Sulphathiazole	0.6737	0.9997	0.9997	0.6737	0.7647	0.7647	0.6737	0.6152	0.6737
Sulphisoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Limiting global purity	0.628	0.001	0.938	0.628	0.432	0.714	0.628	0.419	0.644

Table 4.3 (continued).

Compound	Cyano	HL	CN-HL	Cyano	AQ	CN-AQ	HL	AQ	HL-AQ
Sulphacetamide	0.8423	0.9875	0.9875	0.8423	1.0000	1.0000	0.9875	1.0000	1.0000
Sulphachloropyridazine	0.9999	0.9909	0.9999	0.9999	0.9999	0.9999	0.9909	0.9999	0.9999
Sulphadiazine	0.0955	0.9872	0.9872	0.0955	1.0000	1.0000	0.9872	1.0000	1.0000
Sulphadimethoxine	1.0000	0.9980	1.0000	1.0000	1.0000	1.0000	0.9980	1.0000	1.0000
Sulphaguanidine	1.0000	0.9654	1.0000	1.0000	0.9967	1.0000	0.9654	0.9967	0.9967
Sulphamerazine	0.1452	0.9106	0.9106	0.1452	0.9932	0.9932	0.9106	0.9932	0.9932
Sulphamethazine	0.2246	0.9975	0.9975	0.2246	1.0000	1.0000	0.9975	1.0000	1.0000
Sulphamethizole	1.0000	0.9972	1.0000	1.0000	1.0000	1.0000	0.9972	1.0000	1.0000
Sulphamethoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphamonomethoxine	0.9998	0.9910	0.9998	0.9998	1.0000	1.0000	0.9910	1.0000	1.0000
Sulphanilamide	0.9999	0.9651	0.9999	0.9999	0.9964	0.9999	0.9651	0.9964	0.9964
Sulphapyridine	0.3334	0.7022	0.7022	0.3334	0.6901	0.6901	0.7022	0.6901	0.7022
Sulphaquinoxaline	1.0000	0.9977	1.0000	1.0000	1.0000	1.0000	0.9977	1.0000	1.0000
Sulphathiazole	0.9997	0.7647	0.9997	0.9997	0.6152	0.9997	0.7647	0.6152	0.7647
Sulphisoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Limiting global purity	0.001	0.432	0.621	0.001	0.419	0.685	0.432	0.419	0.530

Table 4.4. Optimal peak purities for the parallel columns, using an optimised mobile phase composition for each column.

Compound	Phenyl	Cyano	Phenyl	C18-HL	Phenyl	C18-AQ	Cyano	C18-HL	Cyano	C18-AQ	C18-HL	C18-AQ
Sulphacetamide	1.0000	0.0859	1.0000	0.9386	1.0000	0.9895	0.0859	0.9692	0.0859	0.9895	0.9269	0.9991
Sulphachloropyridazine	0.9896	0.9635	0.9566	0.6423	0.9379	0.9993	0.9635	0.2248	0.9635	0.9993	0.7220	0.9949
Sulphadiazine	0.9999	0.0955	0.9999	0.9265	1.0000	0.9563	0.0955	0.9677	0.0955	0.9563	0.9077	0.9984
Sulphadimethoxine	1.0000	1.0000	1.0000	0.9909	1.0000	0.9999	1.0000	0.9958	1.0000	0.9999	0.9886	1.0000
Sulphaguanidine	0.9954	1.0000	0.9976	0.9379	0.9979	0.9885	1.0000	0.9523	1.0000	0.9885	0.9332	0.9928
Sulphamerazine	0.8510	0.0000	0.4285	0.9103	0.2787	0.9881	0.0000	0.9093	0.0000	0.9881	0.9097	0.9921
Sulphamethazine	1.0000	0.0000	1.0000	0.9621	1.0000	0.9894	0.0000	0.9882	0.0000	0.9894	0.9491	0.9995
Sulphamethizole	0.9999	1.0000	1.0000	0.9617	1.0000	0.9892	1.0000	0.9879	1.0000	0.9892	0.9489	0.9993
Sulphamethoxazole	0.9896	1.0000	0.9569	1.0000	0.9382	0.9999	1.0000	1.0000	1.0000	0.9999	0.9999	1.0000
Sulphamonomethoxine	1.0000	0.9633	1.0000	0.6422	1.0000	0.9996	0.9633	0.2247	0.9633	0.9996	0.7218	0.9951
Sulphanilamide	0.9951	0.9999	0.9973	0.9378	0.9976	0.9883	0.9999	0.9523	0.9999	0.9883	0.9332	0.9925
Sulphapyridine	0.8210	0.3334	0.9552	0.6913	0.9664	0.6368	0.3334	0.6622	0.3334	0.6368	0.6959	0.4813
Sulphaquinoxaline	1.0000	1.0000	1.0000	0.9907	1.0000	0.9998	1.0000	0.9956	1.0000	0.9998	0.9884	0.9999
Sulphathiazole	0.6737	0.9997	0.3847	0.7647	0.2479	0.6152	0.9997	0.7465	0.9997	0.6152	0.7628	0.4871
Sulphisoxazole	1.0000	1.0000	1.0000	0.9999	1.0000	1.0000	1.0000	0.9999	1.0000	1.0000	0.9999	1.0000
Optimal partial purity	0.691	0.999	0.909	0.701	0.962	0.608	0.928	0.565	0.999	0.588	0.538	0.964
Optimal global purity	0.691		0.638		0.585		0.524		0.588		0.512	
Acetonitrile (%, v/v)	12.5	10.0	10.4	12.9	10.0	15.9	10.0	11.6	10.0	15.9	13.3	13.5

4.4.4. Separation performance of serial columns

The limiting peak purities in Tables 4.1 and 4.3 constitute a boundary that cannot be exceeded using single and parallel columns, respectively. In this work, we show that it is possible to go beyond by simply connecting the columns in series, even when the resolution expectancies for each column are very deficient. A serial combination of columns behaves like a totally new column with new selectivity, and therefore, different limitations. However, it should be noted that the serial coupling of two or more columns implies several restrictions related to the maximal pressure and analysis time, which increases with the column length. The restrictions in this work were: total pressure below 275 bar and analysis time below 50 min. This means that there is a limitation in the total length of the coupled columns. For this reason, it was not possible to combine the columns at their maximal available lengths.

We initially considered the coupling of only two columns as in the previous section, which gives rise to six pairs of coupled columns. Fig. 4.6 illustrates the effect on the resolution of the nature and length of the column combination and mobile phase composition, as contour maps. In this study, for convenience, the length of the combined (hybrid) column was fixed at 10 cm, so that when the length of one column in the combination increased (x in increments of 1 cm), the length of the other column decreased as $(10 - x)$ cm. Even with this limitation, the pressure and analysis time constraints were exceeded for some experimental conditions (the white regions in the diagrams).

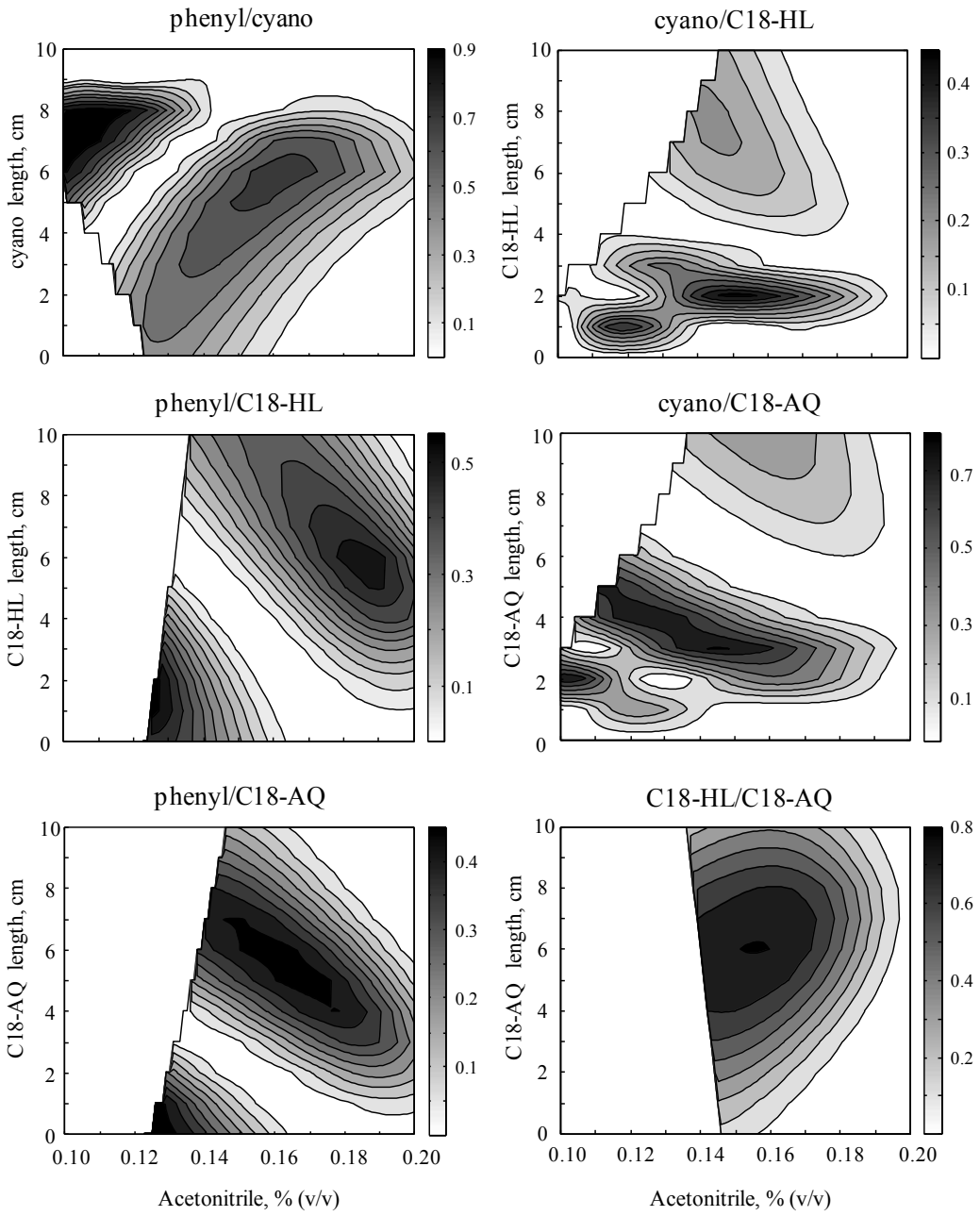

Figure 4.6. Resolution capability depicted as contour maps for serial columns at variable column length and mobile phase composition. The total length was 10 cm (see text for details).

Table 4.5. Limiting peak purities for the serially-coupled columns, considering both column length and mobile phase composition. [a]

Compound	Two columns						Four columns
	Phe-CN	Phe-HL	Phe-AQ	CN-HL	CN-AQ	HL-AQ	Phe-CN-HL-AQ
Sulphacetamide	1.0000	1.0000	1.0000	0.9962	1.0000	1.0000	1.0000
Sulphachloropyridazine	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphadiazine	1.0000	1.0000	1.0000	0.9951	1.0000	1.0000	1.0000
Sulphadimethoxine	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphaguanidine	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphamerazine	0.9994	0.9993	1.0000	0.9949	0.9981	0.9992	1.0000
Sulphamethazine	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphamethizole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphamethoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphamonomethoxine	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphanilamide	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphapyridine	0.9999	0.9713	0.9713	0.9970	1.0000	0.9648	1.0000
Sulphaquinoxaline	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphathiazole	0.9999	0.8030	0.9168	0.9999	0.9999	0.9484	0.9999
Sulphisoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Limiting global purity	0.999	0.779	0.890	0.983	0.998	0.914	1.000

[a] Phe = phenyl, CN = cyano, HL = C18-HL, and AQ = C18-AQ

As can be seen, the resolution is highly affected by both the nature and length of each combined column. The contour maps clearly illustrate that, in all situations, maximal resolution is achieved with hybrid columns (i.e., intermediate situations in the diagrams), so it is confirmed that these are as a sort of new columns with, in some instances, extraordinarily better performance. It should be noted that the surfaces in the maps are not parallel to the axes, indicating that there is an interaction between the solvent content and the column length, which affects peak resolution.

The contour maps illustrate also the importance of the selection of the mobile phase to optimise the coupled columns, which was discussed in previous work [8,9]. The optimal values of length and mobile phase composition for the coupled columns were (optimal resolutions, P_{opt}, are given in parenthesis): 3 cm phenyl + 7 cm cyano and 10.0% acetonitrile (0.990), 8 cm phenyl + 2 cm C18-HL and 12.6% (0.578), 9 cm phenyl + 1 cm C18-AQ and 12.6% (0.493), 8 cm cyano + 2 cm C18-HL and 15.1% (0.469), 7 cm cyano + 3 cm C18-AQ and 14.6% (0.807), and 4 cm C18-HL + 6 cm C18-AQ and 15.5% (0.805). Therefore, the best performance was found by coupling the phenyl and cyano columns, as was also the case for the parallel columns. However, the increase in resolution with the serial columns is more outstanding.

We next considered the serial coupling of two columns limiting the maximal length of the hybrid column to 15 cm, together with the above mentioned constraints on the pressure and analysis time. No other restriction was imposed on the column lengths. Table 4.5 shows the limiting peak purities for the serially-coupled columns, considering the column length and mobile phase composition. For almost all compounds and columns, limiting peak purities are now significantly higher than those reached by each individual column

(compare with the data in Table 4.1), particularly for the phenyl/cyano and cyano/C18-AQ combinations. If these values are compared with those in Table 4.3, where the limiting peak purities are given for parallel combinations of the same columns, it can be concluded that the chances of separation in most cases are remarkably larger.

Table 4.6 shows the optimal elementary and global resolutions for each pair of serial columns. The listed values correspond to the following column lengths and mobile phase compositions: 2 cm phenyl + 6 cm cyano and 10.2% acetonitrile, 4 cm phenyl + 1 cm C18-HL and 10.5%, 3 cm phenyl + 2 cm C18-AQ and 10.0%, 7 cm cyano + 2 cm C18-HL and 14.6%, 6 cm cyano + 3 cm C18-AQ and 13.7%, and 2 cm C18-HL + 3 cm C18-AQ and 16.0%. Almost full resolution was obtained with the best combination (P = 0.993), with minimal elementary resolution for sulphacetamide, sulphadiazine, sulphamerazine and sulphathiazole (p = 0.998). The separation capability of the system was thus exploited up to an impressive 99.4% level (P_{opt} = 0.993 against P_{lim} = 0.999). For the other five column combinations, it was exploited up to: 83.6% for phenyl/C18-HL, 94.5% for phenyl/C18-AQ, 49.4% for cyano/C18-HL, 88.5% for cyano/C18-AQ, and 96.0% for C18-HL/C18-AQ. The latter combination yielded an acceptable separation (P = 0.877) with minimal resolution for sulphapyridine (p = 0.952) and sulphathiazole (p = 0.948). We should remind that with the parallel columns, the optimal resolutions were far smaller from the corresponding limiting values (compare Tables 4.3 and 4.4).

Table 4.6. Optimal peak purities for the serially-coupled columns, considering the column length and mobile phase composition.[a]

Compound	Phe-CN	Phe-HL	Phe-AQ	CN-HL	CN-AQ	HL-AQ
Sulphacetamide	0.9983	1.0000	1.0000	0.9956	1.0000	0.9987
Sulphachloropyridazine	1.0000	1.0000	1.0000	0.9746	0.9988	0.9998
Sulphadiazine	0.9980	1.0000	1.0000	0.9917	0.9999	0.9940
Sulphadimethoxine	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphaguanidine	1.0000	0.9995	0.9995	0.9987	0.9997	0.9950
Sulphamerazine	0.9984	0.9236	0.9688	**0.8903**	0.9479	0.9991
Sulphamethazine	0.9998	1.0000	1.0000	1.0000	1.0000	0.9948
Sulphamethizole	1.0000	1.0000	1.0000	1.0000	1.0000	0.9945
Sulphamethoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Sulphamonomethoxine	1.0000	1.0000	1.0000	0.9749	0.9990	1.0000
Sulphanilamide	1.0000	0.9991	0.9991	0.9984	0.9993	0.9946
Sulphapyridine	0.9999	**0.8788**	0.9481	**0.8177**	0.9515	0.9523
Sulphaquinoxaline	1.0000	1.0000	1.0000	0.9999	1.0000	0.9998
Sulphathiazole	0.9983	**0.8026**	0.9168	**0.7143**	0.9000	0.9484
Sulphisoxazole	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Optimal global purity	0.993	0.651	0.841	0.486	0.809	0.877
Acetonitrile (%, v/v)	10.2	10.5	10.0	14.6	13.7	16.0

[a] Critical compounds are marked in bold.

4.4.5. Optimisation of both resolution and analysis time

So far, the optimisation of the separation conditions has been exclusively based on the maximal peak purity. However, another factor that should be taken into account is the analysis time. Pareto plots allow balancing both factors [21]. Fig. 4.7a shows the Pareto fronts for diverse situations: the use of a single column (phenyl, which is the best), or the optimised serial coupling of two to four columns (which give rise to close Pareto fronts). Fig. 4.7b shows the Pareto front in a search not aimed to a given number of coupled columns (i.e., the combination of two to four columns was allowed). Therefore, this plot can be considered as the Pareto front of the three Pareto fronts in Fig. 4.7a. As observed in Fig. 4.7b, there are several optimal solutions using two to four columns with peak purity $P \geq 0.95$, with analysis times in the 22–35 min range. The solutions with higher peak purity (and also longer analysis time) involve a combination of the four columns, whereas more economical solutions (closer to the analyst aim of sufficiently short analysis time), with also high peak purity, imply only two or three columns.

All solutions in the Pareto front involving the phenyl and cyano columns corresponded to the same lengths: 2 cm phenyl + 6 cm cyano (Fig. 4.7c). Similarly, for the alternative 2 cm C18-HL + 3 cm C18-AQ combination, there were several solutions yielding $P \approx 0.9$. Therefore, the optimal solutions only differed in the mobile phase composition.

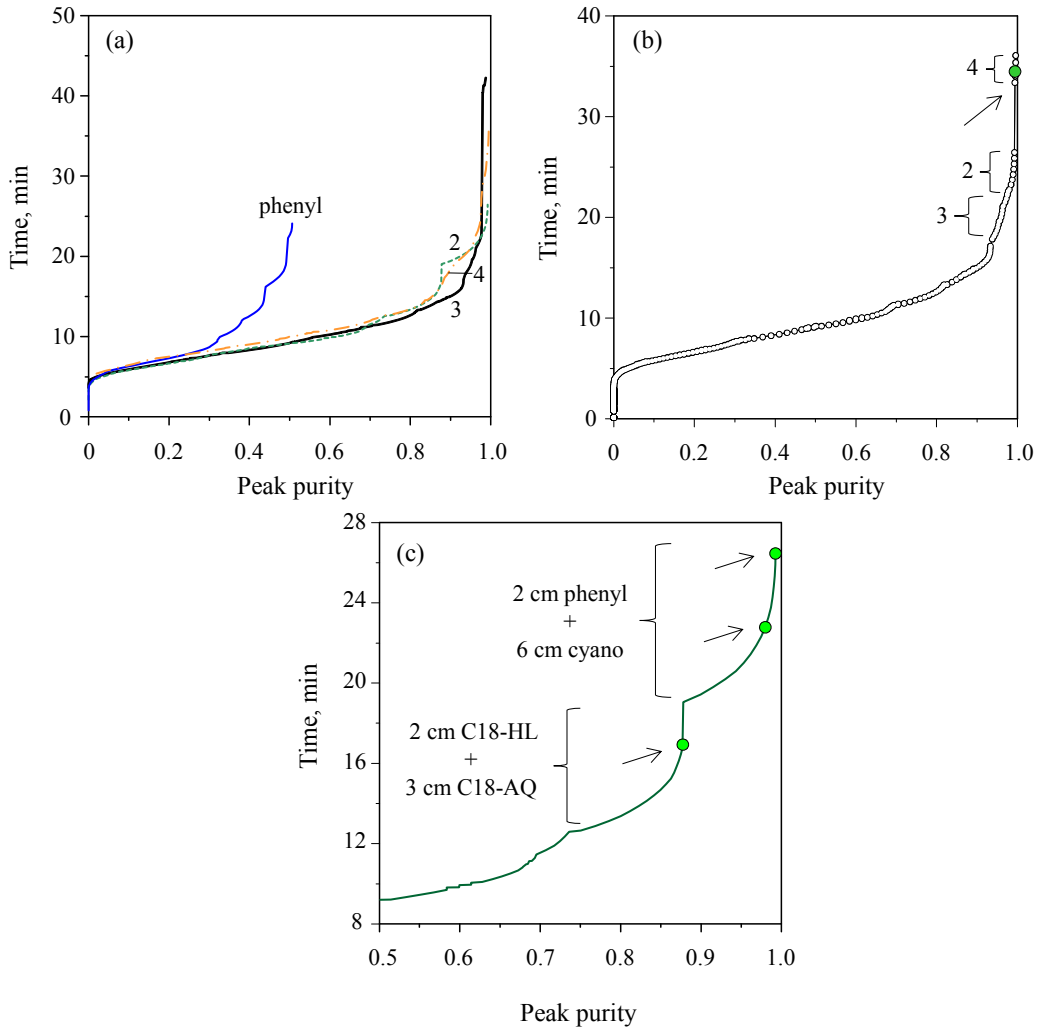

Figure 4.7. Pareto fronts indicating the optimal global peak purities versus the analysis time for the serial columns: (a) Comparison of the performance using the best single column (phenyl) and the combinations of two (- - -), three (—) and four (-.-) columns, (b) optimisation without constraining the number of columns (the regions at higher resolution correspond to the combination of four, two and three columns), and (c) optimisation constrained to two columns (the nature of the combined columns is indicated for the largest resolutions).

Fig. 4.8a and b show the chromatograms for the optimal phenyl/cyano combination using 10.2 and 10.9% acetonitrile, respectively, Fig. 4.8c shows the chromatogram for an optimal C18-HL/C18-AQ combination, and Fig. 4.8d corresponds to the optimal combination of the four columns studied in this work. As can be observed, besides the analysis time, the chromatograms show differences in resolution and elution order for sulphamerazine (compound 6), sulphapyridine (compound 12) and sulphathiazole (compound 14).

4.5. Conclusions

There are two ways to make two or more columns in mono-dimensional separations cooperate, namely parallel and serial configurations. In this work, the performance of both approaches in the separation of 15 solutes with mobile phases characterised by one experimental factor (i.e., the solvent content) was compared under isocratic elution using the same training set. In order to optimise parallel columns of different nature, a previous approach (developed to search complementary mobile phases [20]) was adapted. The performance of the different configurations was evaluated through the limiting resolutions and the percentage of separation capability of the chromatographic systems reached at the optimal conditions.

In the case of study, serial columns outperformed parallel columns. It could be said that this conclusion is not surprising. The outstanding result is that the resolution power is appreciably extended for the serial columns. In previous work, we showed that a higher diversity in the mobile phase composition (e.g., the concentration of two modifiers [22], or the solvent content and pH [23]) made the complementarity approach succeed in the full resolution of highly complex samples using a single column.

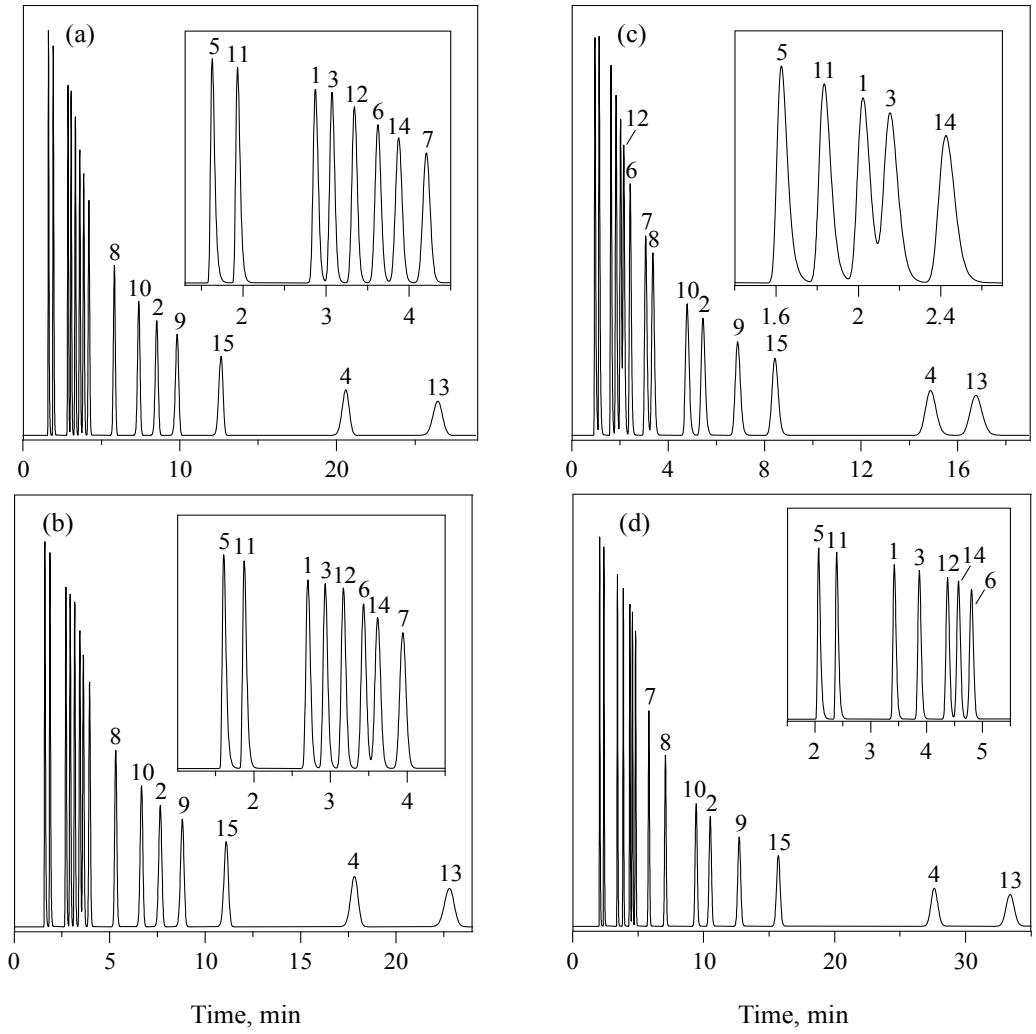

Figure 4.8. Chromatograms for the mixture of 15 sulphonamides using serial columns: (a) 2 cm phenyl + 6 cm cyano and 10.2% acetonitrile (P = 0.993), (b) 2 cm phenyl + 6 cm cyano and 10.9% acetonitrile (P = 0.980), (c) 2 cm C18-HL + 3 cm C18-AQ and 16.0% (P = 0.877), and (d) 2 cm phenyl + 6 cm cyano + 1 cm C18-HL + 2 cm C18-AQ and 13.0% acetonitrile (P = 0.994). Chromatograms (a) to (c) correspond to the conditions marked with arrows in Fig. 4.7c, and chromatogram (d) to the condition marked in Fig. 4.7b. See Section 4.3 for compound identity.

The example studied in this work makes use of only one factor in the mobile phase. In these conditions, the variations in selectivity that can be induced by the mobile phase are much smaller, and so are the chances of success. Certainly, the inclusion of a second factor in the mobile phase (in both parallel and serial columns) would increase significantly the separation power, but the price to pay would be an increase in the experimental effort.

The effect of the changes in the column length is very different in the serial and parallel approaches. With parallel columns, there is no change in selectivity, whereas the column length highly affects the selectivity in serial separations. Therefore, although the comparison of both approaches was intended under the same conditions to avoid any possible advantage, indeed the serially-coupled columns approach has an intrinsic advantage: there is an additional separation factor (the column length), which however has no consequences in the experimental effort. In practice, each serial combination of short columns of different nature and length operates as a new column, with its own selectivity. This increases enormously the wealth of columns available in a laboratory, from which we can select the best for our particular problem.

Experimentally speaking, the use of parallel columns is simpler, since it implies conventional separations without any special requirement. However two independent injections using two HPLC instruments, or two consecutive analyses with the same instrument, are needed. Thus, the working time is longer. Meanwhile, serial columns only require one chromatographic pump and performing one injection by sample.

We show that even using columns offering very poor performance when used isolatedly, their serial combination can give rise to baseline separation. Although the combinations offering the best resolution in this work involved the connection of four columns, it was possible to find excellent compromise

solutions with only two columns of different polarity, as phenyl and cyano columns, or C18-HL and C18-AQ. Interestingly, the best combination had a longer contribution of the faster column.

4.6. References

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CHAPTER 5

UPDATING CHROMATOGRAPHIC PREDICTIONS BY ACCOUNTING AGEING FOR SINGLE AND TANDEM COLUMNS

5.1. Abstract

In high-performance liquid chromatography method development, computer-assisted optimisation is of great help to expedite the search of the best separation conditions. This is especially important for complex samples with multiple peaks, but also for simple samples containing close peaks. The most efficient methodologies are based on the modelling and further prediction of the chromatographic behaviour for each compound in the sample. However, when the column suffers some ageing after the modelling process, predictions may differ significantly from the actual separation. Repeating the modelling process can be an arduous, and thus, undesirable task. This is especially troublesome when several columns are involved, as is the case of the use of columns connected in series to increase the selectivity of the separation, owing to the high experimental effort needed to re-model some or even all columns. In this work, we propose a shortcut to correct the time and peak profiles in these situations, after evaluating the effects of ageing. The application of these corrections will contribute to extend the column lifetime. In the proposed approach, the original models are corrected by introducing parameters accounting for column ageing, which are obtained using the data from a small subset of compounds from those used to model the brand-new column (before ageing). The ageing parameters are fitted from the discrepancies between the data predicted with the original retention models for the brand-new column and the experimental data measured for the aged column. From the gathered information, new chromatograms more in line with the aged column performance are predicted. The approach was developed and tested to predict the chromatographic behaviour of 15 sulphonamides, analysed with individual and serially-coupled columns, using isocratic and gradient elution. The agreement between the predictions and experimental data in the aged columns was excellent.

5.2. Introduction

The possibility of achieving high resolution in high-performance liquid chromatography has experienced a great boost with the development of two- and three-dimensional separations [1–4], on the one hand, and the serial coupling of columns of several nature and length in the so-called stationary phase modulation [5–10], on the other. In both cases, the selectivity is significantly increased by the combination of two or more columns with different separation mechanisms.

Our laboratory has been involved since 2012 in the improvement of separations in reversed-phase liquid chromatography (RPLC), using serially-coupled (or tandem) columns [8,11–15]. The successful development of such column systems required solving technical problems related to the robustness of connections, reduction of extra dead volumes, or deterioration of column packings. These problems are substantially reduced by coupling the columns with zero dead volume connectors that can be attached to any standard column without apparent deterioration [8]. Exploiting appropriately the resolution possibilities offered by serially-coupled columns required also the development of tools to achieve accurate predictions of the retention and shape of chromatographic peaks, in both isocratic and gradient elution [8,11–14]. Using powerful interpretive optimisation strategies, the correct combination of columns and the best mobile phase composition or gradient program can be readily found for a given separation.

The commercialisation of short columns with multiple lengths from several manufacturers has expanded the possibilities of success of the serially-coupled columns approach, using conventional instrumentation. The idea is adapting the separation system by fine tuning the nature and length of the coupled columns. Each column combination can be considered as a new hybrid column with new

separation features, which often outperforms the results offered by each column participating in the combination.

The serially-coupled columns approach is indeed powerful, but it may require considerable investment, since multiple columns of different lengths are needed for each stationary phase. However, recently, we have demonstrated that the combination of only two columns of fixed length may be enough to resolve relatively complex samples satisfactorily, provided that an optimised gradient program is applied [15]. It should also be considered that the search of the best separation conditions with serially-coupled columns requires modelling the chromatographic behaviour of two or more stationary phases, although there is no need to develop models for each column length, since these can be derived from the models obtained for a column with a specific length taken as reference [8,14]. This may involve still considerable experimental work, although the obtained results can make up for the effort.

There is another problem to be solved. With the routine use, chromatographic columns inevitably lose some separation performance, which is translated in shifts in retention and the reduction of column efficiency (peak broadening and tailing). Several reasons explain the deterioration of columns: (i) intensive use; (ii) operation under stressing conditions (e.g., extreme pH values, or high temperatures); and (iii) backpressure exceeding the recommended operative levels for fast separations with short columns, or for coupled columns (this deterioration source can be especially significant for the first segments in the coupled systems).

As a result, the column packing may suffer gradual changes. Therefore, periodic monitoring of the decrease in separation power is required over the column lifetime [16–20]. For this purpose, variations in the plate count and dead time are good indicators on how well a column is performing. As long as

the changes are not excessive, the deteriorated column may still offer useful separations, sufficient for our analytical purposes. The extension of the column lifetime will decrease the analysis cost. However, optimal conditions for the deteriorated column(s) require re-modelling the chromatographic behaviour with new experiments, and this has to be done preferably with minimal effort.

In this work, an approach is described to predict chromatograms for single and coupled aged columns, using the models developed at the beginning of the column lifetime, modified with some parameters that account for the degradation level. The method is assayed for a set of 15 sulphonamides and three types of stationary phases (phenyl, C18 and cyano, isolated or coupled), using isocratic and gradient elution. These columns had been used for several analyses along six years, and suffered diverse degrees of loss in performance. Excellent correlations were found between the predicted and experimental chromatograms in aged conditions.

5.3. Theory

In this section, the fundamentals of the simulation tools used in this work to predict the retention and peak profiles, for the brand-new and aged columns, are described for chromatograms obtained for single and serially-coupled columns, in both isocratic and gradient elution. When the columns are affected by certain degradation, the outline requires some modifications which will be explained and justified in Results and discussion (Section 5.4.).

5.3.1. Prediction of retention times in isocratic elution

When a solute *j* is eluted through a column *i*, the time to reach the peak maximum (retention time. $t_{R,ij}$) is a linear combination of the time needed to go through the external tubing and connections (i.e., the extra-column contribution, t_{ext}), and the time to cross the column ($t_{int,ij}$):

$$t_{R,ij} = t_{ext} + t_{int,ij} \tag{5.1}$$

The retention time varies with the mobile phase composition. In isocratic elution, the dependence between retention and volume fraction of organic solvent (φ) is usually expressed as a function of the logarithm of the retention factor (k_{ij}). In this work, we used decimal logarithms:

$$\log k_{ij} = \log k_{0,ij} + c_{1,ij}\,\varphi + c_{2,ij}\,\varphi^2 \tag{5.2}$$

Eq. (5.2) has particular coefficients for each solute *j* and column *i*, where:

$$k_{ij} = \frac{t_{R,j} - t_{0,i}}{t_{0,i} - t_{ext}} \tag{5.3}$$

$t_{0,i}$ being the dead time for each column (i.e., time needed for an unretained compound to go through the column). The extra-column contribution is cancelled in the numerator of Eq. (5.3), but this contribution should be subtracted from the dead time in the denominator, particularly for serially-coupled columns. Eq. (5.2) will be altered in Results and discussion to account for column degradation.

When solute *j* is eluted isocratically through serially-coupled columns, the retention time ($t_{Rc,j}$) will be a simple combination of the extra-column contribution (which will include now the time associated to the migration

through the connections between columns), and the retention time inside each coupled column *i*, as follows:

$$t_{\mathrm{Rc},j} = t_{\mathrm{ext}} + \sum_{i=1}^{m} t_{\mathrm{int},ij} \tag{5.4}$$

The retention inside each column as a function of mobile phase composition is described by Eq. (5.2), with particular coefficients for each solute and column.

5.3.2. Prediction of retention times in gradient elution

In gradient elution, the prediction of the retention is far more complex, since k_{ij} changes as the organic solvent content is varied along the column. Therefore, to get information about the retention time under gradient elution for a particular column *i* ($t_{\mathrm{g},ij}$), an integral equation should be solved:

$$t_{0,i} = \int_{0}^{t_{\mathrm{g},ij}-t_{0,i}} \frac{dt}{k_{ij}(\varphi(t))} \tag{5.5}$$

where $\varphi(t)$ is the gradient program, and $k_{ij}(\varphi)$ is described by a retention model, such as Eq. (5.2). In this case, Eq. (5.5) should be solved numerically, splitting the equation in a series of *n* integrals:

$$t_{0,i} = \int_{0}^{t_{\mathrm{d}}} \frac{dt}{k_{ij}(\varphi_0)} + \int_{t_{\mathrm{d}}}^{t_1-t_{01}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_1-t_{01}}^{t_2-t_{02}} \frac{dt}{k_{ij}(\varphi(t))} + \ldots + \int_{t_{n-1}-t_{0n-1}}^{t_n-t_{0n}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_n-t_{0n}}^{t_{\mathrm{g},ij}-t_{0,i}} \frac{dt}{k_{ij}(\varphi(t))} \tag{5.6}$$

Each term in Eq. (5.6) corresponds to a column fraction through which solute *j* migrates during a given time interval of the gradient program. The initial time fraction corresponds to the dwell time (t_{d}), during which the solute elutes isocratically until the gradient front reaches the solute. The other terms take into account the progressively longer delay needed for the gradient front to

reach the solute, as it migrates along the column. This internal delay is considered globally by the difference $t_n - t_{0n}$ in the upper integral limit in Eq. (5.6). If the gradient is discretised in a number of isocratic steps, the following approximation can be used instead of Eq. (5.6):

$$\begin{aligned} t_{0,i} &\approx \frac{t_\mathrm{d}}{k_{ij}(\varphi(t_0))} + \frac{t_1 - t_\mathrm{d}}{k_{ij}(\varphi(t_1 - \tau_1))} + \frac{t_2 - t_1}{k_{ij}(\varphi(t_2 - \tau_2))} + \ldots + \frac{t_{\mathrm{g},ij} - t_n}{k_{ij}(\varphi(t_n - \tau_n))} = \\ &= \frac{t_\mathrm{d}}{k_{ij}(\varphi(t_0))} + \frac{\Delta t}{k_{ij}(\varphi(t_1 - \tau_1))} + \frac{\Delta t}{k_{ij}(\varphi(t_2 - \tau_2))} + \ldots + \frac{\Delta t}{k_{ij}(\varphi(t_n - \tau_n))} \end{aligned} \tag{5.7}$$

where the terms in the denominators refer to the correct solvent composition at the solute location when the solute is migrating along the column; the τ parameters consider the progressive delays. For this work, an accuracy of Δt = 0.01 min was adopted. The advantage of using the discretised expression is that it is applicable to any retention model and gradient profile, even those without analytical solution.

The time at which the solute reaches the column outlet (i.e., the gradient retention time $t_{\mathrm{g},ij}$) corresponds to the time at which the cumulative summation up to that term matches the column dead time. For a better accuracy, changes in the marker migration with the solvent content, due to changes in viscosity, have been also considered to find the term where the cumulative sum matches $t_{0,i}$.

Eqs. (5.6) and (5.7) can be used either to predict the retention for a single column, or for the first column of a set of serially-coupled columns. For subsequent columns (i = 2, 3, …), the $t_{\mathrm{g},ij}$ value associated to the migration through column i was calculated as

$$t_{0,i} = \int_{t_{\mathrm{p},ij}}^{t_1 - t_{01}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_1 - t_{01}}^{t_2 - t_{02}} \frac{dt}{k_{ij}(\varphi(t))} + \ldots + \int_{t_{n-1} - t_{0n-1}}^{t_n - t_{0n}} \frac{dt}{k_{ij}(\varphi(t))} + \int_{t_n - t_{0n}}^{t_{\mathrm{g},ij} - t_{0,i}} \frac{dt}{k_{ij}(\varphi(t))} \tag{5.8}$$

where $t_{p,ij}$ is the gradient time when the solute reaches the inlet of the column, after crossing all previous columns. When the solute enters a new column, the corresponding retention model ($k_{ij}(\varphi)$) is adopted, and Eq. (5.8) is solved by searching the integral term at which the cumulative summation matches the dead time for that column ($t_{0,i}$).

The time $t_{gc,j}$ at which a solute j elutes under gradient elution with serially-coupled columns is again described as a linear combination of the extra-column contributions and the retention associated to each connected column ($t_{g,int,ij}$):

$$t_{gc,j} = t_{ext} + \sum_{i=1}^{m} t_{g,int,ij} \tag{5.9}$$

where m is the number of connected columns. Since the involved retention models are specific for each solute and column, the change of retention model (Eq. (5.2)), when a solute is transferred to the next column, should be considered. This will happen at a particular time for each solute. Further details for the mathematical treatment are given in previous reports [12–14].

5.3.3. Prediction of peak profiles

In this work, the peak profiles were predicted based on a linearly-modified Gaussian model [21], which allows realistic simulations of chromatographic peaks. To avoid baseline increments outside the peak region, exponential decays substituted the signal at both peak sides below 10% peak height [22]. The algorithm used to build the chromatographic peaks requires knowledge of the peak width and asymmetry (the latter measured as the ratio between the left, B_{ij}, and right, A_{ij}, half-widths, which are the time dimensions of the preceding and following part of the peak at each side of the peak apex). Since chromatographic signals lack of defined limits, peak width measurements have

to be carried out at a certain peak height. This can be between the inflection points, at a given height ratio, or between tangent lines at both peak decays intersecting the base line. In this work, we used the 10% peak height ratio criterion to make half-widths measurements sensitive to peak asymmetry, avoiding at the same time an undesirable influence of the baseline noise.

For single columns in isocratic elution, the half-widths were modelled as follows to predict the peak shape at varying mobile phase composition [23]:

$$A_{ij} = a_{0,i} + a_{1,i}\, t_{\mathrm{R},ij\varphi} + a_{2,i}\, t^2_{\mathrm{R},ij\varphi} \tag{5.10}$$

$$B_{ij} = b_{0,i} + b_{1,i}\, t_{\mathrm{R},ij\varphi} + b_{2,i}\, t^2_{\mathrm{R},ij\varphi} \tag{5.11}$$

with particular coefficients for each column *i*. Eqs. (5.10) and (5.11) include the extra-column contributions. In contrast to Eq. (5.2) (which should be fitted for each solute and column), these equations can be fitted either for each solute eluted at several φ values, or if they share similar kinetics (as is the case of the set of sulphonamides), for several solutes eluted at one or more φ values. Finally, for each column and solute, the asymmetry factor, f_{ij}, and width, w_{ij}, are calculated as:

$$f_{ij} = \frac{B_{ij}}{A_{ij}} \tag{5.12}$$

$$w_{ij} = A_{ij} + B_{ij} \tag{5.13}$$

In gradient elution, the Jandera's approximation was used to obtain the peak width [24]. This indicates that for a solute leaving the column under gradient elution, the peak width will be that one the solute would exhibit if it migrated isocratically at the solvent composition, when it reached the column outlet.

With serially-coupled columns, it should be considered that the peak profile in a given column is affected by the transit of the solute through previous columns. For each column, the half-widths can be predicted from Eqs. (5.10) and (5.11). Based on the property of additivity of variances (although here it is not strictly valid), the peak variances $A^2_{\mathrm{c},j}$ and $B^2_{\mathrm{c},j}$, for the column combination can be approximated as [11]:

$$A^2_{\mathrm{c},j} = A^2_{\mathrm{ext}} + \sum_{i=1}^{n} A^2_{\mathrm{int},ij} \tag{5.14}$$

$$B^2_{\mathrm{c},j} = B^2_{\mathrm{ext}} + \sum_{i=1}^{n} B^2_{\mathrm{int},ij} \tag{5.15}$$

where A^2_{ext} and B^2_{ext}, and $A^2_{\mathrm{int},ij}$ and $B^2_{\mathrm{int},ij}$ are the extra- and intra-column contributions, respectively. The values of $A^2_{\mathrm{c},j}$ and $B^2_{\mathrm{c},j}$ are needed to calculate the asymmetry and width of the column combination as follows:

$$f_{\mathrm{c},j} = \left(\frac{B^2_{\mathrm{c},j}}{A^2_{\mathrm{c},j}} \right)^{1/2} = \left(\frac{B^2_{\mathrm{ext}} + \sum_{i=1}^{n} B^2_{\mathrm{int},ij}}{A^2_{\mathrm{ext}} + \sum_{i=1}^{n} A^2_{\mathrm{int},ij}} \right)^{1/2} \tag{5.16}$$

$$w^2_{\mathrm{c},j} = (A_{\mathrm{c},j} + B_{\mathrm{c},j})^2 = (A_{\mathrm{ext}} + B_{\mathrm{ext}})^2 + \sum_{i=1}^{n} (A_{\mathrm{int},ij} + B_{\mathrm{int},ij})^2 \tag{5.17}$$

Eqs. (5.10) and (5.11) are fitted with data corresponding to a certain column length (the reference column). To correct the variances to the desired column length for each coupled column, the extra-column contributions should be first subtracted. Further details for the mathematical treatment are given in previous reports [12–14].

The required modifications needed to correct the predictions for accounting column degradation are explained in Results and discussion.

5.4. Experimental

5.4.1. Reagents and columns

A set of 15 sulphonamides, whose separation was optimised in 2012, was considered: (1) sulphacetamide, (2) sulphachloropyridazine, (3) sulphadiazine, (4) sulphadimethoxine, (5) sulphaguanidine, (6) sulphamerazine, (7) sulphamethazine, (8) sulphamethizole, (9) sulphamethoxazole, (10) sulphamonomethoxine, (11) sulphanilamide, (12) sulphapyridine, (13) sulphaquinoxaline, (14) sulphathiazole, and (15) sulphisoxazole.

The injected concentration for all sulphonamides was ca. 20 μg/mL. The corrections proposed in this work are aimed to introduce ageing in the prediction of the chromatographic behaviour. In such studies, the best separation conditions are searched assuming that they will be appropriate for a range of analyte concentrations. Otherwise, the predicted separation conditions would be useless for an analysis, since each sample will have a characteristic peak height pattern. For this reason, it is a usual practice to optimise the conditions using normalised peaks.

The columns considered in this work were: phenyl (two 5 cm long), C18 (two 2 cm and one 5 cm), and cyano (3.5 and 7.5 cm), which were supplied by Advanced Chromatography Technologies Ltd. (ACE, Aberdeen, Scotland, UK). The retention behaviour of the 15 sulphonamides with these columns was carefully modelled in 2012, carrying out isocratic runs with acetonitrile-water mobile phases, buffered at pH 3.5. Along the period 2012–2017, these columns

were intensively used to analyse a variety of compounds. As a result, they suffered performance degradation in different extent. This degradation happened unintentionally in spite of careful use, and the 15 sulphonamides were the first solutes analysed when the columns were brand-new.

5.4.2. Apparatus and software

The studies carried out in 2012 in isocratic and gradient elution involved an HPLC instrument from Agilent (Waldbronn, Germany), equipped with an isocratic pump (HP 1200, Model G1310A), a quaternary pump (HP 1200, Model G1310A), an autosampler with 2 mL vials (HP 1100, Model G1313A), a thermostated column compartment (HP 1100, Model G1316A), and a variable wavelength UV-visible detector (HP 1200, Model G1315 C).

The studies carried out in 2017 involved a different HPLC instrument, also from Agilent, consisting of a binary pump (HP 1200, Model G1312B), an autosampler (HP1260, Model G1329B) with 2 mL vials, a thermostated column compartment (HP1260, Model G1316A) and a diode-array UV-visible detector (HP 1290, Model G7117B).

For both studies, temperature was fixed at 25 °C, and absorbance was monitored at 254 nm. The injection volume was always 20 µL. The flow rate was programmed at 1 mL/min. The dead time for all columns was determined by injection of 20 µg/mL of KBr from Acros Organics (Fair Lawn, NJ, USA) and elution with 10 and 15% (v/v) acetonitrile. A Crison pH meter (Model MicropH 2002, Barcelona), and a combined glass electrode from Orion (Model 8102, Barcelona), containing Ag/AgCl electrodes with a salt bridge filled with a 3.0 M KCl solution, were used to measure the pH of eluents fixed using dihydrogen phosphate as buffer.

The mathematical treatment was performed using MATLAB 2016b (The MathWorks Inc., Natick, MA, USA) routines. The chromatographic peaks were processed with the MICHROM software [25], to obtain the peak parameters (retention time and peak half-widths).

5.5. Results and discussion

5.5.1. Loss of column performance

Along 2012–2017, we developed an approach to carry out the interpretive optimisation of the separation conditions using serially-coupled columns in RPLC. The aim was predicting not only the best combination of columns, but also the best mobile phase composition in isocratic elution, or the best program (linear or multi-linear) in gradient elution. The most time-consuming and expensive step in the optimisation of separations, with either single or serially-coupled columns, is the modelling of the chromatographic behaviour. In this step, using either isocratic or gradient elution, individual standards (or mixtures of standards) are injected under different experimental conditions, and the retention and peak half-widths (or other related properties) are measured for the peaks of all compounds. With this information, prediction models such as Eqs. (5.2), (5.10) and (5.11) (or other similar) are fitted. These models are further used to predict the optimal separation conditions.

In 2012, we modelled the chromatographic behaviour of 15 sulphonamides, using the data from four to six isocratic mobile phases (depending on solute retention), which ranged from 10 to 20% (v/v) acetonitrile. Models for several columns containing diverse stationary phases were obtained. The agreement between the experimental and predicted chromatograms was excellent in all cases with both single and serially-coupled columns [8,11–13]. Along the

following years, the same column set was used for analysing samples containing other compounds, such as diuretics and amino acids. Recently, we have been again interested in analysing sulphonamides, and we decided to use the same columns taking advantage of the models fitted in 2012. However, the chromatograms were not as expected: the peaks were broader and the retention of sulphonamides was shifted to shorter times of variable magnitude for each solute (see Figs. 5.1 and 5.2). Consequently, the experimental conditions yielding full separation in 2012 were not optimal anymore. Favourable resolution might still be possible, but it would require repeating the modelling step, which was costly and unattractive under an experimental standpoint. On the other hand, if the columns were discarded, their useful life would be shortened. We thought then that modifying the retention models without repeating the whole experimental work by evaluating the column degradation with few experiments could be the solution.

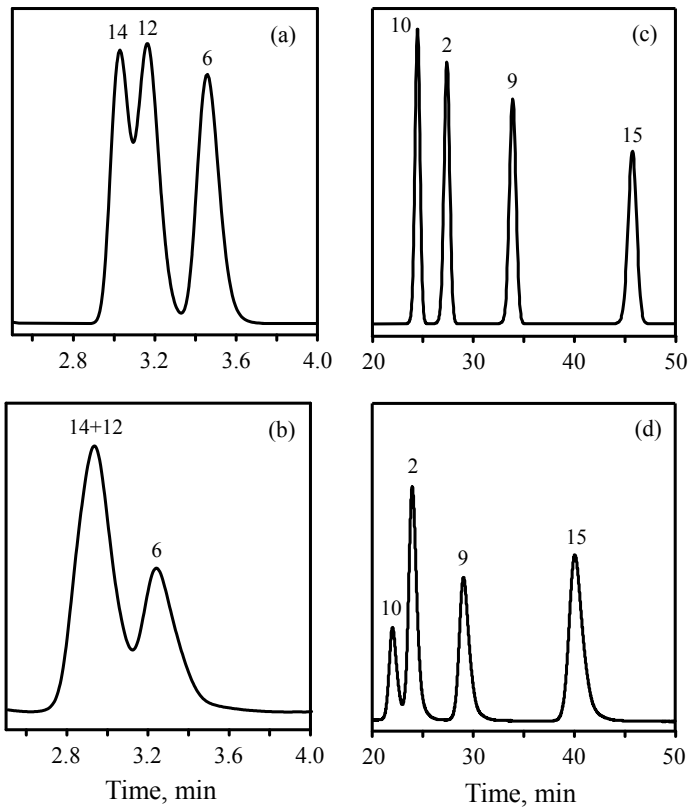

Figure 5.1. Chromatograms obtained for a mixture of sulphonamides in 2012 (a,c), and 2017 (b,d). (a,b) Elution through a single 5 cm C18 column using an acetonitrile gradient run from 10 to 20% in 60 min, and (c,d) isocratic elution with 10% acetonitrile through a serially-coupled column consisting of 5 cm C18 + 5 cm phenyl + 7.5 cm cyano. Peak identities are given in Section 5.4.1.

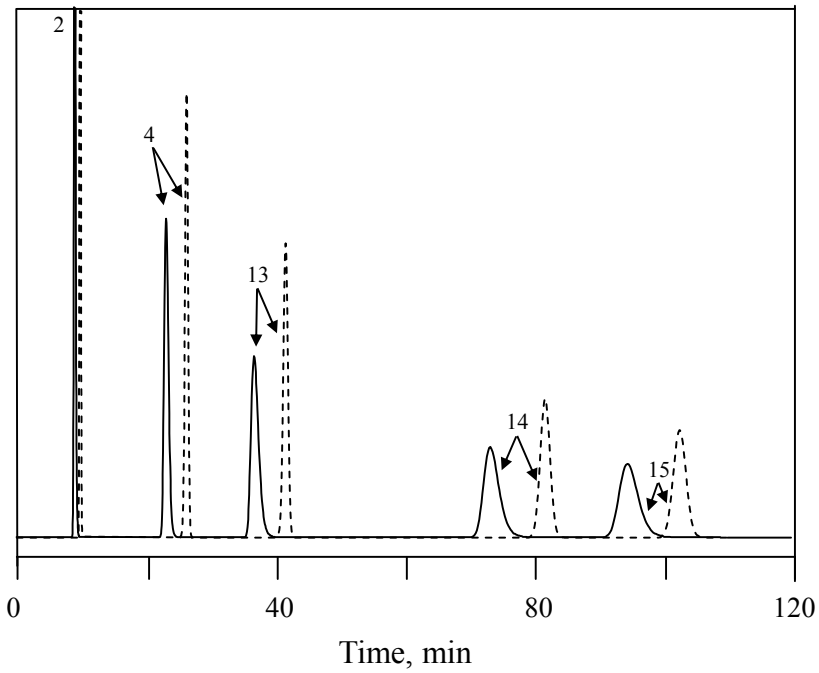

Figure 5.2. Effect of ageing in a serially-coupled column consisting of 5 cm C18 + 5 cm phenyl + 7.5 cm cyano, obtained in 2012 (dashed line) and 2017 (solid line), using isocratic elution with 10% acetonitrile. Peak identities are given in Section 5.4.1.

5.5.2. Study of the performance loss

5.5.2.1. Degradation level of the aged columns for the set of sulphonamides

In a previous report [15], we checked that the combination of only two or three columns of arbitrary intermediate length could yield good separations, provided appropriate elution conditions were optimised. Thus, for this work, we selected the best combination using only three columns: 5 cm phenyl, 5 cm C18, and 7.5 cm cyano. Fig. 5.3 depicts the half-widths of the chromatographic peaks versus the respective retention times for the three columns using all

assayed isocratic mobile phases, as measured in 2012 (Fig. 5.3a to c) and 2017 (Fig. 5.3d to f). The ageing in 2017 is evidenced by broader signals and peak shifts to shorter times. The chromatographic peaks in both 2012 and 2017 were fronting for the phenyl and cyano columns (especially for the latter). The phenyl column suffered the smallest degradation, while the cyano column showed an important reduction in performance.

