UNIVERSIDAD DE SALAMANCA

Facultad de Ciencias Químicas

Departamento de Química Analítica, Nutrición y Bromatología

MÉTODOS RÁPIDOS BASADOS EN ESPECTROMETRÍA DE MASAS PARA LA DETECCIÓN Y CUANTIFICACIÓN DE BIOMARCADORES

RAPID METHODS BASED ON MASS SPECTROMETRY FOR THE DETECTION AND QUANTIFICATION OF BIOMARKERS

Tesis Doctoral

Patricia Martín Santos

2020

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Memoria que para optar al Grado de Doctor por la Universidad de Salamanca presenta la licenciada Patricia Martín Santos.

Salamanca, 17 de junio de 2020

Fdo.: Patricia Martín Santos

D. José Luis Pérez Pavón, Catedrático de Química Analítica de la Universidad de Salamanca, y D. Miguel del Nogal Sánchez, Profesor Titular de Química Analítica de la Universidad de Salamanca, ambos directores del trabajo "Métodos rápidos basados en espectrometría de masas para la detección y cuantificación de biomarcadores", realizado por la licenciada Patricia Martín Santos para optar al Grado de Doctor por la Universidad de Salamanca, autorizan la presentación del mismo al considerar que se han alcanzado los objetivos inicialmente previstos.

Salamanca, 17 de junio de 2020

Fdo.: José Luis Pérez Pavón

Fdo.: Miguel del Nogal Sánchez

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Esta Tesis Doctoral se presenta como compendio de las publicaciones especificadas a continuación:

1. **"Determination of ketones and ethyl acetate-a preliminary study for the discrimination of patients with lung cancer"**

   Patricia Martín Santos, Miguel del Nogal Sánchez, Ángel Pedro Crisolino Pozas, José Luis Pérez Pavón, Bernardo Moreno Cordero.
   Analytical and Bioanalytical Chemistry 409 (2017) 5689–5696.

2. **"Quantitative and qualitative analysis of polycyclic aromatic hydrocarbons in urine samples using a non-separative method based on mass spectrometry"**

   Patricia Martín Santos, Miguel del Nogal Sánchez, José Luis Pérez Pavón, Bernardo Moreno Cordero.
   Talanta 181 (2018) 373–379.

3. **"Liquid-liquid extraction-programmed temperature vaporizer-gas chromatography-mass spectrometry for the determination of polycyclic aromatic hydrocarbons in saliva samples. Application to the occupational exposure of firefighters"**

   Patricia Martín Santos, Miguel del Nogal Sánchez, José Luis Pérez Pavón, Bernardo Moreno Cordero, Rubén Verde Fernández.
   Talanta 192 (2019) 69–78.

4. **"A sensitive and automatic method based on microextraction by packed sorbents for the determination of polycyclic aromatic hydrocarbons in saliva samples"**

   Patricia Martín Santos, Camilo Jiménez Carracedo, Miguel del Nogal Sánchez, José Luis Pérez Pavón, Bernardo Moreno Cordero.
   Microchemical Journal 152 (2020) 104274.

5. **"Determination of polycyclic aromatic hydrocarbons in human biological samples: A critical review"**

   Patricia Martín Santos, Miguel del Nogal Sánchez, José Luis Pérez Pavón, Bernardo Moreno Cordero.
   Trends in Analytical Chemistry 113 (2019) 194-209.

6. **"Non-separative method based on a single quadrupole mass spectrometer for the semi-quantitative determination of amino acids in saliva samples. A preliminary study"**

   Patricia Martín Santos, Miguel del Nogal Sánchez, José Luis Pérez Pavón, Bernardo Moreno Cordero.
   Talanta 208 (2020) 120381.

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SIGLAS Y ACRÓNIMOS

AA-dμ-SPE	Extracción en fase sólida microdispersiva asistida por aire *Air-assisted dispersive micro-solid phase extraction*
AALLME	Microextracción líquido-líquido asistida por aire *Air-assisted liquid-liquid microextraction*
ANN	Redes neuronales artificiales *Artificial neuronal network*
ASAP	Sonda de análisis de sólidos a presión atmosférica *Atmospheric pressure solid analysis probe*
BTEX	Benceno, tolueno, etilbenceno, xileno *Benzene, toluene, ethylbenzene, xylene*
CCD	Detección de conductividad sin contacto *Contactless conductivity detection*
CE	Electroforesis capilar *Capillary electrophoresis*
DER	Reacción de derivatización *Derivatization reaction*
DHS	Generación de espacio de cabeza dinámico *Dynamic headspace sampling*
DLLME	Microextracción líquido-líquido dispersiva *Dispersive liquid-liquid microextraction*
d-SPE	Extracción en fase sólida dispersiva *Dispersive solid-phase extraction*
EC	Electroquímico *Electrochemical*
ECF	Cloroformiato de etilo *Ethyl chloroformate*
ESI	Ionización por electrospray *Electrospray ionization*

F	Fluorescencia *Fluorescence*
FID	Detección de ionización en llama *Flame ionization detection*
GC	Cromatografía de gases *Gas chromatography*
HCA	Análisis de agrupamiento jerárquico *Hierarchical cluster analysis*
HF-LPME	Microextracción en fase líquida por fibra hueca *Hollow-fiber liquid-phase microextraction*
HPLC	Cromatografía líquida de alta resolución *High-performance liquid chromatography*
HS	Generación de espacio de cabeza *Headspace sampling*
IARC	Agencia Internacional para la Investigación sobre el Cáncer *International Agency for Research on Cancer*
ICS	Sociedad Internacional de Quimiometría *International Chemometrics Society*
KNN	Técnica de los k vecinos más próximos *k-nearest neighbors algorithm*
LC	Cromatografía de líquidos *Liquid chromatography*
LDA	Análisis discriminante lineal *Linear discriminant analysis*
LIF	Fluorescencia inducida por láser *Laser induced fluorescence*
LLE	Extracción líquido-líquido *Liquid-liquid extraction*
LOD	Límite de detección *Limit of detection*

LOQ	Límite de cuantificación *Limit of quantification*
LPME	Microextracción en fase líquida *Liquid-phase microextraction*
MDL	Límite de detección multivariante *Multivariate detection limit*
MEPS	Microextracción con sorbentes empaquetados *Microextraction by packed sorbents*
MHE	Extracción de espacio de cabeza múltiple *Multiple headspace extraction*
MIPs	Polímeros de impresión molecular *Molecular imprinted polymers*
MS	Espectrometría de masas *Mass spectrometry*
MS/MS	Espectrometría de masas en tándem *Tandem mass spectrometry*
NMR	Resonancia magnética nuclear *Nuclear magnetic resonance*
P&T	Purga y trampa *Purge and trap*
PAHs	Hidrocarburos policíclicos aromáticos *Polycyclic aromatic hydrocarbons*
PCA	Análisis de componentes principales *Principal component analysis*
PCR	Regresión por componentes principales *Principal component regression*
PCs	Componentes principales *Principal components*
PLS	Regresión por mínimos cuadrados parciales *Partial least squares regression*

PLS-DA	Análisis discriminante-mínimos cuadrados parciales *Partial least squares-discriminant analysis*
PTV	Inyector de temperatura programada *Programmed temperature vaporizer*
q-MS	Cuadrupolo simple *Single quadrupole*
Q-TOF-MS	Espectrometría de masas con analizador de tiempo de vuelo *Quadrupole-time of flight-mass spectrometry*
QuEChERS	Rápido, sencillo, barato, eficaz, robusto y seguro *Quick, easy, cheap, effective, rugged and safe*
RAMs	Materiales de acceso restringido *Restricted access materials*
RMSEV	Error cuadrático medio de validación *Root mean square error*
ROC	Característica operativa del receptor *Receiver operating characteristic*
RSD	Desviación estándar relativa *Relative standard deviation*
SALLE	Extracción líquido-líquido asistida por sales *Salting-out assisted liquid-liquid extraction*
SDME	Microextracción en gota *Single-drop microextraction*
SHS	Generación de espacio de cabeza estático *Static headspace sampling*
SIM	Modo de seguimiento de iones seleccionados *Selected ion monitoring*
SIMCA	Modelado independiente de clases *Soft independent modelling of class analogy*
SPE	Extracción en fase sólida *Solid-phase extraction*

SPME	Microextracción en fase sólida *Solid-phase microextraction*
SVM	Técnica de las muestras apoyadas en la frontera *Support vector machines*
SVs	Vectores soportados *Support vectors*
UPLC	Cromatografía líquida de ultra-alta resolución *Ultra high-performance liquid chromatography*
USEPA	Agencia de Protección Ambiental de Estados Unidos *United States Environmental Protection Agency*
UV	Ultravioleta *Ultraviolet*
VOCs	Compuestos orgánicos volátiles *Volatile organic compounds*
WHO	Organización Mundial de la Salud *World Health Organization*

INTRODUCCIÓN GENERAL

1 Estructura y Objeto de la Tesis

1.1. Estructura de la Tesis Doctoral

Con el objetivo de cumplir los requisitos para la presentación de la Tesis Doctoral en el formato de compendio de publicaciones, esta memoria se ha estructurado en varios apartados. En primer lugar, la Tesis consta de una introducción general en la que se pone de manifiesto la coherencia y relación directa entre las publicaciones presentadas. En la misma se muestran los objetivos, las hipótesis de trabajo y las diferentes metodologías utilizadas. Además, se describen los biomarcadores objeto de estudio, las técnicas usadas como pretratamiento de muestra, las configuraciones instrumentales y, por último, las técnicas quimiométricas empleadas.

Posteriormente, la Tesis consta de tres capítulos generales (capítulos I, II y III) diferenciados según los biomarcadores de estudio y dentro de los cuales se encuentran los diferentes trabajos realizados. En el primero de ellos se lleva a cabo la determinación de nueve cetonas y acetato de etilo, conjunto de analitos incluidos dentro de los compuestos orgánicos volátiles (*volatile organic compounds*, VOCs), en muestras de orina. El segundo capítulo está enfocado a la cuantificación de hidrocarburos policíclicos aromáticos (*polycyclic aromatic hydrocarbons*, PAHs) en muestras biológicas. En este capítulo se incluyen cuatro apartados: uno de ellos enfocado la determinación de estos compuestos en muestras de orina mediante un método sin separación cromatográfica, dos enfocados a la determinación en muestras de saliva que difieren en la técnica de extracción utilizada y un último apartado que corresponde a una revisión bibliográfica de los trabajos en los cuales se determinan PAHs en muestras biológicas. Por último, en el tercer capítulo se aborda la determinación de aminoácidos en muestras de saliva.

Para cada uno de los trabajos que conforman esta Tesis se incluye una copia completa de la publicación original precedida de un resumen en el que se

especifican los objetivos concretos que se desean alcanzar, la metodología empleada, los resultados conseguidos y las conclusiones.

1.2. Introducción

Esta Tesis Doctoral está enfocada al desarrollo de nuevas metodologías analíticas, separativas y no separativas, basadas en la utilización de la espectrometría de masas (*mass spectrometry*, MS) en dos modalidades diferentes. En la primera de ellas se encuentra acoplada a la técnica de cromatografía de gases (*gas chromatography*, GC), que permite la separación de los analitos objeto de estudio de manera previa a su detección. Así, se obtiene información de los compuestos de manera individual. Gracias a la sensibilidad alcanzada mediante esta metodología es posible detectar, identificar y cuantificar compuestos que se encuentran en la matriz analizada en muy bajas concentraciones. En la segunda modalidad la columna del cromatógrafo de gases actúa como una mera línea de transferencia, por lo que no se lleva a cabo la separación previa de los compuestos. En esta modalidad, por tanto, se estudia una señal de perfil del conjunto de todos ellos. La información de interés contenida en dicha señal de perfil se obtiene gracias al uso de técnicas quimiométricas. La ausencia de separación cromatográfica hace que esta modalidad se lleve a cabo con una mayor rapidez, siendo posible utilizarla para un cribado inicial de muestras, mientras que el uso de la metodología con separación se aplica con fines de confirmación, principalmente.

Como se ha dicho previamente esta memoria se ha dividido en tres partes diferenciadas en función del conjunto de biomarcadores estudiados. La primera de ellas se enfoca en la determinación de VOCs en orina, que han sido relacionados con diferentes tipos de enfermedades incluyendo el cáncer de pulmón. En el segundo caso se aborda la determinación de PAHs, algunos de los cuales han sido clasificados como posibles cancerígenos para la especie humana,

en muestras de orina y saliva. Cabe destacar que a pesar de que estos compuestos han sido fundamentalmente descritos como marcadores de exposición en ciertos ambientes laborales, la exposición a los mismos sucede de manera habitual en ambientes cotidianos debido a la dieta o el tabaquismo. En este capítulo se incluye además un artículo de revisión en el que se revisan los trabajos descritos en bibliografía enfocados a la determinación de PAHs en diferentes matrices biológicas humanas. La última parte se centra en el desarrollo de metodologías para la determinación de aminoácidos en muestras de saliva. De nuevo, el interés que recibe este tipo de compuestos es debido a que los niveles de concentración de los mismos varían cuando se padecen distintos tipos de enfermedades, incluyendo el cáncer. Por tanto, todas las partes descritas se dirigen a la puesta a punto de un método o métodos analíticos que cumplan las características previamente descritas para el análisis de biomarcadores en muestras biológicas.

Debido al uso de diversas matrices biológicas, así como a la diferente naturaleza de los analitos objeto de estudio, las etapas de tratamiento de muestra que van a utilizarse en este trabajo (extracción, preconcentración y obtención de derivados) son muy variadas.

En la primera parte de esta Tesis (capítulo I) la etapa de extracción de los VOCs objeto de estudio, nueve cetonas y acetato de etilo, en muestras de orina se lleva a cabo mediante la técnica de generación de espacio de cabeza (*headspace sampling*, HS). El uso de esta técnica de extracción sencilla, con una mínima manipulación de la muestra, permite el análisis de los compuestos volátiles sin interferencias de los compuestos no volátiles que puedan estar presentes en la matriz. Además, permite la automatización del proceso reduciendo los errores asociados a esta etapa del análisis.

En la segunda parte de la Tesis se lleva a cabo la determinación de PAHs en diferentes matrices biológicas, orina y saliva. Debido a la naturaleza no volátil de estos compuestos la extracción de las diferentes muestras se aborda desde una

perspectiva diferente a la descrita previamente. En primer lugar, la extracción de PAHs tanto en orina (capítulo II.1.1) como en saliva (capítulo II.2.1) se lleva a cabo utilizando una extracción líquido-líquido (*liquid-liquid extraction*, LLE). Esta técnica permite llevar a cabo la extracción de los analitos de una manera rápida. En segundo lugar, se pone a punto un método completamente automático para la determinación de PAHs en saliva basado en una microextracción con sorbentes empaquetados (*microextraction by packed sorbents*, MEPS) (capítulo II.2.2). Esta técnica se basa en una miniaturización del proceso de extracción en fase sólida convencional (*solid-phase extraction*, SPE) y permite el uso de pequeños volúmenes de muestra y de disolvente orgánico, y la reutilización de los cartuchos de extracción un número elevado de veces.

En la tercera parte de la Tesis (capítulo III) se lleva a cabo una reacción de derivatización para la determinación de aminoácidos en muestras de saliva con el fin de mejorar las características cromatográficas de los mismos. El uso de cloroformiato de etilo (*ethyl chloroformate*, ECF) como agente derivatizante permite que la reacción se lleve a cabo en la matriz acuosa que se desea analizar. Una vez obtenidos los correspondientes derivados compatibles con la técnica de análisis posterior, la extracción de los mismos se lleva a cabo utilizando una extracción LLE.

Cuando la metodología empleada en el análisis se basa en la modalidad sin separación cromatográfica es necesario aplicar diferentes técnicas quimiométricas a las señales de perfil obtenidas para extraer la máxima información útil. En esta Tesis se utilizan estas técnicas con fines cuantitativos y cualitativos. En el primer caso, se emplea la regresión por mínimos cuadrados parciales (*partial least squares regression*, PLS), que permite la cuantificación de diferentes compuestos en mezclas complejas. En el segundo caso, las señales de perfil obtenidas se estudian mediante el uso de técnicas de reconocimiento de pautas supervisadas y no supervisadas. La técnica no supervisada utilizada en esta Tesis para la exploración de datos es el análisis de componentes principales

(*principal component analysis*, PCA). Las técnicas supervisadas empleadas en esta memoria para la discriminación de muestras son el modelado independiente de clases (*soft independent modelling of class analogy*, SIMCA), el análisis discriminante lineal (*linear discriminant analysis*, LDA), el análisis discriminante-mínimos cuadrados parciales (*partial least squares-discriminant analysis*, PLS-DA) y la técnica de las muestras apoyadas en la frontera (*support vector machines*, SVM). Las principales características y diferencias entre las mismas se explicarán con más detalle en el apartado 5 de esta memoria.

1.3. Objetivos e hipótesis de trabajo

El objetivo general de esta Tesis Doctoral es la puesta a punto de metodologías analíticas enfocadas a la identificación y cuantificación de marcadores de enfermedades o de exposición en diferentes matrices biológicas. Además, se tratará de simplificar la etapa de tratamiento de muestra y reducir el tiempo total del análisis siempre que sea posible. Las metodologías propuestas para la cuantificación podrán ser separativas, aquellas donde se utiliza la cromatografía para separar los compuestos de manera previa a la detección, y no separativas, aplicadas en aquellas situaciones donde los analitos de interés se encuentren en una concentración suficiente para ser detectados sin llevar a cabo una separación cromatográfica. De manera adicional en algunos de los casos se propone como objetivo la discriminación de muestras en base a las concentraciones de los marcadores de estudio.

La espectrometría de masas permite simplificar de forma significativa los procedimientos analíticos debido a la enorme cantidad de información que proporciona. Esto hace que las etapas de tratamiento de muestra puedan reducirse de forma considerable. Además, los datos que proporciona la técnica cuando no está acoplada a sistemas cromatográficos pueden permitir en muchos casos la

obtención de información cualitativa, semicuantitativa o cuantitativa para resolver el problema analítico planteado.

La hipótesis de partida de esta Tesis es la viabilidad de utilizar el acoplamiento de un inyector de temperatura programada (*programmed temperature vaporizer*, PTV) a un cromatógrafo de gases-espectrómetro de masas, o bien directamente el acoplamiento de un inyector de temperatura programada con un espectrómetro de masas para la determinación de especies volátiles y no volátiles en muestras de orina y saliva.

El objetivo concreto de la primera parte es la puesta a punto de un método basado en HS-PTV-GC-MS para la cuantificación de cetonas y acetato de etilo en muestras de orina. Teniendo en cuenta las aplicaciones previas descritas en bibliografía en las cuales se ha utilizado esta configuración instrumental para el análisis de diferentes VOCs en matrices biológicas, se espera que sea adecuada cuando se aplique a estos diez compuestos en muestras de orina. Una vez que la metodología se haya puesto a punto, se plantea la posibilidad de encontrar diferencias significativas en la concentración de alguno de los compuestos de interés cuando se comparan muestras pertenecientes a individuos sanos y pacientes con cáncer de pulmón.

La segunda parte de esta Tesis se centra en el análisis de PAHs en muestras de orina y saliva. Se plantean diferentes objetivos dentro de la misma. Uno de ellos es el desarrollo de un método rápido sin separación cromatográfica, basado en LLE-PTV-MS, para la cuantificación de PAHs en muestras de orina. En este trabajo se plantea como hipótesis que esta metodología es aplicable a la determinación de compuestos semivolátiles o no volátiles. La sensibilidad alcanzada debe ser suficiente para cuantificar estos compuestos en muestras de orina con un rango de concentración similar al descrito habitualmente para individuos expuestos laboralmente. En vista de los buenos resultados descritos en bibliografía cuando se aplican técnicas quimiométricas a las señales de perfil

obtenidas en MS se propone aquí el uso de estas con fines cuantitativos, para la cuantificación de PAHs en orinas, y cualitativos, para la discriminación de las muestras en función de la presencia o ausencia de PAHs, así como para una posible clasificación de muestras futuras.

Otro de los objetivos de esta segunda parte de la Tesis es el desarrollo de metodologías separativas con diferentes técnicas de extracción y la comparación de los resultados obtenidos. En concreto, se compararon dos metodologías: **LLE**-PTV-GC-MS y **MEPS**-PTV-GC-MS. Basándonos en la naturaleza de los compuestos que se determinan (no polares y con baja volatilidad) y en los resultados satisfactorios que estas técnicas han proporcionado previamente en la determinación de compuestos similares en otras matrices, se espera que sean adecuadas cuando se apliquen a la determinación de PAHs en muestras de saliva.

La tercera y última parte está dedicada a la determinación de aminoácidos en muestras de saliva. En este caso, el objetivo es la puesta a punto de una metodología rápida y no separativa en la cual se lleva a cabo una reacción de derivatización (*derivatization reaction*, DER) y posteriormente se analizan los derivados mediante LLE-PTV-MS. La efectividad de la reacción de grupos amino y ácido con ECF en medio acuoso ha sido ampliamente descrita hasta la fecha. Por esta razón, se espera que sea adecuada para la cuantificación de aminoácidos en muestras de saliva. Para ello, se aplicarán técnicas quimiométricas (PLS) a las señales de perfil obtenidas, que han mostrado previamente su utilidad cuando se aplican a la cuantificación de compuestos en situaciones complejas en las cuales existe solapamiento en los espectros de masas.

1.4. Metodología de trabajo

Para el desarrollo de los diferentes apartados de esta Tesis Doctoral se utiliza la misma metodología. Está formada por las siguientes etapas:

1. **Selección y optimización de la etapa de pretratamiento de muestra**

Las muestras biológicas son, por lo general, matrices bastante complejas. Este hecho junto con la diferente naturaleza de los compuestos analizados hace que esta etapa sea de gran importancia. Como se ha mencionado anteriormente, a lo largo del desarrollo de los diferentes trabajos se utilizan diversas técnicas para la extracción de los analitos de la matriz, entre ellas las técnicas HS, LLE y MEPS, así como una reacción de derivatización para la obtención de derivados compatibles con la técnica de análisis. Las características de estas técnicas se explicarán con más detalle en el apartado 3 de esta memoria.

2. **Optimización de la configuración instrumental utilizada (PTV-GC-MS o PTV-MS)**

En esta Tesis Doctoral se utiliza un PTV para llevar a cabo la inyección. En este proceso se optimizan el modo de inyección, el tiempo de inyección y la temperatura de inyección, entre otros. Posteriormente, con el fin de conseguir una adecuada separación y detección de los analitos de interés es necesario optimizar los parámetros que afectan a la configuración GC-MS. En concreto, debido al uso de metodologías separativas y no separativas, la optimización del cromatógrafo de gases se abordará mediante dos estrategias. En el caso de las metodologías separativas se optimizarán los parámetros que permitan una adecuada separación de los analitos en el menor tiempo posible y con una resolución adecuada. En las metodologías no separativas se evaluarán las condiciones adecuadas para la obtención de la señal de perfil de las muestras correspondientes, bien sea utilizando una columna desactivada o aumentando la temperatura de la columna capilar, eliminando así su capacidad de separación.

Así mismo, para una adecuada detección es necesario optimizar las condiciones del espectrómetro de masas, entre ellas la velocidad de barrido y el tiempo de registro de cada ion (*dwell time*).

Una vez optimizado el método se seguirá alguna de las siguientes estrategias en función del objetivo que se desee.

3. Análisis cuantitativo

Inicialmente se evaluará la existencia o ausencia de efecto matriz, ya que el resultado de dicho estudio determinará la estrategia de calibración que se debe utilizar: calibración externa o adición estándar en la metodología separativa, y calibración multivariante (PLS) en agua o en muestras biológicas en la metodología no separativa. A continuación, se llevará a cabo la calibración siguiendo la estrategia adecuada y se determinarán las características analíticas del método. La validez del mismo se comprobará analizando muestras dopadas y comparando el valor de la concentración añadida con el valor predicho por el correspondiente calibrado. Una vez validados los modelos se llevará a cabo la predicción de las concentraciones de los analitos en las nuevas muestras.

4. Análisis cualitativo

Inicialmente, con el fin de llevar a cabo una exploración de datos se utiliza el análisis de componentes principales (PCA). Posteriormente, se aplican las técnicas de reconocimiento de pautas supervisadas (SIMCA, LDA, PLS-DA, SVM). Para ello, se construyen modelos con un grupo de muestras de entrenamiento y se evalúa su validez mediante la clasificación de muestras conocidas. Por último, se lleva a cabo la clasificación de muestras externas al conjunto de entrenamiento.

2 Biomarcadores

A lo largo de los años, la determinación de diferentes compuestos en matrices biológicas ha ganado una gran importancia. Esta determinación ha sido enfocada tanto a compuestos endógenos, que pueden variar de concentración en función del estado de salud del individuo, como a compuestos exógenos o de exposición, que se introducen en el organismo mediante diferentes vías tras una exposición a los mismos, y que en muchas ocasiones pueden dar lugar a diferentes enfermedades.

La Organización Mundial de la Salud (*World Health Organization*, WHO) establece dos definiciones relacionadas con los biomarcadores y la biomonitorización humana[1]:

- Un biomarcador es un compuesto químico, un metabolito de un compuesto químico o el producto de una interacción entre un compuesto y una molécula o célula determinada que se mide en el cuerpo humano.
- La monitorización humana es la medida directa de la exposición de los individuos objeto de estudio a sustancias tóxicas en el medio ambiente mediante el análisis de esas sustancias o sus metabolitos en muestras biológicas, como sangre u orina.

En esta Tesis se han estudiado diferentes biomarcadores englobados dentro de los grupos descritos anteriormente. Por un lado, se ha llevado a cabo el estudio de diversos biomarcadores endógenos, donde se incluye un conjunto de cetonas y acetato de etilo (capítulo I), así como los aminoácidos (capítulo III). Por otro lado, se han determinado hidrocarburos policíclicos aromáticos (capítulo II) como conjunto de biomarcadores de exposición.

2.1. Compuestos orgánicos volátiles

La determinación de compuestos orgánicos volátiles (VOCs) en diferentes muestras biológicas es una de las estrategias más prometedoras en

metabolómica, ya que proporcionan información acerca del estado de salud de los individuos. Estos compuestos son moléculas gaseosas que pueden originarse en el cuerpo, VOCs endógenos, o provenir de fuentes externas como la dieta, medicación o exposición ambiental, VOCs exógenos[2].

En particular, en esta Tesis se determinó un conjunto de VOCs, incluyendo nueve cetonas y acetato de etilo, en muestras de orina (capítulo I). Las cetonas son compuestos orgánicos que se caracterizan por poseer un grupo funcional carbonilo unido a dos átomos de carbono. En cambio, el acetato de etilo es un éster. Estos compuestos se han clasificado como endógenos, pero pueden provenir también de otras fuentes como la alimentación, productos de cuidado personal y el medioambiente[3].

Entre los trastornos y enfermedades descritos en bibliografía que involucran cambios en la concentración habitual de estos analitos se encuentran algunas relacionadas con el intestino, el riñón, el hígado, así como el asma y el cáncer, entre otras[3-5].

Las matrices biológicas descritas habitualmente para la determinación de estos analitos han sido la sangre[6], la orina[7,8], la saliva[9] y el aire exhalado[4,6].

En la figura 2.1 se muestra la estructura química de los VOCs determinados en esta Tesis (capítulo I). Además, en la tabla 2.1 se resumen algunas de las propiedades de los mismos[10-12].

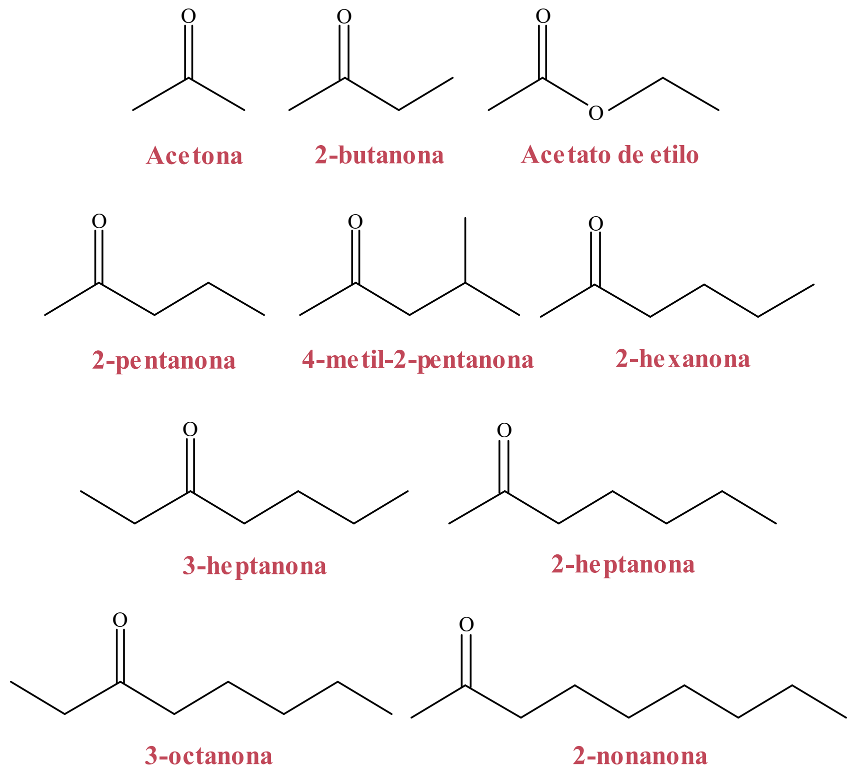

Figura 2.1. *Estructura química de las nueve cetonas y acetato de etilo determinados en orina en esta Tesis*

Tabla 2.1. *Propiedades de las nueve cetonas y acetato de etilo determinados en orina*

Compuesto	Fórmula química	Peso molecular/ $g\ mol^{-1}$	$T_{ebullición}$/ °C	Solubilidad en agua/ $mg\ mL^{-1}$	Densidad/ $g\ mL^{-1}$
Acetona	C_3H_6O	58.08	56	1000	0.791
2-butanona	C_4H_8O	72.11	80	223	0.805
Acetato de etilo	$C_4H_8O_2$	88.11	77	80	0.900
2-pentanona	$C_5H_{10}O$	86.13	102	43	0.809
4-metil-2-pentanona	$C_6H_{12}O$	100.16	116	19	0.797
2-hexanona	$C_6H_{12}O$	100.16	127	17.5	0.83
3-heptanona	$C_7H_{14}O$	114.19	147	4.3	0.818
2-heptanona	$C_7H_{14}O$	114.19	151	4.3	0.807
3-octanona	$C_8H_{16}O$	128.21	168	2.6	0.820
2-nonanona	$C_9H_{18}O$	142.24	195	0.371	0.832

2.2. Hidrocarburos policíclicos aromáticos

Los hidrocarburos policíclicos aromáticos (PAHs) son compuestos formados por dos o más anillos aromáticos fusionados, con una baja volatilidad y poco solubles en agua, disminuyendo su solubilidad al aumentar el número de anillos aromáticos. Estos compuestos pueden unirse a partículas en suspensión, pudiendo encontrarse así en el aire o llegando a suelos, plantas, sedimentos y aguas medioambientales[13,14].

Estos contaminantes medioambientales se producen en la combustión incompleta de materiales orgánicos como el aceite, el petróleo y la madera. El origen puede ser antropogénico (calefacción residencial, producción de asfalto, refinerías, vehículos, etc.) o natural (incendios forestales, actividades volcánicas, etc.). Una vez formados, pueden entrar en el organismo humano mediante ingestión, por contacto con la piel o mediante inhalación, donde se metabolizan originando los correspondientes derivados[15].

El interés de la determinación de estos compuestos se debe a la clasificación de algunos PAHs como cancerígenos (clase 1), posibles cancerígenos (clase 2A) o probables cancerígenos (clase 2B) por la Agencia Internacional para la Investigación sobre el Cáncer (*International Agency for Research on Cancer*, IARC)[16]. Además, están contemplados en la lista de compuestos tóxicos de la Agencia de Protección Ambiental de Estados Unidos (*United States Environmental Protection Agency*, USEPA)[17].

El objetivo fundamental de la determinación de los PAHs en muestras biológicas es relacionar su concentración con una exposición previa a los mismos. Para ello, se han analizado diferentes matrices como orina[18], sangre[19], saliva[20], aire exhalado[21] y pelo[22], entre otras.

En esta Tesis se llevó a cabo la determinación de PAHs en orina y saliva (capítulo II). En la figura 2.2 se muestra la estructura química de los compuestos estudiados.

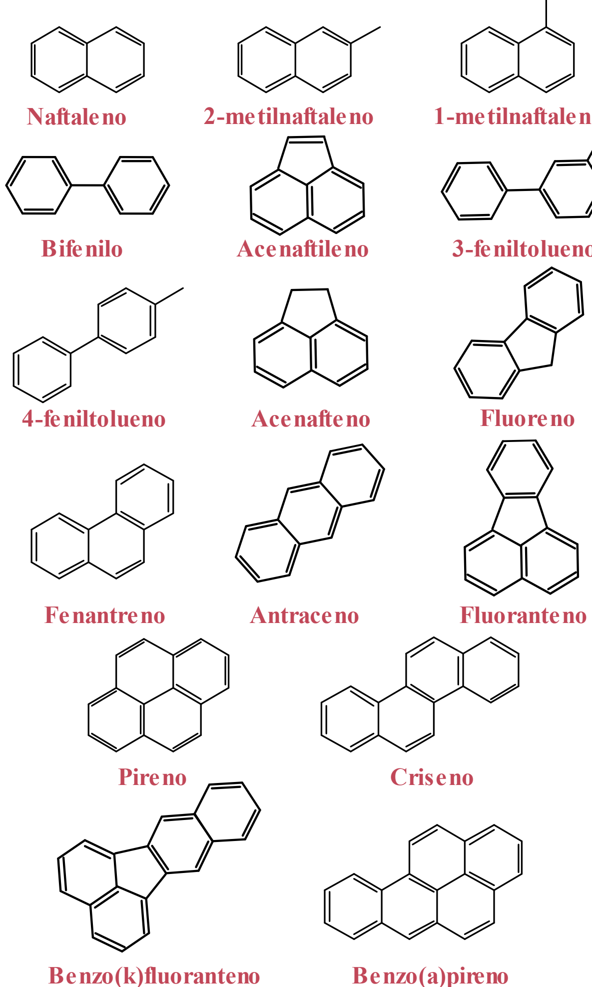

Figura 2.2. *Estructura química de los hidrocarburos policíclicos aromáticos determinados en orina y saliva en esta Tesis*

Además, en la tabla 2.2 se muestran algunas propiedades de estos compuestos[10-12].

Tabla 2.2. *Propiedades de los hidrocarburos policíclicos aromáticos determinados en orina y saliva*

Compuesto	Fórmula química	Peso molecular/ g mol^{-1}	$T_{ebullición}$/ °C	Solubilidad en agua/ mg mL^{-1}	Densidad/ g mL^{-1}
Naftaleno	$C_{10}H_8$	128.17	218	0.031	1.162
2-metilnaftaleno	$C_{11}H_{10}$	142.20	241	0.025	1.006
1-metilnaftaleno	$C_{11}H_{10}$	142.20	245	0.025	1.02
Bifenilo	$C_{12}H_{10}$	154.21	256	0.007	1.041
Acenaftileno	$C_{12}H_8$	152.19	280	0.004	0.899
3-feniltolueno	$C_{13}H_{12}$	168.23	273	0.013	1.018
4-feniltolueno	$C_{13}H_{12}$	168.23	268	0.004	1.015
Acenafteno	$C_{12}H_{10}$	154.21	279	0.004	1.200
Fluoreno	$C_{13}H_{10}$	166.22	295	0.002	1.202
Fenantreno	$C_{14}H_{10}$	178.23	340	0.001	1.179
Antraceno	$C_{14}H_{10}$	178.23	342	0.001	1.250

Tabla 2.2. *Propiedades de los hidrocarburos policíclicos aromáticos determinados en orina y saliva (continuación)*

Compuesto	Fórmula química	Peso molecular/ g mol^{-1}	$T_{ebullición}$/ °C	Solubilidad en agua/ mg mL^{-1}	Densidad/ g mL^{-1}
Fluoranteno	$C_{16}H_{10}$	202.25	384	0.0002	1.250
Pireno	$C_{16}H_{10}$	202.25	404	0.0001	1.270
Criseno	$C_{18}H_{12}$	228.30	448	0.002	1.300
Benzo(k)fluoranteno	$C_{20}H_{12}$	252.30	480	0.0008	1.286
Benzo(a)pireno	$C_{20}H_{12}$	252.30	496	0.002	1.351

2.3. Aminoácidos

Los aminoácidos son compuestos de gran importancia en el cuerpo humano, siendo las unidades estructurales básicas que componen las proteínas y, además, intermedios en diferentes procesos metabólicos. Por ello, su determinación es de gran interés ya que pueden aportar información acerca del estado de salud del individuo.

Estos compuestos orgánicos, que contienen un grupo amino y un grupo carboxilo, pueden clasificarse en dos grupos. Se denominan aminoácidos esenciales a aquellos que no pueden ser sintetizados por el organismo y proceden, por lo tanto, de los alimentos ingeridos. Dentro de estos se encuentran la valina, leucina, treonina, lisina, triptófano, histidina, isoleucina, fenilalanina y metionina. En cambio, aquellos aminoácidos que pueden ser sintetizados en el propio organismo se denominan aminoácidos no esenciales e incluyen la alanina, prolina, glicina, serina, cisteína, asparagina, glutamina, tirosina, ácido aspártico, ácido glutámico y arginina[23].

Son numerosos los trabajos descritos en bibliografía donde se relacionan las alteraciones en los niveles de aminoácidos con diferentes enfermedades. Entre ellas, se encuentran el cáncer[24], la fenilcetonuria[23,25], tirosinemia[23,25], desórdenes en el ciclo de la urea[23,25] y la homocistinuria[23], entre otras.

Entre las matrices biológicas utilizadas de forma más habitual para la determinación de aminoácidos se encuentran la orina[26,27], la sangre[28] y la saliva[27].

En esta Tesis se llevó a cabo la determinación de aminoácidos en muestras de saliva (capítulo III). En la figura 2.3 se muestra la estructura química de los aminoácidos estudiados.

Alanina Glicina Valina Leucina

Isoleucina Prolina Asparagina

Glutamina Serina Metionina

Fenilalanina Ornitina

Lisina Histidina

Tirosina Triptófano

Figura 2.3. *Estructura química de los aminoácidos determinados en saliva en esta Tesis*

Además, en la tabla 2.3 se muestran algunas propiedades de estos compuestos[10-12].

Tabla 2.3. *Propiedades de los aminoácidos determinados en saliva*

Compuesto	Fórmula química	Peso molecular/ g mol^{-1}	$T_{descomposición}$/ °C	Solubilidad en agua/ mg mL^{-1}	Densidad/ g mL^{-1}
Alanina	$C_3H_7NO_2$	89.09	297	166.5	1.40
Glicina	$C_2H_5NO_2$	75.07	236	225	1.161
Valina	$C_5H_{11}NO_2$	117.15	300	58.5	1.23
Leucina	$C_6H_{13}NO_2$	131.17	300	24	1.29
Isoleucina	$C_6H_{13}NO_2$	131.17	284	40	1.0
Prolina	$C_5H_9NO_2$	115.13	222	1500	1.38
Asparagina	$C_4H_8N_2O_3$	132.12	235	29.4	1.543
Glutamina	$C_5H_{10}N_2O_3$	146.14	186	41.3	1.364
Serina	$C_3H_7NO_3$	105.09	225	359.7	1.600
Metionina	$C_5H_{11}NO_2S$	149.21	273	34	1.34
Fenilalanina	$C_9H_{11}NO_2$	165.19	271	14.11	1.34

Tabla 2.3. *Propiedades de los aminoácidos determinados en saliva (continuación)*

Compuesto	Fórmula química	Peso molecular/ g mol^{-1}	$T_{descomposición}$/ °C	Solubilidad en agua/ mg mL^{-1}	Densidad/ g mL^{-1}
Ornitina	$C_5H_{12}N_2O_2$	132.16	215	620	1.2
Lisina	$C_6H_{14}N_2O_2$	146.18	215	1000	1.1
Histidina	$C_6H_9N_3O_2$	155.16	282	38.2	1.4
Tirosina	$C_9H_{11}NO_3$	181.19	298	0.38	1.46
Triptófano	$C_{11}H_{12}N_2O_2$	204.23	282	10	1.4

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3 Técnicas de pretratamiento de muestra

3.1. Generación de espacio de cabeza

La generación de espacio de cabeza (HS) es una técnica mediante la cual es posible separar los compuestos volátiles contenidos en una matriz[1]. Se denomina espacio de cabeza a la fase gaseosa formada que se encuentra en contacto con la muestra, que puede ser líquida o sólida y se encuentra contenida en un vial cerrado. Esta técnica de extracción se encuentra habitualmente acoplada a la GC, la cual es apropiada para el análisis de muestras gaseosas, aunque también es posible el acoplamiento con otras técnicas de separación[2].

Existen diversas estrategias para llevar a cabo la generación de espacio de cabeza. En la primera de ellas, los compuestos volátiles se vaporizan parcialmente desde la muestra a la fase gaseosa superior y vuelven de nuevo a la muestra. Tras un tiempo, el sistema alcanza un equilibrio en el que la concentración de los analitos volátiles en la fase gaseosa permanece constante. Tomando una alícuota de la fase gaseosa, es posible analizar los compuestos volátiles de la muestra sin interferencia de aquellos no volátiles que se encuentran en la matriz. En este procedimiento, ambas fases presentes en el vial de análisis se encuentran bajo condiciones estáticas, y la muestra se transfiere una vez que se ha alcanzado el equilibrio. Por ello, se denomina generación de espacio de cabeza estático (*static headspace sampling*, SHS)[2].

La modalidad SHS se ha utilizado también combinada con microextracción en fase líquida (*liquid-phase microextraction*, LPME)[3]. La técnica fue utilizada por primera vez en 2001 por Theis y colaboradores[4]. En ella, una gota de disolvente extractante situada en la aguja de una jeringa se suspende en el espacio de cabeza de la muestra que contiene los analitos, estableciéndose una distribución de estos entre la fase gaseosa y el disolvente, y evitando así las interferencias debidas a los componentes no volátiles presentes en la misma. Posteriormente, la fase orgánica se introduce en el instrumento analítico para su análisis.

Otra modalidad tradicional de HS es la generación de espacio de cabeza dinámico (*dynamic headspace sampling*, DHS), que es principalmente un método de extracción de gas y separación de los constituyentes volátiles de la matriz mediante el uso de un flujo continuo de un gas inerte sobre la muestra o mediante un burbujeo utilizando vidrio sinterizado con una alta densidad de poros. Esta técnica fue sugerida por primera vez por Swinnerton y colaboradores[5,6] en 1962. Posteriormente, Bellar y Lichtenberg[7] la desarrollaron y pasó a denominarse con el nombre de purga y trampa (*purge and trap*, P&T). Una vez que los volátiles se encuentran en el gas extractante, es necesaria una preconcentración posterior antes de introducirlos en la columna cromatográfica. Esta puede hacerse utilizando una trampa fría o un cartucho empaquetado que contiene un material adsorbente. Posteriormente, los analitos se transfieren a la columna mediante desorción térmica.

Una alternativa a estas propuestas es la microextracción en fase sólida (*solid-phase microextraction*, SPME), introducida por Belardi y colaboradores[8] en 1989. La extracción se lleva a cabo mediante la inmersión de una fibra cubierta con una fase estacionaria inmovilizada en un líquido o mediante el muestreo del espacio de cabeza localizado encima de un líquido o un sólido. En este último caso, la técnica se denomina HS-SPME. Una vez que los analitos quedan adsorbidos en la fibra, se transfieren a la columna cromatográfica mediante desorción térmica.

En la técnica clásica de SHS y en HS-SPME se analiza una alícuota del espacio de cabeza que se genera en un vial cerrado. A diferencia de estas, en la técnica de purga y trampa (P&T) se lleva a cabo una extracción exhaustiva de gas. Es posible describir una modalidad adicional de HS, denominada extracción de espacio de cabeza múltiple (*multiple headspace extraction*, MHE), en la cual se lleva a cabo una extracción exhaustiva de los analitos como una secuencia de medidas estáticas y no como un proceso continuo como el descrito en la técnica de purga y trampa (P&T)[9].

La sensibilidad alcanzada cuando se utiliza HS-GC depende de la capacidad de la columna cromatográfica y del detector utilizado[10].

El HS está constituido por una muestra gaseosa más o menos diluida. Antes de llevar a cabo la inyección de esta en el GC es necesario estudiar cómo introducir el mayor volumen posible de muestra para alcanzar la sensibilidad deseada, y que dicha introducción sea rápida para reducir el ancho de banda inicial del cromatograma. Algunas de las técnicas empleadas para solucionar este problema han sido descritas en el libro de B. Kolb[2]. Entre ellas, se encuentran las trampas criogénicas, en las cuales los compuestos volátiles se retienen a baja temperatura, y las trampas de adsorción, mediante las cuales los compuestos quedan retenidos al hacer pasar la muestra por un tubo de adsorción que puede contener diferentes rellenos.

Una alternativa a estas técnicas, que ha sido utilizada en esta Tesis Doctoral, es el uso de un inyector de temperatura programada (PTV). Cuando se utiliza este dispositivo, que podría ser comparable a la trampa por condensación criogénica, el ancho inicial de banda está determinado por el tiempo de transferencia de la muestra. Además, se requiere un aumento rápido de temperatura para asegurar la preconcentración de los compuestos volátiles en una banda estrecha en la columna. El PTV permite trabajar en diferentes modalidades de inyección, en las cuales es posible programar la temperatura. En esta Tesis Doctoral, el modo seleccionado para la inyección permite retener los analitos a una temperatura baja, eliminándose el disolvente, y posteriormente, transferirlos a la columna mediante una rampa rápida de calentamiento del inyector.