In order to monitor the current state of the columns, five representative sulphonamides (sulphachloropyridazine, sulphadimethoxine, sulphaquinoxaline, sulphathiazole and sulphisoxazole) were selected. This selection was not random: these sulphonamides were the most hydrophobic, the most hydrophilic, and the closest to the median, first and third quartile, according to their polarity, of the set of 15.

Fig. 5.4a to c shows the retention factors for the subset of sulphonamides, eluted with the three columns when they were brand-new (empty circles and dashed lines), and after ageing (full circles and solid lines). The changes in retention behaviour are evident for the three columns, but more significant for the cyano column (Fig. 5.4c), with reductions in the retention below half the initial values. Interestingly, when a log transform was applied to the retention factors (Fig. 5.4d to f), almost parallel shifts of similar magnitude were observed for all sulphonamides. Mean values of $\Delta \log k$ between 2012 and 2017 were –0.0094, –0.0366, and –0.357 for the phenyl, C18 and cyano columns, respectively. The similarity in $\Delta \log k$ for sulphonamides of diverse polarity suggests that the same behaviour should be expected for the other 10 intermediate sulphonamides studied in this work.

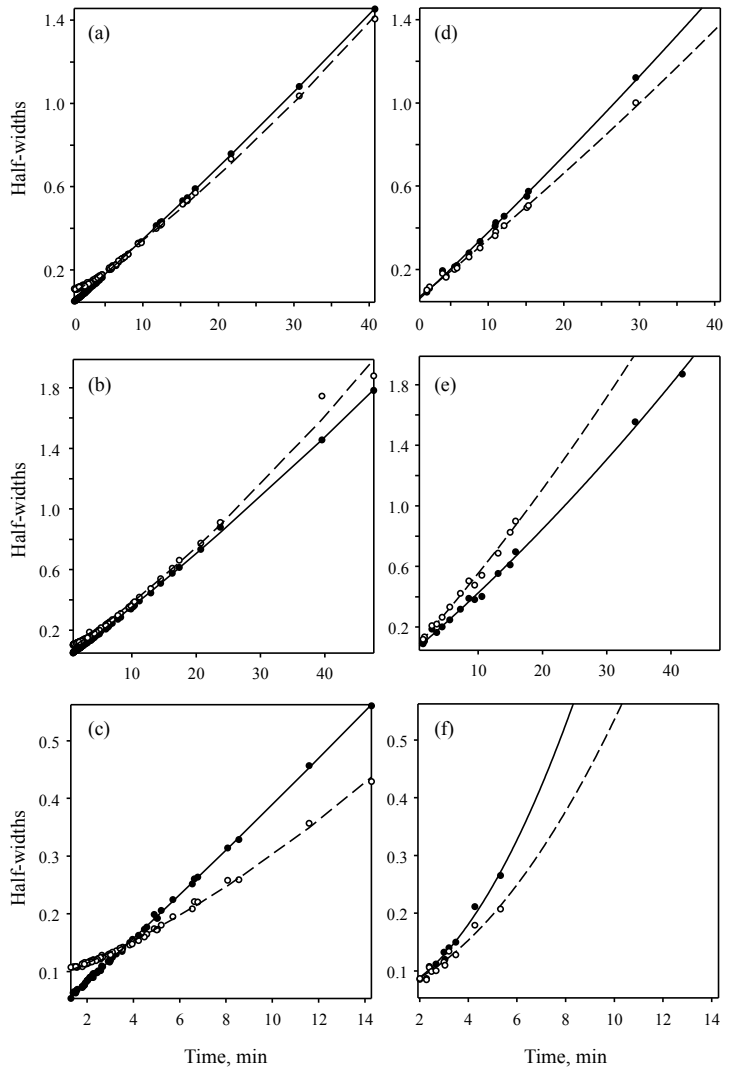

Figure 5.3. Half-widths plots obtained with the data measured in 2012 (a to c) and 2017 (d to f), for: (a,d) Phenyl, (b,e) C18, and (c,f) cyano columns. The data are referred to the same reference length (5 cm for phenyl and C18, and 7.5 cm for cyano). The plots in 2012 were obtained with data for the 15 sulphonamides, whereas those in 2017 were obtained with the data for the five sulphonamides selected for this work, using all assayed mobile phases in both cases. Left (*A*, ●) and right (*B*, ○) half-widths.

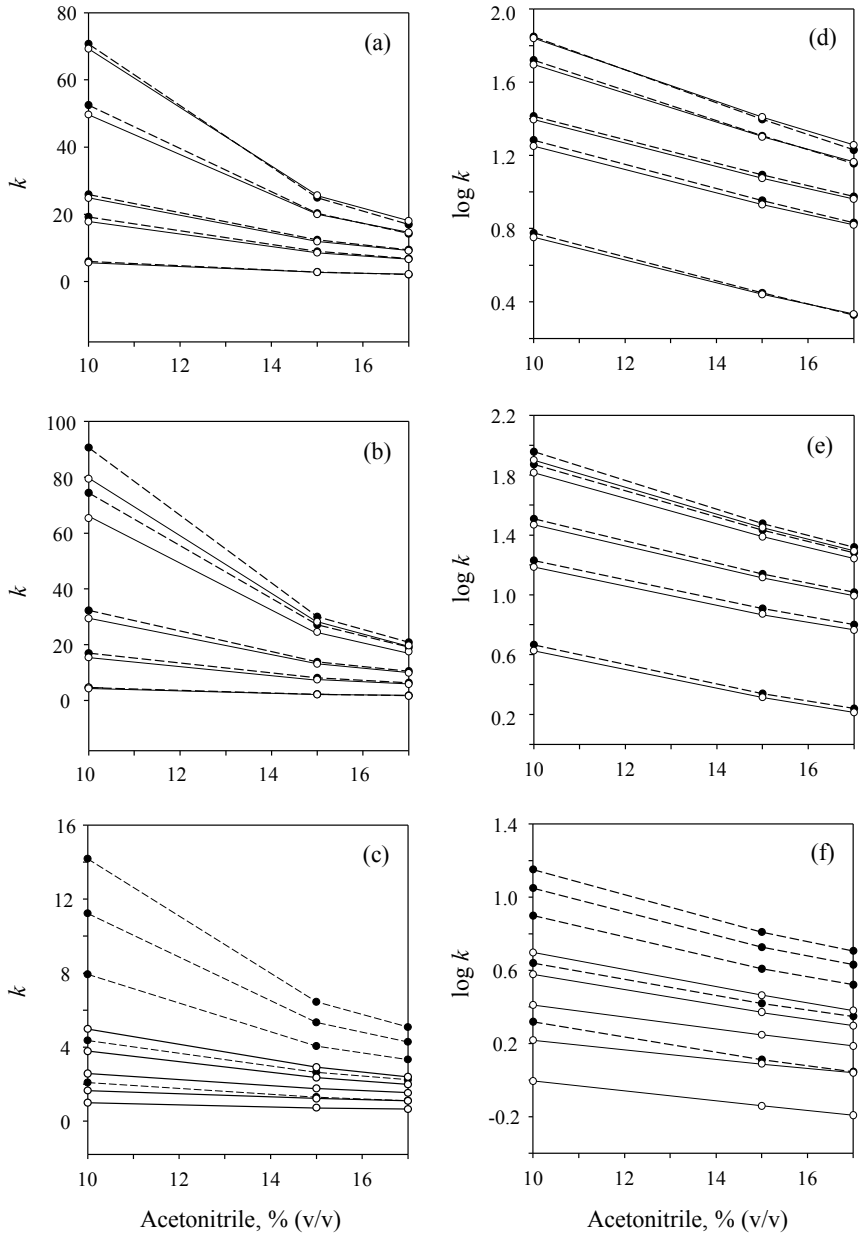

Figure 5.4. Retention factor, expressed as *k* and log *k*, for sulphachloropyridazine, sulphadimethoxine, sulphaquinoxaline, sulphathiazole, and sulphisoxazole (from bottom to top), measured for the phenyl (a,d), C18 (b,e) and cyano (c,f) columns in 2012 (dashed line) and 2017 (solid line).

Column degradation is also manifest by observing the variation in dead time between 2012 and 2017. To compare the dead time values, it should be taken into account that the column lengths in 2012 and 2017 were different, and a translation to a common length is needed. Thus, in 2012, the columns were obtained by coupling two or three brand-new columns of the same nature to get more accurate measurements (5 cm + 5 cm for the phenyl column, 5 cm + 2 cm + 2 cm for the C18 column, and 3.5 cm + 7.5 cm for the cyano column). In 2017, the columns consisted of isolated column segments (5 cm phenyl, 5 cm C18, and 7.5 cm cyano columns). In Fig. 5.5, the experimental values of dead time measured in 2012 are compared with those in 2017 at three mobile phase compositions, the values referred to the lengths used in 2017.

It is observed that the cyano column suffered the largest changes in dead time. This agrees with the shifts in retention shown in Fig. 5.4. It could be thought that the cyano column was more fragile, but one also must be aware that when the columns were operated in series, the cyano column was often located the first in the arrangement, suffering larger backpressure. Meanwhile, the C18 column was often the last column in the arrangement, owing to its larger hydrophobicity, but it should be also mentioned that the C18 column was used in more analyses, owing to its universal usefulness. All these facts could explain the relative magnitude of the deterioration of each column.

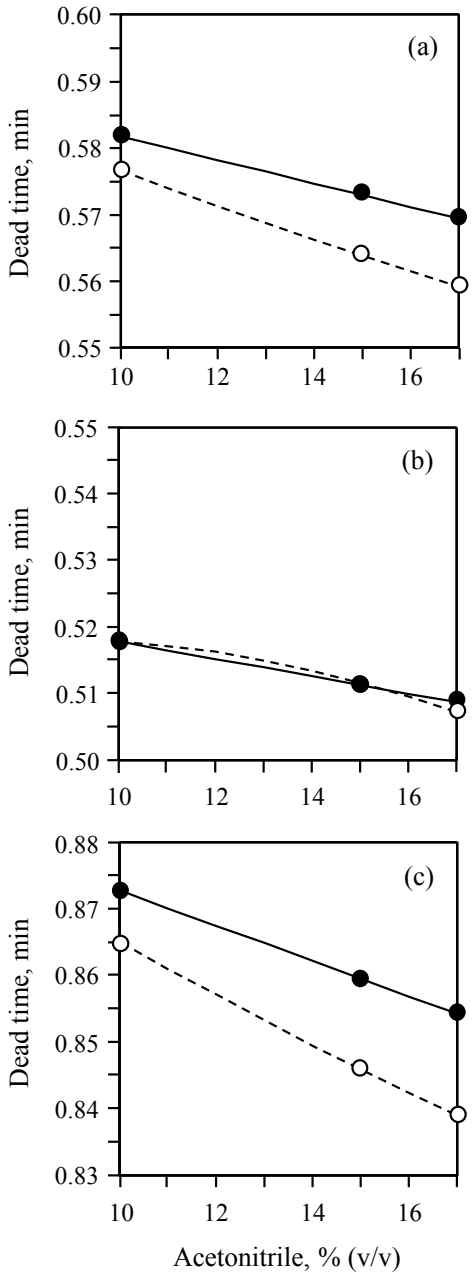

Figure 5.5. Dead time values measured for the phenyl (a), C18 (b) and cyano (c) columns in 2012 (empty circles and dashed lines), and 2017 (full circles and solid lines).

5.5.2.2. Correction of the retention models to make predictions for aged columns

In this work, we propose transferring the retention times measured in 2012 to the aged columns (2017), based on the discrepancies observed before and after column degradation. For this purpose, a minimal number of experiments and the models already developed at the start of the column lifetime should be ideally used. The models including the ageing effects should allow finding new optimal conditions, more in line with the new separation performance. The approach is applied to the separation of sulphonamides using single columns, and columns coupled in series, in both isocratic and gradient elution.

The observed changes in the chromatographic behaviour of solutes eluted with the aged columns will yield a change in the parameters of the retention model. The dead time will also change. For a given solute *j* and column *i*, the effects on retention can be described as follows:

$$\begin{aligned} \log k_{ij}^{*} &= \log k_{0,ij} + \Delta \log k_{0,i} + (c_{1,ij} + \alpha_{1,i})\varphi + (c_{2,ij} + \alpha_{2,i})\varphi^2 = \\ &= \log k_{ij} + \Delta \log k_{0,i} + \alpha_{1,i}\ \varphi + \ \alpha_{2,i}\,\varphi^2 \end{aligned} \tag{5.18}$$

where k_{ij}^{*} and k_{ij} are the retention factors for solute *j*, eluted with the aged and brand-new columns, respectively, and $\Delta\log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$ account for the changes in the parameters of the retention model describing the behaviour in the aged column. It should be noted that the three ageing parameters depend only on the column (i.e., they are common for all sulphonamides and have characteristic values for a given column, according to its degradation level). This outline is supported by the observation of the parallel lines in Fig. 5.4, with the same shift for all solutes. The validity for the remaining 10 sulphonamides (besides the five used to characterise the degradation level)

will be shown in Section 5.5.7., by comparing experimental and predicted chromatograms under diverse elution programs.

Possible reasons of the changes in the retention model parameters are the following:

(i) Loss of stationary phase, which gives rise to several effects, generally including an increase in silanophilic interactions and changes in stationary phase solvation, with a decrease in hydrophobicity in RPLC. For sulphonamides, the loss of stationary phase mainly yielded a change in the intercept of Eq. (5.2) ($\log k_{0,ij}$), which introduced a certain offset ($\Delta \log k_{0,i}$), similar for all solutes.

(ii) Modifications in the relative weight of the interactions of solutes with the column, as the solvent composition changes, due to a change in the column nature. This effect was of low magnitude for sulphonamides and was accounted through the parameters α_1 and α_2, which represent the variation of the elution strength. In our treatment, we will assume that these parameters are identical for all solutes.

(iii) Changes in the packing structure by effect of the high pressure, improper handling and dissolution of silica, which will affect the column dead time ($t_{0,i}$). This will have consequences in the retention times and in the prediction of gradients (as described in Eqs. (5.5)–(5.9), where a correct dead time value is essential). Moreover, peak width and asymmetry will be affected.

5.5.3. Accuracy of the predictions for the brand-new columns

If the modelling process carried out for an interpretive optimisation is correct, and the column keeps its original separation performance, predictions often offer accuracy with errors below 1–2%. In contrast, predictions may differ significantly from the actual separation when the column suffers some deterioration. This means that the initial parameters of the chromatographic models for all solutes (Eqs. (5.2), (5.10) and (5.11)) should be adapted to the new situation. Once this is made, the errors in the predictions should tend again to the uncertainty level obtained with the brand-new column.

Fig. 5.6 shows the quality of the predictions of retention time for the three brand-new columns (in 2012). The prediction error calculated with regard to the mean retention time was 0.46%, 0.33% and 1.11% for the phenyl, C18 and cyano columns, respectively. The somewhat larger scattering for the cyano column is explained by the shorter mean retention.

5.5.4. Estimation of the column ageing parameters

Our purpose was to correct the models developed in 2012 to give accurate predictions in conditions of degradation, by considering parameters accounting for the ageing in the retention models. The ageing parameters in Eq. (5.18) ($\Delta\log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$) were obtained using the data of a set of five representative sulphonamides, eluted with three mobile phases covering the whole retention range for the set of 15 sulphonamides (see Section 5.4.1.).

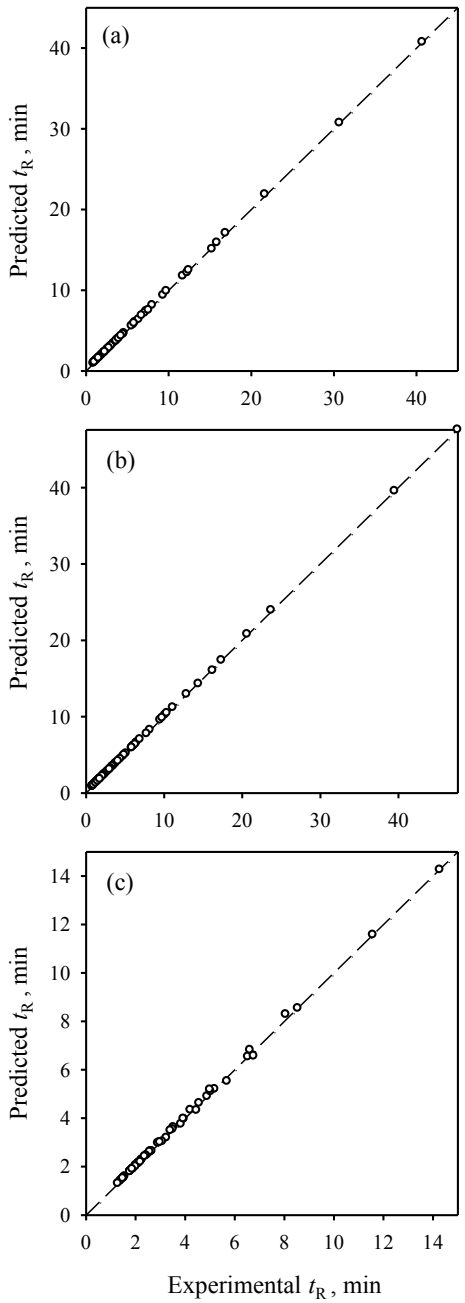

Figure 5.6. Correlation plots showing the quality of the predictions of retention time (t_R) in 2012, for: (a) Phenyl, (b) C18, and (c) cyano columns.

The calculation process consisted of the non-linear fitting of the data, minimising the sum of the squared differences between:

(i) the experimental retention times measured in 2017 for the set of five sulphonamides eluted with the three mobile phases (5×3 = 15 data); and

(ii) the respective predictions, obtained by including the ageing parameters in the retention models fitted for each solute in 2012. For this purpose, the dependence of the dead time with the solvent content for the aged columns, and the extra-column times accounting the change in the instrument in 2017 were needed. The dwell time in the case of gradient prediction was also needed.

As commented, the three ageing parameters ($\Delta \log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$) were assumed to be the same for all solutes in a given column, whereas $\log k_{0,ij}$, $c_{1,ij}$ and $c_{2,ij}$ were specific for each solute and column, and correspond to the fitted values in Eq. (5.2) when the columns were brand-new. Therefore, along the minimisation process, only the parameters $\Delta \log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$ (Eq. (5.18)) were optimised to reach the best agreement between the experimental and predicted retention times.

The minimisation was carried out considering retention times, instead of retention factors, in order to get a uniform error distribution in times. The retention times were calculated by working out $t_{R,j}$ from Eq. (5.3), using the values predicted for 2017 (k_j^*, $t_{0,i}^*$ and t_{ext}^*).

5.5.5. Prediction of retention for the aged phenyl, C18 and cyano columns

5.5.5.1. Adaptation of the retention models developed for the brand-new columns to the aged conditions

In Section 5.5.4., the algorithm to transfer the predictions of retention data to columns that had suffered some ageing is described. According to this correction procedure, Fig. 5.7 depicts the quality of the updated predictions of retention data for the cyano column, considering different levels of corrections in the retention model, including only $\Delta \log k_{0,i}$; both $\Delta \log k_{0,i}$ and $\alpha_{1,i}$; and a full correction considering the three ageing parameters ($\Delta \log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$). Fig. 5.8 shows the quality of the updated predictions for the phenyl and C18 columns, after the correction of only $\Delta \log k_{0,i}$, or both $\Delta \log k_{0,i}$ and $\alpha_{1,i}$. In these figures, only the data for the subset of five sulphonamides eluted at three mobile phase compositions are plotted (the results for the full set of 15 sulphonamides will be shown in Section 5.5.7.).

The needed dead time and extra-column time values were those found experimentally for the brand-new (2012) and degraded (2017) conditions. The dead time values measured in 2012 and 2017 are plotted in Fig. 5.5. An increase in dead volume is observed for columns of the same length. The extra-column times were 0.140 min and 0.0510 min for the HPLC instruments used in 2012 and 2017, respectively.

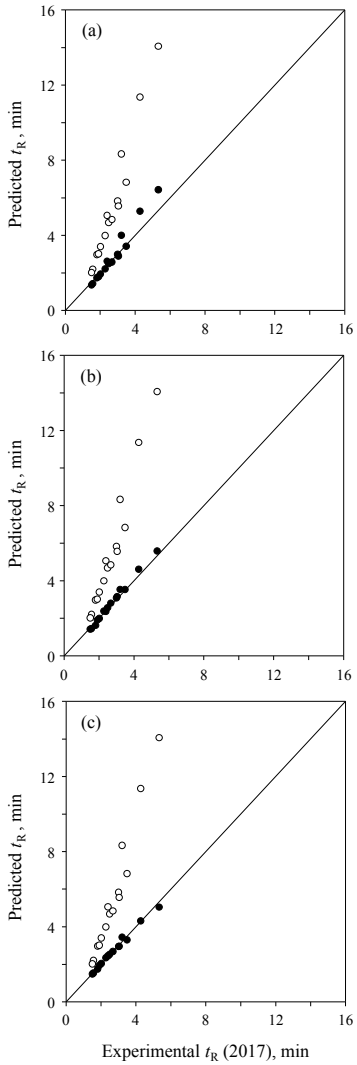

Figure 5.7. Progressive inclusion of ageing parameters in the modelling of retention for the cyano column: (a) only $\Delta \log k_{0,i}$, (b) $\Delta \log k_{0,i}$ and $\alpha_{1,i}$, and (c) $\Delta \log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$. Empty circles represent the predicted values for each solute and mobile phase using the models fitted in 2012 versus the experimental values measured in 2017. Full circles depict the transferred predictions from 2012 to 2017. The diagonal depicts a situation where the retention times in 2012 and 2017 fully agree.

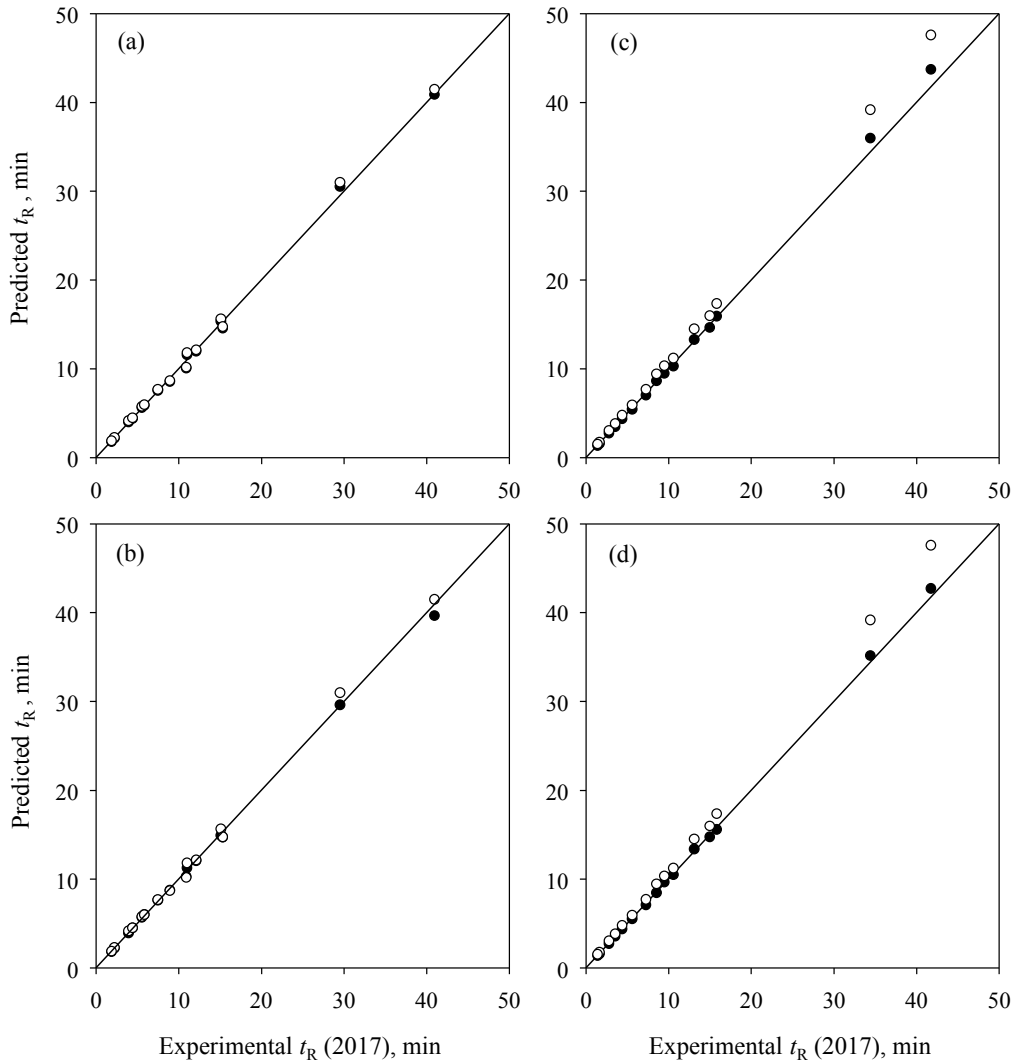

Figure 5.8. Progressive inclusion of ageing parameters in the modelling of retention for the phenyl (a,b) and C18 (c,d) columns, considering: (a,c) $\Delta \log k_{0,i}$, and (b,d) $\Delta \log k_{0,i}$ and $\alpha_{1,i}$. See Fig. 5.7 for other details.

The empty circles represent the predicted values for each solute and mobile phase using the models fitted in 2012 (y-axis) versus the experimental values measured in 2017 (x-axis). The full circles depict the updated predictions from 2012 to 2017, calculated according to Eq. (5.18), where the ageing parameters were obtained as explained in Section 5.5.4. The diagonal of the plots depicts a situation, where the predictions carried out with the models obtained in 2012 (brand-new columns) corrected with the ageing parameters, fully agree with the experimental values measured in 2017 (degraded columns).

Therefore, the larger the distance of the original predictions obtained with retention models without considering ageing parameters (empty circles) to the diagonal, the more important the degradation. According to these considerations, the cyano column (Fig. 5.7) suffered the most intense degradation, followed by the C18 column (Fig. 5.8c and d). The phenyl column experienced very small degradation.

Considering the number of ageing parameters included in Eq. (5.18), it should be indicated that the predictions in 2017 (aged conditions, full circles) become progressively more accurate as the number of parameters increases (from Fig. 5.7a to Fig. 5.7c). It may be observed that the most important correction is accounted by parameter $\Delta \log k_{0,i}$ (followed by $\alpha_{1,i}$), since its inclusion moves considerably the predictions closer to the diagonal. The effect of the third parameter ($\alpha_{2,i}$) is residual and can be neglected. It is observed that by applying the corrections, the updated predictions from 2012 to 2017 agreed satisfactorily with the experimental data measured in 2017.

5.5.5.2. Uncertainty in the estimation of the ageing parameters

As indicated, different levels of correction of the updated retention model were evaluated, assuming the addition of only one ($\Delta\log k_0$), two ($\Delta\log k_0$ and α_1), or three ($\Delta\log k_0$, α_1 and α_2) ageing parameters for updating predictions from 2012 to 2017. In Table 5.1, the uncertainty in the parameters, expressed as the standard deviation in their estimation, is indicated:

$$Sp_k = \sqrt{\frac{\sum_{i=1}^{ne}(\hat{t}_{R_i} - t_{R\,exp,i})^2}{ne - np}} \times \mathrm{Diag}_k \left(\mathbf{J}'\times\mathbf{J}\right)^{-1} \tag{5.19}$$

where *ne* is the number of experiments in the training design, *np* the number of column ageing parameters ($\Delta\log k_{0,i}$, $\alpha_{1,i}$ and $\alpha_{2,i}$) in the retention model (*np* = 1, 2 or 3 depending on the treatment), $\hat{t}_{R_i}$ the predicted retention time for experiment *i*, $t_{R\,exp,i}$ the experimental retention time for experiment *i* and Diag_k, an operator which extracts the element *k*,*k* of the $\left(\mathbf{J}'\times\mathbf{J}\right)^{-1}$ matrix, where **J** is the Jacobian matrix, defined as:

$$\mathbf{J} = \begin{pmatrix} \frac{\partial t_{R_1}}{\partial p_1} & \frac{\partial t_{R_1}}{\partial p_2} & \cdots & \frac{\partial t_{R_1}}{\partial p_{np}} \\ \frac{\partial t_{R_2}}{\partial p_1} & \frac{\partial t_{R_2}}{\partial p_2} & \cdots & \frac{\partial t_{R_2}}{\partial p_{np}} \\ \vdots & \vdots & \ddots & \vdots \\ \frac{\partial t_{R_{ne}}}{\partial p_1} & \frac{\partial t_{R_{ne}}}{\partial p_2} & \cdots & \frac{\partial t_{R_{ne}}}{\partial p_{np}} \end{pmatrix}$$

The quality of each fitted model was evaluated using the following statistics:

(a) *Correlation coefficient for the prediction of retention times*

$$R = \sqrt{1 - \frac{\sum_{i=1}^{ne} (\hat{t}_{R_i} - t_{R\,exp,i})^2}{\sum_{i=1}^{ne} (t_{R\,exp,i} - \bar{t}_{R\,exp})^2}} \tag{5.20}$$

$\bar{t}_{R\,exp}$ being the mean retention time in the experimental design

(b) Adjusted correlation coefficient for the prediction of retention times

$$R_{adj} = \sqrt{1 - (1 - R^2) \times \frac{ne - 1}{ne - np - 1}} \tag{5.21}$$

(c) Mean relative errors

$$RE1 = \frac{\sum_{i=1}^{ne} \left| \frac{\hat{t}_{R_i} - t_{R\,exp,i}}{t_{R\,exp,i}} \right|}{ne} \times 100 \tag{5.22}$$

$$RE2 = \frac{\sum_{i=1}^{ne} \left| \hat{t}_{R_i} - t_{R\,exp,i} \right|}{\sum_{i=1}^{ne} t_{R\,exp,i}} \times 100 \tag{5.23}$$

*RE*1 refers each deviation to the respective experimental value; for this reason, it is affected by the variations in magnitude of $t_{R\,exp,i}$. In contrast, *RE*2 refers each deviation to the mean experimental retention time. In this way, the variations in magnitude of $t_{R\,exp,i}$ do not affect the result.

(d) Maximal absolute error

$$AE_{\max} = \max_{i=1}^{ne} \left| \hat{t}_{R_i} - t_{R\,exp,i} \right| \tag{5.24}$$

(e) Sum of squared residuals

$$SSR = \sum_{i=1}^{ne} (\hat{t}_{R_i} - t_{R\,exp,i})^2 \tag{5.25}$$

(f) Snedecor's F statistic

$$F_{Snedecor} = \frac{\sum_{i=1}^{ne} (\hat{t}_{R_i} - \hat{t}_{Rmean,i})^2}{\frac{\sum_{i=1}^{ne} (t_{R\,exp,i} - \hat{t}_{R_i})^2}{ne - np}} \tag{5.26}$$

The optimal number of parameters for each column can be established from the adjusted correlation coefficient or the Snedecor's F statistic, which reach maximal values at the optimal model complexity, which is marked in bold in Table 5.1. The least degraded column (phenyl) only required the $\Delta\log k_0$ correcting term, whereas the C18 column (whose degradation level is significant) required $\Delta\log k_0$ and α_1. The most degraded column (cyano) needed the three correcting terms.

If the discussion attends to the magnitude of the errors, it can be observed that the higher the complexity of the model, the smaller the error. However, this improvement is artificial since it implies the presence of overfitting, which would lead to larger prediction errors if an external validation set was inspected

instead of the training data. Finally, all correcting terms can be considered significant according to the inclusion of the zero value in the respective confidence intervals, but as commented, beyond the optimal number of correcting terms, the models are not attending the true pattern.

Up to this point, correcting terms were obtained using the data from five standards, measured at three mobile phase compositions. Table 5.2 shows the effect of reducing the number of training experiments, intending to reduce the experimental effort to a minimal. This study was performed using the optimal number of parameters concluded from Table 5.1. Note that in Table 5.2 the designs involved different number of experiments, and for this reason, the Snedecor's *F* values cannot be compared. With a training set composed of five standards measured at two mobile compositions (5×2 design), similar prediction performance was yielded for the three columns, with regard to the results found using the full training set (5×3 design). A further reduction to a minimal number of experiments (two standards at two solvent levels, i.e., 2×2 design) gave satisfactory results, unless column deterioration was important (case of the cyano column). Hence, a 5×2 design can be considered safe for most situations.

Table 5.1. Ageing parameters ($\Delta\log k_0$, α_1 and α_2) with their uncertainties expressed as standard deviation obtained by non-linear regression, and fitting statistics in the prediction of the 15 sulphonamides, estimated from a 5×3 experimental design for the three assayed columns.[a]

		Number of correcting column parameters			
Column	Statistical parameters	0	1	2	3
C18					
	$\Delta\log k_0$		–0.052	**–0.0975**	–0.156
	Uncertainty		±0.003	**±0.0014**	±0.005
	α_1			**0.422**	1.379
	Uncertainty			**±0.004**	±0.005
	α_2				–3.68
	Uncertainty				±0.18
	Correlation coefficient	0.983814	0.999457	**0.999872**	0.999881
	Adjusted correlation coefficient	0.983814	0.999415	**0.999851**	0.999848
	Mean relative error *RE*1	7.74	3.35	**1.10**	1.05
	Mean relative error *RE*2	10.19	2.68	**0.97**	0.96
	Maximal absolute error	5.62	0.72	**0.57**	0.57
	Sum of squared residuals	61.944	2.106	**0.506**	0.491
	Snedecor's *F*	569.6	13218.5	**49830.1**	47411.9

Table 5.1 (continued).

Phenyl					
	$\Delta\log k_0$		**–0.027**	–0.028	–0.093
	Uncertainty		**±0.003**	±0.006	±0.005
	α_1			0.006	1.06
	Uncertainty			±0.04	±0.05
	α_2				–4.03
	Uncertainty				±0.4
	Correlation coefficient	0.994924	**0.999427**	0.999440	0.999471
	Adjusted correlation coefficient	0.994924	**0.999383**	0.999347	0.999327
	Mean relative error *RE*1	7.27	**2.09**	2.11	2.08
	Mean relative error *RE*2	6.86	**2.03**	2.04	2.03
	Maximal absolute error	2.61	**0.83**	0.83	0.85
	Sum of squared residuals	16.174	**1.842**	1.842	1.824
	Snedecor's *F*	1539.4	**11923.6**	11068.9	10321.8
Cyano					
	$\Delta\log k_0$		–0.426	–0.705	**–1.552**
	Uncertainty		±0.019	±0.016	**±0.016**
	α_1			2.39	**16.0**
	Uncertainty			±0.13	**±0.3**
	α_2				**–51.6**
	Uncertainty				**±1.8**
	Correlation coefficient	0.000000	0.942330	0.992476	**0.996579**
	Adjusted correlation coefficient	0.000000	0.937747	0.991216	**0.995644**
	Mean relative error *RE*1	81.44	12.15	5.12	**3.55**
	Mean relative error *RE*2	94.78	11.77	4.53	**2.93**
	Maximal absolute error	8.27	0.56	0.24	**0.24**
	Sum of squared residuals	174.808	1.789	0.270	**0.153**
	Snedecor's *F*	12.4	170.0	812.5	**1322.4**

[a] The optimal number of correcting column parameters for each column is marked in bold.

Table 5.2. Ageing parameters ($\Delta\log k_0$, α_1 and α_2) with their uncertainties expressed as standard deviation obtained by non-linear regression, and fitting statistics in the prediction of the 15 sulphonamides, estimated from a 5×3, 5×2 and 2×2 experimental design for the three assayed columns.

Column	Statistical parameters	Experimental design		
		5×3	5×2	2×2
C18				
	$\Delta\log k_0$	–0.0027	–0.0940	–0.098
	Uncertainty	±0.0014	±0.0007	±0.019
	α_1	0.422	0.39	0.43
	Uncertainty	±0.004	±0.03	±0.10
	α_2			
	Uncertainty			
	Correlation coefficient	0.999872	0.999875	0.999998
	Adjusted correlation coefficient	0.999862	0.999840	0.999994
	Mean relative error *RE*1	1.10	1.26	0.92
	Mean relative error *RE*2	0.98	1.01	0.17
	Maximal aAbsolute error	0.57	0.57	8.54
	Sum of squared residuals	0.506	0.455	7.369
	Snedecor's *F*	49830.1	30397.0	289839.3

Table 5.2 (continued).

Phenyl				
	$\Delta\log k_0$	–0.027	–0.028	–0.0186
	Uncertainty	±0.003	±0.003	±0.0010
	α_1			
	Uncertainty			
	α_2			
	Uncertainty			
	Correlation coefficient	0.999427	0.999386	0.999986
	Adjusted correlation coefficient	0.999383	0.999309	0.999979
	Mean relative error $RE1$	2.09	2.37	1.78
	Mean relative error $RE2$	2.03	2.31	0.51
	Maximal absolute error	0.83	0.86	0.14
	Sum of squared residuals	1.842	1.763	2.955
	Snedecor's F	11923.6	7068.8	99213.0
Cyano				
	$\Delta\log k_0$	–1.552	–0.57	0
	Uncertainty	±0.016	±0.19	±80000
	α_1	16.0	0	0
	Uncertainty	±0.3	±3	±1200000
	α_2	–51.6	6	0
	Uncertainty	±1.8	±13	±5000000
	Correlation coefficient	0.996579	0.997571	0.999898
	Adjusted correlation coefficient	0.995644	0.996354	Singular
	Mean relative error $RE1$	3.55	4.00	5.29
	Mean relative error $RE2$	2.93	3.29	3.35
	Maximal absolute error	0.24	0.24	0.23
	Sum of squared residuals	0.153	0.133	6.412
	Snedecor's F	1322.4	722.5	157.4

5.5.6. Modelling the peak profiles

The above corrections concern only the retention. However, chromatographic predictions should also attend to the peak profiles (width and asymmetry). In this work, the peak profiles are predicted taking advantage of the global character of the half-widths versus retention time relationships (Eqs. (5.10) and (5.11)) for the set of 15 sulphonamides. Here, the term global means that the relationships should be valid for any compound in a family, provided that the kinetic interactions are similar. Global half-widths models can be fitted either with the full set of available compounds or with a representative subset, eluted with one or several mobile phases.

The global character of the half-widths models, together with the fact that these can be readily obtained with a small set of compounds, makes the transference of half-widths data from the brand-new conditions to the degraded ones unnecessary. Instead, in this work, the degradation in peak profile was accounted by replacing the original half-widths models for the brand-new columns (Fig. 5.3a to c) by models developed for the aged columns (Fig. 5.3d to f). According to all above, the half-widths models for the aged columns were fitted with the experimental data (half-widths and retention times) taken in 2017 for the aged column and the five selected sulphonamides (in 2012, the whole set of 15 sulphonamides were used to build the half-widths models). The prediction of half-widths for a given compound would imply the prediction of the retention time in the degraded conditions using the retention model including the ageing parameters. This value is projected in the new relationship of half-width versus retention time for the aged column to obtain the corresponding half-widths.

5.5.7. Predicted chromatograms for coupled columns

To check the accuracy of the approach, the chromatograms for a mixture of the 15 sulphonamides, using 5 cm C18 + 5 cm phenyl + 7.5 cm cyano connected in tandem under several elution programs, were obtained. In Figs. 5.9 and 5.10, the experimental chromatograms obtained in 2017 are compared with those predicted with and without considering the ageing. Fig. 5.9 shows the predicted and experimental chromatograms using an isocratic run and a linear gradient. Fig. 5.10 corresponds to two different multi-linear gradients.

The experimental chromatograms were obtained using a similar concentration of ca. 20 μg/mL for all sulphonamides. This concentration implies a different molar concentration for each compound, which together with the diverse molar absorptivity, gives rise to different peak areas. Meanwhile, the predicted chromatograms were calculated considering normalised peaks. For the sulphonamides, column ageing is evidenced as shifts in the retention times to smaller values, and broader (and often more skewed) peaks. In all cases, the agreement between predicted and experimental chromatograms for 2017 is very satisfactory.

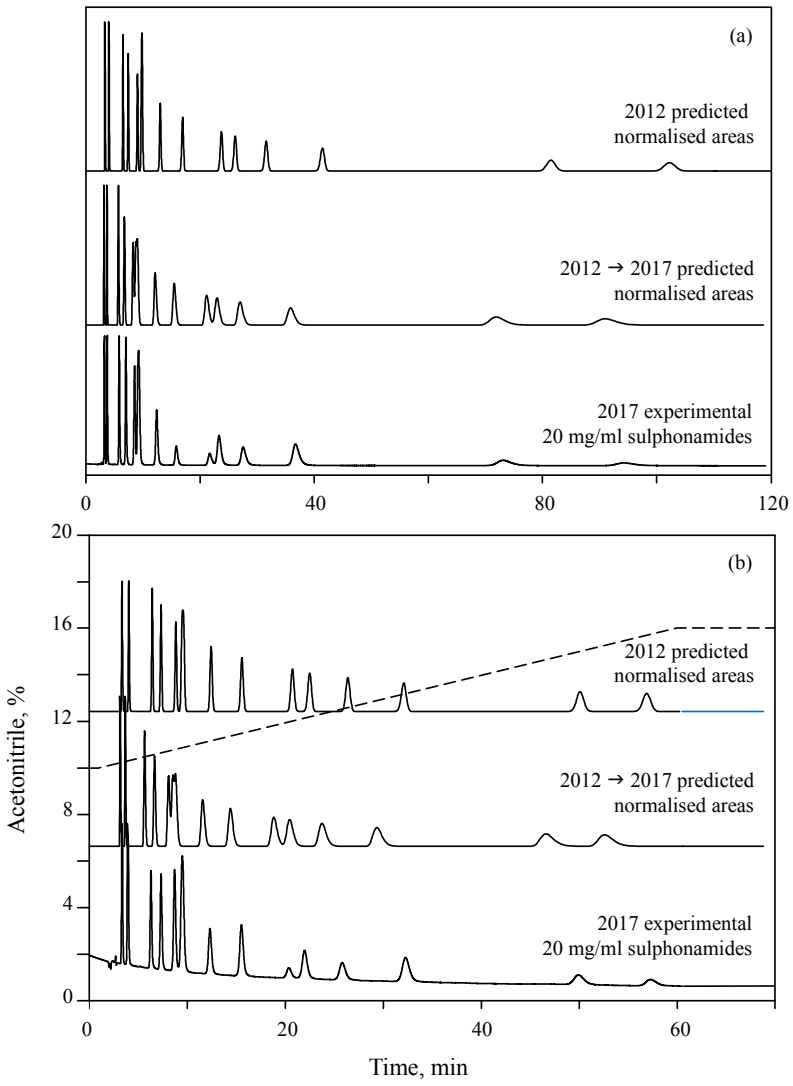

Figure 5.9. Chromatograms for the serially-coupled columns built with 5 cm C18 + 5 cm phenyl + 7.5 cm cyano using: (a) isocratic elution with 10% acetonitrile, and (b) a linear gradient between 10% and 16% acetonitrile run in 60 min. Comparison of the experimental chromatogram obtained in 2017 (bottom) with the predicted chromatograms ignoring (top), or considering (middle) the column degradation. The correction of the retention model and peak shape involves calibration data from five sulphonamides. In (b), the gradient program is overlaid.

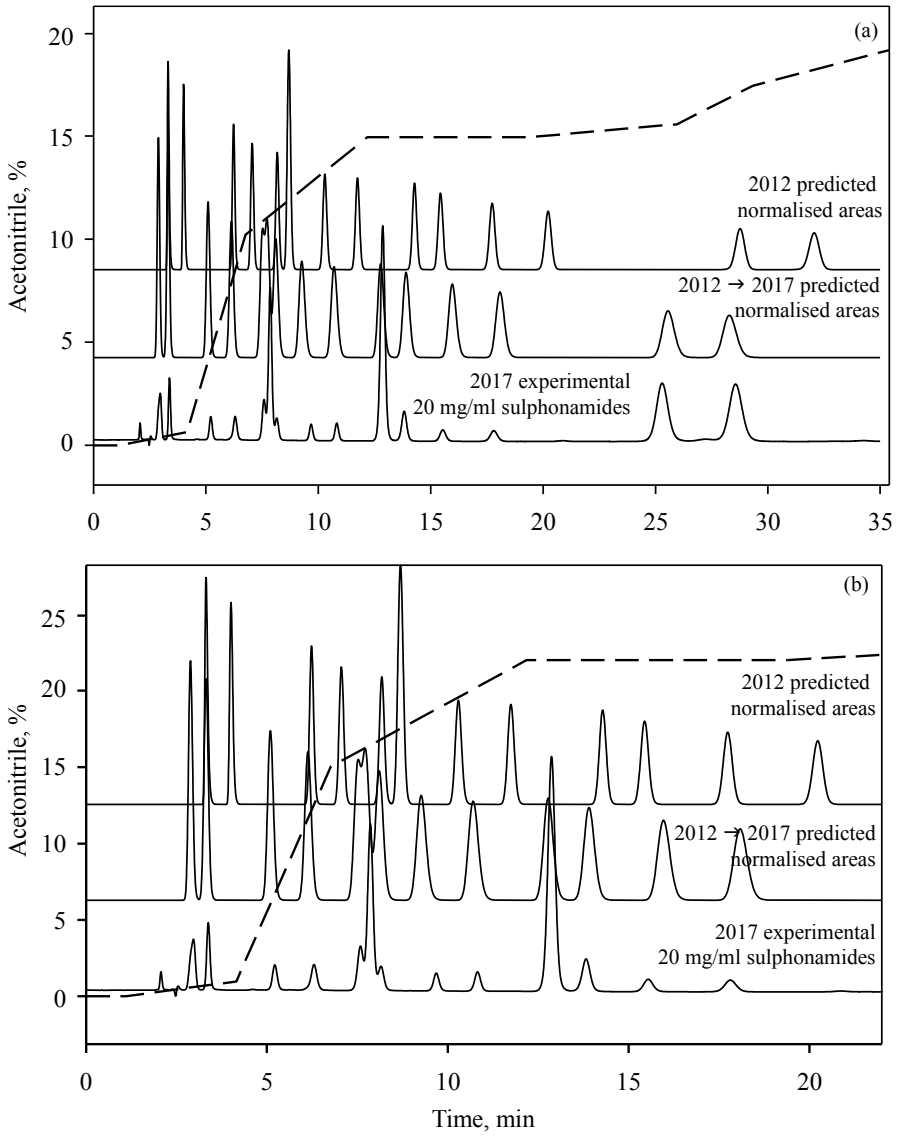

Figure 5.10. Chromatograms for the serially-coupled columns using two multilinear gradients. The gradient programs in (a) and (b) are overlaid. See Fig. 5.9 for other details.

5.6. Conclusions

Interpretive optimisation of the separation conditions is based on the prediction of chromatographic data (time and half-widths), using models previously obtained. This work gives response to a situation where the chromatographic column suffered some degradation after the modelling step had finished. Moreover, another chromatograph was used for the analyses. It was, thus, necessary to modify the models that describe the retention and half-widths, obtained for the brand-new columns, to make an accurate prediction of the separation performance when the column has suffered degradation. A shortcut is proposed to modify the predictions obtained for a brand-new column, by introducing parameters that account for the effects of ageing. Based on the comparison of the log k versus solvent content plots for the brand-new and aged conditions, these parameters were assumed to be column dependent (common for all analytes).

The calculation of the ageing parameters involves a small set of compounds (2 to 5 among those measured with the brand-new columns), and is based on the minimisation of the discrepancies between the experimental data measured for the aged column, and the respective predictions with the chromatographic models obtained with the brand-new column after including ageing parameters. Collaterally, the data used to correct the retention allows establishing new half-widths versus retention time relationships for the aged columns. From the gathered information, predictions more in line with the deteriorated column performance are forecasted for the whole family of compounds analysed with that column or set of coupled columns.

The described approach suggests that, whenever the columns keep sufficient performance, we can take advantage of the extensive experimental work carried

out when the system was initially modelled with the brand-new columns. With this information and a minimal extra experimental effort, highly accurate predictions in the degraded situation are possible. The full modelling of the chromatographic behaviour is thus only made with the brand-new columns.

In the case of study, we predicted chromatograms accounting degradation for a set of 15 sulphonamides analysed with three columns (phenyl, C18 and cyano). These were brand-new in 2012 and, after intensive use they suffered different deterioration in 2017. The main purpose of this study was to predict the behaviour when the compounds were separated with serially-coupled columns showing some degradation. The predictions for the current column performance (2017), based on the correction of the models taken in 2012 using the ageing parameters, were satisfactory in all cases.

In serial coupling, each connected column should be considered independently (even in the case of containing the same stationary phase and length as other connected column), since the degradation level for each column may be different. Coupling aged and new columns to separate the mixture is also possible, whenever the system keeps reasonably good efficiency.

As commented, the column degradation that has been shown here happened unintentionally, and the 15 sulphonamides were the first solutes analysed when the columns were brand-new. It is unlikely that this behaviour be specific for sulphonamides. However, the conclusions obtained should not be generalised to other types of compounds. In this work, it is shown that the approach worked with a family of related compounds with three different columns. It can be thought that the corrections will similarly work with compounds of intermediate molar weight and similar interactions. For some compounds, such as protonated basic compounds for which the retention mechanism is mixed, the corrections could be more complex and perhaps not competitive in terms of

experimental effort, but for many others the corrections may be accurate enough as is the case in our work. Certainly, other sets of compounds would require a specific study.

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CHAPTER 6

ANALYSIS OF AMINO ACIDS USING SERIALLY-COUPLED COLUMNS

6.1. Abstract

Single conventional columns in reversed-phase liquid chromatography are insufficient for analysing the isoindoles of primary amino acids, due to their limited functionality. An interesting possibility for increasing the separation power is the combination of several columns of different nature, where the length is modified by coupling small segments. This approach may require a considerable investment to have multiple lengths for each stationary phase. However, the combination of only two columns of fixed length can be enough to resolve satisfactorily relatively complex mixtures, provided that an optimised gradient program is applied. In this work, a mixture of 19 primary amino acid isoindoles found in proteins was analysed. Four stationary phases were assayed: C18, pentafluorophenyl-C18, C4 and cyano. The mixture of isoindoles was successfully resolved in practical times using a pentafluorophenyl-C18 column coupled to a C4 column, in spite of the extremely poor performance obtained when each column is used isolatedly, independently of the length. The extreme diversity in the polarities of the isoindoles and the need of extrapolating the retention behaviour in certain regions of the solvent content domain makes the modelling of the retention behaviour of the isoindoles particularly difficult. Nevertheless, the predicted optimal separations were very satisfactory.

6.2. Introduction

The chromatographic analysis of amino acids plays an important role in several fields, such as the control of foods and natural products, and pharmaceutics [1–3]. It is not only a suitable tool for the accurate quantification of proteins, peptides and free amino acids, it also provides detailed information regarding the amino acid composition aimed to protein and peptide identification. A protein/peptide should be hydrolysed to its individual constituents before amino acid analysis. Once the hydrolysis is carried out, the chromatographic procedure is the same as that practised for free amino acids, which typically require derivatisation, due to their hydrophilic nature and lack of suitable chromophore [4].

Along the years, a lot of effort has been invested in the development of reversed-phase liquid chromatographic (RPLC) procedures to analyse amino acids. The main problem is the inability to get sufficiently good resolution in practical analysis times, using conventional columns and instrumentation for HPLC. It is thus not surprising that a number of authors have been interested in developing optimisation protocols to improve the chromatographic performance in amino acid analysis [5–17]. The most successful optimisation strategy is the *in silico* scanning of the performance of a large number of arbitrary conditions by inspecting their computer-predicted chromatograms [18–21]. For each predicted chromatogram, the quality of the separation is evaluated with the assistance of a chromatographic objective function (COF), which may collect, besides the resolution, the qualifications with regard to other secondary aims, such as the analysis time or desirable peak shapes [19,22,23].

In previous work [24] (described in Chapter 10), we proposed the use of both time and spectral information from a diode array detector, to optimise the

separation in shorter analysis times of the isoindoles of primary amino acids found in proteins, formed with *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC). The approach made use of a two-way COF based on the multivariate selectivity concept (figure of merit derived from the net analyte signal), which searched the best conditions to resolve numerically overlapped peaks using the orthogonal projection approach and alternating least squares. In this approach, estimates of the spectra of amino acids are projected on the data matrix to obtain estimates of the concentration profiles, subjected to some constraints to guarantee the solution has chemical meaning. The refined concentration profiles are then projected in the data matrix and the process repeated up to convergence. The initial estimates of the spectra are obtained by monitoring the dissimilarity of each spectrum in the data matrix, compared to a collection of the purest spectra in an additive process. More details of the approach can be found in Ref. [24].

We explore here another solution to improve the resolution of the set of amino acids: the use of serially-coupled columns [25–28]. In general, the traditional strategy of using a single conventional column in RPLC is often insufficient to get full resolution, at least in practical analysis times, due to the limited functionality of conventional stationary phases. The serial coupling of columns gives rise to an additive effect of interactions, which can be modulated by controlling the nature and length of each coupled column to get the desired separation. The effect of combining different columns is similar to multiplying the number of those available in the laboratory, each column set behaving as a new column.

The idea of combining columns to get good resolution appeared early in the development of chromatography. Some authors found that by serially coupling two or more columns of different selectivity, the resolution significantly

improved with regard to the individual columns. In the field of the analysis of amino acids, there are two early references reporting the use of combined columns, where phenylthiohydantoin derivatives of 20 common amino acids were resolved with a combination of Zorbax-C8, cyano, benzyl and phenethyl columns [5], and dihydroxyphenylalanine was analysed in urine and plasma samples using a combination of C8 and C18 columns [29].

In 2006, Nyiredy et al. commercialised a patent with Bischoff Chromatography, under the trademark POPLC®, which breathed new life to the idea of coupling columns to increase the resolution in RPLC [30]. The system attracted considerable attention and several authors reported some applications. The POPLC® system, initially developed for isocratic elution, was later adapted to gradient elution [31], and the POPLC® system substituted by conventional columns [32,33]. A review article giving more details on the recent developments about coupling columns has been published [28].

Recently, we have proposed the use of zero dead volume fingertight couplers to facilitate the connection of columns from different manufacturers [33]. We have also developed numerical optimisation tools that allow the reliable search of the best column combination and elution conditions, considering both the isocratic and gradient modes [33–37]. In this work, this approach is applied to the optimisation of the separation of a mixture of the 19 primary amino acids, derivatised with OPA and NAC. It is shown how the combination of only two conventional columns showing different interaction mechanisms can satisfactorily resolve the mixture of amino acids, in spite of the poor performance obtained with each of the columns that are coupled when used separately. For this study, conventional HPLC instrumentation, readily available in any laboratory, has been used.

In the field of conventional HPLC, a drawback of the use of the initially developed POPLC® system and further developments is the strong initial investment to have columns of different lengths and nature in order to build hybrid columns of the desired selectivity. In this work, we show that using simple instrumentation and conventional columns, it is possible to improve significantly the quality of the separation by coupling only two columns of fixed lengths, provided that a careful selection of the gradient program is carried out.

6.3. Experimental

6.3.1. Reagents

The following L-amino acids from several manufacturers were analysed: (1) Aspartic acid (Guinama, Barcelona), (2) glutamic acid (Sigma, Roedermark, Germany), (3) asparagine (Scharlab, Barcelona), (4) serine (Scharlab), (5) glutamine (Scharlab), (6) histidine (Scharlab), (7) glycine (Guinama), (8) arginine (Guinama), (9) threonine (Guinama), (10) alanine (Scharlab), (11) cysteine (Guinama), (12) tyrosine (Scharlab), (13) valine (Scharlab), (14) methionine (Guinama), (15) isoleucine (Guinama), (16) tryptophan (Fluka, Buchs, Switzerland), (17) phenylalanine (Guinama), (18) leucine (Fluka), and (19) lysine (Fluka). The amino acids were dissolved in a small amount of 1 M hydrochloric acid (Panreac, Barcelona), then diluted with water, and stored at 4 ºC. The concentration of the stock solutions for all amino acids was ca. 2.0×10^{-3} M.

The reagent used for the derivatisation of amino acids contained *o*-phthalaldehyde, *N*-acetylcysteine (Fluka), boric acid (Probus, Badalona, Spain), sodium hydroxide (AnalaR, Poole, UK), and ethanol (Merck,

Darmstadt, Germany), all of analytical grade. The separation of amino acid isoindoles was carried out using a procedure recommended in the literature [11], which employs mobile phases prepared with acetonitrile (HPLC grade, Scharlab), and water buffered at pH 6.5 with 0.01 M sodium dihydrogen phosphate (Sigma) and sodium hydroxide (AnalaR). Methanol (Scharlab) was used to clean the chromatographic system.

6.3.2. Derivatisation of amino acids with OPA-NAC

The amino acids were derivatised following a procedure developed in Ref. [38]. The derivatisation reagent was obtained by mixing first an ethanol solution of OPA with an aqueous buffer at pH 9.5, prepared with boric acid and sodium hydroxide. An aqueous solution of NAC was then added to get the following concentrations: 2.5×10^{-4} M for OPA, 4.0×10^{-4} M for NAC and 0.1 M for the buffer. The OPA-NAC reagent was renewed weekly and stored at 4 ºC, protected from light with aluminium foil.

To study the chromatographic behaviour, aliquots of each amino acid were mixed with 3 mL of the OPA-NAC reagent to form the corresponding isoindoles, and then diluted with water up to 10 mL. The final concentration for each amino acid derivative was ca. 2.0×10^{-5} M. To obtain the chromatograms of mixtures of the isoindoles of the 19 amino acids, a solution containing ca. 2.0×10^{-5} M of each amino acid was first prepared. The amino acids were then derivatised by mixing 1 mL of this solution with 9 mL of the OPA-NAC reagent. Therefore, the final concentration of each amino acid isoindole in the mixture was ca. 2.0×10^{-6} M.

6.3.3. Apparatus, column and software

The chromatographic analyses were carried out with an Agilent (Waldbronn, Germany) liquid chromatographic instrument, equipped with the following modules: a quaternary pump run at 1 mL/min, an autosampler with 2 mL vials, a multiple-variable wavelength UV-visible detector (Series 1200) set at 335 nm, and a temperature controller (Series 1100) fixed at 25 ºC. The injection volume was 20 μL.

The chromatographic behaviour of the isoindoles in four columns (7.5 cm length, 4.6 mm internal diameter and 5 μm particle size), containing different stationary phases, was examined. The columns were supplied by Advanced Chromatography Technologies, Ltd. (ACE, Aberdeen, Scotland, UK) and were the following: C18 and C4 (both giving rise to a separation based on hydrophobic interactions), pentafluorophenyl (PFP)-C18 (which combines PFP and C18 mechanisms to separate mixtures not readily resolved with either phase alone), and cyano (with increased retention for polar compounds).

The system was controlled by an OpenLAB CDS LC ChemStation (Agilent B.04.03). The routines for data treatment were developed in MATLAB 2016b from The MathWorks Inc. (Natick, MA, USA).

6.4. Results and discussion

6.4.1. Modelling the chromatographic behaviour of isoindoles

6.4.1.1. Experimental design

In order to model the behaviour of the chromatographic system (combination of column and mobile phase composition), the isoindoles were eluted isocratically with acetonitrile-water mixtures in the 5–27.5% (v/v) range of organic solvent, to get a training set of retention data. In this step, the derivatives were injected individually in each assayed column. We chose isocratic elution (instead of gradients) for the modelling step, to get more accurate prediction models in the wide scanned range of organic solvent compositions. In this way, the solvent composition range was more uniformly sampled, and adapted to each solute needs. As a result, more reliable optimal separation conditions for the coupled columns could be found.

Fig. 6.1 illustrates the concentration ranges of acetonitrile in the mobile phase used in the modelling step, for each amino acid and assayed chromatographic column. The concentration range extends to higher values as the polarity of the compounds decreases. As shown in Fig. 6.1, for the two columns showing stronger retention in the case of study (C18 and PFP-C18), the acetonitrile content in the mobile phase ranged between 2.5% and 27.5%. For the C4 and cyano columns, the assayed domain was shorter: 2.5–22.5% and 2.5–17.5%, respectively.

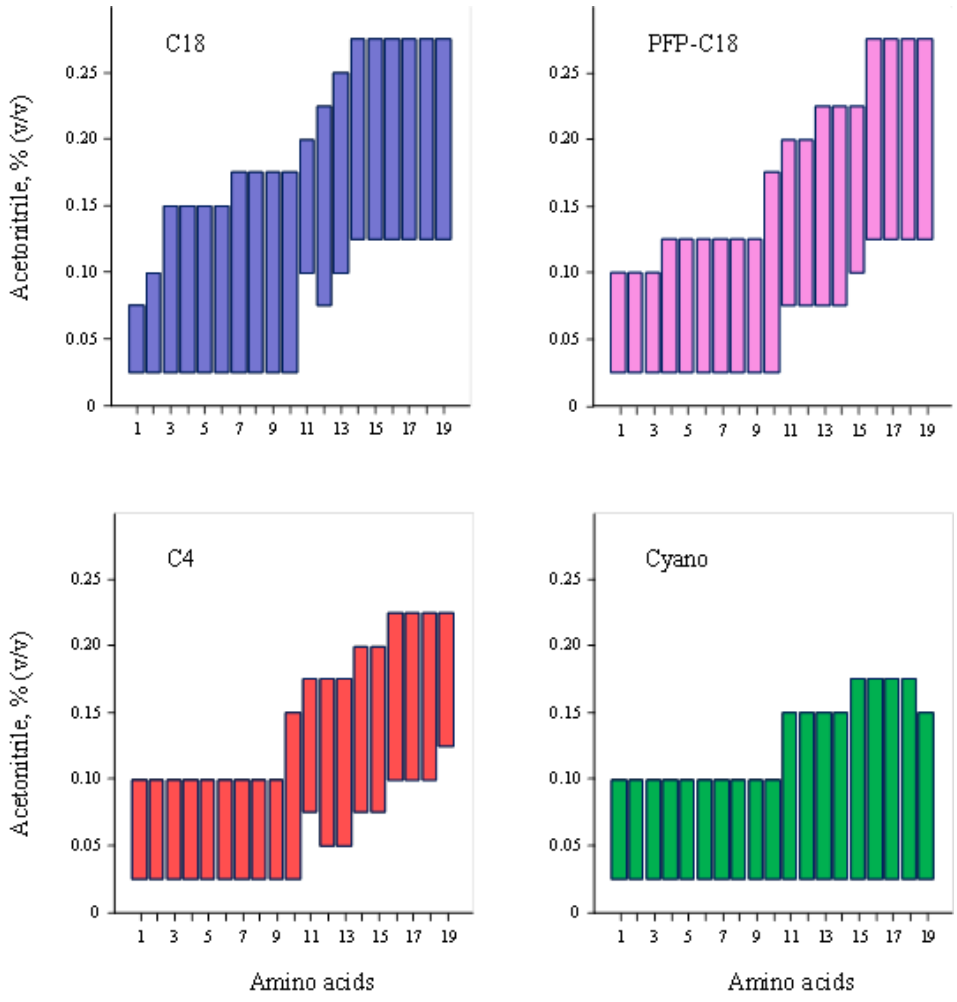

Figure 6.1. Acetonitrile concentration ranges in the isocratic mobile phases used for the modelling of the retention and peak shape of the isoindoles of amino acids. The number on the abscissa axis corresponds to the amino acid codes given in Section 6.3.1.

6.4.1.2. Accuracy of the retention model

Since nearly aqueous eluents were used (starting from 2.5% acetonitrile), the Neue-Kuss equation was selected for modelling the retention factor (k) of the isoindoles of amino acids [39]:

$$k = k_0 (1 + c\varphi)^2 e^{-\frac{B\varphi}{1+c\varphi}} \quad (6.1)$$

where k_0 is the retention factor extrapolated to a phase constituted only of water, c is a curvature parameter related to the presence of adsorption, and B a measurement of the elution strength, being thus related to the distribution of the solute between the stationary and mobile phases due to hydrophobic interactions.

The Neue-Kuss equation has demonstrated excellent performance in isocratic elution in wide domains of organic solvent, being one of the most accurate equations currently available for predicting the retention in RPLC. Other equations usually valid in RPLC were checked to produce a retention minimum at intermediate acetonitrile contents for some isoindoles, effect which is particularly evident for the logarithmic-quadratic model, since the fastest eluents produce nearly null retention. This behaviour was found detrimental for the predictions at high solvent contents, and for this reason, the logarithmic-quadratic model was discarded. The Neue-Kuss equation did not show these artefacts.

The retention data for each isoindole, eluted with mobile phases in the composition ranges indicated in Section 6.4.1.1., for each assayed column at fixed temperature (25 ºC) and flow-rate (1 mL/min), were fitted to Eq. (6.1). The fitting errors, calculated as:

$$\varepsilon = \frac{\sum \left| t_{R,i} - \hat{t}_{R,i} \right|}{\sum t_{R,i}} \times 100 \qquad (6.2)$$

are given in Table 6.1, together with the correlation coefficient of the fitted retention model, for each compound. In Eq. (6.2), $t_{R,i}$ and $\hat{t}_{R,i}$ are the experimental and predicted retention times. The use of this equation is justified by the fact that the prediction errors are related to values of very different magnitude. The mean fitting errors were 2.4%, 1.6%, 2.0% and 4.4%, for the C18, PFP-C18, C4 and cyano columns, respectively.

6.4.2. Resolution of the isoindoles of primary amino acids using single columns

The separation of the set of 19 amino acids, derivatised with OPA-NAC, was first tried using four 7.5 cm single columns of different nature (C18, pentafluorophenyl-C18, C4 and cyano) that were available in our laboratory. The modelling of the chromatographic behaviour described in Section 6.4.1., and the tools developed in Refs. [35,36], were applied to obtain the optimal separation conditions. The particular peak widths and asymmetries, and both resolution and analysis time, were considered along the optimisation. The predictions of the chromatographic performance (retention and peak shape) were carried out inside the whole studied range of mobile phase compositions (2.5–27.5% acetonitrile), for the four columns.

Table 6.1. Relative fitting errors calculated according to Eq. (6.2) and R^2 coefficient in the prediction of the retention time, for each amino acid and assayed column.

Amino acid	C18		PFP-C18		C4		Cyano	
	ε (%)	R^2	ε (%)	R^2	ε (%)	R^2	ε (%)	R^2
Aspartic acid	– [a]	– [a]	4.99	0.9234	– [a]	– [a]	0.04	0.9996
Glutamic acid	2.79	0.9965	4.91	0.9515	3.71	0.9730	0.13	0.9986
Asparagine	0.75	0.9999	0.79	0.9999	2.34	0.9933	0.41	0.9946
Serine	0.67	0.9999	0.95	0.9998	2.60	0.9908	0.62	0.9858
Glutamine	0.56	0.9999	0.87	0.9999	2.31	0.9967	0.99	0.9854
Histidine	0.42	0.9999	1.01	0.9999	1.35	0.9991	0.11	0.9967
Glycine	0.65	0.9999	1.13	0.9998	1.67	0.9980	0.67	0.9966
Arginine	0.37	0.9999	0.74	0.9999	0.95	0.9997	0.94	0.9907
Threonine	0.53	0.9999	0.02	1.0000	3.18	0.9936	0.96	0.9966
Alanine	0.57	0.9999	0.18	0.9999	0.70	0.9999	0.65	0.9987
Cysteine	0.89	0.9999	0.39	0.9999	0.66	0.9999	1.76	0.9995
Tyrosine	1.49	0.9997	0.73	0.9999	3.10	0.9983	0.95	0.9995
Valine	1.94	0.9996	4.88	0.9973	3.50	0.9976	9.47	0.9367
Methionine	2.26	0.9996	1.13	0.9999	0.97	0.9999	4.87	0.9888
Isoleucine	6.45	0.9969	3.48	0.9987	1.19	0.9998	4.54	0.9891
Tryptophan	6.41	0.9971	2.83	0.9992	1.98	0.9993	5.21	0.9916
Phenylalanine	0.49	0.9999	2.88	0.9991	2.13	0.9992	4.67	0.9870
Leucine	0.55	0.9999	2.97	0.9992	2.13	0.9993	3.79	0.9923
Lysine	4.42	0.9983	2.05	0.9996	5.24	0.9946	7.81	0.9836
Mean error	2.37	0.9990	1.63	0.9999	2.04	0.9994	4.43	0.9929

[a] The retention time of the isoindole was close to the dead time.

The global resolution was measured as peak purity (area fraction free of overlapping, *P*) [23]. This is a resolution measurement with an excellent behaviour, which ranges between 0 for full overlapping to 1 for full resolution. Among other advantages, the individual nature of the peak purities and their intrinsic normalisation facilitate their combination for quantifying the resolution in the whole chromatogram. In this work, the global resolution was calculated as the product of purities for all peaks in a chromatogram.

The performance of the expected separation along the mobile phase composition range examined in this work, using the four columns, can be visualised in Fig. 6.2, where the resolution diagrams for the isocratic elution are depicted. As can be seen, none of the assayed columns is capable of fully resolving the mixture of amino acids. However, the predicted resolutions and mobile phase compositions for the mixture of isoindoles using isocratic elution must be taken with caution, since they are based on extrapolated retentions for certain composition ranges, particular for each amino acid (see Fig. 6.1).

In the case of amino acids, gradient elution is required to decrease the analysis time. By attenuating the extreme differences in retention behaviour, the weight of the extrapolated retentions is reduced, which is translated in more reliable predictions. The optimal gradient separations predicted for the C18, PFP-C18 and C4 single columns are depicted in Fig. 6.3. As observed, the predicted resolution is still rather poor (P = 0.372, 0.189 and 0.010, respectively). The chromatogram for the cyano column is not given due to its extremely poor resolution (P was close to zero for all assayed gradients).

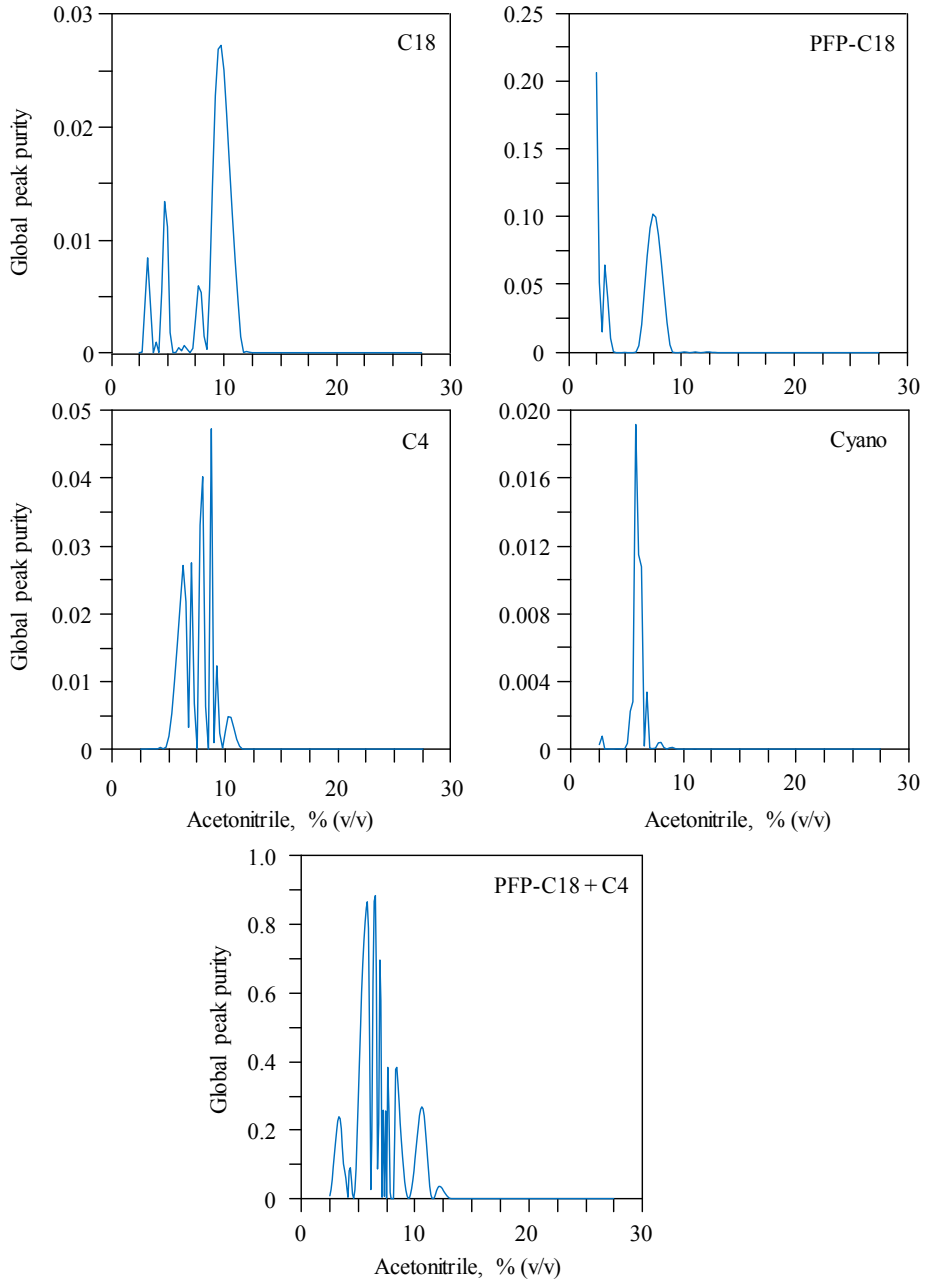

Figure 6.2. Resolution diagrams for the isocratic elution of a mixture of the isoindoles of the 19 primary amino acids, using the 7.5-cm single C18, PFP-C18, C4 and cyano columns, and the combination of 7.5 cm PFP-C18 + 7.5 cm C4.

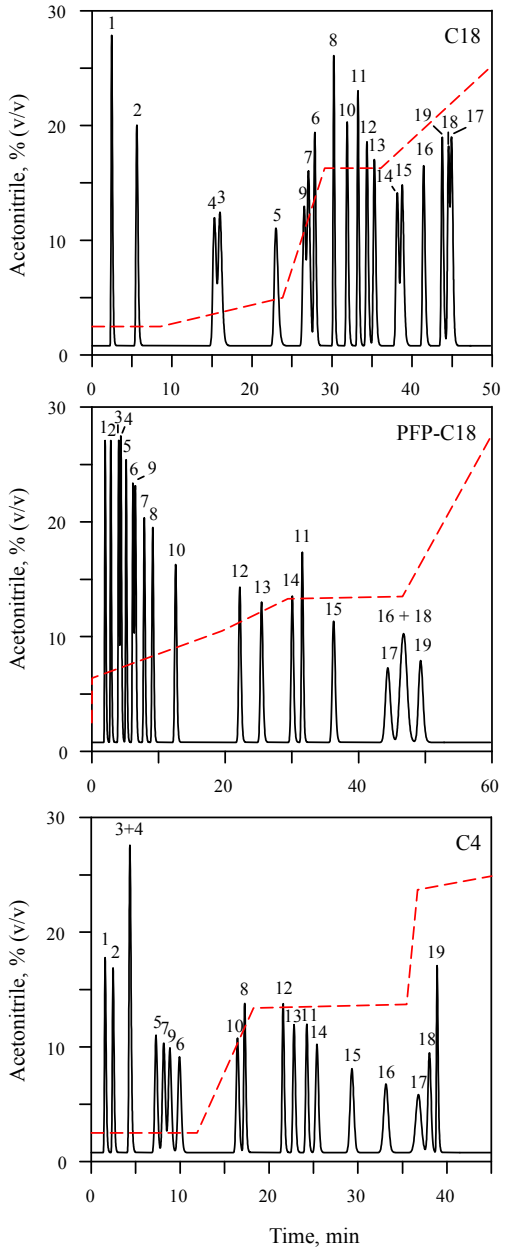

Figure 6.3. Optimal chromatograms for the separation of the isoindoles of the 19 primary amino acids, using multi-linear gradients and the single C18, PFP-C18 and C4 columns. The applied gradients are depicted on the chromatograms.

6.4.3. Resolution of the isoindoles with serially-coupled columns

None of the 7.5 cm conventional columns available in our laboratory was able to resolve satisfactorily the mixture of primary amino acids. This means that each stationary phase by itself did not offer the appropriate interactions to succeed in the separation. However, as shown below, it was possible to find combinations of coupled columns, whose interactions cooperate to improve the separation. Fortunately, no additional experiment is required for the coupled columns optimisation, since the same information on the chromatographic behaviour obtained for each single column (Section 6.4.1) is needed. In this work, we selected first the best column combination in isocratic elution, and then find the best gradient [35,36].

6.4.3.1. Search of the most convenient column combination in isocratic elution

As mentioned, in our laboratory there was only one column length available for each assayed stationary phase (7.5 cm). Nevertheless, we decided to inspect *in silico* the results that could be obtained by combining other lengths to build serially-coupled columns, which would be translated into a richer variety in the ratio of interactions. This study allowed establishing a reference of comparison for appraising the quality of the results found by combining the available 7.5 cm columns. Thus, this initial *in silico* search took into account not only the nature, but also the length of the coupled columns, together with the acetonitrile content in the mobile phase. We considered lengths in the range from 0 to 20 cm, stepped in 2 cm intervals for each column. The search was constrained imposing a maximal back-pressure of 275 bar, and a maximal length of the column combinations of 25 cm.

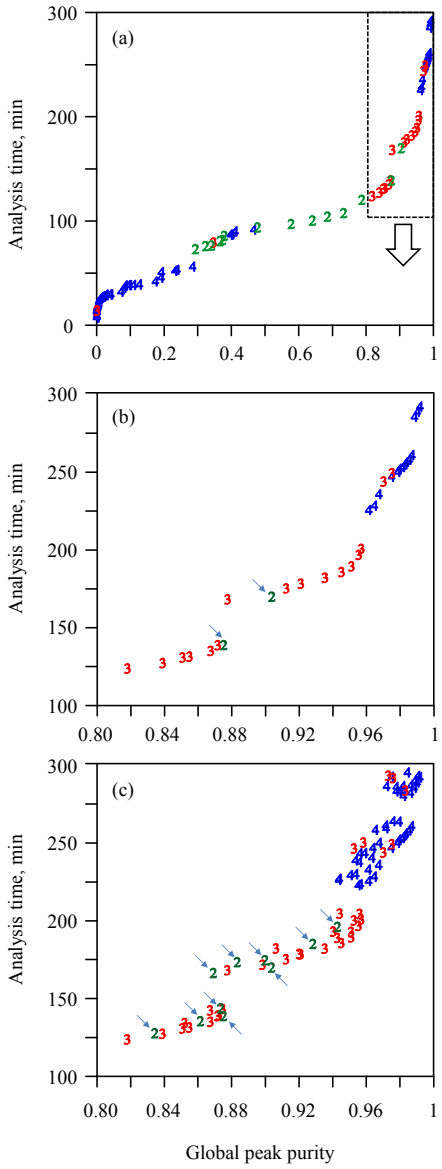

Figure 6.4. (a) Pareto front in isocratic elution for serial combinations of columns, (b and c) depict the magnified high resolution region; (c) shows also neighbour solutions to the Pareto front. The numbers on the plots indicate the number of columns that are combined. The solutions implying only two columns are marked with an arrow.

The optimal solutions (performance of different column combinations and acetonitrile content in the isocratic mobile phase) are shown in Fig. 6.4 as Pareto plots. Each point in the plots represents the analysis time and resolution for a given separation condition. The labels (2, 3 and 4) indicate the number of combined columns (the identity of the columns and mobile phase for the most interesting solutions is discussed below). The plots in Fig. 6.4a and b show only those solutions constituting the so-called Pareto front, namely, those solutions where the resolution cannot be improved without increasing the analysis time [40]. The solutions above the boundary constituting the Pareto front (not shown) would not be optimal. As observed, the highest predicted resolution (which was close to $P = 1$) corresponded to the combination of the four columns: C18, PFP-C18, C4, and cyano, although there were acceptable solutions with only two combined columns.

As commented in the introduction, the aim of this work was to investigate whether the combination of only two columns of fixed length could yield satisfactory resolution. Such combinations are simpler, and in addition, predictions should be less susceptible to error than the solutions implying a higher number of columns. As commented above, the range of polarity of the studied amino acids is rather large, making a common domain of organic solvent for all amino acids not possible (see Fig. 6.1). Thus, some predictions may imply extrapolations, compromising the accuracy.

Fig. 6.4b shows that in the region of higher resolution, between 0.8 and 1, there are only two combinations involving two columns that belong to the Pareto front. Fig. 6.4c plots together with the solutions constituting the Pareto front, those located in its neighbourhood. Although these solutions are not strictly Pareto-optimal, they still offer convenient resolution. Such results

indicate that there are multiple solutions with a low number of columns from which making the choice.

Considering all solutions involving the combination of only two columns, under the established conditions, we found 48 offering optimal resolution with $P > 0.85$, which correspond to chromatograms with sufficiently good separation among all peaks. The most interesting solutions, with $P > 0.94$, were the following (column combination, optimal acetonitrile content, global resolution and analysis time are given): 8 cm PFP-C18 + 6 cm C4 and 8% acetonitrile ($P = 0.943$, 196 min); 10 cm PFP-C18 + 2 cm C4 and 6.8% acetonitrile ($P = 0.941$, 297 min); and 12 cm PFP-C18 + 8 cm C4 and 8.3% acetonitrile ($P = 0.942$, 271 min). As observed, all these solutions correspond to the same combination: PFP-C18 + C4, which vastly outperforms the resolution obtained with each of the columns that are coupled (PFP-C18 and C4, see Fig. 6.2).

One of the column combinations in the upper region of the Pareto plot, close to the front, was 8 cm PFP-C18 + 8 cm C4. It was expected that the resolution for the combination of the 7.5 cm columns should be similar. Indeed, we found that the estimated resolution for the 7.5 cm PFP-C18 + 7.5 cm C4 column was $P = 0.882$. The resolution diagram in Fig. 6.2 (bottom) shows that the optimal isocratic separation with this column combination was obtained using 6.5% acetonitrile.

6.4.3.2 Optimisation of gradient elution

The extrapolated analysis time (above 300 min) was still too high, in isocratic elution, with the selected combination of columns (7.5 cm PFP-C18 + 7.5 cm C4), as was also the case with the single columns. Therefore, we proceeded to perform the optimisation of gradient elution to decrease the analysis time. We first applied a simple linear gradient of acetonitrile. This type of gradient requires defining the search by selecting the initial and final organic solvent content for the optimisation, besides the gradient time. Alternatively, one or two of these parameters can be fixed.

We investigated again the 2.5–27.5% acetonitrile range, and fixed the analysis time to 60 min. Fig. 6.5 shows the Pareto fronts and selected optimal chromatograms for the separation of the isoindoles of the 19 primary amino acids, using linear gradients. Although the column order in the serially-coupled columns should have no effect on the retention in isocratic elution, there are significant differences in gradient elution, since the range of acetonitrile content for each column is totally different [35]. Fig. 6.5a and b illustrates the separation obtained with the 7.5 cm PFP-C18 + 7.5 cm C4 combination, and Fig. 6.5c and d corresponds to the reversed order, the 7.5 cm C4 + 7.5 cm PFP-C18 combination. The resolution is higher when the PFP-C18 column is located before the C4 column (P = 0.912 versus 0.727), but at the expense of somewhat longer analysis time. Although the analysis time was indeed decreased with regard to isocratic elution, it remained still too high. Thus, we decided to apply multi-linear gradients to reduce further the separation between the peaks eluting at close times.

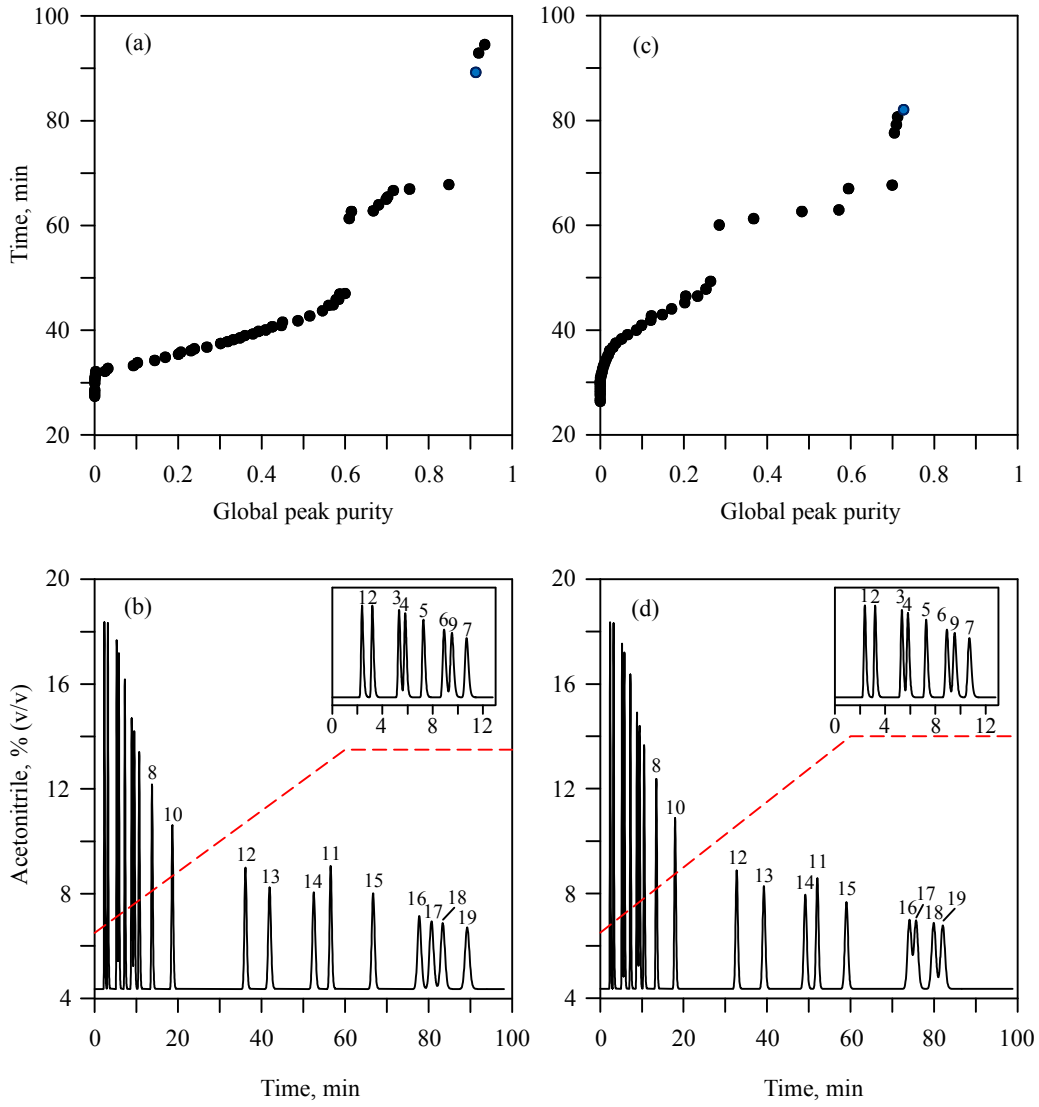

Figure 6.5. Pareto fronts (a,c), and selected optimal chromatograms (b,d), for the separation of the isoindoles of the 19 primary amino acids using coupled columns and linear gradients: (a,b) 7.5 cm PFP-C18 + 7.5 cm C4 (P = 0.912), (c,d) 7.5 cm C4 + 7.5 cm PFP-C18 (P = 0.727). Selected acetonitrile gradient (depicted on the chromatogram): (b) 6.5% to 13.5% in 60 min, and (d) 6.5% to 14.0% in 60 min.

The optimisation of multi-linear gradients is appreciably more complex, but has the advantage of the extra reduction in the analysis time. In this case, not only the initial and final organic solvent content may be optimised, but also the coordinates of the nodes along the gradient and the total analysis time. We decided to fix the final organic solvent content (27.5%), and the analysis time (60 min), and investigate only the position of the initial acetonitrile content and the coordinates of the nodes inside the 5.0–27.5% range. The position of each node was restricted so that even distributions of the nodes in the time domain were favoured. Also, we considered a high enough number of nodes, so that the final concentration and/or time could be corrected throughout the optimisation (Fig. 6.6). The optimal chromatograms and multi-linear gradients found for both column orders (7.5 cm PFP-C18 + 7.5 cm C4 and 7.5 cm C4 + 7.5 cm PFP-C18) are depicted in Fig. 6.6a and c, respectively. The expected resolution and analysis time were $P = 0.885$ and 58 min for the former combination, and $P = 0.740$ and 57 min for the latter.

The accuracy of the predictions can be checked by comparing the predicted (Fig. 6.6a and c) and experimental (Fig. 6.6b and d) chromatograms. The experimental chromatograms were obtained by injecting a sample containing the 19 isoindoles (see Section 6.3.2.). It can be seen that the peaks for all amino acids were well resolved and the predictions were satisfactory, except for lysine (peak 19). The experimental peak for this amino acid appeared at longer time than expected, especially in the chromatogram shown in Fig. 6.6b. This had no consequence in the resolution, since lysine is usually the most retained compound. Also, the peak of lysine did not overlap that of leucine (the previous peak), in any case.

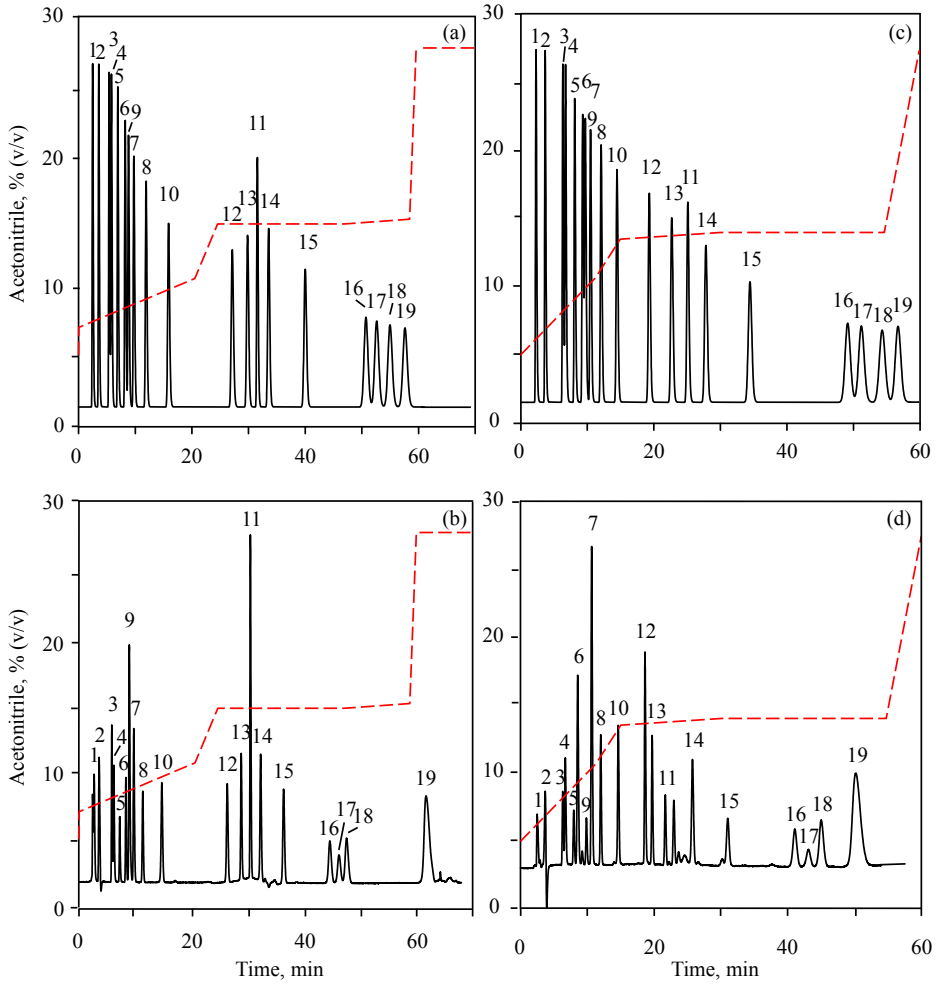

Figure 6.6. Predicted (a,c) and experimental chromatograms (b,d) for the separation of the isoindoles of the 19 primary amino acids, using coupled columns and multi-linear gradients: (a,b) 7.5 cm PFP-C18 + 7.5 cm C4 (P = 0.885), (c,d) 7.5 cm C4 + 7.5 cm PFP-C18 (P = 0.740). Selected acetonitrile gradient: (a, b) 5% to 7% at 0.1 min, 10.6% at 20.7 min, 14.7% at 24.7 min up to 47.2 min, 15% at 58.8 min, and 27.5% at 60 min, and (c, d) 5% to 10.7% at 11.3 min, 13.6% at 15 min, 14.1% at 30.1 min up to 54.7 min, and 27.5% at 60 min.

The origin of this discrepancy (as demonstrated in subsequent experiments) is the degradation of the lysine isoindole. Indeed, the retention time of the peak for lysine was observed to increase when the time between the formation of the isoindole and the injection increased. Also a longer delay between the injection and detection gave rise to a larger peak shift. Note that in Fig. 6.6d, where the peak shift for lysine is smaller, the analysis time is about 10 min smaller with regard to Fig. 6.6b. Also, for this chromatogram, the elapsed time between the derivatisation and injection was minimal (2 min).

To study the reproducibility of the analysis, nine identical runs were carried out, where the mixture containing ca. 2.0×10^{-5} M of each amino acid was derivatised with OPA-NAC, as described in Section 6.3.2. The solutions of the isoindoles were injected immediately after formation, and eluted using the 7.5 cm PFP-C18 + 7.5 cm C4 coupled column and the gradient program described in Fig. 6.6b. The mean retention times and reproducibilities are shown in Table 6.2. The relative standard deviation for the retention time was below 1.7% except for lysine, which was 3.3%.

Table 6.2. Reproducibility (n = 9) in retention times of isoindoles, using the 7.5 cm PFP-C18 + 7.5 cm C4 serial combination and the optimised multi-linear gradient (Fig. 6.6b).

Compound	t_R (min) [a]	Compound	t_R (min) [a]
Aspartic acid	2.45 ± 0.02	Cysteine	30.5 ± 0.3
Glutamic acid	3.61 ± 0.05	Tyrosine	26.4 ± 0.6
Asparagine	5.87 ± 0.09	Valine	28.9 ± 0.6
Serine	6.234 ± 0.103	Methionine	32.3 ± 0.4
Glutamine	7.27 ± 0.13	Isoleucine	36.4 ± 0.6
Histidine	8.37 ± 0.15	Tryptophan	44.6 ± 0.7
Glycine	9.8 ± 0.2	Phenylalanine	46.3 ± 0.8
Arginine	11.4 ± 0.2	Leucine	47.6 ± 0.8
Threonine	9.0 ± 0.2	Lysine	60 ± 2
Alanine	14.8 ± 0.3		

[a] Mean values of retention times and standard deviations are given.

6.5. Conclusions

Interpretive optimisation is the most efficient way for finding the best separation conditions in liquid chromatography. It highly reduces the required time to get the optimal conditions, and the found solutions are more reliable with respect to the still usual "trial and error" strategies. In this work, we show a complex optimisation problem, where the range of organic solvent is highly dependent on the eluted compound, due to the large diversity in the polarities of the analytes in the sample.

Nearly aqueous mobile phases, with organic solvent contents as small as 2.5%, were needed to elute with enough retention the most polar derivatives, especially for the columns showing weak retention. It was found that the Neue-Kuss equation yielded the best predictions for all columns. However, still there was a problem with the prediction of the retention times of amino acids using isocratic elution, owing to the extreme variations in retention and the fact that the retention in specific regions for each amino acid should be extrapolated. This was even more problematic for the serially-coupled columns, since the uncertainties in the predictions are larger. This problem was alleviated using gradient elution, which is needed to decrease the analysis time of amino acids. As observed, the predictions obtained in this elution mode were satisfactory.

The research reported in this work shows that although it was not possible to solve the mixture of amino acids using isolatedly any of the conventional columns available in our laboratory, the expectancies of resolution increased substantially when two of these columns were combined. An interesting result is that the combination of the interactions of the PFP-C18 and C4 columns resulted in a much better resolution of the isoindoles of amino acids with regard to each of these columns when used isolatedly. Also, the resolution was improved with regard to a C18 column. However, it was not possible to further

decrease the analysis time below 50 min, owing to the close elution of the isoindoles of the most retained amino acids (tryptophan, phenylalanine, leucine and lysine), which exhibit similar polarity and wider peaks. Naturally, with more sophisticated instrumentation (UHPLC) and columns containing smaller particles, similar separations should be achieved in a smaller analysis time.

On the other hand, the requirement of having columns of multiple lengths to build hybrid columns can be less critical in other separation techniques (e.g., capillary electrochromatography or nanochromatography), which are attractive due to the possibility of miniaturisation and integration of analytical techniques. In this case, the length can be modulated by cropping the capillary columns, and the nature can be tailor-made by modifying the stationary phase chemistry, even along the column during the synthesis [41–43].

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PART 2

DEVELOPMENT OF NEW CHROMATOGRAPHIC OBJECTIVE FUNCTIONS

CHAPTER 7

A CHROMATOGRAPHIC OBJECTIVE FUNCTION TO CHARACTERISE CHROMATOGRAMS WITH UNKNOWN COMPOUNDS OR WITHOUT STANDARDS AVAILABLE

7.1. Abstract

Getting useful chemical information from samples containing many compounds is still a challenge to analysts in liquid chromatography. The highest complexity corresponds to samples for which there is no prior knowledge about their chemical composition. Computer-based methodologies are currently considered as the most efficient tools to optimise the chromatographic resolution, and further finding the optimal separation conditions. However, most chromatographic objective functions (COFs) described in the literature to measure the resolution are based on mathematical models fitted with the information obtained from standards, and cannot be applied to samples with unknown compounds. In this work, a new COF based on the automatic measurement of the protruding part of the chromatographic peaks (or peak prominences) that indicates the number of perceptible peaks and global resolution, without the need of standards, is developed. The proposed COF was found satisfactory with regard to the peak purity criterion when applied to artificial peaks and simulated chromatograms of mixtures built using the information of standards. The approach was applied to mixtures of drugs containing unknown impurities and degradation products and to extracts of medicinal herbs, eluted with acetonitrile-water mixtures using isocratic and gradient elution.

7.2. Introduction

A main goal in the development of an analytical procedure in liquid chromatography (LC) is getting the maximal resolution through the fine-tuning of the experimental conditions, with the assistance of an optimisation protocol. When an analytical sample contains a large number of compounds, the application of trial and error assays to get complete resolution can be time-consuming, expensive, or even unfeasible and, in addition, without any guarantee of success. Computer-based methodologies are currently the most efficient tools to search the optimal separation conditions in LC [1–4]. A practical way for determining such conditions is the use of global measurements to appraise the separation quality, using chromatographic objective functions (COFs) [5–8].

Several approaches have been proposed to find out the conditions offering the best separation in situations of extremely low chromatographic resolution, where classical COFs are not able to detect even baseline resolved peaks. These approaches are based on counting the peaks in the chromatogram [9–12], and are focused on the well resolved peaks, in contrast to conventional COFs that attend mainly to those least resolved. This means that they are oriented to quantify the degree of success in the separation, and not the failure (as classical COFs do). The most popular of such approaches was proposed by Berridge [9], who defined a COF combining the number of detected peaks, a term that accounts for the Snyder's resolution between adjacent peak pairs (R_S) (see Section 7.3.1), and a term penalising long analysis times, in a weighted summation.

More recently, we proposed an approach that allows the optimisation of the resolution level of chromatograms in cases where conventional global criteria, such as the worst resolved peak pair or the product of elementary resolutions

are not able to detect any separation [10,12]. The approach makes use of a function that counts the number of peaks exceeding a given threshold of peak purity (i.e., the well resolved peaks). The experimental conditions able to resolve the same amount of peaks were discriminated by either quantifying the partial resolution of those peaks that exceeded the established threshold, or by improving the separation of peaks below the threshold.

A higher level of complexity in the optimisation of the chromatographic resolution is represented by samples containing unknown compounds (some or even all), or without standards available. This is the case of chromatographic fingerprints [11,13–15]. Most COFs reported in the literature are based on mathematical models that should be fitted with the chromatographic information obtained from standards through design of experiments. Therefore, they cannot be applied to this type of sample, or are hardly adapted in situations when only a few standards are available. Trying to find a solution to optimise the resolution of fingerprints of environmental samples, Duarte and Duarte suggested a measurement based on a valley-to-peak ratio to assess the resolution [11]. The approach presents, however, certain limitations when the chromatograms contain peaks showing far different heights and/or significant baseline noise. In this work, we propose a different COF to face these situations, which is based on the automatic measurement of the peaks, which is applicable to experimental highly complex chromatograms. The approach, which is related to the peak purity, attends to the protruding part of each visible peak with regard to the valleys that delimit it. The performance of the new COF is evaluated with regard to the peak purity for cases of study of increasing complexity.

7.3. Theory

7.3.1. Classical approaches to measure the elementary resolution

Elementary resolution criteria describe the separation between two solutes exhibiting consecutive peaks, or each particular solute from all other solutes in the sample [1–8,16–20]. For this purpose, several COFs of diverse complexity have been proposed. The simplest one, which only considers the peak position, is the selectivity factor:

$$\alpha_{i,i+1} = \frac{k_{i+1}}{k_i} \tag{7.1}$$

where k_i and k_{i+1} (with $k_{i+1} > k_i$) are the retention factors of two consecutive peaks. Other COFs consider the width of the individual peaks, or the full profile including the size. The Snyder's resolution R_S is the most popular [1,7,8]:

$$R_S = \frac{t_{R,i+1} - t_{R,i}}{B_i + A_{i+1}} \tag{7.2}$$

where $t_{R,i+1}$ and $t_{R,i}$ are the retention times of two consecutive peaks, and B_i and A_{i+1} are the right and left half-widths for peaks i and i+1, respectively.

Other interesting COFs measure different types of valley-to-peak ratios, which require locating a point in the chromatogram related to the valley between two consecutive peaks (which has been called the "valley point") (Fig. 7.1a) [20]. This point is not necessarily the minimum in the valley. The following equation is applied:

$$\nu_{i,i+1} = 1 - \frac{h_v}{h_T} \tag{7.3}$$

where h_v is the signal height (measured from the baseline) at the time of the valley point, and h_T the height measured at this time from the baseline to the

straight-line obtained by joining the maxima of the two neighbouring peaks. Fig. 7.1a illustrates three types of valley-to-peak ratio measurements. In the first measurement (using h_{v1} and h_{T1}), the valley point is straightforwardly found by locating the minimal height between the two consecutive peaks [21]. This definition is similar to the ratio proposed by Duarte and Duarte for the quantification of the resolution of soil fingerprints [11].

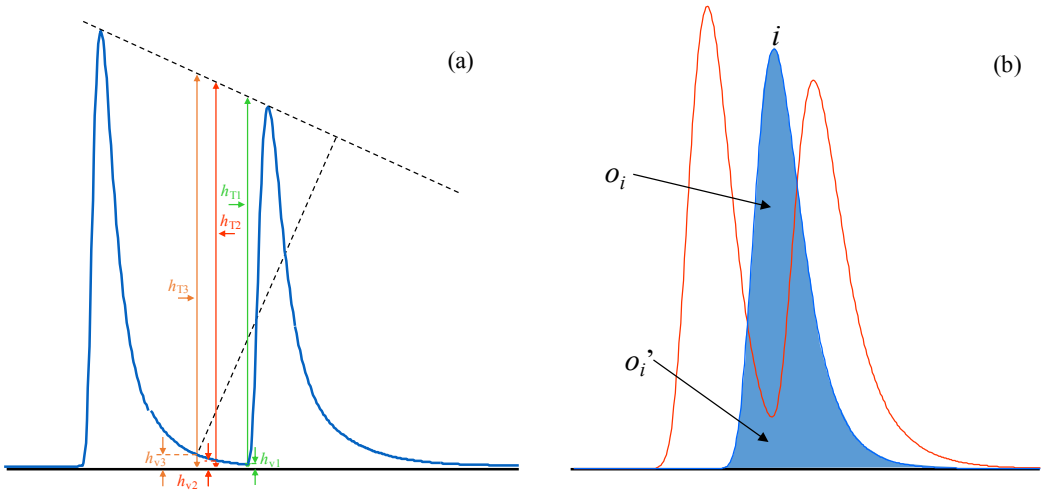

Figure 7.1. Chromatogram illustrating the parameters needed to calculate: (a) valley-to-peak ratios according to three different criteria (V_1, V_2 and V_3, see Eq. (7.3) and text), and (b) the peak purity (Eq. (7.4)).

7.3.2. Peak purity

Different COFs may score the same chromatogram differently, which brings as a consequence the selection of different chromatograms as optimal. However, the analyst may easily discriminate the resolution performance and find some chromatograms more suitable than others. The reason of these differences is that most COFs are too simple to account for all the features that the analyst considers in the assessments of the separation quality. However, some COFs may satisfactorily correlate to the analyst's appraisal of good resolution. An example of such a COF is the peak purity (Fig. 7.1b) [1–3,19]:

$$p_i = 1 - \frac{o_i'}{o_i} \tag{7.4}$$

where o_i' is the area under the peak overlapped by a hypothetical chromatogram built with the peaks of the accompanying compounds in the sample, and o_i the total peak area. A similar COF was proposed early by Schoenmakers [1], although it was scarcely applied since it required the knowledge of both the position and profile for each peak in the chromatogram. Therefore, its calculation was laborious and needed numerical computation. Nowadays, this is not a problem.

The peak purity offers unique features:

(i) It correlates with the information the analyst wishes: the interference level.

(ii) It accounts the overlapping in the whole peak providing a realistic evaluation of the separation, since the full signal including profile and size is monitored.

(iii) It is a normalised function, which facilitates the combination of the peak measurements in the whole chromatogram, and the inclusion of other quality criteria.

(iv) It qualifies individual peaks, instead of peak pairs, which makes peak weighting and exclusion simpler, and avoids identity problems related to peak reversals. This feature has enabled the proposal of new optimisation strategies, based on information not accessible using other COFs.

7.3.3. Proposal of a new COF

The objective of this work is the proposal of a new COF able to score the performance of chromatograms of samples where some or all compounds are unknown, or where standards are not available, making the application of the peak purity criterion unfeasible. The new COF is also valid for situations where all compounds are known. We have called this COF "peak prominence", since it quantifies the protruding part of each peak in a chromatogram with regard to the valleys that delimit it, or incidentally the baseline (see Figs. 7.2a and Fig. 7.3). The resolution level can be calculated as the prominence ratio:

$$\mathrm{pr}_i = \frac{a_{\mathrm{pr},i}}{a_i} \tag{7.5}$$

where $a_{\mathrm{pr},i}$ is the area of the protruding part of the peak (Fig. 7.2a), and a_i represents its total area (Fig. 7.2b). Alternatively, a peak height ratio can be used.

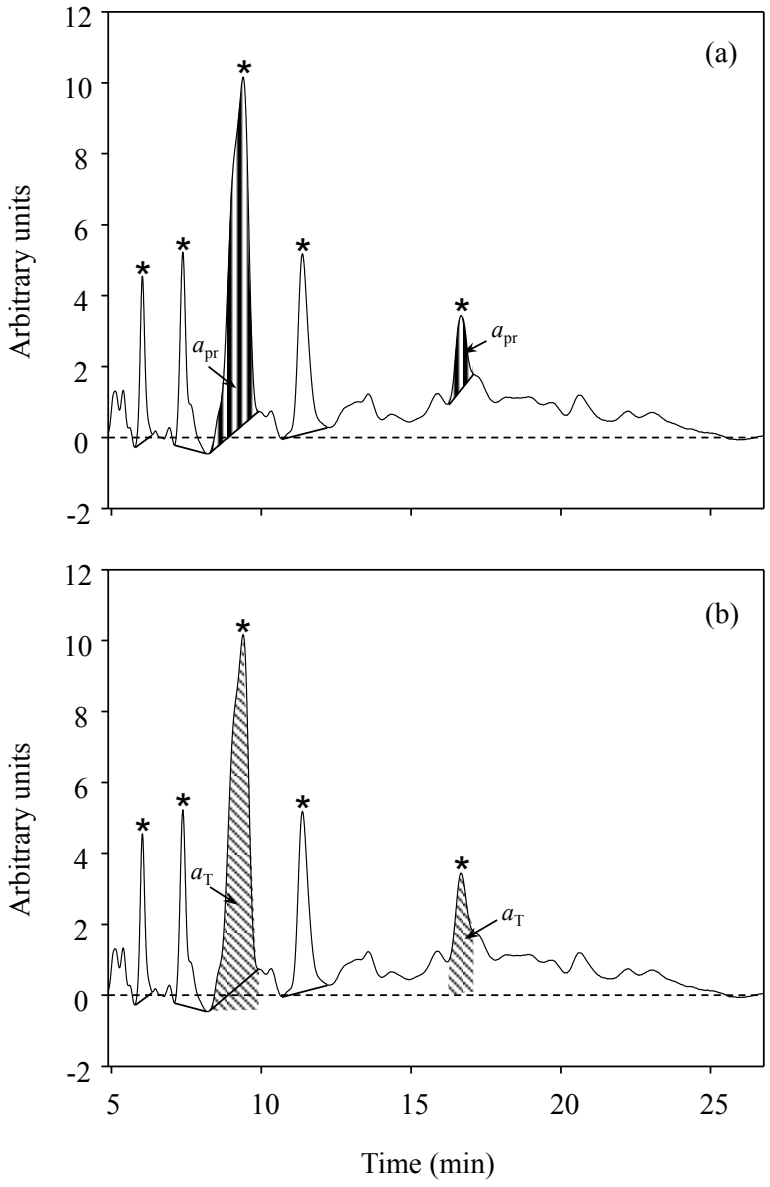

Figure 7.2. Chromatogram illustrating the way peak prominence areas (a), and total peak areas (b), are measured to calculate Eq. (7.5).