La generación de espacio de cabeza es una técnica de pretratamiento de muestra muy asentada y existen innumerables aplicaciones que proponen su utilización. Los campos de aplicación incluyen el análisis de muestras alimentarias[11,12], bebidas[13,14], medioambiente[15,16] y cosméticos y productos de cuidado personal[17], entre otros.

Esta técnica también ha sido ampliamente utilizada en el campo del bioanálisis debido a las ventajas que aporta frente a otras técnicas de pretratamiento de muestra cuando se analizan matrices tan complejas como la orina, la saliva o el plasma. Por un lado, la eliminación de los interferentes no volátiles de la matriz al analizar únicamente los compuestos volátiles, y por otro, la mínima manipulación de muestra, que en muchas ocasiones se reduce a la introducción de la muestra en un vial de análisis.

Algunos ejemplos de la aplicación de HS en muestras biológicas son la determinación de fluoruro[18] y de biomarcadores hidroxilados de benceno, tolueno, etilbenceno y xileno (*benzene, toluene, ethylbenzene, xylene*, BTEX)[19] en muestras de orina, la determinación de etanol y compuestos inhalantes[20] y de aldehídos en muestras de sangre[21], así como la determinación de tiocianato[22] y de disolventes[23] en muestras de saliva. En todos estos trabajos, la metodología empleada incluye una etapa de separación de los analitos previa a la detección. Asimismo, se han descrito aplicaciones sin esta etapa de separación, como aquellas en las que el análisis de orina y saliva se lleva a cabo utilizando un acoplamiento HS-PTV-MS[24,25].

En esta Tesis se propone una metodología que utiliza la generación de espacio de cabeza para la determinación de cetonas y acetato de etilo en muestras de orina (capítulo I.1).

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3.2. Extracción líquido-líquido

La extracción líquido-líquido (LLE) es una de las técnicas de pretratamiento de muestra más consolidadas en los laboratorios de análisis debido a la sencillez del procedimiento y al no ser necesario ningún equipamiento específico.

Esta técnica, conocida también como extracción con disolventes, consiste en llevar a cabo una separación de compuestos en base a su solubilidad relativa en dos líquidos inmiscibles diferentes. Los pasos que incluye este procedimiento son los siguientes. En primer lugar, se lleva a cabo la adición de un disolvente sobre la muestra que contiene los analitos que se quieren extraer y que ha de ser inmiscible con la misma. A continuación, se mezclan estos dos líquidos, proceso que puede mejorarse mediante agitación. En esta etapa los analitos permanecerán en la muestra original o pasarán al disolvente añadido en función de la diferencia de solubilidad. Finalmente, se obtienen dos fases, una de las cuales contendrá los analitos objeto de estudio, que quedarán separados de aquellos que no son solubles en la misma fase y que podrían interferir en el análisis[1].

Generalmente, esta técnica se ha aplicado a la extracción de compuestos orgánicos en agua o matrices acuosas, utilizando como disolvente de extracción un disolvente orgánico apolar, inmiscible con el agua. De esta manera, los compuestos no polares se extraen en la fase orgánica debido a su mayor afinidad por la misma.

A lo largo de los años, la técnica LLE ha sido ampliamente empleada en diversos campos. Algunas de las aplicaciones descritas en bibliografía se han centrado en el ámbito medioambiental, para la determinación de clorofenoles en aguas de desecho[2], alimentario, para la determinación de fungicidas en uvas y vinos[3], y biológico, para la determinación de fármacos en sangre[4].

A pesar de ser uno de los métodos de rutina más empleados, la versión clásica de LLE presenta diversos inconvenientes. El principal es debido al uso de disolventes no polares, ya que su eficacia para la extracción de compuestos orgánicos altamente polares es limitada. El uso de otros disolventes más polares, parcial o totalmente miscibles con la matriz acuosa, no es posible ya que no se consigue una separación de fases adecuada. Además, suelen utilizarse grandes volúmenes de dichos disolventes, muchos de ellos tóxicos, alcanzando así una sensibilidad insuficiente para el análisis de trazas[5]. También es necesario destacar que en ocasiones la extracción es tediosa, requiriendo tiempos elevados, así como una elevada manipulación de muestra. Con el fin de reducir estos inconvenientes, a partir de esta técnica han surgido otras miniaturizadas englobadas dentro de la microextracción en fase líquida (LPME), que tratan de reducir al mínimo el volumen de disolvente utilizado siendo así más compatibles con el medio ambiente.

Estas técnicas pueden dividirse principalmente en tres categorías. La primera que surgió fue la microextracción en gota (*single-drop microextraction*, SDME), en la cual una gota de disolvente orgánico (1-10 µL) se suspende en una jeringa que se introduce en el interior de una muestra líquida (inmersión directa) o se deja en la fase gaseosa (espacio de cabeza). Tras la extracción, la gota vuelve de nuevo al interior de la jeringa y se transfiere al instrumento de análisis[5]. Esta técnica es de gran uso en la actualidad a la vista de las recientes aplicaciones[6,7] y revisiones[8] descritas en bibliografía. La microextracción líquido-líquido dispersiva (*dispersive liquid-liquid microextraction*, DLLME) se concibe como un sistema ternario formado por la muestra acuosa que contiene los analitos, un disolvente de extracción insoluble en agua y un disolvente dispersor soluble en agua. La mezcla de los disolventes de extracción y dispersión se inyecta rápidamente en la muestra acuosa. Tras centrifugar, se recoge el disolvente de extracción, conteniendo ahora los analitos de estudio, para su posterior análisis[5,9]. Son numerosos los trabajos recientes en los que se utiliza esta técnica de

extracción[10-12], indicando así la utilidad de la misma. La tercera técnica surgida a partir de LLE es la denominada microextracción en fase líquida por fibra hueca (*hollow-fiber liquid-phase microextraction*, HF-LPME). En esta técnica, surgida para evitar la inestabilidad que puede existir en la gota de la técnica SDME, los analitos se extraen primero en una membrana líquida soportada que se encuentra en los poros de una fibra hueca hidrofóbica porosa. Posteriormente, se extraen en el disolvente correspondiente, que se encuentra en la cavidad central de la fibra[5]. Se destaca que en este sistema el disolvente de extracción no se encuentra en contacto directo con la matriz que contiene los analitos. En la actualidad, la validez de esta técnica para la determinación de una amplia variedad de analitos en diversas matrices queda demostrada en las recientes aplicaciones[13-15] y trabajos de revisión descritos[16,17].

Como se ha descrito previamente, una de las principales desventajas de la técnica LLE es la limitada eficacia que presenta en la extracción de compuestos polares. En este aspecto, la mejora de la técnica ha sido posible gracias a la adición de sales. El procedimiento consiste en añadir una elevada concentración de sales sobre la mezcla de agua con un disolvente orgánico miscible o parcialmente miscible. Debido a la solvatación de los iones con las moléculas de agua se consigue una separación de las fases inicialmente miscibles (acuosa y orgánica). De este modo, el disolvente orgánico forma una fase separada que contiene los analitos de interés[18-20]. Este fenómeno recibe el nombre de separación de fases inducida por sales, y la técnica de extracción basada en este principio se denomina extracción líquido-líquido asistida por sales (*salting-out assisted liquid-liquid extraction*, SALLE)[21].

Las aplicaciones en las que se ha utilizado esta técnica de extracción incluyen campos semejantes a los descritos en LLE, como la determinación de fenoles y clorofenoles en aguas medioambientales[22], conservantes y edulcorantes artificiales en zumos[23] y la determinación de estimulantes en meconio[24].

La técnica SALLE fue modificada posteriormente para adaptarla a las exigencias medioambientales y analíticas del momento[25]. A la nueva técnica se le asignó el nombre de QuEChERS, que hace referencia a las principales ventajas que ofrece: rápido (*quick*), sencillo (*easy*), barato (*cheap*), eficaz (*effective*), robusto (*rugged*) y seguro (*safe*). Esta técnica mantenía la simplicidad y eficacia del procedimiento original, utilizando una menor cantidad de muestra, de disolventes orgánicos, así como material básico de laboratorio.

A diferencia de la técnica SALLE, en QuEChERS se estudiaron combinaciones de sales para evitar la co-extracción de compuestos polares de la matriz y, posteriormente, se llevó a cabo un procedimiento simple de limpieza basado en una extracción en fase sólida dispersiva (*dispersive solid-phase extraction*, d-SPE)[25].

Originalmente la técnica se desarrolló con el fin de determinar residuos de pesticidas en alimentos[25]. Sin embargo, son muchas las aplicaciones actuales que utilizan esta técnica para extraer diferentes compuestos en diferentes matrices[26]. Algunos ejemplos descritos en bibliografía utilizan la técnica QuEChERS para la determinación de PAHs en suelos[27] y ftalatos en leche materna y orina[28].

En esta Tesis se ha utilizado la técnica LLE para el desarrollo de metodologías analíticas enfocadas a la determinación de hidrocarburos policíclicos aromáticos en muestras de orina (capítulo II.1.1) y saliva (capítulo II.2.1), y para la determinación de aminoácidos en saliva (capítulo III.1).

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3.3. Microextracción con sorbentes empaquetados

La microextracción con sorbentes empaquetados (MEPS) es una técnica de preparación de muestra desarrollada y patentada en 2004 por el grupo de Abdel-Rehim[1,2], basada en la miniaturización y automatización del proceso de extracción en fase sólida convencional (SPE).

Esta técnica se utiliza para la extracción de analitos que se encuentran en muestras líquidas. Para ello, se utiliza una jeringa en la cual se coloca una aguja que contiene un cartucho con una pequeña cantidad de sorbente (entre 0.5 y 4 mg). La extracción se consigue gracias a la diferente interacción de los analitos con la muestra líquida y el sorbente. De este modo, al aspirar las muestras a través de la jeringa, los analitos de interés atraviesan el cartucho y quedan retenidos en el sorbente. En la figura 3.1 se muestra un esquema de los componentes utilizados para la extracción con MEPS.

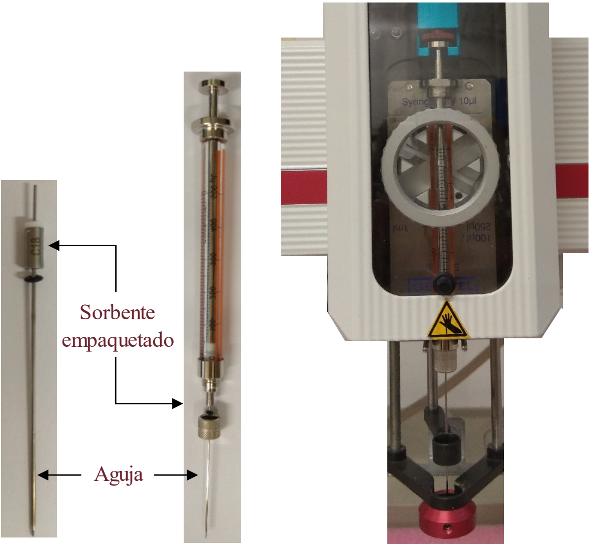

Figura 3.1. *Cartucho C18, jeringa de MEPS y disposición de la jeringa en el sistema de inyección automática*

Como se observa en la figura 3.1, para una correcta fijación de la aguja, esta se asegura a la jeringa con una tuerca de cierre. De esta manera, cuando el cartucho deja de funcionar correctamente o sea necesario utilizar un sorbente de diferente naturaleza, el cartucho puede sustituirse fácilmente.

Para llevar a cabo la extracción de los analitos con éxito es fundamental una adecuada elección del sorbente. Los materiales más utilizados son sílice, fases enlazadas a sílice (C2, C8 y C18) e intercambiadores fuertes de cationes y aniones. Así mismo, pueden utilizarse materiales de acceso restringido (*restricted access materials*, RAMs) o polímeros de impresión molecular (*molecularly imprinted polymers*, MIPs).

Inicialmente, el cartucho ha de ser acondicionado para activar el sorbente y asegurar una retención reproducible de los analitos. La extracción se consigue mediante una serie de aspiraciones y expulsiones consecutivas de la muestra líquida a través del cartucho, consiguiéndose la retención de los analitos en el sorbente empaquetado. A continuación, el cartucho se lava con agua o con una disolución de lavado adecuada para eliminar los posibles interferentes que puedan quedar retenidos, y se lleva a cabo una etapa de secado. Después, los analitos se eluyen haciendo pasar un pequeño volumen de disolvente o mezcla de disolventes orgánicos a través de la jeringa. Si el sistema está automatizado, el eluyente obtenido se inyecta directamente en un sistema de análisis. En caso contrario, si se utiliza un dispositivo manual, el eluyente se deposita en un vial de muestreo y solamente una parte se inyecta en el sistema para su posterior análisis. Por último, puede ser necesaria una etapa de lavado que elimine los analitos que no hayan sido desorbidos en su totalidad en el proceso de elución para evitar posibles contaminaciones entre muestras. En la figura 3.2 se muestra un esquema del proceso de extracción mediante MEPS.

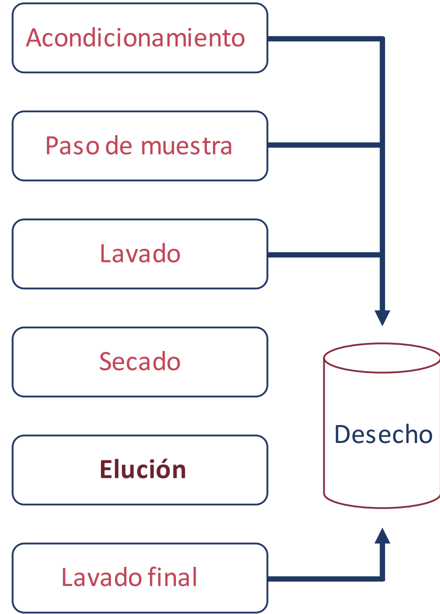

Figura 3.2. *Esquema del proceso de extracción mediante MEPS*

Dentro de la técnica de MEPS existen dos modalidades de trabajo que difieren en la etapa de extracción de muestra[3]. En la primera de ellas, conocida con el nombre de *extract-discard*, la muestra se pasa por el cartucho una vez y se desecha, repitiéndose este proceso con diferentes alícuotas hasta completar el volumen de muestra deseado. En la segunda, conocida con el nombre de *multiple draw-eject*, un volumen fijo de muestra se deposita en un vial y se realizan múltiples tomas y expulsiones de esta siempre del mismo vial. Esta segunda alternativa es de gran interés cuando se desea extraer la mayor cantidad posible de analitos de volúmenes muy pequeños, siendo muy útil su aplicación en el campo del bioanálisis[4,5].

En ambas modalidades, el proceso de MEPS puede realizarse de forma manual o automática. En el primer caso, el desplazamiento del émbolo es automático, pero las diferentes etapas del proceso de extracción se llevan a cabo

de forma manual. Es necesario destacar que en este modo de trabajo es posible llevar a cabo un muestreo *in situ*[6], procediendo a la elución de los analitos de manera posterior en el laboratorio. En la segunda modalidad, la jeringa de MEPS forma parte de un sistema de inyección automática (figura 3.1), por lo que el desplazamiento del émbolo en todas las etapas del proceso se lleva a cabo de manera automática. En este caso, todo el eluyente es inyectado en el sistema de análisis, por lo que es posible eluir los analitos en un volumen de disolvente muy pequeño. Los cartuchos utilizados en ambas modalidades son semejantes. Sin embargo, existen diferentes agujas en cuanto a grosor y forma de punta dependiendo de si el acoplamiento del MEPS automático se realiza a un cromatógrafo de gases o de líquidos.

La técnica de MEPS surge con la intención de agilizar y hacer más eficientes otras ya existentes, como SPE y SPME. El desarrollo de metodologías analíticas con un tratamiento de muestra rápido es fundamental, ya que en este se invierte la mayor parte del tiempo de análisis. Además, al reducir las etapas de preparación de muestra se reducen los errores asociados a las mismas.

Los principales aspectos mejorados con respecto a SPE hacen referencia a la reducción del volumen de muestra utilizado y del volumen de disolvente orgánico necesario para la elución de los analitos. En esta técnica mejorada, se destaca la posibilidad de automatizar el proceso cuando se acopla a un automuestreador. Además, la técnica es simple y más rápida que las técnicas convencionales SPE y LLE, pudiendo alcanzar la misma sensibilidad[7]. La reutilización de cartuchos de MEPS cuando se lleva a cabo un lavado adecuado del mismo mejora la técnica con respecto a SPE, donde es necesario el uso de cartuchos independientes para cada análisis.

La técnica SPME, de manera semejante a MEPS, puede utilizarse en combinación con metodologías basadas en cromatografía de gases o de líquidos (*liquid chromatography*, LC). Sin embargo, su habilidad para extraer analitos

poco concentrados utilizando bajos volúmenes de muestra es limitada. El muestreo utilizando la fibra de SPME es muy sensible dependiendo de la naturaleza de la muestra a analizar (sangre, orina, plasma). Sin embargo, como se ha indicado previamente, en MEPS es posible reutilizar el cartucho un número elevado de veces cuando este se lava adecuadamente[7].

Por todo ello, la técnica de MEPS es más ecológica y económica que muchos otros métodos de tratamiento de muestra y se encuentra dentro de las técnicas de preparación de muestra contempladas dentro de la Química Analítica "verde"[8].

En los últimos años, son muchas las aplicaciones en las que se ha utilizado esta técnica de tratamiento de muestra, como se describe en la revisión bibliográfica de Pereira y colaboradores[9]. La mayoría de las aplicaciones actuales se centran en el análisis de diferentes compuestos en matrices biológicas, en las que se ha utilizado MEPS para determinar ácidos fenilcarboxílicos[10] en suero, disruptores endocrinos en orina[4] y anestésicos locales en plasma y saliva[11], entre otros. En el ámbito medioambiental, se ha aplicado a la determinación de ésteres de ftalatos[12] y aminas aromáticas[13] en muestras de agua. Estos últimos compuestos, que pueden proceder de tintes azoicos, también se han analizado en textiles mediante una técnica basada en MEPS[14]. Algunos ejemplos del uso de la técnica en matrices alimentarias son la determinación de ocratoxina A[15] y derivados furánicos[16] en vinos, y de benzodiacepinas en tónica, spritz y zumo[17], entre otros.

A pesar de que la técnica se ha utilizado principalmente como tratamiento de muestra de manera previa a un análisis que incluía una etapa de separación cromatográfica, se han descrito aplicaciones en las que se ha eliminado dicha etapa mediante el acoplamiento directo de la técnica MEPS a un espectrómetro de masas[18,19].

En esta Tesis se ha puesto a punto una metodología basada en la técnica de MEPS para la determinación de hidrocarburos policíclicos aromáticos en muestras de saliva (capítulo II.2.2).

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3.4. Reacciones de derivatización

En ocasiones, es necesario el uso de reacciones de derivatización mediante las cuales el compuesto original se transforma en el correspondiente derivado.

La finalidad habitual de las reacciones de derivatización es conseguir una buena separación de los analitos en el sistema de análisis y mejorar la sensibilidad del método[1,2]. A veces, estas reacciones pueden también mejorar la estabilidad de los compuestos de interés y la capacidad de identificación de estos. En cromatografía de gases es habitual el uso de este tipo de reacciones para aumentar la volatilidad de los compuestos originales y para modificar su polaridad. Para ello, es de gran importancia seleccionar la reacción de derivatización adecuada en función de los analitos que se desea analizar y de la metodología que se va a utilizar para el análisis.

Las reacciones más habituales son aquellas en las cuales se modifica la estructura química del compuesto original[1]. Para llevar a cabo la reacción se añade un agente derivatizante adecuado que produce una alteración en uno o varios grupos funcionales. Las reacciones más simples incluyen el agente derivatizante, el analito de interés, el derivado deseado de ese analito y productos secundarios no deseados obtenidos en reacciones secundarias o fruto de la degradación térmica. Por ello, es necesario estudiar y seleccionar los parámetros adecuados de manera que la formación de productos secundarios sea mínima.

Algunas posibles alternativas a este tipo de derivatización son aquellas que implican el uso de reacciones fotoquímicas, convirtiendo el compuesto original en uno o más derivados con propiedades de detección mejoradas para sistemas con detección ultravioleta (*ultraviolet*, UV), de fluorescencia (*fluorescence*, F) o electroquímicos (*electrochemical*, EC), entre otros[1]. Estas reacciones normalmente tienen lugar en un proceso en línea después de la separación y son de gran interés en la cromatografía líquida.

En concreto, este apartado se centrará, fundamentalmente, en la reacción de derivatización que se ha utilizado en esta Tesis para la determinación de aminoácidos mediante cromatografía de gases.

La determinación de aminoácidos mediante GC ha sido de gran interés desde los años sesenta[3]. Estos compuestos contienen grupos funcionales polares y son inestables a temperaturas elevadas. Por ello, es necesario llevar a cabo una reacción de derivatización para obtener los correspondientes derivados, estables y volátiles, que puedan ser analizados mediante GC.

Entre las reacciones descritas para este análisis, la sililación ha sido una de las más utilizadas[4,5]. Sin embargo, no todos los derivados obtenidos (arginina y glutamato) son estables[4]. Otras desventajas son la necesidad de aplicar calor para que la reacción tenga lugar[6], y la sensibilidad de los reactivos y derivados a la humedad, lo que dificulta la determinación de estos compuestos en matrices biológicas[4,5]. Por ello, es necesario el uso de reacciones alternativas que permitan un análisis eficiente de estos compuestos.

Entre las alternativas utilizadas se encuentran la reacción de acilación con anhídrido de pentafluoropropilo e isopropanol[7], con anhídrido trifluoroacético e isopropanol[8] y con diferentes combinaciones de anhídrido trifluoroacético y heptafluorobutírico y el alcohol adecuado para la mezcla utilizada[9]. El inconveniente que se presenta cuando finaliza la derivatización en estas reacciones es la necesidad de eliminar el reactivo y, además, hacer un cambio de disolvente, lo que dificulta una posible automatización del proceso.

A principios de los años noventa, Hušek propuso una alternativa a las reacciones descritas previamente para una rápida derivatización de aminoácidos en un solo paso[10-12].

Hasta la fecha, la reacción entre los cloroformiatos de alquilo y los grupos amino para la obtención del correspondiente carbamato, mostrada en la figura 3.3, era ya muy conocida[13].

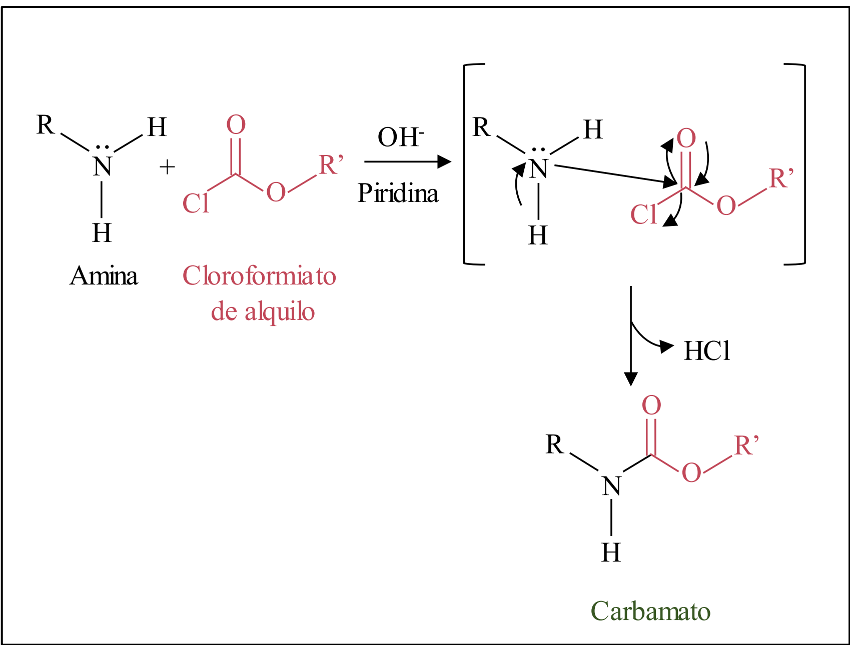

Figura 3.3. *Mecanismo de formación de un carbamato mediante la reacción entre una amina y un cloroformiato de alquilo*

Además, este autor había propuesto en un estudio previo la reacción de esterificación del grupo carboxilo de ácidos grasos con cloroformiatos de alquilo[14]. Esta reacción se llevó a cabo en medio acuoso en presencia de alcohol y de piridina como catalizador.

A partir de estos estudios previos, Hušek propuso por primera vez la reacción simultánea de ambos grupos funcionales[10-12]. Esta consistió en la obtención de los N(O,S)-alquil alcoxicarbonil ésteres de los aminoácidos

mediante el uso de cloroformiatos de alquilo en presencia de un alcohol y piridina como catalizador. Este método de derivatización ofrece muchas ventajas para la determinación de aminoácidos mediante GC, ya que se lleva a cabo en medio acuoso, a temperatura ambiente, en condiciones suaves y es prácticamente instantánea.

Inicialmente, Hušek propuso que el anhídrido mixto formado en la reacción debía descarboxilar (-CO_2) para producir el éster que contenía el grupo alquilo derivado del cloroformiato de alquilo. Sin embargo, el estudio posterior de Wang y colaboradores[15] en el cual se hicieron reaccionar los aminoácidos con diferentes combinaciones de cloroformiatos de alquilo y alcoholes, puso de manifiesto que el anhídrido mixto reaccionaba con el alcohol para formar el éster que contiene el grupo alquilo procedente de este alcohol. El mecanismo de la reacción se muestra en la figura 3.4.

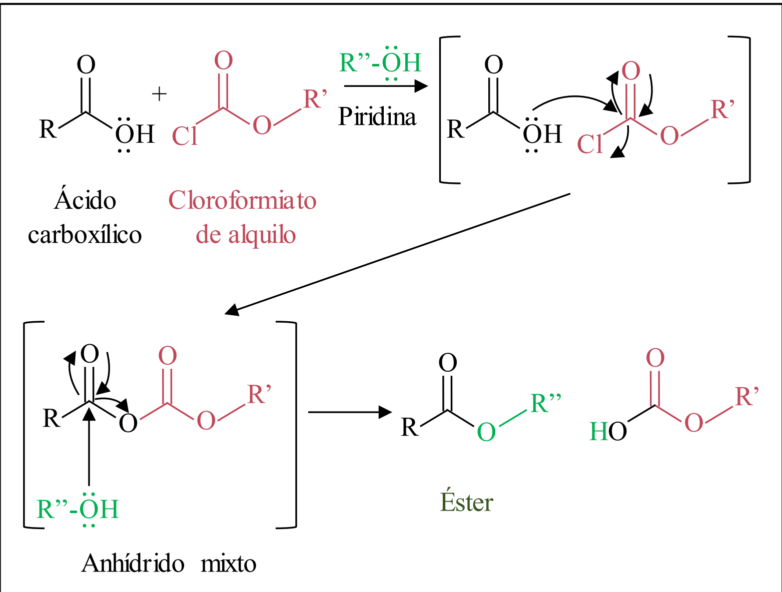

Figura 3.4. *Mecanismo de formación de un éster mediante la reacción entre un ácido carboxílico y un cloroformiato de alquilo*

Mediante la vía mostrada en la figura 3.5 (vía A) se obtiene el producto mayoritario de la reacción entre un grupo carboxilo y un cloroformiato de alquilo en presencia de alcohol y piridina. Sin embargo, también se observó la obtención de una pequeña cantidad del éster con un grupo alquilo similar al del cloroformiato de alquilo utilizado. Este producto minoritario podría proceder de dos posibles vías. En la primera, se produciría por la descarboxilación del anhídrido mixto (figura 3.5, vía B). En la segunda, se plantea que una pequeña cantidad de alcohol producido *in situ* por la hidrólisis del cloroformiato podría reaccionar con el anhídrido mixto (figura 3.5, vía C).

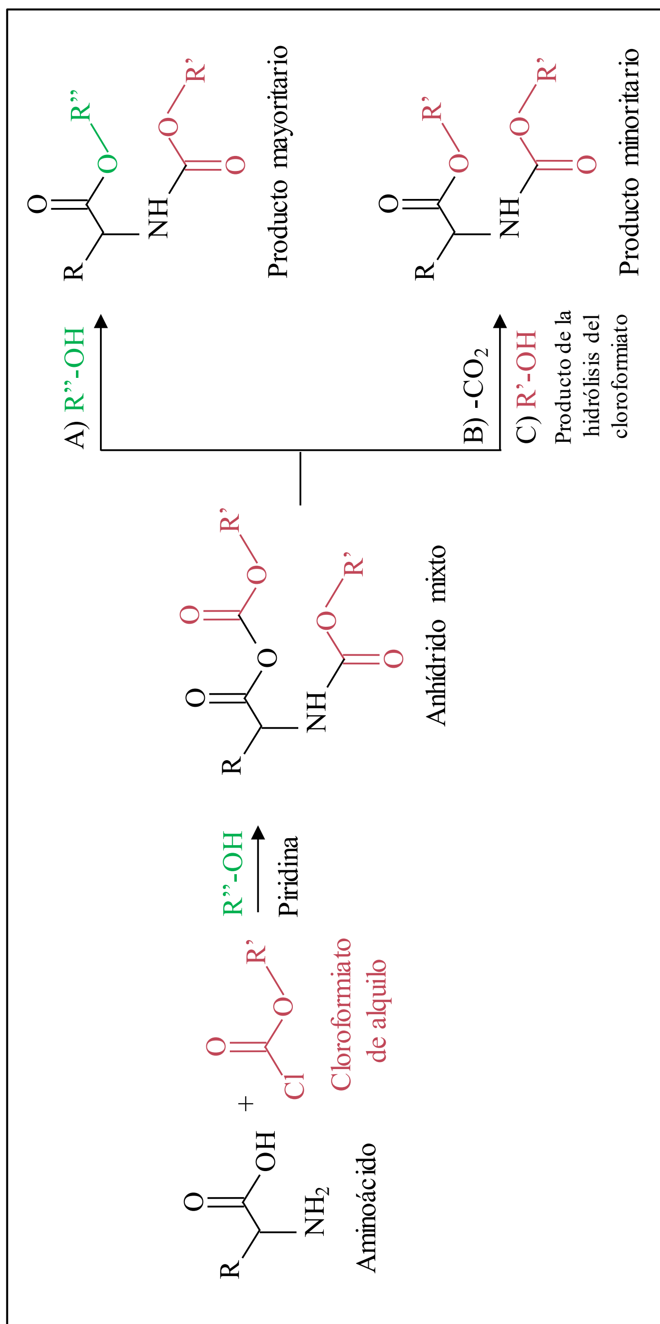

Figura 3.5. *Reacción de derivatización de los aminoácidos con cloroformiato de alquilo*

El inconveniente de obtener dos productos en la reacción se soluciona fácilmente utilizando el mismo grupo alquilo en el cloroformiato y en el alcohol, de modo que ambas vías proporcionen el mismo producto. Así, el cloroformiato de metilo iría acompañado de metanol, el cloroformiato de etilo de etanol, y así sucesivamente.

Debido a la simplicidad y rapidez de este procedimiento, y a la pronta caracterización de los patrones de fragmentación de los correspondientes derivados mediante espectrometría de masas[16], la reacción de aminoácidos con cloroformiatos de alquilo se extendió rápidamente como técnica de derivatización para determinar estos compuestos mediante GC[17,18]. Hasta la actualidad, son muchos los trabajos publicados en los que se han empleado diferentes cloroformiatos como el de metilo[19-21], el de etilo[21-23], propilo[24,25] e isobutilo[21,26,27] en matrices biológicas, medioambientales y alimentarias.

En esta Tesis, se ha puesto a punto una metodología para la determinación de aminoácidos en muestras de saliva (capítulo III.1) basada en la reacción de derivatización de los mismos con cloroformiato de etilo en presencia de etanol y piridina.

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4 Configuración instrumental

El desarrollo de las metodologías analíticas que componen esta Tesis Doctoral se ha llevado a cabo utilizando un automuestreador (Combi PAL o MPS2 MultiPurpose Sampler) con un módulo que permite la generación de espacio de cabeza (HS), la inyección de muestras líquidas y la microextracción con sorbentes empaquetados (MEPS). Este módulo está acoplado a un cromatógrafo de gases (GC) que dispone de un inyector de temperatura programada (PTV), y a un espectrómetro de masas cuadrupolar (qMS). En la figura 4.1 se muestra una imagen de la configuración instrumental utilizada en todas las metodologías analíticas desarrolladas.

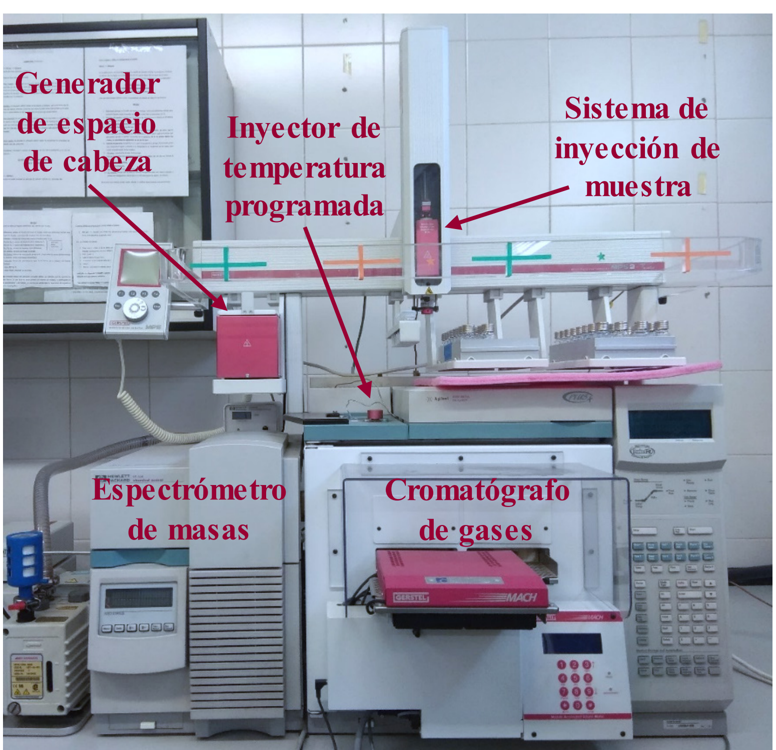

Figura 4.1. *Configuración instrumental MPS2-PTV-GC-MS*

4.1. Sistema de inyección de muestras

En esta Tesis se han utilizado dos sistemas de introducción de muestras que trabajan de manera semejante: el automuestreador Combi PAL (CTC Analytics AG, Suiza) y el automuestreador MPS2 MultiPurpose Sampler (Gerstel, Mülheim an der Ruhr, Alemania). Este tipo de automuestreadores hace posible la selección de diferentes modalidades de introducción de muestra, entre las que se encuentran la generación de espacio de cabeza (capítulo I.1), la inyección de muestras líquidas (capítulos II.1.1, II.2.1, III.1) y la microextracción con sorbentes empaquetados (capítulo II.2.2) utilizadas en esta Tesis. Estos dispositivos disponen de un brazo automuestreador automático sobre el cual pueden acoplarse diferentes módulos con la correspondiente jeringa en función del tipo de introducción de muestra que se utilice en cada caso. El tipo de bandeja utilizada para la colocación de los viales con la muestra también varía en función de la técnica empleada.

En el desarrollo de estos trabajos, la inyección de muestra se llevó a cabo utilizando una jeringa termostatada de 2.5 mL para la técnica de HS, una jeringa de 100 µL para la inyección de líquidos y una jeringa de 500 µL con un cartucho de C18 para la extracción con MEPS.

El horno donde se llevó a cabo la generación de espacio de cabeza dispone de seis posiciones para viales. Su temperatura puede programarse entre 35 y 200 ºC y permite una agitación orbital de las muestras en un rango de velocidades comprendidas entre 250 y 750 rpm.

4.2. Inyector de temperatura programada

La inyección de las muestras se llevó a cabo utilizando un PTV correspondiente al modelo CIS-4 (Gerstel, Baltimore, MD). En la figura 4.2 se muestra un esquema del dispositivo utilizado.

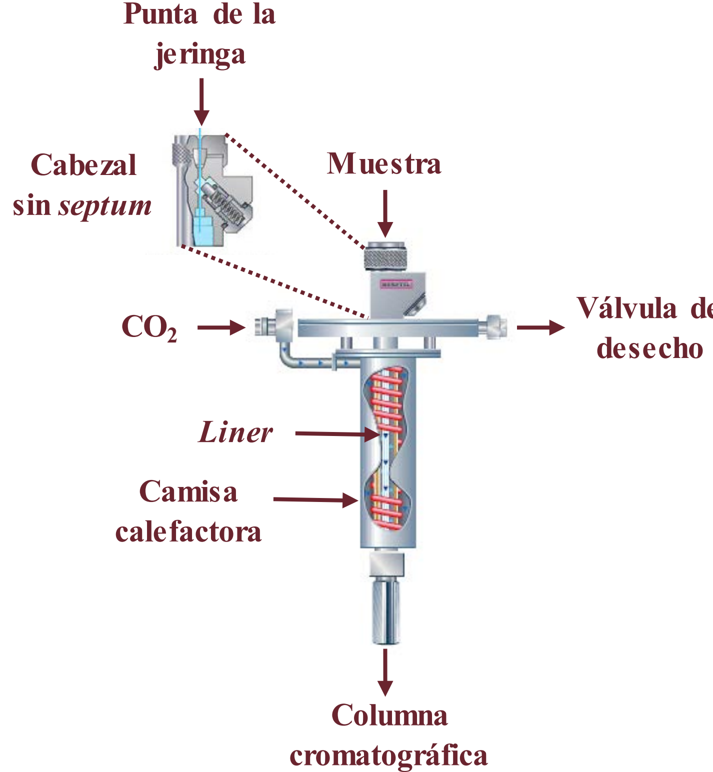

Figura 4.2. *Esquema de un inyector de temperatura programada*

El cabezal del inyector cuenta con una válvula de sellado que se desplaza en el momento de la inyección para que la jeringa entre en el interior del mismo. En todos los casos, la inyección se llevó a cabo en el modo de venteo de disolvente o *solvent vent*. El enfriamiento del sistema se consigue mediante el uso de CO_2 líquido, que permite alcanzar una temperatura de hasta –78 ºC. El calentamiento se consigue mediante el uso de una camisa calefactora que permite llevar a cabo un aumento lineal y homogéneo de temperatura en el cuerpo del inyector. Para ello, es posible seleccionar hasta dos rampas de temperatura consecutivas comprendidas entre 2 y 12 ºC s^{-1}. El inyector está equipado con una cámara de vaporización o *liner* (71 mm × 2 mm) que puede tener diferentes tipos de relleno.

En esta Tesis se utilizaron dos rellenos diferentes. El primero de ellos consistió en un relleno de Tenax-TA® (capítulos I.1, II.1.1, II.2.1, II.2.2), que se trata de un polímero poroso de óxido de 2,6-difenileno. Este tipo de relleno tiene baja afinidad por el agua y el metanol y se utiliza habitualmente en la retención y desorción térmica de compuestos comprendidos en el rango de C5-C20. El segundo relleno utilizado fue de lana de vidrio desactivada (capítulo III.1), cuyo empaquetado puede actuar también como filtro de partículas y se emplea para compuestos estables con puntos de ebullición moderados o altos.

4.3. Cromatógrafo de gases

En todas las metodologías desarrolladas se ha utilizado un cromatógrafo de gases Agilent 6890. Debido a la distinta naturaleza de los compuestos que se analizan y a las diferentes metodologías utilizadas, se han utilizado dos columnas cromatográficas y un tubo desactivado. La primera de ellas fue una columna capilar de baja polaridad DB-VRX (20 m × 0.18 mm × 1 µm, (-10-260) ºC, J&W Scientific, USA), utilizada en la determinación de VOCs en muestras de orina (capítulo I.1). La segunda, se trata de una columna HP-5MS UI (30 m × 0.25 mm × 0.25 µm, 60 ºC-325 ºC, J&W Scientific, USA), ultra inerte y de baja polaridad, especialmente diseñada para la determinación de compuestos ácidos, básicos o semivolátiles. Esta columna se ha utilizado para la determinación de PAHs en orina (capítulo II.1.1) y saliva (capítulos II.2.1, II.2.2), y de aminoácidos en saliva (capítulo III.1). Por último, debido a la baja volatilidad de los PAHs, estos compuestos se retenían en las columnas descritas anteriormente incluso cuando se trabajaba de forma isotérmica a las máximas temperaturas permitidas por las columnas. Por ello, fue necesario utilizar un tubo capilar Ultimate Plus Deactivated Fused Silica (30 m × 0.25 mm, límite 360 ºC, J&W Scientific, USA), de sílice desactivada, útil en el análisis de compuestos semivolátiles en fluidos corporales. En particular, en esta Tesis se utilizó para la determinación de PAHs

en muestras de orina (capítulo II.1.1). En la figura 4.3 se muestra una imagen con las columnas capilares utilizadas.

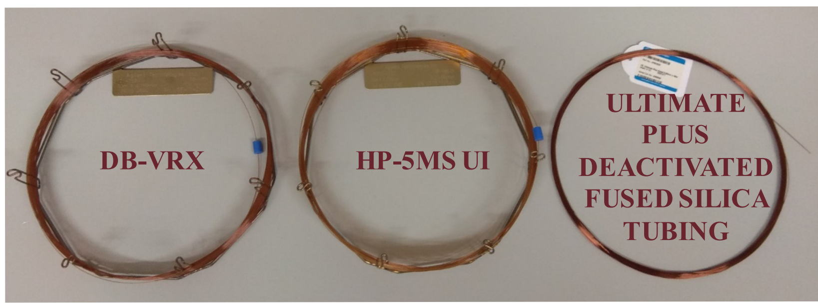

Figura 4.3. *Columnas capilares DB-VRX, HP-5MS UI y tubo capilar de sílice desactivada*

El cromatógrafo de gases utilizado permite establecer las siguientes rampas máximas: 70 ºC min^{-1} hasta 175 ºC, 45 ºC min^{-1} desde 175 hasta 300 ºC y 35 ºC min^{-1} desde 300 hasta 450 ºC. Se utilizó helio N60 (99.9999 %, Air Liquide) como gas portador.

4.4. Espectrómetro de masas

El detector utilizado es un espectrómetro de masas cuadrupolar HP 5973 N (Agilent Technologies, Alemania). Se utiliza una fuente de ionización electrónica con un voltaje de 70 eV. Las temperaturas de la fuente de ionización y el cuadrupolo son 230 y 150 ºC, respectivamente.

Este equipo permite seleccionar tres modos de adquisición de datos. En el primero de ellos, *scan*, se hace un barrido de un intervalo amplio de masas especificado de manera previa. En el segundo, modo de seguimiento de iones

seleccionados (*selected ion monitoring*, SIM), se seleccionan los iones característicos de los compuestos de estudio, registrándose un pequeño número de iones seleccionados en diferentes intervalos en el tiempo de análisis. Adicionalmente, es posible trabajar en el modo SIM/*scan* combinado, que permite la adquisición de datos en ambos modos en un único análisis.

La identificación de los compuestos se lleva a cabo utilizando la base de datos NIST'08 (NIST/EPA/NIH, Mass Spectral Library, versión 2.0).

5 Técnicas quimiométricas

La Quimiometría, según la Sociedad Internacional de Quimiometría (*International Chemometrics Society*, ICS), es la ciencia que se dedica a relacionar las medidas realizadas en un sistema o proceso químico con el estado del sistema mediante la aplicación de métodos matemáticos y estadísticos[1,2]. Estas técnicas, por tanto, se utilizan de manera habitual con el objetivo de diseñar procedimientos de medida y obtener la máxima información útil a partir del análisis de datos.

Dentro de esta disciplina se encuentran los métodos de diseño experimental, la calibración simple y multivariante, las técnicas de reconocimiento de pautas y los sistemas expertos e inteligencia artificial, entre otros.

El diseño experimental utilizado en la planificación de experimentos es una alternativa a la técnica clásica en la que se estudia cada variable de manera independiente. Este permite estudiar el efecto de todas las variables (y sus interacciones) en la respuesta analítica y modelar la relación entre ellas, obteniendo la mayor información útil posible utilizando el menor número de experimentos[2].

Los métodos de reconocimiento de pautas son herramientas utilizadas para clasificar o agrupar muestras según las características de las mismas. Así, todas aquellas muestras que se clasifican o agrupan en una misma clase se caracterizan por tener una o varias propiedades comunes a todas ellas[3,4]. Estos métodos pueden dividirse en técnicas no supervisadas y supervisadas.

Las técnicas no supervisadas determinan las relaciones entre las muestras sin utilizar información previa de a qué clase pertenece cada una de las muestras. El objetivo es establecer el número de posibles grupos e identificarlos. Así, permite tener una idea de la utilidad de la información contenida en los mismos. Entre ellas, podemos encontrar el análisis de agrupamientos, en particular, el agrupamiento jerárquico (*hierarchical cluster analysis*, HCA), mediante el cual

se agrupan las muestras según unos índices de similitud, y el análisis de componentes principales (PCA), que utiliza combinaciones lineales de las variables originales para reducir la dimensión del conjunto de datos de estudio.

En las técnicas supervisadas se describen las características propias que definen cada clase mediante el uso de un conjunto de muestras cuya pertenencia a una clase se conoce previamente, seleccionando las variables que la caracterizan. De este modo, se desarrollan reglas para la clasificación posterior de muestras desconocidas. El éxito en la predicción depende de que dichas reglas, así como los límites marcados por ellas, sean adecuados. La estrategia que se sigue cuando se utilizan técnicas supervisadas es la siguiente[5]:

- Inicialmente se genera un modelo matemático que relaciona los valores de las variables analizadas en el conjunto de muestras (objetos), denominado conjunto de entrenamiento o *training set*, con la clase a la que pertenecen.