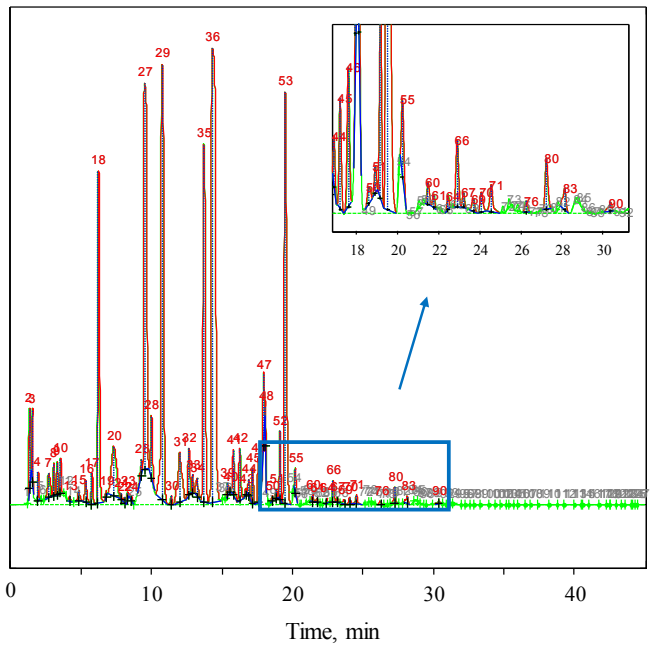

Figure 7.3. Fragment of a fingerprint of chamomile showing peaks that exceeded the selected threshold (marked with a number). Tangents that define the prominence region for each peak are depicted.

7.3.4. Global resolution

There is a significant difference between the new approach and the conventional valley-to-peak ratio criteria described in Section 7.3.1. The latter depend on the measurement of two neighbouring peaks and the valley in between, whereas the proposed COF is based on the measurement of one peak between two valleys (or the baseline). The peak prominence shares the good features of the peak purity: it is sensitive to signal profile and size, it is an easily interpretable normalised measurement, and it scores individual peaks, which facilitates the combination of the elementary resolution and peak counting. However, it offers important differences: it is straightforwardly

applicable to experimental chromatograms, it does not require standards, and it attends to all visible peaks, each one corresponding to one or more underlying compounds.

The global separation in a chromatogram is evaluated by reducing the elementary resolutions (r_i) to a single value (R) depicting the overall separation [1,19,22]. In the most widely applied strategy, due to its simplicity, the worst elementary resolution is searched. In other cases, the optimisation is extended to more peaks, by combining the respective elementary resolutions. In this work, the non-normalised product was applied to calculate the global peak purity:

$$R_1 = \prod_{i=1}^{n} r_i \tag{7.6}$$

where n represents the number of individual peaks. Owing to the type of situations where the peak prominence criterion is aimed to be used (i.e., highly complex multi-analyte samples), the global resolution was calculated according to:

$$R_2 = \left(\prod_{i=1}^{n_{\mathrm{pr}}} \frac{a_{\mathrm{pr},i}}{a_i} \right)^{1/n_{\mathrm{pr}}} \tag{7.7}$$

where n_{pr} is the number of detected peaks exceeding a pre-established threshold. R_1 and R_2 vary between 0 (when at least one peak is fully overlapped) and 1 (when all peaks are baseline resolved). The closer the function to unity, the better the separation.

It should be noted that Eqs. (7.6) and (7.7) tend to be dominated by the peaks exhibiting low resolution. This means that an optimisation based on these products will discard quickly conditions where two or more peaks remain

unresolved. Meanwhile, a resolution criterion based on the separation of the critical peak may seem reasonable, but it is insensitive to the resolution reached by the remaining peaks. Therefore, chromatograms with similar resolution scores may exhibit significant differences for the non-critical peaks. Hence, criteria attending to all peaks are preferable.

The evaluation of the information provided by the chromatograms of complex mixtures of unknown compounds requires considering other features, such as the number of perceptible peaks (i.e., the peak count) [9–12]. The analysis time is also useful to appraise the results, but it has not been considered in this work.

7.4. Experimental

7.4.1. Reagents, experimental conditions and columns

Two sets of probe compounds and samples of medicinal herbs were studied:

(i) The first set consisted of six compounds: acebutolol, atenolol, carteolol, celiprolol, metoprolol, and pindolol, purchased from Sigma (St. Louis, MO, USA) and Rhône-Poulenc Rorer (Alcorcón, Madrid). Stock solutions containing 100 μg/mL were prepared in nanopure water obtained with a Barnstead water purification system from Thermo Scientific (Dubuque, IA, USA), assisted by an ultrasonic bath from Elmasonic (Singen, Germany). These solutions were diluted with water up to approximately 20 μg/mL, before analysis. The analyses were carried out with a 4.6 mm×150 mm Zorbax Eclipse XDB-C18 analytical column supplied by Agilent.

(ii) The second set consisted of 30 compounds with different acid-base behaviour (acidic, basic, neutral and amphoteric), and were analysed with a polymeric column with an extended pH range [10].

(iii) Extracts from three types of medicinal herbs (horsetail, decaffeinated tea and chamomile), purchased in small bags in a local supermarket, were analysed with the Zorbax Eclipse XDB-C18 column.

The separations were carried out with isocratic mobile phases and gradients prepared with HPLC grade acetonitrile from Scharlau (Barcelona), 0.01 M anhydrous sodium dihydrogen phosphate from Sigma (Roedermark, Germany) to buffer the pH, and nanopure water. For the first set of probe compounds and the extracts of medicinal herbs, the pH was set at approximately 3 with the aid of 0.01 M HCl and 0.01 M NaOH from Scharlau. For this purpose, a pH-meter from Crison (Model MicropH 2002, Barcelona), and a combined glass electrode from Orion (Model 8102, Barcelona) containing Ag/AgCl reference electrodes with 3.0 M KCl aqueous solution as salt bridge, were used. The data for the second set of probe compounds, which were eluted isocratically with acetonitrile-water at several pH values, were taken from our laboratory database. The corresponding experimental details can be consulted in Ref. [10].

7.4.2. Preparation of the extracts of medicinal herbs

The extracts of medicinal herbs to be injected into the chromatograph were prepared following the recommendations by Dumarey et al. [23]. First, 0.5 g of each herb was ground. Then, an infusion was prepared by treating 0.2 g with 20 mL of boiling nanopure water. The extract was filtered through a 0.2 μm membrane filter (Pall Gelman Laboratory, Karlstein/Main, Germany) to fill the 2 mL vials of the autosampler for chromatographic analysis.

7.4.3. Apparatus and software

The chromatographic analyses were carried out with an Agilent (Waldbronn, Germany) liquid chromatographic system, equipped with the following modules: a quaternary pump run at 1 mL/min, an autosampler, and a multiple-variable wavelength UV-visible detector (Series 1200). A temperature controller (Series 1100) set at 25 ºC was also used. The injection volume was 10 μL. The probe compounds were detected at 225 nm, and the fingerprints at 210 nm. The system was controlled by an OpenLAB CDS LC ChemStation (Agilent B.04.03).

The routines for data treatment were developed in MATLAB 2010b from The MathWorks Inc. (Natick, MA, USA).

7.5. Results and discussion

The aim of this study was to propose and evaluate an automatic algorithm to measure the separation performance of highly complex chromatograms containing unknown compounds, such as mixtures of drugs with unknown impurities and degradation products, or chromatographic fingerprints from extracts of medicinal herbs. The new approach is based on the measurement of peak prominences (Section 7.3.3), and was evaluated by scoring simulated chromatograms in comparison with the peak purity criterion, which in previous work has been demonstrated to offer an excellent performance [3,10,12,24–26]. However, the peak purity is only accessible when the standards for all compounds in a sample are available. Therefore, the comparison study of the peak prominence and peak purity criteria was performed by processing simulated chromatograms within a range of overlapping/resolution situations, built with artificial peaks or obtained with the chromatographic information

from probe compounds. In a next step, the peak prominence criterion was applied to real samples: a solution containing six drugs and unknowns of diverse origin, and extracts of medicinal herbs.

7.5.1. Inadequacy of classical COFs to quantify chromatograms with unknown compounds

As commented, most COFs in the literature are based on information obtained from standards. Thus, for instance, the peak purity criterion (Eq. (7.4)) requires the identity of the peaks being evaluated and the chromatogram of their interferences (Fig. 7.2b). Something similar happens with the R_S criterion (Eq. (7.2)). Modelling the chromatographic behaviour of standards allows the prediction of the signals for each compound in a sample, for a variety of experimental conditions (most of them non-assayed). By combining appropriately these signals for all compounds for a given condition, simulated chromatograms of the mixture can be obtained, for which the resolution is evaluated.

Strictly, the above COF definitions are not applicable when there are no standards available. However, the R_S criterion and other COFs, such as the selectivity factor (Eq. (7.1)), or the valley-to-peak criteria (Eq. (7.3)), can be applied directly to an experimental chromatogram, without any prior knowledge of the underlying signals, although the results can be highly unreliable, since the measurements on the overlapped peaks can be subjected to a high experimental error. Moreover, the selectivity factor does not address the peak profile and size, and is not useful for complex samples. On the other hand, the R_S, selectivity factor and valley-to-peak criteria offer measurements associated to peak pairs, which give rise to discontinuities when peak reversals occur, besides the problem of the peak identity.

7.5.2. Evaluation of a COF based on the peak prominence

The performance of the new COF to characterise chromatograms with diverse overlapping degree, including unknown compounds, was evaluated through its application to cases of increasing complexity. As commented, peak purity scores were taken as reference. When the peak purity is applied to the optimisation of the separation of mixtures, a threshold of elementary purity, p = 0.95, is usually arbitrarily established to denote virtually full separation. Since our purpose is detecting peaks, a less demanding threshold is required. The peak count threshold in this work was taken as p = 0.5 (see Section 7.5.2.3), which represents an incipient, but still perceptible separation, for two peaks with the same height. This figure could be slightly increased if a more neat separation is wished.

The peaks should be first delimited to measure the peak prominences. For this purpose, an algorithm was built to locate automatically optimal points to find the tangent line that isolates a given peak by joining the valleys at each peak side (Fig. 7.2a and Fig. 7.3). The peak count was taken as the number of peaks whose prominence area exceeded 0.05% of the sum of prominence areas for all peaks in the chromatogram. This value was obtained from replicated chromatograms of real samples of different nature, and represents the threshold below which the relative prominence areas start to diverge among replicates. The peaks below this area threshold are processed in a non-reproducible way by the algorithm, and may correspond to minor impurities or degradation products, instrumental spikes or noise. Once the significant peaks have been found, the area for each prominence ($a_{\mathrm{pr},i}$, Fig. 7.2a) and the corresponding peak total area (a_i, Fig. 7.2b) are measured to compute the elementary resolution according to Eq. (7.5).

The cases of study in the evaluation of the new COF were the following: chromatograms built with artificial peaks (Section 7.5.2.1), and simulated chromatograms based on previous modelling of the chromatographic behaviour for six probe compounds eluted considering only one factor (Section 7.5.2.2), and for 30 probe compounds of different character (acidic, basic, neutral and amphoteric) eluted considering two factors (Section 7.5.2.3). Peak simulation was carried out according to the linearly-modified Gaussian model in the peak region, which was replaced by exponential decays for height ratios below 10% [27,28].

7.5.2.1. Case of study I: Crossing of artificial peaks

A comparative study was first carried out considering a simple case that consisted of chromatograms built by summing three artificial peaks. The retention times for two peaks with efficiencies (N) of 1950 and 1900, and asymmetries measured at 10% peak height (f) of 1.3 and 1.4, were fixed at 11 and 12 min, respectively. The third peak, with N = 2000 and f = 1.2, was gradually shifted between 7 and 15 min to create several overlapping situations.

Fig. 7.4 depicts the resolution diagrams obtained according to the peak purity (Fig. 7.4a) and peak prominence criteria (Fig. 7.4b). For both COFs, two types of scores were obtained: the number of peaks exceeding the threshold (the peak count) and the global resolution according to Eqs. (7.6) and (7.7), respectively.

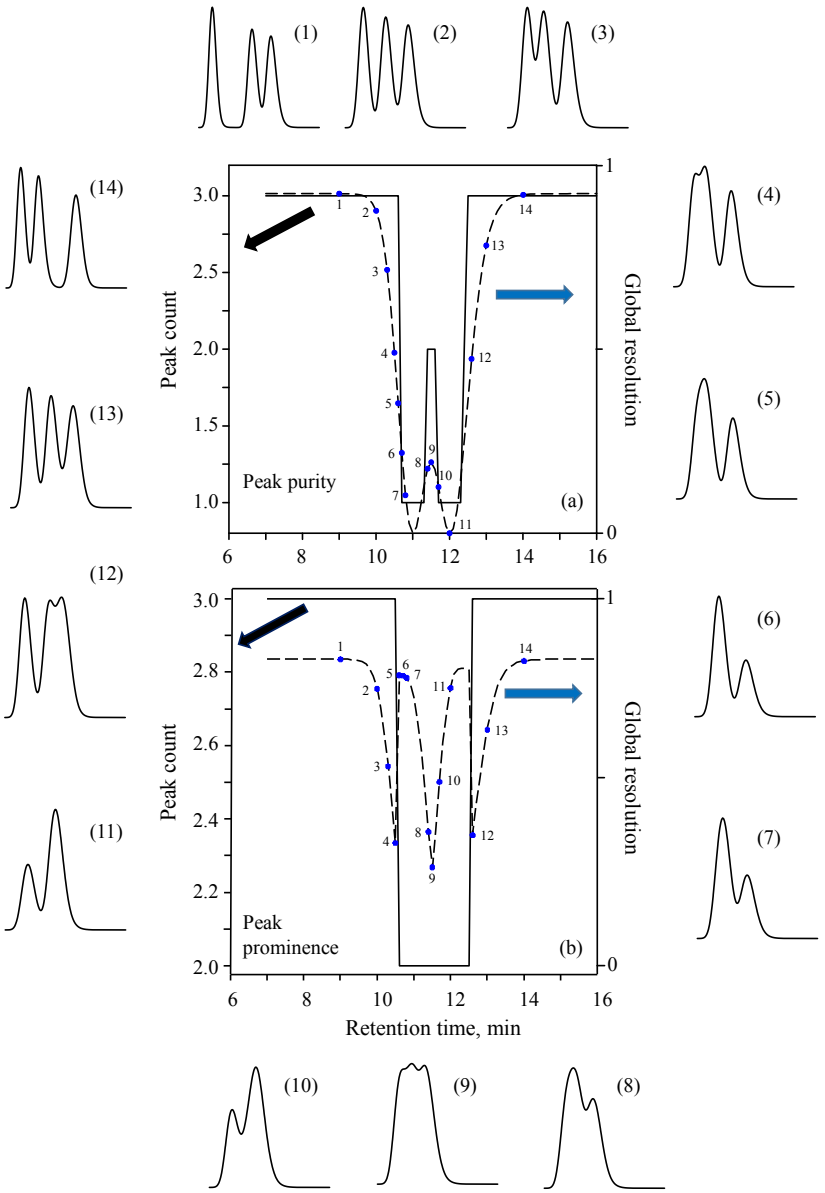

Figure 7.4. Resolution diagrams for chromatograms built with three artificial peaks, based on the peak purity (a), and peak prominence (b). Some representative chromatograms and their scores are given. The number of peaks is represented as full line, and the resolution as dashed line. The time axis represents the retention time of the shifted peak.

Since one peak was artificially shifted along the time axis, there was only one factor that affected the resolution. Hence, it was possible to draw two-dimensional diagrams, where the ordinates correspond to the separation performance (either measured as peak count or resolution), and the abscissas to the controlled factor (the retention time of the shifted peak). Peak distributions associated to several representative cases were drawn around the diagrams to facilitate the evaluation.

For both criteria, a good correlation is observed between the chromatograms and the peak count and resolution. However, the plots illustrate some differences between them. Thus, for instance, the peak count values for the peak purity may be below those for the peak prominence. The reason is that the peak purity has a comprehensive knowledge of the underlying signal, and consequently, it makes use of this information to evaluate whether what seems to be a single peak corresponds to one or more compounds. This implies that configurations where there are unresolved peaks merged in a cluster may be scored with a peak count smaller than the number of perceptible peaks, since only the peaks exceeding the threshold associated to a single compound are included in the peak count. This is observed in the chromatograms (5) to (8) in Fig. 7.4, where the major cluster includes two compounds. In contrast, the peak count based on the peak prominence attends to the number of peaks, since it has no information about the number of underlying compounds.

Meanwhile, the resolution drop occurs at about the same overlapping degree for both criteria, which correlate pretty well until full overlapping. Beyond this point, the peak purity continues its decrease, while the peak prominence recovers practically its initial value, since what was previously shown as two poorly resolved peaks merges into a single peak, at smaller peak distances.

7.5.2.2. Case of study II: Separation of a mixture of six probe compounds using one experimental factor

A similar study was carried out using the retention times and peak half-widths measured for experimental chromatographic peaks for six probe compounds. The compounds were injected individually to avoid any confusion due to peak reversals. The data were used to model the chromatographic behaviour of the compounds, and obtain a series of simulated chromatograms of the mixtures in a range of conditions. Diagrams were finally drawn representing the peak count and resolution versus the mobile phase composition (Fig. 7.5). The conclusions were similar to those obtained for the chromatograms built with the three artificial peaks. The peak purity (Fig. 7.5a) informs about the existence of unresolved peaks, whereas the peak prominence (Fig. 7.5b) offers a larger sensitivity to detect changes in the peak distribution, responding only to the perceptible peaks.

Fig. 7.6 represents the scores obtained by adding the peak count and the resolution value, according to both the peak purity and peak prominence, for the example of the mixture of six probe compounds. The differences in the plots illustrate the peculiarities of each criterion. However, in spite of the different basis of both assessments, the range of conditions resolving maximally the mixture and the drops producing peak merging (with incidental reversals) agreed for both criteria.

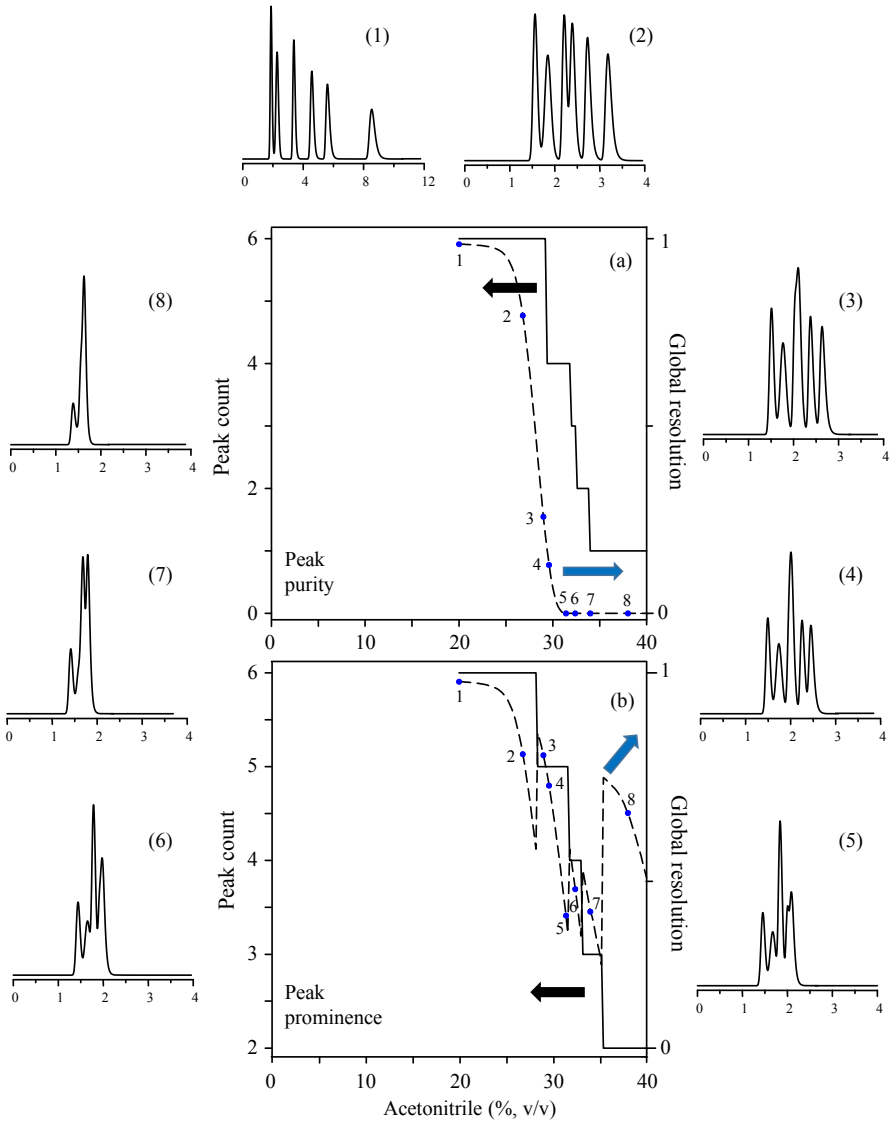

Figure 7.5. Resolution diagrams for simulated chromatograms for mixtures of six probe compounds using only one factor (the mobile phase composition), based on the peak purity (a), and peak prominence (b). Other details are given in Fig. 7.4.

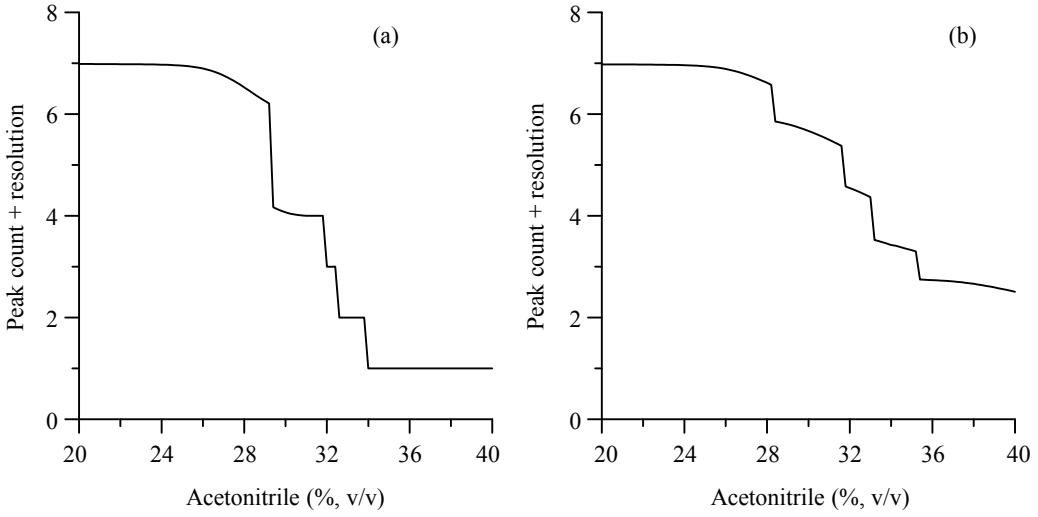

Figure 7.6. Performance diagram, based on the peak purity (a), and peak prominence (b), for the mixtures of six probe compounds. The combined contributions of the peak count and resolution are plotted.

7.5.2.3. Case of study III: Separation of a mixture of 30 probe compounds using two experimental factors

The performance of the peak prominence criterion was also evaluated for chromatograms of mixtures of considerable larger complexity. Simulations were based on the retention times and peak half-widths for 30 probe compounds, eluted according to two experimental factors: the organic solvent content and pH in the mobile phase. The full representation of this information would require three-dimensional diagrams. However, for the sake of simplicity and clarity, contour maps were drawn considering only the peak count as target function. The capability of the peak prominence to recognise the peaks was monitored again through the scores associated to several chromatograms. The

contour map for the mixture of 30 probe compounds, depicting the peak count based on the peak prominence criterion, is shown in Fig. 7.7. The maximal number of detected peaks was 28. In general, the peak count agreed with the number of peaks observed in the chromatograms.

As indicated, the peak count for the peak prominence was taken as the number of peaks that exceeded 0.05% of the sum of peak prominence areas, which agree with the perceptible peaks. Meanwhile, in Sections 7.5.2.1 and 7.5.2.2, the detection threshold for the peak purity criterion was set to a minimal value of elementary peak purity of $p = 0.5$. Fig. 7.8 depicts contour maps for the mixture of 30 probe compounds, according to this criterion, considering thresholds between $p = 0.1$ and 0.6. By comparing these contour maps with the contour map for the peak prominence in Fig. 7.7, it can be concluded that they become fairly similar for a threshold of peak purity $p = 0.5$ (Fig. 7.8e). The most important conclusion is that with this threshold, the maximal peak count and the experimental regions for maximal resolution agreed remarkably well for both peak purity and peak prominence.

7.5.3. Application of the proposed COF to chromatograms of real samples with unknowns

In previous sections, the peak prominence approach was shown to measure the chromatographic performance satisfactorily, when applied to artificial mixtures of up to 30 compounds. In the assayed samples, the identity of all compounds (i.e., the number of peaks) was known. But it should not be forgotten that the purpose of this work was the development of a measurement that could be applied to samples where the peak identity is unknown. Therefore, the approach was next applied to real samples.

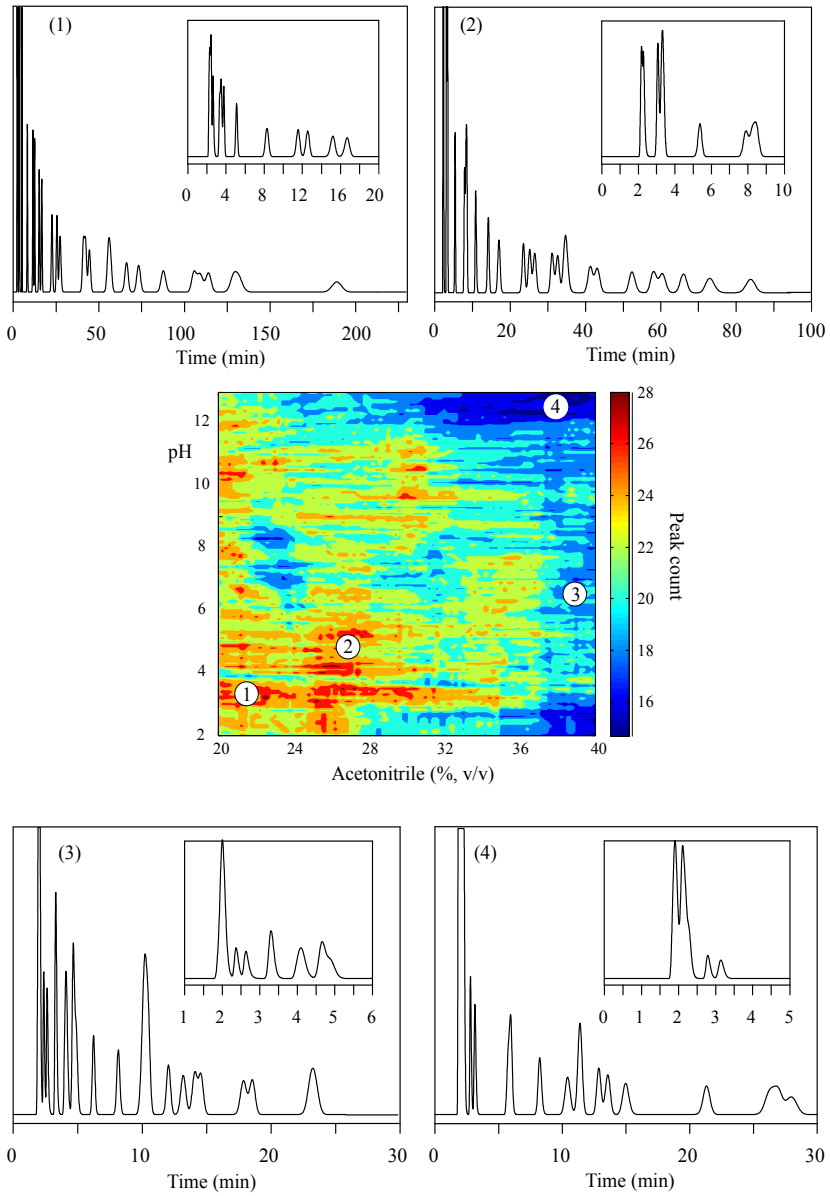

Figure 7.7. Contour map depicting the peak count, based on the peak prominence, for simulated chromatograms of mixtures of 30 probe compounds using two factors (the mobile phase composition and pH). The scores for representative chromatograms are marked on the contour map.

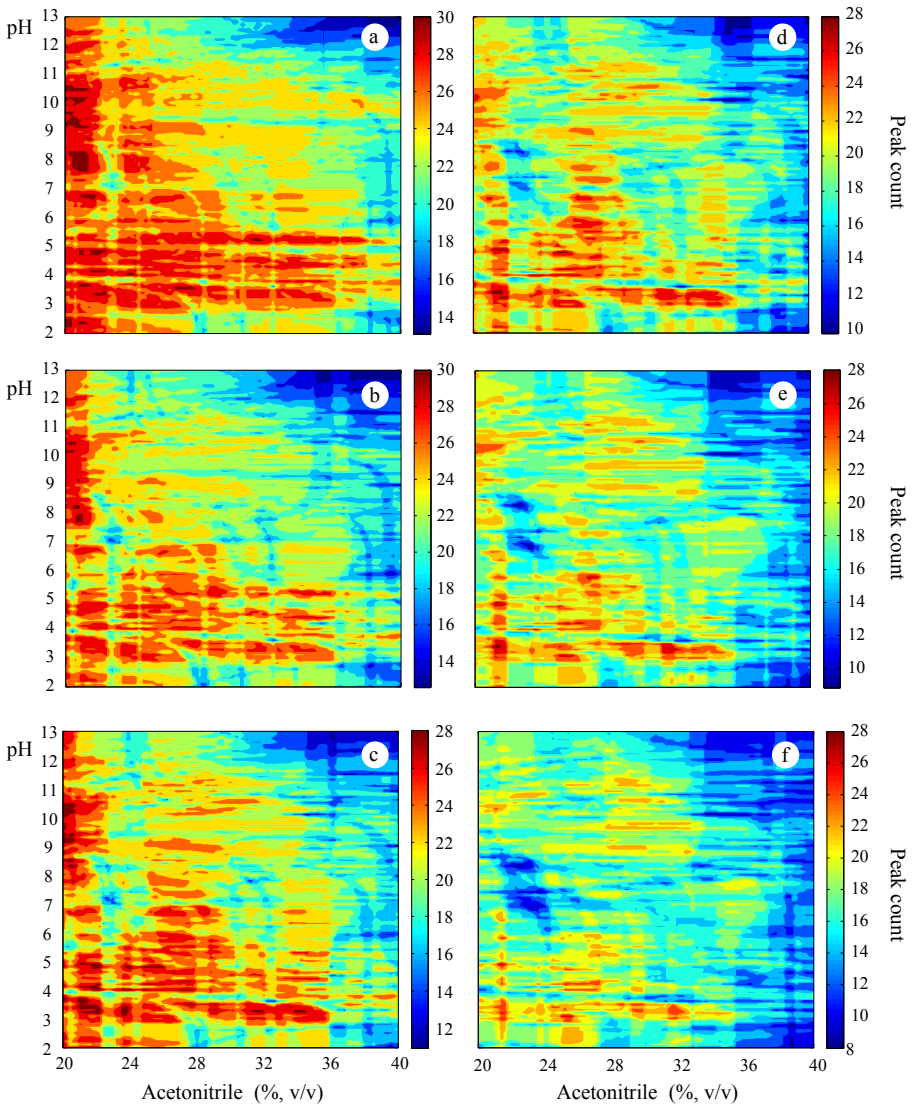

Figure 7.8. Contour maps depicting the peak count, based on the peak purity, for simulated chromatograms for mixtures of 30 probe compounds using two factors. The contour maps correspond to different thresholds of elementary peak purity (p) (Eq. (7.4)): (a) 0.1, (b) 0.2 (c) 0.3, (d) 0.4, (e) 0.5, and (f) 0.6.

Signals were processed using MATLAB homemade routines, as follows:

(i) The base line was first subtracted using smoothing cubic splines.

(ii) A raw peak table was generated applying the standard MATLAB routine FINDPEAKS of the signal processing toolbox, using its default parameters.

(iii) The peak limits were established using an iterative algorithm built for this work, which searches the tangent points delimiting the two valleys at the sides of each prominence (Figs. 7.2a and 7.3).

(iv) With the achieved information, a report giving the peak limits, retention times, total area and height of the whole peaks and the prominences, and other properties, was generated.

It should be noted that, even with simple mixtures, more peaks than those expected from the number of known compounds may be observed in the chromatograms. The major peaks will correspond to compounds at relatively high concentration or offering high signals, while smaller peaks may correspond, as commented, to impurities, degradation products, instrumental spikes, or noise.

In a first study, we mixed the solutions of the six probe compounds previously studied, and separated the sample solution using several experimental conditions, either isocratically (Fig. 7.9), or under gradient elution (Fig. 7.10). After applying the above protocol and considering again a threshold of 0.05% of relative peak prominence area for the smallest significant peak, the peak count and global resolution according to Eq. (7.7) were obtained.

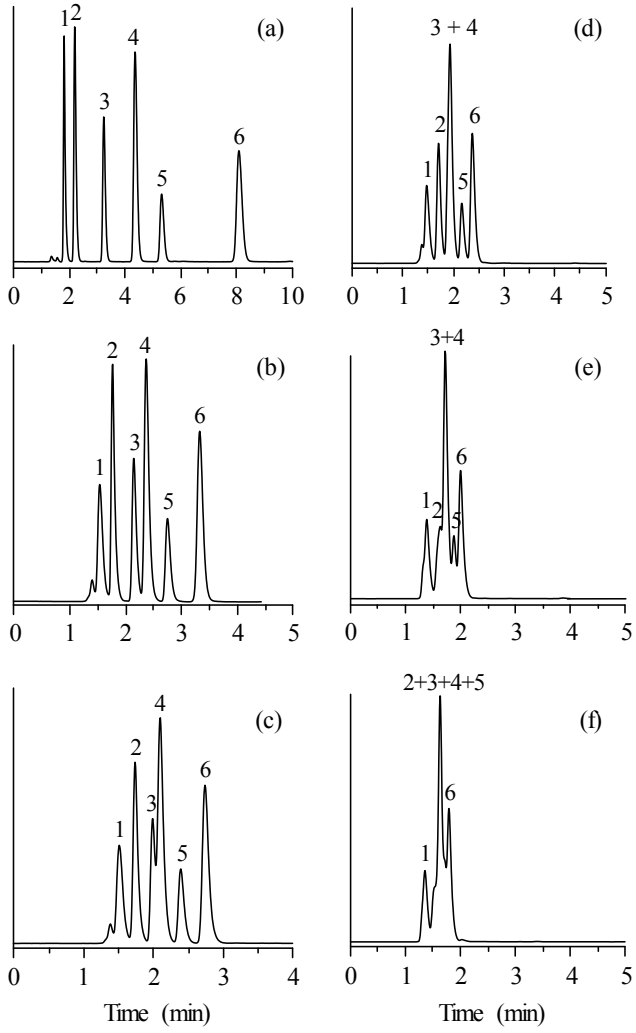

Figure 7.9. Chromatograms for real mixtures of the six probe compounds obtained with isocratic elution. Acetonitrile contents (% v/v): (a) 20, (b) 25, (c) 27.5, (d) 30, (e) 32, and (f) 35. Compound: (1) acebutolol, (2) atenolol, (3) carteolol, (4) celiprolol, (5) metoprolol, and (6) pindolol.

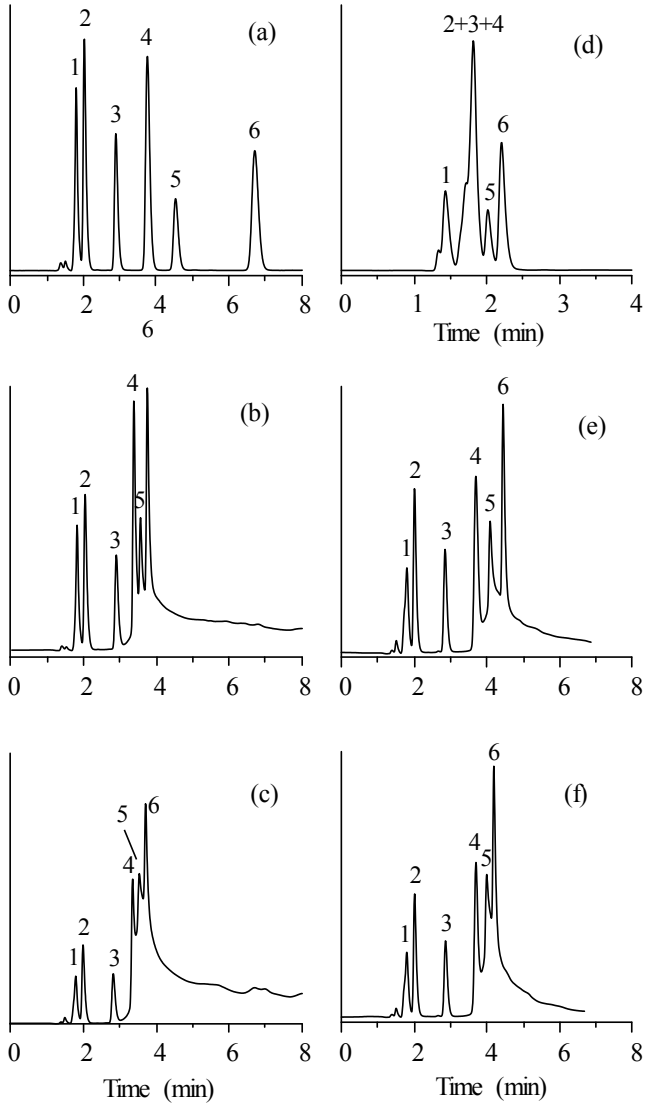

Figure 7.10. Chromatograms for real mixtures of the six probe compounds obtained with gradient elution. Acetonitrile gradients: (a) 20.0% (5 min) and 20.0–40.0% (10 min), (b) 20.0–40.0% (12 min), (c) 20.0–60.0% (12 min), (d) 29.7–31.5% (4.5 min), (e) 29.7–31.5% (4.5 min) and 31.5–40.0% (12 min), and (f) 35.0% (8.1 min) and 35.0–37.7% (8.5 min). Compound identities are given in Fig. 7.9.

The results are given in Table 7.1. The proposed algorithm found 5 to 16 peaks, depending on the elution conditions, which were confirmed with the visual inspection of the enlarged chromatograms. The observed peaks in the chromatograms should correspond to the six main compounds and several impurities and/or degradation products.

The proposed approach was also applied to characterise chromatographic fingerprints of medicinal herbs. Fig. 7.11 shows a raw fingerprint corresponding to an extract of chamomile in boiling water (Fig. 7.11a), and the processed chromatogram after baseline detrending using smoothing cubic splines (Fig. 7.11b). Fig. 7.12 shows the processed chromatograms for extracts of horsetail, decaffeinated tea and chamomile, eluted with a gradient changing the acetonitrile content from 2.0 to 37% in 25 min. The initially detected peaks in the elution window using the MATLAB routine FINDPEAKS, the peaks exceeding the selected threshold of 0.05% of relative peak prominence area, and the global resolution according to Eq. (7.7) for the peaks above the threshold were, respectively: horsetail (113, 50, 0.801), decaffeinated tea (124, 76, 0.601), and chamomile (104, 64, 0.620).

Table 7.1. Characterisation of chromatograms of real mixtures of the six probe compounds, according to the peak prominence criterion.

Acetonitrile (v/v)	Peak count	R_2 [a]
Isocratic elution		
20%	9	0.928
25%	7	0.867
27.5%	7	0.713
30%	7	0.726
32%	7	0.543
35%	5	0.488
Gradient elution		
20.0% (5 min), 20.0–40.0% (10 min)	8	0.955
20.0–40.0% (12 min)	16	0.352
20.0–60.0% (12 min)	14	0.232
29.7–31.5% (4.5 min)	8	0.601
29.7–31.5% (4.5 min), 31.5–40.0% (12 min)	11	0.649
35.0% (8.1 min), 35.0–37.7 (8.5 min)	10	0.569

[a] For the accepted peaks according to Eq. (7.7).

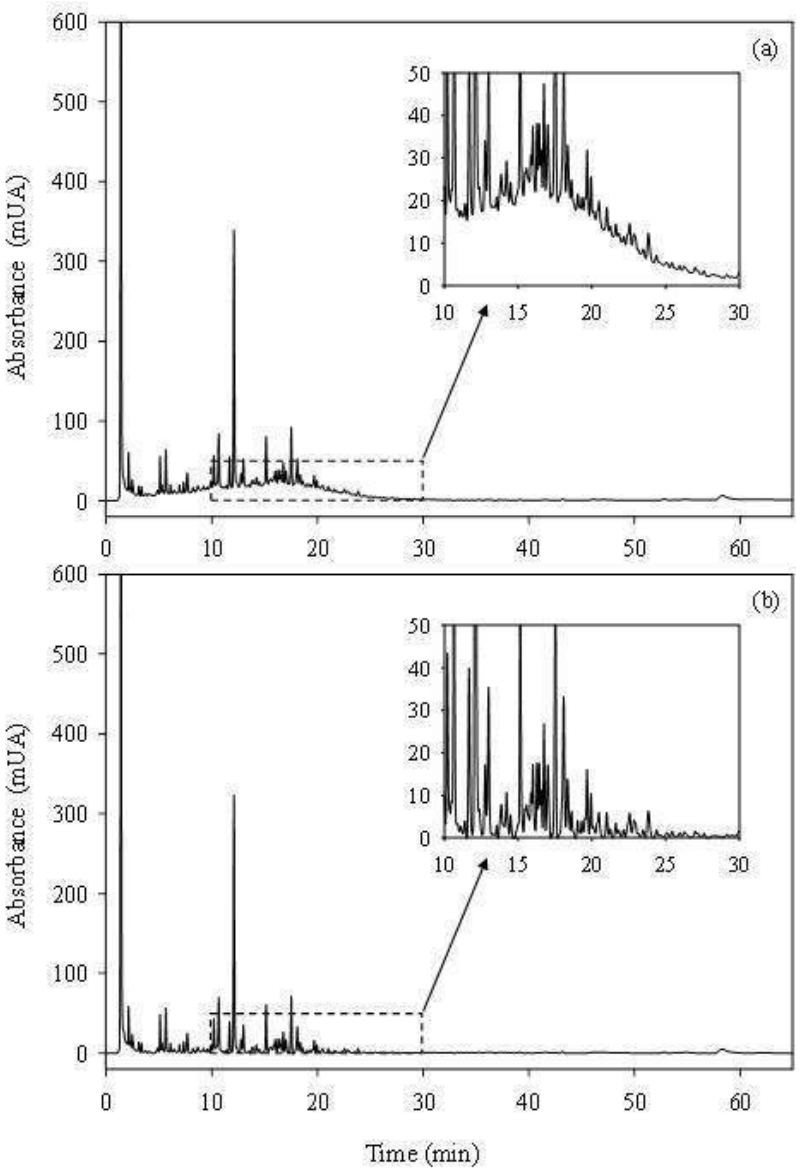

Figure 7.11. Chromatographic fingerprint for chamomile before being processed (a), and after baseline detrending using smoothing cubic splines. Gradient: 35–60% (v/v) acetonitrile in 12 min.

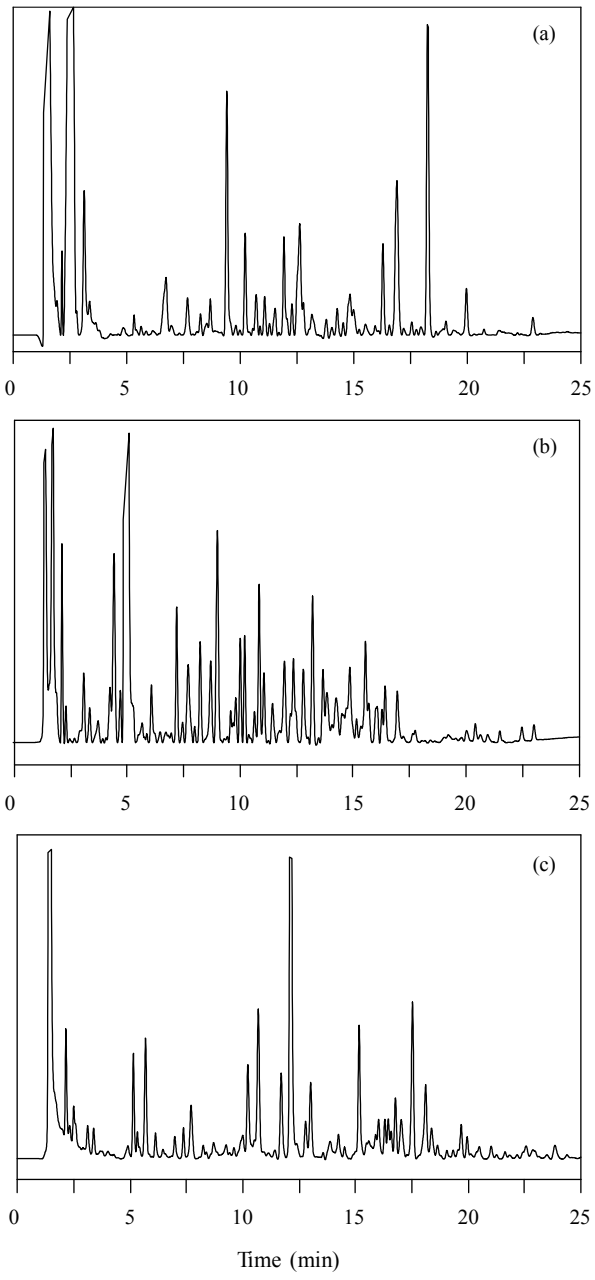

Figure 7.12. Chromatographic fingerprints for horsetail (a), decaffeinated tea (b), and chamomile (c), obtained with a gradient of acetonitrile (v/v): 2.0–37% (25 min).

7.6. Conclusions

Classificatory analysis applied to samples with unknown compounds, or with compounds for which there are no standards available in the laboratory, such as the extracts of medicinal herbs, requires chromatograms as informative as possible, with the maximal number of well resolved peaks. In this work, an algorithm has been developed to measure the level of information in such chromatograms in an unsupervised fashion, based on the concept of peak prominence, which is the protruding part of a peak from the two valleys delimiting it. Owing to the good features of the peak purity criterion and the parallelism between the peak prominence and peak purity, a comparative study of both COFs was considered a proper way to appraise the advantages and limitations of the new COF. The study was carried out with artificial chromatograms of diverse complexity with a number of known peaks.

The proposed COF has two contributions: the number of peaks exceeding a threshold (i.e., the number of significant peaks) and their resolution level (i.e., the visibility of the protruding fractions). Altogether, both features offer a comprehensive description of what is observed in a chromatogram. The primary criterion is the peak count. Only in situations where the number of significant peaks coincides, the resolution will be the criterion to make the decision.

For chromatographic fingerprints with multiple peaks of very different size, the feature to be monitored is the number of peaks exceeding a pre-selected threshold. Since the real number and nature of the components in these samples is unknown, the use of a threshold is necessary to discriminate among the significant peaks and those peaks that are not

reproducible in the data processing (e.g., associated to peak artefacts, impurities or noise). There is a certain risk of including peaks associated to noise or baseline perturbations. The fact is whether this risk gives rise to the wrong decision. This will depend on the type of sample. With complex samples, if the threshold is established as an absolute area, the risk is not relevant. On the contrary, if the sample is relatively simple with only some unknown minor peaks, the decision should be made attending to the resolution restricted only to the main peaks.

In future work, the peak prominence approach will be further developed and applied to screen the experimental conditions that yield a maximal number of peaks in extracts of medicinal herbs. In a next step, our purpose is to develop a procedure to optimise the best separation conditions, in order to get maximally informative fingerprints, using either internal standards or peaks present in the sample whose correspondence among experiments is unequivocal.

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CHAPTER 8

AN APPROACH TO EVALUATE THE INFORMATION IN CHROMATOGRAPHIC FINGERPRINTS: APPLICATION TO THE OPTIMISATION OF THE EXTRACTION AND CONSERVATION CONDITIONS OF MEDICINAL HERBS

8.1. Abstract

A new approach is reported for high-performance liquid chromatography to measure the level of information in fingerprints. For this purpose, the concept of peak prominence, which is the protruding part of each visible peak with regard to the valleys that delimit it, was used. The peaks in the fingerprints are ranked according to the areas of the peak prominences, and a threshold is established to discriminate between the significant peaks and those that are irreproducible. The approach was applied to evaluate the impact of several extraction conditions (solvent nature and composition, time and temperature of the treatment, amount of sample, and time and temperature of conservation of the extracts) on the number of significant peaks found in the fingerprints of a medicinal herb (a green tea sample), using Plackett-Burman designs. Acetonitrile, ethanol and methanol were used for the extraction, and a linear gradient for chromatographic analysis, where the acetonitrile content was increased from 5.0 to 42.5% (v/v) in 45 min. The maximal number of significant peaks in the fingerprints was obtained using a methanol-water mixture as extraction solvent, high ultrasonication time and high temperature. The reported approach can be generalised to other complex samples and situations.

8.2. Introduction

Chromatographic fingerprint analysis is nowadays a good alternative for the analytical control of medicinal herbs [1–6]. For authentication of these samples, classification analysis is used, where the sample is compared with one or more reference profiles [7–10]. To facilitate the recognition, chromatographic fingerprints containing a large number of visible peaks are desirable. A main objective in these analyses is to achieve satisfactory resolution, which is achieved by controlling the separation conditions [11,12]. Also, the extraction protocol can influence the results.

In a previous work, we developed a chromatographic objective function, based on the automatic measurement of the protruding part of the chromatographic peaks (or peak prominences) to measure the resolution of samples with unknown compounds [13]. In this work, peak prominence is shown as a tool to recognise which peaks are significant for quantifying the information in a chromatographic fingerprint.

The ultimate goal of the research developed in our laboratory with chromatographic fingerprints of medicinal herbs is the development of a protocol for maximising the provided information, using gradient elution. Before addressing the optimisation of the gradient program, it was necessary to investigate the effect of the extraction conditions prior to the chromatographic analysis on the quality of the obtained information. In this work, we describe a protocol to evaluate the extraction yield of the components of a medicinal herb, which is translated into a greater number of significant peaks in the chromatographic fingerprints. Given the number of factors involved, the study was conducted based on design of experiments.

8.3. Experimental

8.3.1. Reagents and column

Acetonitrile, ethanol and methanol (HPLC grade) of Scharlau (Barcelona) were used to obtain the extracts. The chromatographic separation was carried out with acetonitrile-water gradients, built by mixing acetonitrile (HPLC grade) and an aqueous phase consisting in a 0.01 M solution of anhydrous dihydrogen phosphate of Sigma (Roedermark, Germany). Barnstead ultrapure water was obtained from a purification system of Thermo Scientific (Dubuque, IA, USA). The pH of the aqueous phase was fixed at 3.0 by addition of 0.01 M HCl of Scharlau (Barcelona). All solutions and solvents were filtered through 0.45 μm Nylon membranes of Micron Separations (Westboro, MA, USA).

Chromatographic analyses were performed using a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm i.d.) of Merck (Darmstadt, Germany).

8.3.2. Apparatus

A modular instrument of Agilent (Waldbronn, Germany), consisting of a quaternary pump (HP1100 Series), an autosampler (HP1200 Series) equipped with 2 mL vials, a thermostated column compartment (HP1260 Series) at 25 °C, and a variable wavelength UV-visible detector (HP1100 Series) fixed at 210 nm, were used. The injection volume was 10 μL. The flow rate was kept constant at 1 mL/min. The dead time, determined by injection of 20 mg/mL of KBr of Acros Organics (Fair Lawn, NJ, USA), was 1.33±0.08 min.

A Crison pH meter (Model MicropH 2002, Barcelona), and a combined glass electrode of Orion (Model 8102, Barcelona), containing Ag/AgCl electrodes with a salt bridge filled with a 3.0 M KCl solution, were used to measure the pH of the dihydrogen phosphate solution that built the gradient.

8.3.3. Recommended sample preparation

Green tea of Pompadour (Alicante), purchased in small bags in a supermarket, was analysed to show the approach. The recommended procedure is as follows:

(i) 1 g of green tea powder is weighed,

(ii) 15 mL of an aqueous-organic mixture containing 30% (v/v) methanol is added,

(iii) the sample solution is introduced in an ultrasonic bath and sonicated during 60 min at 80°C,

(iv) centrifugation is carried out at 3000 rpm during 5 min,

(v) the extract is filtered through a Nylon membrane (Pall Gelman Laboratory, Karlstein/Main, Germany) with a pore size of 0.45 μm,

(vi) the autosampler vials are filled with the extract for chromatographic analysis, using an acetonitrile linear gradient from 5.0 to 42.5% in 45 min.

8.4. Data treatment

Getting useful chemical information from samples containing multiple components is still a big challenge for analysts [14]. An approach is here reported to obtain the maximal amount of significant information in chromatographic fingerprint analysis. For significant information, we mean the number of visible and reproducible peaks in the chromatograms with sufficient signal-to-noise ratio and resolution. The approach was applied to optimise the conditions of sample extraction and conservation of the extract of a medicinal herb. In general terms, the data treatment consisted of the following steps: baseline removal in the chromatograms using smoothing cubic splines, peak detection, measurement of peak properties and selection of the significant peaks

that exceeded a given prominence threshold. The whole process is carried out in an unsupervised fashion from the table of times and signals defining the sample chromatograms.

8.4.1. Baseline removal

The baseline of the chromatograms of the extracts of medicinal herbs is remarkably irregular, with no neat patterns that allow an easy removal. The origin of the irregularities is the applied gradient, the existence of matrix peaks and the accumulation of unresolved peaks. The presence of these disturbances biases the quantification, making the measurement of the information contained in the fingerprints and its interpretation difficult. Therefore, before processing any chromatogram, all contributions of the baseline should be carefully removed.

With this purpose, smoothing cubic splines, which is a class of non-parametric regression method capable of modelling irregular profiles of all kinds with excellent accuracy, were applied [15,16]. Smoothing cubic splines approximate a series of points to a function by defining a number of nodes, whose flexibility can be regulated by tension factors. This allows the adaptation of the baseline at small scale, which is essential for the detection of peaks of small magnitude, discarding at the same time non-significant variations associated to noise. A correct selection of the spline tension factor is important to avoid the introduction of artefacts due to an insufficient level of generalisation of the baseline pattern. This kind of splines is rather popular in engineering, computational and environmental sciences, but has been scarcely used in Analytical Chemistry. Fig. 8.1 shows as example the chromatograms of a green tea extract before and after removal of the baseline.