- A continuación, se determina la capacidad predictiva del modelo, por ejemplo, en función del porcentaje de muestras clasificadas correctamente. Para ello, se pueden utilizar dos tipos de validación. En la validación cruzada o *cross validation* se genera un modelo en el que se excluye una muestra o un conjunto pequeño de ellas del *training set* y se predicen utilizando dicho modelo. Este procedimiento se repite hasta que todas las muestras se han predicho al menos una vez. La validación externa o *external validation* comprueba la capacidad de predicción del modelo utilizando un conjunto de muestras conocidas (*test set*) que no se han utilizado para construir dicho modelo. Estas muestras se utilizan, por tanto, como muestras desconocidas y posteriormente se compara el valor predicho por el modelo con el valor real.

- Por último, los diferentes modelos generados se utilizan para predecir muestras desconocidas.

Entre las técnicas de reconocimiento de pautas supervisadas se destacan el análisis discriminante lineal (LDA), el análisis discriminante con mínimos cuadrados parciales (PLS-DA), el modelado independiente de clases (SIMCA), la técnica de las muestras apoyadas en la frontera (SVM), la técnica de los k vecinos más próximos *(k-nearest neighbors algorithm*, KNN) y las redes neuronales artificiales (*artificial neuronal network*, ANN).

Dentro de la Quimiometría se engloban también los métodos de calibración. Mientras que en la calibración univariante se obtienen modelos con los que se predice una variable respuesta a partir de una variable correlacionada con la primera (regresión lineal simple), la calibración multivariante utiliza más de una variable predictora o respuesta instrumental. Esta última se utiliza generalmente cuando existe más de un compuesto cuya respuesta no se puede medir de manera individual y se basa fundamentalmente en concentrar toda la información de las variables medidas en un número menor de ellas sin perder información relevante. Dentro de la calibración multivariante se engloban la regresión por mínimos cuadrados parciales (PLS) y la regresión por componentes principales (*principal component regression,* PCR).

Las técnicas quimiométricas utilizadas en este trabajo son:

- Diseño experimental
- PCA
- SIMCA, LDA, PLS-DA y SVM
- PLS

A continuación, se describen brevemente los fundamentos de las diferentes técnicas quimiométricas utilizadas.

5.1. Diseño experimental

Al inicio de una nueva investigación es importante conocer qué variables o factores están involucradas en el proceso de trabajo, cuáles afectan de una manera significativa a la respuesta estudiada, cómo pueden optimizarse y qué intervalo de valores han de utilizarse.

Como se ha dicho previamente, la planificación de experimentos tradicional asume que el valor de una variable que optimiza la respuesta es independiente del resto de variables, sin tener en cuenta que pueda existir una interacción entre ellas. Por ello, puede no ser adecuada en ciertas ocasiones. Como alternativa, el diseño experimental estudia la influencia de las variables en la respuesta analítica ("efecto de una variable") y permite detectar las interacciones existentes entre las mismas. Además, se utiliza para modelar la relación entre las diferentes variables y la respuesta obteniendo la información útil del conjunto de variables analizadas utilizando el menor número posible de experimentos[2]. La calidad de los parámetros de estudio depende solo del diseño que se utiliza, y es óptima (menor varianza) cuando la matriz utilizada en el diseño es ortogonal[2], es decir, cuando las concentraciones de los analitos de interés no están correlacionadas.

El diseño experimental puede utilizarse como técnica de criba para la selección de variables[6] y optimización posterior de las mismas[7], así como para la calibración en situaciones complejas como la cuantificación de analitos en una mezcla[8].

5.1.1. Diseños de selección y optimización de variables

Para construir un diseño experimental, la primera etapa consiste en determinar las variables o factores que influyen en la respuesta analítica. Estas pueden ser de tipo cuantitativo o cualitativo. En ocasiones, se dispone de información previa acerca de los factores que producen un efecto significativo en

la respuesta analítica. En los casos en que esto no se cumple, es útil incluir en el diseño todos los posibles factores con el fin de realizar una selección de los mismos.

El siguiente paso es establecer los niveles que se deben estudiar para cada factor con el fin de no omitir posibles puntos de interés (intervalos pequeños) o estudiar niveles excesivos e inútiles (intervalos muy amplios).

Con el fin de reconocer los factores que afectan significativamente a la respuesta analítica es habitual el uso de diseños factoriales completos y fraccionados.

Los diseños factoriales completos permiten estudiar todas las combinaciones de variables y niveles seleccionados y se denotan con la expresión n^k, siendo n el número de niveles de cada variable y k, el número de variables estudiadas. El resultado de dicha expresión proporciona el número de experimentos del diseño. Los más habituales son aquellos donde cada factor se estudia a dos niveles diferentes. En la práctica, es raro que existan interacciones de tres o más variables que resulten significativas. En general, se obtiene información aceptable de la investigación objeto de estudio considerando los efectos de las variables y las interacciones de dos factores. Por esta razón, puede ser suficiente utilizar una parte del diseño completo, lo que se denomina diseño factorial fraccionado.

Los diseños factoriales fraccionados, a diferencia de los completos, realizan solo una parte del correspondiente diseño completo. Estos diseños se denotan como 2^{k-p}, siendo p el grado de fraccionamiento del diseño. Esta expresión, de nuevo, indica el número de experimentos que se deben realizar.

Una vez seleccionadas aquellas variables con un efecto significativo sobre la respuesta es necesario establecer las condiciones que proporcionan una

mejor respuesta analítica. En este caso, los diseños se llevan a cabo utilizando tres o más niveles para cada factor con el fin de poder detectar curvaturas.

Existen dos estrategias para optimizar las variables de estudio: secuencial y simultánea. En la primera, se lleva a cabo un número muy reducido de experiencias y permite seleccionar los siguientes experimentos, repitiendo el proceso hasta encontrar las condiciones más favorables. Un ejemplo de la misma es la optimización mediante el método "simplex"[9]. En la segunda estrategia se fija previamente el número de experimentos que se van a realizar. Algunos ejemplos son los diseños de Plackett-Burman[10-12] y los diseños compuestos centrales[10,12].

Una alternativa a los diseños factoriales que se utiliza para la optimización de variables es el diseño Box-Behnken[12], empleado cuando la respuesta óptima no se encuentra en los extremos de la región experimental utilizada y no se dispone de información previa. El número mínimo de variables que se estudian con este tipo de diseño es tres. Estas han de ser continuas y se estudian a tres niveles diferentes que se sitúan en la parte inferior, central o superior de un cubo, localizados en todos los puntos medios de los lados y en el centro de dicho cubo. El diseño está constituido por experimentos donde se combinan los niveles extremos de dos o tres variables con el nivel medio de las restantes e incluye, además, un número determinado de muestras centrales.

En la figura 5.1 se muestran los valores que toman las variables de los 13 experimentos que forman un diseño Box-Behnken para tres factores.

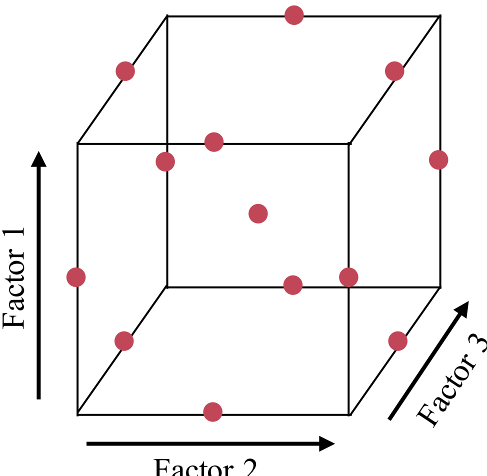

Figura 5.1. *Diseño Box-Behnken*

En este diseño, todos los puntos experimentales diferentes al central se encuentran situados a la misma distancia del centro del cubo (localizados en una esfera) de manera que todas las muestras influyen de manera similar en los parámetros que se calculan a partir del mismo.

5.1.2. Diseños para calibración

Cuando se aborda la cuantificación de compuestos en una mezcla es necesario generar un conjunto de muestras de calibración adecuado para resolver el problema. Considerando, por ejemplo, una mezcla de dos compuestos (1 y 2), pueden darse dos situaciones diferentes como se observa en la figura 5.2.

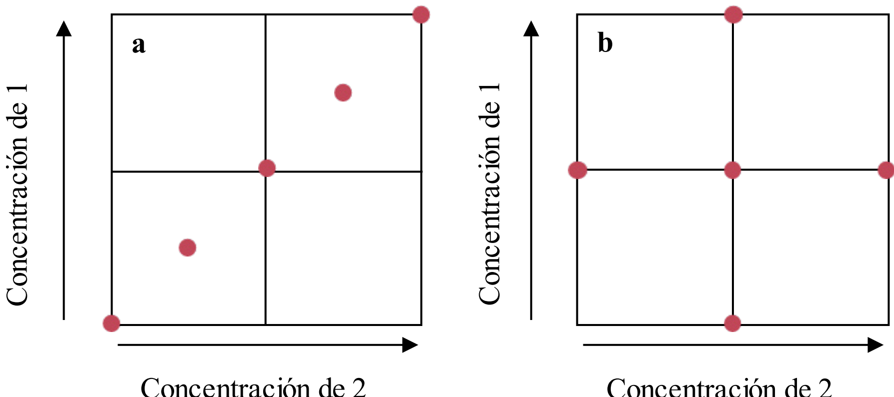

Figura 5.2. *Diseño experimental con concentraciones correlacionadas (a) y no correlacionadas (b)*

En el primer caso (5.2.a) las concentraciones de ambos compuestos se encuentran correlacionadas de manera que una variación en la señal analítica no permite determinar si la misma es debida a uno u otro compuesto. Este tipo de diseño solo es válido si las futuras muestras de predicción se encuentran correlacionadas del mismo modo.

El segundo diseño (5.2.b) es adecuado en situaciones donde se desconoce la naturaleza de las muestras que se van a predecir, que es la situación habitual. En este caso, el diseño se genera con concentraciones no correlacionadas y ortogonales entre sí.

A continuación, se describen los pasos que se deben seguir en una de las posibles estrategias para la construcción de un diseño experimental de calibración a partir de diseños Plackett-Burman, donde se tienen mezclas de multicomponentes[5,13-15] con varios niveles de concentración:

1. Determinar el número de compuestos de estudio, k.

2. Establecer el rango de concentración (mínimo y máximo) para cada compuesto.
3. Seleccionar el número de niveles de concentración, h, que se utilizan para cada compuesto. Para asegurar la ortogonalidad del diseño, h ha de ser un número primo o potencia de un número primo.
4. Determinar el número de mezclas, n, requeridas por el diseño ($n=h^2$).

Bajo estas condiciones, el número máximo de compuestos con concentraciones no correlacionadas que se pueden estudiar para diseños con 3, 4 y 5 niveles de concentración son 4, 5 y 12, respectivamente.

El conjunto de experimentos de calibración que se van a utilizar se diseña codificando los diferentes niveles de concentración utilizados y mediante el uso de un permutador cíclico[14,15] y un vector diferencia[14,15] adecuados que relacionen dichos niveles entre sí. El diseño obtenido proporciona las concentraciones que se deben utilizar en cada experimento y está formado por tantas columnas como número de compuestos se estudien y tantas filas como experimentos se realicen. Las concentraciones de cada columna son ortogonales con todas las demás. A modo de ejemplo, en la tabla 5.1 se muestra el desarrollo de un diseño experimental para 6 compuestos y 5 niveles.

Tabla 5.1. *Diseño experimental ortogonal para 6 compuestos y 5 niveles*

	Experimento	Compuesto 1	2	3	4	5	6
	1	0	0	0	0	0	0
Nivel rep.	2	0	-2	-2	2	-1	2
Bloque 1	3	-2	-2	2	-1	2	0
	4	-2	2	-1	2	0	-1
	5	2	-1	2	0	-1	-1
	6	-1	2	0	-1	-1	1
	7	2	0	-1	-1	1	2
Nivel rep.	8	0	-1	-1	1	2	1
Bloque 2	9	-1	-1	1	2	1	0
	10	-1	1	2	1	0	2
	11	1	2	1	0	2	2
	12	2	1	0	2	2	-2
	13	1	0	2	2	-2	1
Nivel rep.	14	0	2	2	-2	1	-2
Bloque 3	15	2	2	-2	1	-2	0
	16	2	-2	1	-2	0	1
	17	-2	1	-2	0	1	1
	18	1	-2	0	1	1	-1
	19	-2	0	1	1	-1	-2
Nivel rep.	20	0	1	1	-1	-2	-1
Bloque 4	21	1	1	-1	-2	-1	0
	22	1	-1	-2	-1	0	-2
	23	-1	-2	-1	0	-2	-2
	24	-2	-1	0	-2	-2	2
	25	-1	0	-2	-2	2	-1

En la figura 5.3 se representa la distribución de niveles de concentración para los compuestos 1 y 2 de una mezcla con 5 niveles de concentración. El número de experimentos que hay que realizar es 25.

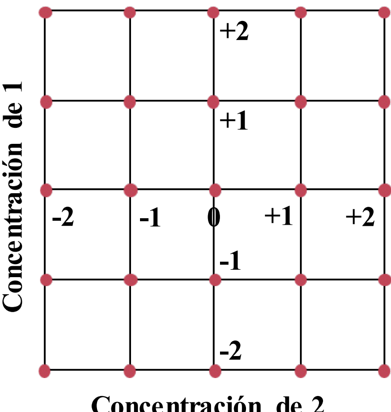

Figura 5.3. *Distribución de los niveles de concentración de los compuestos 1 y 2 en los 25 experimentos del diseño experimental*

Las correlaciones de cualquier diseño experimental pueden comprobarse determinando los coeficientes de correlación entre las concentraciones de los diferentes compuestos. En el ejemplo de la figura, el valor es siempre cero.

5.2. Técnicas de reconocimiento de pautas

5.2.1. Análisis de componentes principales

El análisis de componentes principales (PCA) es una técnica de reconocimiento de pautas no supervisada.

En Química, a menudo es necesario trabajar con tablas extensas que contienen mucha información que puede estar "parcialmente oculta o latente" debido a que los datos son demasiado complejos para que se interpreten de manera sencilla. El PCA es un método de proyección que ayuda a visualizar la información contenida en una tabla de datos[16].

Esta técnica permite conocer las diferencias entre las muestras, las variables que contribuyen a estas diferencias y si esas variables contribuyen de manera semejante (correlación) o de manera independiente unas de otras. Además, permite detectar patrones de muestras y agrupaciones particulares, y cuantificar la cantidad de información útil contenida en los datos[16].

El análisis mediante PCA trata de reducir la dimensión del conjunto de datos original construyendo unas variables nuevas, que son ortogonales entre sí (componentes principales, *principal components*, PCs) y que son combinaciones lineales de las variables originales. La matriz de datos original **X** está formada por **I** objetos o muestras y **J** variables (dimensión **I**×**J**). En el análisis, mostrado en la figura 5.4, esta matriz se descompone en el producto de dos matrices **T** y **P** (**T**×**P**), que contienen la información relacionada con las muestras y variables, respectivamente. Además, en la descomposición se obtiene también una matriz residual **E**.

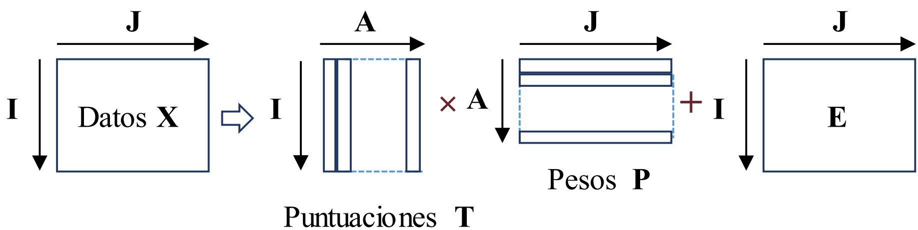

Figura 5.4. *Análisis de componentes principales (PCA)*

La matriz **T** contiene las puntuaciones (*scores*), valores de cada muestra en las variables nuevas, de I objetos en A componentes principales, que describen las propiedades de las muestras. La matriz **P** contiene los pesos (*loadings*), coeficientes en las variables originales para generar las nuevas variables, de J variables sobre A componentes principales, que describen las relaciones entre variables. Estas matrices consisten en una serie de vectores columna y fila,

respectivamente, según se observa en la figura 5.4, y están formadas por tantos vectores como PCs puedan calcularse. El número máximo de PCs viene limitado por la matriz de datos originales **I×J**, siendo el menor de ambos valores el limitante[17]. Existe un tercer atributo que caracteriza los PCs, denominado varianza, que es la medida del error que indica cuánta información se tiene en cuenta en cada PC. Esta puede darse en forma de varianza residual, indicando cuánta variación en los datos queda aún por explicar cuando un PC ya se ha tenido en cuenta, o como varianza explicada, a menudo medida como porcentaje total de varianza en los datos, que mide la proporción de variación considerada por el PC que se está estudiando[12].

El objetivo principal es que el número de PCs seleccionado explique la parte determinante de la matriz de datos con residuales lo más pequeños posibles[18]. Cuando se analiza una mezcla de compuestos[5] el número de PCs debería coincidir con el de compuestos existentes. Sin embargo, debido al ruido y otros factores, este supuesto no siempre se cumple. Los primeros PCs son los más importantes, ya que el primero explica el mayor porcentaje de varianza de los datos; el segundo, el mayor porcentaje de la varianza restante y así sucesivamente. Seleccionando el número de PCs se selecciona la información importante y se separa de aquella relacionada con el ruido.

Es posible visualizar esta técnica de una manera más clara mediante una interpretación gráfico-geométrica. En esta visualización un objeto o muestra se define como un punto en el hiperespacio constituido por J variables. Con esta técnica se intenta reducir la dimensión construyendo variables nuevas (ejes) y creándose un nuevo espacio en el que las muestras quedan situadas de forma similar a la anterior. De manera semejante a la varianza, cada variable explica la máxima información posible. La nueva matriz de puntuaciones está constituida por las proyecciones de cada objeto en los nuevos ejes o componentes principales. La representación de las puntuaciones de los objetos sobre los PCs permite reconocer los agrupamientos de las muestras. Esto se muestra en la figura 5.5.

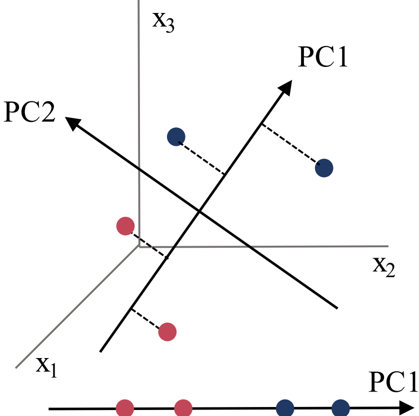

Figura 5.5. *Representación de las puntuaciones de dos clases de objetos en los dos primeros componentes principales*

5.2.2. Modelado independiente de clases

El modelado independiente de clases (SIMCA) es una de las técnicas de reconocimiento de pautas supervisadas más utilizadas[2].

Esta técnica utiliza PCA para construir un modelo independiente para cada clase cuando se aplica a un determinado grupo de muestras de entrenamiento. El número de PCs utilizados en cada una puede fijarse si se tiene información previa o puede establecerse en aquel valor que explique un porcentaje determinado de la varianza de los datos. Antes de usar los modelos para predecir nuevas muestras es necesario evaluar su especificidad, por ejemplo, para saber si existe solapamiento entre las clases o están suficientemente distantes[12,19].

En la figura 5.6 se representan dos clases de objetos, A y B, con los correspondientes modelos obtenidos al aplicar PCA y las regiones de confianza alrededor de cada clase. El modelo obtenido para la clase A (puntos rosas) consta

de dos PCs (un plano), mientras que el obtenido para la clase B (puntos azules) consta de un único PC (una recta).

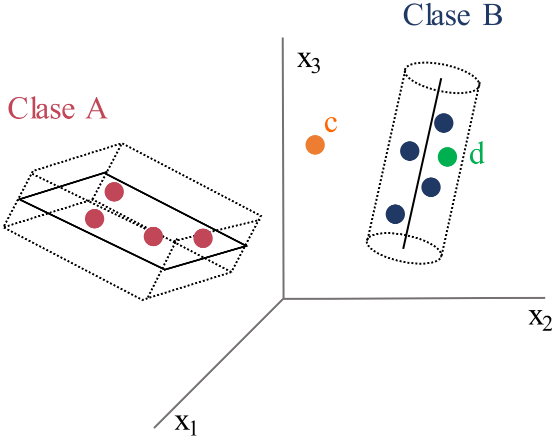

Figura 5.6. *Modelos de dos clases de objetos A y B en el espacio de sus componentes principales, regiones de confianza y predicción de dos muestras*

Una vez que se han creado los modelos, las muestras desconocidas que se desean clasificar se comparan con los modelos de cada clase y se asignan de acuerdo con su proximidad a las muestras del conjunto de entrenamiento. A diferencia de otras técnicas en que cada muestra se asigna a única una clase, en SIMCA, además, es posible que el resultado de una clasificación sea la no pertenencia a ninguna clase o la pertenencia a más de una. En la figura 5.6 el objeto d (punto verde) sería asignado a la clase B mientras que el c (punto naranja) no pertenecería a ninguna de las clases, por lo que se consideraría una muestra extraña o *outlier*.

La sensibilidad de una clase y su modelo se define como el porcentaje de muestras que perteneciendo a esa clase son clasificadas correctamente por el modelo matemático. La especificidad es el porcentaje de muestras que perteneciendo a otra clase se clasifican como muestras ajenas al modelo.

Estos índices se visualizan utilizando los diagramas de *Coomans*, que son representaciones bidimensionales de las distancias de las muestras a los modelos donde se incluyen los límites para ambas clases. De este modo, es posible ver si una muestra pertenece a una de las clases, a ambas o a ninguna. En la figura 5.7 se muestra un ejemplo de este tipo de diagrama.

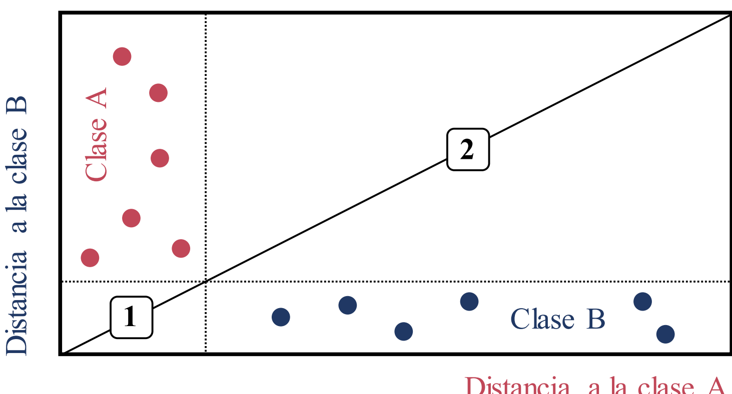

Figura 5.7. *Diagrama de Coomans*

Como puede observarse en el diagrama, la distancia a la clase A y a la clase B se representan en los ejes de abscisas y ordenadas, respectivamente. Las muestras pertenecientes a alguna de estas clases se clasifican en el espacio correspondiente a dicha clase. La parte inferior izquierda (1) corresponde a aquellas muestras clasificadas en ambas clases. La especificidad del modelo aumenta cuanto menor es el número de muestras clasificadas en este espacio. La parte superior derecha (2) corresponde a las muestras ajenas a los modelos, consideradas como *outliers*.

Si la técnica se utiliza solamente para clasificar las muestras desconocidas de acuerdo con ambas clases, las que se encuentren por encima de la diagonal se asignarán a la clase A y aquellas por debajo a la clase B.

5.2.3. Análisis discriminante lineal

El análisis discriminante lineal (LDA) es una técnica de reconocimiento de pautas supervisada[2].

Mediante esta técnica de clasificación se trata de buscar una función discriminante lineal que sea una combinación lineal de las variables originales. Los coeficientes de la nueva función se eligen de modo que esta refleje la diferencia entre las clases tanto como sea posible. Así, se busca que la varianza sea máxima entre diferentes clases y mínima dentro de cada clase. Esta transformación permite una reducción de la matriz de datos original, preservando la máxima información discriminatoria posible, para obtener un espacio con una dimensión inferior[9].

El objetivo de la técnica es determinar los mejores parámetros de ajuste para la clasificación de muestras con el modelo desarrollado[12]. El modelo se obtiene siguiendo tres pasos. En primer lugar, se determina la separabilidad entre las diferentes clases, por ejemplo, teniendo en cuenta la distancia entre las medias de las diferentes clases, denominada varianza entre clases. El segundo paso consiste en calcular la distancia entre la media y cada una de las muestras que pertenecen a esa clase, denominada varianza dentro de una clase. Por último, se construye el espacio de dimensión inferior utilizando un algoritmo que busca funciones discriminantes lineales (combinaciones lineales) de las variables originales que maximicen la varianza entre clases y minimice la varianza dentro de la clase[20,21]. De este modo, las muestras que pertenecen a la misma clase se encuentran juntas mientras que las diferentes clases se encuentran separadas entre sí.

En la figura 5.8 se representa un ejemplo gráfico cuando se tiene un conjunto de muestras que pertenecen a dos clases diferentes.

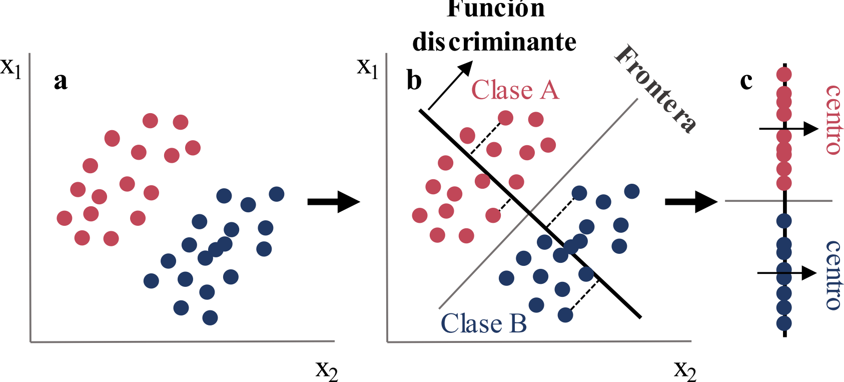

Figura 5.8. *(a) Dos clases de muestras definidas por las variables x_1 y x_2. (b) Frontera que separa ambas clases. (c) Proyecciones de las muestras en la función discriminante y cálculo de los centros de cada clase*

Como se observa en la figura, las muestras (u objetos) representadas en rosa podrían considerarse diferentes a las representadas en azul. Sin embargo, ninguna de las variables originales, x_1 y x_2, es capaz de discriminar por separado las diferentes muestras. En esta figura es posible dibujar una frontera entre ambos grupos de manera que las muestras quedan divididas en dos conjuntos: por encima de la línea, aquellas que pertenecen a la clase A y por debajo, aquellas que pertenecen a la clase B. De manera gráfica, esto se representa mediante las proyecciones de las muestras sobre la función discriminante, como se observa en las figuras 5.8.b y 5.8.c. De este modo, es posible calcular de una manera sencilla el centro de cada clase y la distancia de muestras desconocidas al mismo, asignándose dichas muestras al grupo más cercano[17]. Es necesario tener en cuenta que con esta técnica, a diferencia de SIMCA, aquellas muestras con características de ambas clases o incluso aquellas que no pertenezcan a ninguna serán igualmente asignadas exclusivamente a una de ellas[22].

Cuando el conjunto de datos contiene más variables que muestras en el grupo de entrenamiento con el que se obtiene el modelo es necesario realizar previamente un análisis mediante PCA para reducir la dimensionalidad de los datos (PCA-LDA). A diferencia de SIMCA, donde se realizaba un PCA para cada clase, esta técnica utiliza un espacio de proyección común para todas las clases del grupo de entrenamiento.

El análisis discriminante lineal se utiliza cuando la variabilidad dentro de los grupos tiene la misma estructura, es decir, si las matrices de covarianza de todas las clases son similares. Sin embargo, en algunos casos esta afirmación no se cumple y es posible utilizar diferentes alternativas, como el análisis discriminante cuadrático o el uso del cálculo de distancias diferentes a la euclídea, como la de Mahalanobis, para un mejor modelado de las clases.

5.2.4. Análisis discriminante-Mínimos cuadrados parciales

El análisis discriminante con mínimos cuadrados parciales (PLS-DA) es una técnica de reconocimiento de pautas supervisada que se lleva a cabo con el objetivo de conseguir una separación máxima entre clases[23]. PLS-DA combina la reducción de dimensionalidad utilizando PLS y el análisis discriminante en un único algoritmo, y es especialmente aplicable al modelado de un conjunto de datos con una gran dimensión en la que se tienen más variables que muestras[24].

Esta técnica consiste en una regresión clásica PLS donde la respuesta variable es de tipo categórico. Esta variable permitirá identificar las clases del conjunto de datos y tendrá tantos niveles como clases existen. En un conjunto de muestras con dos clases se asignan, por ejemplo, los valores +1 (clase A) y -1 (clase B), expresando así la pertenencia a las mismas[24]. Con estas nuevas variables se construyen tantos factores PLS (nuevos ejes) como sean necesarios en el conjunto de muestras de entrenamiento para obtener el modelo.

A modo de ejemplo, en la figura 5.9 se representa el modelo obtenido (5.9.a) para un conjunto de datos con dos clases de muestras.

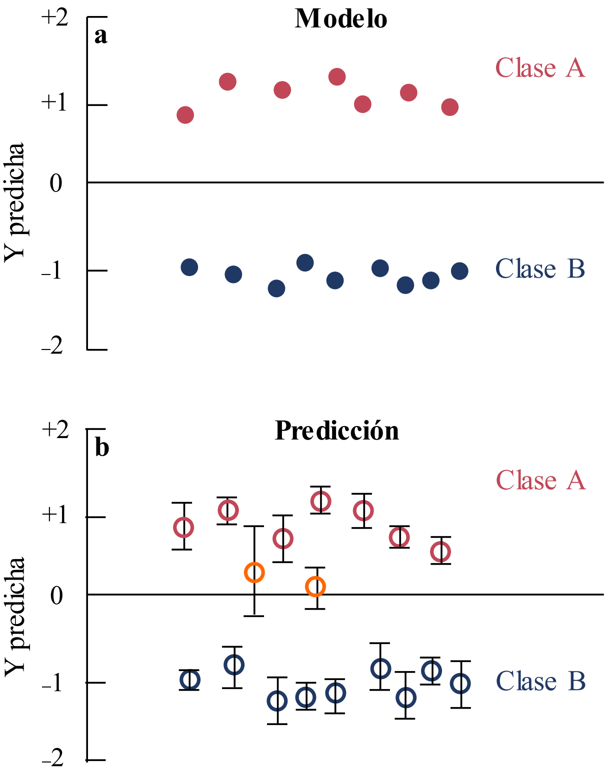

Figura 5.9. *(a) Modelo PLS-DA y (b) predicción de un conjunto de muestras pertenecientes a dos clases*

En este modelo, las muestras pertenecientes a la clase A (puntos rosas) tienen un valor teórico asignado +1 mientras que a las muestras de la clase B (puntos azules) se les asigna un valor teórico de -1. En la figura 5.9.a se observa el valor predicho por el modelo para estas muestras.

Los factores PLS se construyen tratando de encontrar un compromiso adecuado entre la descripción del conjunto de variables explicativas y la predicción de muestras[23].

Posteriormente, se lleva a cabo la predicción de la clase para las muestras desconocidas utilizando el modelo previamente generado. Para una perfecta pertenencia a las clases, los valores de predicción deberían ser equivalentes a los que se han fijado inicialmente (+1 y −1). Sin embargo, en la práctica los valores de predicción obtenidos tanto en el conjunto de muestras de entrenamiento como en el de predicción difieren de esos valores teóricos. Por ello, es necesario establecer en cada caso unas reglas de decisión que permitan determinar a qué clase pertenece la muestra de acuerdo con su valor de predicción.

En la figura 5.9.b se muestra un ejemplo de la aplicación del modelo formado por dos clases a la predicción de un conjunto externo de datos. Como se observa en el mismo, puede considerarse que las muestras con valores de predicción cercanos a +1 pertenecen a la clase A (círculos rosas), mientras que aquellos con valores cercanos a −1 pertenecen a la clase B (círculos azules). Si los valores de predicción son cercanos a cero y tienen valores de incertidumbre elevados (círculos naranjas), no deberían considerarse dentro de ninguna de las clases ya que la desviación estimada (incertidumbre) alrededor del valor de predicción incluiría el cero[12].

5.2.5. Técnica de las muestras apoyadas en la frontera

La técnica de las muestras apoyadas en la frontera (SVM) es una técnica de reconocimiento de pautas supervisada.

Cuando se dispone de un conjunto elevado de muestras pertenecientes a diferentes clases, la clasificación de las mismas en base a sus características puede ser un proceso largo. Por ello, es interesante disponer de una estrategia fiable que permita construir un modelo con un número reducido de muestras.

El objetivo de esta técnica es la determinación de una función que describa un hiperplano que proporcione una óptima separación de clases[12]. Para ello, se utiliza un pequeño número de muestras distribuidas en las proximidades de la frontera que separa las clases. Estas muestras se denominan vectores soportados (*support vectors*, SVs) o muestras localizadas ("apoyadas") en la frontera.

En la figura 5.10 se ha representado un conjunto de muestras pertenecientes a dos clases diferentes. El modelo de clasificación solo utiliza aquellas situadas en la frontera que separa las clases, representadas en la figura como una X. El resto de las muestras del conjunto de entrenamiento no participan en la construcción del modelo. Esto puede ser interesante si se desea repetir un modelo con el tiempo.

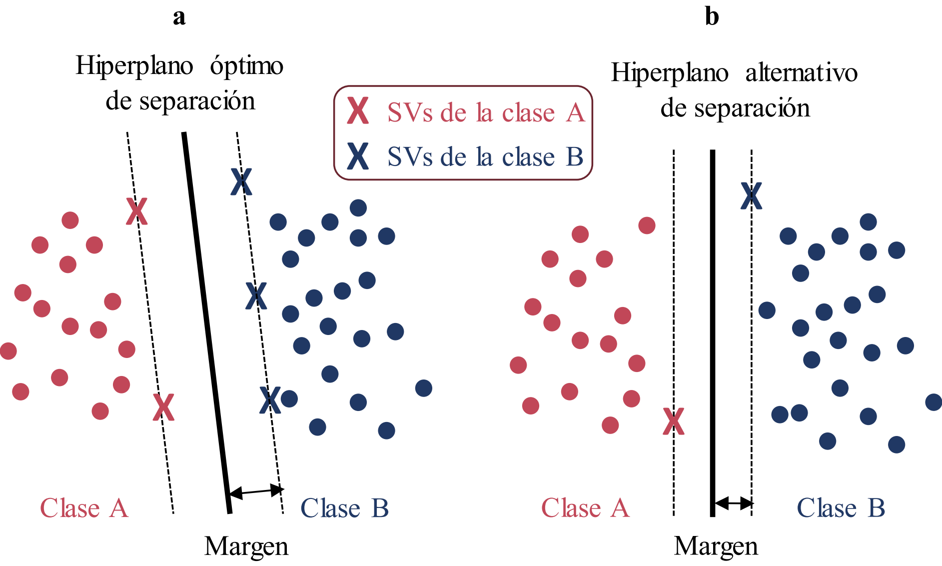

Figura 5.10. *Muestras de la frontera (SVs) seleccionadas para construir los modelos de clasificación y márgenes de los hiperplanos de separación*

En la construcción del modelo de clasificación es necesario destacar que, dado un conjunto de muestras, es posible obtener diferentes hiperplanos que proporcionan una separación entre las clases. En concreto, en esta figura se han destacado dos posibles hiperplanos de separación (figuras 5.10.a y 5.10.b).

El hiperplano óptimo de separación es aquel que hace mínimo el error de clasificación y a la vez maximiza el margen, es decir, hace máxima la distancia entre dicho hiperplano y las muestras más próximas al mismo (SVs)[25]. En el caso particular de la figura es el observado en la parte izquierda de la misma (5.10.a).

Originalmente, la técnica de SVM se desarrolló para proporcionar clasificaciones en conjuntos de datos separables linealmente, pero es también aplicable a conjuntos de datos no lineales. A modo de ejemplo, en la figura 5.11 se observa un conjunto de muestras pertenecientes a dos clases que no son separables linealmente.

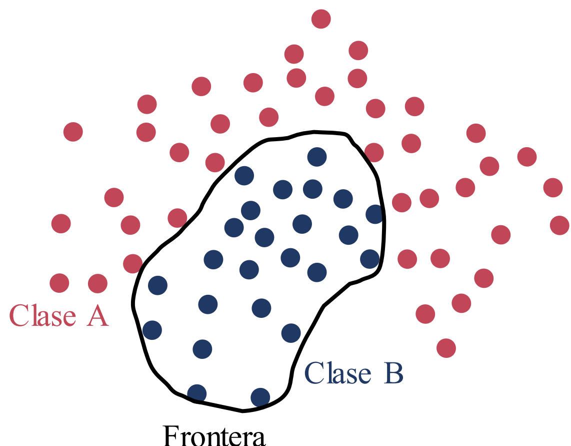

Figura 5.11. *Conjunto de muestras pertenecientes a dos clases no separables linealmente*

Para la construcción de estos modelos se utilizan otro tipo de funciones que permiten determinar fronteras más complejas. Entre las más utilizadas se encuentran la función radial, polinomial con distintos órdenes y sigmoidal.

Una vez determinada la función óptima que define el hiperplano de separación, el modelo se utilizará para la clasificación de futuras muestras ajenas al mismo.

5.3. Calibración

5.3.1. Regresión por mínimos cuadrados parciales

La regresión por mínimos cuadrados parciales, una de las técnicas más importantes de la regresión multivariante, tiene como objetivo el modelado de dos matrices **X** (respuesta analítica) y **c** (concentración) simultáneamente para encontrar las variables latentes u ocultas en la primera que permita una mejor predicción de las variables latentes en la segunda matriz[12]. La regresión mediante PLS maximiza la covarianza entre ambas matrices.

Los componentes PLS de esta técnica son semejantes a los componentes principales obtenidos en PCA, aquí denominados factores. Por lo tanto, es adecuado decir que se basa en el análisis de componentes principales. En esta técnica no es necesario tener información de todos los compuestos presentes en las muestras de estudio, aunque hay que realizar una estimación del número de PCs significativos. Este número no debe coincidir necesariamente con el número de compuestos de la muestra[13].

En esta técnica se tienen en cuenta tanto los errores del conjunto de datos **X** como los relacionados con la estimación de la concentración **c**.

En este tipo de regresión pueden distinguirse dos técnicas, PLS1 y PLS2. En la primera de ellas, que es la utilizada en esta Tesis, el modelo de calibración

se obtiene utilizando un vector concentración para cada compuesto que compone la mezcla, esto es, una única variable **c**. En este caso, el número de factores PLS óptimo puede ser diferente para cada analito. En la segunda, en cambio, se utiliza una matriz de concentraciones, es decir, se obtiene el modelo utilizando un conjunto de variables **c**. En este tipo de regresión todos los analitos se modelan a la vez, por lo que ha de seleccionarse un número de factores PLS común como valor de compromiso para todos los analitos.

5.3.1.1. PLS1

En esta técnica se dispone de dos bloques de matrices: **X**, que recoge la información de la respuesta analítica y cuya dimensión es $\mathbf{I}\times\mathbf{J}$ (muestras y variables, respectivamente), y **c**, referida a las concentraciones y con dimensión $\mathbf{I}\times\mathbf{1}$. Estas matrices se descomponen en sus componentes principales mediante las expresiones[13]:

$$\mathbf{X}=\mathbf{T}\times\mathbf{P}+\mathbf{E} \qquad (1)$$

$$\mathbf{c}=\mathbf{T}\times\mathbf{q}+\mathbf{f} \qquad (2)$$

Estas matrices se muestran en la figura 5.12. El producto de las matrices $\mathbf{T}\times\mathbf{P}$ y $\mathbf{T}\times\mathbf{q}$ se aproxima a los valores medidos con la configuración instrumental utilizada y a las concentraciones reales de cada muestra, respectivamente. La matriz de puntuaciones **T** es común a ambos bloques, **X** y **c**. **E** y **f** son las matrices de error.

En la figura 5.12 se muestra la descomposición de dichas matrices.

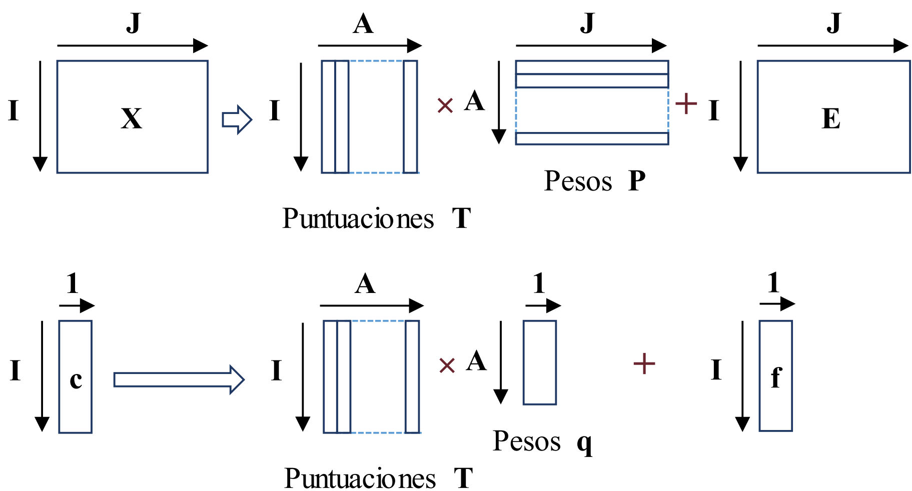

Figura 5.12. *Matrices de PLS1*

En la descomposición de ambas matrices se obtiene una matriz de puntuaciones o *scores* (**T**), una matriz de pesos o *loadings* (**P** y **q**) y una matriz residual (**E** y **f**). Como se observa en la figura 5.12, las matrices de puntuaciones y pesos son una serie de vectores columna y fila, respectivamente, formadas por tantos vectores como factores (**a**) puedan calcularse.

A diferencia de otras técnicas en las que existe una única matriz de puntuaciones para todos los compuestos de estudio, en esta técnica cada compuesto genera una matriz de puntuaciones diferente.

5.3.2. Validación de los modelos

Una vez obtenidos los modelos PLS es necesario llevar a cabo una validación de los mismos. Mediante esta validación se obtiene información sobre el número de factores significativos necesarios para caracterizar el conjunto de datos de estudio y sobre la capacidad de dichos modelos para predecir muestras futuras. Existen, principalmente, tres métodos de validación. A continuación, se describe cada uno de ellos.

- **Auto predicción**

Es el método de validación más sencillo. Para cada compuesto de estudio se calcula el error (**E_{cal}**) teniendo en cuenta la concentración real y predicha por el modelo mediante la siguiente expresión:

$$\mathbf{E_{cal}}=\sqrt{\frac{\sum_{i=1}^{I}(c_i-\hat{c}_i)^2}{I}} \qquad (3)$$

I es el número de muestras que componen el modelo, c_i es la concentración real de la muestra y $\hat{c}_i$ es la concentración predicha por el modelo. Esta aproximación es poco utilizada ya que aumentando el número de componentes del modelo el ajuste puede ser perfecto.

- **Validación cruzada (*cross validation*)**

En esta aproximación, una muestra o un número pequeño de ellas se separan del conjunto de calibración. A continuación, se obtiene un modelo con el conjunto de muestras reducido y se predicen las otras muestras. El procedimiento más habitual es aquel en el cual se deja fuera una única muestra, se predice con el modelo y se repite el proceso I veces hasta que todas las muestras han sido predichas una vez. En este caso, la expresión utilizada para el cálculo del error (**E_{CV}**) es la siguiente:

$$\mathbf{E_{CV}}=\sqrt{\frac{\sum_{i=1}^{I}(c_i-{}^{CV}\hat{c}_i)^2}{I}} \qquad (4)$$

En esta ecuación, ${}^{CV}\hat{c}_i$ es la concentración predicha de la muestra i del conjunto de muestras utilizado en la validación cruzada.

Este tipo de validación es útil para tener información del ruido instrumental o de diferentes errores, como de dilución o pesada. Sin embargo, si existe correlación entre las concentraciones utilizadas en el diseño experimental, como un aumento de concentración de un analito a medida que aumenta otro, la

predicción de muestras futuras en las que no existe dicha correlación no será adecuada.

- **Validación externa (*external validation*)**

En los casos anteriores la validación se lleva a cabo utilizando muestras pertenecientes al conjunto con el cual se construye el modelo de calibración. Sin embargo, también es posible comprobar la calidad predictiva del modelo con un conjunto de muestras independiente (*test set*), es decir, que no ha intervenido en ninguna etapa de construcción del modelo. En este caso, el error (**E_{test}**) se determina de la siguiente manera:

$$\mathbf{E_{test}} = \sqrt{\frac{\sum_{i=1}^{I} (c_i - {}^{test}\hat{c}_i)^2}{I}} \qquad (5)$$

En esta ecuación, I es el número de muestras que contiene el conjunto de validación externa y ${}^{test}\hat{c}_i$ es la concentración predicha de la muestra i del *test set*.

A partir de los valores absolutos obtenidos en las ecuaciones (3-5) es posible expresar el error en términos relativos utilizando la siguiente expresión:

$$\mathbf{E\%} = \frac{E}{\bar{c}} \qquad (6)$$

En esta expresión, $\bar{c}$ es la media de las concentraciones de todas las muestras del conjunto.

Finalmente, es posible determinar la tendencia de los datos del modelo y del conjunto de validación mediante el sesgo (*bias*). Para ello se utiliza la siguiente expresión:

$$\mathbf{S} = \frac{\sum_{i=1}^{I} (\hat{c}_i - c_i)}{I} \qquad (7)$$

siendo I el número de muestras del modelo o del conjunto de validación.

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CAPÍTULO I

Determinación de compuestos orgánicos volátiles en muestras de orina

I.1 Artículo de investigación

Determinación de cetonas y acetato de etilo. Estudio preliminar para la discriminación de pacientes con cáncer de pulmón

Resumen

El cáncer de pulmón es una de las principales causas de muerte en el mundo. Por ello, la detección temprana de la enfermedad es de vital importancia para que el tratamiento se lleve a cabo de una manera eficaz. En este sentido, el análisis de compuestos orgánicos volátiles (VOCs) ha sido de gran interés para la detección de diferentes tipos de cáncer ya que se encuentran involucrados en diferentes vías metabólicas.

En los últimos años, el análisis de orina, saliva y aire exhalado ha sido de gran interés en la monitorización rutinaria de trastornos metabólicos debido a su muestreo no invasivo e indoloro, así como a la posibilidad de llevar a cabo esta toma de muestra tan a menudo como sea necesario. Entre los diversos estudios metabólicos basados en el análisis de orina pueden destacarse aquellos relacionados con cáncer de pecho, pulmón, próstata e hígado, entre otros. Existe una mayor diversidad de VOCs en la orina que en otros fluidos biológicos.

Teniendo en cuenta las bajas concentraciones de VOCs encontradas en orina, normalmente en el rango de ppb-ppm, los analitos de interés deben ser extraídos de la matriz y preconcentrados. Entre las técnicas de pretratamiento de muestra más utilizadas para el estudio de biomarcadores potenciales de cáncer en orina se encuentran la técnica de espacio de cabeza (HS), así como la microextracción en fase sólida en la modalidad de espacio de cabeza (HS-SPME), que ha sido la más utilizada. El uso del inyector de temperatura programada (PTV) para la introducción de muestras en la columna cromatográfica no ha sido muy común en este campo. Su uso no requiere ningún tratamiento de muestra adicional ni extiende el tiempo de análisis. Sin embargo, ofrece beneficios en cuanto a simplicidad y automatización, así como una buena sensibilidad.

Según la revisión bibliográfica realizada previamente, la metodología más utilizada para separar compuestos con grupos carbonilo en orina ha sido la cromatografía de gases (GC), aunque también se ha descrito el uso de

cromatografía de líquidos (LC). La espectrometría de masas (MS) ha sido la técnica de detección más utilizada.

El objetivo principal de este trabajo fue la propuesta de biomarcadores en orina que permitiesen la diferenciación entre muestras de individuos sanos y pacientes con cáncer de pulmón. Para ello, se llevó a cabo el desarrollo de un método basado en HS-PTV-GC-MS para el análisis de nueve cetonas y acetato de etilo en orina. Cabe destacar que, debido al bajo número de muestras disponibles, este trabajo ha de considerarse un estudio preliminar y no un estudio clínico.

Inicialmente, se estudiaron los diferentes parámetros que afectaban al espacio de cabeza incluyendo temperatura y tiempo de generación de espacio de cabeza, agitación y volumen de muestra. Con el fin de obtener la máxima señal analítica para los compuestos de interés se seleccionó un volumen de 5 mL de orina, previamente descongelada y centrifugada durante 10 minutos a 4500 rpm, conteniendo 3.0 g de NaCl (saturación). La generación de espacio de cabeza se llevó a cabo manteniendo una temperatura constante de 80 ºC durante 10 minutos con agitación de la muestra.

A continuación, se optimizaron los parámetros correspondientes a la inyección utilizando un PTV (temperatura, tiempo y flujo de venteo, y modo de inyección). La inyección se llevó a cabo en el modo *solvent vent* o de venteo de disolvente a 25 ºC durante 0.09 minutos con un flujo de venteo de 50 mL min^{-1}, eliminándose el disolvente y quedando los analitos retenidos en un *liner* de Tenax-TA®. Posteriormente, se elevó la temperatura hasta 250 ºC, fijándose 1 minuto como tiempo de inyección, para desorber los analitos del *liner* y transferirlos a la columna cromatográfica.

Con el fin de obtener la mejor separación de analitos y definición de picos cromatográficos se optimizaron los parámetros que afectan a la separación mediante cromatografía de gases (rampa cromatográfica) y a la detección

mediante espectrometría de masas (valores de velocidad de barrido en el modo *scan* y tiempo de permanencia en el modo de iones seleccionados (SIM)). El tiempo de análisis por muestra cuando se utilizó el programa de temperatura óptimo del cromatógrafo de gases fue de 4.78 minutos. El uso del modo SIM/*scan* combinado en la detección mediante MS permitió la adquisición de datos en ambas modalidades en un solo análisis, sin pérdida de sensibilidad o calidad de espectros con respecto al análisis individual de cada modo de adquisición de datos. Se establecieron seis grupos en el modo SIM y tres grupos en el modo *scan* con diferentes valores de tiempo de permanencia y velocidad de barrido, respectivamente.

Con el fin de estudiar la posible existencia de efecto matriz se compararon las señales obtenidas para los diferentes analitos cuando se analizaron cinco muestras de orina de diferentes individuos y una muestra de agua ultrapura, todas ellas dopadas con los compuestos al mismo nivel de concentración. Los valores de recuperación oscilaron entre 83 y 115 %, no observándose diferencias significativas. Por ello, se concluyó que no existía efecto matriz y la cuantificación de los analitos se llevó a cabo mediante calibración externa en agua ultrapura.

Las características analíticas del método se determinaron mediante el análisis de agua ultrapura dopada con seis niveles diferentes de concentración. Las rectas de calibración mostraron comportamiento lineal, sin fallo de ajuste, con coeficientes de determinación (R^2) satisfactorios y siempre superiores a 0.99. Los límites de detección (*limit of detection*, LOD) en agua ultrapura se encontraron entre 0.001 (2-nonanona) y 0.139 (acetona) µg L^{-1}. Los límites de cuantificación (*limit of quantification*, LOQ) oscilaron entre 0.004 (2-nonanona) y 0.465 (acetona) µg L^{-1}. Estos límites fueron iguales o inferiores a aquellos descritos previamente en bibliografía para el análisis de orina en muestras de pacientes con cáncer del pulmón en los cuales fue necesaria una etapa de

derivatización o tratamiento de extracción adicional, lo que extendía el tiempo de análisis.

El estudio de repetibilidad y reproducibilidad se llevó a cabo utilizando dos niveles diferentes de concentración en agua ultrapura y los valores obtenidos en términos de desviación estándar relativa (*relative standard deviation*, RSD, %) fueron iguales o inferiores al 10 y al 11 %, respectivamente. De manera adicional, la repetibilidad se estudió en orina realizándose seis análisis de la misma y obteniéndose un valor de RSD igual o inferior al 8 % para todos los analitos.

Para comprobar la aplicabilidad del método se doparon cinco muestras de orina con una concentración conocida de analitos. La exactitud se evaluó mediante la comparación de la concentración añadida y la diferencia entre el resultado obtenido en la predicción de la correspondiente orina dopada y sin dopar. Los valores obtenidos se encontraron entre el 82 y el 109 %.

El método puesto a punto se utilizó para el análisis de 24 muestras de orina (4 mujeres, 20 hombres) de voluntarios sanos (12 muestras) y de pacientes con cáncer de pulmón (12 muestras). Las muestras de pacientes con cáncer de pulmón correspondieron a 5 pacientes con carcinoma escamoso, 6 pacientes con adenocarcinoma y un paciente con carcinoma microcítico (de células pequeñas). Se realizó un análisis de componentes principales (PCA) y se observó una adecuada separación entre los diferentes grupos de muestras (grupo de individuos sanos y grupo de pacientes). El porcentaje de varianza explicada (acumulada) para los tres primeros componentes principales fue 45, 68 y 83 %, respectivamente.

Con el fin de saber cuál de los analitos de estudio proporcionaba diferencias significativas se llevó a cabo un test U de Mann-Whitney. Para asegurar que las diferencias encontradas se debían solo a la presencia o ausencia de cáncer de pulmón, los grupos de muestras correspondientes a individuos sanos

y pacientes fueron homogéneos en lo referente a edad y sexo. El rango de edad fue semejante en ambos grupos y ambos estaban formados por 2 mujeres y 10 hombres, lo que está de acuerdo con la prevalencia del cáncer de pulmón, normalmente superior en hombres que en mujeres. Se encontraron diferencias significativas para tres de los diez compuestos: acetato de etilo, 3-heptanona y 3-octanona. La concentración de estos analitos fue siempre mayor en pacientes que en individuos sanos. El poder de discriminación del acetato de etilo y de la 3-octanona fue superior al correspondiente proporcionado por la 3-heptanona. Este resultado estuvo de acuerdo con aquel encontrado cuando se obtuvieron las correspondientes curvas características operativas del receptor (*receiver operating characteristic*, ROC).

Finalmente, con el objetivo de proponer una estrategia para asignar futuras muestras de orina a uno de los grupos de muestras estudiados (pacientes o sanos) se utilizó el test de rangos y signos de Wilcoxon. Se seleccionaron 7 muestras de orina de voluntarios sanos como grupo de referencia. Se calculó la mediana de las concentraciones para los tres analitos que habían proporcionado diferencias significativas (acetato de etilo, 3-heptanona y 3-octanona). A continuación, se utilizó el test para determinar si la concentración de estos analitos encontrada en el resto de las muestras de orina (pacientes y sanos) difería de la mediana del grupo de referencia. Cuando se llevó a cabo la clasificación de muestras de individuos sanos, prácticamente todas las muestras se asignaron correctamente, ya que la mediana obtenida para cada biomarcador con el modelo de referencia no fue significativamente inferior ($p>0.05$) a la concentración correspondiente de la muestra estudiada. Sólo una muestra de un voluntario sano se asignó de manera incorrecta (falso positivo) en función de la concentración de 3-octanona. En el caso de las muestras de pacientes el contenido de acetato de etilo y 3-octanona fue siempre mayor al correspondiente de la mediana del conjunto de referencia. Tres de las muestras correspondientes a pacientes se

asignaron de manera incorrecta (falsos negativos) cuando se utilizó la 3-heptanona.

Se han descrito trabajos previos en los que se utilizó una configuración semejante sin separación cromatográfica para la diferenciación de muestras de pacientes con cáncer de pulmón y voluntarios sanos, analizando volátiles en orina y saliva. En el caso de la orina, se consiguió dicha diferenciación atendiendo a las concentraciones de 2-pentanona y 2-etil-1-hexanol. Los límites de detección en estas metodologías no separativas empeoraron al eliminar la etapa de separación, siendo superiores a las concentraciones encontradas en algunas orinas en el trabajo que aquí se propone, razón por la cual no se ha utilizado. A pesar de ello, cabe destacar que en el presente trabajo no se encontraron diferencias significativas entre los grupos de muestras en base a la concentración de 2-pentanona. En vista de los diferentes resultados encontrados en estos trabajos, el estudio debería extenderse a un mayor número de muestras para poder confirmar o descartar este compuesto como biomarcador de cáncer de pulmón.

Entre las principales conclusiones de este trabajo se destaca que se han encontrado diferencias significativas de concentración para el acetato de etilo, la 3-heptanona y la 3-octanona en muestras de orina de pacientes con cáncer de pulmón e individuos sanos, observándose mayor concentración en orina de pacientes. La caracterización de una muestra utilizando varios biomarcadores y no uno de manera individual para su clasificación posterior dentro de un grupo de muestras se considera una buena estrategia en la discriminación de muestras.

El test de rangos y signos de Wilcoxon podría ser utilizado para la asignación de futuras muestras, siempre teniendo en cuenta los diferentes resultados obtenidos en función de cada biomarcador y considerando este trabajo como un estudio preliminar debido al bajo número de muestras.

Finalmente, la configuración instrumental utilizada en esta metodología reduce la manipulación de muestra y, por lo tanto, los errores relacionados con esa etapa del análisis.

I.1 Artículo de investigación

Determinación de cetonas y acetato de etilo. Estudio preliminar para la discriminación de pacientes con cáncer de pulmón

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RESEARCH PAPER

Determination of ketones and ethyl acetate—a preliminary study for the discrimination of patients with lung cancer

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Abstract In this work, ten possible volatile biomarkers of lung cancer (acetone, 2-butanone, ethyl acetate, 2-pentanone, 4-methyl-2-pentanone, 2-hexanone, 3-heptanone, 2-heptanone, 3-octanone, and 2-nonanone) have been analyzed to evaluate their different concentration levels in urine samples from lung cancer patients (n = 12) and healthy controls (n = 12). The volatile compounds were generated with a headspace autosampler and analyzed with a gas chromatograph equipped with a programmed temperature vaporizer and mass spectrometry detector (HS-PTV-GC-MS). With the aim of evaluating the aforementioned differences, a Mann-Whitney U test and box-plots were obtained. Very good discrimination between cancer and control groups was achieved for three (ethyl acetate, 3-heptanone, and 3-octanone) of the ten analytes studied. With a view to assigning samples to the group of healthy or ill individuals, the Wilcoxon signed-rank test has been used. In spite of the small number of urine samples assayed, the results may suggest that the studied compounds could be considered useful tools in order to discern samples and they could be employed as a complementary test in a diagnosis.

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Keywords Volatile biomarkers · Urine · Lung cancer · Mass spectrometry

Introduction

Lung cancer is one of the most common causes of death in the world [1]. Early detection is important in the successful treatment of the disease. The analysis of volatile organic compounds (VOCs) is one of the most encouraging metabolomics approaches for the detection of different types of cancer [2, 3]. In this sense, the study of VOCs has gained scientific importance because they are involved in many metabolic pathways [4]. Different VOCs have been correlated with the presence of lung cancer [5–8], among other diseases, and they may therefore serve as biomarkers.

In recent years, the analysis of urine [9], saliva [10], and breath [11] has attracted much scientific interest for the routine monitoring of metabolic disorders, due to its non-invasive and painless sampling, and to the fact that it can be performed as often as needed. Urinary metabolomics studies have been applied to breast, lung, prostate, colorectal, and liver cancer among others [12]. There is more diversity of VOCs in the urine than in other biological fluids where these analytes can be measured [13].

Concentrations of most of VOCs in urine are in the ppm-ppb range [2]. Analytes of interest must be extracted from the matrix and preconcentrated. Within the different sample pretreatment techniques in studies of potential cancer biomarkers in urine, headspace-solid phase microextraction (HS-SPME) is the most used [14–17]. Sometimes, only headspace extraction has been used [18, 19]. Another alternative in this field, which has not become very common yet, is the use of a programmed temperature vaporizer (PTV) inlet to inject the samples [18]. This setup does not involve any additional sample

treatment and does not extend the time required to perform the analysis. It offers benefits in terms of simplicity and automation possibilities, together with very good sensitivity.

Gas chromatography (GC) is the most useful choice for determining carbonyl compounds in urine [13–20]. In several of the methodologies proposed, a derivatization step is used to reduce the polarity of the analytes of interest, which extends the time of the analysis [15, 19]. Beside GC, liquid chromatography (LC) has also been used [21–23]. The main detection technique that has been employed in VOC cancer biomarker studies in urine is mass spectrometry (MS) [13–21, 23]. Sometimes, ion mobility spectrometry [24, 25], selected ion flow tube-mass spectrometry (SIFT-MS) [26], and electronic noses based on mass spectrometry [27] have also been used.

In this work, urine samples from healthy volunteers and patients with lung cancer were analyzed and the concentrations of nine ketones and ethyl acetate were compared in order to obtain some significant differences. The methodology used employs a headspace autosampler in combination with a gas chromatograph equipped with a PTV and a MS detector (HS-PTV-GC-MS). This preliminary study cannot be considered a clinical study due to the small number of samples available.

Materials and methods

Reagents and standard solutions

The standards of 2-butanone, ethyl acetate, 2-pentanone, 4-methyl-2-pentanone, 2-hexanone, 3-heptanone, 2-heptanone, 3-octanone and 2-nonanone, as well as methanol (HPLC grade) and the reagents needed for creatinine measurements (creatinine standard and picric acid) were supplied by Sigma-Aldrich (Steinheim, Germany). Acetone and sodium chloride were purchased from Scharlau (Barcelona, Spain). The purity of all the reagents was at least 98%.

A stock solution which contained all the compounds was prepared in methanol and stored at 4 °C in a refrigerator. It was diluted in UHQ water to prepare the working solutions and used to spike the water and urine samples at the different concentrations analyzed (0.026–1947 μg/L). UHQ water was obtained with a Wasserlab Ultramatic water purification system (Noain, Spain).

Urine samples and sample preparation

Urine from 24 adults (4 women, 20 men) whose age ranged from 55 to 85 were collected in a disposable sterile specimen collection cup and stored at −20 °C. Sample nos. 1–12 corresponded to healthy individuals unaffected by diseases; sample nos. 13–24 were from patients with lung cancer at the Internal Medicine Unit of the Virgen de la Vega Hospital in Salamanca. The division of urines from patients was as follows: patient nos. 13–17 had squamous cell carcinoma (non-small cell lung cancer); sample nos. 18–23 had adenocarcinoma (non-small cell lung cancer); and no. 24 corresponded to a patient who had small cell lung cancer. The characteristics of the patients and healthy controls whose urines were analyzed are shown in Table S1 (in the Electronic Supplementary Material (ESM)).

After completely thawing the urine samples at room temperature, they were transferred to a 12-mL polypropylene tube (Scharlau, Spain) and were centrifuged for 10 min at 4500 rpm. Measurements of urine samples were conducted by transferring 5 mL of urine, 3.0 g of NaCl (supersaturation) and 62.5 μL of a mixture of methanol:UHQ water (5:10,000) to a 10-mL headspace vial (Agilent Technologies, DE, Germany) which was sealed with a Teflon®/silicone septum (Agilent Technologies, DE, Germany). To analyze the spiked samples, 5 mL of urine, 3.0 g of NaCl, and 62.5 μL of the corresponding stock solution were subjected to the same process.

This study was authorized by the Hospital Ethics Committee.

Creatinine measurements

It is well-known that the concentration of endogenous compounds in urine can vary daily depending on the diet and the volume of urine excreted. With the aim of avoiding a 24-h collection of samples, the normalization of analyte concentration to total creatinine concentration (mmol) is required. The values of the normalized concentrations are expressed as nmol analyte/mmol creatinine.

For this purpose, a modification of the Jaffè method [28] has been used. Creatinine reacts with picric acid to form a reddish complex which can be detected with a photometric detector (λ = 500 nm) in order to measure creatinine concentrations. The equipment used was a spectrophotometer Shimadzu UV/Vis-160, equipped with a processing and recording unit, using a quartz cell (Suprasil) with a 10 mm path length.

HS-PTV-GC-MS measurements

Headspace sampling was performed with a PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), equipped with a tray for 32 consecutive samples and an oven with positions for 6 sample vials. The oven was kept at 80 °C for 10 min with agitation at 750 rpm. The injection of the sample was carried out by using a 2.5-mL syringe at 110 °C. The time lapse between consecutive samples was set at 18 min.

The injection of the samples after headspace sampling was performed with a programmed temperature vaporizer (PTV) inlet (CIS-4, Gerstel, Baltimore, MD, USA) in solvent-vent mode. A liner (71 mm × 2 mm) packed with Tenax-TA® was used. The initial temperature of the injector was set at 25 °C

for 0.09 min, with a vent flow of 50 mL/min at a pressure of 5.00 psi, with the aim of eliminating the solvent. After 0.09 min, the split valve was closed and the liner was flash-heated at a rate of 12 °C/s until it reached 250 °C (injection time, 1.00 min), desorbing the analytes from the liner and transferring them to the column. In the final step, after the injection, the split valve is reopened. Purge flow was adjusted at 150 mL/min and the liner temperature was held at 250 °C for 5 min to clean the liner. Liquid CO_2 was used to accomplish the cooling of the PTV system.

Chromatographic analyses were performed on an Agilent 6890 GC device equipped with a low polarity DB-VRX capillary column (20 m × 0.18 mm × 1 μm) from J&W Scientific (Folsom, CA, USA). The carrier gas was helium N50 (99.999% pure, Air Liquide). The initial oven temperature was 35 °C, held for 0.5 min. Then, temperature was increased by following two consecutive ramps. First, temperature was raised at a rate of 60 °C/min to 175 °C; then, it was programmed at 45 °C/min to 240 °C. This temperature was held for 0.5 min. The total chromatographic run time was 4.78 min.

The detector was a quadrupole mass spectrometer (HP 5973 N) equipped with an inert ion source. It was operated in electron-ionization mode with an ionization voltage of 70 eV. Temperatures for ion source and quadrupole were 230 and 150 °C, respectively. The analyses were performed in synchronous SIM/scan mode, which allowed the collection of both SIM and full scan data in a single run. A solvent delay of 0.60 min was established. Six selected-ion monitoring (SIM) groups with different *m/z* and dwell time values were used for quantification and three scan groups (*m/z* 25–125) with different data acquisition speed values were used for compound identification. The conditions for SIM/scan analyses are shown in Table S2 (in the ESM). The different compounds were identified by comparison of the experimental spectra with those found in the NIST08 database (NIST/EPA/NIH Mass Spectral Library, version 2.0). The selected settings for synchronous SIM/scan analysis resulted in no significant decline of peak integrity, sensitivity, or spectral quality in comparison to SIM or scan-only acquisitions.

Data analysis

Enhanced ChemStation software [29] and OriginPro v8.0724 statistical package [30] have been used.

Results and discussion

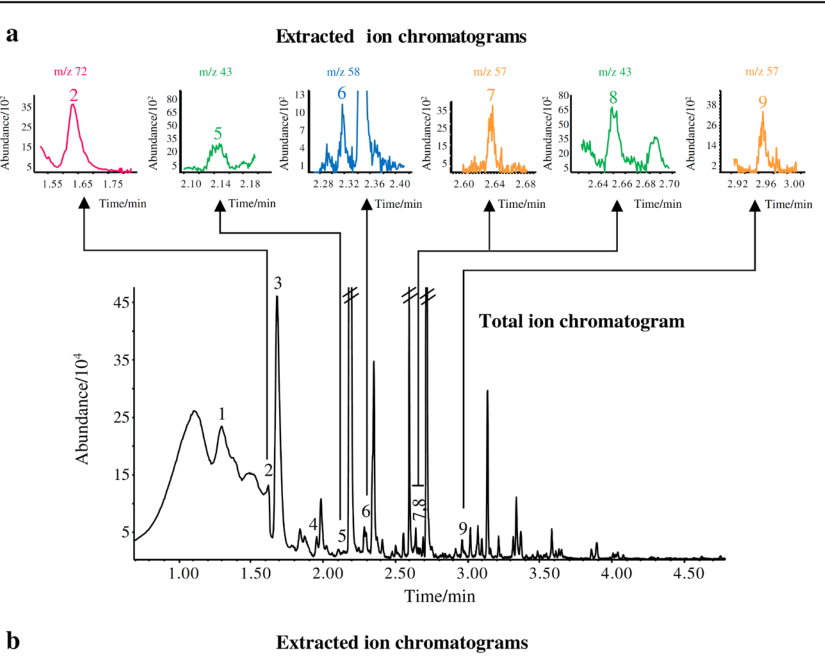
Study of the signals obtained

In the present work, ten possible volatile biomarkers of lung cancer in urine were studied. Figure 1 shows an example of the scan (Fig. 1a) and SIM (Fig. 1b) chromatograms obtained when a urine sample from a lung cancer patient with squamous non-small cell lung cancer (no. 13) was analyzed. At the bottom of Fig. 1a, the total ion chromatogram is shown. Some of the analytes studied (nos. 2, 5–9) are difficult to distinguish due to their low intensity. Consequently, the base peak of these analytes has been extracted for identification purposes. The extracted ion chromatograms are displayed at the top of Fig. 1a. They show a significant level of noise and a poor morphology of the peaks. These drawbacks are solved in the SIM mode (Fig. 1b). The level of noise decreases and the morphology of the peaks improves, as can be seen in the corresponding extracted SIM chromatograms. Analyte no. 10 was not found in this urine sample.

Evaluation of the HS-PTV-GC-MS method

In order to check the possible existence of a matrix effect, urine samples from five different subjects and ultrapure water were spiked at different concentration levels. No significant differences were observed between urine samples and between urine and UHQ water samples (recoveries ranged between 83 and 115% for most of the analytes). Since no matrix effect was observed, quantification was performed with external calibration method in UHQ water.

Ultrapure aqueous solutions of the ten analytes studied were prepared at six different concentration levels in order to obtain the calibration curves, detection limits, and precision. Each level was analyzed in triplicate. The analytical signals used for the calibration curves were the peak areas of the compounds in the extracted ion chromatograms (SIM mode) for the quantitation ions shown in Table S2 (in the ESM). All calibrations showed good linear behavior. Their validity was checked using ANOVA, and it was observed that the models did not exhibit any lack of fit. The values of the coefficient of determination (R^2) were satisfactory for all the compounds (>0.99). Good results were obtained, although peak tailing was observed due to the polar nature of the compounds. The limits of detection (LOD) and quantification (LOQ) were calculated as three and ten times, respectively, the standard deviation of a standard solution ($n = 6$) that provided an S/N ratio of approximately 3, divided by the slope of the calibration straight line. The limits of detection in ultrapure water ranged from 0.001 (2-nonanone) to 0.139 (acetone) μg/L and the quantification limits ranged from 0.004 (2-nonanone) to 0.465 (acetone) μg/L. The analytical characteristics of the method are shown in Table 1. According to the literature, some of these analytes have also been determined in urine samples of lung cancer patients using different methodologies based on HS-SPME-GC-MS [15] and HS-SPME-GC-SRI-TOF-MS [31]. The limits of detection achieved in this work were lower or of the same order as those found with other previously published methodologies [15, 31] in which



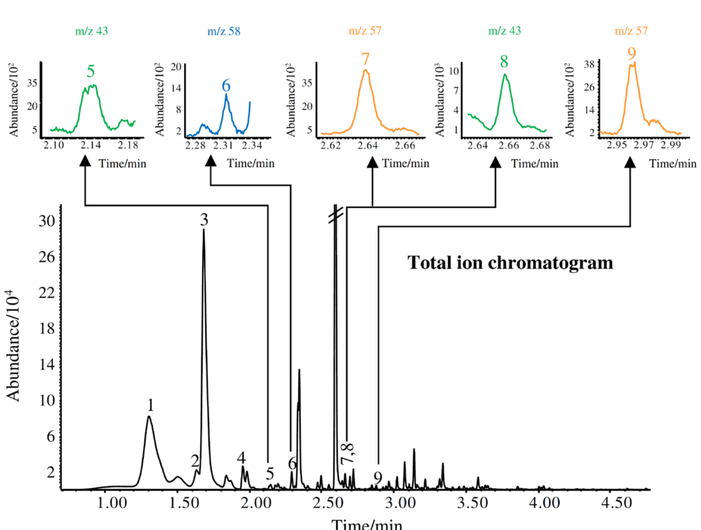


Fig. 1 Scan (**a**) and SIM (**b**) chromatograms of a urine sample (no. 13) from a non-small cell lung cancer patient (squamous cell)

Table 1 Analytical characteristics of the HS-PTV-GC-MS method

Compound	Calibration range (μg/L)	R^2	LOD (μg/L)	LOQ (μg/L)	Repeatability (%)		Reproducibility (%)	
					Low level	High level	Low level	High level
Acetone	LOD-1947	0.9986	0.139	0.465	3	3	5	5
2-butanone	LOD-150	0.9988	0.026	0.087	6	3	10	6
Ethyl acetate	LOD-150	0.9996	0.054	0.181	7	3	6	5
2-pentanone	LOD-150	0.9987	0.007	0.022	8	3	8	5
4-methyl-2-pentanone	LOD-10	0.9988	0.007	0.024	8	2	8	4
2-hexanone	LOD-10	0.9987	0.003	0.011	9	7	7	7
3-heptanone	LOD-10	0.9985	0.002	0.007	7	2	9	6
2-heptanone	LOD-20	0.9988	0.003	0.011	10	3	11	5
3-octanone	LOD-10	0.9986	0.004	0.013	9	4	6	5
2-nonanone	LOD-10	0.9982	0.001	0.004	4	5	5	7

derivatization or additional extraction steps are included, thus increasing the time required to perform the analysis.

Repeatability and reproducibility were determined at two different concentration levels. The low level corresponded to a solution which contained all the compounds in a concentration which provided an S/N ratio of approximately 10 (ranging between 0.026 and 1.06 μg/L), whereas the high level corresponded to a concentration slightly higher than the second level of the calibration curve (from 1.67 to 486 μg/L). This evaluation was carried out by analyzing the spiked solutions six times for each level on the same day (repeatability) and on two consecutive days (reproducibility). The relative standard deviation (RSD, %) obtained for all the compounds was lower or equal to 10% for repeatability and to 11% for reproducibility (Table 1).

In order to evaluate the repeatability in urine, six measurements of a urine sample were conducted. The value of the relative standard deviation was lower than 8% for all the analytes. Good results were obtained and it was not necessary to use any internal standard.

To check the methodology, five urine samples were spiked with the studied analytes at different concentration levels. Accuracy was obtained by comparing the amount added with the difference between the results obtained with and without the addition and it ranged between 82 and 109%.

Urine analysis

The calibration models in UHQ water were used to predict the concentrations of all the analytes in urines from healthy individuals (sample nos. 1–12) and patients with lung cancer (sample nos. 13–24). The predicted concentrations of the studied biomarkers are shown in Table S3 (in the ESM). The analytes have been tagged with numbers according to their retention times (see ESM, Table S2).

A principal component analysis was performed on the data corresponding to Table S3 (in the ESM) and a suitable separation between both types of samples was achieved. Figure 2 shows a score plot of the samples for the first three PCs. The percentage of the cumulative explained variance with the first three PCs was 45, 68, and 83%.

In order to know which biomarkers produce the significant differences, the Mann-Whitney *U* test was used. To ensure that the differences are only due to the presence or absence of lung cancer and differences due to factors like gender and age are eliminated, homogeneous groups of healthy volunteers and patients were formed. Consequently, the ages of healthy subjects and patients were similar. Furthermore, both groups were composed of two women and ten men. This is in agreement with the prevalence of lung cancer, which is higher in men than in women.

Significant differences ($p < 0.05$) were found for three of the ten analytes (ethyl acetate, 3-heptanone, and 3-octanone) with the statistical test mentioned above. The corresponding box-plots representing the concentration of the biomarkers for both groups of samples are shown in Fig. 3. The concentration of these three analytes was always higher in patients with lung cancer than in the control samples. Good discrimination power was achieved when ethyl acetate and 3-octanone were used. The discrimination capacity of 3-heptanone was slightly lower than that obtained with the other two analytes. Similar results were obtained when a receiver operating characteristic curve (ROC curve) analysis was performed. Figure 4 shows ROC curves for ethyl acetate, 3-heptanone, and 3-octanone. The

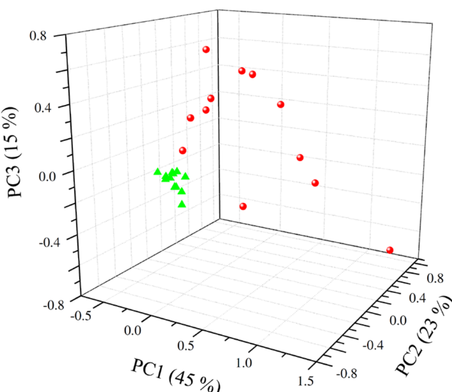


Fig. 2 PCA score plot for 24 urine samples corresponding to patients with lung cancer (red circles) and healthy volunteers (*green triangles*)

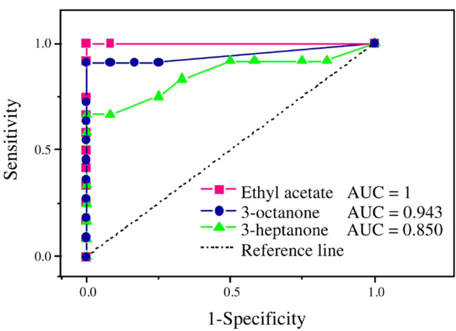


Fig. 4 The receiver operating characteristic (*ROC*) curves for ethyl acetate, 3-heptanone, and 3-octanone in differentiating between controls and lung cancer patients. The area under the curve (*AUC*) is also shown

area under the curve (AUC) was 1, 0.850, and 0.943, respectively. The AUC values for the other seven compounds ranged between 0.506 and 0.687.

With the aim of proposing a strategy which allows the assignment of future urine samples to one group or another, the Wilcoxon signed-rank test was used to test whether a sample median differs from a standard value [32]. Seven urine samples from healthy volunteers (nos. 1–7) were considered as the reference group. The median of the concentrations found for the three analytes which afforded significant differences (ethyl acetate, 3-heptanone, and 3-octanone) was obtained. This reference value was compared with the concentration of the biomarkers predicted in the remaining group of healthy urines (nos. 8–12) and patient urines (13–24). The results obtained are presented in Table 2. In the case of healthy individuals, except sample no. 11 for 3-octanone, all the samples were assigned correctly since the median obtained for each biomarker (reference model) was not significantly lower ($p > 0.05$) than the concentration obtained for the sample studied. Regarding the patient samples, the content of ethyl acetate and 3-octanone was higher than in the reference set in all cases. However, sample nos. 14, 18, and 19 were not correctly assigned when the reference value for 3-heptanone was compared.

Table 3 shows the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for ethyl acetate, 3-heptanone, and 3-octanone. The results show that these three compounds may be useful for differentiating subjects with presence or absence of lung cancer but, by being confined to a small number of samples, the findings presented above for the comparison between control individuals and lung cancer patients should be confirmed with an extensive set of samples.

Comparison with other works

In previous works [27, 33], a non-separative method based on coupling of a headspace sampler, a programmed temperature vaporizer, and a mass spectrometer (HS-PTV-MS) has been proposed for the rapid determination of possible biomarkers of lung cancer in urine [27] and saliva [33] samples. In both cases, the samples were subjected to the headspace generation process and introduced without separation into the mass spectrometer which allowed obtaining the fingerprint of the sample analyzed. In the work involving urine samples, five analytes described in literature as possible biomarkers were selected as

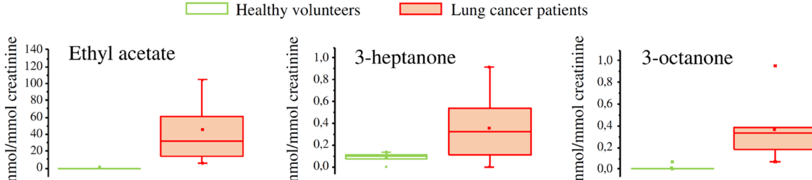


Fig. 3 Box-plot graphics showing the concentration distributions (nmol analyte/mmol creatinine) of the biomarkers distinguishing between healthy subjects and lung cancer patients

Table 2 Classification of the samples using the Wilcoxon signed-rank test

	Compound			
	Sample	Ethyl acetate	3-heptanone	3-octanone
Controls	8	−	−	−
	9	−	−	−
	10	−	−	−
	11	−	−	+
	12	−	−	−
Patients	13	+	+	+
	14	+	−	+
	15	+	+	+
	16	+	+	+
	17	+	+	+
	18	+	−	+
	19	+	−	+
	20	+	+	+
	21	+	+	+
	22	+	+	+
	23	+	+	+
	24	+	+	+

−: The median of the concentrations of the control group (reference model) is not significantly lower ($p > 0.05$) than the concentration of the sample studied

+: The median of the concentrations of the control group (reference model) is significantly lower ($p < 0.05$) than the concentration of the sample studied

test compounds to check the possibilities of the new methodology. Among the studied biomarkers, the best results in the discrimination of controls and patients were obtained when 2-pentanone and 2-ethyl-1-hexanol were used. These results agree with some others reported in literature [34, 35].

The non-separative methodology described above (HS-PTV-MS) has not been used in the present work due to the fact that the concentrations of some biomarkers in a high number of samples are below the detection limits of the method. In view of this limitation, a chromatographic method (HS-PTV-GC-MS) has been used here which provided lower detection limits according to the concentration found in the samples.

Table 3 Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the three compounds whose concentrations showed significant differences between healthy individuals and lung cancer patients

Compound	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ethyl acetate	100	100	100	100
3-heptanone	75	100	100	63
3-octanone	100	80	92	100

2-Pentanone has been also analyzed in this work and significant differences between both sample groups were not found this time, which could mean that future work should be done to confirm or discard this candidate as a real biomarker. Even in works where good results have been obtained using 2-pentanone, the authors recommend extending the analysis to more samples to have significant results [35].

Conclusions

The Mann-Whitney U test revealed metabolomic differences between patients with cancer and healthy individuals. Although the number of samples analyzed in this work does not allow obtaining any clinical conclusion, increased concentrations of ethyl acetate, 3-heptanone, and 3-octanone in urine samples from patients show some kind of useful information about the diagnosis of lung cancer.

With a view to assigning future samples to the groups of healthy individuals or patients, the Wilcoxon signed-rank test has been used. All the samples from healthy individuals were correctly assigned except one of them when 3-octanone was employed (false positive). Regarding the samples from lung cancer patients, there were no false negatives for any of the samples when the biomarkers ethyl acetate and 3-octanone were used. However, for 3-heptanone, 3 of the 12 lung cancer patients studied were misassigned (false negatives).

Taking into account that for the characterization of a sample it is preferred to use a battery of biomarkers rather than a single one, the results obtained here show that ethyl acetate, 3-heptanone, and 3-octanone could be considered useful tools for discerning samples, enhancing the possible group of helpful biomarkers.

The instrumental configuration used minimizes sample manipulation, reducing errors associated with this step of the analysis, and no derivatization reaction is required.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was provided by all the individuals involved in the study.

Ethics approval The study was authorized by the Hospital Ethics Committee.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Determination of ketones and ethyl acetate—a preliminary study for the discrimination of patients with lung cancer

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Table S1 Characteristics of controls and patients

	Healthy controls	Lung cancer patients
No. of subjects	12 (samples nos. 1-12)	12 (samples nos. 13-24)
Gender	Male 10 Female 2	Male 10 Female 2
Average age Standard deviation Range	67 9 (55-83)	71 9 (59-85)
Histopathology		Non-small cell lung cancer (squamous cell) 5 (samples nos. 13-17) Non-small cell lung cancer (adenocarcinoma) 6 (samples nos. 18-23) Small cell lung cancer 1 (sample no. 24)

Table S2 Parameters for the SIM/scan data acquisition mode

Compound	t_R (min)	SIM ions (*m/z*)*	SIM group and dwell time	Scan group
(1) Acetone	1.308	42, **43**, 58		
(2) 2-butanone	1.639	29, 43, **72**	1 (10 ms)	1 (3.46 scan/s)
(3) Ethyl acetate	1.689	43, **61**, 45		
(4) 2-pentanone	1.957	41, **43**, 86	2 (10 ms)	
(5) 4-methyl-2-pentanone	2.152	41, **43**, 58	3 (1 ms)	2 (18.65 scan/s)
(6) 2-hexanone	2.319	43, 57, **58**		
(7) 3-heptanone	2.643	27, 29, **57**	4 (1 ms)	
(8) 2-heptanone	2.662	27, **43**, 58		3 (27.16 scan/s)
(9) 3-octanone	2.965	43, **57**, 72	5 (1 ms)	
(10) 2-nonanone	3.301	41, 43, **58**	6 (1 ms)	

*Quantifier ion in bold

Table S3 Concentration of the analytes in the urine samples expressed as nmol analyte/mmol creatinine. The analytes have been tagged with number according to their retention times (see Table S2)

	Compound									
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	$(175 \pm 9)\cdot 10$	88 ± 4	<LOD	61 ± 3	0.8 ± 0.1	0.098 ± 0.001	0.12 ± 0.03	0.9 ± 0.3	0.06 ± 0.03	0.12 ± 0.04
2	$(144 \pm 8)\cdot 10$	69 ± 5	<LOD	90 ± 3	1.2 ± 0.2	0.320 ± 0.002	0.07 ± 0.04	2.0 ± 0.3	0.07 ± 0.04	0.29 ± 0.05
3	$(30 \pm 2)\cdot 10^2$	75 ± 6	<LOD	109 ± 5	1.5 ± 0.4	0.318 ± 0.004	<LOD	1.6 ± 0.2	<LOD	<LOD
4	$(60 \pm 4)\cdot 10$	27 ± 1	2 ± 1	13 ± 1	0.3 ± 0.1	0.042 ± 0.001	<LOD	0.40 ± 0.04	<LOD	<LOD
5	$(39 \pm 2)\cdot 10^2$	64 ± 4	<LOD	60 ± 3	0.8 ± 0.1	0.227 ± 0.003	0.11 ± 0.07	1.1 ± 0.1	<LOD	<LOD
6	$(108 \pm 7)\cdot 10$	70 ± 3	<LOD	52 ± 2	0.9 ± 0.1	0.174 ± 0.002	0.13 ± 0.05	0.75 ± 0.09	<LOD	<LOD
7	$(36 \pm 2)\cdot 10^2$	111 ± 7	<LOD	115 ± 4	1.3 ± 0.3	0.240 ± 0.003	0.10 ± 0.06	0.8 ± 0.1	<LOD	0.10 ± 0.07
8	$(147 \pm 5)\cdot 10$	51 ± 2	<LOD	66 ± 3	1.3 ± 0.1	0.232 ± 0.001	0.12 ± 0.03	0.78 ± 0.06	<LOD	0.25 ± 0.04
9	$(196 \pm 7)\cdot 10$	18 ± 3	<LOD	14 ± 2	0.7 ± 0.2	0.118 ± 0.002	0.08 ± 0.04	1.12 ± 0.09	<LOD	<LOD
10	$(144 \pm 7)\cdot 10$	45 ± 3	<LOD	45 ± 2	1.0 ± 0.2	0.131 ± 0.002	0.10 ± 0.05	1.4 ± 0.3	<LOD	0.07 ± 0.07
11	$(157 \pm 9)\cdot 10$	24 ± 2	<LOD	40 ± 2	1.0 ± 0.2	0.149 ± 0.002	0.08 ± 0.03	1.8 ± 0.3	0.05 ± 0.03	0.12 ± 0.04
12	$(115 \pm 7)\cdot 10$	61 ± 3	<LOD	31 ± 2	0.6 ± 0.1	0.083 ± 0.002	0.09 ± 0.04	0.78 ± 0.09	<LOD	<LOD
13	$(5 \pm 1)\cdot 10^2$	28 ± 3	104 ± 5	5 ± 2	0.3 ± 0.1	0.124 ± 0.004	0.23 ± 0.09	0.5 ± 0.2	0.35 ± 0.08	<LOD
14	$(21 \pm 1)\cdot 10^2$	103 ± 5	6 ± 2	129 ± 5	0.46 ± 0.09	0.087 ± 0.002	0.10 ± 0.04	0.81 ± 0.08	0.18 ± 0.02	0.35 ± 0.05
15	$(18 \pm 1)\cdot 10^3$	41 ± 4	27 ± 3	77 ± 3	-	-	0.12 ± 0.06	0.9 ± 0.1	0.21 ± 0.05	0.43 ± 0.08
16	$(27 \pm 2)\cdot 10^2$	115 ± 8	42 ± 6	$(21 \pm 1)\cdot 10$	9.4 ± 0.6	0.839 ± 0.006	0.6 ± 0.1	80 ± 5	1.0 ± 0.1	<LOD
17	$(6 \pm 1)\cdot 10^2$	33 ± 7	82 ± 5	22 ± 6	1.9 ± 0.5	0.369 ± 0.007	0.3 ± 0.1	1.3 ± 0.4	0.8 ± 0.1	<LOD
18	$(24 \pm 1)\cdot 10^2$	84 ± 5	14 ± 4	97 ± 6	8.9 ± 0.3	0.367 ± 0.003	0.11 ± 0.08	4.0 ± 0.8	0.18 ± 0.04	0.3 ± 0.1
19	$(10 \pm 1)\cdot 10^2$	17 ± 4	62 ± 7	<LOD	0.6 ± 0.3	0.087 ± 0.006	<LOD	0.5 ± 0.3	<LOD	<LOD
20	$(99 \pm 7)\cdot 10^2$	$(30 \pm 1)\cdot 10$	14 ± 2	$(35 \pm 2)\cdot 10$	4.9 ± 0.3	0.287 ± 0.002	0.9 ± 0.2	2.2 ± 0.3	0.33 ± 0.04	0.6 ± 0.1
21	$(42 \pm 2)\cdot 10^3$	$(4 \pm 1)\cdot 10$	31 ± 8	20 ± 8	0.9 ± 0.3	0.117 ± 0.007	0.3 ± 0.2	0.9 ± 0.3	0.3 ± 0.1	0.4 ± 0.2
22	$(5 \pm 1)\cdot 10^2$	25 ± 5	81 ± 4	16 ± 5	2.2 ± 0.4	0.254 ± 0.006	0.5 ± 0.1	0.7 ± 0.3	0.22 ± 0.09	0.3 ± 0.1
23	$(63 \pm 9)\cdot 10$	37 ± 7	56 ± 4	12 ± 4	<LOD	0.064 ± 0.003	0.3 ± 0.1	0.6 ± 0.3	-	<LOD
24	$(23 \pm 1)\cdot 10^2$	172 ± 8	25 ± 1	$(40 \pm 3)\cdot 10$	3.0 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	2.7 ± 0.4	0.39 ± 0.04	0.5 ± 0.2

-: not quantified due to interferences with other peaks from the matrix

CAPÍTULO II

Determinación de hidrocarburos policíclicos aromáticos en muestras biológicas

Los hidrocarburos policíclicos aromáticos (PAHs) son compuestos orgánicos formados por, al menos, dos anillos aromáticos unidos entre sí. Estos compuestos se liberan al medio ambiente a partir de la combustión incompleta de fuentes naturales (incendios forestales, erupciones volcánicas) y antropogénicas (emisiones de vehículos, humo de cigarrillo, proceso de cocinar). Teniendo en cuenta que algunos de estos procesos están presentes en muchas industrias, los PAHs pueden considerarse marcadores de exposición cuando se detectan niveles elevados de los mismos. En bibliografía se han descrito estos compuestos como marcadores de exposición para trabajadores de hornos de coque, bomberos, trabajadores del aluminio y aquellos expuestos al humo diésel, entre otros. También pueden detectarse dependiendo de la dieta y el consumo de tabaco.