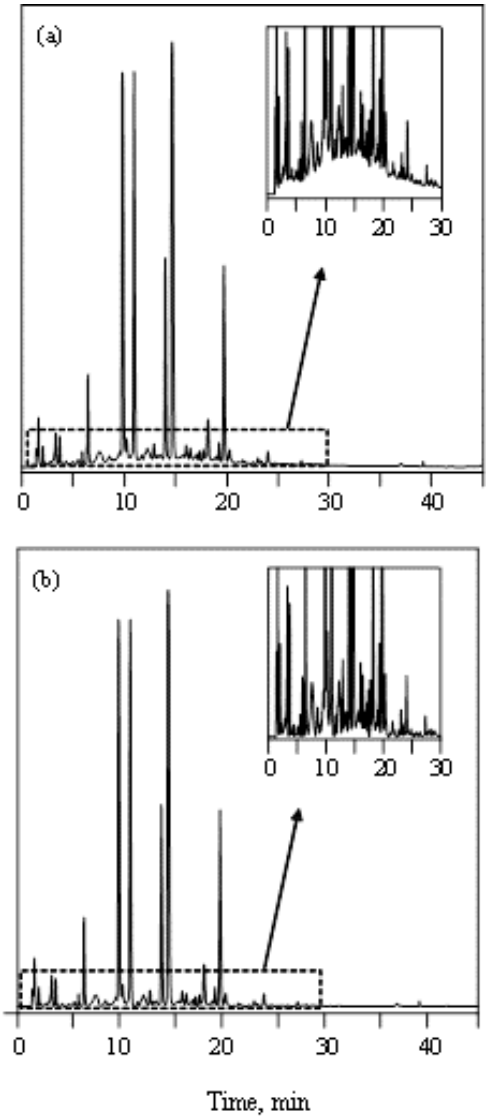

Figure 8.1. Green tea fingerprint before being processed (a) and after removal of the baseline by application of smoothing cubic splines (b).

8.4.2. Peak detection and automatic measurement of peak properties

The standard function "Findpeaks" of the signal processing Matlab toolbox was used in a first step to do a raw analysis of the chromatograms, in order to find the peak maxima. With the default parameters, this function detects a very high number of peaks (often associated with noise). This way of operation was found preferable for relying on the analyst the decision of the selection of the significant peaks, starting from comprehensive primary information.

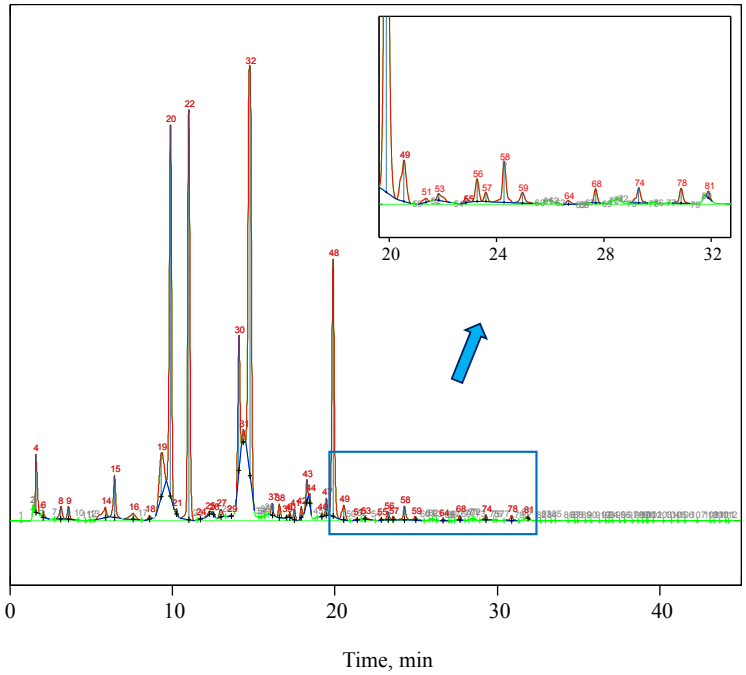

Fig. 8.2. Screenshot of a MATLAB chromatogram corresponding to a green tea extract. Tangents that define the prominence region for each peak are depicted. The peaks are numbered according to their elution order and those that exceeded a relatively peak area of 0.025% are marked in red.

A Matlab function called “Chromscan” was developed in our laboratory to obtain automatically the significant information contained in a complex chromatogram, using the raw vector of maxima indexes given by Findpeaks. Chromscan locates the optimal tangent points defining the limits for peak integration (those delimiting the two valleys at the sides of each prominence) (Fig. 8.2). The program measures the peak areas and other parameters. The integrated areas were defined from the points of tangency of the valleys.

8.4.3. Finding the significant peaks

Even the simplest chromatographic fingerprints include a high number of peaks within a very wide range of sizes. The major peaks correspond to components at relatively high concentration or showing strong signals, while the smallest peaks may correspond to trace or subtrace components, impurities introduced during sample preparation, artefacts or just noise: the smaller the peak, the higher the weight of noise and the probability of being processed in a non-reproducible way.

The presence of noise, together with the high complexity of the signals in the fingerprints, makes their manual processing unfeasible and the results undesirably dependent on the analysts’ subjective criteria. A highly complex signal, such as the chromatogram from a herb extract, presents inevitable irregularities associated to peaks of small magnitude or noise, whose reproducibility cannot be guaranteed between consecutive injections, and consequently, their inclusion may strongly bias the results. These considerations indicate that we need somehow differentiating between peaks corresponding to significant compounds in the fingerprints and those affected by non-significant components or noise. Since Findpeaks detects peaks in a wide range of magnitudes, a threshold must be selected to discriminate which

peaks are too variable among replicates, and the measurements are not consistent and should be discarded.

As an example, Fig. 8.2 depicts a screenshot of a chromatogram of a green tea extract, processed by Chromscan. The peaks are numbered according to their elution order, which will be called “peak index”. In Table 8.1, the peaks are sorted according to the magnitude of their areas. Since the order number matches the number of peaks detected below a certain threshold, it will be called “number of (detected) peaks”. In Fig. 8.2, the peaks depicted in red are those above the threshold, whose selection will be discussed below.

The evaluation of the information level obtained for each fingerprint was performed assisted by two types of plots obtained unsupervisedly, where the relative or absolute area of each peak in the fingerprint is represented in decreasing order (Fig. 8.3). In both cases, due to the wide range in peak magnitude, the ordinate axis is in logarithmic scale. The abscissas indicate the number of detected peaks. The semi-logarithmic plots in Fig. 8.3 correspond to three replicates of green tea extracts in 30% ethanol. Each replicate was obtained by repeating the whole analytical process: sample weighting, extraction, chromatographic separation and automatic data treatment. Note the high agreement among the lines in the plot of relative areas (Fig. 8.3a), up to a specific value (marked with a dashed line), beyond which the lines diverge. The value where the lines start to diverge can be adopted as a threshold to discriminate the significant peaks in the fingerprints.

Table 8.1. Chromatographic peaks in the fingerprint depicted in Fig. 8.2, ordered according to the area of the peak prominences.

Number of peaks[a]	Peak index[b]	Absolute area	Relative area (%)	Cumulative area (%)
1	32	948.5	24.01	24.01
2	20	577.1	14.61	38.61
3	22	533.3	13.50	52.11
4	48	372.5	9.429	61.54
5	30	269.3	6.817	68.36
6	19	229.3	5.803	74.16
7	31	163.7	4.144	78.31
8	4	75.85	1.920	80.23
9	15	71.82	1.818	82.04
10	43	59.32	1.502	83.55
11	14	42.55	1.077	84.62
12	37	30.45	0.771	85.39
13	49	29.68	0.751	86.14
14	16	28.35	0.718	86.86
15	47	28.07	0.710	87.57
16	44	27.37	0.693	88.27
17	8	26.14	0.662	88.93
18	38	25.33	0.641	89.57
19	27	24.69	0.625	90.19
20	25	23.85	0.604	90.80
21	58	22.65	0.573	91.37
22	9	21.56	0.546	91.92
23	42	17.02	0.431	92.35
24	21	16.63	0.421	92.77
25	29	13.12	0.332	93.10
26	41	12.85	0.325	93.42
27	6	12.80	0.324	93.75
28	56	12.39	0.314	94.06
29	26	12.28	0.311	94.37
30	46	11.80	0.299	94.67

[a] Peak order according to the areas. [b] Peak order according to the elution order.

Table 8.1 (continued).

Number of peaks[a]	Peak index[b]	Absolute area	Relative area (%)	Cumulative area (%)
31	36	11.12	0.282	94.95
32	18	10.29	0.260	95.21
33	74	10.09	0.256	95.47
34	40	9.89	0.250	95.72
35	53	9.71	0.246	95.96
36	35	9.16	0.232	96.20
37	78	8.76	0.222	96.42
38	59	8.38	0.212	96.63
39	45	7.85	0.199	96.83
40	3	7.73	0.196	97.02
41	2	7.43	0.188	97.21
42	39	6.64	0.168	97.38
43	68	6.57	0.166	97.55
44	57	6.18	0.156	97.70
45	28	6.46	0.164	97.87
46	81	6.09	0.154	98.02
47	5	5.87	0.148	98.17
48	24	5.09	0.129	98.30
49	33	4.23	0.107	98.41
50	51	4.22	0.107	98.51
51	55	4.04	0.102	98.61
52	7	3.57	0.090	98.71
53	80	3.55	0.090	98.80
54	72	2.90	0.073	98.87
55	1	2.48	0.063	98.93
56	10	2.47	0.061	98.99
57	61	2.33	0.059	99.05
58	34	2.23	0.056	99.11
59	64	2.04	0.052	99.16
60	70	2.02	0.051	99.21
61	52	2.00	0.051	99.26
62	12	1.99	0.050	99.31

[a] Peak order according to the areas. [b] Peak order according to the elution order.

Table 8.1 (continued).

Number of peaks[a]	Peak index[b]	Absolute area	Relative area (%)	Cumulative area (%)
63	62	1.98	0.050	99.36
64	71	1.97	0.050	99.41
65	76	1.81	0.046	99.46
66	17	1.60	0.040	99.50
67	107	1.54	0.039	99.54
68	23	1.46	0.037	99.57
69	77	1.34	0.034	99.61
70	67	1.25	0.032	99.64
71	60	1.10	0.028	99.67
72	85	1.07	0.027	99.69
73	63	1.05	0.027	99.72
74	69	0.92	0.023	99.74
75	54	0.86	0.022	99.77
76	83	0.75	0.019	99.78
77	91	0.70	0.018	99.80
78	73	0.65	0.017	99.82
79	11	0.64	0.016	99.84
80	75	0.63	0.016	99.85
81	50	0.62	0.016	99.87
82	84	0.52	0.013	99.88
83	82	0.45	0.011	99.89
84	102	0.41	0.010	99.90
85	79	0.39	0.0099	99.92
86	86	0.38	0.0096	99.92
87	95	0.32	0.0081	99.93
88	96	0.29	0.0074	99.94
89	104	0.25	0.0064	99.94
90	13	0.24	0.0062	99.95
91	89	0.17	0.0042	99.95
92	110	0.16	0.0041	99.96

[a] Peak order according to the areas. [b] Peak order according to the elution order.

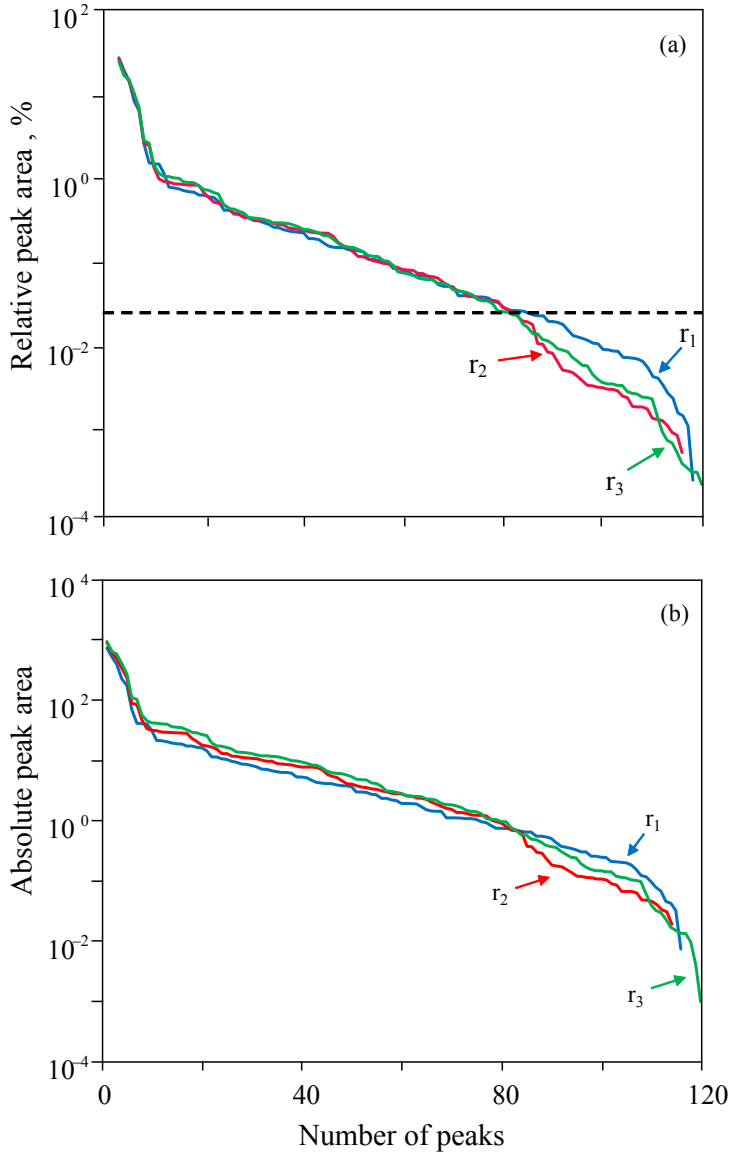

Figure 8.3. Semi-logarithmic plots of relative (a) and absolute (b) prominence areas of the peaks in a fingerprint extract, obtained with 30% ethanol. The lines $r_{1–3}$ correspond to replicates.

The divergence indicates the lack of reproducibility when the data processing algorithm is applied, owing to the influence of noise in low magnitude signals. The agreement among replicates is poorer in the semi-logarithmic plots of absolute areas (Fig. 8.3b), since the differences are magnified at low values owing to the logarithmic scale. This makes the establishment of the proper threshold more difficult.

From the observation of the plots for replicates of several samples of green tea and other medicinal herbs, this threshold was established at approximately a relatively peak area of 0.025%. Beyond this threshold, the replicates began to differentiate among them. This value corresponds to a cumulative relative area of 99.7% (see Table 8.1). As will be seen below, when the amount of processed sample is changed, absolute areas should be considered to evaluate the information.

In this work, the described approach was applied to optimise the extraction of a green tea sample and the conservation conditions of the extracts to maximise the number of significant peaks in the fingerprints. The semi-logarithmic plots of relative and absolute areas were found appropriate to evaluate the performance of different extraction treatments of components in the analysed sample and other types of medicinal herbs. The higher the number of significant peaks in a chromatogram, the larger the amount of contained information. Consequently, the recognition of the samples through their fingerprints can be expected to be more reliable.

8.5. Results and discussion

8.5.1. Preliminary considerations

A wide literature survey highlighted the disparity of extraction conditions reported in the literature by different analysts to process samples of medicinal herbs for fingerprint analysis [5,17–29] (see Table 8.2). In previous analyses carried out in our laboratory, extracts of herbal medicines were obtained in hot water using a microwave oven [13]. For the study presented in this work, we considered the possibility of improving the extraction yield by using a hydro-organic mixture or pure solvents as extractants, and optimising some factors involved in the process: the nature and concentration of the solvent used in the extraction, the time and temperature of the treatment, the sample weight and the time and temperature of conservation of the extract, always with the aim of maximising the information in the fingerprints.

The HPLC separation of the green tea sample was carried out using the linear gradient described in Section 8.3.3. After this time, the acetonitrile concentration was increased up to 100% (v/v) and maintained during 5 min to remove incidental highly non-polar compounds that could be retained on the column. Gradient elution was selected due to the asymmetrical distribution of the peaks in the chromatogram, which concentrated the peaks of higher intensity mostly in the first minutes when isocratic elution was applied. The chromatograms obtained with the selected gradient, using pure acetonitrile, ethanol or methanol for the extraction, are shown in Fig. 8.4.

Table 8.2. Some reported treatments in the literature for the preparation of extracts of medicinal herbs for further chromatographic fingerprint analysis.

Weight (g)	Solvent	Sonication temperature	Sonication time	Procedure	Ref.
0.36	70% ethanol (5 mL)	Room temperature	90 min	Sample weighed into a 25 mL volumetric flask, ultrasonically extracted and centrifuged at 10000 rpm (10 min).	[17]
2.00	50% ethanol (20 mL)	Room temperature	30 min	Sample weighed into a 50 mL centrifuge tube, ultrasonically extracted and centrifuged at 3000 rpm (5 min).	[18]
0.15	50% ethanol (25 mL)	Room temperature	60 min	Sample weighed into a 25 mL volumetric flask and ultrasonically extracted, 5 mL was centrifuged at 3000 rpm (5 min).	[19]
0.15	Pure acetonitrile, methanol and tetrahydrofuran (1 mL)	Room temperature	60 min	Sample weighed into a 5 mL centrifuge tube, ultrasonically extracted and centrifuged at 5800 rpm (5 min).	[20]
0.50	Pure methanol (10 mL)	Room temperature	20 min	Sample weighed into a 50 mL centrifuge tube, ultrasonically extracted and centrifuged at 4000 rpm (10 min).	[5]
1.0	Pure methanol (10 mL)	Room temperature	30 min	Sample ultrasonically extracted.	[21]
0.50	Pure methanol (50 mL)	Room temperature	30 min	Sample weighed into a 50 mL centrifuge tube, ultrasonically extracted and filtered. This process was repeated twice. The combined filtrate was evaporated to dryness in vacuo and the residue dissolved and diluted to 10 mL.	[22]
0.25	30% methanol (25 mL)	Room temperature	20 min	Sample ultrasonically extracted.	[23]

Table 8.2 (continued).

Weight (g)	Solvent	Sonication temperature	Sonication time	Procedure	Ref.
0.50	75% methanol (10 mL)	Room temperature	60 min	Sample weighed into a 50 mL centrifuge tube, ultrasonically extracted and centrifuged at 3000 rpm (10 min).	[24]
0.20	Pure methanol (5 mL)	Room temperature	30 min	Sample weighed into a 10 mL centrifuge tube, ultrasonically extracted and centrifuged at 2000 rpm (5 min). The residue was extracted twice with 5 mL of methanol and the supernatant transferred into a 50 mL centrifuge tube, refluxed with 10 mL of water (30 min) and centrifuged at 2000 rpm (5 min).	[25]
1.0	70% methanol (10 mL)	Room temperature	20 min	Sample ultrasonically extracted and filtered. The extraction was repeated twice more. The combined filtrate was evaporated to dryness in vacuo and the residue dissolved in 5 mL of 70% methanol.	[26]
1.0	50% ethanol (25 mL)	80ºC	-	Sample weighed into a 100 mL volumetric flask, treated in a water bath at 80°C (20 min) and filtered. The solution was dried in vacuo at 40ºC and the dried extract dissolved in 100 mL of 35% methanol.	[27]
5.0	50% ethanol (60 mL)	Room temperature	-	The solution was left over 24 h. The extract was filtered and concentrated in a rotary evaporator below 80°C. The process was repeated 10 times. The extracts were placed in an oven at 37ºC.	[28]
2.5	Pure methanol (25 mL)	40-50ºC	60 min	Sample weighed into a 50 mL centrifuge tube, ultrasonically extracted, filtered and evaporated under reduced pressure at 50°C.	[29]

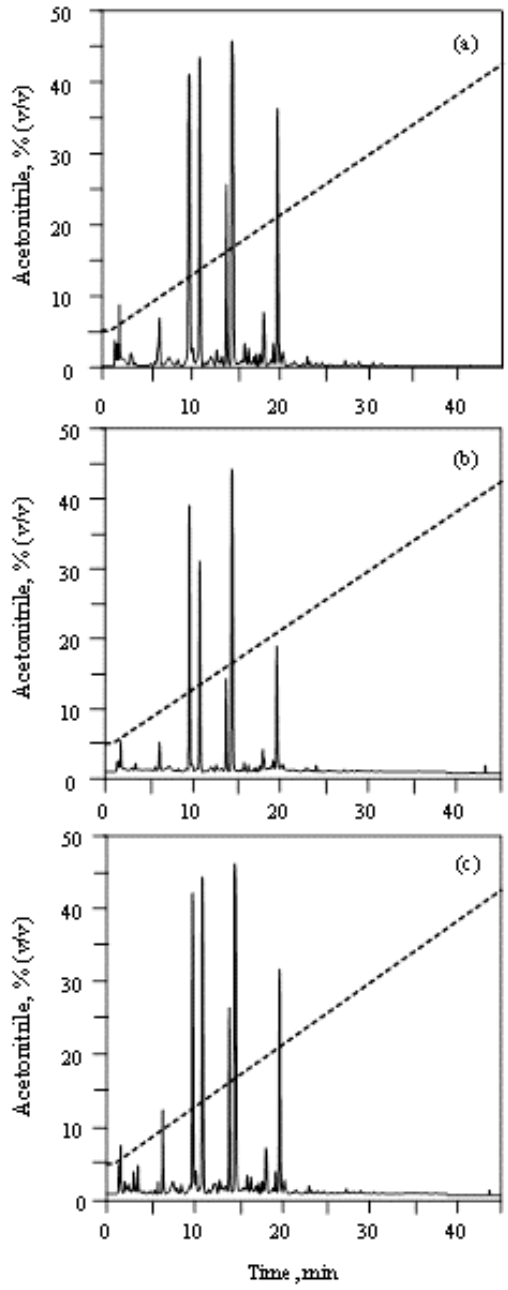

Figure 8.4. Chromatograms of a green tea extract, obtained with a gradient of 5.0-42.5% acetonitrile applied in a time window of 45 min. Pure organic solvents were used as extractants: (a) acetonitrile, (b) ethanol, and (c) methanol.

8.5.2. Experimental outline

Initially, we thought in evaluating all factors altogether in a single step by performing a Plackett-Burman design [30]. In the literature, these screening designs have been applied to examine the effect of different factors in a variety of studies. Some recent reports appear in Refs. [31–36]. Plackett-Burman designs have the advantage of exploring a high number of factors with a minimal number of experiments, allowing the estimation of main effects. As limitations, we can find their inability to discriminate first-order interactions of primary effects, and that necessarily, the number of experiments should be a multiple of 4. Confounding of main factors with first-order interactions was perfectly valid for our purposes, since we were only interested in ranking the factors.

However, the incompatibility among some of the involved factors and the sequential nature of the analytical process did not advice a comprehensive study using designs that considered all factors simultaneously, in order to set the most favourable levels. Therefore, these were divided in two groups of three factors:

(i) First group: Concentration of the extraction solvent, and time and temperature of the treatment in the ultrasonic bath, which were considered altogether in a first step. This study was carried out in blocks for three types of hydro-organic mixtures including either acetonitrile, ethanol or methanol. Additional blocks with pure water and the three pure organic solvents were also considered.

(ii) Second group: Amount of sample, and time and temperature of conservation of the extracts, which were studied in a second step.

The simplest experimental designs for screening the three factors in each group are fractional factorial designs with $2^{3-1} = 4$ experiments. With a proper generator, Plackett-Burman designs with 4 experiments are equivalent to fractional factorial designs, and allow obtaining each of the three main factors confounded with primary interactions of the other two. In this work, these designs were built based on the reformulation collected in Ref. [37], where the design matrices are listed from $n = 4$ to 48.

8.5.3. Type and concentration of the extraction solvent and ultrasonication conditions

As explained, the best solvent for the extraction of the components in the analysed green tea was investigated together with the most appropriate experimental conditions for the treatment in the ultrasonic bath. First, three series of experiments following Plackett-Burman designs with $n = 4$ were carried out with hydro-organic mixtures, each series using a different solvent (acetonitrile, ethanol and methanol). For this purpose, Design I in Table 8.3 was applied. The experimental factors (*A*, *B*, *C*) and studied levels were:

(i) Factor *A*: Percentage of organic solvent in the hydro-organic mixture, with minimal (–) and maximal (+) levels of 30 and 70% of pure solvent, respectively.

(ii) Factor *B*: Time of the ultrasonication treatment, with minimal (–) and maximal (+) levels of 30 and 60 min, respectively.

(iii) Factor *C*: Temperature of the ultrasonication treatment, with minimal (–) and maximal (+) levels of 30 and 80 ºC, respectively.

As can be seen in Table 8.3, the designs consisted of four non-replicated experiments for each solvent, so the total number of experiments was 12 (3 solvents × 4 experiments by design). In the table, each row corresponds to a different experiment and each column indicates the studied factor, which was set at its minimal (–) or maximal (+) nominal levels. Other factors (amount of extracted sample, and centrifugation time and speed) were fixed. The amount of extracted sample was considered, together with other factors, in a further step. It should be indicated that the chromatographic separation was carried out immediately after the extraction.

The effect of the pure solvents on the extraction yield was next investigated, using the same type of design (Table 8.3). In these series of experiments, for factor *A*, the maximal (+) level was 100% of organic solvent (pure acetonitrile, ethanol or methanol), and the minimal (–) level was as low as 0% of organic solvent (100% water). The maximal and minimal levels for the time and temperature of the ultrasonication treatment were the same as those for the hydro-organic mixtures: 30 and 60 min, and 30 and 80 ºC, respectively. It should be noted that the experiments using pure water were common for the designs with the three solvents. Therefore, the total number of experiments involving pure solvents was 8 (3 pure organic solvents × 2 experiments + water × 2 experiments), rearranged in three designs of 4 experiments.

As the same amount of sample was processed in the three series of experiments, the number of significant peaks in the fingerprints for the different conditions was based on the comparison of semi-logarithmic plots of relative peak areas. Table 8.4 lists the number of peaks exceeding the established threshold (99.7% of cumulative relative peak area in the chromatograms, see Figure 8.3a), corresponding to the experiments described in Table 8.3 for the three solvents. Since the experiments (– + –) and (– – +) for the pure solvents

were performed using water to obtain the extracts, the number of peaks detected above the threshold was the same.

Table 8.3. Factorial designs applied to the selection of solvent and ultrasonication conditions (Design I: Factors *A*, *B* and *C*), using hydro-organic mixtures or pure solvents with acetonitrile, ethanol or methanol, and amount of sample and conservation of the sample extracts (Design II: Factors *D*, *E* and *F*), using methanol.[a]

Factor *A* or *D*	Factor *B* or *E*	Factor *C* or *F*
+	+	+
+	–	–
–	+	–
–	–	+

[a] Factor *A* = solvent percentage; Factor *B* = ultrasonication time; Factor *C* = ultrasonication temperature; Factor *D* = sample weight; Factor *E* = extract conservation time; Factor *F* = extract conservation temperature. Minimal (–) and maximal (+) levels.

Table 8.4. Number of peaks exceeding the established threshold in the fingerprints for the experiments in the factorial designs in Table 8.3, using hydro-organic mixtures and pure solvents.

Design I with hydro-organic mixtures				
Factors *A B C*	+ + +	+ − −	− + −	− − +
Acetonitrile	62	45	73	70
Ethanol	48	48	80	73
Methanol	73	49	70	89
Design I with pure solvents				
Factors *A B C*	+ + +	+ − −	− + −	− − +
Acetonitrile	21	16	56	73
Ethanol	49	25	56	73
Methanol	67	58	56	73
Design II				
Factors *D E F*	+ + +	+ − −	− + −	− − +
Methanol	92	102	69	69

8.5.4. Interpretation of the results and prediction of behaviours

Regardless of the adopted threshold, 30:70 methanol-water was the solvent that yielded more peaks in the fingerprints of green tea samples. It was observed that the number of peaks was significantly reduced with a higher concentration of organic solvent in the hydro-organic mixtures, and especially with the pure solvents.

The experimental designs in the series described above can be outlined as cubes, where each edge represents an experimental factor and each vertex, an experiment. To be advantageous, an experimental design should maximise the information by reducing the number of experiments (using Plackett-Burman designs, only 4 of the 8 possible experiments are carried out). Despite this partial inspection, it is possible to establish models to predict how many peaks would be expected using experimental conditions non-assayed in the design, based on the data from the assayed experiments.

Since Plackett-Burman designs give only information about the main effects, the following models were fitted by multiple linear regression:

$$\mathrm{NP} = c_0 + c_1 \times A + c_2 \times B + c_3 \times C \qquad (8.1)$$

where NP is the number of peaks exceeding the established threshold, and A, B and C are the experimental factors. The study was carried out with the coded levels (+1 and –1), which makes the evaluation of the effects of the different factors (i.e., the coefficients $c_{0–3}$) comparable to each other. If the coefficient for a given factor is positive, it will favour a higher number of peaks. If its value is negative, it will not cooperate to obtain peaks.

Three threshold values of cumulative relative peak area were used to check whether the results were consistent: the applied threshold to obtain the data in Table 8.4 (99.7% of cumulative relative peak area), a value that included only the main peaks (99.2%), and another that included noise and poorly reproducible processing (99.99%). The coefficients of Eq. (1) for the series with the hydro-organic mixtures and pure solvents are indicated in Table 8.5. As can be observed, the values of the coefficients suggest similar conclusions for the thresholds measured considering 99.7% and 99.2% of cumulative relative peak area, as expected when the measurements consider reliable peaks. The coefficients suggest that a smaller organic solvent concentration is preferable. The ultrasonication has a positive effect on the extraction (i.e., higher ultrasonication times are preferable). The treatment at higher temperature produces also a positive effect, especially for methanol.

Table 8.6 shows the predicted number of peaks for different experimental conditions, using Eq. (8.1) and the coefficients in Table 8.3 for both the hydro-organic mixtures and pure solvents, and a threshold of 99.7%. The maximal number of peaks corresponds to the extraction with 30% methanol, used at the maximal assayed values of ultrasonication time (60 min) and temperature (80 °C), with an expected number of peaks of 91. This corresponds to an unassayed condition set in the experimental design (Table 8.3). It should be commented that using measurements processed by setting a threshold of 99.99% (Table 8.5), the results were inconsistent, since they were affected in a greater extent by noise, with a negative impact on the reproducibility of the algorithm. Nevertheless, with these measurements, it can be also concluded that the temperature increased significantly the number of peaks with methanol.

Table 8.5. Coefficients in Eq. (8.1) after performing multiple linear regression of the data in Table 8.4.

Solvent	Hydro-organic mixtures				Pure solvents			
	c_0	c_1	c_2	c_3	c_0	c_1	c_2	c_3
Design I								
Cumulative relative peak area = 99.2%								
Acetonitrile	40.3	–8.3	4.8	7.3	27.8	–15.8	-0.8	3.8
Ethanol	37.5	–6.0	0.0	1.5	32.3	–11.3	1.3	5.8
Methanol	42.8	–6.8	5.3	9.8	42.5	–1.0	0.0	4.5
Cumulative relative peak area = 99.7%								
Acetonitrile	62.5	–9.0	5.0	3.5	41.5	–23.0	–3.0	5.5
Ethanol	62.3	–14.2	1.75	10.3	50.8	–13.8	1.8	10.3
Methanol	70.3	–9.3	1.3	10.8	63.5	–1.0	–2.0	6.5
Cumulative relative peak area = 99.99%								
Acetonitrile	95.0	–7.0	6.0	2.0	76.0	–24.0	2.5	4.5
Ethanol	90.0	–17.5	–10.0	–6.5	88.0	–12.0	3.5	5.5
Methanol	109.5	–7.5	–1.0	6.0	95.3	–4.8	0.8	2.7
Design II								
Methanol	83	14	–2.5	–2.5				

Table 8.6. Predicted number of peaks for the eight extreme experimental conditions covering the region of Designs I and II (Table 8.3), and using the coefficients in Table 8.5, for the hydro-organic mixtures, pure solvents and optimal extraction conditions, for a threshold of cumulative relative area of 99.7%.

Factors	Acetonitrile		Ethanol		Methanol		Factors	Methanol
A B C	Hydro-organic mixtures	Pure	Hydro-organic mixtures	Pure	Hydro-organic mixtures	Pure	*D E F*	
+ + +	62	21	48	49	73	67	+ + +	92
+ + –	55	10	52	29	52	54	+ + –	97
+ – +	52	27	45	46	71	71	+ – +	97
– + +	80	67	77	77	91	69	– + +	64
– – +	70	73	73	73	89	73	– – +	69
– + –	73	56	80	56	70	56	– + –	69
+ – –	45	16	48	25	49	58	+ – –	102
– – –	63	62	77	53	68	60	– – –	74

By combining the information of the designs using the pure solvents with the designs with the hydro-organic mixtures, we could know whether hydro-organic mixtures were preferable or not against pure solvent to perform the extractions. With the coefficients in Table 8.5, the number of peaks was predicted along the methanol content axis (0, 30, 70 and 100% methanol levels) with the best levels in temperature and sonication time. A parabolic trend was observed with the maximum at 30% methanol.

8.5.5. Amount of processed sample and conservation conditions of the extract

The above series allowed establishing the best conditions associated with the type of solvent and its concentration, as well as the ultrasonication time and temperature. Methanol was found the best solvent, used in 30% mixtures with water, using a high ultrasonication time and temperature (60 min and 80 ºC). However, the following factors should be still investigated:

(i) Factor *D*: Amount of treated sample, with minimal (–) and maximal (+) levels of 0.1 and 1.0 g, respectively.

(ii) Factor *E*: Conservation time of the extracts, with minimal (–) and maximal (+) levels of 1 and 5 days, respectively.

(iii) Factor *F*: Conservation temperature, with minimal (–) and maximal (+) cooling levels of 4ºC and –10ºC, respectively.

The assayed design (Design II) is shown in Table 8.3, and the results in Table 8.4. In Fig. 8.5, the number of peaks above different relative and absolute peak areas is plotted. It should be noted that in this new series of experiments, the amount of sample changes between experiments, and consequently, the use of semi-logarithmic plots of relative peak areas (Fig. 8.5a) is inappropriate owing to the different weight of noise. Therefore, the conclusions were based on the absolute areas (Fig. 8.5b). It can be observed that a larger processed mass (1 g, two upper lines) provided about 20 peaks more than 0.1 g (two lower lines). The cumulative relative peak areas were, however, useful in establishing comparison thresholds. Thus, if a threshold of relative peak area of 0.025% (99.7% cumulative relative area) is established, 69 peaks exceeded the threshold for the 0.1 g sample (bottom of Table 8.4). When 1 g was processed instead, the detected peaks amounted 92 and 102, depending on the conservation conditions.

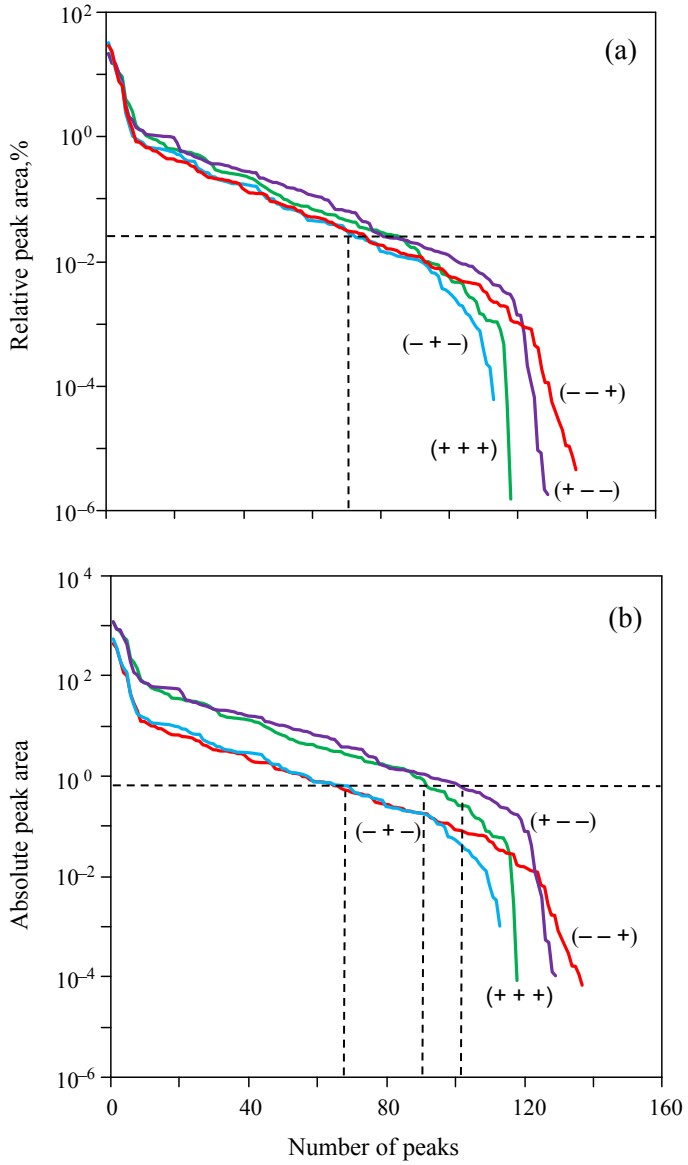

Fig. 8.5. Semi-logarithmic plots of relative (a) and absolute (b) prominence areas corresponding to the investigation of the effect of the sample amount and conservation conditions of the extract. The experimental conditions are indicated in Table 8.3.

A multiple linear regression model was fitted from the data in Table 8.4, in order to evaluate the effect of factors *D*, *E* and *F* on the number of significant peaks:

$$NP = c_0 + c_1 \times D + c_2 \times E + c_3 \times F \quad (8.2)$$

Table 8.5 shows the corresponding model coefficients (Design II), and Table 8.6 the number of predicted peaks in the assayed conditions and in those not included in the design. The coefficients in Table 8.5 indicate that the factor with the strongest effect on achieving a high number of peaks is the amount of treated sample. The conservation time and temperature of the extracts have relatively little effect on the production of significant peaks.

However, the results indicate that if the sample is refrigerated at 4°C, the number of peaks is somewhat larger than when it is frozen at –10°C. The larger number of peaks at higher temperature is probably produced by the degradation of some compounds, which gives rise to new peaks in the fingerprints exceeding the established threshold. Since the degradation processes of the sample extracts are undesirable, the analyses should be carried out immediately after the extraction. Otherwise, the extracts should be kept frozen.

8.6. Conclusions

The study developed for this work is aimed to improve the level of information in chromatographic fingerprints, evidenced by the number of significant peaks. The quantification of the peaks in the chromatograms was based on a function that measures the peak prominences automatically. A new approach was developed where peaks are ranked according to the prominence areas and those peaks exceeding an established threshold (99.7%) of cumulative relative peak area are selected.

Following a preliminary study to establish an acceptable gradient program, we proceeded to study the factors affecting the extraction of the sample components in a medicinal herb and the stability. The examined factors were type and concentration of organic solvent, ultrasonication time and temperature, amount of sample, and time and temperature of conservation of the extracts. The use of the three usual solvents reported in the literature for this type of extractions was investigated (acetonitrile, ethanol and methanol). The proposed methodology to describe the information content of fingerprints, based on the measurement of peak prominences, showed conclusive results. The extraction solvent giving rise to the maximal number of peaks in the analysed sample was 30:70 methanol-water at high temperature (80 °C) and long ultrasonication time (60 min).

The algorithm ascribes a larger "significance" to peaks with larger area. Peaks with low area (lower than a threshold) are omitted from consideration. It is known, however, that some important phytochemicals, such as those that define biological activity of the herbs, are found in small amounts, and may be missed using the proposed algorithm. Also, heating, especially for a long time under sonication, results in the degradation of thermolabile phytochemicals. Thus, if the purpose is to obtain a representative image of the green tea composition, the conditions would not be appropriate, since heating will increase the number of peaks. However, if peaks belong to genuine compounds or to the products of thermodegradation it is not relevant if the process is reproducible, and a larger number of peaks can improve sample recognition.

The described approach was developed using a green tea sample, but it is suitable for finding the best extraction and conservation conditions for other types of medicinal herbs, in order to get fingerprints with the maximal information. Currently, research is being developed in our laboratory to

optimise the gradient program to analyse fingerprints of medicinal herbs or from other sources, by applying the proposed chromatographic objective function based on the measurement of peak prominences. However, the approach should still demonstrate its usefulness to distinguish different samples from each other.

Finally, it should be commented that the approach to find the significant peaks in a chromatogram can be generalised to other complex samples. Thus, it can be seen as a general method rather than a particular solution for a specific problem.

8.7. References

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CHAPTER 9

STUDY OF THE PERFORMANCE OF A GLOBAL RESOLUTION CRITERION TO CHARACTERISE COMPLEX CHROMATOGRAMS WITH UNKNOWNS OR WITHOUT STANDARDS

9.1. Abstract

The search of the best conditions in liquid chromatography is routinely carried out with information provided by chemical standards. However, sometimes there are samples with insufficient knowledge about their chemical composition. In other cases, the identities of the components are known, but there are no standards available, and in other cases the identities of peaks in chromatograms taken under different conditions are ambiguous. Most resolution criteria used to measure the separation performance cannot be applied to these samples. In this work, a global resolution function valid for all situations was developed based on automatic measurements of peak prominences (area fraction exceeding the line that joins the valleys delimiting each peak). Relative performance is evaluated against the peak purity criterion (which measures the area free of overlapping). Peak purity provides a truly comprehensive measurement of global resolution since the underlying signals for each compound are used. However, it is only accessible through *in silico* simulations. In contrast, peak prominences are not based on a comprehensive knowledge of the individual signals, and can be obtained from experimental chromatograms besides *in silico* simulations. Therefore, this criterion is suitable for the direct evaluation of the resolution of chromatograms of high complexity. A comparison study was carried out based on the agreement of the gradients chosen as Pareto-optimal by both criteria, using the information from standards of the 19 primary amino acids found in proteins. The developed global resolution function was applied with success to chromatographic fingerprints of medicinal herbs.

9.2. Introduction

Scientific and technological advances are increasingly demanding more powerful analytical techniques. Therefore, the development of not only more sophisticated instrumentation and materials, but also strategies and chemometric methodologies, are necessary to solve new problems. Reversed-phase liquid chromatography is nowadays the most extended chromatographic technique, due to its wide field of application, reliability, robustness, and sensitivity [1]. However, its efficiency is limited in comparison with other analytical techniques such as gas chromatography, capillary electrophoresis, and other electromigration techniques, which is especially detrimental for analysis of samples with complex compositions. Arbitrarily selected experimental conditions rarely provide enough selectivity. This problem can be minimised with the use of highly selective detectors such as mass spectrometry (MS). However, despite the high selectivity of this detection technique, the inclusion of a well-designed separation step is still needed [2–4]. Moreover, MS detection is still out of reach for most laboratories. Thus, an optimisation of separation conditions is always helpful.

Independently of the peaks in a chromatogram being known or unknown, it is required that their mutual separation be as large as possible for both identification and quantification. An extreme case where relative peak distribution and magnitude is the relevant feature is found in chromatographic fingerprints and classification studies. In this case, better resolution will offer more informative chromatograms.

A satisfactory separation implies obtaining sufficient chromatographic resolution in an acceptable analysis time. This is achieved by the appropriate adjustment of the experimental conditions. When complex samples are analised, the determination of the optimal conditions by “trial and error” can

become an expensive, slow and inefficient process. *In silico* simulations are the most efficient tools to discover the best separation conditions [5–7]. A practical way to carry out a search is measurement of the separation quality using a chromatographic objective function (COF) [6–19]. Usually, this type of function requires information about the retention and profile of chromatographic peaks to establish models in order to predict chromatograms in a wide range of conditions, without the need of performing further experimental assays.

Two main strategies have been traditionally used to evaluate the quality of the separation: (i) monitoring the expected resolution for the worst resolved peak pair, and (ii) combining the resolution values for all peaks in a chromatogram; both cases apply for a set of unassayed conditions involving an experimental design. The second strategy is more interesting when the aim is obtaining complete (or almost complete) separation for all peaks in a chromatogram. However, conventional COFs are not appropriate in all instances to quantify the resolution level. Since these COFs are dominated by the worst resolved compounds, when at least one compound appears significantly overlapped under all experimental conditions, the global resolution will always be close to zero, even when all other compounds were fully resolved.

We have proposed new approaches to find the best separation in situations of extremely low chromatographic resolution; they consist of monitoring the sufficiently resolved peaks in the chromatogram, which has been called peak count [20–22]. These approaches are aimed to quantify the degree of success in the separation (the well resolved peaks), contrary to conventional COFs, which quantify the degree of failure (they attend mainly to the worst resolved peaks).

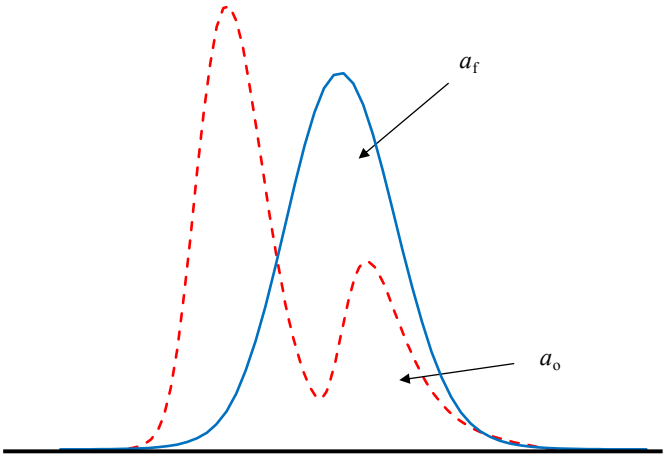


Fig. 9.1. Peak purity criterion. The free fraction area (a_f) and overlapped area with other peaks in the chromatogram (a_o) is shown. The total peak area (a_T) is the sum of a_f and a_o.

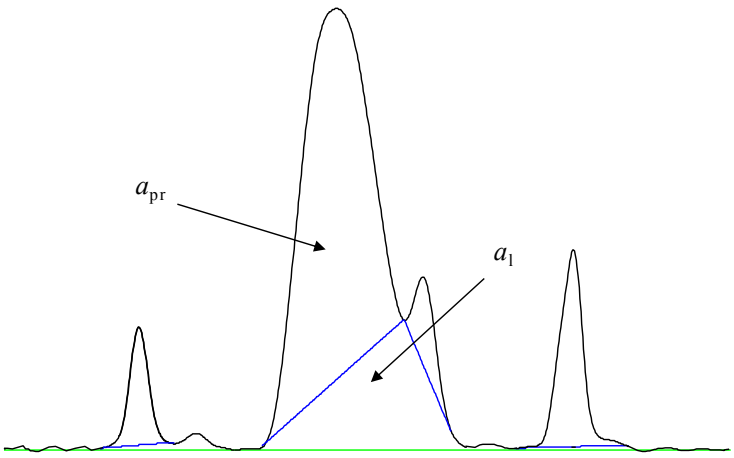


Fig. 9.2. Peak prominence criterion. The line that joins the valleys delimiting each peak divides it in two regions of area a_{pr} and a_l. The total peak area (a_T) is approximated to the sum of a_{pr} and a_l.

A further level of complexity consists in optimising the chromatographic resolution for samples where some or even all compounds are unknown, or for which no chemical standards are available. As indicated above, most COFs reported in the literature are based on predictive models, which must be fitted using chromatographic information obtained from standards for all constituents. In the absence of standards, predicting the best separation conditions is not possible using conventional COFs. This is the case of the Snyder's R_S resolution and the peak purity (area free of overlapping, see Fig. 9.1), which require simulated chromatograms for their evaluation. In contrast, other conventional COFs, such as the so-called "selectivity" (α), and valley-to-peak functions [21] can be straightforwardly measured from experimental chromatograms, without knowledge of the underlying signals of each compound. However, when two or more peaks are overlapped, these functions can offer misleading conclusions. In addition, they are related to peak pairs, and reversals in the elution order give rise to discontinuities. This is also the case for the Snyder's R_S resolution.

In previous work [23,24] (Chapters 7 and 8), we proposed a new COF to solve such problematic situations, which was called "peak prominence". This criterion is based on measurement of the protruding part of chromatographic peaks (area fraction exceeding the line that joins the valleys delimiting each visible peak, see Fig. 9.2), and is applicable to experimental chromatograms without the need of standards. In that work, peaks in the sample were ranked according to the areas of peak prominences, and a threshold of cumulative area was established to discriminate between the significant peaks and those that were irreproducible in replicated injections. The number of significant peaks in fingerprint chromatograms was then used to optimise the extraction and conservation conditions of medicinal herbs [24].

This work represents another step in the development of peak prominence as a resolution criterion. The main objective was to check to what extent the results found as optimal by peak prominence agree with those given by a reference function, which intrinsically has an exhaustive knowledge about the number of eluting compounds and profile of the peak of each compound. Peak purity was chosen for the comparison, since it provides accurate and reliable estimations of resolution, and matches closely with the assessment of resolution of experienced analysts [11]. The study implies development of a proper global function and inspection of its performance in a number of controlled situations gradually closer to reality. Finally, the selected function was adapted to cope with complex chromatograms containing unknown compounds or without available standards.

9.3. Experimental

Two types of samples were considered:

(i) Solutions of the 19 primary amino acids found in proteins, for which standards were available: (1) Aspartic acid, (2) glutamic acid, (3) asparagine, (4) serine, (5) glutamine, (6) histidine, (7) glycine, (8) arginine, (9) threonine, (10) alanine, (11) cysteine, (12) tyrosine, (13) valine, (14) methionine, (15) isoleucine, (16) tryptophan, (17) phenylalanine, (18) leucine, and (19) lysine. Previous to chromatographic separation with acetonitrile-water mixtures, the amino acids were derivatised with *o*-phthalaldehyde (OPA) and *N*-acetylcysteine (NAC) so they could be monitored at 335 nm. The set of amino acids was used to study performance of the peak prominence criterion compared to the peak purity. The purpose of using these compounds was to have experimental

information about the characteristics of the peaks for each analyte. Experimental information on chromatographic behaviour of the amino acid derivatives was taken from our laboratory database. More details on the chromatographic procedure are given elsewhere [25].

(ii) Extracts of decaffeinated and horsetail teas (bought at a local supermarket), were prepared according to the recommendations given by Dumarey et al. [26]. These samples were used as representatives of cases where no standards are available to build predictive models of retentions and peak profiles. Fingerprints of the extracts were obtained with a modular Agilent chromatograph (Model HP 1100, Waldbronn, Germany), consisting of a quaternary pump, autosampler, thermostatted column compartment, and UV-visible detector set at 210 nm. The injection volume was 10 μL, and the mobile phase flow rate was kept constant at 1 mL/min. Analyses were carried out with a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm i.d. with 5 μm particle size, Agilent), using linear gradients of acetonitrile, buffered with 0.01 M monosodium dihydrogen phosphate (Sigma) and 0.01 M HCl (Scharlab) at pH 3.

9.4. Data treatment

A MATLAB (2016b version, The MathWorks Inc., Natick, MA, USA) function was developed for automatic measurement of relevant information in complex chromatograms. This function detects and measures all peaks present in a chromatogram up to a certain critical threshold, which depends on the noise level. It also merges secondary signals, and discards spikes and noise peaks. A number of peak properties are measured for those peaks surviving the refinement process. One of them is peak prominence, which is obtained from

the optimised tangent line between the two valleys defining each peak. More details are given elsewhere [23,24].

9.5. Results and discussion

A study was carried out to check whether the peak prominence criterion was able to discover the same optimal conditions as those selected by peak purity, which in previous work has shown excellent performance [11,19,22,25,27,28]. Obviously, when all peaks are resolved, both criteria will agree, but in conditions where there are fewer visible peaks than compounds, the differences may become notorious. With this aim, several synthetic study cases involving the separation of OPA-NAC derivatives of amino acids using 1081 linear acetonitrile-water gradients were generated. Note that for this study we needed a sufficiently complex set of compounds with standards available to be able to predict resolution according to the peak purity criterion.

Three definitions of global peak prominence were examined, investigating the following situations: existence of peaks in a chromatogram with the same or different areas, presence of noise and unknown compounds. The best global resolution function was further refined to account situations where the number of components is not well defined, as is the case of chromatographic fingerprints. Since the information from standards is required, the peak purity of fingerprints cannot be calculated.

9.5.1. Selection of optimal conditions for a sample with standards available

Chromatographic training data for the amino acid derivatives consisted of a number of isocratic experiments using acetonitrile in the range 5–27.5% (v/v) [25]. This information yielded very accurate predictions for linear gradients, suitable for the studies designed for this work. The predicted chromatograms were computed following a methodology described elsewhere [27,28]. Complexity of the sample gave rise to generation of a variety of situations with chromatograms involving multiple peak configurations. This allowed evaluating performance of the peak prominence criterion under controlled situations. Similar conclusions could be achieved with any other complex sample, provided that full information is available from standards.

The search of optimal chromatographic conditions requires attending simultaneously to both resolution and analysis time, opposed to each other: high analysis times (which are undesirable) tend to provide better resolution (which is favourable); conversely, short analysis times do not favour best resolution. This behaviour implies that instead of a single solution for the optimal separation, there is usually a collection of valid solutions, some of them preferable because analysis time is shorter and others preferable because resolution is higher. These solutions (linear gradients in this work) can be represented in a plot where the axes correspond to the opposite quality measurements to be enhanced: chromatographic resolution and analysis time. Such type of plots were suggested by Pareto et al. in the context of multi-objective optimisations [29]. The purpose of the plots is to reveal the Pareto-optimal solutions. A solution is qualified as Pareto-optimal when a response cannot be improved without worsening another [30].

Fig. 9.3a shows a Pareto plot for the separation under gradient elution of a mixture containing the OPA-NAC derivatives of the 19 primary amino acids,

assuming that peak areas for all compounds are the same. In our example, each point in the plot corresponds to a particular linear gradient, which is characterised by its global peak purity –calculated as product of the individual peak purities–, and analysis time. The assayed 1081 gradients assumed a gradient time of 60 min and variable initial and final concentrations of acetonitrile in the 5–27.5% range, which corresponds to the extreme concentrations in the isocratic experimental design used for modelling the chromatographic behaviour.

The border region in Fig. 9.3a constitutes the Pareto front (Fig. 9.3b), which includes the gradients fulfilling the optimality principle: those experimental conditions (in our case linear gradients), for which resolution cannot be improved without increasing analysis time, from which an analyst can make his/her selection. It can be observed that almost complete resolution (global peak purity > 0.95) is only possible at long analysis times. To appraise the separation performance, two points were marked with arrows on the Pareto front, corresponding to situations of incomplete and nearly complete resolution (global peak purity of 0.3 (point c) and above 0.9 (point d), respectively). The corresponding chromatograms are shown in Figs. 9.3c and d, respectively.

In the horizontal intermediate region of the Pareto front, the best chromatograms keep an approximately constant analysis time, but very different resolution from low to high. This situation is usual in chromatography, and the solution selected as optimal is the last high resolution condition in the right side of the horizontal region (point (d) in Fig. 9.3b). If the analyst tries to decrease the analysis time beyond point (d), the resolution decreases drastically and there is no longer a practical optimal solution.