Algunos PAHs han sido clasificados por la Agencia Internacional para la Investigación del Cáncer (IARC) como posibles o probables carcinógenos humanos, aumentando así la preocupación por la exposición a los mismos en todo el mundo. Por ello, existen numerosos trabajos cuyo objetivo es estudiar la relación entre las concentraciones de PAHs presentes en personas expuestas a los mismos y el cáncer y otros problemas de salud. Además, la Comisión Europea ha establecido niveles máximos de PAHs en algunas matrices como comida o productos primarios con aroma a humo.

Los PAHs pueden llegar al cuerpo humano mediante diferentes vías entre las que se encuentran la inhalación de aire contaminado, ingestión o absorción a través de la piel. Una vez en el cuerpo, estos compuestos pueden ser sometidos a diferentes biotransformaciones metabólicas sucesivas como oxidación, hidroxilación o hidratación. De este modo, se generan diferentes derivados de PAHs. Por esta razón, existen muchos trabajos en los que se describe la cuantificación simultánea de metabolitos hidroxilados.

La determinación de PAHs se ha llevado a cabo en diferentes matrices incluyendo aceite vegetal, agua, pescado ahumado, leche y gasolina, así como en

matrices biológicas entre las que se encuentran la orina, sangre, plasma, saliva, aire exhalado, leche materna y pelo, entre otras.

II.1 Determinación de hidrocarburos policíclicos aromáticos en muestras de orina

II.1.1 Artículo de investigación

Análisis cuantitativo y cualitativo de hidrocarburos policíclicos aromáticos en muestras de orina usando un método no separativo basado en espectrometría de masas

Resumen

Existen pocas aplicaciones descritas para la determinación de PAHs sin metabolizar en muestras de orina. Según la bibliografía revisada, las concentraciones habituales de PAHs encontradas en la población expuesta a dichos compuestos se encuentra principalmente en el rango de ppb. Debido a esta baja concentración estos analitos deben ser extraídos y preconcentrados antes de su análisis. La extracción de PAHs en muestras de orina se ha abordado hasta la fecha utilizando diferentes técnicas, entre las cuales se encuentran la microextracción en fase sólida (SPME), la microextracción en fase sólida en la modalidad de espacio de cabeza (HS-SPME), la extracción en fase sólida (SPE) y la microextracción líquido-líquido dispersiva (DLLME).

Las técnicas de separación utilizadas hasta la fecha para el análisis de PAHs en muestras de orina se han basado, fundamentalmente, en cromatografía de gases (GC) con detección mediante espectrometría de masas (MS) y de ionización en llama (*flame ionization detection*, FID). También se ha utilizado la cromatografía líquida de alta resolución (*high performance liquid chromatography*, HPLC) con detección mediante fluorescencia (F).

Una alternativa interesante para el análisis de PAHs en orina que no ha sido muy explorada hasta la fecha es aquella en la cual se utiliza la espectrometría de masas sin separación cromatográfica. Algunas aplicaciones descritas incluyen el análisis de derivados hidroxilados de PAHs utilizando SPE con ionización por electrospray (*electrospray ionization*, ESI) y detección mediante espectrometría de masas en tándem (*tandem mass spectrometry*, MS/MS), así como SPME-MS donde la extracción se lleva a cabo en el interior de un capilar de vidrio con una posterior ionización por ESI (*glass-capillary* nanoESI). A pesar de los buenos resultados obtenidos con estas técnicas su coste es elevado, por lo que no se encuentran disponibles en los laboratorios comunes.

El objetivo de este trabajo fue el desarrollo de un método no separativo rápido y simple para el análisis de 11 PAHs en muestras de orina. Al eliminar la

etapa de separación cromatográfica se pretende reducir el tiempo de análisis requerido por muestra. La sensibilidad del método desarrollado debe de ser adecuada para determinar PAHs en el rango de concentración habitual encontrado en la orina de la población expuesta a los mismos. El método se basó en la extracción líquido-líquido (LLE) seguida del análisis mediante PTV-qMS (*single quadrupole*, q). Posteriormente, los datos obtenidos se analizaron mediante técnicas quimiométricas.

Con el fin de obtener información cuantitativa y cualitativa, el estudio se dividió en dos partes. El método desarrollado se utilizó para determinar la concentración de 11 PAHs en muestras de orina (análisis cuantitativo) y para discriminar muestras con presencia y ausencia de PAHs (análisis cualitativo).

Inicialmente, se optimizaron los parámetros que afectaban a la LLE incluyendo volumen de muestra, naturaleza y volumen de disolvente de extracción, y tiempo de extracción. En condiciones óptimas, 6 mL de orina previamente centrifugada, 225 μL de metanol (para las muestras no dopadas) o 225 μL de una disolución con los 11 PAHs (para las muestras dopadas) y 1 mL de hexano se agitaron en vórtex (2 minutos) y se centrifugaron para conseguir la separación de fases. El extracto orgánico resultante se recogió y se transfirió a un vial para su posterior análisis.

Con respecto a las condiciones instrumentales, se pusieron a punto dos métodos. El primero de ellos fue un método no separativo basado en PTV-qMS que podría ser utilizado como método de criba de muestras en función de la presencia o ausencia de PAHs y para la semicuantificación de los mismos. Para ello, se utilizó en el cromatógrafo de gases una columna de sílice desactivada que actuó como interfase entre el inyector PTV y el MS, evitando la separación de los compuestos de interés. El tiempo requerido para el análisis fue de 3.70 minutos. El segundo fue un método separativo basado en PTV-GC-qMS que incluía separación cromatográfica de los analitos y podría ser utilizado posteriormente

para confirmar los resultados obtenidos con el método anterior. El tiempo de análisis fue de 8.25 minutos. Las condiciones experimentales del inyector fueron semejantes en ambos métodos.

Los resultados que se indican a continuación corresponden a los datos obtenidos con el método no separativo. Una vez analizadas las muestras, el análisis de la información adquirida se enfocó de dos maneras diferentes. En la primera de ellas se llevó a cabo un análisis dirigido para la cuantificación de 11 PAHs en muestras de orina. Para ello, se utilizó la calibración mediante mínimos cuadrados parciales (PLS1). Con el fin de obtener un modelo de calibración adecuado para cada analito teniendo en cuenta la variabilidad de todos los compuestos que pueden contribuir a la señal, se utilizaron 5 muestras de orina diferentes para construir los modelos. Estas orinas fueron previamente analizadas mediante el método separativo y ninguno de los 11 PAHs fue detectado. Las variables independientes en la regresión mediante PLS1 fueron la suma de las intensidades para todos los iones detectados durante la adquisición de datos. Las variables dependientes fueron las concentraciones de PAHs añadidas en las muestras de orina. Se utilizó un diseño experimental con el fin de obtener el conjunto de muestras de orina para calibración. Cada orina se dopó con los 11 analitos de estudio a 5 niveles diferentes de concentración distribuidos de manera uniforme.

Los PAHs de interés presentaban algunas masas solapadas en sus correspondientes espectros, lo que permitió estudiar las posibilidades del método en condiciones complejas.

Para construir los modelos PLS se utilizó la validación cruzada, mediante la cual se generan los modelos de calibración dejando una muestra fuera del mismo, que se predice con dicho modelo repitiéndose el proceso completo hasta que todas las muestras se han predicho una vez. De esta manera, es posible seleccionar el número óptimo de factores PLS para cada calibrado que

proporciona un mínimo error cuadrático medio en la validación (*root mean square error*, RMSEV). Los errores relativos (E %) encontrados inicialmente en la validación cruzada oscilaron entre 14 y 76 %. Sin embargo, su reducción fue posible gracias a la aplicación del criterio de incertidumbre de Martens, que elimina aquellas variables m/z cuyos coeficientes de regresión tienen valores de incertidumbre mayores que el propio valor. Tras aplicar este criterio, el número de factores PLS de los modelos se redujo, simplificándose así dichos modelos, y los errores relativos oscilaron entre el 7 y el 38 %. El mayor error se obtuvo para el bifenilo (38 %) y el acenafteno (35 %), que presentaban masas solapadas en sus espectros haciendo difícil su cuantificación individual. Sin embargo, este error disminuyó hasta un 15 % cuando se construyó un nuevo modelo PLS considerando la suma de concentraciones de ambos analitos.

La repetibilidad y reproducibilidad del método se evaluaron con orina dopada a dos niveles de concentración. Los valores de RSD obtenidos fueron satisfactorios oscilando entre el 9 y el 14 %, respectivamente.

Los límites de detección multivariantes (*multivariate detection limit*, MLD) se determinaron mediante dos estrategias diferentes basadas en la varianza de la concentración predicha por el modelo. La primera fue desarrollada por Faber y Bro y la segunda se basó en la incertidumbre de la predicción proporcionada por el software Unscrambler®. Los valores obtenidos fueron semejantes en ambos casos y se encontraron en el rango de ppb. Teniendo en cuenta que los valores de concentración descritos en bibliografía para la población expuesta a PAHs fueron de ese orden el método propuesto podría ser de utilidad en el análisis de dichos compuestos en muestras de orina.

Para comprobar la validez del método se llevó a cabo el análisis de 5 muestras de orina que no habían participado en los modelos de calibración. No se encontró ninguno de los analitos de interés en estas muestras. Este resultado fue confirmado utilizando la metodología separativa basada en PTV-GC-qMS.

Estas muestras se doparon con todos los analitos a dos niveles diferentes de concentración y se llevó a cabo su predicción con los modelos PLS de calibración. Los errores relativos encontrados oscilaron entre el 6 y el 20 %, mostrando la aplicabilidad del método no separativo para la cuantificación, o al menos semicuantificación, de PAHs en orina.

El segundo enfoque del trabajo tenía como objetivo la discriminación de muestras mediante un análisis no dirigido. Para ello, se utilizaron técnicas de reconocimiento de pautas no supervisadas y supervisadas para la discriminación de muestras con presencia o ausencia de PAHs. En las técnicas de reconocimiento de pautas supervisadas, se dividió el grupo de muestras en dos: entrenamiento (49 muestras: 15 no dopadas y 34 dopadas) y validación (30 muestras: 12 no dopadas y 18 dopadas). En total, se analizaron 79 muestras de orina (27 no dopadas y 52 dopadas) cuya señal de perfil se sometió a un proceso de normalización interna.

La técnica de reconocimiento de pautas no supervisada utilizada en este trabajo fue el análisis de componentes principales (PCA) y se llevó a cabo en el conjunto completo de muestras. Se obtuvo una varianza explicada (acumulada) del 45, 75, 88 y 93 % para los cuatro primeros PCs, respectivamente. Se observó una clara separación entre muestras dopadas y no dopadas. Solo 5 muestras de orinas sin PAHs se encontraban cerca de las orinas dopadas.

Las técnicas de reconocimiento de pautas supervisadas utilizadas en este trabajo fueron las siguientes: modelado independiente de clases (SIMCA), análisis discriminante lineal (LDA), análisis discriminante-mínimos cuadrados parciales (PLS-DA) y técnica de las muestras apoyadas en la frontera (SVM).

En la técnica SIMCA se construyó un modelo de clasificación con las muestras de entrenamiento seleccionando las relaciones m/z con valores óptimos para el poder discriminante (superior a 5.5) y el poder de modelado (superior a 0.95), que proporcionaron una buena separación entre ambos grupos de muestras (dopadas y no dopadas). A continuación, se llevó a cabo la clasificación de las

muestras que formaban el grupo de validación. Todas las muestras se clasificaron correctamente excepto una muestra dopada, reconocida como *outlier*.

Debido a que el número de variables m/z fue mayor que el número de muestras en el grupo de entrenamiento se utilizó PCA-LDA para llevar a cabo el análisis discriminante lineal. Se utilizó el grupo de muestras de entrenamiento para construir modelos con 3, 4, 5 y 6 PCs, obteniendo una clasificación correcta del 100 % de las muestras de entrenamiento usando los modelos con 5 y 6 PCs. Las m/z que más contribuían en el modelo correspondieron con el pico base de los espectros correspondientes a los analitos de estudio. Para evitar un sobreajuste de los datos se seleccionó el modelo con 5 PCs como óptimo. Se aplicó el modelo a la clasificación del conjunto de muestras de validación externa y todas ellas fueron asignadas correctamente.

En el análisis mediante PLS-DA se construyó inicialmente un modelo de clasificación con las muestras de entrenamiento utilizando todas las variables m/z analizadas. Posteriormente, este modelo se mejoró utilizando el criterio de incertidumbre de Martens, reduciendo el número de variables. Cuando se aplicó el modelo óptimo con el fin de predecir el conjunto de muestras de validación todas se clasificaron satisfactoriamente.

Finalmente, se llevó a cabo un análisis de los datos mediante SVM. Se utilizó una función lineal con el conjunto de muestras de entrenamiento y se optimizó, mediante validación cruzada, el parámetro C que controla la compensación entre los errores encontrados en la clasificación de las muestras del modelo y la maximización del margen que separa los grupos de muestras que se quieren diferenciar. Para valores bajos de C el número de vectores soportados (SVs) en el modelo final era mayor mientras que valores altos suponían un aumento del número de muestras del conjunto de entrenamiento mal clasificadas. Una vez seleccionados los valores óptimos para C y SVs (3.3 y 10,

respectivamente) se utilizó el modelo para predecir las muestras de validación. Todas ellas fueron clasificadas correctamente.

En conclusión, gracias a las señales de perfil obtenidas en el análisis de PAHs en orinas mediante PTV-qMS se ha obtenido información cuantitativa y cualitativa. Por un lado, el uso de un diseño experimental multivariante para llevar a cabo la calibración ha permitido la determinación simultánea de 11 PAHs en muestras de orina, resaltando la fiabilidad de la técnica para la semicuantificación en mezclas. Por otro lado, el uso de técnicas de reconocimiento de pautas es una solución simple y efectiva para la diferenciación de orinas con presencia o ausencia de PAHs. No se encontraron falsos negativos o positivos en ningún caso. Solo una muestra dopada se clasificó como *outlier* en SIMCA.

Teniendo en cuenta que los límites de detección de la técnica son del orden de concentración descrito para personas expuestas a PAHs, este método podría ser utilizado como criba para una discriminación rápida entre muestras con presencia o ausencia de PAHs.

Finalmente, cabe destacar la rapidez del método debida a la ausencia de separación cromatográfica, a la simple preparación de muestra y a la obtención de información cuantitativa y cualitativa en un único análisis. Esto hace que el acoplamiento PTV-qMS sea una herramienta prometedora para el análisis de compuestos no volátiles en muestras de orina.

II.1.1 Artículo de investigación

Análisis cuantitativo y cualitativo de hidrocarburos policíclicos aromáticos en muestras de orina usando un método no separativo basado en espectrometría de masas

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Quantitative and qualitative analysis of polycyclic aromatic hydrocarbons in urine samples using a non-separative method based on mass spectrometry

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ABSTRACT

In this work, a method for the quantitative and qualitative analysis of 11 polycyclic aromatic hydrocarbons (PAHs) in urine samples is reported. The method is based on the coupling of a programmed temperature vaporizer (PTV) with a quadrupole mass spectrometer (qMS), via a deactivated fused silica tubing. Before the PTV-qMS analysis, the samples were subjected to a liquid-liquid extraction (LLE).

The method was rapid since no chromatographic separation was performed. The samples were introduced directly into the PTV, and the analytes were trapped in the Tenax-TA® packed liner while the solvent was purged. After that, all the compounds reached the mass spectrometer, obtaining the fingerprint of the analysed samples.

Urine samples free of PAHs and the same samples spiked with the compounds were analysed. The resulting profile signals were used to quantify the analytes using multivariate calibration, and to classify the samples according to the presence or absence of the PAHs. In the latter task, non-supervised and supervised pattern recognition techniques were employed. The calibration models worked satisfactorily and errors lower or equal to 15% were obtained, in most cases, when an external validation set was analysed. Regarding the classification of the samples, most of the supervised pattern recognition techniques provided excellent results (100% success), where all of the samples were classified correctly.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that consist of, at least, two fused aromatic rings. These ubiquitous contaminants are released into the atmosphere by incomplete combustion from both natural (forest fires, volcanic eruptions) and anthropogenic (vehicle emissions, cigarette smoke, cooking) sources. Since these processes are present in many industries, PAHs have been considered as exposure markers where higher levels of these compounds can be detected, for example, in different types of workers such as coke oven workers [1–5], firefighters [6,7], aluminium workers [8], and those workers exposed to diesel exhaust [9]. These compounds have also been detected depending on diet [10,11] and smoking habits [12].

Several PAHs have been classified by the International Agency for Research on Cancer (IARC) as possible or probable human carcinogens [13], raising great health concerns all over the world. For this reason, many studies are aimed at associating the risk of cancer [14,15] and the presence of other adverse health effects [16–18] with the concentration of PAHs found in people exposed to these compounds. In addition, the European Commission has established maximum levels for PAHs in several matrixes, for instance in food [19] and primary smoke products [20]. The maximum levels permitted are in the range of μg kg^{-1}.

Once PAHs have entered the human body by the inhalation of contaminated air, ingestion or dermal absorption, they can be subjected to successive metabolic biotransformations, including oxidation, hydroxylation and hydration, and generate derivatives of the corresponding PAHs. This is why most studies report the simultaneous quantification of hydroxylated metabolites [2,4–7,10,12]. However, the determination of unmetabolized PAHs is less explored. Very few applications have been found in the literature for determining unmetabolized parent compounds in urine [1,3,4,8,9,11]. The concentrations of PAHs for people exposed to these analytes found in literature have been reported to be mostly in the range of μg L^{-1} [1,3,4,21–24]. In addition, the analysis of PAHs has been performed in other matrices during the last few years, including hair [25], blood and plasma [26], edible vegetable oil [27], water [28], smoked fish [29], milk [30] and gasoline [31].

Because these compounds are present at trace concentrations, they must be extracted from the matrix and preconcentrated before analysis.

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The issue of extracting PAHs from urine has been approached by using headspace-solid phase microextraction (HS-SPME) [1,4,9,21–23], dispersive liquid-liquid microextraction (DLLME) [32], solid phase microextraction (SPME) [3] and solid phase extraction (SPE) [8,24,33].

A number of analytical methods, including those based on gas chromatography-mass spectrometry (GC-MS) [1,3,4,9,22–24,33], gas chromatography-flame ionization detection (GC-FID) [21,32] and high performance liquid chromatography-fluorescence detection (HPLC-F) [8], have been developed to analyse PAHs in urine samples.

An interesting alternative that has not been particularly explored, to date, is the use of mass spectrometry detection without chromatographic separation for the analysis of PAHs in urine samples. Some examples of this approach include the analysis of the hydroxylated metabolites of polycyclic aromatic hydrocarbons using solid phase extraction-electrospray ionization tandem mass spectrometry (SPE-ESI-MS/MS) [34] and solid phase microextraction-glass capillary nanoelectrospray ionization with a hybrid triple quadrupole/linear ion trap mass spectrometer (SPME-nanoESI-MS) [35]. Although good results have been obtained with these techniques, they are expensive and not available in all laboratories.

In this study, a rapid, simple and non-separative method for the analysis of 11 PAHs in urine samples is proposed. The aim of this work was to reduce analysis time, as well as to obtain low detection limits that allow the determination of the PAHs in the urine of people exposed to these analytes in the common concentration range. The method is based on liquid-liquid extraction (LLE) and subsequent analysis with a programmed temperature vaporizer and a quadrupole mass spectrometer (PTV-qMS) followed by chemometric techniques. In order to assess the potential of the proposed method, the study was divided into two different tasks with the aim to obtain both quantitative and qualitative information. To this end, the method was used to determine the concentration of 11 PAHs in urine samples (quantitative analysis) and to discriminate those samples with and without PAHs (qualitative analysis). To the best of our knowledge, this is the first time a programmed temperature vaporizer coupled with quadrupole mass spectrometer has been used to analyse unmetabolized PAHs in urine samples.

2. Experimental

2.1. Reagents and stock solutions

The standards of naphthalene, 2-methylnaphthalene, biphenyl, 4-phenyltoluene, fluorene, phenanthrene and fluoranthene were supplied by Acros Organics (Geel, Belgium). The standards of acenaphthylene, acenaphthene, chrysene, benzo(k)fluoranthene and methanol were supplied by Sigma-Aldrich (Steinheim, Germany). N-hexane was purchased from Scharlab (Barcelona, Spain). The purity of all the reagents was at least 96%.

Stock solutions of 100 mg L^{-1} of each analyte were prepared in methanol, except for chrysene, which was prepared in acetone. Individual stock solutions of each analyte (6–20 mg L^{-1}) for subsequent dilutions were prepared in methanol. All the solutions were stored at 4 °C.

2.2. Urine samples

Human urine samples were collected from 27 adults (13 women, 14 men), aged between 27 and 83 years, in a disposable sterile specimen collection cup. The samples were frozen and stored at −20 °C in the dark prior to use. The pH range varied from 4.6 to 8.1. A GC-qMS analysis confirmed the absence of the studied analytes in the samples. These 27 urine samples were spiked at different concentration levels (1.09–58.05 µg L^{-1}) by adding 225 µL of the corresponding solution containing the 11 PAHs of study in order to obtain the spiked urine group.

Before liquid-liquid extraction, the urine samples were thawed at room temperature and transferred to a 12-mL polypropylene tube (Scharlab). The urines were centrifuged at 1811 × g for 10 min and after that, the sediment was eliminated.

Written informed consent was obtained from each volunteer.

2.3. Liquid-liquid extraction

Liquid-liquid extraction of the urine samples was carried out by transferring 6 mL of urine, 225 µL of methanol (non-spiked samples) or 225 µL of a solution with the PAHs (spiked samples) and 1 mL of hexane to a 15-mL glass centrifuge tube with a PTFE screw cap (Scharlab). After vortexing for 2 min (maximum setting), the sample was centrifuged at 2415 × g for 5 min to separate the organic and aqueous phases. The organic extract was collected and placed in a GC vial (Scharlab).

2.4. PTV-qMS conditions

The vial that contained the organic extract was placed in a PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) equipped with two trays, each with 21 positions for holding the samples. The injection of the sample was carried out with a programmed temperature vaporizer (PTV) inlet (CIS-4, Gerstel, Baltimore, MD) using a liner (71 mm x 2 mm) packed with Tenax-TA®. The injection volume was 30 µL. The operating mode selected was solvent vent. The injector was set at 115 °C for 0.46 min, with a vent flow of 150 mL min^{-1} at 6.00 psi. After eliminating the solvent, the split valve was closed and the liner was heated (12 °C s^{-1}) until 340 °C (injection time: 1.75 min) for desorbing and transferring the analytes to the column. Then, the split valve was opened and the final temperature was held for 2.5 min for cleaning the system. A purge flow of 150 mL min^{-1} was used. The cooling of the PTV system was accomplished with liquid CO_2.

An Agilent 6890 GC device was equipped with an ultimate plus deactivated fused silica tubing (30 m x 0.250 mm) from J&W Scientific (Folsom, CA, USA) as the interface between the PTV inlet and the qMS, which was maintained at 340 °C throughout the signal recording time of 3.70 min. Thus, the analytes reached the detector without separation. Additionally, approximately 2.20 min were needed to re-establish the initial conditions of the PTV inlet; therefore, the analysis time per sample was in the region of 6 min. The carrier gas was helium N50 (99.999% pure, Air Liquide).

The detector used was a quadrupole mass spectrometer (HP 5973 N) equipped with an inert ion source. It was operated in electron-ionization mode (ionization voltage: 70 eV). Ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Data acquisition was performed in full scan mode (0.71 scan s^{-1}). A solvent delay of 1.10 min was established. The m/z range was 35–300 amu.

2.5. PTV-GC-qMS conditions

This method was used to check the urine samples for the presence or absence of the PAHs included in the study.

The experimental conditions for the PTV inlet were the same as those described for the non-separative methodology. To perform the GC-qMS measurements, the GC device was equipped with a HP5-MS UI capillary column (30 m x 0.250 mm x 0.25 µm) from J&W Scientific (Folsom, CA, USA). The initial oven temperature was 60 °C (0.5 min). This was increased at a rate of 60 °C min^{-1} to 175 °C and then further increased at 45 °C min^{-1} to 325 °C. This temperature was held for 2.5 min. The total chromatographic run time was 8.25 min. Additionally, about 8 min were needed to achieve the initial conditions of the programmed temperature vaporizer and gas chromatograph; therefore, the time between sample runs was 17 min.

The analyses were performed in a synchronous SIM/scan mode, allowing the collection of both SIM and full scan data in a single run. A

solvent delay of 2.50 min was established. The m/z range was 35–300 amu. One scan group with a data acquisition speed value corresponding to 15.82 scan s^{-1} was used for compound identification. The target compounds were identified by comparison of the experimental spectra with those found in the NIST'08 database (NIST/EPA/NIH Mass Spectral Library, version 2.0). In the SIM mode, a dwell time value of 1 ms was established for all the m/z ratios (the three most abundant ones for each analyte).

2.6. Data analysis

Data collection was performed with the Enhanced ChemStation software [36] from Agilent Technologies. Chemometric techniques were implemented with the Unscrambler® statistical package [37].

3. Results and discussion

Both targeted and non-targeted analyses were performed on the profile signals obtained from direct injection of the sample into the quadrupole mass spectrometer via a deactivated fused silica tubing.

3.1. Targeted analysis: quantification of eleven PAHs

3.1.1. Partial Least Squares (PLS) calibration

With the aim of obtaining a suitable calibration model for each analyte, and taking into account the variability of the compounds that contribute to the signal, 5 different urine samples were used to build the models. Independent variables in the partial least squares regression (PLS1) were the sum of the intensities of all the ions detected during data acquisition. Dependent variables were the added concentrations of the studied compounds. The 5 urine samples were previously analysed using the method based on GC-qMS and none of the 11 PAHs were detected.

The calibration standards set for the urine samples was designed using a multilevel multifactor design. An eleven-component experimental design (11 PAHs studied) at five uniformly distributed concentration levels was used. Thus, the total number of calibration samples was 25. All samples had uncorrelated concentrations, which meant that orthogonality between components was ensured [38]. A cyclic generator (−2, 1, 2, 1, −2), a repeater of 0, and a difference vector (0 2 3 1) were used to obtain the design. Table S1 (see Supplementary Information) shows the concentration data for the 11 PAHs. Each urine sample was spiked at 5 different concentration levels.

The selected analytes showed overlapping mass spectra in some m/z ratios, such as 76, 115, 150–155 and 165–170, among others, which allowed them to be used to check the possibilities of the method under complex situations.

PLS models were built for each analyte using the whole m/z range (35 − 300). Cross-validation (leave one out) was used to select the optimum number of PLS components corresponding to the minimum root mean standard error of validation (*RMSEV*). Selected PLS components and the relative error (E %) for each analyte are shown in Table 1. This error is expressed as (1):

$$E(\%) = \frac{RMSEV}{\bar{c}} x100 \quad (1)$$

where $\bar{c}$ is the average of the added concentration for each analyte studied.

The E % values ranged between 14% and 76% in the cross-validation step, which were reduced by using the Martens uncertainty criterion [37] as a variable selection method. This eliminated all the m/z ratios whose regression coefficients had uncertainty values higher than the absolute value from the model. After applying this criterion, the optimum number of PLS factors decreased, simplifying the models, and the relative error (E %) ranged between 7% and 38%, as shown in Table 1. Table S2 shows the m/z ratios selected for each analyte after applying this criterion. In Fig. 1 the PLS model with all the m/z ratios (Fig. 1A) and the model obtained after using the Martens uncertainty criterion (Fig. 1B) for benzo(k)fluoranthene are shown. The highest values for E % corresponded to biphenyl (38%) and acenaphthene (35%). Both analytes have a very similar mass spectrum with significant overlapping in almost all of the m/z ratios, implying that the individual quantification of both analytes provided high errors. However, a relative error of 15% was obtained when a new model was built considering the sum of concentrations for both analytes instead of individual concentrations.

Table 1
Number of optimum PLS factors and relative predictive error (E %) in the calibration step (cross-validation) with all the m/z variables and with the m/z selected by the Martens uncertainty criterion. The relative predictive error (E %) and bias when the optimum PLS1 models were used to predict the external validation set are also shown.

	Calibration set				External validation set	
	All the m/z variables (35–300)		Martens uncertainty criterion			
Compound	PCs	E %	PCs	E %	E %	Bias
(1) Naphthalene	12	17	5	10	19	2.69
(2) 2-methylnaphthalene	13	45	5	10	18	0.12
(3) Biphenyl	2	75	4	38	50	1.25
(4) Acenaphthene	1	76	5	35	47	−1.35
(3+4) Biphenyl +acenaphthene	13	16	4	15	17	−0.17
(5) Acenaphthylene	11	33	2	21	20	−0.02
(6) 4-phenyltoluene	18	28	2	11	12	0.22
(7) Fluorene	16	63	3	11	14	0.08
(8) Phenanthrene	18	27	5	7	10	0.02
(9) Fluoranthene	20	23	2	8	14	0.34
(10) Chrysene	14	21	3	9	13	−0.22
(11) Benzo(k) fluoranthene	14	14	3	8	6	0.13

The repeatability and reproducibility of the method were evaluated at two different concentration levels using spiked urine samples. The concentration levels provided a S/N ratio of approximately 10 and 100. The most abundant m/z ratio for each compound was used. Repeatability was checked by extracting the sample and injecting it into the system 8 times on the same day. In all cases, the relative standard deviation (RSD) was lower than or equal to 9%, indicating a satisfactory repeatability. The results are shown in Table 2. To evaluate reproducibility, extraction and injection were performed 8 times per day on 2 days; values less than 14% were obtained. Since good results were obtained in terms of repeatability and reproducibility, no internal standard was used.

The multivariate detection limits (MDL) were obtained using two different strategies based on the variance of the concentration predicted by the model [39]. The first strategy was developed by Faber and Bro [40], and the second one is based on the prediction uncertainty provided by the Unscrambler [37,41]. Eight urine samples, spiked with the analytes and providing a S/N ratio of approximately 10, were used to calculate the MDL. The detection limits were in the μg L^{-1} range, as shown in Table 2, and similar values were obtained with both strategies. Taking into account that the concentration values found in other works [1,3,4,21–24] are normally in the range of μg L^{-1}, corresponding to urine samples from people exposed to these types of compounds, the proposed method could therefore be suitably applied.

In previous works where unmetabolized PAHs in human urine were determined [1,3,4,8,9,21–24,33], the preconcentration technique most frequently used was based on an adsorption/desorption process in a fibre at high temperature, which increased the overall analysis time (53–80 min). When DLLME [32] and nanoparticles [24] were used, the time needed for the extraction of the analytes was found to be similar to

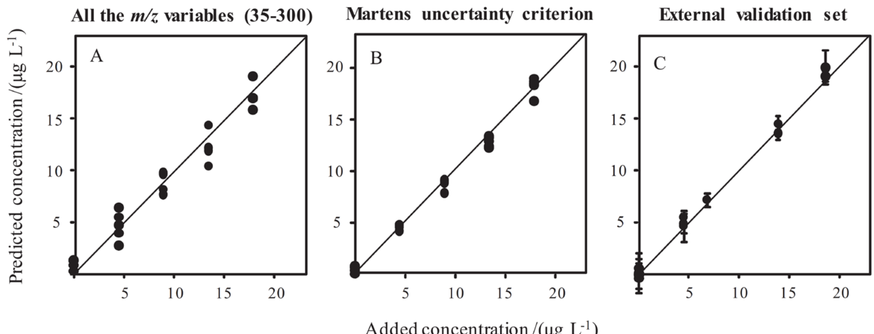


Fig. 1. Correlation plots of predicted vs added concentrations for benzo(k)fluoranthene: (A) in the calibration step (cross-validation) when all the *m/z* variables (35 − 300) were used; (B) in the calibration step (cross-validation) after applying the Martens uncertainty criterion; (C) in the validation step when the model with the selected variables was used to predict an external set of samples.

the one used in this work (2 min). In all the cited works, chromatographic methods were reported, and the corresponding chromatographic run time was longer (18–66 min) than the run time employed in the present study (3.70 min).

One of the advantages of separative methods is that lower detection limits (ng L^{-1}-μg L^{-1} range) can be achieved. However, the entire analysis time is at least five times longer than the time required for the non-separative method proposed here, making this method a promising tool to consider for the satisfactory determination of PAHs in urines from exposed people in the common concentration range of μg L^{-1}.

3.1.2. Sample analysis

The method was applied to the analysis of 5 urine samples (3 women, 2 men) which had not been included in the calibration step. None of the analytes were found in the samples. These results were confirmed using the separative methodology (GC-qMS). To assess the potential of the method using samples containing the analytes included in this study, the aforementioned urine samples were spiked at different concentration levels ranged between 1.09 and 58.05 μg L^{-1} (each urine was spiked at 2 different levels). The relative error (E %) and the bias are shown in Table 1, and ranged between 6% and 20% and − 0.22 and 2.69 μg L^{-1}, respectively (for biphenyl and acenaphthene, only the model considering the sum of the concentrations was taken into account, as previously described). The predicted concentrations obtained for this validation set, when using the PLS model for benzo(k)fluoranthene after applying the Martens uncertainty criterion, are shown in Fig. 1C. These results support the applicability of the non-separative method for the quantification, or at least semi-quantification, of these compounds in urine.

3.2. Non-targeted analysis: discrimination of samples

Both non-supervised and supervised pattern recognition techniques were used to discriminate the presence of the analytes in the samples. The 27 non-spiked urine samples, and the same samples spiked at different concentration levels within the calibration range previously mentioned (Table S1), were used.

When using the supervised pattern recognition techniques, the set of samples was divided into two groups: training and validation sets. The sets of samples are shown in Table 3. The training set (49 samples) consisted of 15 non-spiked urine samples and 34 spiked urine samples. The validation set (30 samples) consisted of 12 non-spiked urine samples and 18 spiked urine samples. Consequently, the total number of samples in this part of the work was 79 (49 + 30), 27 of them corresponding to non-spiked urine samples and the 52 remaining samples corresponding to spiked urine samples.

Before using the pattern recognition techniques, the profile signals were subjected to an internal normalization process, which consists of

Table 2
Repeatability, reproducibility and multivariate detection limits.

	Repeatability		Reproducibility		Detection limits (μg L^{-1})	
Compound	S/N 10	S/N 100	S/N 10	S/N 100	MLD1*[a]	MLD2*[b]
(1) Naphthalene	5	5	11	5	8.21	13.32
(2) 2-methylnaphthalene	5	7	14	8	1.63	2.16
(3) Biphenyl	6	6	10	5	1.54	2.67
(4) Acenaphthene	8	7	12	6	4.89	4.86
(3+4) Biphenyl+acenaphthene	7	6	11	5	6.91	3.00
(5) Acenaphthylene	5	4	9	3	1.42	3.99
(6) 4-phenyltoluene	6	3	11	3	0.64	2.06
(7) Fluorene	6	9	11	9	0.83	1.01
(8) Phenanthrene	4	4	10	5	3.40	0.69
(9) Fluoranthene	2	2	6	2	0.62	1.05
(10) Chrysene	3	3	8	4	0.58	1.59
(11) Benzo(k)fluoranthene	4	5	11	6	2.61	3.02

[a]MLD1*: Strategy based on the prediction uncertainty provided by The Unscrambler.
[b]MLD2*: Strategy developed by Faber and Bro.

Table 3
Samples used for the non-targeted analysis.

	Training set	Validation set	Total
Non-spiked samples	**15**	**12**	**27**
Spiked samples	13 samples spiked at two levels = 26 2 samples spiked at four levels = 8 Total = 26+8 = **34**	6 samples spiked at two levels = 12 6 samples spiked at one level = 6 Total = 12+6 = **18**	**52**
Total	15+34 = **49**	12+18 = **30**	**79**

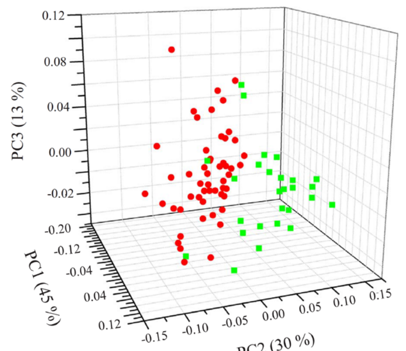


Fig. 2. PCA score plot for the 79 urine samples. Red circles correspond to urine samples spiked at different concentrations with the 11 PAHs and green squares to the non-spiked urine samples.

the expression of each m/z ratio as a percentage of the maximum value for each sample.

3.2.1. Principal component analysis (PCA)

PCA was performed on all the samples (27 non-spiked and 52 spiked urine samples, see Table 3). The cumulative explained variance with the first four PCs was 45%, 75%, 88% and 93%. As shown in Fig. 2, there was a clear separation between both groups when the first three PCs were plotted. Only five samples, corresponding to the urine that did not contain any of the PAHs, seemed to be very close to the spiked samples.

3.2.2. Soft independent modelling of class analogy (SIMCA)

In the first step, a classification model was built with the samples from the training set. When all the m/z variables (35 − 300) were considered, some samples (27 of the 49 samples) from both groups were in the region of the Coomans diagram common to the two classes. The other samples were located within the limits of their classes. To improve the model, only m/z ratios with a discriminant power higher than 5.5 and a modelling power higher than 0.95 were selected. This diminished the number of variables from 266 to 40. The selected variables are shown in Table S3. The optimal number of PCs was 4 and 5 for non-spiked and spiked groups, respectively. This model allowed the correct classification of all the samples in the training set (filled red circles for spiked urine samples and filled green squares for non-spiked urine samples) as shown in Fig. 3 (1% as the significance level). When the model was used to classify the validation set (empty red circles for spiked urines and empty green squares for non-spiked urines), all samples were within the limits of their class (Fig. 3). Only one sample (one spiked sample) was recognized as an outlier.

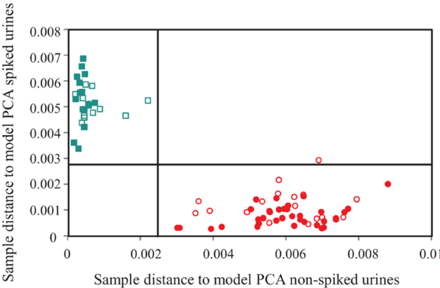


Fig. 3. Coomans plot for the classification model constructed with the training set (filled red circles for spiked urine samples and filled green squares for non-spiked urine samples) and for the prediction of the external validation set (empty red circles for spiked urines and empty green squares for non-spiked urines).

3.2.3. Linear discriminant analysis (LDA)

Due to the fact that the number of variables (m/z ratios) was larger than the number of samples in the training set, PCA-LDA had to be used. Models with 3, 4, 5 and 6 PCs were performed using the samples from the training set. The results are shown in Table 4. The best results (100% success) were obtained with 5 and 6 PCs. In order to know the contribution of each of the variables (m/z), the corresponding loadings used to compute the first three PC scores for the analysis are shown in Fig. 4A. As can be seen in the figure, some of the m/z ratios with the largest proportion coincided with the base peak of the spectrum corresponding to the understudy analytes (m/z 128, 142, 152, 153, 154, 178, 202 and 252 among others). To avoid overfitting of the data, the model with 5 PCs was used to predict the validation set. None of the samples were misclassified, as can be seen in Fig. 4B.

3.2.4. Partial least squares-discriminant analysis (PLS-DA)

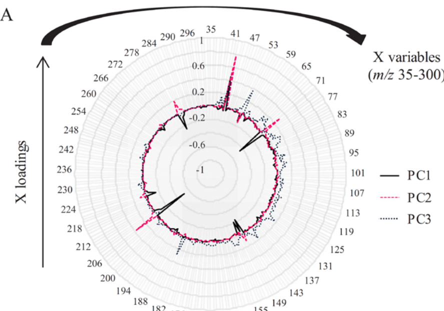
In the first step, a classification model was built with all the variables (m/z 35–300). To improve the model, the Martens uncertainty criterion was used, reducing the variables from 266 to 79. The selected variables are shown in Table S3. The optimal number of PLS factors in the model was 5. When the model was applied to the validation set, a satisfactory classification was obtained as shown in Fig. 5.

3.2.5. Support vector machines (SVM)

Linear function was used on the training set. Different values of parameter C ranging between 0 and 5 were tested using cross-validation (6 segments). The parameter C controls the trade-off between errors in the training samples and margin maximization. The highest accuracy value was 98%, which was achieved with 3.3 ≤ C ≤ 4.4. Lower values of C involved a high number of SVs in the final model and higher values produced an increase in the number of misclassified samples in the training set. A value of C = 3.3 was selected. The number of SVs in the model with this value was 10 of the 49 samples in the training set (7 spiked samples and 3 non-spiked samples). When this model was used to predict the validation set, all samples were classified correctly.

Table 4
Misclassification of samples in the training set when PCA-LDA is used. The results obtained with the best model are in bold.

PCs	False positives	False negatives	% Correct assignation
3	2	0	93
4	2	0	93
5	**0**	**0**	**100**
6	0	0	100



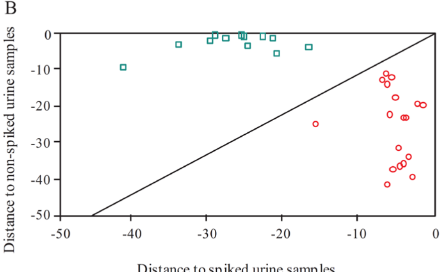


Fig. 4. Linear discriminant analysis (PCA-LDA). (A) Plot of the PCA loadings of each of the 266 variables (*m/z* 35–300) for the first three PCs. (B) Plot of the discriminant scores for the prediction of the external validation set with the PCA-LDA (5 PCs) model (empty red circles correspond to the spiked urine samples and empty green squares to the non-spiked urine samples).

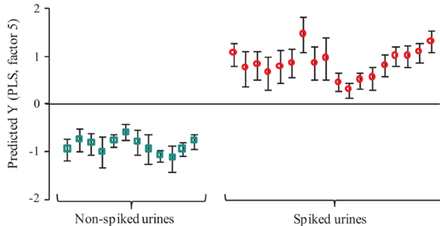


Fig. 5. Predicted values and deviation of the external validation set when PLS-DA was used (empty red circles correspond to the spiked urine samples and empty green squares to the non-spiked urine samples).

4. Conclusions

The urinary fingerprints provided by the PTV-qMS analysis have allowed both quantitative and qualitative information to be obtained. On one hand, the use of a multivariate experimental design for calibration has permitted the determination of 11 PAHs in urine samples; the results show that a PTV-qMS coupling with multivariate calibration constitutes a reliable technique for simultaneous quantification in mixtures. On the other hand, the use of pattern recognition chemometric techniques is a simple and effective solution for the detection of PAHs in urine samples. Neither false negatives nor false positives were found in any of the cases, while only one spiked sample was detected as an outlier when using SIMCA. The method could be used as a screening tool for the rapid discrimination between individuals with PAHs concentrations in the range of μg L^{-1}, which is the usual concentration level found in people exposed to these types of compounds.

Furthermore, the method is very rapid, since no chromatographic separation is performed, allowing a considerable amount of time to be saved. In addition, both qualitative and quantitative information are obtained simultaneously in the same analysis (only one analysis per sample). Sample preparation is simple, since the procedure only requires a liquid-liquid extraction prior to analysis. These characteristics make the method quick, allowing a large number of samples to be processed due to its high sample throughput.

Additionally, it should be emphasized that the PTV-qMS coupling can be considered as a promising tool for the analysis of non-volatile compounds in urine samples.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.01.032.

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1 **Supplementary Information**

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6 Quantitative and qualitative analysis of polycyclic aromatic 7 hydrocarbons in urine samples using a non-separative method based 8 on mass spectrometry

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26 **Table S1**

27 Concentration data of the 11 PAHs* used in the calibration step.

	Concentration (µg L^{-1})*										
solution	1	2	3	4	5	6	7	8	9	10	11
1	29.03	4.31	2.19	4.12	4.29	4.33	2.65	2.18	3.06	4.74	8.93
2	29.03	0	0	8.24	8.59	2.16	2.65	1.09	1.53	7.1	17.86
3	0	0	4.38	4.12	2.15	8.65	1.32	1.09	4.59	9.47	13.4
4	0	8.62	1.09	2.06	8.59	4.33	1.32	3.26	6.12	7.1	8.93
5	58.05	2.16	4.38	2.06	4.29	2.16	3.97	4.35	4.59	4.74	17.86
6	14.51	8.62	2.19	6.18	2.15	2.16	5.29	3.26	3.06	9.47	17.86
7	58.05	4.31	1.09	8.24	2.15	6.49	3.97	2.18	6.12	9.47	0
8	29.03	2.16	1.09	6.18	6.44	8.65	2.65	4.35	6.12	0	13.4
9	14.51	2.16	3.28	4.12	8.59	6.49	5.29	4.35	0	7.1	0
10	14.51	6.47	4.38	8.24	6.44	4.33	5.29	0	4.59	0	8.93
11	43.54	8.62	3.28	8.24	4.29	8.65	0	3.26	0	4.74	13.4
12	58.05	6.47	2.19	0	8.59	8.65	3.97	0	3.06	7.1	13.4
13	43.54	4.31	4.38	6.18	8.59	0	0	2.18	4.59	7.1	4.47
14	29.03	8.62	4.38	0	0	6.49	2.65	3.26	4.59	2.37	0
15	58.05	8.62	0	4.12	6.44	0	3.97	3.26	1.53	0	4.47
16	58.05	0	3.28	6.18	0	4.33	3.97	1.09	0	2.37	8.93
17	0	6.47	0	6.18	4.29	6.49	1.32	0	1.53	4.74	0
18	43.54	0	2.19	2.06	6.44	6.49	0	1.09	3.06	0	0
19	0	4.31	3.28	0	6.44	2.16	1.32	2.18	0	0	17.86
20	29.03	6.47	3.28	2.06	2.15	0	2.65	0	0	9.47	4.47
21	43.54	6.47	1.09	4.12	0	2.16	0	0	6.12	2.37	17.86
22	43.54	2.16	0	0	2.15	4.33	0	4.35	1.53	9.47	8.93
23	14.51	0	1.09	0	4.29	0	5.29	1.09	6.12	4.74	4.47
24	0	2.16	2.19	8.24	0	0	1.32	4.35	3.06	2.37	4.47
25	14.51	4.31	0	2.06	0	8.65	5.29	2.18	1.53	2.37	13.4

28 *11 PAHs of study: 1: naphthalene; 2: 2-methylnaphthalene; 3: biphenyl; 4: acenaphthene; 5:
29 acenaphthylene; 6: 4-phenyltoluene; 7: fluorene; 8: phenanthrene; 9: fluoranthene; 10:
30 chrysene; 11: benzo(k)fluoranthene.