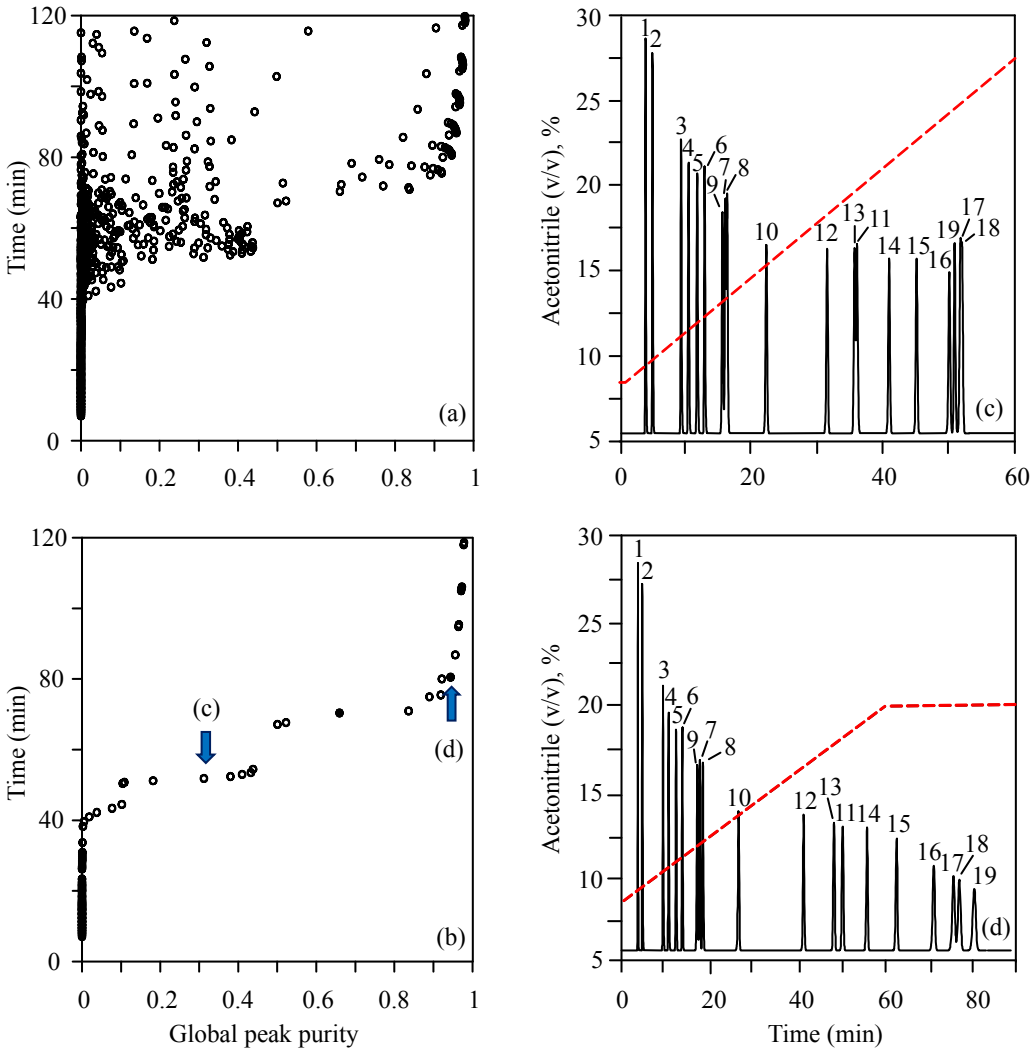


Fig. 9.3. Performance of the separation of a sample containing the 19 primary amino acids, derivatised with OPA-NAC, using linear gradients in a selected experimental design: (a) full Pareto plot, (b) Pareto front, and (c) and (d) chromatograms corresponding to the situations marked with arrows on the Pareto front. Amino acids identities are given in Section 9.3. The gradient program is represented on each chromatogram.

9.5.2. Peak prominence versus peak purity

Peak prominence and peak purity criteria are based on different principles and have different scopes of application. Peak purity is calculated from simulated chromatograms obtained using information from chemical standards. It is possible to predict the individual contribution for each compound, and from this and the contribution for the accompanying compounds, the associated resolution. By combining the peak purities for all compounds, a global measurement expressing quality of the separation of the whole chromatogram is obtained. In this way, it is possible to predict how the chromatograms would change under different experimental conditions (*in silico* simulation). In contrast, the peak prominence is measured directly from experimental chromatograms (which is not possible with the peak purity). Since the aim of this study was the comparison of both functions (peak prominence versus peak purity), information obtained from standards (the set of amino acid derivatives) was used. In this study, when peak prominences were computed, the chromatogram of the mixture was straightforwardly processed as if we only had that chromatogram.

A common way to reduce information from several peaks to a single value is the product of the resolutions of all peak pairs, which qualifies the global resolution reached in a chromatogram. Alternatively, the product of the individual resolutions associated with all peaks can be used. This global criterion is used in Fig. 9.3. However, only a comprehensive knowledge of the compounds eluting under an apparent peak allows a proper appraisal of the global resolution. When the peak purity criterion is applied, the number of expected compounds in the mixture is always known, and so the number of underlying peaks (being visible or not). In contrast, when only the overall chromatogram of a sample is available, it is not possible to know whether a

peak hides one or more underlying compounds. Consequently, it can be expected that the products of peak purities and peak prominences show strong differences for chromatograms presenting significant overlaps, where the number of visible peaks is smaller than the existing compounds.

9.5.3. Global resolution function based on peak prominence

As the product of elementary values is not a good choice to compare both resolution criteria, the sum of normalised elementary resolutions was used instead. Similarly to the product, the higher the summation, the more are the resolved compounds. However, the product is extremely sensitive to peak overlapping, whereas the summation allows improvements of resolution in spite of existing overlapped peaks. Another advantage of the summation is that the maximal global value for a set of experimental conditions will give an indication of the number of compounds in the sample (i.e., the sample will contain that number –or more– compounds). The sum of resolutions has been used in the literature and participates, for instance, in the COFs reported by Berridge [31] and Duarte et al. [21].

For peak prominence, three variants were considered for the global resolution function:

$$PR1_j = \sum_{i=1}^{nd} a_{\mathrm{pr},i,j} \tag{9.1}$$

$$PR2_j = \sum_{i=1}^{nd} \frac{a_{\mathrm{pr},i,j}}{a_{\mathrm{T},i,j}} \tag{9.2}$$

$$PR3_j = nd \times \frac{\sum_{i=1}^{nd} a_{\mathrm{pr},i,j}}{\sum_{i=1}^{nd} a_{\mathrm{T},i,j}} \tag{9.3}$$

nd being the number of detected peaks, $a_{\mathrm{pr},i,j}$ the area of the protruding part of peak *i*, and $a_{\mathrm{T},i,j}$ its total area (Fig. 9.2), for a chromatogram obtained with gradient *j*. Eq. (9.1) considers directly areas of the protruding part of the peaks, and makes sense when applied to a numerical optimisation based on simulated chromatograms, where all peaks have unit areas. Eqs. (9.2) and (9.3) are aimed to make the resolution function insensitive to differences among peak areas, and additionally, achieve better equivalence between peak prominence and peak purity in the selection of optimal gradients. Eq. (9.2) considers normalised values referred to each individual peak, so that if the peaks are fully resolved, *PR*2 matches the number of eluted compounds. Eq. (9.3) also provides a normalised measurement, but in this case referred to the whole chromatogram, so that if it is multiplied by the number of peaks, it gives values similar to *PR*2, but giving more importance to the resolution of major components.

For comparison purposes, the global peak purity was calculated as:

$$P_j = \sum_{i=1}^{n} p_{i,j} \tag{9.4}$$

where $p_{i,j}$ is the elementary peak purity for compound *i* and gradient *j*, and *n* the number of eluted compounds.

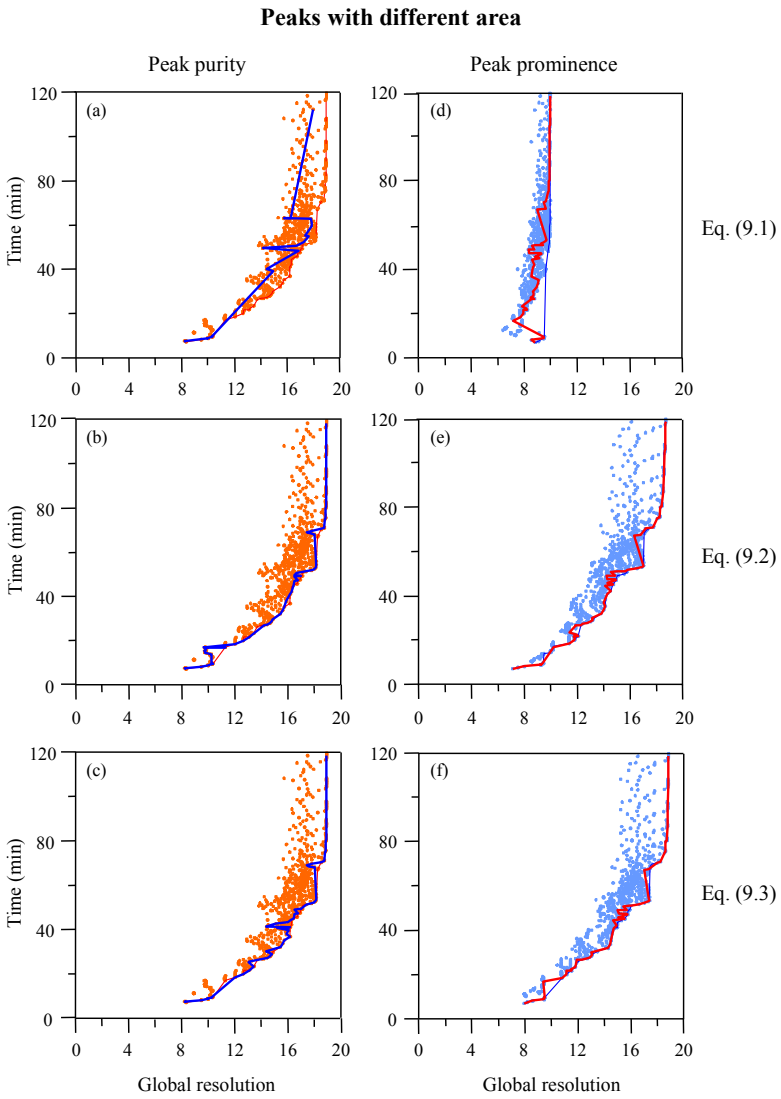


Figure 9.4. Pareto plots for chromatograms of samples containing the 19 amino acids with differentiated peak areas. Global resolution criteria: (a,b,c) sum of peak purities, (d) sum of areas of the protruding part of each peak, (e) sum of peak prominences, and (f) number of detected peaks multiplied by the ratio of the sum of the protruding parts of each peak and sum of total areas. The Pareto fronts for each criterion are shown as thin lines, and the projections of the gradients from one criterion to the other as thick lines.

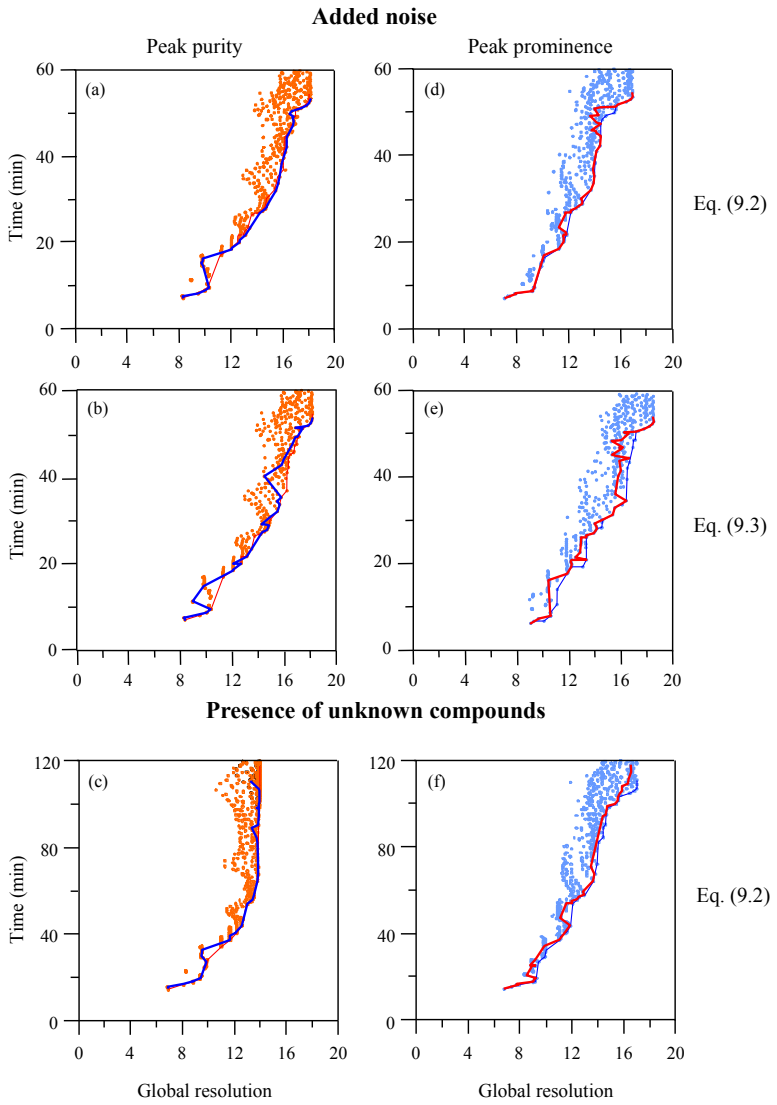


Fig. 9.5. Pareto plots for chromatograms of samples containing the 19 amino acids with differentiated peak areas, added noise and unknown compounds: (a,b,c) sum of peak purities, (d,f) sum of peak prominences, and (e) number of detected peaks multiplied by the ratio of the sum of the protruding parts of each peak and sum of total areas. In (c,f), five compounds were considered as unknowns. See Fig. 9.4 and text for other details.

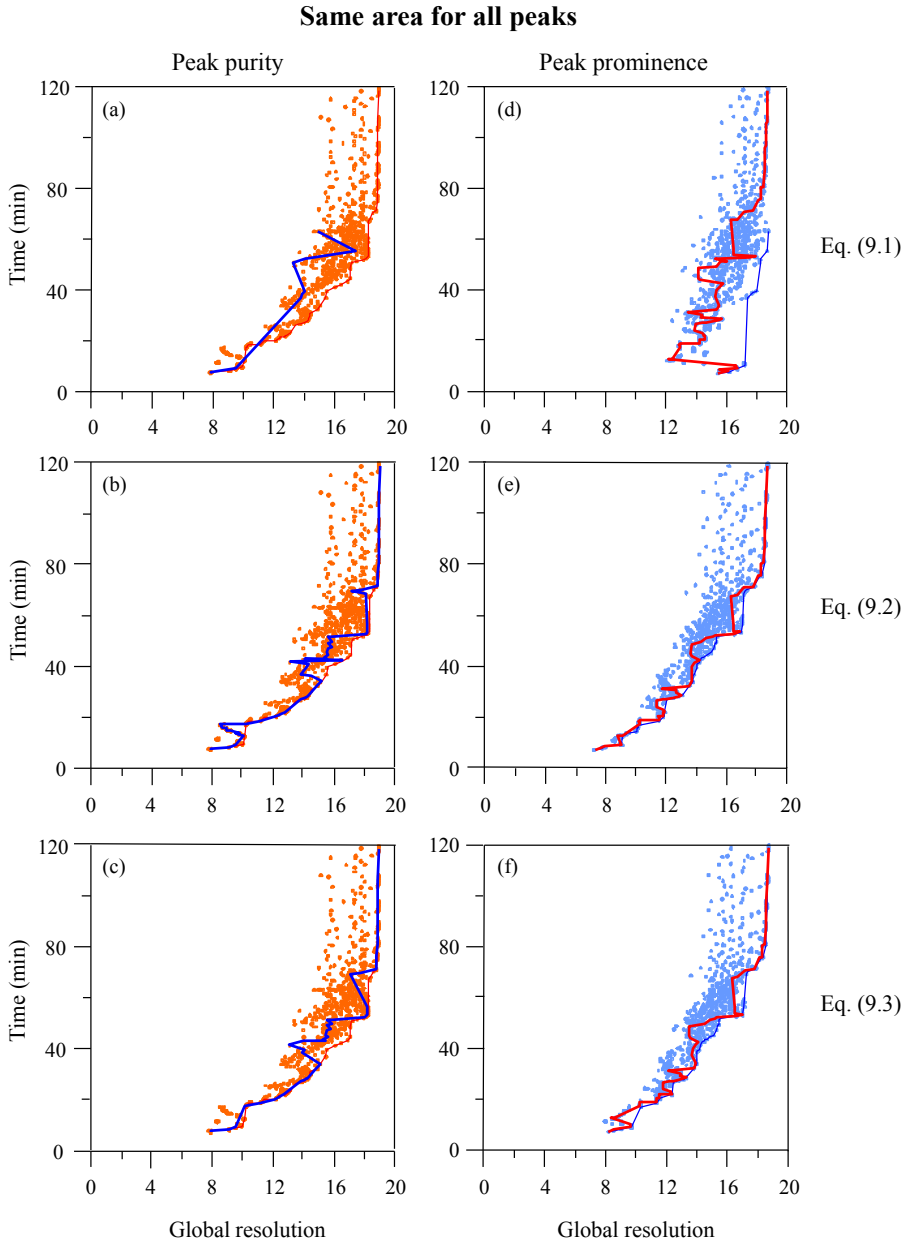


Fig. 9.6. Pareto plots according to different global resolution criteria, corresponding to the separation of the OPA-NAC derivatives of the 19 amino acids assuming normalised peak areas. See Fig. 9.4 and text for other details.

9.5.4. Comparison methodology to check the good performance of peak prominence

In Section 9.5.3, three different definitions of global peak prominence (Eqs. (9.1) to (9.3)), and one definition for the peak purity (Eq. (9.4)) were proposed. The plot in Fig. 9.4 represents Pareto plots for peak purity (left) and peak prominence according to the three definitions (right). The most suitable definition for peak prominence will be that one for which the optimal selected gradients (those for the Pareto front) agree the best with those gradients selected by peak purity under conditions of incomplete resolution. To facilitate a comparison between peak prominence and peak purity, a sort of "projection" of the Pareto fronts for one criterion was made on the Pareto plot for the other criterion, and vice versa. For this purpose, the gradients selected as Pareto optimal in one criterion (drawn as a thin line) were searched on the Pareto plot for the other criterion, and the corresponding points were joined with a thick line. If the thin and thick lines agreed in the Pareto plots for both criteria (prominence and purity), the analyst would select the same gradients as optimal, or at least gradients performing similarly, using both criteria. In other words, a global function for peak prominence will be considered ideal if the "projection" of the gradients representing the performance of the Pareto front for peak prominence matches with the Pareto front obtained for peak purity.

The three left Pareto plots for peak purity in Fig. 9.4 are exactly the same (same dots and thin line), but the projection of the Pareto front (thick line) for each definition of peak prominence (Eqs. (9.1) to (9.3)) is different. Each plot in Figs. 9.4, 9.5 and 9.6 shows what happens in different situations. The goal is to find what definition of peak prominence (which lacks information about the number of compounds under the global signal) selects the same optimal

gradients as the criterion that has full knowledge about the real number of compounds in the sample.

It can be expected that in the region of the Pareto plot where the number of visible peaks coincides with the number of compounds, the Pareto front for one criterion and the projection of the other will agree, meaning that the same gradients are selected as optimal by both criteria. Therefore, the region where the resolution is poorer (the number of peaks is less than the number of compounds) is more meaningful for the comparison of both criteria.

9.5.5. Study of peak prominence performance

In this section, the most suitable definition of global peak prominence is investigated in situations progressively closer to reality. For this purpose, the set of 1081 predicted chromatograms for the mixture of the OPA-NAC derivatives of the 19 amino acids, obtained using linear gradients, were processed. Those gradients yielding analysis times exceeding 120 min were not considered in the Pareto plots.

9.5.5.1. Effect of peak area

In a first step of the study, an ideal situation implying noise-free predicted chromatograms was considered. Fig. 9.4 shows the corresponding Pareto plots, where areas of the involved peaks were different (a similar figure for chromatograms containing peaks with the same area, is given in Fig. 9.6). Relative peak areas, in the range 0.04–0.95, were randomly selected for each amino acid. The same values were used for the 1081 assayed linear gradients and kept for the next studies. As shown in Figs. 9.4 and 9.6, correspondence between the Pareto fronts and the “projections” for both resolution criteria is

very satisfactory when Eqs. (9.2) and (9.3) were used, especially for the former, but very poor for Eq. (9.1), in spite of involving normalised resolution measurements. Since the results obtained with Eq. (9.1) were not acceptable, this global function was discarded for further studies.

9.5.5.2. Effect of noise

Fig. 9.5 shows the Pareto plots obtained according to peak purity (Fig. 9.5a,b) and peak prominence criteria (Fig. 9.5c,d), for peaks of different size including significant noise, which was fixed for all gradients in the experimental design. The signal-to-noise ratio was 6.45 for the smallest peak obtained with the slowest gradient, expressing the noise band as $2 \times 1.96 \times 0.015$, where 0.015 was the standard deviation of the normal noise.

It can be observed again that Eq. (9.2) gave rise to a better matching with the peak purity compared to Eq. (9.3). Therefore, Eq. (9.2) seems to be the most suitable measurement of global resolution due to high agreement between the Pareto front for peak purity and the projection of optimal gradients for peak prominence, and vice versa.

9.5.5.3. Presence of unknown compounds

Fig. 9.5c,f constitutes a case of study even closer to reality since it includes, besides peaks of different size and noise, the presence of unknowns: Compounds 2, 4, 7, 13, and 17 (see identities in Section 9.3) were randomly chosen among those with peaks of low magnitude, in order to simulate the presence of unknown impurities or matrix components. Interference of the five unknown compounds was taken into account for calculating peak purity, but measurement of global resolution was limited to the remaining 14 compounds.

In contrast, the peak prominence straightforwardly attended to all visible peaks, independently of being analytes, impurities, or matrix components.

For peak purity, as there were only 14 target compounds to be resolved (the remaining five were unknowns), the maximal sum of elementary peak purities tended to 14. Meanwhile, for peak prominences, the maximal number of visible peaks tended to 18. In spite that the measurements of global resolution are different, the Pareto fronts for both criteria agreed satisfactorily in the regions of incomplete resolution, where the differences between them are magnified. This means that the prominence criterion selects as optimal practically the same gradients as peak purity, and consequently, it is possible to carry out an optimisation using the peak prominence criterion with a guarantee of finding the same optimal conditions as peak purity, independently of the resolution level.

9.5.6. Measurement of the mean resolution from Eq. (9.2)

9.5.6.1. Chromatograms containing a limited number of peaks

From the above discussion (Section 9.5.5), the sum of peak prominences according to Eq. (9.2) was concluded to be the best choice for monitoring resolution in complex chromatograms. In order to convert this measurement to a mean resolution and make it independent of the number of peaks, the sum of peak prominences should be somehow normalised. For instance, it can be divided by the number of compounds in the sample (19 for the case of study involving the amino acid derivatives).

However, for many samples, the number of compounds is unknown, although still limited. This is the situation that happens when a non-excessively complex sample with similar concentrations for all solutes is analysed using a

set of gradients, so that the elution conditions are forced to reduce the analysis time. One way of transforming the sum of prominences into a normalised resolution in such a situation is by dividing the sum of prominences by the maximal number of detected peaks, considering all assayed experimental conditions. In the separation example of the amino acid derivatives, taking into account different areas and the 1081 inspected gradients, the maximal observed sum of prominences was 18.85, which means that there should be at least 19 compounds in the sample. In this way, the maximal representative resolution would be 18.85/19 = 0.992 (almost full resolution). Similarly, the respective summations for all other gradients can be converted to a normalised resolution by dividing by 19.

9.5.6.2. Chromatograms containing an undefined number of peaks

Up to now, we considered cases where the number of compounds present in the sample was known, or at least, limited. However, the ultimate goal of this research was to measure the resolution in chromatograms of real complex samples, where the identity of some or even all compounds is unknown, and the number of peaks is not well defined (i.e., the number varies largely depending on the experimental and detection conditions, and with sample concentration). This is the case of chromatographic fingerprints, such as those obtained from medicinal herbs [32–34]. Appraising properly the resolution level in such samples is particularly difficult, owing to the extreme disparity in signal size. Fig. 9.7 depicts the fingerprints of extracts of decaffeinated and horsetail teas. In this type of sample, there is a high number of analytes in a wide range of concentrations.

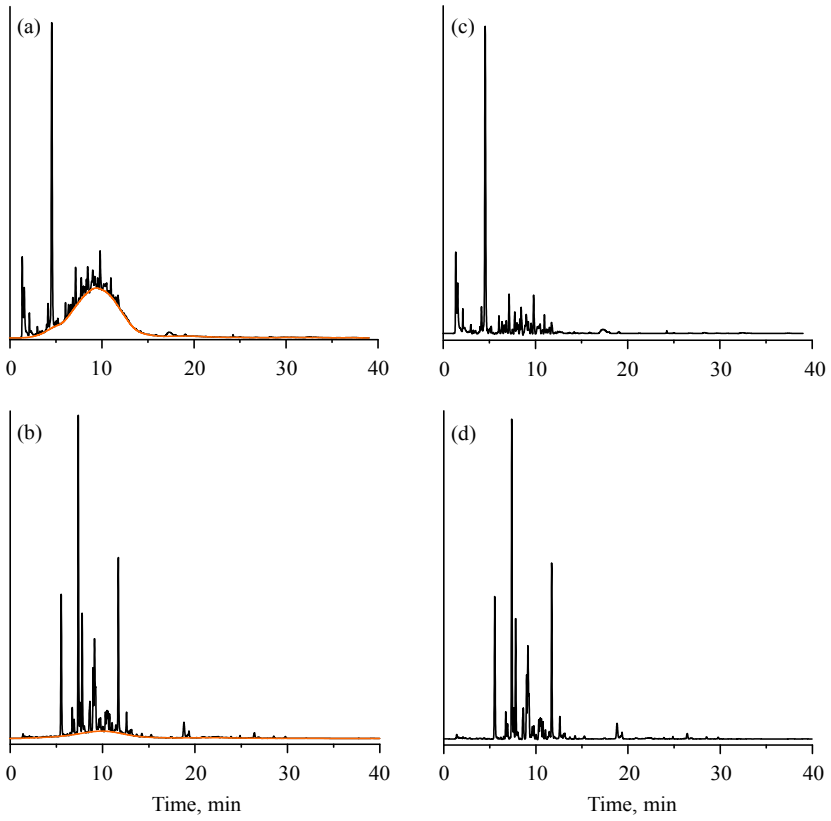


Figure 9.7. Chromatographic fingerprints for extracts of decaffeinated tea (a,c), and horsetail tea (b,d) obtained with a 20–60% (v/v) acetonitrile linear gradient using a gradient time of 10 min. (a,b) Raw chromatograms depicting the baseline found by the BEADS algorithm, and (c,d) chromatograms obtained after subtracting the baseline.

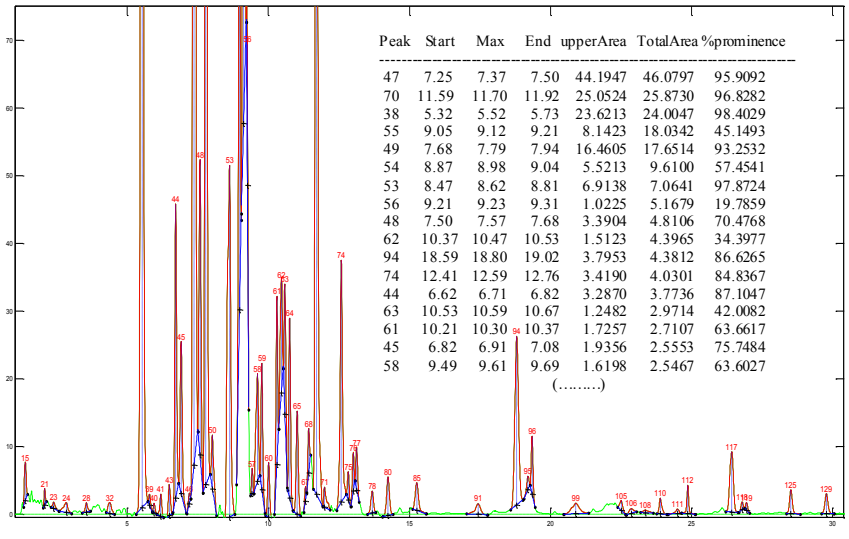
For such samples, the selected global function for the peak prominence criterion (Eq. (9.2)) requires additional adaptations. Without them, resolution values would depend on the number of considered peaks. It should be noted that, owing to the undefinedness in the number of analyte peaks in the fingerprints, the sum of peak prominences does not provide unequivocal values,

since it is conditioned by the number of terms included in the summation. Thus, if the detected peaks are prematurally cropped (i.e., the detection criterion only selects major peaks), significant peaks will be neglected, and oppositely, if the peaks are cropped too late, noise peaks will be included in the measurement. In contrast, a normalised resolution (if it could be calculated somehow) would be less prone to such variability.

A solution to this issue could be computing the summation in Eq. (9.2), after sorting the peak prominences according to a second property, such as the respective peak areas, or alternatively, the area of the protruding part of the peaks. The operation of sorting the results allows introducing a secondary factor (namely, signal size), which does not participate in the calculation of peak prominence, but whose importance in estimating the resolution is decisive when the number of peaks is not well defined, and the peaks span a wide range of magnitude. Thus, if the sorting is carried out by decreasing peak areas, the influence of residual peaks tends to be neglected. If the sorted summation is done according to the protruding part of the peaks, less visible peaks will have smaller influence. Both options are valid and the results are similar.

Raw chromatograms for the extracts of two tea samples analysed in our laboratory are shown in Fig. 9.7 (left). As observed, both chromatograms present humps, which are constituted by the accumulation of tens of thousands of unresolved co-eluting compounds, as described by Kuhnert et al. [35]. The presence of humps is a very common phenomenon in samples of natural products and cannot be left aside. Depending on the way these humps are processed, the conclusions can be very different. Processing a chromatogram whose baseline preserves the hump will attend less to the protruding parts of the peaks over the hump, whereas processing a chromatogram corrected with an apparent baseline that cancels the hump would give more importance to those

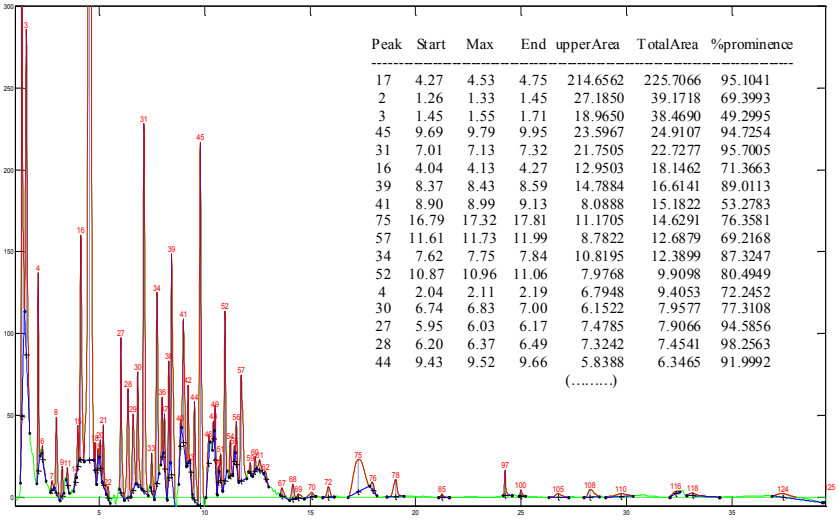
peaks protruding over the hump. Thus, an optimisation based on hump-corrected chromatograms will tend to magnify the visibility of the peaks over the hump. Obtaining a maximal amount of the protruding peaks (over the hump) is clearly the desirable situation. Therefore, the apparent baselines should be subtracted prior to any measurement of resolution.



Peak	Start	Max	End	upperArea	TotalArea	%prominence
47	7.25	7.37	7.50	44.1947	46.0797	95.9092
70	11.59	11.70	11.92	25.0524	25.8730	96.8282
38	5.32	5.52	5.73	23.6213	24.0047	98.4029
55	9.05	9.12	9.21	8.1423	18.0342	45.1493
49	7.68	7.79	7.94	16.4605	17.6514	93.2532
54	8.87	8.98	9.04	5.5213	9.6100	57.4541
53	8.47	8.62	8.81	6.9138	7.0641	97.8724
56	9.21	9.23	9.31	1.0225	5.1679	19.7859
48	7.50	7.57	7.68	3.3904	4.8106	70.4768
62	10.37	10.47	10.53	1.5123	4.3965	34.3977
94	18.59	18.80	19.02	3.7953	4.3812	86.6265
74	12.41	12.59	12.76	3.4190	4.0301	84.8367
44	6.62	6.71	6.82	3.2870	3.7736	87.1047
63	10.53	10.59	10.67	1.2482	2.9714	42.0082
61	10.21	10.30	10.37	1.7257	2.7107	63.6617
45	6.82	6.91	7.08	1.9356	2.5553	75.7484
58	9.49	9.61	9.69	1.6198	2.5467	63.6027
				(.....)		

Fig. 9.8. Screenshot for horsetail tea.

For this purpose, we developed a modification [36] of the BEADS algorithm [37], which assists in its application to real samples. Fig. 9.7 shows the corrected chromatograms on the right. Corresponding screenshots of the chromatograms processed with the developed MATLAB function are shown in Figs. 9.8 and 9.9.



Peak	Start	Max	End	upperArea	TotalArea	%prominence
17	4.27	4.53	4.75	214.6562	225.7066	95.1041
2	1.26	1.33	1.45	27.1850	39.1718	69.3993
3	1.45	1.55	1.71	18.9650	38.4690	49.2995
45	9.69	9.79	9.95	23.5967	24.9107	94.7254
31	7.01	7.13	7.32	21.7505	22.7277	95.7005
16	4.04	4.13	4.27	12.9503	18.1462	71.3663
39	8.37	8.43	8.59	14.7884	16.6141	89.0113
41	8.90	8.99	9.13	8.0888	15.1822	53.2783
75	16.79	17.32	17.81	11.1705	14.6291	76.3581
57	11.61	11.73	11.99	8.7822	12.6879	69.2168
34	7.62	7.75	7.84	10.8195	12.3899	87.3247
52	10.87	10.96	11.06	7.9768	9.9098	80.4949
4	2.04	2.11	2.19	6.7948	9.4053	72.2452
30	6.74	6.83	7.00	6.1522	7.9577	77.3108
27	5.95	6.03	6.17	7.4785	7.9066	94.5856
28	6.20	6.37	6.49	7.3242	7.4541	98.2563
44	9.43	9.52	9.66	5.8388	6.3465	91.9992
				(.....)		

Fig. 9.9. Screenshot for decaffeinated tea.

In Fig. 9.10, mean resolution values for the two corrected fingerprints are plotted, after sorting the sum of peak prominences according to the respective peak areas. As can be seen, the horsetail extract not only has a smaller drop in resolution when new peaks are added, but also includes more detected peaks.

In order to make resolution values for intrinsically different samples comparable, a common reference is needed. In previous work [24], we observed that sorted relative areas of the protruding parts of the peaks of replicates of fingerprints diverged beyond 99.95% of the cumulative sum. The same is valid for the total peak areas. Thus, for the measurement of global resolution, a logical choice consists in summing the sorted peak prominences according to Eq. (9.2) up to this level, which would include most minor peaks in the computation of the resolution. However, this threshold can be decreased or increased according to user needs and sample features.

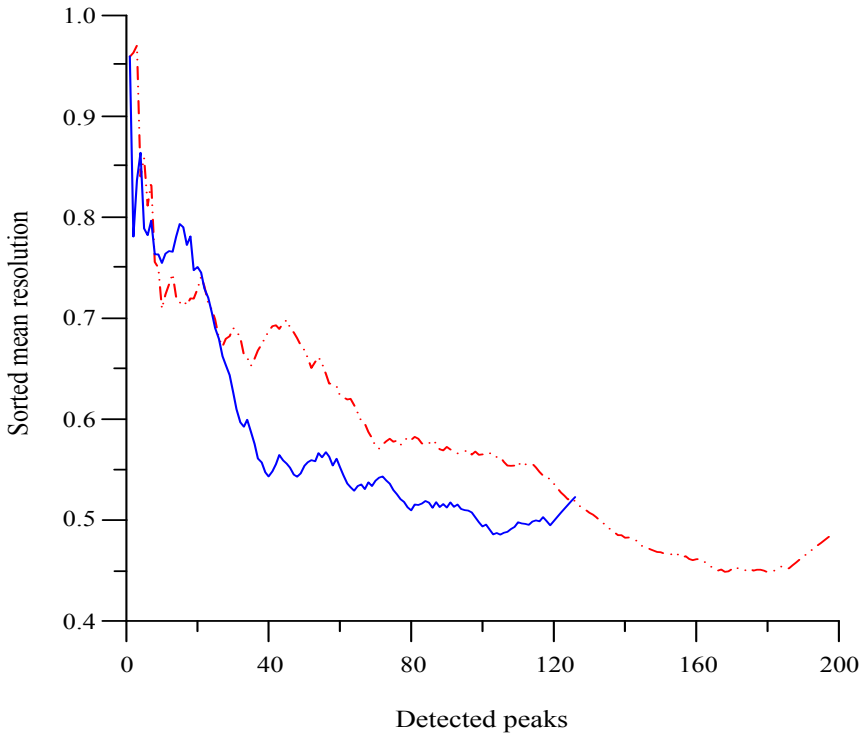


Fig. 9.10. Sorted mean resolution plot for the fingerprints of the extracts of medicinal herbs: decaffeinated tea (continuous line), and horsetail tea (dotted dashed line). See text for meaning.

For the horsetail and decaffeinated tea (Fig. 9.7), the number of peaks included at a threshold of 99.95% total peak area was 120 and 102, respectively. The sum of peak prominences sorted according to the areas was 64.33 and 50.05, and therefore, the mean global peak prominence was 64.33/120 = 0.536 and 50.05/102 = 0.491 for the two samples. Note, however, that if our objective is selecting the chromatogram giving more resolved peaks, the sum of peak prominences at a given threshold is the best measurement.

9.6. Conclusions

This work demonstrates that experimental conditions selected as the best by the global peak prominence agrees with those chosen by global peak purity. Peak purity is a function that has shown excellent features for measuring the resolution level in a chromatogram. This criterion is able to find the best separation conditions, even when complete resolution is not achieved, as is the case of the OPA-NAC derivatives of the amino acids found in proteins at short analysis times. However, it requires comprehensive information on the individual signals under each chromatogram to be computed. Peak prominence is based on very different principles, but shares some of the best features of the peak purity criterion, with the additional advantage of being evaluable directly from experimental chromatograms of the sample, without going through steps of modelling, prediction and simulation based on the information obtained from standards, as required for peak purity.

The representation of Pareto plots allowed thorough inspection of a large number of separation conditions, and the evaluation of several global resolution functions versus analysis time. After discarding the product of peak prominences as a resolution measurement, three functions represented by Eqs. (9.1) to (9.3) were compared with the sum of peak purities. The mutual projections of Pareto plots in cases of increased realism allowed a pairwise comparison of resolution functions, strategy that can be useful in evaluating the performance of other COFs.

Our comparison study showed that Eq. (9.2) was the best definition of global peak prominence, since the same gradients were selected as optimal by both criteria (peak prominence and peak purity), which is particularly noteworthy in the low resolution region. This agreement allows having a valid resolution criterion in situations where it is not possible to evaluate peak purity.

Some of such situations include measuring quality of a separation in a "trial and error" optimisation, or when the sample contains unknown matrix components, or when there are no standards available for some (or all) analytes. The studies carried out in this work indicate that a resolution function based on measurement of peak prominences can be applied to any type of sample, in the presence and absence of standards, with chromatograms including peaks of similar size or with very different magnitude, in the presence or absence of noise, in the presence of unknown matrix components, even when the number of constituents is not well defined (e.g., natural products with extreme disparity in the concentration of components).

This work is dedicated to developing and validating a global resolution function that can be used for screening studies and optimisation in a design of experiments (DOE) framework. The validation has been outlined through optimisation studies, by checking whether the experimental conditions selected as optimal by the peak prominence agree with those selected by peak purity, in spite of lacking information of underlying peaks. The results suggest that the sum of peak prominences is a good choice for optimising fingerprint chromatograms. The practical use of the developed function for optimisation purposes with fingerprints obtained by gradient elution is currently being developed in our laboratory, and it will be the subject of a future report.

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CHAPTER 10

OPTIMISATION OF CHROMATOGRAPHIC RESOLUTION USING OBJECTIVE FUNCTIONS INCLUDING BOTH TIME AND SPECTRAL INFORMATION. COMPOUNDS EXHIBITING SMALL SPECTRAL DIFFERENCES

10.1. Abstract

Current liquid chromatography instruments may yield two-way signals with full spectra collected as a function of time. These signals can be processed to recover chromatographic peaks in case of low resolution in the time order, without any peak shape assumption. A two-way chromatographic objective function (COF) that incorporates both time and spectral information using diode array detection, based on the multivariate selectivity concept (figure of merit derived from the net analyte signal), was previously reported. The approach was applied to improve the chromatographic analysis of a mixture of 25 phenolic compounds with appreciable spectral differences, which remained unresolved in the time order. This work is aimed to illustrate the usefulness of the two-way COF to reduce the analysis time for compounds exhibiting small spectral differences. The COF locates experimental conditions where the spectrochromatograms can be recovered with low error, using the combination of the orthogonal projection approach and alternating least squares. The probe compounds (19 *o*-phthalaldehyde/ N-acetylcysteine amino acid derivatives) were satisfactorily resolved using multi-linear and multi-isocratic gradients of acetonitrile-water, but at too long analysis times. By increasing the elution strength, the analysis time decreased, but producing overlapped peaks. The approach found the experimental conditions where the information for these peaks could be accurately recovered based on their spectral information. This demonstrates that the developed two-way COF succeeds to find convenient separation conditions for mixtures containing overlapped peaks exhibiting small spectral differences.

10.2. Introduction

The most efficient optimisation outlines are based on the *in silico* scanning of the performance of a large number of arbitrary conditions, by inspecting their computer-predicted chromatograms [1–6]. For each predicted chromatogram, the quality of the separation is evaluated with the assistance of a chromatographic objective function (COF) that may consider, besides the resolution, qualifications with regard to other secondary aims, such as the analysis time or desirable peak shapes [1,7–9]. In the optimisation of chromatographic resolution, a usual practice consists of attending only to the time order, processing one-way data, which is supported by tradition and economy reasons: affordable chromatographs equipped with single wavelength detectors can offer enough information for mixtures of moderate complexity. However, the hyphenation of liquid chromatography (LC) with diode array detection, which allows collecting the full spectra as a function of time giving rise to two-way signals, offers interesting possibilities.

In previous work, we proposed two-way COFs using both time and spectral information from a diode array detector, suitable for optimising the separation in LC [10]. When needed, the signals from the overlapped peak pairs were recovered using the orthogonal projection approach (OPA) and alternating least squares (ALS) [11–13]. The methodology was useful in the search of experimental conditions for the analysis of a mixture of 25 phenolic compounds eluted from a C18 column with acetonitrile-water mixtures, under isocratic and gradient conditions. Some phenolic compounds remained unresolved under all examined conditions. The two-way COF was useful to select the most favourable conditions that allowed recovering the signals from all peaks in the chromatograms.

The success of the approach depends, besides the degree of overlapping, on the spectral differences of the unresolved compounds. The assayed phenolic compounds showed appreciable spectral differences, but this is not often the case for the analysed compounds. Therefore, the usefulness of the two-way approach should be still evaluated when it is applied to the resolution of a mixture of compounds with small spectral differences. The success depends on the presence of selective spectral windows for the compounds under co-elution. If this is the case, the accurate recovery of the signals of the overlapped peaks can be expected, provided that minimal differences exist between the spectra and elution profiles of the co-eluting compounds.

This possibility was checked with a mixture of *o*-phthalaldehyde/ N-acetylcysteine derivatives of primary proteic amino acids, eluted under gradient elution [14,15]. In the assayed conditions, the amino acid derivatives could be resolved only in prohibitively long analysis times, even using multi-linear and multi-isocratic gradients. If the analysis time was reduced, significant overlapping was obtained for several compounds. The developed two-way optimisation approach was able to find separation conditions at shorter times, where the signal recovery of the overlapped chromatographic peaks was successful, thanks to the spectral differences among the co-eluted amino acid derivatives.

10.3. Experimental conditions and data treatment

The analysed sample consisted of a mixture of the *o*-phthalaldehyde/ N-acetylcysteine derivatives of proteic primary amino acids, which was eluted with isocratic mobile phases, and linear, multi-linear and multi-isocratic gradients, using mixtures of acetonitrile from Scharlau (HPLC grade, Barcelona) and water. The amino acids (obtained from several manufacturers) were: (1) Aspartic acid, (2) glutamic acid, (3) aspargine, (4) serine, (5) glutamine, (6) histidine, (7) glycine, (8) arginine, (9) threonine, (10) alanine, (11) cysteine, (12) tyrosine, (13) valine, (14) methionine, (15) isoleucine, (16) tryptophan, (17) phenylalanine, (18) leucine, and (19) lysine. Amino acids derivatisation was carried out previously to the chromatographic separation, according to the procedure detailed in Ref. [14]. The concentration of the amino acids in the injected solutions was 6.0×10^{-6} M, except for cysteine (1.8×10^{-5} M), which showed lower absorptivity.

The chromatographic column was an Inertsil ODS3 (250 mm × 4.6 mm, 5 μm particle size) from Análisis Vínicos (Tomelloso, Ciudad Real). Mobile phases were buffered using trisodium citrate dihydrate and hydrochloric acid (Merck, Darmstadt, Germany) at pH 6.5. A liquid chromatographic instrument (Agilent, Waldbronn, Germany), equipped with a quaternary pump and a diode array detector, was used. Other experimental conditions are described in Ref. [10]. The computation routines were developed in MATLAB 2015a (The MathWorks Inc., Natick, MA, USA).

The training data used to model the chromatographic behaviour were obtained using a set of 10 isocratic mobile phases ranging from 5 to 27.5% (v/v) acetonitrile, in 2.5% steps. The concentration windows in the experimental design were adapted to each amino acid derivative, according to

their polarity, so that the measured retention times were always below 30 min. Under each range of conditions, duplicated injections were carried out.

10.4. Results and discussion

The aim of this work is resolving a sample, taking advantage of the small spectral differences among some analytes. For comparison purposes, the results obtained by applying a conventional separation in the time order, using the one-way peak purity (P_1), which quantifies the analyte peak area fraction free of interferences, will be first discussed. The separation based on the two-way multivariate selectivity (SEL_2), which measures the sinus of the angle defined by the orthogonal vector contribution of the analyte signal to the projection space of its interferences, with respect to the analyte signal, will be then considered. For both COFs, the global resolution was calculated as the product of the contributions of each analyte. More details on the data treatment are found in Refs. [10,16].

As probe compounds, a mixture of *o*-phthalaldehyde/N-acetylcysteine amino acid derivatives was used, which were separated with a column yielding long analysis times. The two-way approach could handle the problem and allowed finding significantly shorter analysis times than those provided with a conventional separation.

10.4.1. Separation considering only the time information

The unfeasibility of analysing amino acids using isocratic elution and conventional C18 columns is well known. With the C18 Inertsil column, the analysis time may be also still too long with gradient elution. Figs. 10.1a, 10.2a and 10.3a depict the Pareto fronts for the optimisation of the separation of the 19 amino acid derivatives with a 250 mm Inertsil column, using linear, multi-linear and multi-isocratic gradients of acetonitrile-water, respectively. The gradients were optimised only attending to the time resolution (P_1). The Pareto fronts show the performance of the optimal gradients giving rise to different resolution and analysis time. The chromatograms corresponding to the solutions marked with an arrow are shown in Figs. 10.1b, 10.2b and 10.3b.

The selected optimal single linear gradient increased the acetonitrile content from 8.5 to 20.5% in 90 min, with a final isocratic step (Fig. 10.1b). Satisfactory resolution was obtained (P_1 = 0.973), although in an unpractical analysis time of ca. 100 min. The optimal multi-linear gradient contained four nodes and the acetonitrile content ranged from 5.0 to 27.5% in 60 min (Fig. 10.2b). The resolution performance was P_1 = 0.987, and the analysis time was reduced to 63 min. The optimal multi-isocratic gradient contained also four nodes and the acetonitrile content ranged from 5.0 to 27.5% in 60 min (Fig. 10.3b). The resolution was P_1 = 0.999 and the analysis time, 58 min.

For the three types of gradients, almost complete resolution was obtained, especially for the multi-isocratic gradient. By applying multi-linear and multi-isocratic gradients, the analysis time was reduced with respect to the single linear gradient, but it was still above 50 min.

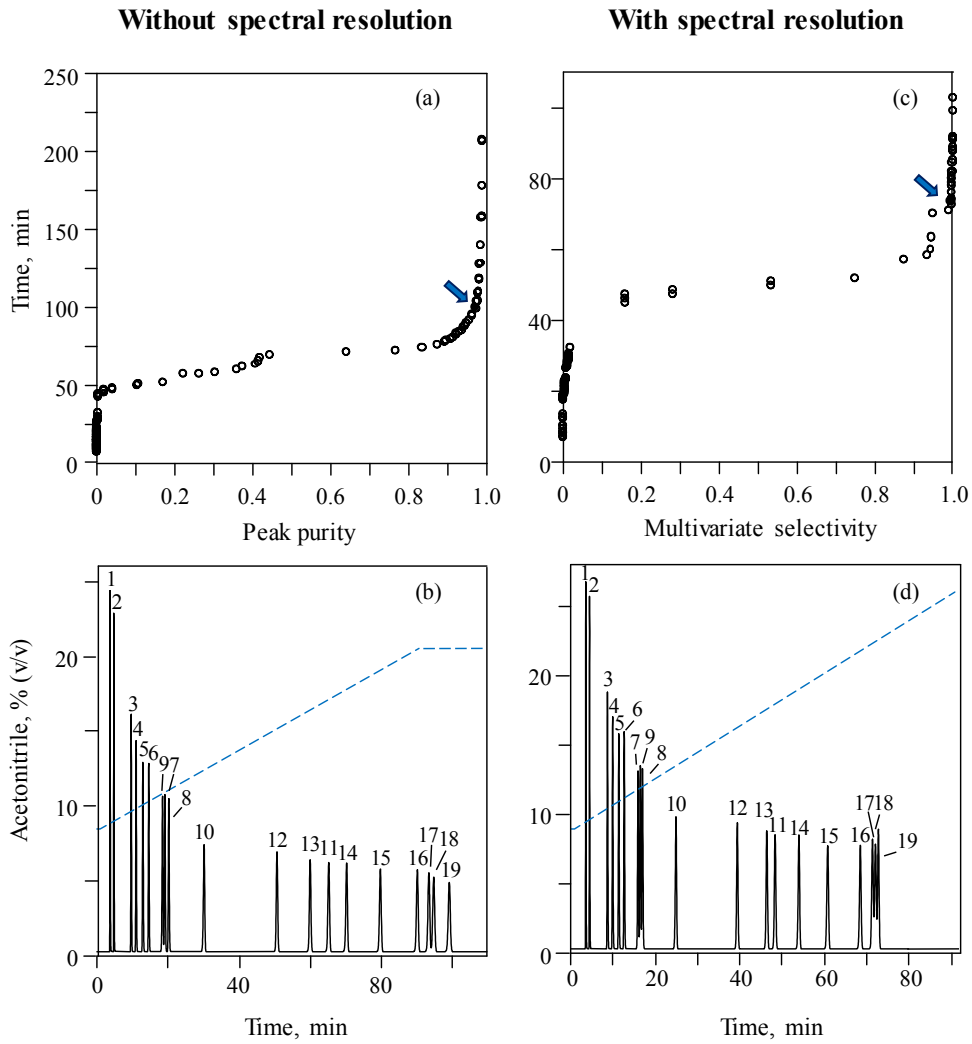

Figure 10.1. Pareto fronts depicting analysis time versus global resolution (a,c), and selected optimal chromatograms (b,d), for the separation of 19 amino acid derivatives using linear gradients. COFs: (a,b) peak purity (P_1), (c,d) multivariate selectivity (SEL_2). Chromatograms (b) and (d) (P_1 = 0.973 and SEL_2 = 0.996, respectively) correspond to the conditions marked with arrows in (a) and (c), respectively. The gradient programs are overlaid on the chromatograms. See the Experimental Section for compound identity.

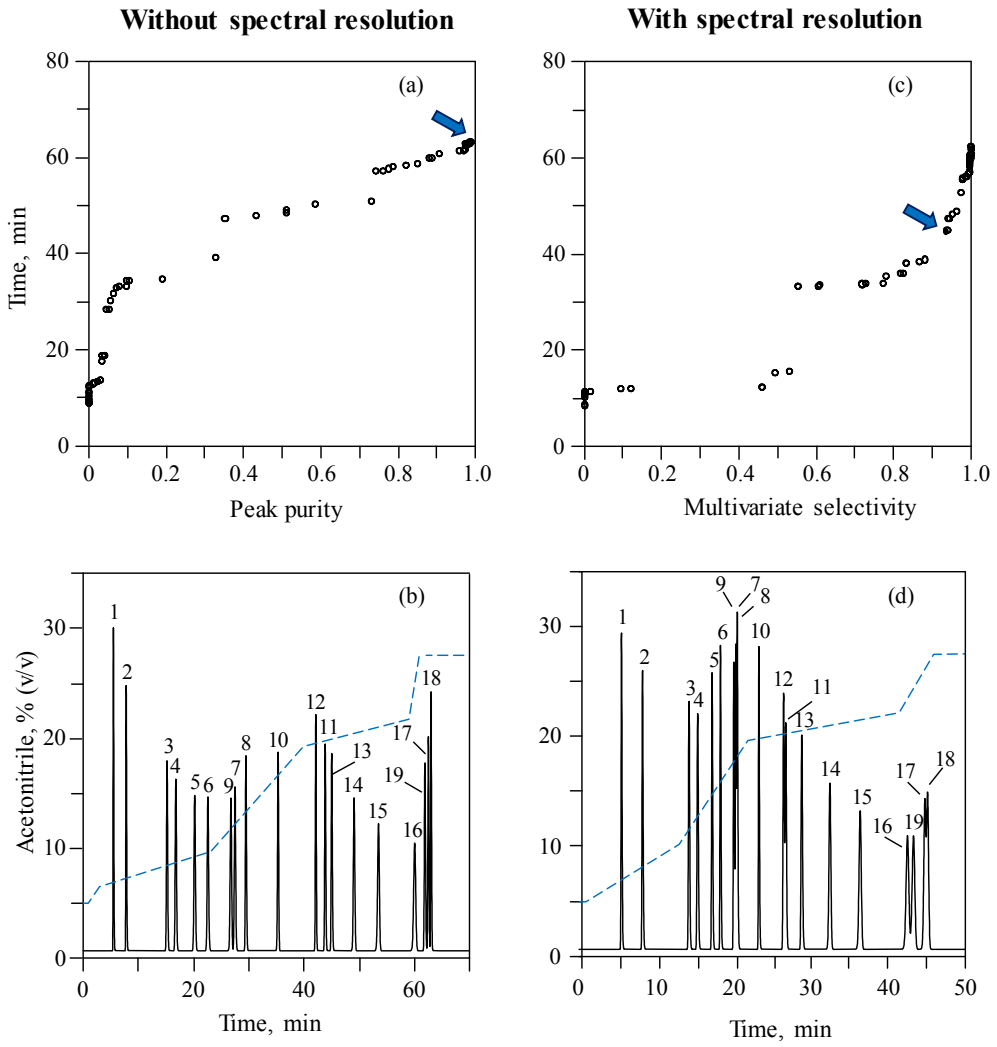

Figure 10.2. Optimisation of the separation of 19 amino acid derivatives using multi-linear gradients, and P_1 and SEL_2 as COFs. Global resolution values for chromatograms (b) and (d) were: P_1 = 0.987 and SEL_2 = 0.939, respectively. Other details are given in Fig. 10.1.