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42 **Table S2**

43 Selected *m/z* ratios for the PAHs in the PLS1 models after applying the Martens
44 uncertainty criterion.

Compound	*m/z* ratios selected by the Martens uncertainty criterion
(1) Naphthalene	64, 91, 102, 127, 128
(2) 2-methylnaphthalene	91, 115, 119, 141, 142
(3) Biphenyl	150, 151, 152, 153, 154, 155
(4) Acenaphthene	150, 151, 152, 153, 154, 155
(3+4) Biphenyl+acenaphthene	109, 123, 137, 153, 154, 177
(5) Acenaphthylene	152, 154
(6) 4-phenyltoluene	107, 168
(7) Fluorene	163, 165, 166, 180, 230
(8) Phenanthrene	76, 88, 89, 174, 176, 177, 178, 179, 245
(9) Fluoranthene	100, 101, 174, 175, 198, 199, 202
(10) Chrysene	226, 228, 229
(11) Benzo(k)fluoranthene	69, 124, 126, 248, 250, 251, 252, 253

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67 **Table S3**

68 The *m/*z ratios selected for each chemometric technique.

Chemometric technique	*m/z* selected
SIMCA	44, 45, 50, 55, 62-64, 74-76, 81-83, 87, 95, 97, 104, 109, 111, 123, 140, 149, 150, 152, 153, 170, 185, 186, 244, 258, 259, 264, 267, 277, 281-283, 287, 298, 299
PLS-DA	44, 45, 49-52, 58, 62-65, 74-78, 87, 88, 100-102, 104, 113-115, 126-128, 139-142, 149-156, 165-170, 174, 176, 178, 179, 185, 186, 200-202, 217, 221-223, 226, 228, 229, 238-240, 242, 244, 249-253, 264, 267, 275, 281, 282, 284, 295

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II.2 Determinación de hidrocarburos policíclicos aromáticos en muestras de saliva

A pesar de los numerosos estudios descritos para la determinación de PAHs en diferentes muestras biológicas, en muy limitadas ocasiones se ha llevado a cabo la determinación de estos compuestos en muestras de saliva. El mayor número de aportaciones se ha realizado en muestras de orina y sangre, aunque también se han determinado estos compuestos en plasma, suero y aire exhalado, entre otros. Las principales aplicaciones descritas en orina se centran en la determinación de los correspondientes derivados de PAHs, ya que estos compuestos sufren procesos de biotransformación tras su absorción que dependen directamente de cada individuo. La matriz saliva, en cambio, permite el análisis de los PAHs sin metabolizar. La principal ventaja que presenta la saliva con respecto a la segunda matriz más utilizada, la sangre, es la toma de muestra, ya que se lleva a cabo de una manera simple y no invasiva, mientras que el análisis de sangre requiere el uso de agujas, así como nociones especiales para la recolección de la muestra.

Como se ha dicho anteriormente, existen muy pocos estudios enfocados a la determinación de PAHs y sus derivados en muestras de saliva. En primer lugar, el análisis de los compuestos sin metabolizar se ha llevado a cabo utilizando una técnica basada en una extracción en fase sólida microdispersiva asistida por aire (*air-assisted dispersive micro-solid phase extraction*, AA-dµ-SPE) seguida del análisis mediante GC-FID. Otro conjunto de PAHs de la misma naturaleza se ha analizado en saliva utilizando una sonda de análisis de sólidos a presión atmosférica (*atmospheric pressure solid analysis probe*, ASAP) acoplada a un espectrómetro de masas con un analizador de tiempo de vuelo (*quadrupole-time of flight-mass spectrometry*, Q-TOF-MS). Esta técnica también se ha utilizado en el análisis de PAHs nitrogenados, oxigenados e hidroxilados. Recientemente se ha propuesto un nuevo método basado en una microextracción líquido-líquido asistida por aire (*air-assisted liquid-liquid microextraction*, AALLME) seguida del análisis mediante GC-MS para la determinación de PAHs sin metabolizar en muestras de saliva[1]. Por último, se ha llevado a cabo la

extracción de hidroxi-PAHs mediante LLE analizándose posteriormente mediante cromatografía líquida de ultra-alta resolución (*ultra-high performance liquid chromatography*, UPLC) acoplada a espectrometría de masas en tándem (MS/MS).

Las concentraciones de PAHs descritas en salivas se encontraron en el rango de ppb-ppm.

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II.2.1 Artículo de investigación

Determinación de hidrocarburos policíclicos aromáticos en muestras de saliva mediante extracción líquido-líquido-inyección a temperatura programada-cromatografía de gases-espectrometría de masas. Aplicación a la exposición ocupacional de bomberos

Resumen

El objetivo principal de este trabajo fue el desarrollo de un método basado en LLE-PTV-GC-MS para la determinación de PAHs en muestras de saliva de bomberos y voluntarios no expuestos. Según nuestra información, este método se propone por primera vez para la determinación de PAHs no metabolizados en muestras de saliva.

En primer lugar, se llevó a cabo la optimización de las variables que afectan a la extracción LLE con el fin de obtener la máxima extracción de los analitos de la saliva, así como la mejor separación posible entre la saliva y el disolvente orgánico utilizado. Para ello, se utilizó una muestra de saliva dopada con los analitos de estudio. Se estudiaron cuatro disolventes orgánicos diferentes: acetato de etilo, hexano, metil tert-butil éter y éter dietílico. Todos ellos se estudiaron en dos situaciones distintas: sin adición de NaCl y en sobresaturación de NaCl. Los mejores resultados se obtuvieron con acetato de etilo sin adición de NaCl.

La extracción se llevó a cabo poniendo en contacto la saliva y el disolvente, y agitando en vórtex. Se compararon diferentes tiempos de agitación comprendidos entre 0.5 y 2 minutos. A partir de 1 minuto de agitación no se observó un aumento significativo en la señal analítica, por lo que se seleccionó como óptimo.

Para llevar a cabo el estudio del volumen de saliva utilizado fue necesario tener en cuenta que el volumen que puede ser recolectado varía entre diferentes individuos. Además, en la extracción se observó la formación de una interfase entre la saliva y el disolvente orgánico, que variaba según la saliva, y que en algunos casos dificultaba la recolección posterior del extracto orgánico que contenía los analitos. Teniendo en cuenta estos factores, se probaron volúmenes comprendidos entre 100 y 500 μL. Como era de esperar, se observó un aumento de la señal analítica con el aumento del volumen de muestra. El uso de volúmenes superiores a 500 μL aumentaba el tamaño de la interfase formada impidiendo una

adecuada toma de muestra del extracto orgánico. Por ello, se seleccionó 500 µL como volumen óptimo para los siguientes análisis.

Por último, se optimizó el volumen de acetato de etilo utilizado para la extracción. Para ello, se compararon los resultados obtenidos cuando se llevó a cabo la extracción de saliva dopada con volúmenes comprendidos en el rango de 750 y 2000 µL. El uso de volúmenes inferiores no fue posible debido a la formación de la interfase mencionada anteriormente. Como era de esperar, los mejores resultados se obtuvieron cuando se utilizó el menor volumen, 750 µL, que se seleccionó como óptimo.

El estudio del efecto matriz se llevó a cabo comparando las señales obtenidas cuando se analizaron una muestra de agua ultrapura y 5 muestras de saliva, todas ellas dopadas con los mismos niveles de concentración de PAHs. No se encontraron diferencias significativas, concluyendo que no existía efecto matriz.

A continuación, se evaluó la eficiencia de la extracción comparando la señal obtenida al extraer una muestra de agua ultrapura dopada con la señal correspondiente de una disolución en acetato de etilo que contenía una concentración equivalente a una extracción completa de los analitos. Los valores obtenidos se encontraron entre el 91 y el 104 %, mostrando una alta eficiencia de extracción.

Teniendo en cuenta la ausencia de efecto matriz y los buenos resultados obtenidos en el estudio de la eficiencia de extracción, la optimización de las variables que afectaban al acoplamiento PTV-GC-MS se llevó a cabo analizando una disolución de acetato de etilo que contenía los PAHs de estudio.

Los parámetros que afectaban al PTV se optimizaron en dos etapas. El tiempo de inyección se optimizó de manera individual, mientras que el resto de las variables o factores (temperatura de venteo, tiempo de venteo, flujo de venteo

y volumen de inyección) se optimizaron utilizando un diseño de experimentos para poder estudiar las posibles interacciones entre ellos.

Se estudiaron diferentes tiempos de inyección comprendidos entre 0.5 y 2 minutos. Los analitos más volátiles no se vieron afectados para tiempos elevados de inyección, mientras que los menos volátiles necesitaron al menos 1 minuto para una adecuada desorción del *liner*. Por ello, el tiempo de inyección se fijó en 1 minuto.

El resto de las variables que afectaban a la inyección se optimizaron utilizando un diseño experimental de tipo Box-Behnken. Para cada variable o factor estudiado se fijaron 3 niveles con el objetivo de obtener la correspondiente superficie de respuesta de la señal analítica. Una vez recopilados los valores de respuesta para cada factor se estudiaron tres modelos de ajuste de datos. El primero de ellos incluía los factores individuales, el segundo incluía los factores y sus interacciones, y el tercero incluía los factores, sus cuadrados y las interacciones entre ellos. Ningún modelo mostró fallo de ajuste. Además, todos los modelos proporcionaron resultados semejantes, seleccionándose como óptimo el más simple, es decir, aquel que solo incluía los factores individuales. Con este modelo, la temperatura y tiempo de venteo (analitos más volátiles), así como el volumen de inyección (todos los analitos) fueron las variables más significativas. El flujo de venteo no fue significativo en ninguno de los casos. Con el objetivo de tener una máxima señal analítica para los compuestos de interés y una menor señal correspondiente al acetato de etilo (disolvente) que asegure una eficiencia máxima en el proceso de venteo de disolvente, los valores óptimos fueron los siguientes: 115 ºC para la temperatura de venteo, 0.45 minutos para el tiempo de venteo y 30 μL como volumen de inyección. Teniendo en cuenta que el flujo de venteo no fue significativo se seleccionó un valor de 150 mL min^{-1} para un menor consumo de gas.

Partiendo de una temperatura inicial optimizada de 60 ºC, el cromatógrafo de gases utilizó las máximas rampas de temperatura permitidas para conseguir una adecuada separación de los PAHs.

La detección mediante MS se llevó a cabo en el modo SIM/*scan* combinado, que ya se describió previamente en el capítulo I. De nuevo, se optimizaron los parámetros que afectaban a ambos modos de adquisición de datos (valores de velocidad de barrido en el modo *scan* y tiempo de permanencia en el modo de iones seleccionados (SIM)). En el modo *scan* se establecieron dos grupos con diferentes valores de velocidad de barrido, mientras que en el modo SIM el tiempo de permanencia óptimo fue de 1 ms para todos los analitos.

La evaluación del método se llevó a cabo utilizando disoluciones de acetato de etilo que contenían los PAHs a diferentes niveles de concentración. Como se explicó previamente, esto fue posible debido a la ausencia de efecto matriz y a la adecuada eficacia en el proceso de extracción. La cuantificación de los PAHs se llevó a cabo utilizando calibrados para los cuales se analizaron disoluciones preparadas a siete niveles diferentes de concentración. Todos ellos mostraron comportamiento lineal con coeficientes de determinación adecuados y sin fallo de ajuste. Los valores obtenidos para los LODs y LOQs oscilaron entre 0.002-0.057 μg L^{-1} y 0.007-0.191 μg L^{-1}, respectivamente.

El estudio de repetibilidad y reproducibilidad se llevó a cabo a dos niveles diferentes de concentración. Los valores obtenidos en términos de RSD fueron iguales o inferiores al 11 y 19 %, respectivamente. El estudio de repetibilidad se llevó a cabo en muestras de saliva dopadas con los analitos de interés, obteniéndose valores semejantes a los correspondientes en acetato de etilo.

Para comprobar la validez del método 4 muestras de saliva que no contenían los analitos de interés se doparon a 4 niveles de concentración conocida dentro de los límites del calibrado. Esta concentración fue posteriormente predicha utilizando los calibrados en acetato de etilo y comparada con el valor

real añadido. Los valores de recuperación obtenidos oscilaron entre el 79 y el 122 %, lo que pone de manifiesto que el método propuesto puede ser aplicado satisfactoriamente a la cuantificación de PAHs sin metabolizar en muestras de saliva.

Por último, este método se utilizó para analizar un total de 55 muestras de saliva que se dividieron en los siguientes conjuntos:

- 45 muestras de saliva de bomberos recogidas:
    - Inmediatamente después de participar en la extinción de un fuego (10 muestras).
    - A las seis horas tras la extinción del fuego (11 muestras).
    - A diferentes horas una vez que habían pasado doce horas tras la extinción del fuego (15 muestras).
    - Al menos dos días después de haber participado en la extinción del fuego (9 muestras).
- 10 muestras de voluntarios no expuestos al humo de un incendio.

Todas las muestras correspondieron a individuos no fumadores.

Con el objetivo de saber si existía una separación entre las muestras de bomberos y de los voluntarios no expuestos, en primer lugar, se llevó a cabo un análisis mediante PCA. Las muestras recogidas en bomberos inmediatamente después de la actividad se separaron claramente de las muestras recogidas una vez pasadas doce horas desde la actividad, tras dos días de la actividad y en voluntarios no expuestos, todas ellas agrupadas en un único conjunto. Las muestras recogidas a las seis horas desde la extinción del fuego se distribuyeron entre estos dos grupos de muestras.

En un segundo paso, se llevó a cabo la cuantificación de PAHs en estas muestras de saliva. En todas ellas se cuantificó el fluoreno, sin diferencias significativas entre muestras de bomberos y no expuestos. El fenantreno se

cuantificó en algunas de las muestras pertenecientes a ambos grupos, siendo siempre mayor la concentración correspondiente a las muestras de bomberos. Estos resultados están de acuerdo con la bibliografía revisada que indica la presencia de dichos compuestos en diferentes matrices alimentarias, así como en el agua o recipientes de almacenaje de comida, pudiendo entrar en el cuerpo por ingestión. El naftaleno, acenaftileno, antraceno, fluoranteno y pireno se detectaron solo en muestras de bomberos, observándose una disminución de la concentración en las muestras recogidas a las seis horas con respecto a las recogidas inmediatamente después del fuego. Todos estos resultados están de acuerdo con la separación observada entre grupos en el análisis mediante PCA.

Como se ha dicho previamente, la bibliografía disponible para la determinación de PAHs en muestras de saliva es muy limitada. Las ventajas principales del método aquí propuesto frente a aquellos basados en AA-dμ-SPE-GC-FID y LLE-UPLC-MS/MS son la disminución del tiempo de análisis, así como la mejora en los límites de detección. Con respecto a los métodos basados en ASAP-Q-TOF-MS, a pesar de que el tiempo de análisis fue inferior debido a la ausencia de tratamiento de muestra y separación cromatográfica, estos se utilizaron principalmente con fines de identificación o semicuantificación debido al empeoramiento de la sensibilidad.

Los trabajos de bibliografía enfocados a la determinación de PAHs en muestras de orina y aire exhalado de bomberos estuvieron de acuerdo con los resultados encontrados aquí. Mientras que en muestras de orina se observó un aumento y posterior disminución en los niveles de PAHs debido a los procesos de biotransformación, en las muestras de aire exhalado los mayores niveles se encontraron inmediatamente después de la exposición.

En conclusión, el análisis de componentes principales reveló una buena discriminación entre las muestras tomadas tras la exposición a un incendio y aquellas correspondientes a voluntarios no expuestos, muestras tomadas sin

exposición durante los dos días previos y tomadas una vez pasadas doce horas desde la exposición. Las muestras recogidas a las seis horas de la exposición se encontraron entre ambos grupos.

El método propuesto se utilizó para cuantificar PAHs en todas las muestras de saliva, habiéndose detectado algunos de ellos en ambos grupos de muestras y otros solamente en muestras correspondientes a bomberos.

Cabe destacar que la extracción de los analitos fue rápida, simple y muy efectiva mientras que el análisis posterior fue rápido y sensible, siendo estas las ventajas principales observadas frente a otros métodos descritos en bibliografía.

Es importante enfatizar la relevancia de este trabajo para la determinación de PAHs sin metabolizar en saliva como posibles biomarcadores de exposición, cuya presencia se asocia de manera más directa a la exposición previa que aquellos derivados obtenidos tras los procesos de biotransformación, que dependen de cada individuo.

II.2.1 Artículo de investigación

Determinación de hidrocarburos policíclicos aromáticos en muestras de saliva mediante extracción líquido-líquido-inyección a temperatura programada-cromatografía de gases-espectrometría de masas. Aplicación a la exposición ocupacional de bomberos

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Liquid-liquid extraction-programmed temperature vaporizer-gas chromatography-mass spectrometry for the determination of polycyclic aromatic hydrocarbons in saliva samples. Application to the occupational exposure of firefighters

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ABSTRACT

We present the development and validation of a sensitive method for the reliable determination of sixteen polycyclic aromatic hydrocarbons (PAHs) in saliva samples, which can be used as exposure markers. This method was based on a liquid-liquid extraction and programmed temperature vaporizer-gas chromatography-mass spectrometry analysis (LLE-PTV-GC-MS). Since no matrix effect was found, quantification was performed using external calibration. The detection limits were lower than or equal to 0.057 $\mu g\, L^{-1}$ for all analytes, and repeatability and reproducibility (expressed as relative standard deviation, RSD) were always lower than or equal to 11% and 19%, respectively. The method was used to quantify polycyclic aromatic hydrocarbons in the saliva samples taken from firefighters and unexposed volunteers, detecting the presence of seven of the sixteen analytes analysed. Two of the compounds (fluorene and phenanthrene) were found in the both exposed and unexposed individuals, while the remaining five analytes (naphthalene, acenaphthylene, anthracene, fluoranthene and pyrene) were only detected in samples taken from the firefighters. Good discrimination between the firefighters and the unexposed volunteers was obtained through a principal component analysis.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) comprise a class of contaminants ubiquitous in the environment, which have received considerable attention due to their association with different types of cancer including oral [1], lung [1–3], skin [3,4] and bladder cancer [3,5] among others. The International Agency for Research on Cancer (IARC) [6] has listed a number of PAHs as possible carcinogens, and PAHs have also been considered to be priority pollutants by the United States Environmental Protection Agency (U.S. EPA) [7].

These compounds are composed of two or more fused aromatic rings and are the result of incomplete combustion of organic materials. The main sources of exposure originate from industrial emissions, air, water, soil and foodstuffs [8], entering the body via ingestion, dermal contact or inhalation. Although working environments are often the main place for exposure to occur, it has been reported that diet also highly contributes to exposure to PAHs [8].

Several studies have described the determination of PAHs in biological matrices, including urine [9,10], blood [11–14], plasma [14], serum [15,16] and semen [17]; however, very few have proposed the determination of these analytes in saliva samples [18–20]. Most publications analysing urine and semen samples are focused on determining PAH derivatives, since these compounds are subjected to biotransformation processes that directly depend on each individual after they are absorbed. Nevertheless, the use of saliva presents a non-invasive way to carry out sample collection, whereas blood, plasma and serum samples are obtained using needles and require special considerations for their handling and use. It also has the potential for cross contamination to occur among different individuals. For these reasons, saliva samples can provide a suitable medium for the determination of unmetabolized PAHs prior to biotransformation, and can also be easily collected and handled.

Polycyclic aromatic hydrocarbons have previously been analysed in saliva samples as unmetabolized compounds [18,19], as their nitro- and oxo-derivatives [19] and as hydroxylated metabolites [20]. These

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analytes were analysed using techniques such as air-assisted dispersive micro-solid phase extraction (AA-dμ-SPE) based on magnetic graphitic carbon nitride nanocomposites and gas chromatography-flame ionization detection (GC-FID) [18], atmospheric pressure solid analysis probe (ASAP) coupled to quadrupole-time of flight mass spectrometry (Q-TOF-MS) [19,20] and liquid-liquid extraction (LLE)-ultrahigh performance liquid chromatography (UPLC) with mass-triple quadrupole detection (MS/MS) [20]. Once detected, the concentration of the PAHs in saliva samples was reported to be in the range of ppm-ppb.

Saliva samples from firefighters and unexposed volunteers were analysed with the aim of determining the concentration of PAHs. The method was based on liquid-liquid extraction-programmed temperature vaporization-gas chromatography-mass spectrometry (LLE-PTV-GC-MS). To our knowledge, this is the first time this method has been used to determine unmetabolized PAHs in saliva samples.

2. Experimental

2.1. Chemicals and standard solutions

Naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, biphenyl, 3-phenyltoluene, 4-phenyltoluene, fluorene, phenanthrene, fluoranthene, pyrene and *tert*-butyl methyl ether were supplied by Acros Organics (Geel, Belgium). Acenaphthylene, acenaphthene, anthracene, chrysene, benzo(k)fluoranthene, benzo(a)pyrene, ethyl acetate, methanol and diethyl ether were supplied by Sigma-Aldrich (Steinheim, Germany). Acetone, hexane and sodium chloride were purchased from Scharlab (Barcelona, Spain) and acetonitrile was supplied by Merck (Darmstadt, Germany). The purity of all the compounds was at least 95%. UHQ water was obtained from a Wasserlab Ultramatic water purification system (Noain, Spain).

Stock solutions (100 mg L^{-1}) of each compound were prepared in methanol, except for chrysene and benzo(a)pyrene, which were prepared in acetone and acetonitrile, respectively. All solutions were stored at 4 °C and were subsequently diluted to prepare the working solutions.

2.2. Saliva samples

Unstimulated saliva samples were collected from 55 participants, who were non-smokers, in individual 10-mL glass vials, sealed with a silicone septum cap and frozen at − 20 °C until analysed. A group of 11 firefighters who had worked in three different firefighting activities (a fire induced specifically for the purpose of practicing, a fire in a train and a fire in a supermarket) volunteered to provide 45 saliva samples. Ten of these saliva samples were collected immediately after (zero hours) firefighting activities (samples 1–10), 11 of the samples were collected six hours after the fire had been extinguished (samples 11–21), 15 samples were collected at different times once a 12-h period of exposure had elapsed (samples 22–36) and the nine remaining samples were taken from firefighters that had not partaken in firefighting activities two days prior to sample collection (samples 37–45). Ten saliva samples were taken from volunteers unexposed to smoke (samples 46–55).

The saliva samples were thawed at room temperature and homogenized before analysis.

Written informed consent was obtained from all volunteers.

2.3. Extraction procedure and instrumental conditions

Five hundred μL of each saliva sample, 25 μL of methanol and 750 μL of ethyl acetate were transferred to an 11.5-mL glass centrifuge tube with a PTFE screw cap (Scharlab) and vortexed for 1 min at maximum speed. Then, the mixture was centrifuged at 2415 ×*g* for 5 min and the ethyl acetate extract was collected and placed in a GC vial (Scharlab).

The extraction procedure was optimized using a saliva sample that did not contain any of the analytes which was spiked by adding 25 μL of a methanolic solution containing all analytes (10 μg L^{-1}).

A 30-μL volume of the ethyl acetate extract was injected into a programmed temperature vaporizer inlet (CIS-4, Gerstel, Baltimore, MD). A liner (71 mm x 2 mm) packed with Tenax-TA® was used. The inlet was operated in the solvent vent mode and the conditions were as follows: the temperature of the injector was initially set at 115 °C for 0.46 min with a vent flow of 150 mL min^{-1} (6.00 psi). After venting the solvent, the split valve was closed, and the liner was quickly heated (12 °C s^{-1}) until 340 °C to transfer the analytes from the injector to the column. The injection time was 1 min. To clean the system, the split valve was opened, and the final temperature of the liner was held for 5 min. Liquid CO_2 (Air Liquide) was used to cool the PTV system.

The instrument used for GC-MS analysis was an Agilent Technologies 6890 gas chromatograph equipped with a HP 5973 N mass selective detector quadrupole mass spectrometer. The gas chromatograph was fitted with a HP5-MS UI capillary column (30 m x 0.250 mm x 0.25 μm, working range −60 to 325 °C) from J&W Scientific (Folsom, CA, USA). A flow rate of 2 mL min^{-1} of helium N50 (99.999% pure; Air Liquide) was used as the carrier gas. The initial oven temperature was set at 60 °C and held for 0.5 min. Then, the temperature was increased at 60 °C min^{-1} to 175 °C and further increased at 45 °C min^{-1} to 325 °C, held for 2.5 min. The total run time was 8.25 min. In addition, an interval of 16 min was set between the injection of each sample, because the column took approximately 7 min to cool down from the final temperature of 325 °C to the initial temperature of 60 °C. The detector was equipped with an inert ion source. The source and quadrupole temperatures were kept at 230 and 150 °C, respectively. The electronic beam energy of the quadrupole mass spectrometer was 70 eV. The analyses were performed in a synchronous SIM/scan mode, acquiring both SIM and full scan data in a single run. A solvent delay of 2.45 min was established. The *m/z* range selected was 35–300 amu. Identification was performed by comparing the acquired mass spectra to the corresponding found in the NIST'08 database (NIST/EPA/NIH Mass Spectral Library, version 2.0). Two full scan groups from 2.45 to 6.20 min (9.40 scan s^{-1}) and from 6.20 to 8.25 min (5.19 scan s^{-1}) were used. In the selected ion monitoring (SIM) mode, one quantitation and two qualifier ions were monitored for quantifying purposes. Four SIM groups with a dwell time value of 1 ms and different *m/z* were employed (see Table S1 in Supplementary Information).

2.4. Data analysis

Data collection was performed with the Enhanced ChemStation, from Agilent Technologies [21]. The Box-Behnken experimental design and the principal component analysis were performed using The Unscrambler, v. 10.2, statistical package [22].

3. Results and discussion

The variables affecting the liquid-liquid extraction procedure and the PTV-GC-MS analysis were optimized. Then, calibration curves were obtained and the accuracy and precision of the developed method were evaluated. Finally, the method was used to analyse the 55 saliva samples.

3.1. Optimization of the extraction conditions

The extraction procedure was optimized by using a saliva sample spiked with all 16 analytes (10 μg L^{-1}).

The aim of this study was to determine the appropriate conditions for maximising the extraction of the various analytes from the saliva, as well as obtaining good separation between the saliva sample and the organic extract. Four different solvents were tested: ethyl acetate, hexane, *tert*-butyl methyl ether and diethyl ether. All solvents were

tested in both a state of supersaturation and without the addition of NaCl. The solution with ethyl acetate as the solvent, without NaCl, produced the best results.

PAHs were extracted from the spiked saliva sample in the ethyl acetate solution by vortexing the mixture. The optimal vortexing time was determined by testing different lengths of time ranging from 0.5 to 2 min. Subsequently, the vortex time was set at 1 min, since no significant increase in the analytical signals was obtained at longer times.

The difficulty of obtaining an appropriate volume of each saliva sample to be analysed can vary depending on the ability of each individual to salivate. Furthermore, the organic extract can be easier or more difficult to collect depending on the nature of the saliva sample, due to the formation of an interface between the aqueous and organic phases. Therefore, sample volume is an important variable to be taken into account. In this study, volumes of saliva between 100 and 500 µL were studied. As expected, the analytical signals increased as the volume increased; the highest analytical signals with 500 µL of saliva sample. Volumes greater than 500 µL caused the interface to spread, hindering the suitable sampling of the organic extract and its subsequent injection into the PTV-GC-MS system. Accordingly, a volume of 500 µL was chosen to carry out the experiments.

Finally, the optimal volume of ethyl acetate was determined by adding different volumes of ethyl acetate, between 750 and 2000 µL, to the spiked saliva sample. It was observed that as expected, the highest analytical signals were obtained when a volume of 750 µL was used. In addition, smaller volumes were tested but not analysed, because extract collection was made difficult, as well as its subsequent injection into the system.

3.2. *Matrix effect and extraction efficiency*

The matrix effect was studied by comparing the analytical signals obtained when ultrapure water and five saliva samples were spiked with all the compounds at different concentration levels (1.6–65.2 $\mu g\,L^{-1}$). No significant differences were found between the six different samples indicating the lack of a matrix effect.

Recoveries of the extraction procedure were determined by comparing the analytical signals obtained by liquid-liquid extraction of ultrapure water spiked with all the analytes to the signals obtained for a standard in ethyl acetate injected directly into the PTV-GC-MS system at an equivalent concentration. The extraction efficiency was very high, with recovery values ranging between 91% and 104%.

Since no matrix effect was found between the water and saliva samples, and all of the analytes were extracted from the matrix with high percentages as shown by the values obtained in the recovery study, the optimization of the variables affecting injection with a programmed temperature vaporizer and the analysis by gas chromatography and mass spectrometry were carried out by using a solution that contained all of the analytes in ethyl acetate (7 $\mu g\,L^{-1}$).

3.3. *Optimization of the instrumental conditions*

3.3.1. *Programmed temperature vaporizer*

The injection time was individually optimized, while the rest of the variables (vent temperature, vent time, vent flow and injection volume) were optimized at the same time by means of an experimental design due to the possible interactions between them.

First, different injection times ranging between 0.5 and 2 min were studied. While the more volatile analytes, those with lower retention times in the chromatogram, were not very affected by longer injection times, the analytes with higher retention times needed at least 1 min to be desorbed from the liner and subsequently introduced into the column. One minute was selected as the optimal length of time, since no significant differences were found for times longer than this.

To study the four remaining factors affecting the injection conditions, a Box-Behnken experimental design was employed. This design allowed response surfaces to be computed using three levels for each of the factors studied (vent temperature, 100–115–130 °C; vent time, 0.3–0.45–0.60 min; vent flow, 150–175–200 $mL\,min^{-1}$, and injection volume, 20–30–40 µL). The levels used in the experimental design were selected according to our previous work where a programmed temperature vaporizer was used to determine PAHs in urine samples [23].

Table 1
Box-Behnken experimental design for the optimization of the injection conditions in the solvent vent mode. A: vent temperature, B: vent time, C: vent flow, D: injection volume.

Experiment	Factors			
	A/°C	B/min	C/mL min^{-1}	D/µL
1	130	0.60	175	30
2	130	0.45	150	30
3	115	0.60	150	30
4	115	0.45	200	40
5	115	0.30	175	20
6	115	0.60	175	40
7	100	0.45	175	20
8	100	0.45	200	30
9	115	0.30	175	40
10	115	0.45	150	40
11	115	0.30	150	30
12	115	0.60	175	20
13	100	0.60	175	30
14	115	0.60	200	30
15	100	0.45	150	30
16	115	0.30	200	30
17	100	0.45	175	40
18	130	0.30	175	30
19	115	0.45	200	20
20	130	0.45	175	40
21	115	0.45	150	20
22	130	0.45	200	30
23	130	0.45	175	20
24	100	0.30	175	30
25[a]	**115**	**0.45**	**175**	**30**
26[a]	**115**	**0.45**	**175**	**30**
27[a]	**115**	**0.45**	**175**	**30**

[a] Center samples are in bold.

The design matrix used is shown in Table 1. To detect lack of fit of the data to the model generated, the center sample was measured in triplicate. The rest of the experiments were measured once. The peak areas of the corresponding quantifier ions were the experimental responses chosen (see Table S1 in Supplementary Information) for the 16 PAHs studied, as well as the area for *m/z* 43, corresponding to the solvent ethyl acetate.

Three different models of data fitting were tested using the response values and the factors. The first model included the individual factors, the second model included the factors and their interactions and the third one included the factors, their squares and the interactions among them. None of the models showed lack of fit. Since no significant differences were found when the interactions and the squares of the factors were included, the simplest model that included only the main factors was chosen as being the best model.

The main conclusions obtained using this model were the following: the vent temperature and time for the most volatile analytes and the injection volume for all of the compounds were determined to be the most significant variables; and vent flow was not significant in any of the cases.

To better visualize these results, response surfaces for all the analytes were generated by maintaining a constant vent flow of 150 $mL\,min^{-1}$. In Fig. 1, response surfaces for ethyl acetate (solvent used, Fig. 1A and B), 2-methylnaphthalene (Fig. 1C and D) and benzo(k)fluoranthene (Fig. 1E and F) are represented. These compounds were selected to show the behaviour of both volatile (2-methylnaphthalene) and less volatile compounds (benzo(k)fluoranthene). Analytes with

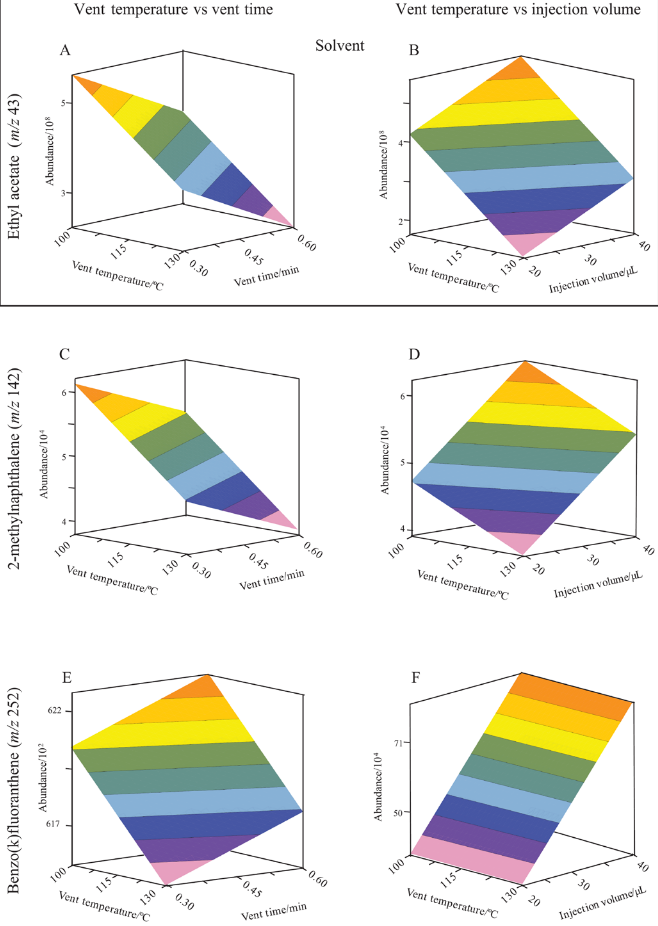


Fig. 1. Response surfaces of vent temperature vs vent time and vent temperature vs injection volume for ethyl acetate (m/z 43), 2-methylnaphthalene (m/z 142) and benzo(k)fluoranthene (m/z 252).

moderate volatility showed a similar behaviour to less volatile compounds. Ethyl acetate was also included in the study because, in addition to obtaining maximum analytical signals for the compounds studied, the minimum signal of the solvent must also be ensured (maximum efficiency of the solvent venting process).

When the factors of vent temperature and vent time were considered, the highest analytical signals for the most volatile compounds (Fig. 1A and C) were obtained when the lowest values were selected (100 °C and 0.30 min, respectively). However, in the case of the less volatile compounds (Fig. 1E), the highest signals were obtained for the lowest value of vent temperature (100 °C) and the highest value of vent time (0.60 min). It can be observed that the variation of the signals produced for the most volatile analytes is more pronounced than that corresponding to the less volatile compounds, which remained almost constant. The best conditions for removing the solvent (Fig. 1A) were a vent temperature of 130 °C and a vent time of 0.60 min.

When vent temperature and injection volume (Fig. 1B, D and F) were studied, all of the compounds showed a significantly higher signal when the injection volume was increased. For benzo(k)fluoranthene (Fig. 1F), the maximum signals were obtained when the injection volume was 40 μL, regardless of the vent temperature selected. Nevertheless, the analytical signal depends on the vent temperature for the most volatile compounds (Fig. 1B and D). The maximum signals were found when an injection volume of 40 μL and a vent temperature of 100 °C were selected. Similar results were found when comparing the vent time and the injection volume. The best conditions for solvent

Table 2
Analytical characteristics of the LLE-PTV-GC-MS method.

Compound	Calibration range (µg L^{-1})	R^2	Repeatability (RSD, %)		Reproducibility (RSD, %)		LOD (µg L^{-1})	LOQ (µg L^{-1})
			Low level	High level	Low level	High level		
Naphthalene	LD-18.82	0.9971	4	2	5	3	0.048	0.161
2-methylnaphthalene	LD-16.73	0.9954	10	1	8	2	0.013	0.043
1-methylnaphthalene	LD-17.76	0.9957	11	2	11	3	0.018	0.060
Biphenyl	LD-18.12	0.9972	10	2	19	4	0.014	0.048
Acenaphthylene	LD-11.62	0.9980	5	1	9	3	0.009	0.029
3-phenyltoluene	LD-17.56	0.9960	5	2	6	3	0.008	0.025
4-phenyltoluene	LD-18.63	0.9954	4	1	5	3	0.013	0.043
Acenaphthene	LD-18.12	0.9960	8	2	9	6	0.025	0.083
Fluorene	LD-17.25	0.9937	9	2	9	8	0.012	0.041
Phenanthrene	LD-12.29	0.9936	8	1	12	3	0.011	0.036
Anthracene	LD-12.08	0.9968	11	1	10	4	0.027	0.091
Fluoranthene	LD-11.96	0.9980	10	1	10	3	0.002	0.007
Pyrene	LD-11.73	0.9973	8	1	8	4	0.024	0.079
Chrysene	LD-19.22	0.9970	9	1	7	2	0.015	0.049
Benzo(k)fluoranthene	LD-34.85	0.9942	7	1	7	3	0.034	0.113
Benzo(a)pyrene	LD-45.62	0.9946	8	1	7	3	0.057	0.191

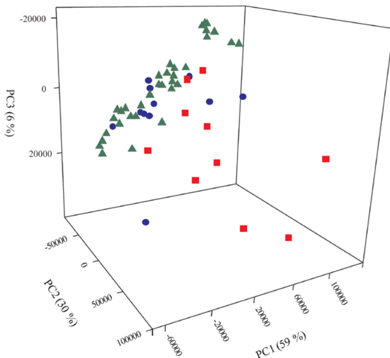


Fig. 2. Principal component analysis score plot for all the saliva samples analysed. Red squares: samples taken from firefighters collected immediately after the firefighting activity. Blue circles: samples taken from firefighters six hours after the firefighting activity. Green triangles include three different types of samples: samples taken from firefighters at different times once a 12-h period of exposure had elapsed, samples taken from firefighters who had not partaken in firefighting activities two days prior to sample collection and samples from unexposed.

removal (Fig. 1B) were a vent temperature of 130 °C and an injection volume of 20 µL.

Considering the results obtained, the best experimental conditions for the venting process were selected to remove as much solvent as possible without significantly losing the most volatile analytes. The following values were selected as being optimal: 115 °C for the vent temperature, 0.45 min for the injection time and 30 µL as the injection volume. Since vent flow was not significant in any of the cases, the lower value of 150 mL min^{-1} was chosen as being optimal value for less gas consumption.

3.3.2. Gas chromatography-mass spectrometry

With the aim of performing a fast separation of the 16 PAHs by gas chromatography, the maximum ramps permitted by the gas chromatograph configuration were selected. Under these conditions, the initial column temperature was optimized. Initial temperature values ranging between 50 and 80 °C were assessed in order to determine the optimal temperature. Although higher values reduced the time needed to reach the initial oven conditions for the next analysis, a widening of the initial peaks was observed. Consequently, 60 °C was chosen as the initial oven temperature, which afforded an adequate separation of the analytes without excessively prolonging the analysis time.

The MS detector was set on the simultaneous SIM/scan mode, allowing both chromatograms SIM and scan to be obtained for identification and quantification purposes in a single run. In the scan mode, it was necessary to optimize the sampling rate. This variable is related to the number of times that each m/z is recorded. Different values between 2.73 and 15.82 scan s^{-1} were studied. The optimum conditions were chosen according to the best definition of the peaks, as well as the S/N ratio. Two full scan groups analysing m/z between 35 and 300 were selected: the first group ranged from 2.45 to 6.20 min using a sampling rate of 9.40 scan s^{-1}, and the second group ranged from 6.20 to 8.25 min using a sampling rate of 5.19 scan s^{-1}. The peak width for the analytes included in the first group was narrower than the peak width for the compounds in the second group. In the SIM mode, different dwell times (1–30 ms) were assessed. Although the noise of the chromatographic peaks decreased when the dwell time value was increased, poorer peak definition was observed because of the few points defining it. Thus, 1 ms was chosen as the optimum value. Fig. S1 (see Supplementary Information) shows the corresponding extracted ion chromatograms for one of the analytes studied, 1-methylnaphthalene (m/z 142), when different numbers of scan s^{-1} and different dwell time values were used. The selected values are highlighted in Fig. S1 (see Supplementary Information).

3.4. Evaluation of the method

Since a matrix effect was not observed and the extraction efficiency was very high (ranging between 91% and 104%), solutions of the 16 PAHs prepared in ethyl acetate were used to obtain the calibration curves, as well as the detection and quantification limits. For calibration purposes, seven concentration levels were analysed in triplicate (see Table 2). The analytical signal used for constructing the calibration curves were the peak areas of each compound when their quantitation ion was extracted in the SIM mode (see Table S1 in Supplementary Information). As can be seen in Table 2, all calibrations showed good linear behaviour with satisfactory coefficients of determination (R^2). The models obtained did not show any lack of fit.

A standard solution that provided an S/N ratio of approximately 3 for all of the compounds was analysed for calculating the detection and quantification limits. These values were calculated as 3 and 10 times, respectively, the standard deviation when that solution was analysed 5

Table 3
Concentration of PAHs in saliva samples (µg L^{-1}).

			Concentration (µg L^{-1})						
		Saliva sample	Naphthalene	Acenaphthylene	Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene
	Zero hours	1	< LD	0.24 ± 0.01	0.437 ± 0.008	1.1 ± 0.2	< LD	0.32 ± 0.01	0.23 ± 0.07
		2	0.38 ± 0.07	0.66 ± 0.05	0.939 ± 0.008	2.4 ± 0.3	0.360 ± 0.004	0.69 ± 0.05	0.63 ± 0.06
Firefighters		3	< LD	0.05 ± 0.01	0.469 ± 0.008	0.53 ± 0.09	< LD	0.21 ± 0.01	< LD
		4	< LD	0.17 ± 0.01	0.809 ± 0.008	0.59 ± 0.09	< LD	0.21 ± 0.01	< LD
		5	< LD	< LD	0.677 ± 0.008	0.59 ± 0.09	< LD	0.19 ± 0.01	< LD
		6	< LD	0.20 ± 0.01	0.95 ± 0.06	1.6 ± 0.2	0.217 ± 0.004	0.66 ± 0.05	0.51 ± 0.06
		7	0.43 ± 0.07	0.73 ± 0.05	1.48 ± 0.06	3.3 ± 0.3	0.379 ± 0.004	1.10 ± 0.05	0.86 ± 0.06
		8	0.33 ± 0.07	0.43 ± 0.01	1.23 ± 0.06	2.0 ± 0.3	0.329 ± 0.004	0.94 ± 0.05	0.83 ± 0.06
		9	0.27 ± 0.07	0.40 ± 0.01	1.09 ± 0.06	1.8 ± 0.2	0.282 ± 0.004	0.67 ± 0.05	0.52 ± 0.06
		10	< LD	1.45 ± 0.07	1.30 ± 0.06	1.7 ± 0.2	0.199 ± 0.004	0.46 ± 0.01	0.40 ± 0.07
	Six hours	11 (sample 1, 6 hours later)	< LD	0.43 ± 0.01	0.411 ± 0.008	0.26 ± 0.09	< LD	0.27 ± 0.01	0.22 ± 0.07
		12 (sample 2, 6 hours later)	< LD	< LD	0.371 ± 0.008	< LD	< LD	< LD	< LD
		13 (sample 3, 6 hours later)	< LD	< LD	0.348 ± 0.008	0.18 ± 0.09	< LD	< LD	< LD
		14 (sample 4, 6 hours later)	< LD	< LD	0.356 ± 0.008	0.20 ± 0.09	< LD	< LD	< LD
		15 (sample 5, 6 hours later)	< LD	< LD	0.338 ± 0.008	0.15 ± 0.09	< LD	< LD	< LD
		16 (sample 6, 6 hours later)	< LD	< LD	0.439 ± 0.008	0.26 ± 0.09	< LD	0.20 ± 0.01	< LD
		17 (sample 7, 6 hours later)	< LD	< LD	0.555 ± 0.008	0.48 ± 0.09	< LD	0.31 ± 0.01	0.20 ± 0.07
		18 (sample 8, 6 hours later)	< LD	0.43 ± 0.01	0.480 ± 0.008	1.2 ± 0.2	< LD	1.06 ± 0.05	1.5 ± 0.2
		19 (sample 9, 6 hours later)	< LD	< LD	0.314 ± 0.008	0.22 ± 0.09	< LD	< LD	< LD
		20 (sample 10, 6 hours later)	< LD	1.42 ± 0.07	0.776 ± 0.008	0.33 ± 0.09	< LD	0.28 ± 0.01	0.32 ± 0.07
		21[a]	< LD	< LD	0.294 ± 0.008	0.29 ± 0.09	< LD	0.22 ± 0.01	0.18 ± 0.07
	Different samplings after 12 hours	22	< LD	< LD	0.136 ± 0.008	< LD	< LD	< LD	< LD
		23	< LD	< LD	0.158 ± 0.008	< LD	< LD	< LD	< LD
		24	< LD	< LD	0.443 ± 0.008	< LD	< LD	< LD	< LD
		25	< LD	< LD	0.180 ± 0.008	< LD	< LD	< LD	< LD
		26	< LD	< LD	0.487 ± 0.008	< LD	< LD	< LD	< LD
		27	< LD	< LD	0.611 ± 0.008	< LD	< LD	< LD	< LD
		28	< LD	< LD	0.434 ± 0.008	0.20 ± 0.09	< LD	< LD	< LD
		29	< LD	< LD	0.385 ± 0.008	< LD	< LD	< LD	< LD
		30	< LD	< LD	0.359 ± 0.008	< LD	< LD	< LD	< LD
		31	< LD	< LD	0.206 ± 0.008	< LD	< LD	< LD	< LD
		32	< LD	< LD	0.325 ± 0.008	< LD	< LD	< LD	< LD
		33	< LD	< LD	0.233 ± 0.008	< LD	< LD	< LD	< LD
		34	< LD	< LD	0.594 ± 0.008	0.24 ± 0.09	< LD	< LD	< LD
		35	< LD	0.78 ± 0.05	0.94 ± 0.06	0.17 ± 0.09	< LD	< LD	< LD
		36	< LD	< LD	0.546 ± 0.008	0.22 ± 0.09	< LD	< LD	< LD
	Without firefighting activities	37	< LD	< LD	0.218 ± 0.008	< LD	< LD	< LD	< LD
		38	< LD	< LD	0.632 ± 0.008	0.40 ± 0.09	< LD	< LD	< LD
		39	< LD	< LD	0.96 ± 0.06	0.43 ± 0.09	< LD	< LD	< LD
		40	< LD	< LD	0.808 ± 0.008	0.40 ± 0.09	< LD	< LD	< LD
		41	< LD	< LD	0.95 ± 0.06	0.8 ± 0.2	< LD	0.32 ± 0.01	0.17 ± 0.07
		42	< LD	< LD	0.692 ± 0.008	0.38 ± 0.09	< LD	< LD	< LD
		43	< LD	< LD	0.612 ± 0.008	0.32 ± 0.09	< LD	< LD	< LD
		44	< LD	< LD	0.294 ± 0.008	0.16 ± 0.09	< LD	< LD	< LD
		45	< LD	< LD	0.408 ± 0.008	< LD	< LD	< LD	< LD

(*continued on next page*)

Table 3 (*continued*)

	Saliva sample	Concentration ($\mu g\ L^{-1}$)						
		Naphthalene	Acenaphthylene	Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene
Unexposed volunteers	46	< LD	< LD	0.202 ± 0.008	< LD	< LD	< LD	< LD
	47	< LD	< LD	0.504 ± 0.008	< LD	< LD	< LD	< LD
	48	< LD	< LD	0.569 ± 0.008	0.18 ± 0.09	< LD	< LD	< LD
	49	< LD	< LD	0.316 ± 0.008	< LD	< LD	< LD	< LD
	50	< LD	< LD	0.538 ± 0.008	< LD	< LD	< LD	< LD
	51	< LD	< LD	0.310 ± 0.008	< LD	< LD	< LD	< LD
	52	< LD	< LD	0.750 ± 0.008	0.18 ± 0.09	< LD	< LD	< LD
	53	< LD	< LD	0.452 ± 0.008	0.23 ± 0.09	< LD	< LD	< LD
	54	< LD	< LD	0.346 ± 0.008	0.16 ± 0.09	< LD	< LD	< LD
	55	< LD	< LD	0.558 ± 0.008	0.18 ± 0.09	< LD	< LD	< LD

[a] Sample not collected at zero hours

times, divided by the slope of the corresponding calibration curve. The detection limits ranged from 0.002 to 0.057 $\mu g\ L^{-1}$ and the quantification limits ranged from 0.007 to 0.191 $\mu g\ L^{-1}$ for fluoranthene and benzo(a)pyrene, respectively.

Repeatability (intra-day precision) and reproducibility (inter-day precision) were studied at two different concentration levels: low level (concentration range: 0.006–0.143 $\mu g\ L^{-1}$) and high level (concentration range: 0.72–23.59 $\mu g\ L^{-1}$). Repeatability was evaluated by analysing these solutions five times on the same day. For reproducibility, the solutions were analysed five times per day on two consecutive days. As can be seen in Table 2, the relative standard deviation (RSD) was lower than or equal to 11% and 19% in all cases for repeatability and reproducibility, respectively. Repeatability was also checked in saliva samples when they were spiked with all the analytes at the same concentration levels. The RSD values obtained were similar to those obtained in ethyl acetate. No internal standard was used since no significant variability was found.

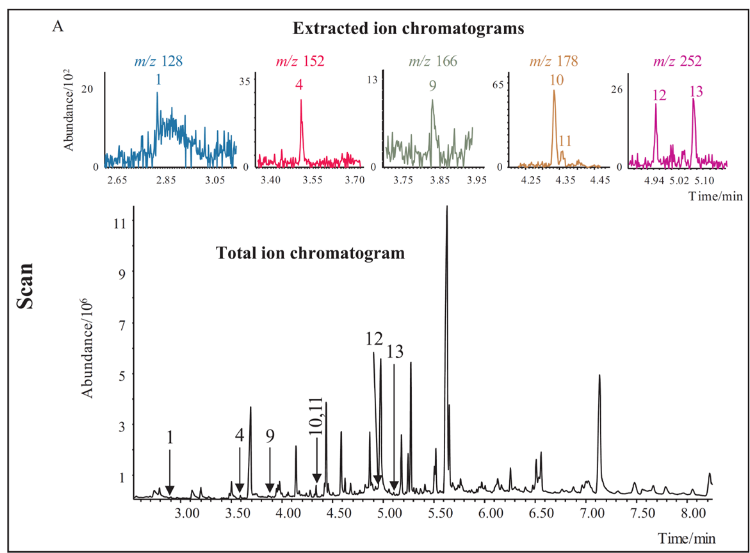
To check the method, 4 saliva samples not containing any of the PAHs were spiked with all of the analytes at four different concentration levels in the calibration range. The recovery was calculated by comparing the predicted concentration and the added concentration. The values ranged between 79% and 122% in all cases. This result highlights the applicability of the proposed method for the quantification of PAHs in saliva samples.

3.5. Analysis of saliva samples

The proposed LLE-PTV-GC-MS method was used to analyse the 55 saliva samples previously described in the experimental section.

First, a principal component analysis (PCA) was performed on the data with the aim of determining whether suitable separation could be achieved between the samples taken from the firefighters and the unexposed volunteers. In Fig. 2, the score plot of the 55 samples for the first three PCs is shown. Red squares correspond to samples 1–10, that were saliva samples collected from firefighters immediately after firefighting activities, the blue circles correspond to samples 11–21, saliva samples from firefighters collected after six hours of firefighting activities and the green triangles correspond to samples 22–55, corresponding to samples from firefighters collected at different times once a 12-h period of exposure had elapsed (samples 22–36) and to samples without firefighting activity two days prior to sample collection (samples 37–45) as well as the samples from the unexposed volunteers (samples 46–55). The percentage of the cumulative explained variance was 59%, 89% and 95%, respectively, for the first three PCs. It can be seen that the samples collected immediately from the firefighters at time zero (the samples in red) and the group of samples including the ones collected at different times once 12 h had elapsed, the samples without firefighting activity two days prior to sample collection and the samples from the unexposed volunteers (the samples in green) were separated. The three types of samples previously described (after 12 h had elapsed, without firefighting activity and unexposed volunteers) were grouped in the same area of the diagram. The samples in blue, taken from firefighters six hours after firefighting activities, were found near the samples from firefighters at zero hours in some cases (samples 11, 18 and 20) and near the other group of samples in other cases (samples 12–17, 19 and 21).

In a second step, quantification of the PAHs considered was carried out. In Table 3, the concentrations of PAHs found in the 55 saliva samples are shown. The order of the firefighters who provided samples 1–10 (collected at zero hours) was maintained in the collection of samples 11–20 (collected six hours later) to allow these two time points to be compared. The sample collected from firefighter number 21 was not collected at zero hours. Fluorene was quantified in all of the samples, showing no significant differences among the group of firefighters and the unexposed volunteers. Phenanthrene was quantified in several of the samples belonging to both groups, however, the samples originating from the firefighters showed higher concentrations of the



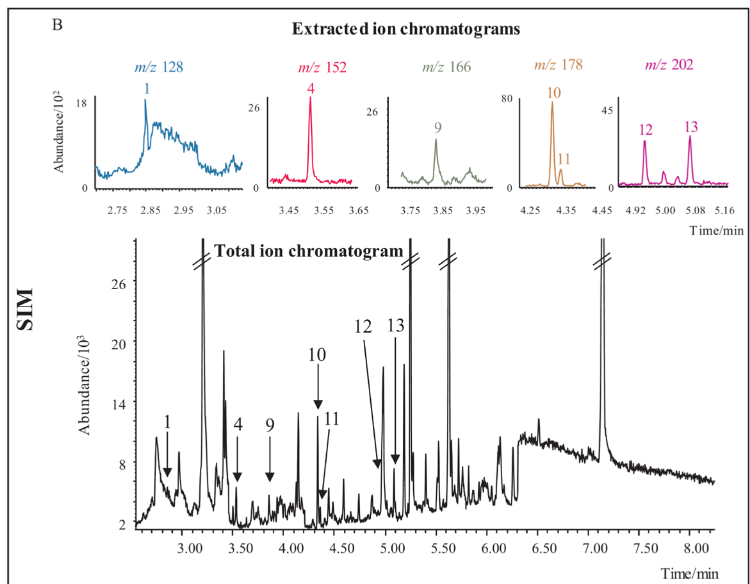


Fig. 3. Scan (A) and SIM (B) chromatograms of a saliva sample collected immediately after a firefighting activity (number two). The following analytes were found: (1) naphthalene, (4) acenaphthylene, (9) fluorene, (10) phenanthrene, (11) anthracene, (12) fluoranthene, (13) pyrene.

compound. Several studies have reported that PAHs are persistent organic pollutants found in the environment, and can enter the body through exposure within the workplace or natural environments, and can also be ingested. The presence of PAHs has been studied in food matrices such as meat [24–27], milk [24,28], wine [24], vegetables and fruits [27,29,30], among others. These analytes have also been found in drinking water [31,32] and their presence has been related to the material of storage containers [33]. Previous studies have also reported the presence of PAHs in saliva samples from smokers and non-smokers [18–20]. The results of all of these studies are in accordance with the presence of PAHs detected in the unexposed volunteers of this study. Naphthalene, acenaphthylene, anthracene, fluoranthene and pyrene were only found in the samples taken from firefighters, where the concentration of these compounds tended to decrease in the samples collected at six hours as compared to the samples collected at zero hours. These results (Table 3) support the results previously obtained by the principal component analysis, which showed differences between the groups of samples studied.

The chromatograms obtained after analysing sample number two are shown in Fig. 3. In the upper part of the figure (Fig. 3A) the total ion chromatogram (scan mode) and their extracted ion chromatograms for *m/z* 128, 152, 166, 178 and 252 are shown. Some of the detected analytes in the total ion chromatogram were difficult to distinguish, due to instrumental noise and their low intensities. However, they were better distinguished when the corresponding quantitation peaks were extracted. In the bottom part of the figure, the corresponding chromatograms in SIM mode are shown (Fig. 3B). Improvement in the morphology of the extracted ions as well as a decrease in the noise of the chromatograms can be seen.

3.6. Comparison with other works

As mentioned previously in the introduction, very few studies have reported the determination of polycyclic aromatic hydrocarbons in saliva samples as unmetabolized compounds [18,19] or their derivatives [19,20]. When PAHs were determined using AA-dμ-SPE-GC-FID [18], the time required for extracting the compounds was similar to the extraction time proposed in this work. However, a longer chromatographic runtime (14 min) greatly lengthens the total time required for sample analysis. In addition, the detection limits reported by this author were approximately ten times higher (0.3–0.6 $\mu g\,L^{-1}$) than those reported here (lower than or equal to 0.057 $\mu g\,L^{-1}$ in all cases).

In the case of the ASAP-MS-Q-TOF method [20], there was no sample treatment step and the time of analysis was approximately 3 min. The main disadvantage of this method is that the detection limits obtained for the two hydroxy-PAHs cited were approximately four orders of magnitude greater than those proposed here. This method was used mainly for identification purposes, and an additional method was used to confirm the results obtained in terms of quantification. The method used for confirmation was based on liquid-liquid extraction (13 min) with UPLC-MS/MS analysis (10 min), which is longer than the time proposed in our procedure (6 min for the extraction process and 8.25 min to analyse the samples by PTV-GC-MS). The detection limits were also higher (0.01 and 0.05 $mg\,L^{-1}$).

ASAP-Q-TOF-MS [19] was also used for identification and semi-quantification purposes with respect to the internal standards previously added. The run time was reduced because no chromatographic separation was performed; however, additional methods are required for the further quantification of samples testing positive.

Finally, regarding the literature available where firefighters have been exposed to PAHs, most of the studies analysed urine and exhaled breath samples in search of biomarkers [34–36]. The results are consistent with those found in this work, since the concentration levels increased over time and further decreased in urine samples (biotransformation processes), while the highest concentration levels found in exhaled breath samples were obtained immediately after exposure.

4. Conclusions

Initially, a principal component analysis, using the peak areas of the compounds studied, revealed good discrimination between the samples collected from firefighters immediately after firefighting activities and the group of samples including those collected from firefighters at different times once 12 h had passed since the fire, those corresponding to firefighters who had not partaken in firefighting activities two days prior to sample collection and the samples from the unexposed volunteers. The samples collected six hours after the fire had been extinguished were found to be distributed among these two groups.

The proposed method was then used to quantify the concentration of polycyclic aromatic hydrocarbons in all samples. Fluorene was quantified in all of the samples and phenanthrene was quantified in several of the samples, including both unexposed volunteers and the firefighters. Naphthalene, acenaphthylene, anthracene, fluoranthene and pyrene were only quantified in the samples taken from the firefighters. The concentrations determined confirmed the results obtained by the principal component analysis.

The extraction step is rapid, simple and very effective and the programmed temperature vaporizer-gas chromatography-mass spectrometry analysis is also fast and sensitive. The main advantages of this method are its short extraction time and chromatographic run time, as well as the low detection limits (lower than or equal to 0.057 $\mu g\,L^{-1}$) as compared to other methods.

It is important to emphasize the relevance of this approach for the determination of unmetabolized polycyclic aromatic hydrocarbons in saliva used as exposure markers, since after entering the body these compounds can be subjected to biotransformations depending on each individual. Consequently, the presence of unmetabolized polycyclic aromatic hydrocarbons is more directly associated with previous exposure to the compounds rather than their corresponding metabolites.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.09.030.

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1 **Supplementary Information**
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4 **Liquid-liquid extraction-programmed temperature vaporizer-gas**
5 **chromatography-mass spectrometry for the determination of polycyclic aromatic**
6 **hydrocarbons in saliva samples. Application to the occupational exposure of**
7 **firefighters.**
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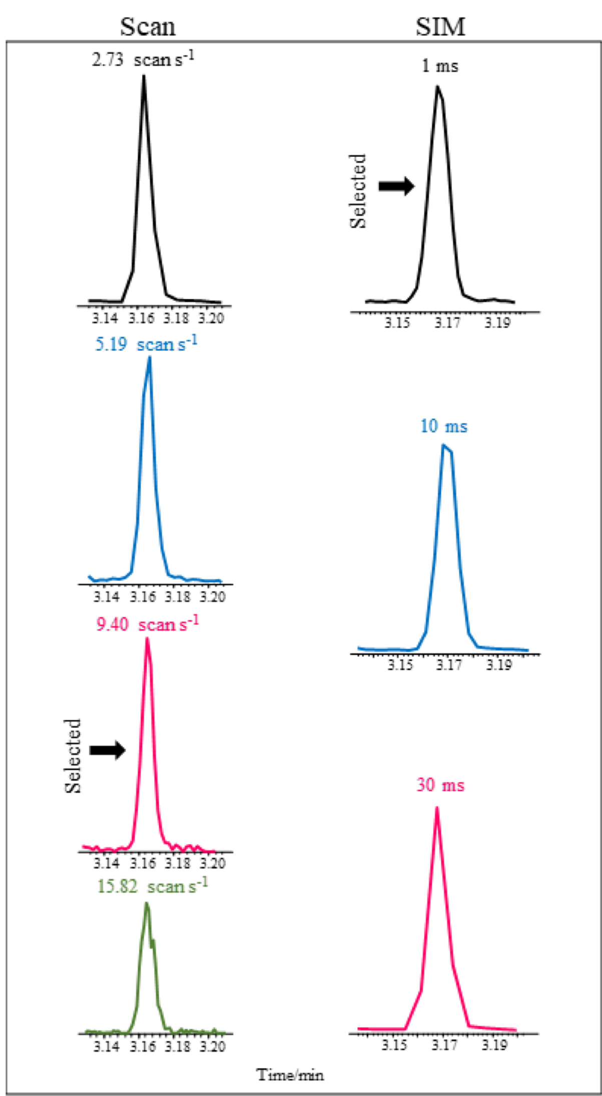
23 **Table S1**
24 Retention time, selected ions for quantification and identification of the 16 PAHs and
25 SIM groups used
26

Compound	t_R/min	Quantifier* and qualifier ions	SIM group
Naphthalene	2.854	**128**; 129; 127	1 (1ms)
2-methylnaphthalene	3.129	**142**; 141; 115	
1-methylnaphthalene	3.175	**142**; 141; 115	
Biphenyl	3.326	**154**; 153; 152	
Acenaphthylene	3.533	**152**; 153; 151	2 (1ms)
3-phenyltoluene	3.580	**168**; 167; 165	
4-phenyltoluene	3.606	**168**; 167; 165	
Acenaphthene	3.616	153; **154**; 152	
Fluorene	3.853	**166**; 165; 167	
Phenanthrene	4.336	**178**; 176; 179	3 (1ms)
Anthracene	4.359	**178**; 179; 176	
Fluoranthene	4.966	**202**; 203; 200	
Pyrene	5.089	**202**; 203; 200	
Chrysene	5.767	**228**; 226; 229	
Benzo(k)fluoranthene	6.387	**252**; 253; 250	4 (1ms)
Benzo(a)pyrene	6.590	**252**; 253; 250	

27
28 *Quantifier ions are in bold
29



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31 **Fig. S1.** Optimization of scan (scan s^{-1}) and SIM (dwell time) parameters for 1-

32 methylnaphthalene (*m/z* 142)

II.2.2 Artículo de investigación

Un método sensible y automático basado en microextracción con sorbentes empaquetados para la determinación de hidrocarburos policíclicos aromáticos en muestras de saliva

Resumen

El objetivo principal de este trabajo fue el desarrollo de un método sensible y automático basado en microextracción con sorbentes empaquetados (MEPS) acoplado a un sistema PTV-GC-MS para la determinación de PAHs sin metabolizar en muestras de saliva. Según nuestra información, es la primera vez que se propone este acoplamiento con este fin. El uso de un espectrómetro de masas cuadrupolar simple (qMS) en lugar de equipos más sofisticados permite la aplicación del método en la mayoría de los laboratorios de todo el mundo.

La extracción se llevó a cabo utilizando un volumen de 1500 µL de saliva dopada con 18 µL de patrón interno y el mismo volumen de metanol o disolución con analitos. El primer paso de la extracción fue un acondicionamiento del sorbente con acetato de etilo y agua ultrapura. A continuación, se retuvieron los analitos de la muestra de saliva en el sorbente. Después, se lavó el cartucho con agua ultrapura, se secó haciendo pasar aire y se procedió a la elución de los compuestos con acetato de etilo. Finalmente, se lavó el cartucho con agua y acetato de etilo para prepararlo para la extracción posterior. Las condiciones utilizadas para el análisis mediante PTV-GC-MS fueron las mismas que aquellas utilizadas en el apartado correspondiente al capítulo II.2.1.

La optimización de los diferentes parámetros que afectaban a la extracción se llevó a cabo utilizando una muestra de agua ultrapura dopada con los analitos de interés, excepto aquellos pasos en los que el uso de saliva fue crítico (velocidad de llenado en la etapa de muestreo, efecto memoria o *carry-over* y recuperación).

El estudio del cartucho y del disolvente de elución óptimos se llevó a cabo simultáneamente. Se compararon las señales analíticas obtenidas cuando los analitos se retuvieron en cartuchos C8 y C18, y posteriormente se eluyeron con los siguientes disolventes orgánicos: acetato de etilo, hexano y metanol. Los mejores resultados se obtuvieron utilizando el cartucho C18 y eluyendo con acetato de etilo.

El volumen de muestra se seleccionó considerando que el volumen total recogido, dependiendo del individuo, no supera normalmente los 7 mL. Además, el aumento del volumen de muestra puede causar el bloqueo del cartucho de MEPS reduciendo el número de experimentos que pueden llevarse a cabo utilizando un único cartucho. Por ello, se probaron volúmenes comprendidos entre 500 y 1500 µL obteniéndose los mejores resultados, como era de esperar, para el mayor volumen, que se seleccionó como óptimo.

En la extracción mediante MEPS la etapa de elución se optimizó de manera que se obtuviera la mayor señal analítica para los analitos de estudio desorbiendo la máxima cantidad posible de los mismos del cartucho. Por ello y con el fin de seleccionar el volumen de elución óptimo, se estudiaron 4 fracciones de elución sucesivas sin ninguna etapa de lavado entre ellas. Se probaron volúmenes de elución comprendidos entre 50 y 110 µL. Se consideró la suma de las señales analíticas para las 4 fracciones como el 100 %. Para todos los volúmenes estudiados, la suma de las tres primeras porciones fue siempre mayor al 98 %. Se observó un aumento en el porcentaje desorbido en la primera fracción al aumentar el volumen de elución. Sin embargo, este porcentaje no era significativo si se comparaba con la disminución observada en la señal analítica para todos los compuestos. Por ello, se estableció un volumen de elución de 50 µL como óptimo.

El efecto memoria se estudió modificando la última etapa de lavado en el proceso de MEPS. Tras el lavado del cartucho con 1500 µL de agua ultrapura se continuó esta etapa de lavado en dos situaciones: con 500 o con 1000 µL de acetato de etilo. En ambas situaciones el resultado fue semejante, observándose efecto memoria solo para el fluoranteno (0.8 %). Con el fin de no prolongar el tiempo total del proceso de MEPS se seleccionó el volumen de 500 µL de acetato de etilo como óptimo.

Para corregir la variabilidad del proceso completo se utilizó el 4-feniltolueno como patrón interno.

Se llevó a cabo el estudio del efecto matriz mediante la comparación de señales de agua ultrapura y de tres muestras de saliva que no contenían PAHs, todas ellas dopadas al mismo nivel de concentración. Se observó efecto matriz principalmente para el fluoranteno, pireno, criseno, benzo(k)fluoranteno y benzo(a)pireno. Este efecto se encontró entre las muestras de saliva y el agua ultrapura, así como entre las distintas muestras de saliva, impidiendo el uso de un calibrado en saliva para cuantificar el resto de las muestras. Por ello, la calibración se llevó a cabo utilizando el método de adición estándar.

A continuación, se procedió a la evaluación del método utilizando salivas dopadas a diferentes niveles de concentración. La cuantificación de PAHs en 9 muestras de saliva se llevó a cabo analizando tres niveles de concentración: muestra no dopada y muestra dopada a dos niveles de concentración. Cada nivel se analizó una vez. El resultado obtenido en este calibrado se comparó con aquel obtenido cuando se analizaron dos réplicas de cada nivel. Los resultados obtenidos fueron semejantes, reduciéndose el volumen de saliva necesario cuando se analizó solamente una réplica. Los valores de los coeficientes de determinación fueron superiores a 0.99 en todos los casos. El fenantreno (15 ± 6 ng L^{-1}) se encontró en una de las muestras analizadas.

La exactitud del método se evaluó mediante la comparación de la concentración añadida sobre diferentes muestras de saliva que no contenían los analitos y aquella predicha utilizando el calibrado. Los valores encontrados oscilaron entre el 78 y el 123 %.

El estudio de la repetibilidad y reproducibilidad del proceso completo del análisis mediante MEPS-PTV-GC-MS se llevó a cabo a dos niveles diferentes de concentración. Los valores obtenidos fueron semejantes en todas las salivas analizadas, con valores comprendidos entre 1-10 % y 2-17 %, respectivamente.

Se calcularon los límites de detección y cuantificación en las 9 muestras de saliva analizadas. A modo de ejemplo, los LODs encontrados para una de ellas oscilaron entre 4.6 ng L^{-1} (fluoranteno) y 79 ng L^{-1} (bifenilo). Los valores fueron similares para todas las salivas y se encontraron en el rango de ppt.

La recuperación del proceso de extracción con MEPS se estudió comparando la señal analítica de los PAHs en una muestra de saliva dopada con aquella obtenida al analizar una disolución en acetato de etilo que contenía los analitos en la concentración equivalente a una preconcentración y elución total. Ambas muestras se filtraron previamente al análisis. Los valores obtenidos oscilaron entre el 24 % (bifenilo) y el 46 % (acenaftileno).

La principal ventaja de este método frente a otros descritos previamente en bibliografía es la automatización del proceso completo de análisis. En la extracción mediante MEPS, así como en la técnica basada en extracción en fase sólida microdispersiva asistida por aire (AA-dμ-SPE), el volumen de disolvente orgánico utilizado en la elución se redujo frente a aquel utilizado en la extracción líquido-líquido (LLE). Sin embargo, el tiempo necesario para extraer los analitos con esta última técnica fue inferior al requerido en el método aquí propuesto. En los métodos sin separación cromatográfica, basados en el análisis de sólidos a presión atmosférica-espectrómetro de masas-tiempo de vuelo (ASAP-Q-TOF-MS), el tiempo de análisis disminuyó considerablemente. Sin embargo, se observó un empeoramiento en la sensibilidad y no fue posible distinguir isómeros con la misma masa molecular.

Como conclusión del trabajo es posible decir que se ha conseguido el objetivo propuesto y se ha puesto a punto de un método sensible y automático basado en MEPS-PTV-GC-MS para la determinación de PAHs sin metabolizar en muestras de saliva.

Además, los buenos resultados obtenidos en lo referente a sensibilidad (del orden de ng L^{-1}), repetibilidad, reproducibilidad y exactitud, así como el uso

de instrumentación simple (qMS) hacen que el método sea de utilidad y pueda ser aplicable en la mayoría de los laboratorios analíticos.

II.2.2 Artículo de investigación

Un método sensible y automático basado en microextracción con sorbentes empaquetados para la determinación de hidrocarburos policíclicos aromáticos en muestras de saliva

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A sensitive and automatic method based on microextraction by packed sorbents for the determination of polycyclic aromatic hydrocarbons in saliva samples

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ABSTRACT

A sensitive method for the quantification of polycyclic aromatic hydrocarbons (PAHs) in saliva samples is proposed. The method is based on the use of microextraction by packed sorbents (MEPS), injection with programmed temperature vaporizer (PTV), gas chromatography (GC) and mass spectrometry with a single quadrupole (MS). As far as we know, the MEPS technique has not been used to date for the analysis of PAHs in saliva samples.

Sample treatment carried out only involves centrifugation and filtration of the saliva sample, which is subjected to the MEPS process without dilution. Standard addition was used as a calibration strategy, due to the matrix effect found using ultrapure water and among the different saliva samples. Nine saliva samples were analysed and phenanthrene was found (15 ± 6 ng L^{-1}) in one of them.

To confirm the accuracy of the method, the concentrations added in the standard additions were compared with the value predicted by the calibration model. The values ranged between 78 and 123%. The limits of detection (LOD) found in the saliva samples ranged between 4.6 and 79 ng L^{-1} and the repeatability and reproducibility were always equal to or lower than 10 and 17%, respectively.

The main advantage of the proposed methodology over the existing ones is the complete automation of the analysis process (extraction, separation and detection). Once the saliva sample is placed in the vial, the entire process occurs on-line.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds formed by two or more fused aromatic rings. Some of these compounds have been considered by the International Agency for Research on Cancer (IARC) as possible carcinogens [1]. In addition, the United States Environmental Protection Agency (US EPA) lists them as priority pollutants [2].

In addition, these compounds have received much attention, due to their effects on humans and their persistence in the environment. Proof of this can be seen by the large number of review articles that have been published addressing diverse aspects related to the presence of PAHs in the environment [3,4] and in food matrices [5,6]. Special attention has also been paid to the determination of PAHs in biological samples [7–11]. The main routes of entry of these compounds into the body are ingestion, dermal contact and inhalation. Once in the body, these compounds can be subjected to different metabolic biotransformations and PAH derivatives can be generated. Specifically, in biological samples, different analytical methods have been developed, mainly in urine samples [12,13], blood [14], serum [15], plasma [16] and exhaled air [17]. Samples of saliva [16,18], hair [19], placenta [20], breast milk [21] and cerebrospinal fluid [22] have been used in lower proportions. Currently, the use of matrices that can be obtained non-invasively as opposed to other types of samples, such as blood and plasma, are becoming greatly important. In this sense, saliva samples are collected and manipulated more easily and allow the determination of unmetabolized PAHs before their biotransformation in the body.

For analysing PAHs in saliva, liquid-liquid extraction (LLE) [18,23] and air-assisted dispersive micro-solid phase extraction (AA-dμ-SPE) [16] have been used as sample preparation procedures. Subsequently, separation is carried out either by using gas (GC) [16,18] or liquid (HPLC) [23] chromatography and detection using a flame ionization

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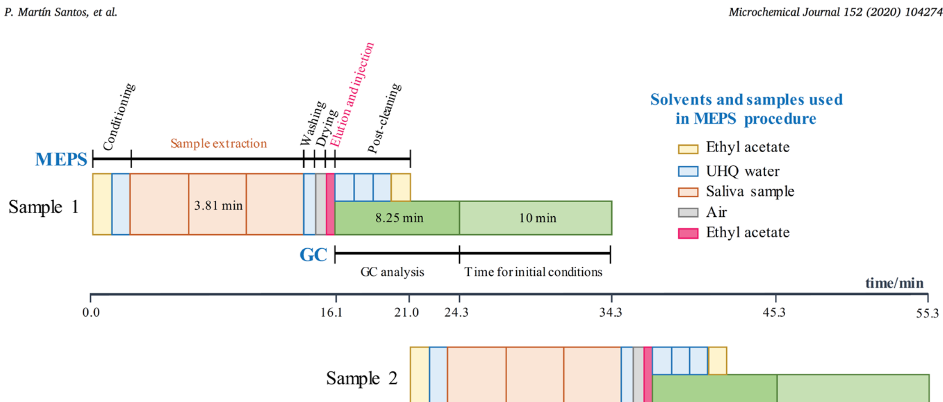


Fig. 1. Scheme of the different steps included in the analysis of two consecutive saliva samples using on-line MEPS-PTV-GC–MS method.

Table 1
Characteristics of the different steps included in the microextraction by packed sorbents procedure.

Step	Sample	Volume/µL	Drawing speed/µL s^{-1}	Discarding speed/µL s^{-1}	Viscosity delay/s
Conditioning	Ethyl acetate	1 × 500	10	100	5
	UHQ water	1 × 500	10	100	5
Sampling	Saliva	3 × 500	5	5	10
Washing	UHQ water	1 × 250	10	100	3
Drying	Air	10 × 500	500	500	0
Elution	**Ethyl acetate**	**1 × 50**	**5**	**5**	**0**
Post-cleaning	UHQ water	3 × 500	10	100	5
	Ethyl acetate	1 × 500	10	100	5

detector (FID) [16] or mass spectrometry (MS) [18,23]. In addition to the previous separative methods, others have been developed without a prior chromatographic separation step, which are based on atmospheric pressure solid analysis probe (ASAP) and quadrupole-time of flight mass spectrometry (Q-TOF-MS) [23,24]. These methods were used for rapidly screening samples and the semi-quantification of analytes. The results obtained were then confirmed using a method based on HPLC-MS/MS [23].

In this work, a sensitive and automatic method for the determination of PAHs in saliva samples is proposed. The method is based on microextraction by packed sorbent (MEPS), programmed temperature vaporization (PTV), gas chromatography (GC) and quadrupole mass spectrometry (MS). As far as we know, the MEPS technique has not been used to date for the analysis of PAHs in saliva samples. The use of a simple instrument (single quadrupole mass spectrometer) instead of more expensive and sophisticated equipment (high-resolution or tandem mass spectrometry) makes the method applicable in laboratories worldwide.

2. Materials and methods

2.1. Chemicals and standard solutions

Pyrene, fluoranthene, phenanthrene, fluorene, 3-phenyltoluene, 4-phenyltoluene (internal standard, IS), biphenyl, 1-methylnaphthalene, 2-methylnaphthalene and naphthalene were supplied by Acros Organics (Geel, Belgium). Benzo(a)pyrene, benzo(k)fluoranthene, anthracene, chrysene, acenaphthylene, acenaphthene, ethyl acetate and methanol were supplied by Sigma-Aldrich (Steinheim, Germany). Hexane and sodium chloride were provided by Scharlab (Barcelona, Spain) and acetonitrile was supplied by Merck (Darmstadt, Germany). The purity of all the analytes was at least 95%.

A stock solution (100 mg L^{-1}) for each analyte was prepared in methanol, except for pyrene, chrysene and benzo(a)pyrene which were prepared in acetonitrile. All solutions were stored at 5 °C and were properly diluted in methanol to prepare the working solutions.

2.2. Saliva samples

Unstimulated saliva samples were taken from 9 volunteers and stored in individual glass vials sealed with a silicone septum cap. Samples were stored at −26 °C until their analysis. After thawing, each saliva sample was centrifuged at 2415 × *g* for 3 min and the supernatant was collected for analysis. Written informed consent was obtained from all the participants.

2.3. MEPS-PTV-GC–MS conditions

A volume of 1500 µL of a saliva sample spiked with 18 µL of the internal standard (5 µg L^{-1}) and 18 µL of methanol (non-spiked saliva sample), or 18 µL of the corresponding solution containing all the analytes (spiked saliva sample), was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter (0.45 µm, Scharlab, Spain) into a 10-mL vial. Then, the vial was placed on a VT32-10 tray and subjected to MEPS extraction.

Automatic extraction of PAHs from saliva samples was carried out using a 500-µL syringe held by a MPS2 Multi-Purpose Sampler. A cartridge containing C18 packed sorbent (SGE Analytical Science, Griesheim, Germany) was placed inside the syringe to retain the analytes. Maestro software (Gerstel) was employed to control sample extraction.

In Fig. 1, the different steps included in the extraction and

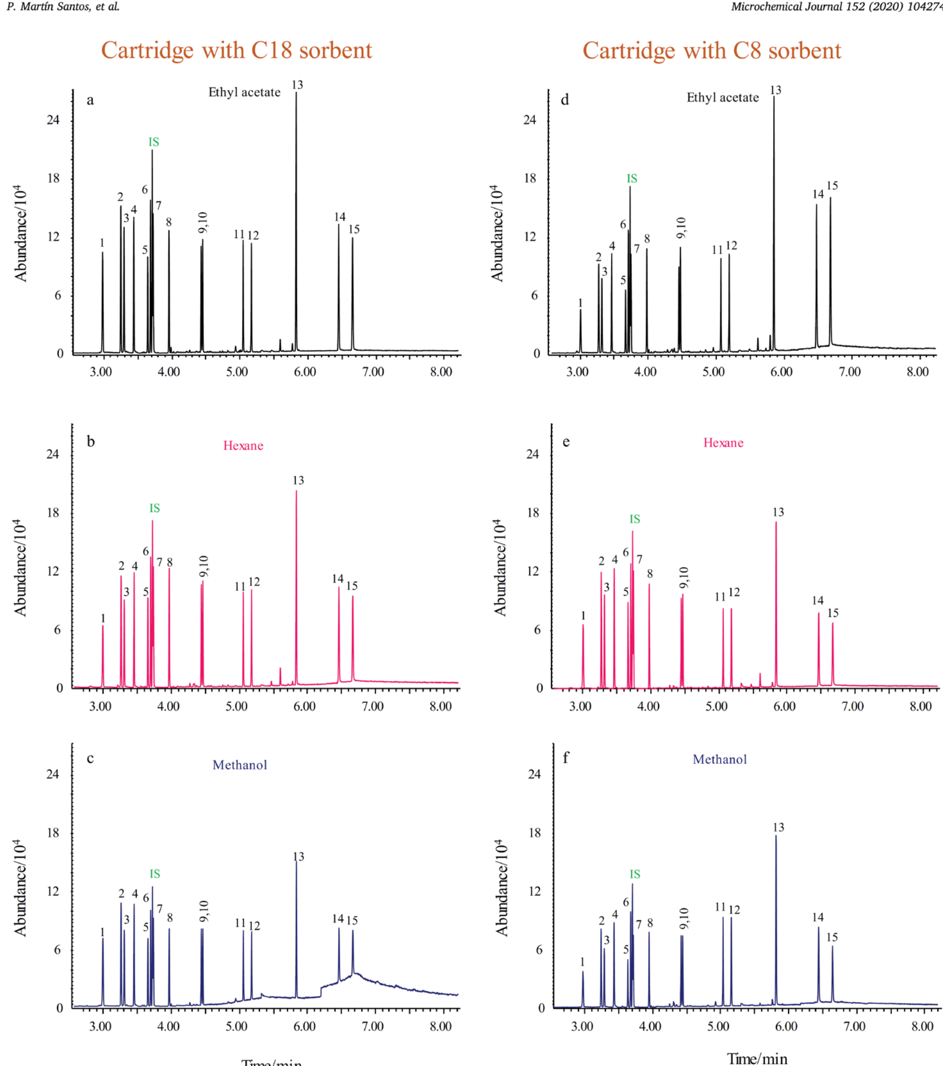
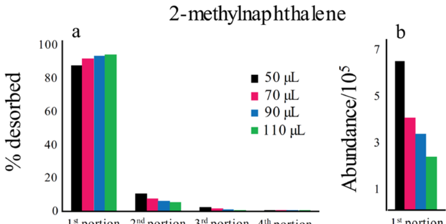
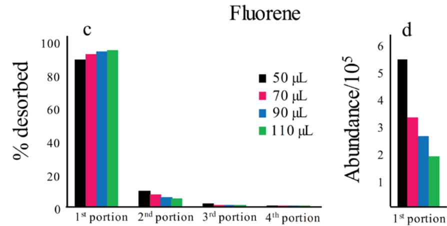


Fig. 2. Comparison of the chromatograms obtained when using C18 (a,b,c) and C8 (d,e,f) sorbents, and ethyl acetate (a,d), hexane (b,e) and methanol (c,f) for elution. The peaks are labelled according to Table 2.

separation processes are shown. First, the sorbent was conditioned with 500 µL of ethyl acetate and 500 µL of ultra-high quality water (UHQ, Wasserlab Ultramatic water purification system, Noain, Spain). Then, the analytes were extracted from the saliva sample (3 cycles of 500 µL). The sorbent was washed with 250 µL of UHQ water to remove possible interferes. Air was then pumped (10 × 500 µL) to dry the cartridge. A volume of 50 µL of ethyl acetate was drawn to elute the analytes and it was directly injected into the PTV system. Post-cleaning of the cartridge included 3 cycles of 500 µL of UHQ water and 1 cycle of 500 µL of ethyl acetate. With the aim of ensuring the complete filling of the syringe, all steps except for drying and elution required a time of viscosity delay. The viscosity delay time of each step, as well as the speed value for drawing and discarding, are shown in Table 1. The time required for extracting each sample was 21 min. As shown in the Fig. 1, the





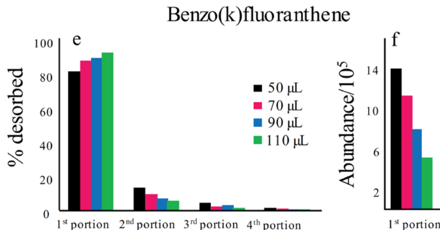


Fig. 3. Desorbed percentage of the first four portions of ethyl acetate (elution solvent) and analytical signal for the first portion of ethyl acetate for 2-methylnaphthalene (a and b), fluorene (c and d) and benzo(k)fluoranthene (e and f).

extraction of PAHs in the second sample overlaps with the chromatographic separation of the first sample.

The PTV-GC–MS instrumental conditions were as previously described [18]. Briefly, injection was performed using a PTV with a liner (71 mm × 2 mm) packed with Tenax-TA®. It was operated in solvent vent mode at 115 °C for 0.46 min, increasing the temperature until 340 °C and establishing 1 min as injection time. The GC programme temperature started at 60 °C (0.5 min), increased at 60 °C min^{-1} to 175 °C, and further increased at 45 °C min^{-1} to 325 °C (2.5 min) for a total runtime of 8.25 min. The time between the analysis of two consecutive samples was set according to the extraction time (21 min), since the automation of the process allowed one saliva sample to be extracted during the GC–MS analysis of the previous sample (Fig. 1). Mass spectrometry detector was used in the SIM/scan synchronous mode. Two full scan groups (2.45–6.20 min at 9.40 scan s^{-1}, and 6.20–8.25 min at 5.19 scan s^{-1}) and a dwell time value of 1 ms for all the m/z ratios selected in the SIM mode were employed.

2.4. Data analysis

The Enhanced ChemStation software from Agilent Technologies [25] was used for data collection.

3. Results and discussion

All the experiments conducted for optimizing the different parameters involved in MEPS were performed using UHQ water (spiked with the analytes), except for those steps in which the use of saliva sample was critical. This was the case of the fill speed in the sampling step, carry-over, and recovery.

3.1. Optimization of MEPS conditions

The following variables were studied: sorbent material, elution solvent, sample volume, elution volume and carry-over.

3.1.1. Sorbent material and elution solvent

Selection of the optimum sorbent material was assayed together with the organic solvent used to elute the analytes. The analytical signals were compared after retaining PAHs with C8 and C18 sorbents and further eluting them with ethyl acetate, hexane, and methanol organic solvents. In Fig. 2, the chromatograms obtained for both sorbents and the different solvents are shown. For most of the analytes, the signal increases slightly when the C18 sorbent and ethyl acetate were used (Fig. 2a). Accordingly, they were selected for further analysis.

3.1.2. Sample volume

For the extraction, the sample was pumped up once and discarded. The effect of sample volume was studied using increasing volumes (500–1500 µL). Saliva production can vary for each volunteer and the volume collected is usually less than 7 mL. Moreover, the number of experiments that can be performed with the same cartridge is reduced when the volume of sample increases, causing the cartridge to become obstructed. Consequently, amounts of saliva greater than 1500 µL were not assayed. As expected, the best results were obtained for the largest volume tested, 1500 µL, which was selected as being the optimum amount.

3.1.3. Elution volume

Elution volumes between 50 and 110 µL of ethyl acetate were studied. For this specific experiment, the original sequence of MEPS was modified by eliminating the post-cleaning step. For each elution volume tested, after eluting and injecting the analytes into the system (1st portion), three additional analyses were performed by drawing and injecting ethyl acetate (2nd, 3rd and 4th portions, respectively). This was done so that the remaining amount of the analytes in the cartridge could be studied without carrying out the post-cleaning step. Considering that the sum of the analytical signals for the four portions was 100%, the percentage corresponding to each portion was calculated. For all the elution volumes tested, the percentage obtained for the sum of the first three portions was higher than 98%. When the elution volume increased, the percentage of analytes desorbed in the first portion also increased. However, this increase was not significant in comparison with the decrease observed in the analytical signal for all compounds. Consequently, an elution volume of 50 µL of ethyl acetate was set as optimum. In Fig. 3 an example of the percentage desorbed in each portion for all the elution volumes tested, as well as the analytical signal obtained for the first portion for 2-methylnaphthalene, fluorene, and benzo(k)fluoranthene, is shown.

3.1.4. Carry-over

In order to study carry-over, ethyl acetate was analysed immediately after the analysis of a saliva sample spiked with the PAHs at the highest concentration level studied (with the corresponding post-

Fig. 4. Study of the matrix effect between UHQ water and three saliva samples spiked with all the analytes at the same concentration level.

Table 2
Characteristics of the standard addition method for one of the saliva samples studied.

Compound	t_R/min	SIM ions[a]	Calibration range/µg L^{-1}	R^2
Naphthalene (No. 1)	2.955	**128**; 129; 127	LD-0.384	0.9971
2-methylnaphthalene (No. 2)	3.225	**142**; 141; 115	LD-0.525	0.9992
1-methylnaphthalene (No. 3)	3.274	**142**; 141; 115	LD-0.341	0.9992
Biphenyl (No. 4)	3.417	**154**; 153; 152	LD-0.234	0.9996
Acenaphthylene (No. 5)	3.623	**152**; 153; 151	LD-0.167	0.9988
3-phenyltoluene (No. 6)	3.666	**168**; 167; 165	LD-0.842	0.9992
Acenaphthene (No. 7)	3.706	153; **154**; 152	LD-0.267	0.9987
Fluorene (No. 8)	3.939	**166**;165; 167	LD-0.309	0.9995
Phenanthrene (No. 9)	4.418	**178**; 176; 179	LD-0.236	0.9999
Anthracene (No. 10)	4.439	**178**; 179; 176	LD-0.241	0.9980
Fluoranthene (No. 11)	5.042	**202**; 203; 200	LD-0.265	0.9978
Pyrene (No. 12)	5.165	**202**; 203; 200	LD-0.307	0.9999
Chrysene (No. 13)	5.829	**228**; 226; 229	LD-0.549	0.9998
Benzo(k)fluoranthene (No. 14)	6.457	**252**; 253; 250	LD-0.679	0.9986
Benzo(a)pyrene (No. 15)	6.664	**252**; 253; 250	LD-0.830	0.9995

[a] Quantifier ion in bold.

cleaning step of the MEPS cartridge). Then, the signals obtained with the saliva sample and with the ethyl acetate were compared. Memory effect was only observed for fluoranthene (0.8%). This effect was similar when post-cleaning of the cartridge included 1500 µL (3 × 500 µL) of UHQ water and one (500 µL) or two (1000 µL = 2 × 500 µL) cycles of ethyl acetate. Consequently, optimum post-cleaning was achieved by washing once with ethyl acetate.

Under the optimum conditions, one C18 cartridge could be used to analyse around 85 saliva samples.

3.2. MEPS-PTV-GC–MS method

All available samples were analysed using the proposed method. To correct the variability of the whole process, 4-phenyltoluene was used as an internal standard.