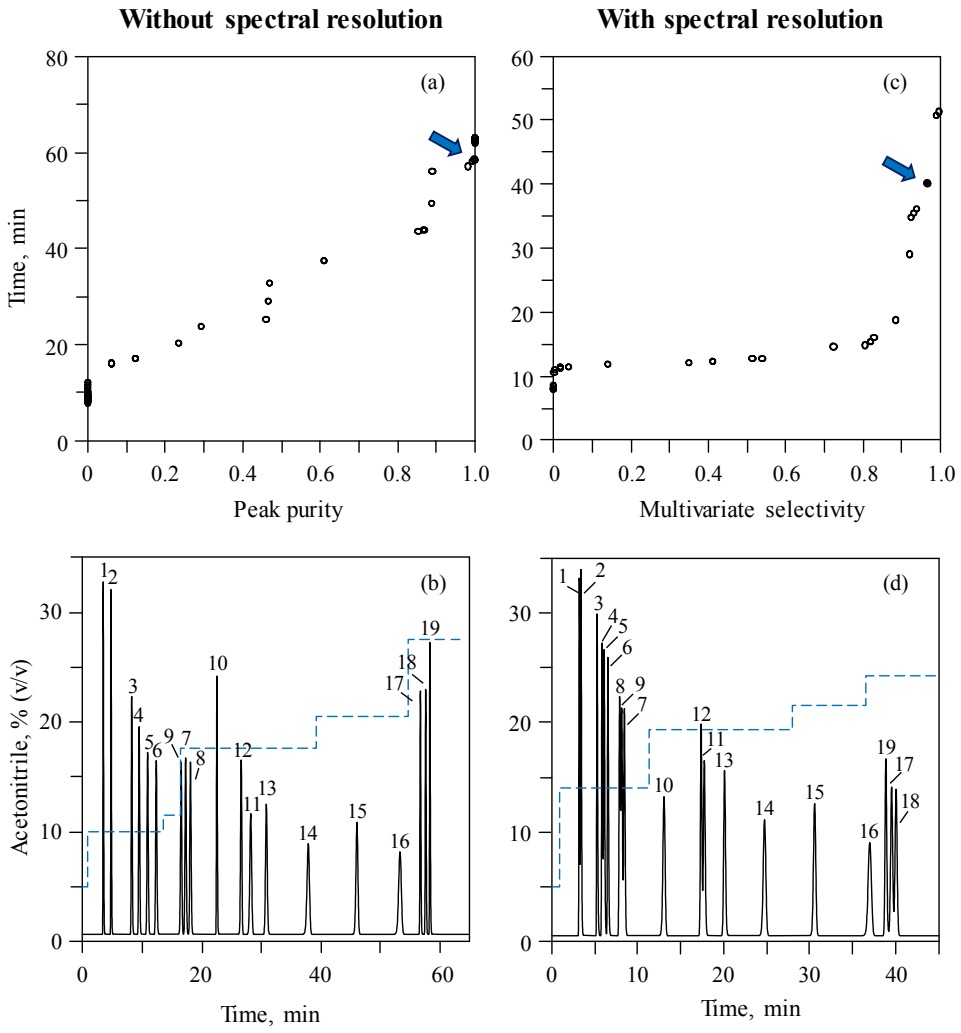

Figure 10.3. Optimisation of the separation of 19 amino acid derivatives using multi-isocratic gradients, and P_1 and SEL_2 as COFs. Global resolution values for chromatograms (b) and (d) were: $P_1 = 0.999$ and $SEL_2 = 0.967$, respectively. Other details are given in Fig. 10.1.

10.4.2. Separation considering both the time and spectral information

A feature that made the separation of the amino acid derivatives with the Inertsil column particularly difficult is the close elution of the three most hydrophobic derivatives (phenylalanine, leucine and lysine, compounds 17 to 19 in Figs. 10.1b, 10.2b and 10.3b). It was not possible to accelerate the elution of these compounds using conventional gradient approaches. However, it is possible to find separation conditions with shorter analysis times, sacrificing the resolution but with peak configurations still sufficiently favourable to recover the signals based on the spectral information.

It can be expected that a COF incorporating spectral information will qualify with higher scores those situations where the components that co-elute in a mixture show some spectral differences that allow signal recovery. In previous work, such a COF was successful to resolve a mixture of 25 phenolic compounds [10]. The spectra of the amino acid derivatives show smaller differences, making the problem more challenging (Fig. 10.4a, b and c) [17]. For several amino acids, the spectra are identical, and the approach should take advantage of the small differences among only a few amino acids, such as phenylalanine, tryptophan and tyrosine, to find conditions of partial co-elution. However, we show below that even with small spectral differences the approach succeeded in the selection of the separation conditions that allowed the signal recovery of the overlapped peaks. The price to pay is the need of larger resolution between the overlapped peaks so to take advantage of selective spectral windows.

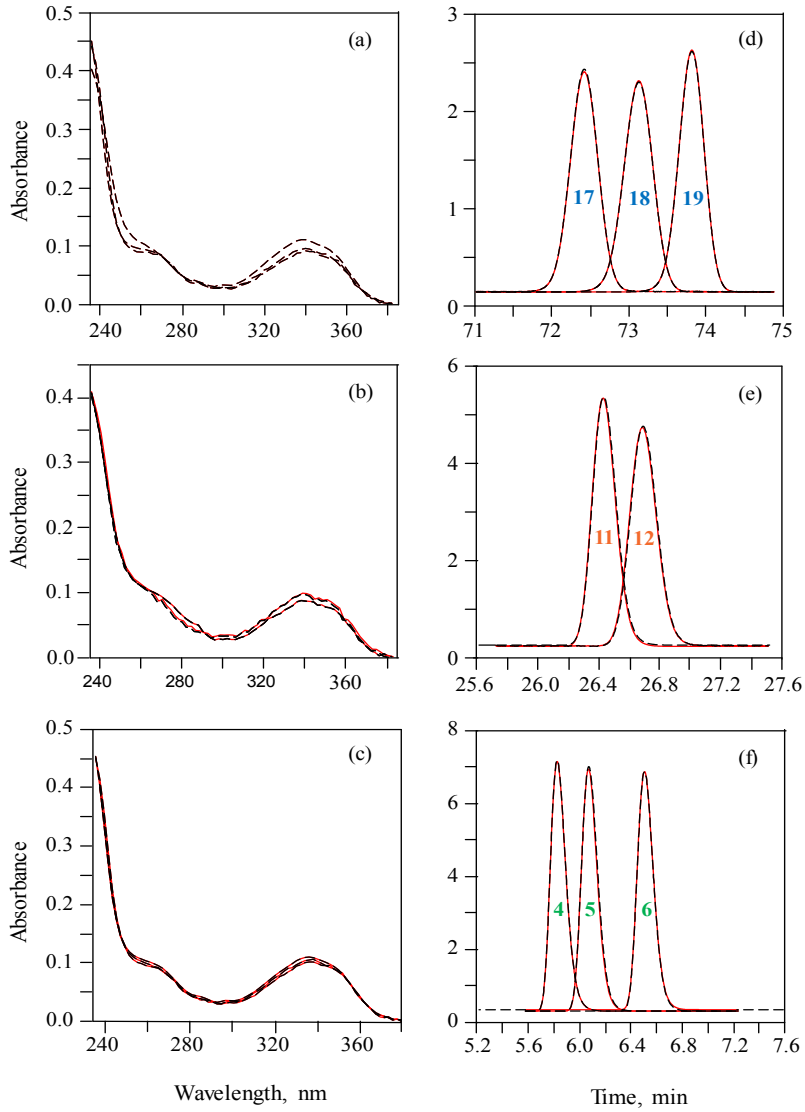

Figure 10.4. Normalised spectra and concentration profiles for three peak clusters contained in the three optimal chromatograms of the 19 amino acid derivatives: (a,d) The linear gradient in Fig. 10.1d, (b,e) the multi-linear gradient in Fig. 10.2d, and (c,f) the multi-isocratic gradient in Fig. 10.3d. Continuous red lines: original individual contribution for each compound. Dashed lines: retrieved contributions after applying OPA-ALS.

In Fig. 10.5, the performance of the one-way (P_1) and two-way (SEL_2) criteria versus the analysis time, using single- (Fig. 10.5a) and multi-linear (Fig. 10.5c) gradients, is compared for the separation of the amino acid derivatives. Note the significant increase in the number of experimental conditions when multi-linear gradients are applied. This means that the latter gradients provide a much wider richness of situations with favourable resolution. A convergent pattern between P_1 and SEL_2 is observed at high resolution, similarly to the separation of phenolic compounds discussed in Ref. [10]. For amino acids, almost full resolution ($P_1 > 0.8$) is possible with some slow gradients, although the resolution was insufficient for most gradients. In contrast, there is a number of intermediate conditions yielding shorter analysis time, which offer high enough spectral resolution for a feasible signal recovery with low error (region with $SEL_2 > 0.8$, delimited by a dashed rectangle in Fig. 10.5b and d), in spite of the low time resolution ($P_1 < 0.5$).

Figs. 10.1c, 10.2c and 10.3c depict the Pareto fronts for the optimisation of the separation of the 19 amino acid derivatives, using linear, multi-linear and multi-isocratic gradients of acetonitrile, respectively, attending to both the time and spectral resolutions according to SEL_2. The chromatograms in Figs. 10.1d, 10.2d and 10.3d correspond to the selected conditions marked in the Pareto fronts, considering high resolution and short analysis time. The SEL_2 values for the selected chromatograms were 0.996, 0.939 and 0.967, respectively. The P_1 values were < 0.5, indicating significant overlapping in the time order. The analysis times were reduced to 74, 46 and 41 min, for the selected optimal linear, multi-linear and multi-isocratic gradients, respectively.

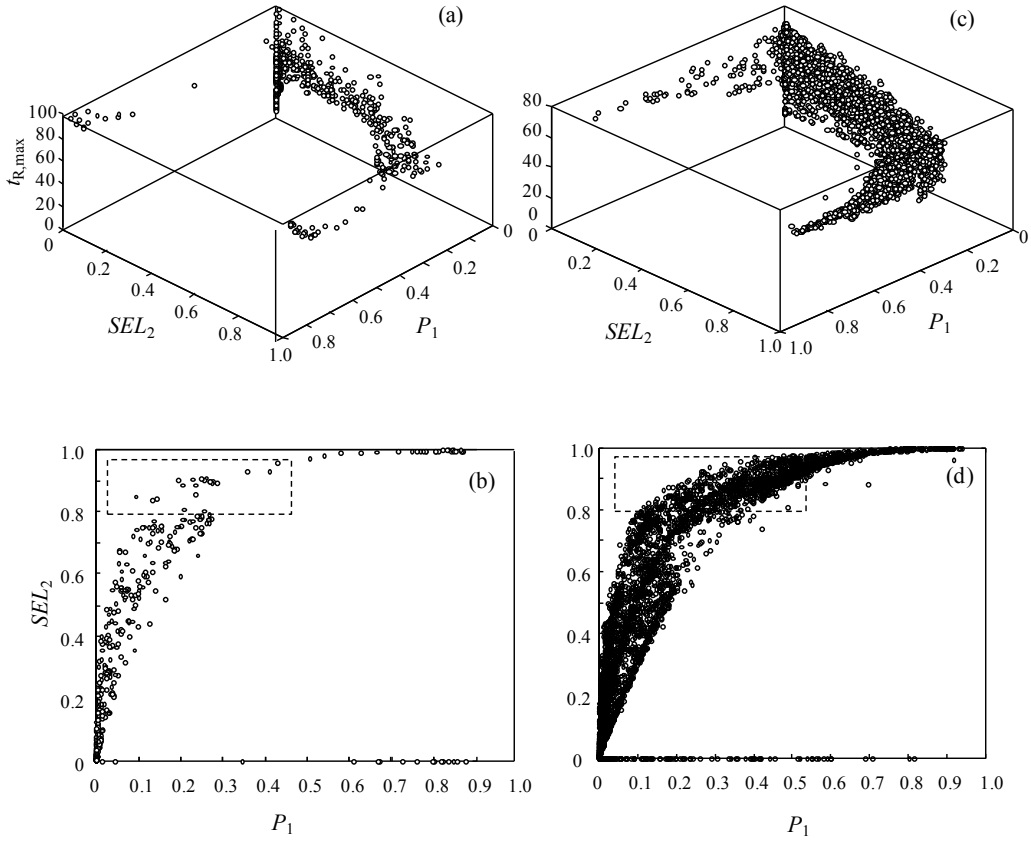

Figure 10.5. Three- and two-dimensional Pareto plots for the separation of the 19 primary amino acid derivatives, eluted using single linear gradients ((a) and (b)), and multi-linear gradients ((c) and (d)) of acetonitrile-water. The two-dimensional plot is a projection on the resolution plane of the three-dimensional plot, and shows the correlation between SEL_2 (which considers both time and spectral information) and P_1 (which considers only the time information). The dashed rectangles in (b) and (d) show the region yielding the best conditions balancing accurate signal recovery and short analysis time.

10.4.3. Reliability of the signal recovery under controlled conditions

Before considering the signal recovery of the individual elution and spectral profiles for the partially overlapped amino acid clusters, through the application of OPA-ALS, its limitations under controlled conditions will be discussed in line with the Manne's theorems [18]. These theorems indicate that: (1) The correct elution profile of an analyte can be recovered when all the analytes inside its elution window are also present outside; and (2) the correct spectrum of an analyte can be recovered if its related elution window is not completely embedded inside the elution window of another analyte.

Fig. 10.6, where the chromatograms and spectra for a mixture of two artificial compounds are depicted, can help to understand the level of complexity in the resolution of the components, based on the observed signal. Each line in the left plots (Fig. 10.6a to c) corresponds to the chromatogram of the mixture monitored at specific wavelengths. The lines in the middle plots (Fig. 10.6d to f) are the spectra contained in the spectrochromatogram when it is sampled at regular time intervals. On the right (Fig. 10.6g to i), the same spectra are depicted once normalised to unity according to the Euclidean norm.

Each row corresponds to a different situation. In the first row (Fig. 10.6a, d and g), the two compounds in the mixture show visible resolution and different spectra. In this situation, the chromatogram can be split in three regions: in the left and right regions, compounds A and X elute isolated, respectively, whereas in the intermediate region both compounds co-elute, dominating one or the other depending on the time of elution. This is the reason of the bands in Fig. 10.6g at different heights, which depict the normalised spectra: the highest bands correspond to the region in the chromatogram where only one compound elutes, whereas the shorter bands are the spectra for mixtures of the two

compounds in the co-eluting region at variable concentration ratios, showing the transition from one compound to another in the chromatogram.

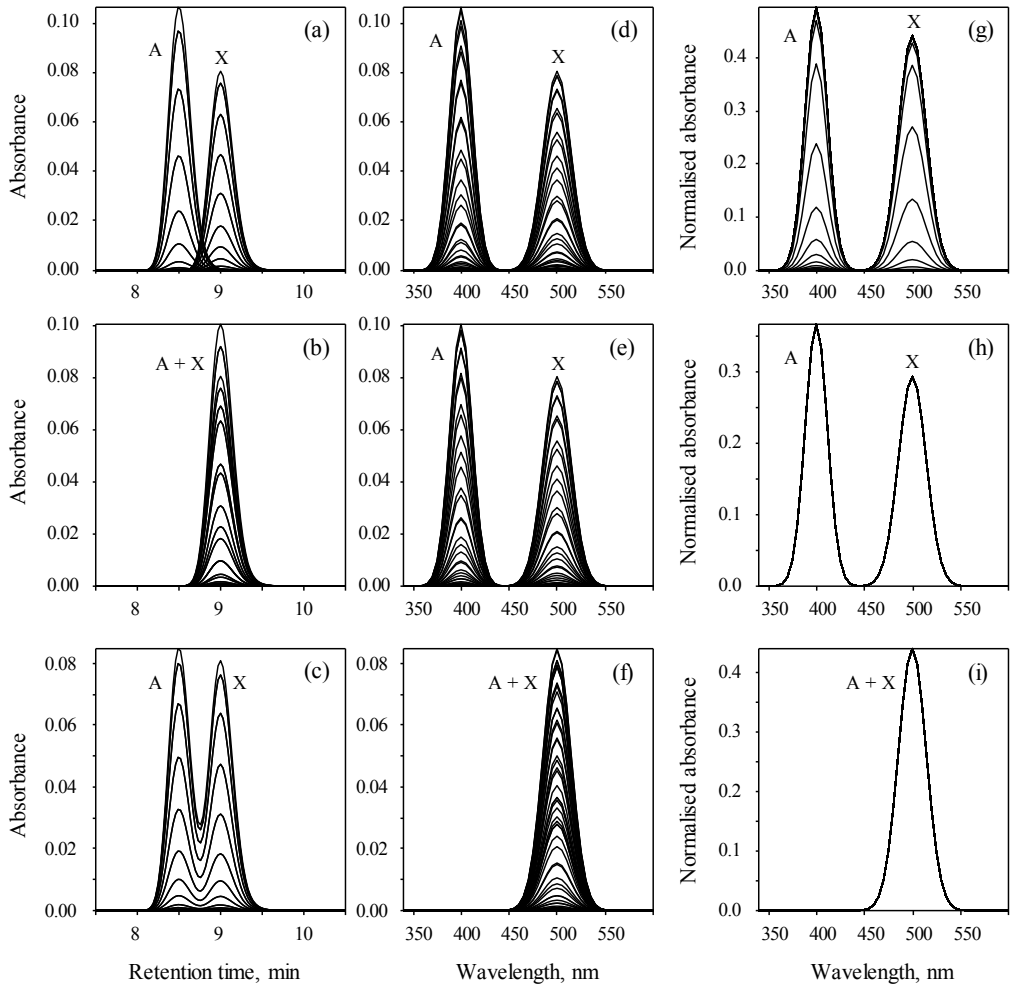

Figure 10.6. Time and spectral data for two overlapped artificial compounds (analyte X and interference A): (a-c) Chromatograms, (d-f) original spectra, and (g-i) normalised spectra.

The second row (Fig. 10.6b, e and h) corresponds to an extreme situation, where two compounds showing the same peak profile in the chromatogram, but different spectra, fully co-elute. The spectra taken at different times of elution (Fig. 10.6e) are similar to the previous situation (Fig. 10.6d), but there is only one band for each compound in the normalised spectra (Fig. 10.6h), because the rate of variation of the concentrations for both compounds is the same. The result is indistinguishable from that obtained for a sample containing only one compound with a spectrum showing two bands. The recognition and quantification of both components will only be possible if the spectra of both compounds are available, or at least, there is some information about the existence of two compounds.

The third row (Fig. 10.6c, f and i) corresponds to two compounds showing the same spectra, but some resolution in the time order. With enough resolution, it will be possible to identify the compounds only based on the information given by the chromatogram (i.e., the time order). However, for highly overlapped peaks, signal recovery would require the introduction of some type of constraint, such as the fitting of the free portions of the signals to a given peak model.

The discussion above illustrates the fact that those COFs attending to both time and spectral information sometimes provide misleading results, and require additional information to solve the problem (e.g., the number of components under a signal and the spectrum of at least one component). In the first situation (Fig. 10.6a, d and g), because there are selective regions and the spectra of the components are different, it is possible to retrieve the contribution of each compound to the chromatographic signal without the need of pure standards. Therefore, the use of the two types of information (time and spectral orders) is useful only in situations where each order exhibits a minimal

difference between the compounds to be resolved. In principle, multivariate deconvolution can be applied to any situation. However, when these methods are blindly applied to extreme situations, such as those in the second and third cases in Fig. 10.6, purity tests will reveal only one component.

10.4.4. Signal recovery of overlapped peaks in the optimal chromatograms

The SEL_2 values for the chromatograms in Figs. 10.1d, 10.2d and 10.3d (0.996, 0.939 and 0.967, respectively) suggest the presence of a number of gradients where the signals of all amino acids can be either resolved or recovered based on the spectral differences. Fig. 10.4 shows the results obtained by applying the OPA-ALS method to three peak clusters showing diverse peak overlapping in the selected gradients. As observed, in spite of the small spectral differences, the results are satisfactory.

In order to check the limits of the approach, artificial signals were generated by adding noise to synthetic combined signals for the peak cluster of phenylalanine, leucine, and lysine (Fig. 10.7). As observed, the approach is adequate at least up to a signal-to-noise ratio of 500 for the case of study.

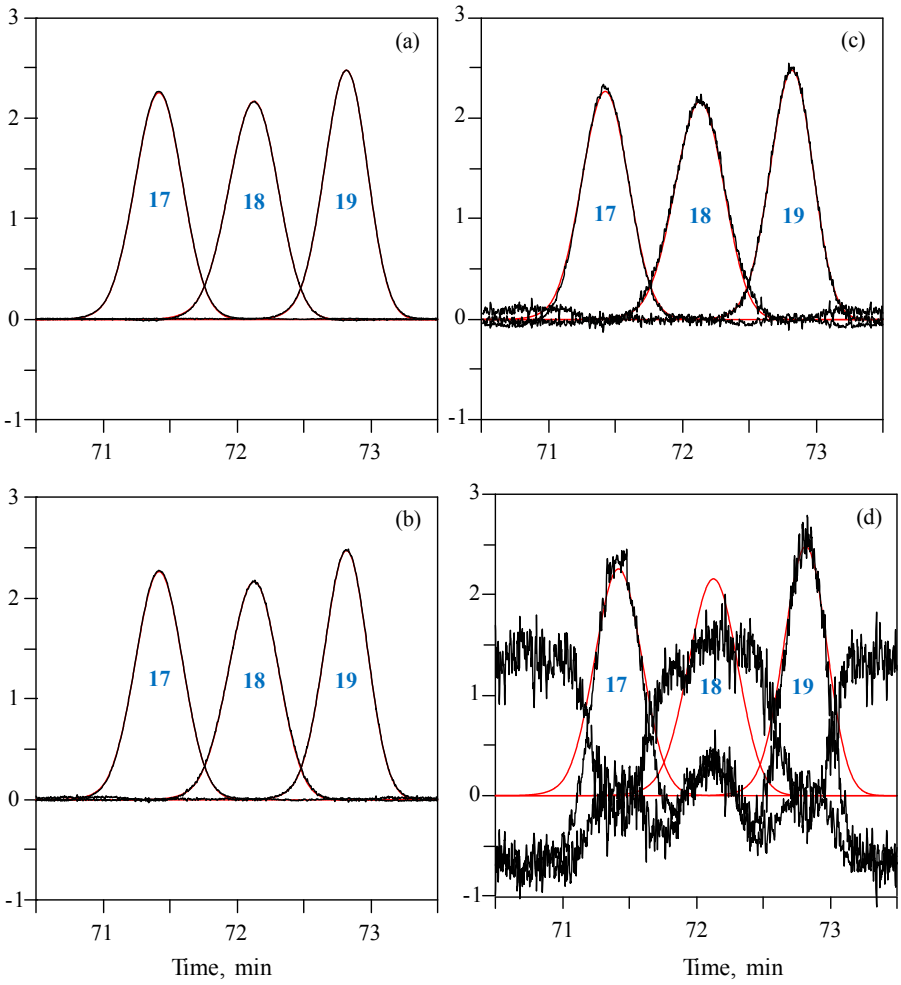

Figure 10.7. Concentration profiles for a peak cluster corresponding to phenylalanine, leucine and lysine, using the linear gradient in Fig. 10.1d. Different noise level was added before carrying out the signal recovery. Signal-to-noise ratios: (a) 5000, (b) 2000, (c) 500, and (d) 100. Continuous red lines: original individual contribution for each compound. Black lines: retrieved contributions after adding the noise and applying OPA-ALS.

10.5. Conclusions

Some separation performance can be sacrificed to achieve shorter analysis time, since the information necessary to restore the overlapped peaks can be obtained from the spectrochromatograms recorded by a diode array detector on a recurrent basis. This is not, in fact, a new idea. Multi-wavelength measurements have been used to resolve overlapped peaks for the sake of quantitative analysis since the mid-1980s [12,13,19–26]. The novelty of the proposed approach is using the spectral information for guiding the optimisation previously to signal recovery.

Measurements of chromatographic resolution only accounting the separation in the time direction, such as P_1, tend to find optimal conditions where the peaks are well separated, since they qualify negatively any peak overlap. In contrast, when the spectral information is also included, situations showing significant overlap may be scored as valid. This work shows that, in spite of the small spectral differences of the analysed amino acid derivatives, the optimisation based on the SEL_2 approach is able to find conditions where the overlapped peaks can be acceptably retrieved using OPA-ALS or a similar technique. Several signal-to-noise levels were examined to appraise the influence of non-idealities. The results were correct in a wide range of conditions, up to a signal-to-noise ratio of at least 500, for SEL_2 above 0.8. Other curve resolution techniques can be expected to succeed in similar or harder situations [13,24–26].

The two-way COF penalises the co-elution of compounds with identical spectra, whereas scores with larger values the co-elution of compounds with spectral differences (the larger the differences, the higher the scores). In this way, co-elution of compounds with at least slight spectral differences is

favoured, and thus, the achievement of selective windows for the co-eluting compounds. In the latter case, the OPA procedure will select truly pure spectra and OPA-ALS will converge in a small number of cycles (or even in one cycle).

The selected sample contained the *o*-phthalaldehyde/N-acetylcysteine derivatives of proteic amino acids. Some compounds present identical spectra and for others these are slightly different. The shortest analysis times were obtained with optimised multi-isocratic gradients considering both the time and spectral resolution. The Pareto plot indicates that this type of gradients is capable of yielding SEL_2 resolutions above 0.8 with an analysis time that can be decreased down to 20 min with the 250 mm Inertsil column (Fig. 10.3c). Also, there is a range of gradients with favourable resolution. Without taking into account the spectral resolution, the shortest analysis time offering acceptable resolution was 60 min (Fig. 10.3a). The situation will be even more favourable if the analysed compounds showed larger differences in their spectra.

10.6. References

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PART 3

FUNDAMENTAL STUDIES ON COLUMN PERFORMANCE

CHAPTER 11

NEW APPROACHES TO EVALUATE THE DISPERSION PARAMETERS IN LIQUID CHROMATOGRAPHY BASED ON THE INFORMATION OBTAINED FROM A SET OF COMPOUNDS

11.1. Abstract

The disadvantages of the classical approach used to characterise column performance in liquid chromatography, based on the observation of Van Deemter plots, are the need of measuring the extra-column variance, the use of data from only one compound eluted with a single mobile phase, which limits the conclusions of the approach, and the uncertainty arising from the handling of position and variance of chromatographic peaks to obtain the theoretical plate height. In this work, a comprehensive study of the factors affecting the uncertainty of the chromatographic data used to build Van Deemter plots is carried out and three complementary approaches are proposed to avoid the described disadvantages. One of the approaches is based on the direct treatment of the peak variances of several probe compounds according to a linear relationship of the variance with the squared retention volume, which includes the extra-column variance as a fitting parameter. In a second approach, the peak variance is fitted, for each probe compound, against the flow rate, and the model parameters are further related to the retention factors for the set of compounds. The third approach is based on the combination of both above approaches, and allows the data treatment in a single step. The three approaches were applied with good results to the characterisation of three C18 columns (Zorbax Eclipse XDB, Spherisorb and Chromolith SpeedROD), using a set of five sulphonamides as probe compounds. In all cases, the prediction errors for the variance were highly satisfactory, being in the 2–4% range. The three approaches are complementary, and allow a complete visualisation of the chromatographic performance, giving information on how the retention factor affects the A, B and C parameters in the Van Deemter equation.

11.2. Introduction

The evaluation of the column properties in liquid chromatography has great interest for the characterisation of stationary phases, development of better materials and enhancement of the resolution for complex samples. Column performance is related to the dispersion of chromatographic peaks, which depends on the processes that take place inside the column along solute elution, such as eddy diffusion, molecular longitudinal and axial diffusion, convection, multiple elution paths, and slow mass transfer between mobile phase and stationary phase [1]. To these factors, the extra-column effects associated to the injector and detector, and inside the capillaries connecting these devices to the column, should be added [2]. Finally, the existence of non-linear effects produced by the interaction of analytes with underivatised silanols may be relevant for some compounds, as those having positive charge. The attraction of these compounds to the anionic free silanols gives rise to two deletorious effects: peak broadening and asymmetry. Peak broadening is a consequence of the slow kinetics of the adsorption-desorption equilibria between the cationic compounds and free silanols. Asymmetry can be explained by the saturation of adsorption sites, which yield non-linear isotherms [3].

Traditionally, the study and quantification of all these effects has been carried out using the chromatographic data of a probe compound eluted at several flow rates (F) to calculate the corresponding theoretical plate height (H). The representation of H at each mobile phase linear velocity (u) gives rise to the so-called Van Deemter plots [4,5], which quantify the factors contributing to the chromatographic peak broadening. These plots have been extensively used to characterise chromatographic columns. However, the approach has a number of disadvantages:

(i) The requirement of the estimation of the extra-column variance, which introduces an additional uncertainty, and likely, bias in the results.
(ii) The use of data from a single probe compound eluted with a single mobile phase to build each plot, which limits the conclusions.
(iii) The need to handle experimental peak parameters depicting the position and variance, which are combined, increasing the uncertainty due to error propagation.

In this work, the factors affecting the uncertainty of the chromatographic data used to build Van Deemter plots are examined. Three alternative approaches are proposed to characterise chromatographic columns, which avoid the commented disadvantages.

11.3. Theory

11.3.1. Measurement of the dispersion in chromatography

The performance of a chromatographic column is related to its retention capability and peak broadening measured by its variance. From a practical point of view, the total variance is divided into two contributions: the variance provided by the column, and the variance provided by the devices external to the column:

$$\sigma_t^2 = \sigma_{\text{col}}^2 + \sigma_{\text{ext}}^2 \tag{11.1}$$

where σ_t^2 is the observed peak variance expressed in time units, and σ_{col}^2 is the variance produced inside the column due to the partition equilibrium, diffusion, kinetic effects, and other dispersion mechanisms. Finally, σ_{ext}^2 quantifies the extra-column contributions, produced in the connection capillaries, injector,

detector, and any other dispersion sources inside the chromatographic system [2].

A common practice is to relate the column variance to the theoretical plate height values, which according to the Martin and Synge plate model adopts the following expression [1]:

$$\sigma_t^2 = \frac{H}{L}(t_R - t_{ext})^2 + \sigma_{ext}^2 \tag{11.2}$$

where the variance is expressed in time units, t_R is the mean retention time for compounds eluted through a chromatographic column (which agrees with the time at the peak maximum for symmetrical peaks), L is the column length, and t_{ext} (the extra-column time) is the mean retention time without column. Gritti and Guiochon found a similar linear behaviour as that in Eq. (11.2) for a group of compounds with closely related elution properties, such as those in a homologous series (Eq. (11.1) in Ref. [6]).

To relate the peak dispersion with the experimental factors, different elution models have been proposed. In linear chromatography, considering a slow mass transfer mechanism and diffusion in the mobile phase, the following model is obtained [7]:

$$H = A + \frac{B}{u} + C\,u \tag{11.3}$$

where A, B and C are parameters that quantify the contributions to the dispersion of the eddy diffusion, longitudinal diffusion, and resistance to mass transfer, respectively.

Other expressions relating the theoretical plate height with the mobile phase linear velocity have been proposed that explain the heterogeneities produced by the flow [4,8]. Among them, the most important were proposed by Knox:

$$H = A u^{1/3} + \frac{B}{u} + C u \tag{11.4}$$

and Giddings:

$$H = \frac{A}{1 + E/u} + \frac{B}{u} + C u \tag{11.5}$$

In both cases, the *A* term is modified as a function of the mobile phase linear velocity. In Eq. (11.5), *E* is a constant that accounts for the cooperative effects of eddy and mobile phase dispersion.

11.3.2. Experimental measurement of the dispersion

As mentioned, the measurement of the theoretical plate height, *H*, is of big relevance for the characterisation of chromatographic columns. This parameter is usually obtained using the following expression:

$$H = \frac{\sigma_t^2}{t_R^2} L \tag{11.6}$$

Eq. (11.6) has the disadvantage of not considering the extra-column contribution to the variance, as shown in Eq. (11.2). A more accurate value of the theoretical plate height of a chromatographic column is the following:

$$H = \frac{\sigma_t^2 - \sigma_{ext}^2}{(t_R - t_{ext})^2} L \tag{11.7}$$

The values of t_R and σ_t^2 are calculated as:

$$t_R = \frac{\mu_1}{\mu_0} \tag{11.8}$$

$$\sigma_t^2 = \frac{m_2}{\mu_0} \tag{11.9}$$

where μ_0 and μ_1 are the zeroth and first-order moments with respect to the origin, and m_2 the second-order moment with respect to the mean. These parameters can be computed through the numerical integration of the net signal after subtraction of the baseline. This requires the establishment of the peak integration limits. The whole process introduces some uncertainty, as will be discussed below. Consequently, several alternative procedures have been proposed to measure mean and variance. Grushka et al. [9] applied the bi-Gaussian model, making use of the half-widths measured at 60.65% peak height:

$$t_R = t_m + \frac{2(B_{60} - A_{60})}{\sqrt{2\pi}} \tag{11.10}$$

$$\sigma_t^2 = \frac{A_{60}^3 + B_{60}^3}{A_{60} + B_{60}} - \frac{2(B_{60} - A_{60})^2}{\pi} \tag{11.11}$$

where t_m is the time at the peak maximum, and A_{60} and B_{60} are the left and right half-widths, respectively (both measured at 60.65% peak height), which matches the standard deviation for both left and right Gaussians.

Foley and Dorsey made instead the assumption that the peak follows an exponentially-modified Gaussian model and proposed the following equation [10]:

$$\sigma_t^2 = \frac{(A_{10} + B_{10})^2}{1.764 \times (B_{10} / A_{10})^2 - 11.15 \times B_{10} / A_{10} + 28} \tag{11.12}$$

where the half-widths are measured at 10% peak height.

To minimise the sources of experimental uncertainty, the mean times and variances in this work were obtained with the equations proposed by Grushka et al., based on the bi-Gaussian peak model [9]. As discussed further in this work, the measurement of the half-widths at 60.65% peak height is affected by less uncertainty than at 10%. In addition, the linear factors contributing to the peak dispersion, which are the basis of the Van Deemter equation (Eq. (11.3)), are more accurately defined at the peak centre, which corresponds to the elution of most molecules of solute along the column, less affected by non-linear interactions. In contrast, the half-widths below 10% peak height, besides being associated to a larger measurement error, are more strongly affected by non-linear factors, such as those produced by the interaction with free silanols that yield non-linear isotherms.

11.4. Experimental

11.4.1. Reagents and columns

In order to check the performance of the different approaches, the chromatographic data of five sulphonamides (sulphachloropyridazine, sulphadimethoxine, sulphamerazine, sulphamethizole, and sulphisoxazole) from Sigma (St. Louis, MO, USA) were used. The behaviour of the probe compounds with three columns was examined: the microparticulate columns Zorbax Eclipse XDB C18 (Agilent, Waldbronn, Germany) and Spherisorb C18 (Waters, Massachusetts, USA), both with column dimensions 150 mm × 4.6 mm I.D. and 5 μm of particle size, and the silica-based monolithic Chromolith SpeedROD C18 column (Merck, Darmstadt, Germany, 50 mm × 4.6 mm I.D).

Isocratic runs were carried out using 25% (v/v) acetonitrile-water mobile phases, buffered at pH 3 with 0.01 M sodium dihydrogen phosphate, and HCl

and NaOH from Scharlab (Barcelona). A pH-meter from Crison (Model MicropH 2002, Barcelona), and a combined glass electrode from Orion (Model 8102, Barcelona), containing Ag/AgCl reference electrode with 3.0 M KCl aqueous solution as salt bridge, were used.

11.4.2. Apparatus

The chromatographic runs were carried out with an Agilent instrument, equipped with the following modules (Series 1260): a quaternary pump working in the 0.1–3.0 mL/min range, an autosampler, a multiple-variable wavelength UV-visible detector set at 271 nm, and a temperature controller set at 25 ºC and 50 ºC. The injection volume was 10 µL. A 10 µL detector cell with 10 mm path-length was used. Two PEEK tubes (0.17 mm diameter) connected injector and column (18 cm length), and column and detector (60 cm length). The chromatographic system was controlled by an OpenLAB CDS LC ChemStation (Agilent B.04.03).

11.4.3. Procedures

The experimental measurement of the extra-column variance was carried out by replacing the column by a zero volume connector, keeping unchanged the other connections. For this purpose, KBr (Acros Organics, Fair Lawn, NJ, USA) and several probe compounds were individually eluted at all assayed flow rates, using the same mobile phase as in the assays carried out in the presence of column. The mean extra-column volume was 52±3 µL.

The measured peak parameters were time at the peak maximum, peak height, left and right half-widths at different heights and peak moments. The mean elution time and peak variance were obtained according to Eqs. (11.8) and (11.9), or Eqs. (11.10) and (11.11). All calculations were performed with

programs written in Basic QB64. The time and height at the peak maximum were determined by adjusting the top of the peak to the polynomially variance modified Gaussian (PVMG) model [11], and the half-widths through parabolic interpolation. The Solver tool of EXCEL from Microsoft (Seattle, WA, USA) was used to carry out the non-linear fittings. Other treatments were carried out with GRAPHER from Golden Software (Golden, CO, USA).

11.5. Results and discussion

As discussed above, the characterisation of chromatographic columns requires the measurement of parameters that define the peak position and dispersion, which are combined according to Eq. (11.7). The accuracy in the measurement of the peak parameters affects significantly the final results, and is influenced by random errors in both the instrumental measurement of the peak signal and the data treatment, including the baseline subtraction. First, the uncertainty associated to the different parameters needed for the computation of the theoretical plate height will be evaluated. Next, alternative approaches will be proposed to evaluate the column performance.

11.5.1. Uncertainty associated to the response time

The response time (related to the sampling frequency or number of experimental points measured per unit time) is an important parameter, which is not usually taken into account in most dispersion studies, but with a decisive influence on the accuracy of the measured variance of chromatographic peaks. A reduction in the sampling frequency may result in an artificial peak broadening, which will yield a systematic bias in the calculation of the theoretical peak height and width. The use of a low sampling frequency makes

the peaks wider and more symmetrical, which may be understood by considering that the signal is weighted in the instrument during the established interval. The effect is larger for peaks eluting at smaller times. Thus, a decrease in the sampling frequency from 80 to 5 Hz has a similar effect for a peak eluting at 0.06 min (Fig. 11.1a), as the decrease from 80 to 20 Hz for a peak eluting at less than 0.02 min (see also Fig. 11.2). Therefore, the sampling frequency should be considered in the measurement of the peak dispersion in the extra-column region even at low flow rates (Fig. 11.3).

The sampling frequency can also affect significantly the measurements for peaks of compounds eluted through a chromatographic column at high flow rates, as observed in Fig. 11.1b. Above 0.6 mL/min (i.e., retention times below 5 min for sulphamerazine), there is a clear discrepancy between the peaks obtained at different frequencies: an additional increase is observed in the variance for low values. This effect would result in a fictitious increase in the *C* term in the Van Deemter equation (Eq. (11.3)), which quantifies the peak deterioration when the flow rate increases.

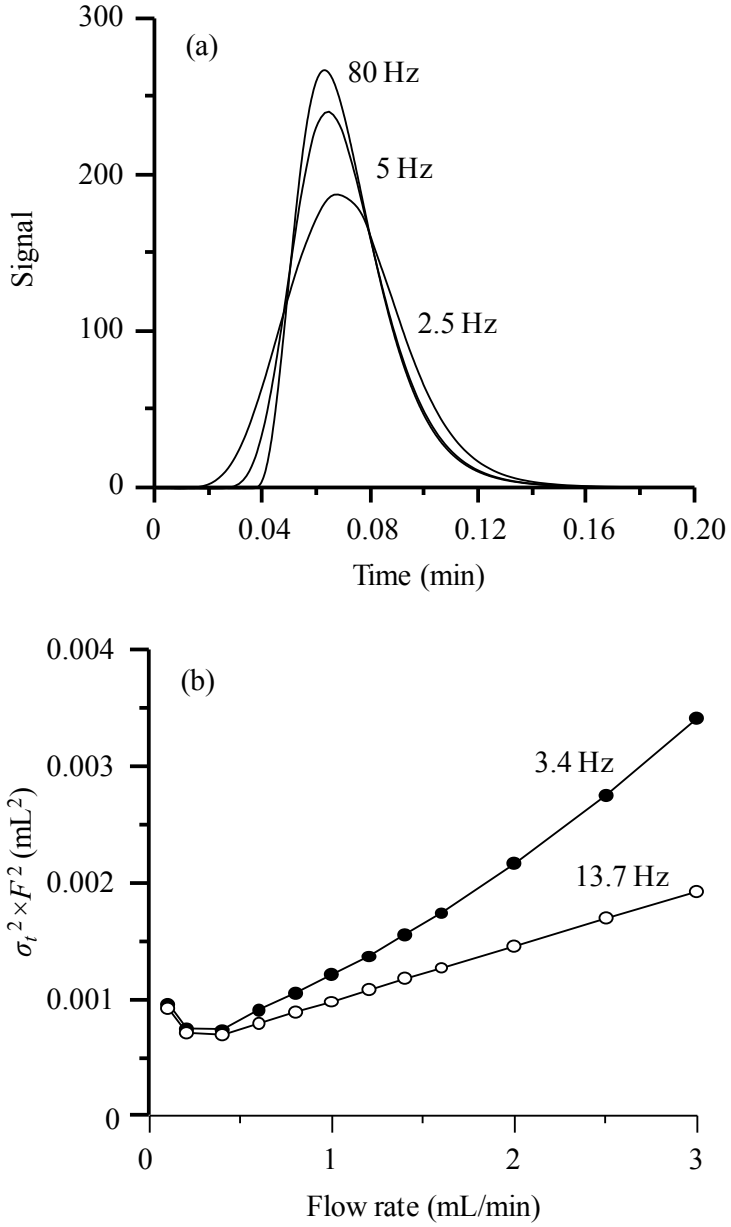

Figure 11.1. Effect of sampling frequency on the peak variance in the absence (a) and presence (b) of column, for sulphamerazine.

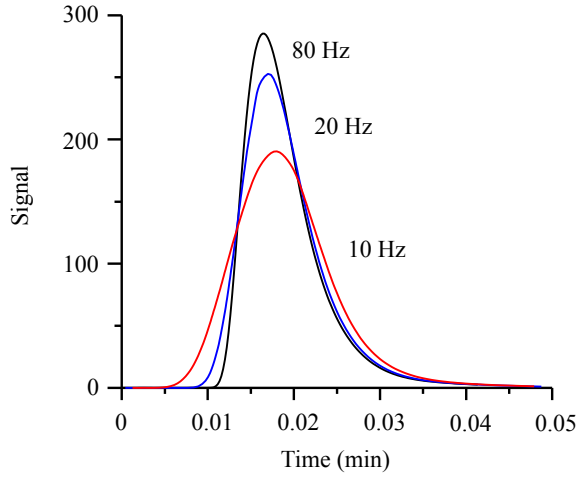

Figure 11.2. Effect of the sampling frequency on the peak variance, in the absence of column, for sulphamerazine elued at a flow rate of 3.2 mL/min..

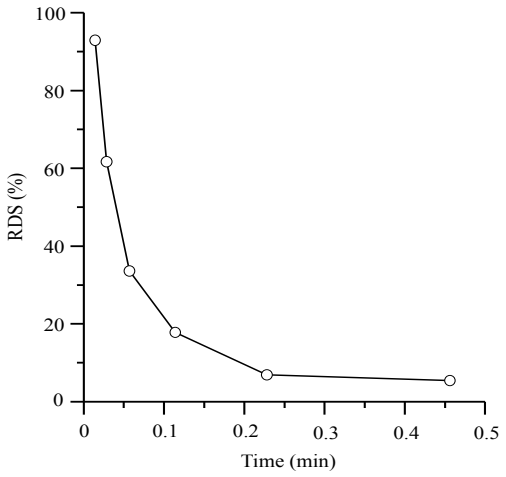

Figure 11.3. Effect of the elution time of sulphamerazine, at different flow rates, on the relative standard deviation of the left half-width measured at 10% peak height, in the absence of column. The relative standard deviation corresponds to the data set obtained by measuring the peak at each flow rate using seven different sampling frequencies (1.25, 2.5, 5, 10, 20, 40 and 80 Hz). Fig. 11.2 shows signals at three of these frequencies.

11.5.2. Uncertainty in the evaluation of the peak dispersion parameters

The computation of the peak variance through numerical integration introduces an additional source of uncertainty, as it is largely affected by the selected peak limits, data noise and method used to subtract the baseline. This is illustrated in Fig. 11.4, where the effect of selected peak limits on the variance (expressed as standard deviation and calculated by numerical integration), and the left half-width measured at 60.65% (A_{60}) and 10% (A_{10}) peak height, using quadratic interpolation and different time intervals, is depicted. To measure these parameters, symmetrical intervals with regard to the peak maximum (measured as the number of half-widths at 60.65% peak height) were established, subtracting previously the baseline. As observed, the measured half-widths are more stable than the standard deviation, so that when a peak is defined considering more than four half-widths, the values remain substantially constant. In contrast, the standard deviation does not reach a constant value. Also, the smaller variation range of the half-width at 60.65% indicates a higher precision in its measurement.

The measurement of the standard deviation, and consequently, the variance, is thus subjected to greater uncertainty than the half-widths. The standard deviation does not reach a stable value, since it is very sensitive to heterogeneities in the baseline. In view of these results, and because the peaks were skewed to the right, the measurement intervals were defined by taking points up to 6 fold the left half-width ($6\times A_{60}$) and 8 fold the right half-width ($8\times B_{60}$), both measured at 60.65% peak height.

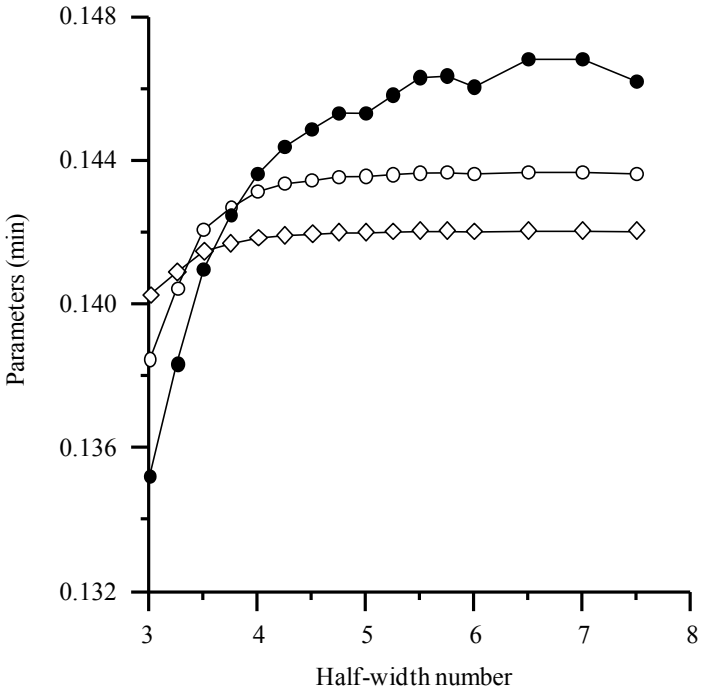

Figure 11.4. Change in the left half-width measured at 60.65% (◊) and 10% (○) peak height, and standard deviation calculated by numerical integration (●), for the peak of sulphachloropyridazine obtained at a flow rate of 1 mL/min. Different time intervals (consisting of the same number of left and right half-widths) were considered. For the representation, the half-widths at 10% peak height were divided by 2.15.

The uncertainty in the measurement of the half-widths, as a function of the peak height, was also studied. To measure the half-widths, previous knowledge of the time and height at the peak maximum is needed. The time at which a certain height is reached on the left and right peak sides is calculated using numerical interpolation. Fig. 11.5 shows how a 0.1% bias in the height value at which both half-widths are measured is propagated on the half-widths values.

The most reliable region to measure the half-widths is found between 10 and 70% peak height.

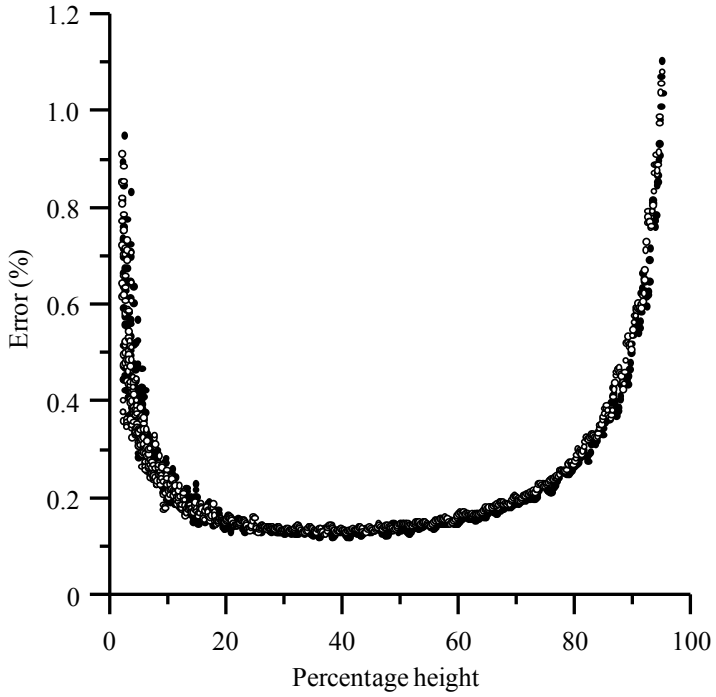

Figure 11.5. Relative error in the measurement of the left (○) and right (●) half-widths for sulphachloropyridazine, at different peak heights and with a constant error in the measured peak heights.

The procedure to obtain variance values was also examined. Fig. 11.6 depicts the effect of the flow rate on the peak variance, calculated according to: (i) Eq. (11.11), (ii) Eq. (11.12), and (iii) numerical integration. As observed, the data obtained with Eq. (11.11) shows less variability. For this reason, the variance was further evaluated with this equation.

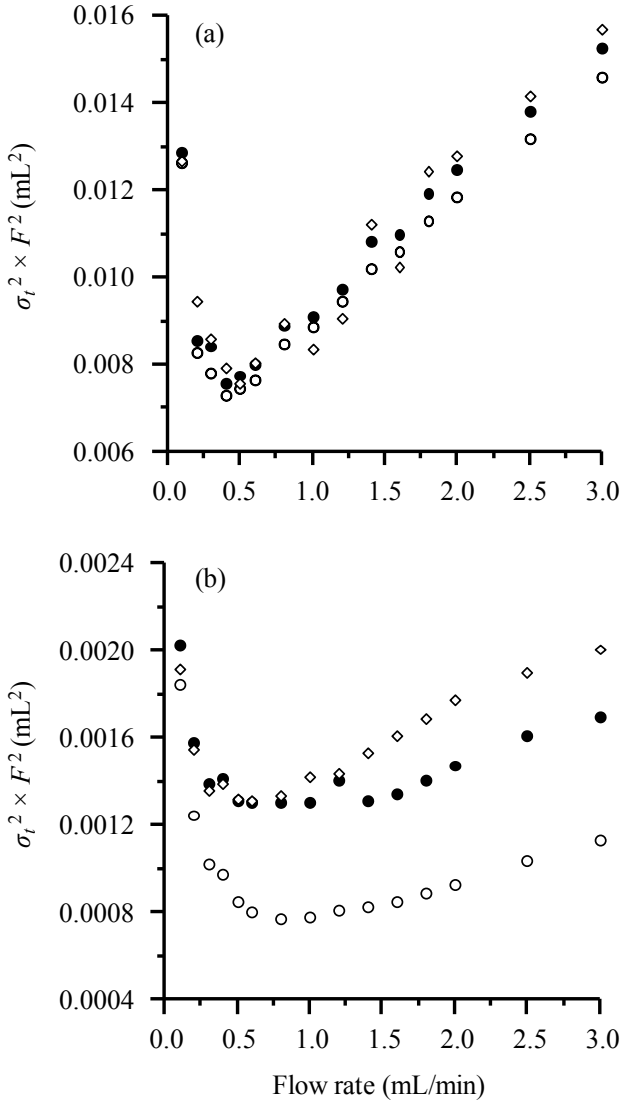

Figure 11.6. Change in the peak variance for sulphisoxazole with the flow rate, calculated according to three procedures: (○) Eq. (11.11), (●) Eq. (11.12), and (◊) numerical integration, for the Zorbax (a) and Chromolith (b) columns. The variance is expressed in volume units.

11.5.3. Uncertainty in the evaluation of the theoretical plate height

To build a Van Deemter plot, *H* values at several linear velocities should be calculated according to Eq. (11.7). As commented, the measurement of the extra-column variance is particularly problematic, especially at high flow rates where the peaks are very narrow and usually defined by a small number of points. The relevance of the extra-column variance on the theoretical plate height is shown in Fig. 11.7, where the *H* values were calculated using two different sets of extra-column variances, measured at varying flow rate in independent experiments. The data used to calculate *H* were obtained using Eqs. (11.10) and (11.11). As observed, the influence of the extra-column variance is larger when the retention time is shorter. The effect would be stronger if the variance values were obtained by numerical integration. The measured extra-column variance may be even larger than the column variance for peaks eluting at very short retention times.

Another effect that should be highlighted is the dependence of the theoretical plate height with the retention time of the probe compounds. In Fig. 11.8, the theoretical plate height obtained for each sulphonamide, eluted at 1 mL/min, is plotted as a function of the inverse retention time (trying to simulate the effect of a variable flow rate). It may be expected that since the flow rate is constant, the theoretical plate height for compounds eluting at different times should not vary. However, the variation is significant, and the behaviour similar to that expected at variable flow rate: those compounds eluting at the smallest retention times yield higher theoretical plates. The change in the retention time by varying the flow rate may also affect the theoretical plate height value, when the data from only one compound are used to build the plots, probably due to a slight additional peak broadening for the peaks eluting at very low retention times.

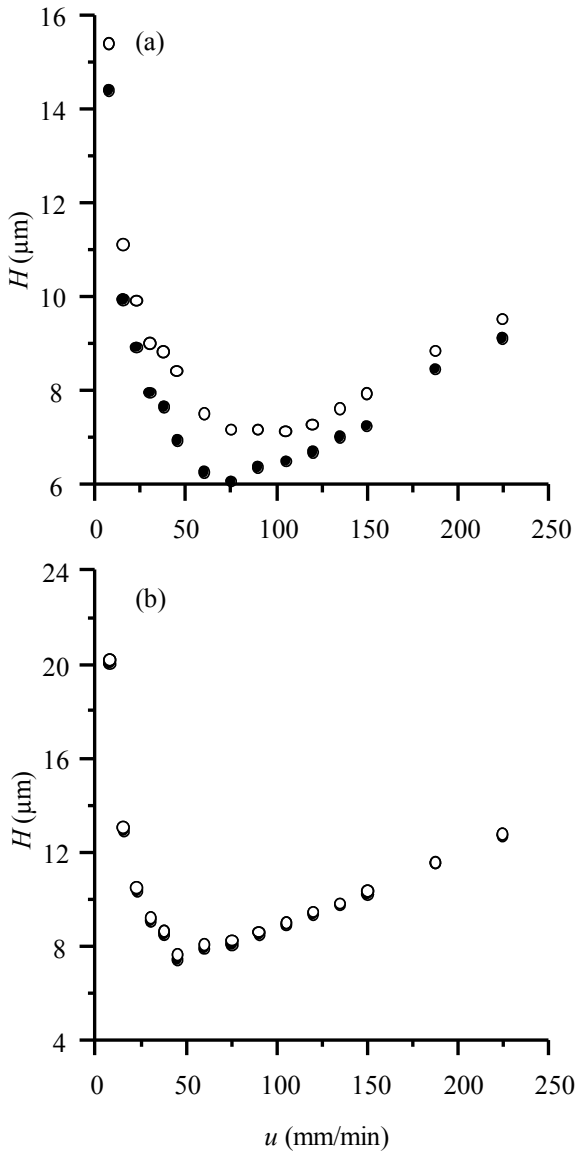

Figure 11.7. Effect of the extra-column variance on the theoretical plate height for sulphamerazine (a) and sulphadimethoxine (b), which elute at 1.1 min and 2.9 min, respectively, at a flow rate of 1 mL/min. The plotted values correspond to the same data set obtained with the Chromolith column and two sets of extra-column variances obtained in the absence of column in independent experiments and represented by symbols (○) and (●).

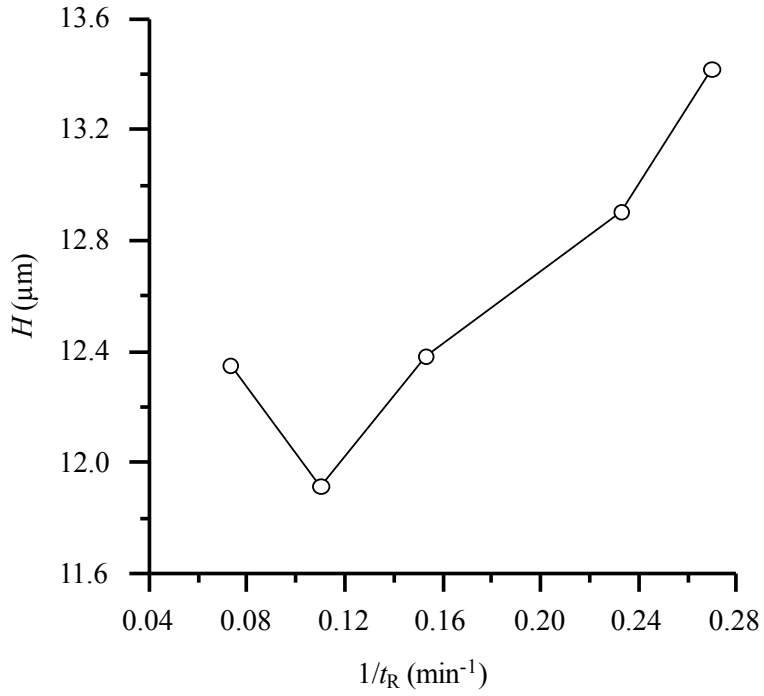

Fig. 11.8. Theoretical plate height values obtained for the five sulphonamides, eluted at constant flow rate of 1 mL/min. Each point corresponds to the inverse retention time for each sulphonamide.

Before examining the dependence of the total variance with the flow rate, it is interesting to evaluate the behaviour of the extra-column variance. In column characterisation, the measurement of the extra-column variance is carried out by replacing the column by a zero-volume connector, assuming that this variance matches the extra-column contribution the solute is experiencing inside the chromatographic system. However, as indicated by Gritti and Guiochon [12], this assumption may not be correct. Hence, the requirement of measuring the extra-column contribution to the total variance is a drawback of

the classical approach for the characterisation of columns, owing to the uncertainty in the measurements and the systematic error introduced in the final result. Moreover, as shown below, the procedure followed to measure the peaks in the extra-column region (i.e., sampling frequency, peak limits, and baseline subtraction) adds significant uncertainty to the variance.

11.5.4. Dependence of the retention time and variance with the flow rate

Both the retention time and peak variance are affected by the flow rate. Therefore, it is interesting to examine the models relating these two variables with the flow rate. As expected, the plots of retention time of the probe compounds versus the inverse flow rate showed linear fittings (with $R^2 > 0.9996$, Fig. 11.9). Meanwhile, according to the extended theoretical plate model [13], the extra-column variance can be expressed in terms of the flow rate as:

$$\sigma_{\text{ext}}^2 = \frac{c_{\text{ext}}}{F} + \frac{a_{\text{ext}}}{F^2} + \frac{b_{\text{ext}}}{F^3} \tag{11.13}$$

a_{ext}, b_{ext} and c_{ext} being model parameters. The accuracy of Eq. (11.13) was checked using values of the extra-column variance, obtained without column at different flow rates. The dependence of the extra-column variance in the time domain, on the inverse flow rate, shows a polynomic trend (Fig. 11.10) with an excellent cubic fit (determination coefficients $R^2 = 0.99991$ for sulphamerazine and $R^2 = 0.99989$ for sulphachloropyridazine) This confirms the adequacy of Eq. (11.13) to describe the extra-column variance against the flow rate.

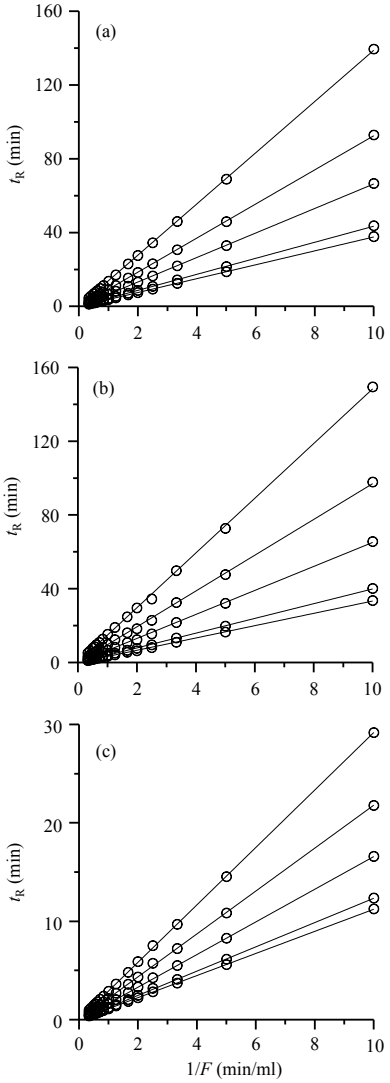

Figure 11.9. Dependence of the retention time with the inverse flow rate for the five sulphonamides and three columns: (a) Spherisorb, (b) Zorbax, and (c) Chromolith. From bottom to top, the lines correspond to the compounds: sulphamerazine, sulphamethizole, sulphachloropyridazine, sulphisoxazole and sulphadimethoxine. For all assayed compounds, the determination coefficient was $R^2 > 0.99995$ for the Spherisorb column, $R^2 > 0.9996$ for the Zorbax column, and $R^2 > 0.9998$ for the Chromolith column.

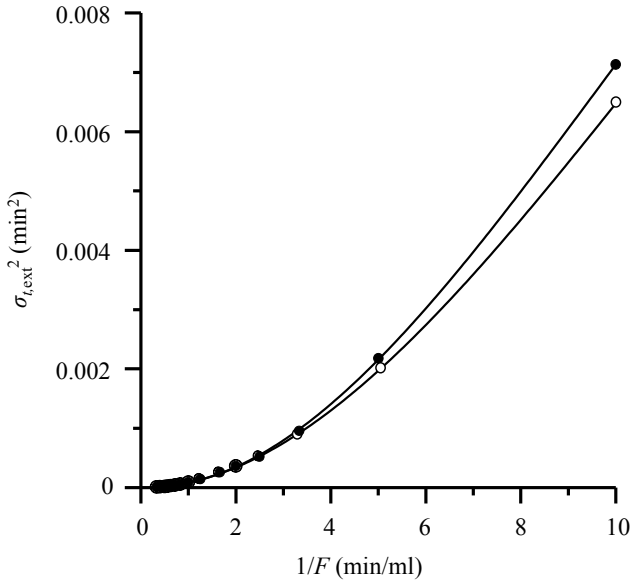

Figure 11.10. Fitting of the extra-column variance to Eq. (11.13) (continuous line). The points correspond to variance values ($\sigma_{t,\mathrm{ext}}^2$) obtained at different flow rates between 0.1 and 3 mL/min, for sulphamerazine (●) and sulphachloropyridazine (○), eluted without column. Peak variance values were calculated with Eq. (11.11).

Finally, the total variance in time units, taking into account Eqs. (11.2), (11.3), and (11.13), can be expressed as:

$$\sigma_t^2 = \frac{c}{F} + \frac{a}{F^2} + \frac{b}{F^3} \tag{11.14}$$

where a, b and c are model parameters that include the extra column contribution. In order to obtain Eq. (11.14), the relationships between the linear velocity (u), flow rate (F), retention time (t_R), retention volume (V_R) and void volume (V_0), were considered:

$$t_R = \frac{V_R}{F} \tag{11.15}$$

$$u = F\frac{L}{V_0} \tag{11.16}$$

By multiplying Eq. (11.14) by the flow rate, the following is obtained:

$$\sigma_t^2 \times F = c + \frac{a}{F} + \frac{b}{F^2} \tag{11.17}$$

The behaviour described by Eq. (11.17) is given in Fig. 11.11a for sulphisoxazole, eluted from the three columns. In all cases, the lines were non-linearly fitted with $R^2 > 0.999$. In Fig. 11.11b, the dependence of the variance expressed in volume units is depicted versus the flow rate, which considering Eq. (11.18) can be expected to show a similar behaviour to that observed for the theoretical plate height in the Van Deemter equation (Eq. (11.3)):

$$\sigma_V^2 = \sigma_t^2 \times F^2 = a + \frac{b}{F} + c\,F \tag{11.18}$$

Figure 11.11b indicates the optimal flow rate, at which the dispersion expressed in volume units is minimal. According to Eq. (11.2), and multiplying the variance in time units by the squared flow rate, the dependence of the variance on the retention volume will be:

$$\begin{aligned}\sigma_V^2 = \sigma_t^2 \times F^2 &= \frac{H}{L}(t_R - t_{ext})^2 \times F^2 + \sigma_{t,ext}^2 \times F^2 \\ &= \frac{H}{L}(V_R - V_{ext})^2 + \sigma_{V,ext}^2\end{aligned} \tag{11.19}$$

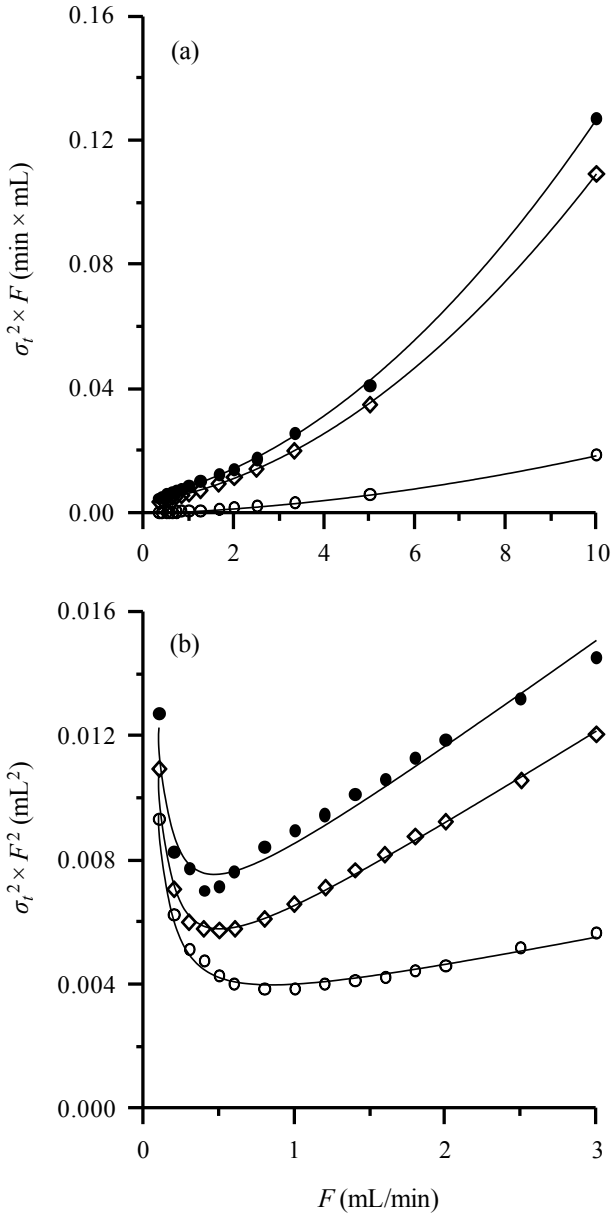

Figure 11.11. Change with the flow rate of parameters derived from the variance, for sulphisoxazole chromatographed with the three columns: Spherisorb (●), Zorbax (◊), and Chromolith (○). The values for the Chromolith column were multiplied by 5 to keep the scale.

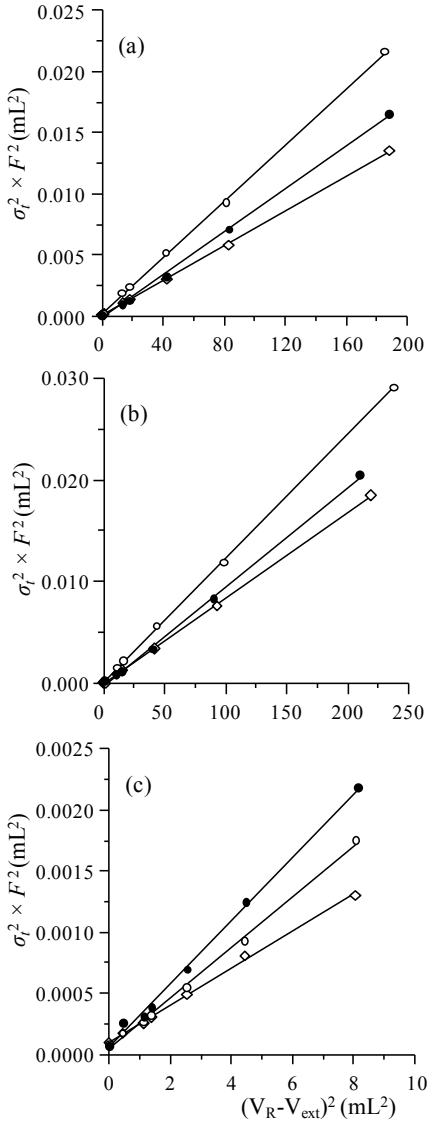

Figure 11.12. Dependence of the variance, expressed in volume units units ($\sigma_V^2 = \sigma_t^2 \times F^2$), with the squared retention volume, for the five sulphonamides eluted with: (a) Spherisorb, (b) Zorbax, and (c) Chromolith. The data corresponding to the dead time and extra-column effects are also included. The measurements were carried out at the flow rates: (○) 0.2, (◊) 0.6 and (●) 2.0 mL/min.

As predicted by Eq. (11.19), the observed behaviour was linear (Fig. 11.12) for the column and compounds used in this work. Moreover, the linearity was extended to the peak obtained for the dead time marker and the peak depicting the extra-column effect. This allows the use of Eq. (11.19) to evaluate the theoretical plate height. In theory, a non-linear behaviour should be expected if the slow mass transfer term (the *C* term in Eq. (11.3)) were affected significantly by the retention factor of solutes [14,15].

11.5.5. New data treatments to evaluate the column performance

In this section, three approaches are described to evaluate the column performance, without the need of previous measurement of the extra-column contribution to the variance. The proposed approaches have been tested using the classical Van Deemter equation (Eq. (11.3)). However, they can be adapted to other dispersion models as Eqs. (11.4) and (11.5).

11.5.5.1. Approach I

The probe compounds used for column characterisation should show similar interaction kinetics within each assayed chromatographic column, as is the case of the five selected sulphonamides. As indicated by Eq. (11.19), the slopes of the straight-lines obtained when the variance is represented against the squared retention volume ($m = H/L$), at constant flow rate (Fig. 11.12), gives information on the theoretical plate height. In the fittings, the extra-column variance, which is constant, appears as a model parameter.

From the slope *m*, the column theoretical plate height can be obtained at each flow rate:

$$m(u) \times L = H = A + \frac{B}{u} + C u \qquad (11.20)$$

Therefore, in this approach, the *A*, *B*, *C* parameters are obtained in two steps. First, the peak variances for a number of compounds showing similar kinetics are linearly fitted to Eq. (11.19), at a fixed flow rate. The value of theoretical plate height is obtained from the slope of the straight-line. The process is repeated at several flow rates. In a second step, the theoretical plate heights at each flow rate are non-linearly fitted to Eq. (11.20).

Approach I (Eqs. (11.2) or (11.19)) assumes a linear behaviour of the variance with the squared retention time or retention volume, which is valid when the parameters in the Van Deemter equation do not depend on the eluted compound. This is correct if the diffusion in the stationary phase is negligible, and the *C* term is associated either to the Taylor-Aris dispersion [15], produced by the flow inside the column (which is independent of the retention factor), or to slow mass transfer in the mobile phase (which is independent of the retention factor when this is significantly above $k = 1$). If the *C* term were associated to slow mass transfer in the stationary phase, Approach I could not be used, since the *C* parameter would depend strongly on the retention factor.

The results for the three columns examined in this work are shown in Fig. 11.13, and compared with those obtained with the conventional calculation of the theoretical plate height through Eq. (11.7), using the data for sulphisoxazole.

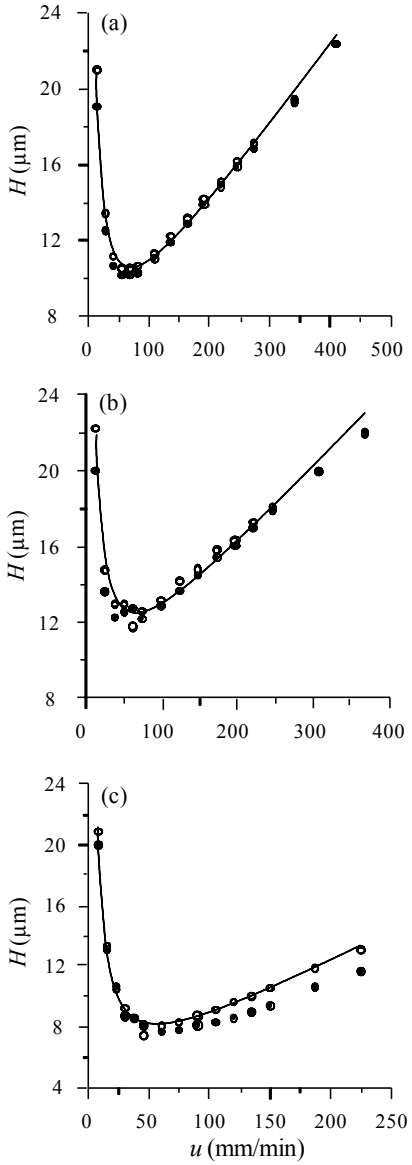

Figure 11.13. Theoretical plate heights obtained from Approach I (○), plotted versus the linear velocities, for the three studied columns: (a) Spherisorb, (b) Zorbax, and (c) Chromolith. The lines correspond to the predicted values from the non-linear fitting to Eq. (11.20). Theoretical plate heights obtained with Eq. (11.7) using the data for sulphisoxazole are also drawn as (●).

11.5.5.2. Approach II

According to Eq. (11.13), the extra-column variance shows a cubic behaviour with respect to the inverse flow rate. From Eqs. (11.13) and (11.16), an equation describing the extra-column variance in volume units can be obtained, as a function of the mobile phase linear flow velocity:

$$\sigma_{V,\text{ext}}^2 \times L = \sigma_{\text{ext}}^2 \times F^2 \times L = a_0 + \frac{b_0}{u} + c_0 u \tag{11.21}$$

Combining Eqs. (11.19) to (11.21), the following expression is obtained:

$$\sigma_V^2 \times L = \left(A + \frac{B}{u} + Cu \right)(V_\text{R} - V_\text{ext})^2 + a_0 + \frac{b_0}{u} + c_0 u \tag{11.22}$$

Rearranging the terms in Eq. (11.22), an equation relating the variance in volume units with the inverse linear velocity is yielded for each compound:

$$\sigma_V^2 \times L = A_V + \frac{B_V}{u} + C_V u \tag{11.23}$$

Taking into account the following equivalence:

$$V_\text{R} - V_\text{ext} = V_0 (1 + k) \tag{11.24}$$

where k is the retention factor, the following is obtained:

$$A_V = A \times V_0^2 (1 + k)^2 + a_0 \tag{11.25}$$

$$B_V = B \times V_0^2 (1 + k)^2 + b_0 \tag{11.26}$$

$$C_V = C \times V_0^2 (1 + k)^2 + c_0 \tag{11.27}$$

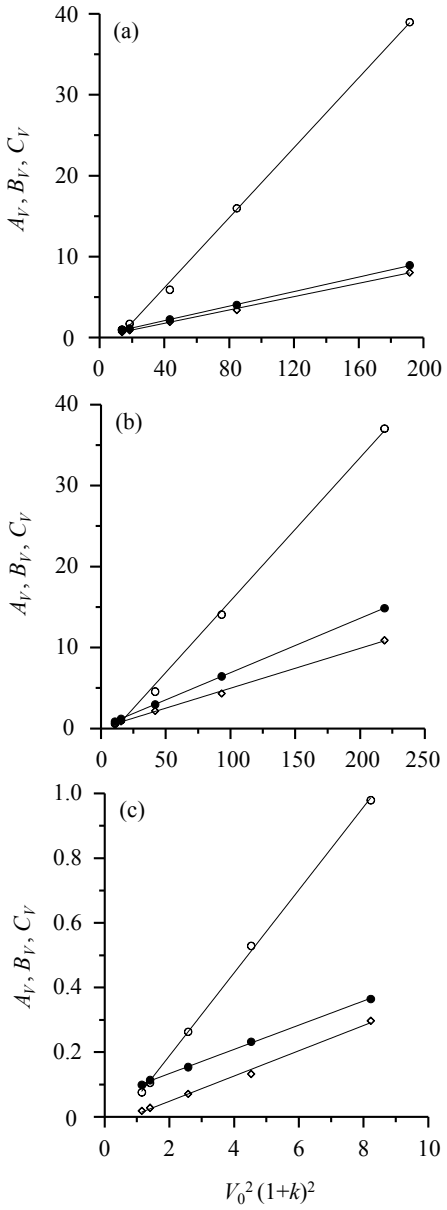

Figure 11.14. Plots of the A_V (●), B_V (○) and C_V (◊) parameters in Eq. (11.23), against the squared retention volume, for each probe compound and the three studied columns: (A) Spherisorb, (B) Zorbax, and (C) Chromolith. The straight-lines correspond to the linear fittings according to Eqs. (11.25)–(11.27). For convenience, the A_V values were divided by 100 and the B_V values by 1000.

Therefore, in this approach, the variance values expressed in volume units for each compound are first fitted to Eq. (11.23), and then, the model parameters (A_V, B_V and C_V) are linearly fitted to Eqs. (11.25)–(11.27) versus a term related to the retention factor for the probe solutes ($V_0^2(1+k)^2$). The slopes of each straight-line are the searched parameters (A, B, C) (Fig. 11.14). A highly linear behaviour was observed for the three columns, which confirms the validity of the assumptions. The determination coefficients where between R^2 = 0.9950 and 0.999996.

11.5.5.3. Approach III

Eq. (11.22) is the basis of the third approach, which involves the direct non-linear fitting of the peak variance in volume units, multiplied by the column length, as a function of the linear velocity and the retention volumes of the probe compounds. The model parameters (A, B and C, and a_0, b_0 and c_0) are obtained in only one step, using the data for a set of compounds eluted at several flow rates. Note that Approach III can be adapted to non-linearities against the retention factor in the A, B and C terms.

Table 11.1. Parameters of the Van Deemter equation (Eq. (11.3)), obtained using the three proposed approaches with the set of sulphonamides as probe compounds, and for the classical approach, for the Zorbax, Spherisorb and Chromolith columns.

	25 ºC				50 ºC			
	A	*B*	*C*	ε_r (%) [a]	*A*	*B*	*C*	ε_r (%) [a]
Zorbax column								
Approach I	7.42	170	0.0402	3.82	6.13	204	0.0408	2.90
Approach II	6.72	177	0.0491	4.33	5.40	219	0.0386	2.12
Approach III	7.46	170	0.0400	2.65	5.96	207	0.0414	2.53
Classical approach [b]	7.52	103	0.0452	5.96	6.99	131	0.0402	3.91
Spherisorb column								
Approach I	4.67	212	0.0428	2.91	4.21	274	0.0328	2.41
Approach II	4.50	216	0.0412	2.42	3.66	290	0.0278	2.05
Approach III	4.61	213	0.0430	2.30	4.08	277	0.0333	1.92
Classical approach [b]	5.14	136	0.0441	6.10	4.52	189	0.0346	4.64
Chromolith column								
Approach I	3.65	129	0.0404	2.17	3.06	189	0.0286	2.18
Approach II	3.74	128	0.0389	2.83	3.28	187	0.0248	2.91
Approach III	3.71	128	0.0399	2.49	3.18	188	0.0276	2.54
Classical approach [b]	4.57	100	0.0262	6.68	3.02	156	0.0209	8.64

[a] Prediction mean errors (ε_r (%)) calculated according to Eq. (11.28).

[b] The theoretical plate heights were calculated with Eq. (11.7). Mean *A*, *B* and *C* values for the five sulphonamides are given.

11.5.6 Application of the proposed approaches to the characterisation of chromatographic columns

The approaches described above were applied to the characterisation of chromatographic columns run at two temperatures, 25 °C and 50 °C. The fitted parameters for Eq. (11.3), according to the three proposed approaches and to the classical Van Deemter approach are given in Table 11.1, for the set of sulphonamides eluted with the three assayed columns. The mean prediction error was calculated according to:

$$\varepsilon_{\mathrm{r}}(\%) = \left(\sum \left|\sigma_i^2 - \hat{\sigma}_i^2\right|\right) / \left(\sum \left|\sigma_i^2\right|\right) \times 100 \qquad (11.28)$$

where σ_i^2 and $\hat{\sigma}_i^2$ are the experimental and predicted variances, respectively.

Although Approach I is the most robust since it does not involve any assumption about the extra-column value, Approach III is the most practical, since all experimental data are fitted in a single step. However, the three approaches are complementary, and allow a complete visualisation of the chromatographic performance. Approach I evaluates the linearity of the variances with the squared retention time or retention volume, and therefore, the influence of the retention factor on the evaluation of the theoretical plate height. Approach II allows a more detailed study of the validity of the Van Deemter equation, and how the retention factor affects each parameter. This approach reveals specific effects during the elution. Thus, for instance, by examining the B_V parameter versus the squared retention volume, a deviation from linearity would indicate a greater importance of the diffusion in the stationary phase. The C_V parameter is even more relevant, since it would deviate from linearity if the slow mass transfer were the fundamental factor in the stationary phase [14,15].

In the case of study, the three approaches yielded parameters with comparable values, but Approaches I and III provided more similar values. In all cases, the prediction errors for the variance were highly satisfactory, being in the 2–4% range. Two important observations arise from the estimation of the dispersion carried out at two temperatures (25 °C and 50 °C, see Table 11.1). First, the B term increased with temperature, which agrees with an increase in the diffusion in the mobile phase. Secondly, the C term decreased with temperature, owing to the increase in the mass transfer kinetics.

Finally, Table 11.2 shows the influence of the extra-column contribution on the dispersion parameters, for each assayed compound. The model parameters were obtained by fitting the data of each compound without considering the extra-column contribution ($a_0 = b_0 = c_0 = 0$). In the table, the parameters obtained using Approach III with the data of all compounds (the five sulphonamides) are also given. This approach provides the parameters for the extra-column contribution (a_0, b_0 and c_0). As expected, for the most retained compounds, the A, B, C parameters for a single compound are similar to those obtained with the whole set of sulphonamides, since the dispersion associated to the column is larger, making the extra-column contribution less relevant. The large variability of the B term, which ranges from $B = 65$ for sulphamerazine to $B = 200$ for sulphadimethoxine is noteworthy. This behaviour may be explained considering that as the retention factor increases, the elution time is larger and the contribution of diffusion becomes more evident, since the residence time of the compound inside the column is longer.

Table 11.2. Retention factors and model parameters for the five sulphonamides analysed with the Spherisorb column.[a]

	k	*A*	*B*	*C*
Sulphamerazine	2.37	6.85	65	0.0512
Sulphamethizole	2.90	6.09	89	0.0499
Sulphachloropyridazine	4.97	5.18	135	0.0472
Sulphisoxazole	7.35	4.78	186	0.0426
Sulphadimethoxine	11.55	4.81	200	0.0436
Set of sulphonamides	–	4.61	213	0.0430

[a] The A, B and C parameters correspond to Eq. (11.22), obtained assuming $a_0 = b_0 = c_0 = 0$. The parameters obtained with the whole set of sulphonamides were calculated according to Approach III.

11.6. Conclusions

The need of measuring the extra-column contribution to the total variance is a drawback of the classical approach used to characterise columns, based on the Van Deemter equation, owing to the uncertainty in its measurement and the systematic error introduced in the final result. The influence of the extra-column variance on the measurement of the theoretical plate height is larger at smaller retention of the assayed compound. Several approaches are currently used to make not significant (below 5%) the extra-column effects by the right selection of unions and connection tubes, injection volume and detector cell volume. However, there are still many situations where the extra-column

contribution is not negligible. The proposed approaches can be applied in all cases with high or low extra-column variance.

For asymmetrical peaks, the calculation of the peak variance using the Grushka and Giddings equation and the half-widths at 60.65% peak height is more stable and subjected to less uncertainty than the method based on peak integration. The sampling frequency also affects the peak shape. Thus, low sampling frequencies result in a fictitious increase in the *C* term of the Van Deemter equation.

When the extra-column contribution is not negligible, the theoretical plate height depends on the retention of the assayed compound. Compounds eluting at low retention times provide higher *H* values. Therefore, the conclusions obtained from a Van Deemter plot may vary depending on the selected probe compound. The alternative approaches proposed in this work allow a full graphical study of the column performance, based on the behaviour of a set of probe compounds, without the need of obtaining previously the extra-column variance. The approaches give information on how the retention factor affects the *A*, *B* and C parameters in the Van Deemter equation, which describe the importance of diffusion and slow mass transfer in the stationary phase. For this work, we have selected a set of sulphonamides, but the validity of the three proposed approaches has been checked with other sets of compounds. A complete study of a column would require its evaluation using several sets of compounds with different interaction behaviour. This will be the topic of future work.

11.7. References

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SUMMARY AND CONCLUSIONS

In the next pages, the general conclusions from each chapter of this PhD. Project are outlined, classified in three sections:

- Optimisation of multi-column systems in mono-dimensional liquid chromatography
- Development of new chromatographic objective functions
- Fundamental studies on column performance

C.1. Optimisation of multi-column systems in mono-dimensional liquid chromatography

C.1.1. Stationary phase modulation in liquid chromatography through the serial coupling of columns

- Liquid chromatography with single columns often does not succeed in the analysis of complex samples, in terms of resolution and analysis time. A relatively simple solution to enhance chromatographic resolution is the modulation of the stationary phase through the serial direct coupling of columns. This can be implemented with any type of column using compatible elution conditions and conventional instruments. The key features of column coupling and published procedures are described and discussed, where two or more columns were coupled in series to solve separation problems. In all reports, the authors could not resolve their samples with single columns, whereas significant enhancement in chromatographic performance was obtained when the columns were combined.
- Particularly interesting is the reduction in the analysis time in the isocratic mode, which alleviates the "general elution problem" of liquid chromatography. This may represent a stimulus for the proposal of new isocratic procedures, especially in combination with mass spectrometric, electrochemical and refractometric detection.

Developments proposed to make the serial coupling of columns useful in routine and research laboratories are outlined, including optimisation strategies that facilitate the selection of the appropriate column combination and elution conditions (solvent content, flow rate or temperature) in both isocratic and gradient modes. In this regard, the availability of zero dead volume couplers, able to connect standard columns, and the commercialisation of short columns with multiple lengths, have expanded the possibilities of success.

- Although a specific guideline about the most suitable columns that should be combined for a particular application cannot be given yet, it can be affirmed that the combination of a small number of common columns (e.g., C18, cyano, amino and phenyl) may succeed in most separations where isolatedly they fail. A wise selection of columns (nature and length) and solvent (isocratic mobile phase or gradient program), through the application of interpretive optimisation approaches, multiplies impressively the probabilities of success. The use of serially-coupled columns has the advantage over two-dimensional liquid chromatography (2DLC) of being accessible to any laboratory, whereas 2DLC requires a very considerable investment and training, and in this sense is more restrictive.

C.1.2. Interpretative approaches to optimise serially-coupled columns in reversed phase liquid chromatography

- A simple solution to expand the possibilities of high-performance liquid chromatography (HPLC) is to serially connect two or more columns containing different stationary phases. Nearly continuous transition of selectivity is yielded, which allows fine-tuning for each particular separation. Additionally, the analysis time is decreased making gradient elution less necessary. The effect of serially combining columns

containing different stationary phases is comparable to multiplying the number of columns in the laboratory to hundreds, thousands or even millions, each of them behaving as a new column. The probability of getting the ideal column for a particular separation is highly increased. However, being the combination of different columns a highly powerful tool to resolve complex samples, its successful development and widespread application required solving some technical problems.

- First, chromatographic columns should be connected without adding extra dead volumes between columns and without affecting the stationary phase nature close to the column connections. We have solved these problems by connecting conventional chromatographic columns through zero dead volume fingertight couplers, which are screwed directly to the columns. This is also a universal and reliable system which allows coupling of columns from different manufacturers.
- Second, a system was needed to find the correct combination and optimise its performance. For this purpose, we adapted some tools developed in our laboratory for the optimisation of single columns. Finally, adequate software to properly exploit all the potential that the separation system offers should be developed.
- This work summarises the developments carried out to implement methods in HPLC using serially-coupled columns. The capabilities of the serial connection of columns is demonstrated taking as example the separation of a mixture of 15 sulfonamides considering four stationary phases of different nature (two C18 columns with different retention behaviour, a phenyl phase and a cyano phase). All these columns showed insufficient performance for the separation of the probe compounds when used as single columns. However, with an optimised combination of columns, excellent separation in terms of resolution and analysis time was obtained. Similar improved separations are expected with other complex samples.

C.1.3. Serial versus parallel columns using isocratic elution: A comparison of multi-column approaches in mono-dimensional liquid chromatography

- There are two ways to make two or more columns cooperate in mono-dimensional separations, namely parallel and serial column configurations. The performance of both approaches in the separation of 15 sulfonamides with mobile phases characterised by one experimental factor (i.e., the solvent content) was compared under isocratic elution using the same training set.
- In order to optimise parallel columns of different nature, a previous approach was adapted, based on the complementarity concept. In this approach, a separation condition focuses on the resolution of some compounds in the sample, while the other compounds remain unresolved, but are optimally resolved in a second (or subsequent) condition(s). The performance of the different configurations was evaluated through the limiting resolutions and the percentage of separation capability of the chromatographic systems reached at the optimal conditions.
- In the case of study, serial columns outperformed parallel columns. Previously, we showed that a higher diversity in the mobile phase composition (e.g., the concentration of two modifiers, or the solvent content and pH) made the parallel approach succeed in the full resolution of highly complex samples using only one column nature. The example studied in this work, however, makes use of only one factor in the mobile phase. In these conditions, the variations that can be induced in selectivity by the mobile phase are much smaller, and so are the chances of success.
- The effect of the changes in the column length is shown to be very different in the serial and parallel approaches: in parallel separations,

there are no changes in the selectivity, whereas the column length highly affects the selectivity in serial separations. Therefore, although the comparison of both approaches was intended under the same conditions to avoid any possible advantage, indeed the serially-coupled columns approach has an intrinsic advantage, owing to its own nature: there is one more separation factor (i.e., the column length).

- Experimentally speaking, the use of parallel columns is simpler, since it implies conventional separations without any special requirement. Nevertheless, the disadvantage of the complementary approaches, besides the need of availing a higher diversity of selectivity (e.g., mobile phases involving two or more factors, such as the concentration of different modifiers, pH and temperature), is the longer analysis time.

- Serial columns offer a powerful way to enhance the resolution in HPLC. In practice, each serial combination of short columns of different nature and length operates as a new column, with its own selectivity. This increases enormously the wealth of columns available in a laboratory, from which we can select the best for our particular problem.

- It is demonstrated that even using columns offering very poor performance when used isolatedly, their serial combination can give rise to baseline separation. Although the examined combinations offering the best resolution involved the connection of four columns, it was possible to find excellent compromise solutions with only two columns of different polarity, as phenyl and cyano columns, or C18-HL and C18-AQ. Interestingly, the best combination had a longer contribution of the faster column.

C.1.4. Updating chromatographic predictions by accounting ageing for single and tandem columns

- Model-based chromatographic optimisations provide the best separation conditions efficiently. Unfortunately, with the routine usage, columns inevitably suffer some deterioration and, as a result, actual chromatograms may differ from the forecasted expectancies; new optimal separation conditions are then needed, but previous models are not valid anymore. The chromatographer could then decide repeating the whole modelling process, but this solution is undesirable and too time consuming, particularly when several coupled columns are involved.

- A shortcut is proposed to correct time and peak profile predictions for aged columns. The original models are corrected by introducing parameters accounting the deterioration, which are obtained with a small subset of compounds. These are selected from those in the training set used before the column had suffered performance decline. The ageing parameters are not solute dependent. These are obtained in such a way that the discrepancies between the data predicted with the original retention models for the brand-new column and the experimental data measured for the aged column are minimised.

- The approach was developed and tested to predict the chromatographic behaviour of 15 sulphonamides, analysed with individual and tandem columns, using isocratic and gradient elution. From the gathered information, predictions more in line with the deteriorated performance were forecasted for the whole family of compounds analysed with that column or set of coupled columns.

- The results suggest that, whenever the columns keep sufficient performance, it is possible to take advantage of the extensive experimental work carried out when the system was initially modelled

with the brand-new columns. With this information and a minimal extra experimental effort, accurate enough predictions in the degraded situation are possible. The full modelling of the chromatographic behaviour is thus only made with the brand-new columns. The agreement between predicted and experimental chromatograms in the aged columns was excellent.

- The main limitations of the proposed approach are that specific solute-dependent effects (magnified when the deterioration level is severe) are not considered, and that if the changes in column chemistry affect the adsorption-desorption kinetics, the half-width predictions will be also solute-dependent. Correcting these effects would imply more experimental effort and an eventual correction would make less sense. Of course, from a quality perspective, the aged columns should be regenerated or replaced if their separation parameters (i.e., efficiencies, selectivities or retention factors) do not meet acceptable criteria.

C.1.5. Analysis of amino acids using serially-coupled columns

- Along the years, a lot of effort has been invested in the development of reversed-phase liquid chromatographic (RPLC) methods to analyse amino acids. Some reports deal with the separation of the *o*-phthalaldehyde/N-acetyl-L-cysteine (OPA/NAC) derivatives of the 19 primary proteic amino acids using gradient elution. These derivatives allow sensitive procedures to analyse the amino acids using fluorescence detection. However, the amino acid derivatives are frequently resolved only in excessively long analysis times, even using multi-linear or multi-isocratic gradients. If the analysis time is reduced, significant overlapping occurs for several compounds. Single columns in RPLC are thus insufficient to solve complex separations, due to their limited functionality.

- A relatively simple solution to resolve the amino acid derivatives in sufficiently short analysis times is the serial connection of two or more columns with different stationary phases. Each combination performs as a totally new column. The full potential of this approach requires, however, the development of a powerful interpretive optimisation strategy. A unique predictor system was developed in our laboratory, which implemented the different strategies with single and serially coupled columns, in both isocratic and gradient elution. An outstanding agreement was found in all cases between experimental and predicted chromatograms.
- The Neue-Kuss equation yielded the best predictions of retention times for all columns. However, there was still a problem with the predictions for amino acids using isocratic elution, owing to the extreme variations in retention and the fact that the retention in specific regions for each amino acid should be extrapolated. This was even more problematic for the serially-coupled columns, with larger uncertainties in the predictions. This problem was alleviated using gradient elution, which decreases the analysis time of amino acids. The predictions obtained in this elution mode were satisfactory.
- The combination of the interactions of PFP-C18 and C4 columns resulted in much better resolution for the isoindoles of amino acids, compared to each column when used isolatedly. Also, the resolution was improved with regard to a C18 column.
- It was not possible to further decrease the analysis time below 50 min, owing to the close elution of the isoindoles of the most retained amino acids (tryptophan, phenylalanine, leucine and lysine), which exhibit similar polarity and wider peaks.

C.2. Development of new chromatographic objective functions

C.2.1. A chromatographic objective function to characterise chromatograms with unknown compounds or without standards available

- Getting useful chemical information from samples containing many compounds is still a challenge to analysts. The highest complexity corresponds to samples for which there is no prior knowledge about their chemical composition or without standards available. Most chromatographic objective functions (COFs) described in the literature to measure the separation performance cannot be applied to this type of samples. A new COF was first proposed for such cases. The approach was applied to improve the analysis of fingerprints of medicinal herbs.

- Chromatographic fingerprints are chromatograms of very complex mixtures of chemical compounds, such as those found in biological and environmental samples, which show multiple peaks of different heights. The larger peaks belong to components in relatively high concentration, while the smaller peaks can correspond to compounds at trace level, degradation products at different concentrations, impurities introduced in particular samples, or instrumental noise. The developed algorithm is unsupervised and measures the level of information in the chromatograms, through the concept of peak prominence, which is the protruding part of the signal with regard to the valleys that delimit a chromatographic peak (or the baseline).

- The ability of the new COF to characterise chromatograms with diverse overlapping was first evaluated using mixtures of six probe compounds, whose behaviour (retention times and peak half widths) was modelled. The built models allowed the simulation of chromatograms in a range of conditions. From these, a diagram was drawn that represented the resolution according to the peak prominence criterion and peak purity (which measures the overlapped area of a peak with other peaks in a

chromatogram) versus the mobile phase composition. The peak prominence offered a large sensitivity to detect changes in the peak configuration, responding only to the perceptible peaks.

- The peak prominence presents several advantageous features:

  (i) It is associated to individual peaks, which facilitates the combination of the elementary resolution and the measurement of peak count.
  (ii) It is sensitive to signal profile and size.
  (iii) It is an easily interpretable normalised measurement.
  (iv) It does not require standards.
  (v) It can be measured from the raw global signal.
  (vi) It attends to all peaks, independently that they correspond to one or more compounds.

- Even with simple mixtures (as those formed by mixing the six probe compounds), more peaks than those expected may be observed in the chromatograms. The global resolution was calculated for only the significant peaks, which were those exceeding a selected threshold of 0.05% of relative peak prominence area. The study suggested that the most significant parameter to characterise chromatographic fingerprints with multiple peaks of very different size is the number of perceptible peaks (peak count). Since the real number and nature of the components in these samples is unknown, it is necessary to establish a threshold to discriminate among the significant peaks and those that are not reproducible in the data processing (artefacts, peaks of impurities, or instrumental noise).

C.2.2. An approach to evaluate the information in chromatographic fingerprints: Application to the optimisation of the extraction and conservation conditions of medicinal herbs

- One main characteristic of fingerprints is that the identity of most or even all compounds is unknown, and there are no standards available. However, they are useful in determining the identity of the samples, by comparing fingerprints for different samples and using similarity analysis and diverse classification methods.
- Due to the high number of factors needed to optimise the extraction conditions for medicinal herbs, factorial design strategies were used. For each experimental factor, a high and a low value were established. The incompatibility of the experimental factors forced to divide them into two groups. In a first step, only the solvent concentration, sonication time and temperature of the treatment were studied. A separate investigation was carried out for acetonitrile, ethanol and methanol as extraction solvents. In a second step, the sample weight, and conservation time and temperature were investigated.
- In order to optimise the extraction conditions, the peaks in the fingerprints were ranked according to the peak prominence areas, establishing a threshold to discriminate between the significant peaks and those that are irreproducible.
- The experiments showed that methanol was the best solvent, and should be used preferably in mixtures with water at 30%, using a sonication time of 60 min and a temperature of 80 ^{o}C.
- The sample weight contributes very significantly to the number of peaks. The number of peaks is also larger when the conservation temperature is higher (4 ^{o}C against –10 ^{o}C), probably due to the degradation of some compounds at higher temperature. In general, the analysis should be performed immediately after obtaining the extracts.

- Although the described approach was developed using a green tea sample, it is suitable for finding the best extraction and conservation conditions for other types of medicinal herbs. It is expected that the peak prominence together with a strategy adapted to this type of samples will be useful to optimise the gradient program for chromatographic analyses, in order to get fingerprints of the highest quality.

C.2.3. Study of the performance of a global resolution criterion to characterise complex chromatograms with unknowns or without standards

- The quantification of chromatograms requires finding out experimental conditions that separate each peak from the others, and is routinely carried out with the information provided by standards in a small number of runs, in a 'design of experiments' fashion. However, there are samples where the identity of the solutes is partially or fully unknown, and consequently, there is not the support of standards. For these situations, the chromatographer lacks of a definite criterion to assist in the selection of the suitable experimental conditions, and computer-based optimisations are not applicable.
- A global resolution function valid for general situations (with or without standards) is proposed and validated. The function is based on the automatic measurement of peak prominences, and is compared with the performance given by the peak purity criterion. A Matlab application was developed to automate the analysis of chromatograms, previous subtraction of the baseline, comprehensively detecting all peaks, and calculating peak properties, such as the peak prominences.

- The separation of a mixture of the OPA/NAC derivatives of the 19 primary proteic amino acids under gradient elution was taken as controlled case. The amino acid derivatives could be resolved only in excessively long analysis times, even using multi-linear or multi-isocratic gradients. If the analysis time was reduced, significant overlapping occurred for several compounds. This overlapping gives access to interesting cases of study for the evaluation of the resolution functions. Using as training data the retention and peak width information of standards in a set of 10 isocratic conditions, the separation in around 1100 gradients, ranging from 5 to 27.5% acetonitrile, was predicted. Several levels of difficulty were considered: with and without differences in peak size, presence of unknown compounds, different noise levels, and real baselines.

- The comparison was carried out based on the coincidence of the gradients chosen as Pareto-optimal using two criteria: the peak purity (which implies a comprehensive knowledge of the individual signals of each compound), and the peak prominence (whose information is only limited to the combined signal, without any prior knowledge of the number of compounds in the sample). When the two Pareto fronts agreed, the same gradients were selected as optimal. Several fingerprint chromatograms of a tea extract, where the same sample was eluted with different gradients, were successfully evaluated.

- The best global resolution function was aimed to make the resolution insensitive to differences among peak areas and achieve better equivalence between peak prominence and peak purity in the selection of optimal gradients. The selected function considered normalised values referred to each individual peak, so that if the peaks were fully resolved, it matches the number of eluted compounds.

C.2.4. Optimisation of chromatographic resolution using objective functions including both time and spectral information: Compounds exhibiting small spectral differences

- The optimisation of the resolution in HPLC is traditionally performed attending only to the time information. However, even in the optimal conditions, some peak pairs may remain unresolved. Such resolution can still be completed by deconvolution, which can be carried out with more guarantees of success by including spectral information.
- A two-way COF that incorporates both time and spectral information using diode-array detection was tested, based on the multivariate selectivity (figure of merit derived from the net analyte signal). This COF is sensitive to situations where the components that coelute in a mixture show some spectral differences. Therefore, it is useful to locate experimental conditions where the spectrochromatograms can be recovered by deconvolution using the combination of orthogonal projection approach and alternating least squares.
- The approach was applied to a mixture of proteic amino acids, which were satisfactorily resolved using an acetonitrile-water gradient, but still at too high analysis times. By increasing the elution strength, the retention times decreased, but yielding overlapping among the peaks. The approach was used to find experimental conditions where the peaks could be deconvolved in spite of the small spectral differences.
- The two-way COF penalises maximally the co-elution of compounds with identical spectra, whereas scores with larger values the co-elution of compounds with spectral differences. This is the case of a sample that contains the OPA/NAC derivatives of proteic amino acids, where some compounds present identical spectra and for others these are slightly different.

- The shortest analysis times were obtained with optimised multi-isocratic gradients considering both the time and spectral resolution. The Pareto plot indicates that this type of gradients is capable of yielding acceptable spectral resolution with an analysis time that can be decreased down to 20 min. Also, there is a range of gradients with favorable resolution. Without taking into account the spectral resolution, the shortest analysis time offering acceptable resolution was 60 min.

C.3. Fundamental studies on column performance

C.3.1. New approaches to evaluate the dispersion parameters in liquid chromatography based on the information obtained from a set of compounds

- The study of column performance is of great interest to characterise stationary phases, develop new materials and increase the resolution of complex samples. Traditionally, this study has been performed through the elution of a probe compound at different flow rates and the representation of Van Deemter plots, which relate the column plate height to the linear mobile phase velocity. The interest of these plots is that they can reveal the different contributions to band broadening. Meanwhile, the disadvantages are the need of measuring the extra-column variance, the use of a unique compound which limits the conclusions of the approach, and the increase of the uncertainty due to the manipulation of two peak parameters (position and variance) to obtain the theoretical plate height.
- When the extra-column contribution is not negligible, the theoretical plate height depends on the retention of the assayed compound. Compounds eluting at low retention times provide larger plate height values. Therefore, the conclusions obtained from a Van Deemter plot may vary depending on the selected probe compound.

- Three complementary approaches were proposed and compared that avoid the above disadvantages. One of the approaches is based on the direct treatment of peak variances of several compounds according to a linear relationship with the squared retention time, including the extra-column variance as a fitting parameter. In another approach, the peak variance (expressed in volume units), for each substance, is fitted against the flow rate, and the model parameters are further related to the retention factors. The third methodology is based on a combination of both approaches.
- The proposed approaches allow a full graphical study of the column performance, based on the behaviour of a set of probe compounds, without the need of obtaining previously the extra-column variance. The approaches give information on how the retention factor affects the *A*, *B* and C parameters in the Van Deemter equation, which describe the importance of diffusion and slow mass transfer in the stationary phase.
- The behaviour of five sulphonamides, eluted from C18 columns of different types, using acetonitrile-water mixtures was studied. The results show that it is possible to characterise the columns with greater reliability using the proposed approaches, without previous knowledge of the extra-column variance.
- First of all, the factors affecting the uncertainty of the chromatographic data used to build Van Deemter plots were examined: the response time (related to the sampling frequency), selected peak limits, data noise and method used to subtract the baseline needed in the evaluation of the peak dispersion parameters, the procedure and peak height to obtain the best variance measurement, and the methodology to measure the theoretical plate height.

CONTRIBUTION OF THE PhD WORK TO PUBLISHED ARTICLES

The chapters in this PhD. work correspond to the following published articles, listed according to the publication date. The percentage of contribution of Tamara Álvarez Segura as PhD. student is indicated with each article.

- T. Álvarez Segura, M.C. García Álvarez-Coque, C. Ortiz Bolsico, J.R. Torres Lapasió, *Interpretive approaches to optimize serially-coupled columns in reversed-phase liquid chromatography*, Current Chromatogr. 2 (2015) 110–121 (Chapter 3).
  Contribution: 100% Tamara Álvarez Segura

- T. Álvarez Segura, C. Ortiz Bolsico, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *Serial versus parallel columns using isocratic elution: A comparison of multi-column approaches in mono-dimensional liquid chromatography*, J. Chromatogr. A 1390 (2015) 95–102 (Chapter 4).
  Contribution: 100% Tamara Álvarez Segura

- T. Álvarez Segura, A. Gómez Díaz, C. Ortiz Bolsico, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *A chromatographic objective function to characterize chromatograms with unknown compounds or without standards available*, J. Chromatogr. A 1409 (2015) 79–88 (Chapter 7).
  Contribution: 100% Tamara Álvarez Segura

- T. Álvarez Segura, E. Cabo Calvet, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *An approach to evaluate the information in chromatographic fingerprints: Application to the optimisation of the extraction and conservation conditions of medicinal herbs*, J. Chromatogr. A 1422 (2015) 178–185) (Chapter 8).

  Contribution: 50% Tamara Álvarez Segura; 50% Elsa Cabo Calvet

- T. Álvarez Segura, J.R. Torres Lapasió, C. Ortiz Bolsico, M.C. García Álvarez-Coque, *Stationary phase modulation in liquid chromatography through the serial coupling of columns: A review*, Anal. Chim. Acta 923 (2016) 1–23 (Chapter 2).
  Contribution: 100% Tamara Álvarez Segura

- T. Álvarez Segura, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *Optimisation of chromatographic resolution using objective functions including both time and spectral information. Part 2: Compounds exhibiting small spectral differences*, Current Chromatogr. 3 (2016) 34–43 (Chapter 10).
  Contribution: 100% Tamara Álvarez Segura

- T. Álvarez Segura, C. Camacho Molinero, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *Analysis of amino acids using serially coupled columns*, J. Sep. Sci. 40 (2017) 2741–2751 (Chapter 6).

  Contribution: 100% Tamara Álvarez Segura

- T. Álvarez Segura, M.D. Peris Díaz, M.C. García Álvarez-Coque, J.J. Baeza Baeza, *New approaches to evaluate the dispersion parameters in liquid chromatography based on the information obtained from a set of compounds*, Current Chromatogr. 4 (2017) 156–166 (Chapter 11).

  Contribution: 100% Tamara Álvarez Segura

- J.A. Navarro Huerta, T. Álvarez Segura, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *Study of the performance of a resolution criterion to characterise complex chromatograms with unknowns or without standards*, Anal. Methods 9 (2017) 4293–4303 (Chapter 9).

  Contribution: 50% Tamara Álvarez Segura Álvarez Segura; 50% José Antonio Navarro Huerta

- T. Álvarez Segura, J.R. Torres Lapasió, M.C. García Álvarez-Coque, *Modelling of column ageing and correction of optimal separations in isocratic and gradient elution for aged single and serially-coupled columns*, J. Sep. Sci. (2018, in press) (Chapter 5).

  Contribution: 100% Tamara Álvarez Segura

Both supervisors (or at least one of them) of this PhD. work (María Celia García Álvarez-Coque and José Ramón Torres Lapasió) appear as co-authors. They are also main researchers of the projects who have funded the investigations. José Ramón Torres Lapasió has also prepared the computer programs used in the data processing included in the different articles.

The article in Chapter 11 was carried out in collaboration with Juan José Baeza Baeza, member of the research group, who proposed the main lines of the work and prepared the computer programs to carry out the data processing.

Casandra Ortiz Bolsico participated in the initial period of the PhD. work of Tamara Álvarez Segura training and supervising her experimental work (preparation of solutions, experimental technique, handling of instruments, data processing and interpretation of the results). In addition, some research work contributed to the training of the Master student Antonio Gómez Díaz, and Degree students Carolina Camacho and Manuel David Peris Díaz.