3.2.1. Matrix effect

To study the possible matrix effect, a comparison was performed between the signals corresponding to UHQ water and the saliva samples, both spiked with the analytes studied at the same concentration level. Three saliva samples without any of the analytes were used. A high matrix effect was observed for five compounds: fluoranthene, pyrene, chrysene, benzo(k)fluoranthene and benzo(a)pyrene. The differences in signal with respect to the UHQ water were between 2 and 363%. In addition, the differences observed among the saliva samples were between 5 and 92%. This prevented a saliva sample for being used to make a calibration model suitable for the rest of the samples. Fig. 4 shows an example of the signals obtained with acenaphthylene, acenaphthene, pyrene and benzo(a)pyrene in the UHQ water and in the three saliva samples. The figure shows analytes with a high matrix effect, and others where this effect does not exist or is less pronounced. In view of these results, the quantification of PAHs in the saliva samples was carried out using the standard addition method.

3.2.2. Evaluation of the method

The concentrations of the PAHs in the standard additions ranged between 0.083 (acenaphthylene) and 5.84 µg L^{-1} (benzo(a)pyrene). The analytical signal used to build the calibration models was the peak area of each analyte in the extracted ion chromatogram in SIM mode. The selected ions are shown in Table 2. To reduce the volume of saliva in the calibrations, three calibration levels were used (non-spiked sample and two additional levels) and each sample was measured only once. This implies that 4.5 mL of sample per individual is necessary (1.5 mL × 3 concentration levels); replicas of the samples were included when a sufficient amount of sample was available. The result given did not differ significantly from the previous one and required 9 mL of sample (1.5 mL × 3 concentration levels × 2 replicates). However, in most cases it is difficult to collect this amount of saliva. Due to these factors, calibrations with three levels of concentration and without replicates were used. In all cases, calibrations with coefficient of determination values greater than 0.99 were obtained. The calibration range and R^2 values for one of the saliva samples studied are shown in Table 2.

Phenanthrene was found in one of the saliva samples studied at a concentration of 15 ± 6 ng L^{-1}. In Fig. 5a, the chromatogram (SIM mode) of this sample and those corresponding to the two standard additions are shown when the base peak of phenanthrene is selected. In

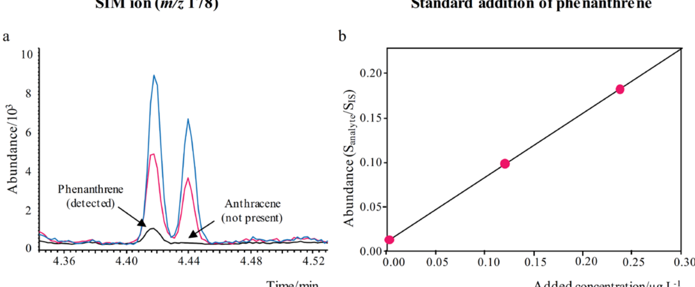


Fig. 5. (a) Extracted ion chromatogram (*m/z* 178) in SIM mode for the saliva sample containing phenanthrene and the two standard additions. (b) Calibration curve of phenanthrene using the standard addition method.

Table 3
Figures of merit of the MEPS-PTV-GC–MS method for one of the saliva samples studied.

Compound	Repeatability Low level	High level	Reproducibility Low level	High level	LOD/ng L^{-1}	LOQ/ng L^{-1}	Accuracy/%
Naphthalene	4	3	8	6	17	58	83
2-methylnaphthalene	4	2	6	5	32	105	111
1-methylnaphthalene	8	1	8	2	35	117	91
Biphenyl	4	1	17	10	79	265	107
Acenaphthylene	9	1	12	5	9.4	31	89
3-phenyltoluene	5	1	9	5	29	96	91
Acenaphthene	6	4	6	5	21	69	88
Fluorene	2	3	12	6	11	38	78
Phenanthrene	4	2	12	6	17	55	120
Anthracene	5	1	8	3	14	47	119
Fluoranthene	2	0	9	5	4.6	15	88
Pyrene	5	1	6	7	16	52	98
Chrysene	4	1	8	8	10	34	104
Benzo(k)fluoranthene	10	1	11	10	35	116	114
Benzo(a)pyrene	2	1	15	11	10	34	108

Table 4
Comparison of published works analysing PAHs and derivatives in saliva samples.

Reference	n° PAHs	Analytical method	LOD/µg L^{-1}
Rajabi et al. [16]	5[a]	AA-dµ-SPE-GC-FID	0.3–0.6[d]
Martín Santos et al. [18]	16[a]	LLE-PTV-GC–MS	0.002–0.057[e]
Carrizo et al. [23]	2[b]	ASAP-Q-TOF-MS	100–510[d]
	2[b]	LLE-HPLC-MS/MS	10–50[d]
Carrizo et al. [24]	31[c]	ASAP-Q-TOF-MS	5–20[e]
This work	15[a]	MEPS-PTV-GC–MS	0.005–0.079[d]

[a] Unmetabolized PAHs.
[b] Hydroxy-PAHs.
[c] Fifteen unmetabolized PAHs, 8 nitro-PAHs and 8 oxo-PAHs.
[d] LOD in saliva sample.
[e] LOD in organic solvent.

addition, the signal of another compound (anthracene) not present in the sample and containing the *m/z* ratio 178 is also shown. In Fig. 5b, the corresponding calibration curve of phenanthrene using the standard addition method is shown. No analytes were found in the rest of the samples.

To check the accuracy of the proposed methodology, several saliva samples in which none of the analytes had been found were used, and the amount added in the standard additions was compared with the value predicted by the model. In all cases, recovery values ranged between 78 and 123% for all PAHs. The results for one of the saliva samples studied are shown in Table 3.

The repeatability and reproducibility were studied at two concentration levels, low (0.035–0.157 µg L^{-1}) and high (0.251–1.25 µg L^{-1}), considering all the steps of the analytical process (MEPS-PTV-GC–MS). For both levels, repeatability was assessed by measuring the samples 4 times on the same day. In the case of reproducibility, samples were measured 4 times on two different days. The results for one of the saliva samples studied are shown in Table 3. The relative standard deviation was between 1 and 10% for repeatability and between 2 and 17% for reproducibility. The values obtained were similar for all the saliva samples analysed.

The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times, respectively, the standard deviation of a saliva sample measured four times (S/N ratio of three for all analytes) and divided by the slope of the corresponding calibration curve. LOD for one of the saliva samples studied are shown in Table 3, with values between 4.6 ng L^{-1} (fluoranthene) and 79 ng L^{-1} (biphenyl). The values obtained were similar for all the saliva samples analysed.

Recovery of the MEPS extraction procedure was studied by comparing the analytical signal of each analyte obtained for a saliva sample spiked with all the analytes (0.13–1.94 µg L^{-1}) with one obtained for a solution in ethyl acetate containing all the analytes at an equivalent concentration (3.9–58.2 µg L^{-1}) for a preconcentration factor of 30 (1500 µL of sample/50 µL of elution volume). Both samples were previously filtered (PTFE). The values obtained ranged between 24% and 46% for biphenyl and acenaphthylene, respectively.

3.3. Comparison with other works

To date, there is a small number of analytical methods developed for the analysis of PAHs in saliva samples. The extraction methods used have been AA-dμ-SPE [16] and LLE [18,23]. The main advantage of the proposed method compared to others is the automation of the entire analysis process. The technique based on AA-dμ-SPE requires low consumption of adsorbent and there is a reduction or elimination of toxic organic solvents in contrast to the 1-mL volume used in LLE. In the proposed work, the volume of organic solvent used for the elution of the analytes was 50 μL. Extraction time for LLE was 6 min while the complete MEPS process per sample lasted 21 min.

The separation and detection techniques used have mainly been GC–MS [18], GC-FID [16], and HPLC-MS/MS [23], although methods without the separation step based on ASAP-Q-TOF-MS [23,24] have also been reported. In the case of this last method, the main advantages are the elimination of the sample preparation step (and the errors associated to it) and a significant reduction in the analysis time. The main drawbacks of these rapid screening methods are that the detection limits become worse by several orders of magnitude (they are in the range of mg L^{-1}) and the impossibility of distinguishing isomers with the same molecular weight (example: fluoranthene and pyrene). Table 4 shows the characteristics of published studies analysing PAHs and their derivatives in saliva samples. As can be seen in this table, the LOD proposed in this work are of the same order (ng L^{-1}) as those described in the work with the lowest values [18] for the detection of PAHs in saliva samples.

4. Conclusions

A sensitive and automatic method for the determination of PAHs in saliva samples has been proposed. Once the saliva sample is placed in the vial, all the steps of the method (extraction, separation and determination) take place on-line.

The detection limits found in saliva were of the order of ng L^{-1}, and the repeatability, reproducibility, and accuracy of the method were satisfactory.

The use of a single quadrupole mass spectrometer facilitates the application of the method in most laboratories as compared to other methods described in literature [23,24] based on high-resolution equipment or tandem mass spectrometry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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II.3 Determinación de hidrocarburos policíclicos aromáticos en muestras biológicas humanas

II.3.1 Artículo de revisión

Determinación de hidrocarburos policíclicos aromáticos en muestras biológicas humanas. Una revisión crítica

Resumen

El aumento del número de publicaciones de investigación y revisión enfocadas a hidrocarburos policíclicos aromáticos pone de manifiesto la creciente preocupación por los mismos. Hasta la fecha, se han descrito numerosos artículos de revisión atendiendo a diferentes aspectos relacionados con estos compuestos. Entre ellos, se encuentran los artículos enfocados a su presencia en plantas, aire, suelo, agua, comida y entornos de trabajo. También se ha descrito su reactividad química y se han evaluado diferentes procedimientos para la eliminación de los mismos del medio ambiente. Por el contrario, el número de artículos de revisión enfocados a la presencia de PAHs en muestras biológicas es muy limitado. Entre ellos, se encuentran aquellos trabajos enfocados a la determinación de derivados de PAHs en muestras de orina, considerando aspectos relacionados con la exposición, metabolismo, excreción y biomonitorización. También se ha llevado a cabo una revisión en busca de nuevos biomarcadores de PAHs en muestras de orina y sangre tras una exposición previa, relacionando estos compuestos con diversos efectos para la salud. En un artículo enfocado principalmente a la determinación de PAHs en la atmósfera se proporcionó, además, una breve información acerca de los métodos descritos para determinar PAHs y sus derivados en muestras de orina. Sin embargo, según nuestra información, no se ha descrito ningún trabajo que resuma las principales metodologías para determinar PAHs en diferentes matrices biológicas.

En esta Tesis Doctoral se incluye un artículo de revisión enfocado a la determinación de hidrocarburos policíclicos aromáticos, sin metabolizar y metabolizados, en muestras biológicas humanas. En este trabajo se han evaluado matrices con un destacado interés, como la orina, la sangre, el plasma, el suero y, en los últimos años, la saliva. Además, se han estudiado otras matrices como aire exhalado, pelo, fluido folicular, placenta, leche materna y fluido cerebroespinal.

El uso de orina para la determinación de PAHs ha sido habitual debido a que la toma de muestra se realiza de forma no invasiva, fácil y rápida. Además, la cantidad de muestra recolectada es elevada en comparación con otras matrices

y existe una relación directa entre la excreción de PAHs y la exposición previa a los mismos. Sin embargo, la baja solubilidad de estos compuestos en matrices acuosas hace que se encuentren en baja concentración en comparación con otros posibles interferentes, lo que dificulta la determinación de los compuestos de interés.

La sangre ha sido ampliamente utilizada para estudiar los niveles de PAHs en diferentes individuos tras una exposición reciente. Sin embargo, estos niveles son habitualmente más bajos que los encontrados en orina debido a que los PAHs se metabolizan tras entrar en el cuerpo. La ventaja que presenta la sangre frente a la orina es la posibilidad de evitar la toma de muestra en un día completo o el ajuste de concentraciones que compense los posibles efectos de dilución en función de la ingesta de líquidos. La principal desventaja radica en la dificultad de la toma de muestra, siendo necesaria la presencia de especialistas al ser una toma de muestra invasiva.

Una posible alternativa a estas matrices que está adquiriendo interés en los últimos años es la saliva. La toma de muestra de esta matriz se lleva a cabo de manera no invasiva, rápida y sencilla, y permite la determinación de PAHs de manera previa a la biotransformación. La clara desventaja que presenta es el bajo volumen de muestra que es posible recoger en función de cada individuo. Además, es necesario tener en cuenta la posible modificación en la concentración de PAHs en función de la ingesta de comida, líquidos o de la técnica utilizada para la toma de muestra.

Desde un punto crítico, en este trabajo de revisión se han evaluado las principales ventajas y desventajas de los diferentes trabajos en cuanto a los métodos de muestreo, técnicas de preconcentración, así como atendiendo a las diferentes estrategias utilizadas para separar y detectar los PAHs. Se revisaron también aquellos trabajos que no incluyeron una etapa de separación de los analitos de manera previa a la detección.

El procedimiento habitual para determinar PAHs en muestras biológicas incluyó diferentes etapas, aunque en ocasiones no todas se llevaron a cabo. En algunos trabajos el procedimiento se inició realizando una hidrólisis. A continuación, en ocasiones se llevaron a cabo procesos de derivatización, digestión o saponificación. Posteriormente y de manera habitual, se procedió a la extracción y preconcentración de los analitos. Una vez extraídos, en algunos estudios se incluyeron procesos de derivatización, congelación, incubación y/o lavado. Finalmente se procedió a la separación, generalmente con técnicas cromatográficas, y a la detección de los analitos, en la mayoría de los casos con técnicas basadas en espectrometría de masas.

Las principales conclusiones que se pueden destacar son las siguientes. En primer lugar, a la hora de determinar PAHs en matrices biológicas humanas existe preferencia por aquellas matrices no invasivas con respecto a las matrices que requieren especialistas en la toma de muestra. Sin embargo, la selección de esta debe llevarse a cabo en función del tipo de analitos que se quiere analizar, teniendo en cuenta que la orina es la más utilizada para determinar los derivados, mientras que los compuestos sin metabolizar y, por tanto, más relacionados con una exposición previa reciente, se han determinado de manera más habitual en saliva, aire exhalado y sangre. Por el contrario, si se desea estudiar la exposición a largo plazo la matriz seleccionada habitualmente es el pelo.

Atendiendo al procedimiento de extracción, a pesar de existir numerosas alternativas (generación de espacio de cabeza y microextracción en fase sólida en la modalidad de espacio de cabeza, entre otras) los mejores resultados correspondieron a aquellas técnicas en las que existe un contacto directo entre los analitos y un material adsorbente o un disolvente de extracción, como la microextracción en fase sólida en la modalidad de inmersión directa o la extracción líquido-líquido.

Finalmente, en la mayoría de los casos las metodologías analíticas propuestas se basaron en métodos separativos. Los métodos basados en cromatografía de gases permitieron el análisis de un mayor número de analitos de manera simultánea. Por el contrario, algunos trabajos basados en cromatografía líquida requirieron el uso de diferentes técnicas de detección debido a la ausencia de respuesta de los PAHs de interés, incrementando así el tiempo de análisis. Los mejores resultados en términos de sensibilidad y selectividad se consiguieron al utilizar la espectrometría de masas.

En el caso de la orina y la saliva, se han propuesto, además, métodos sin etapa de separación previa a la detección. Estos métodos proporcionaron una respuesta rápida con una menor información que los métodos separativos. Sin embargo, dicha información, tratada generalmente con técnicas quimiométricas, fue suficiente para resolver el problema planteado en cada caso. Estos métodos no separativos se utilizaron generalmente como criba en el análisis de un gran número de muestras y, posteriormente, solo aquellas muestras con concentraciones consideradas como anómalas se analizaron con la metodología separativa con fines de confirmación. De este modo se redujo el número de muestras que debían ser analizadas con los métodos separativos y, con ello, el tiempo de análisis.

En conclusión, a la hora de determinar PAHs en matrices biológicas, es necesario establecer el tipo de exposición, el tipo de analitos, la sensibilidad deseada y el tiempo de análisis, entre otros, para así seleccionar la metodología más adecuada que se debe utilizar.

II.3.1 Artículo de revisión

Determinación de hidrocarburos policíclicos aromáticos en muestras biológicas humanas. Una revisión crítica

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Determination of polycyclic aromatic hydrocarbons in human biological samples: A critical review

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A B S T R A C T

Polycyclic aromatic hydrocarbons (PAHs) have raised great concern all over the world due to their carcinogenicity and persistence in nature. Exposure to these analytes can be estimated by determining their concentration in biological matrices, allowing PAH levels in the corresponding samples to be extrapolated to those in the environment. In this article we offer a critical review of the methods used to determine PAHs in different biological samples (urine, blood, plasma, serum, saliva, exhaled breath, hair, follicular fluid, placenta, breast milk and cerebrospinal fluid). We focus on sampling methods, preconcentration procedures and different strategies of analysis of PAHs. Emphasis will be placed on the analysis of both unmetabolized and metabolized PAHs, as these analytes usually require different forms of sample treatment. Also, one type of PAHs can be more interesting than others depending on the objective of the study. Advantages and disadvantages of the main contributions will be discussed.

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1. Introduction

Polycyclic aromatic hydrocarbons, a class of chemicals comprised of at least two fused aromatic rings, have been listed by the International Agency for Research on Cancer (IARC) [1] as possible carcinogens. Several studies have been aimed at assessing health risks to humans due to PAHs exposure, such as cancer [2,3], cardiovascular events and obesity [4,5], diabetes [6,7] and oxidative stress [8,9] among others.

The United States Environmental Protection Agency (U.S. EPA) [10] has considered PAHs to be priority pollutants. Resulting from incomplete combustion of organic matter, they are ubiquitous contaminants of the environment, and are present in aqueous [11,12], air [13,14] and solid samples such as corals [12], solid particles in the air [14] and sediments [12,15], as well as in food samples [16,17].

PAHs can enter the body directly by ingestion. Food is one of the most important sources of exposure, but PAHs can also enter the body by dermal contact or via inhalation. These compounds are then metabolized producing different metabolites. For this reason, studies related to the determination of PAH metabolites and the parent compounds can be found in the literature. In Fig. 1, a scheme of the main sources of PAHs and human exposure is shown. The determination of PAH metabolites has been explored by Oliveira et al. [18] in situations where they are excreted in urine after being metabolized via oxidative pathways, producing phenols, dihydrodiols and triols, among others. These authors consider that monohydroxyl PAHs reflect the internal dose received from environmental exposure. Woudneh et al. [19] have studied hydroxyl polycyclic aromatic hydrocarbons (OH-PAHs), since PAHs rapidly biotransform into these analytes after exposure and further biotransform into reactive electrophiles which bind to DNA causing carcinogenicity and mutagenicity. Guo et al. [20] report that the half-life of OH-PAHs in the human body is about few hours. These authors also report that low molecular OH-PAHs are excreted in urine, whereas high molecular PAHs are excreted in the faeces. Conversely, the alternative of determining unmetabolized analytes has also been justified by several authors. According to Campo et al. [21,22], while the elimination of metabolites depends on the biotransformation rate inside the body, the parent compounds are less susceptible to intra-individual variability. De Craemer et al. [23] also agree that the concentration of unmetabolized PAHs in urine is less sensitive to variability than the corresponding metabolites, and as a consequence their determination does not require creatinine normalization.

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Abbreviations

AA-dμ-SPE	air-assisted dispersive micro-solid phase extraction
AgNPs	silver nanoparticles
ASAP	atmospheric pressure solid analysis probe
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CNL	constant neutral loss
CRM	certified reference material
DDP	diethoxydiphenylsilane
DLLME	dispersive liquid-liquid microextraction
EI	electron ionization
ESI	electrospray ionization
F	fluorescence detection
FID	flame ionization detector
FT-IR	Fourier-transform infrared spectroscopy
GAC	green analytical chemistry
GC	gas chromatography
HPLC	high performance liquid chromatography
HS	headspace
IARC	International Agency for Research on Cancer
LLE	liquid-liquid extraction
LODs	limits of detection
MES	microwave extraction system
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	methyl-*N*-(trimethylsilyl)trifluoroacetamide
MTBSTFA	*N*-(t-butyldimethylsilyl)-*N*-methyltrifluoroacetamide
MWCNT	ionic liquid-mediated multi-walled carbon nanotube
NCI	negative chemical ionization
nMIP	nano-sized molecularly imprinted polymer
OH-PAHs	hydroxyl polycyclic aromatic hydrocarbons
PAHs	polycyclic aromatic hydrocarbons
PCA	principal component analysis
PDA	polydopamine
PDMS	poly(dimethylsiloxane)
PEEK	polyetheretherketone
PID	photoionization detector
PLS	partial least squares
PS	polystyrene
PT-SPE	pipette-tip solid phase extraction
PTV	programmed temperature vaporizer
PUF	polyurethane foam
q	single quadrupole
QqQ	triple quadrupole
SBSE	stir bar sorptive extraction
SEM	scanning electron microscope
SIM	selected ion monitoring
SIMCA	soft independent modelling of class analogy
SLE	solid-liquid extraction
SPE	solid phase extraction
μ-SPE	micro-solid phase extraction
SPLE	selective pressurized liquid extraction
SPME	solid phase microextraction
(S,R)-HFBOPCl	(2S,4R)-*N*-heptafluorobutyryl-4-heptafluorobutoyloxy-propyl chloride
SRM	selected reaction monitoring
SVM	support vector machine
TE	thermal extraction
TOF	time of flight
Tri-Sil TBT	*N*-Trimethylsilylimidazole/N,O-Bis(trimethylsilyl) acetamide/trymethylchlorosilane
TSFS	total synchronous fluorescence
U.S. EPA	United States Environmental Protection Agency
UV	ultraviolet detection

To date, many reviews have been published addressing different aspects related to PAHs such as their presence in the environment, chemical reactivity, their elimination, as well as their determination in different types of samples. Reviews aimed at describing the presence of polycyclic aromatic hydrocarbons in the environment (plants, food, air, soil, workplaces and water) have been published by Srogi [24] and Alegbeleye et al. [25]. In the latter publication the effects of PAHs on human health, soils and aquatic systems were also considered, as well as in work by Abdel-Shafy et al. [26]. Keyte et al. [27] describe the chemical reactivity of these analytes along with their rate and mechanisms. Studies evaluating the elimination of these compounds from the environment using living organisms have been discussed by Alegbeleye et al. [25] and Abdel-Shafy [26]. Lamichhane et al. [28] examined the literature available for removing analytes by sorption from aqueous media, whereas Ncube et al. [29] extended this study by using adsorbents for isolating PAHs from organic solvent extracts. Lawal [30] focused his review on the determination of PAHs in the environment, paying special attention to sampling, sample preparation and extraction and the analytical methods used. Recent reviews have been focused on the presence of PAHs in food matrices such as fruits and vegetables [17] or processed food [31]. With reference to biological samples, Jacob et al. [32] summarized the literature related to the determination of polycyclic aromatic hydrocarbons metabolites (pyrene, phenanthrene and benzo(*a*)pyrene metabolites and DNA adducts) in human urine, considering aspects related to exposure, metabolism, excretion and biomonitoring. Boogaard [33] and Angerer et al. [34] on the other hand reviewed many works oriented towards finding new PAHs biomarkers in urine and blood after exposure, as well as relating PAHs to health effects. More recently, Gao et al. [35] published a review focusing on biochemical aspects, placing special attention on the metabolic pathways of the PAHs after entering the body, their effects and the different biomarkers available in the literature. Information about separation methods for the determination of PAHs and their derivatives in urine has been included in the work published by Hayakawa et al. [36], who reviewed the different steps of the available analytical methods (sampling, extraction, clean-up and analysis) for the determination of PAHs in the atmosphere.

In view of the effects that polycyclic aromatic hydrocarbons have on humans and their persistence in the environment, reliable methods for their determination in biological samples are continually required.

In this review, the methods reported for the determination of parent PAHs and their metabolites in biological samples, such as urine, blood, serum, saliva and hair, among others, are discussed. In addition, aspects including sample treatment and different methods for the separation and detection of these analytes will be addressed. In Fig. 2, the main extraction procedures and separation and detection methods considered in this review are shown. The results found in these studies will be compared and the main benefits and inconveniences of the methods will be discussed.

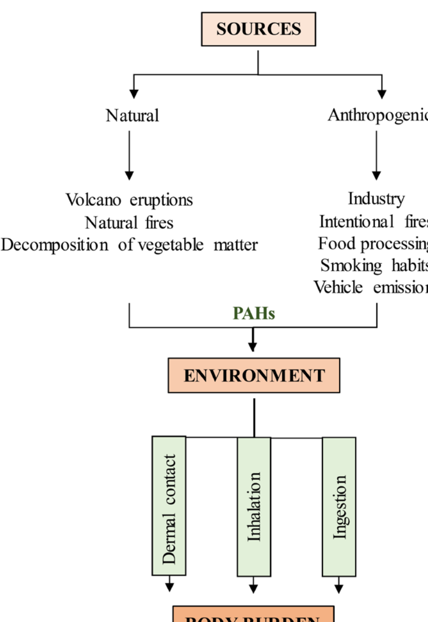


Fig. 1. Sources of PAHs and exposure of humans.

2. Determination of polycyclic aromatic hydrocarbons and their metabolites in human biological samples

The procedure for determining PAHs in biological samples is carried out following different steps (see Fig. 2), although not all are present in each study considered. First, hydrolysis is used because metabolized PAHs are found in samples as conjugated (glutathione, glucuronide and sulphate conjugates) and unconjugated compounds; however, in some studies the process of hydrolysis is eliminated. Prior to extraction other procedures, such as saponification, digestion and derivatization, have been used. Then, the analytes are extracted and preconcentrated. Once the compounds are extracted, some studies include procedures involving derivatization, freezing, incubation and/or clean-up. Finally, the determination of PAHs is carried out using methods that include chromatographic separation, although in some studies the analysis is performed without previous separation of the analytes in the sample.

The review is divided into the extraction techniques and separation and detection methods reported in the literature for each type of sample.

2.1. Urine samples

The use of urine samples to determine analytes of interest is widespread due to non-invasive sampling. The main advantages of the use of such samples is that a large amount of sample can be collected, this matrix is easy and fast to process, and owing to the relationship found between intake or exposure and the excretion of the analytes [18,23]. On the other hand, disadvantages such as the possible existence of a matrix effect, low solubility of some compounds in aqueous solutions that leads to low concentrations, and the inference of other compounds found in higher concentrations have to be taken into account. In addition, if the metabolites produced after the reaction of the parent compounds are to be determined, the rate of metabolism in each individual has to be considered.

Since Jacob et al. [32] published an extensive review in 2002 on the determination of PAHs in urine samples, we will only discuss papers reported during the last fifteen years for determining unmetabolized PAHs and their metabolites in urine samples.

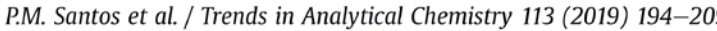
2.1.1. Extraction procedures

Several techniques have been proposed for the extraction and preconcentration of unmetabolized and metabolized PAHs from urine samples prior to their analysis. In Table 1, the characteristics of the most relevant methods proposed are shown.

Headspace-solid phase microextraction (HS-SPME) is one of the techniques that has been most frequently used [21,22,37–44]. The urine sample is heated at a constant temperature and the PAHs are adsorbed in fibre and further desorbed into the system. The boiling point of the analytes of interest (218–536°C, [22]) are usually higher than the temperatures used to generate the HS (below 85°C, since urine is an aqueous matrix) and to desorb the analytes for their injection (below 280°C). These processes are time consuming (30–90 min). Consequently, extraction by HS could be less effective in comparison to techniques which involve direct immersion or those based on contact between liquids, such as dispersive liquid-liquid microextraction (DLLME) or liquid-liquid extraction (LLE). This situation was demonstrated by Campo et al. [23,45,46], where the authors compare HS-SPME and SPME using direct immersion. In this study it was observed that sensitivity for high molecular PAHs (those with five and six aromatic rings) was improved when the procedure with direct immersion was used. This procedure allowed the quantification of a higher number of analytes than the one using HS. Furthermore, direct contact with the sample could reduce fibre performance.

In the method developed by Vatani et al. [41], the fibre used in the adsorption process was an ionic liquid-mediated multi-walled carbon nanotube (MWCNT)-poly(dimethylsiloxane) (PDMS), which was prepared in the laboratory using a sol-gel technique for increasing the surface area with this porous structure. This study showed that the stability and long durability of the proposed fibre allowed it to be used more than 210 times. However, the time required for its preparation was long (more than four days), increasing the time of analysis in comparison with the use of commercially available fibres. The procedure also required the identification of the chemical structure of the fibre using Fourier-transform infrared spectroscopy (FT-IR), and scanning electron microscope (SEM) for determining its morphology.

Laboratory prepared fibres of diethoxydiphenylsilane (DDP) were also used for the determination of metabolized PAHs using HS-SPME with *in situ* derivatization [44]. These fibres provided acceptable results in comparison to commercially available fibres. An alternative to this approach was proposed by Liu et al. [47], where the extraction of the analytes was carried out by SPME without the use of a derivatization step. In this second approach, the time required for the adsorption process was reduced by half (from 90 to 45 min) and sensitivity was also improved (detection limit ranged from 100-2000 ppt to 30–60 ppt). Similar results to those found by Liu et al. [47] were reported by Yang et al. [48] in



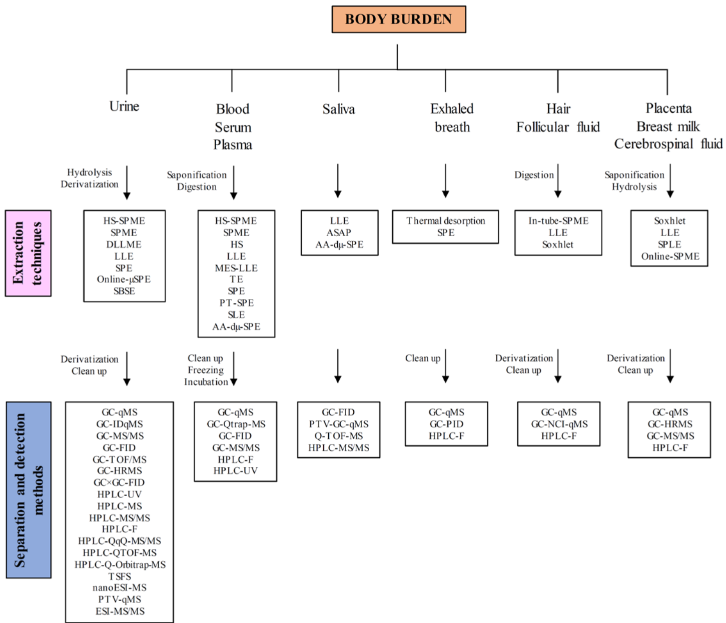


Fig. 2. Basic information on types of samples, extraction, and separation and detection techniques applied for determination of PAHs in human biological samples.

terms of limits of detection (LODs) when SPME followed by elution/spray ionization in a glass capillary was used to determine monohydroxylated PAHs. With this modification, the time required to extract the analytes was drastically reduced (3 min).

Another technique that has been widely employed is solid-phase extraction (SPE) [49–55]. Buratti et al. [49] extracted unmetabolized analytes by SPE with polyurethane foam (PUF) chips that were manually prepared. This manual procedure makes it difficult to obtain similar chips, may worsen reproducibility, and also increases the time needed to carry out the analysis.

Another contribution was based on two SPE phases in series for extracting metabolized and unmetabolized PAHs [50]. After hydrolysis, the sample was passed through two different cartridges for the selective retention of each type of analyte. Then, the samples were jointly eluted and loaded onto a silica column and further eluted with two different solvents. In this way two extracts for the independent analysis of each group of compounds were obtained: one of the groups containing the metabolized compounds (which required further derivatization and incubation) and the other one the unmetabolized PAHs. Since the extraction procedure was carried out simultaneously until the last elution step, this method is less time consuming than ones previously considered where unmetabolized PAHs were independently extracted by HS-SPME [38,40,42] and metabolized PAHs were extracted by SPE [38] and LLE [40,42].

The use of prepared Fe_3O_4 magnetic nanoparticles has also been proposed for SPE by Bianchi et al. [52]. In this work, characterization techniques were required prior to the use of the nanoparticles for extraction of unmetabolized PAHs. The main advantage of this method is that the analytes can be adsorbed in 6 min.

Zhang et al. [54] prepared *in situ* silver nanoparticles (AgNPs) on polydopamine (PDA) coated polystyrene electrospun fibres (PS@PDA-Ag) for their further use in online micro-solid phase extraction (μ-SPE). The results obtained were good due to the large surface area, high porosity and certain resistance to matrix interference, as well as the automation of the extraction procedure. However, the method for preparing the nanoparticles took more than three days. The inconvenience of manually preparing the nanoparticles, the need for the use of characterization techniques, the questionable reproducibility, as well as the increase in time until analysis must be taken into account.

In the study reported by Chauhan et al. [55], OH-PAHs were extracted using SPE based on synthetized nano-sized molecularly imprinted polymer (nMIP) and LLE. The results obtained with both techniques were compared. While the polymer used for extraction required more than 24 h for its preparation, the selectivity to extract the desired analytes was satisfactory only with the SPE technique. The presence of interfering compounds as well as worse sensitivity (reduced approximately by half) was found when LLE was used.

Other alternative methods reported in the literature are based on stir bar sorptive extraction (SBSE) [56] and are used to reduce interference and organic solvent consumption, but require over 3 h

Table 1
Relevant studies for the determination of PAHs in urine samples.

Reference	nº PAHs	Sample preparation procedure	Separation and detection method	Detection limits (ng L^{-1})
Unmetabolized PAHs				
[22]	13	HS-SPME	GC-IDqMS	2.28–22.8
[23][a]	16	SPME	GC-MS/MS	0.5–5.4
[40][a]	13	HS-SPME	GC-IDqMS	2–25
[41][b]	3	HS-SPME	GC-FID	0.5–4
[45][a]	10	SPME	GC-IDqMS	0.5–2.2
[49][b]	11	SPE	HPLC-UV	0.1–0.5
[50]	19	Hydrolysis-SPE	GC-qMS	3–90
[51][b]	18	Enzymatic incubation-SPE	GC-TOF/MS	10–100
[52][b]	16	SPE	GC-qMS	0.04–0.39
[58]	3	DLLME	GC-FID	30–40
[59][c]	11	LLE	PTV-qMS	620–8210
Metabolized PAHs				
[19]	4	Enzymatic hydrolysis-LLE-derivatization	GC-HRMS	13–23.9
[40,60][a]	12	Enzymatic hydrolysis-LLE-derivatization	GC-qMS	100–1400
[44][b]	5	Enzymatic hydrolysis-HS-SPME-*in situ* derivatization	GC-qMS	100–2000
[47]	7	Enzymatic hydrolysis-SPME	GC-qMS	30–160
[48][c]	5	Enzymatic hydrolysis-SPME	nanoESI-hybrid QqQ/Qtrap-MS	10
[50]	34	Hydrolysis-SPE-derivatization-incubation	GC-qMS	6–181
[53]	13	Enzymatic hydrolysis-SPE	HPLC-MS/MS	10–500
[54][b]	3	Enzymatic hydrolysis-online μSPE	HPLC-MS	7–32
[55][b]	5	Acid hydrolysis-SPE	HPLC-F	330–2600
[56][a]	11	Enzymatic hydrolysis-SBSE	HPLC-MS/MS	1–22
[57][a]	6	Pre-column enrichment	HPLC-F	24–96
[61]	11	Enzymatic hydrolysis-LLE-clean up	HPLC-MS/MS	10–25
[62]	10	Enzymatic hydrolysis-LLE-clean up	HPLC-MS/MS	1.72–17.47
[63]	19	Methanol addition, vortexing and centrifugation	HPLC-QqQ-MS/MS	–
			HPLC-QTOF-MS	–
			HPLC-Q-Orbitrap-MS	–
[66]	23	Enzymatic hydrolysis-SPE-derivatization	GC-HRMS	2–10
[67]	10	Enzymatic hydrolysis-LLE-derivatization	GC×GC-FID	30–180
[68]	9	Enzymatic hydrolysis-LLE-derivatization	HPLC-MS/MS	10–200
[69][c]	5	Acid hydrolysis-SPE	TSFS	100–900
[70][c]	1	Acid/enzymatic hydrolysis-SPE	ESI-MS/MS	10

–Information not available.
[a] Quantification limits.
[b] Manually prepared fibre.
[c] Non-separative methods.

to extract the analytes. The use of a pre-column consisting of copper phthalocyanine modified silica gel to enrich the analytes before the analysis has also been proposed [57]. Although the use of these techniques is less common, the limits of quantification achieved were in the same range (ppt) than those found with other techniques, as can be seen in Table 1.

Techniques based on liquid extraction have also been used for the determination of PAHs in urine samples. DLLME has been applied to extract unmetabolized PAHs from urine samples [58], reducing the extraction time to 4 min. The clear disadvantage of this procedure, in comparison with the previously discussed methods based on adsorption processes, is the use of organic solvents, although sometimes the volumes used were in the range of mL. Conventional LLE has been applied as a rapid procedure to extract unmetabolized PAHs [59] in 2 min, and has also been used to extract metabolized PAHs [60–62] using volumes of organic solvents larger than 4 mL. In general, similar results in terms of sensitivity were found when these extraction techniques were compared with the ones previously described, as can be seen in Table 1.

The studies that analysed metabolized PAHs were mainly focused on monohydroxyl metabolites obtained after using either enzymatic (β-glucuronidase/arylsulfatase, β-glucuronidase/sulfatase and β-glucuronidase, overnight period) or acid (HCl, 60 min) hydrolysis. However, other derivatives such as dihydroxylated, glucuronides, cysteine-glycine, sulfates and nitro metabolites of PAHs have also been determined in human urine samples [50,63–65] using extraction techniques similar to the ones previously mentioned.

2.1.2. *Separation and detection methods*

The determination of PAHs in urine samples has been performed using several methodological approaches. Since biological matrices are complex, the importance of using sensitive and efficient techniques should be emphasized because the analytes are present in the matrix at trace concentrations in comparison to the interferers.

Generally, the limits of detection reported for the determination of unmetabolized PAHs (ranging between 0.04 and 100 ppt) are better than those found for metabolized PAHs (ranging between 1 and 2600 ppt), as can be seen in Table 1.

Gas chromatography-mass spectrometry (GC-MS) with a single quadrupole (q) is the technique that has been most frequently used for the determination of unmetabolized compounds. The coupling of HS-SPME and SPME with GC-qMS has been widely used, since it allows sample handling to be minimized and the procedure is fully automatic [22,45]. Methods based on GC coupled to tandem mass spectrometry (GC-MS/MS) [23], after direct immersion SPME, have improved sensitivity and specificity. GC with high-resolution MS using a time of flight mass spectrometer (GC-TOF/MS) [51] was proposed in an attempt to improve selectivity, maintaining the sensitivity in the same ppt range.

A less common alternative has been proposed by some authors attempting to obtain good analytical resolution by using GC with a flame ionization detector (GC-FID) after HS-SPME [41] and DLLME [58] extraction. As can be seen in Table 1, similar results were achieved in terms of sensitivity when methods based on GC-MS and GC-FID were compared using the same extraction technique (HS-SPME) [23,40]. The results were slightly better with GC-FID,

although the number of compounds analysed using the latter method was lower.

The best LODs for the unmetabolized PAHs were obtained when SPE, followed by HPLC with ultraviolet detection (HPLC-UV) [49], and extraction with magnetic nanoparticles, followed by GC-qMS analysis [52], were employed (see Table 1).

With reference to the metabolized PAHs, high separation efficiency for the identification of PAHs can be achieved with GC-MS. With the aim of converting metabolite PAHs into more volatile compounds, as well as increasing the selectivity and sensitivity for methods based on GC-MS, a derivatization step was required prior to the analysis. Several reagents including N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) [40,60], a mixture of *N*-Trimethylsilylimidazole/N,O-Bis(trimethylsilyl)acetamide/trymethylchlorosilane (Tri-Sil TBT) [42], *N*-(t-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) [50], methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) [19,66,67] and acetic anhydride [44] have been used. This step was also included to enhance sensitivity in HPLC-MS/MS using pentafluorobenzyl bromide [68]. Liu et al. [47] did not include a derivatization step for the analysis of OH-PAHs using SPME-GC-MS. In the work published by Amorim et al. [67], the use of two-dimensional GC with FID (GC×GC-FID), together with the derivatization procedure, reduced the interferences that hindered an appropriate peak identification in biological matrices, enhancing resolution in the determination of the studied analytes.

Despite the good results obtained for GC methods with derivatization reaction, the low volatility of the higher molecular weight PAHs, in addition to the polarity of their corresponding metabolites, are drawbacks that sometimes can be more easily resolved with the use of HPLC coupled to different types of detectors: MS [54], MS/MS [53,56,61–63,68] and fluorescence (F) [55,57].

Xu et al. [53] compared the results obtained with a method based on HPLC-MS/MS when the mass spectrometer operated in different modes: selected ion monitoring (SIM), constant neutral loss (CNL) and selected reaction monitoring (SRM). The LODs found when CNL mode was used were worse than those found with the SIM mode (one order of magnitude, approximately). However, SRM mode provided the best LODs, which were one or two orders of magnitude lower than those reported for the SIM mode. In this work, the authors highlighted that the CNL mode may be used for screening unknown OH-PAHs, since the neutral loss of 28 Da is characteristic for most of these compounds.

With the aim of finding potential metabolites of PAHs, Tang et al. [63] performed the analysis using HPLC-MS/MS with triple quadrupole (QqQ) for the screening of untargeted compounds, since this method presents high sensitivity in full scan mode. Then, these authors identified and confirmed the previous results in a targeted screening with two different methods, HPLC with quadrupole time-of-flight-MS (HPLC-Q-TOF-MS) and HPLC with quadrupole orbitrap-MS (HPLC-Q-Orbitrap-MS), simplifying data processing and enhancing analysis efficiency due to the accurate mass determination. No information about LODs was provided in this study.

Fluorescence detection has also been used coupled to HPLC [55,57], producing the best results in the study proposed by Rihs et al. [57] (LODs: 24–96 ng L^{-1}). In this case a pre-column was used to enrich the analytes of study instead of the common SPE used in the work proposed by Chauhan et al. [55] (LODs: 0.33–2.60 μg L^{-1}).

The runtime required for the analysis of unmetabolized and metabolized PAHs in urine samples depended on the method used. The range of time reported was between 13 and 66 min.

In addition to the chromatographic methods previously described, the possibilities of non-separative methods, with runtimes ranging from 2 to 4 min, have also been explored. Martín Santos et al. [59] described a method without chromatographic separation that was based on the coupling of a programmed temperature vaporizer (PTV) to a mass spectrometry detector (qMS) to obtain the profile signals of unmetabolized PAHs. Chemometric techniques, such as partial least squares (PLS) calibration for quantification purposes and principal component analysis (PCA), soft independent modelling of class analogy (SIMCA) and support vector machine (SVM), among others, were used for discrimination purposes. The strategy of this approach was the analysis of an elevated number of samples, since the method was rapid, discriminating the presence or absence of PAHs. Only the samples with PAHs were further analysed to confirm the results using a chromatographic method that required more time, consequently saving both time and money. As expected, the LODs achieved using this technique were worse than those found using chromatographic methods. However, the LODs were sufficient enough since the usual concentrations of PAHs found in urine samples are within the same range.

Calimag-Williams et al. [69] also demonstrated the applicability of non-chromatographic methods as screening approaches for the analysis of five OH-PAHs in urine samples using total synchronous fluorescence (TSFS) followed by chemometric techniques. Although the LODs achieved for the five OH-PAHs considered were better (nearly one order of magnitude, see Table 1) than those reported elsewhere [59] for 11 unmetabolized PAHs, second-order calibration methods were required due to matrix interferences.

In the work published by Yang et al. [48], the analysis of five OH-PAHs was carried out by coupling SPME with glass-capillary nanoelectrospray ionization mass spectrometry (SPME-nanoESI-MS) using a hybrid triple quadrupole/linear ion trap MS for qualitative and quantitative purposes. The LODs (10 ng L^{-1}) were better than those reported previously [59,69], although the equipment used is not often available in all the laboratories.

Li et al. [70] used a polyetheretherketone (PEEK) tube for SPE extraction of nine OH-PAHs from urine samples. But the authors only reported information about the quantification of urinary 1-hydroxypyrene through a non-separative analysis based on the direct coupling of the PEEK tube with ESI-MS/MS in less than 2 min. Although the information available in terms of LODs only referred to one analyte, the value achieved was similar to that obtained with SPME-nanoESI-MS [48] (10 ng L^{-1}, see Table 1), which were the best values reported for non-separative methods.

Certified reference materials (CRM) are only available for validating methods that determine PAHs in urine samples. Despite this, the most common practice for evaluating accuracy has been based on a process that included matrix spiking and prediction of PAH concentration. In all cases, good results in terms of accuracy were given, observing a wider range of percent values provided for metabolites PAHs in comparison with the corresponding parent compounds [40,50,56].

Few protocols have been established for the determination of PAHs in human biological samples and they are mostly applied on the analysis of urine samples. These protocols are based on extraction techniques and separation and detection methods included in this work, such as online-SPE-HPLC-MS/MS [71] and automated LLE-GC-MS-MS [72].

2.2. Blood, plasma and serum samples

Of late, blood has been selected as a useful matrix for assessing an individual's internal level of PAHs after recent exposure. As previously mentioned, even though PAHs are persistent in the environment, once inside the body they are rapidly metabolized and eliminated through urine. For this reason, the concentration of these compounds in blood is typically three orders of magnitude lower than those found in urine; thus urine is the most frequently chosen sample type for the determination of PAHs [73]. However,

the collection of blood samples has traditionally been the more common practice because urine sampling usually requires 24 h collection or concentration adjustment, since the analytes can be highly diluted or highly concentrated in relation to the intake of liquids. The clear disadvantage presented by the analysis of blood is that it is difficult to collect and is invasive, and requires special handling of the needles and adequate storage during transport until it is analysed. A less invasive alternative to blood sampling is the use of capillary blood, as proposed by Gruber et al. [74], where a minimal volume of blood (1 μL) is collected using sterile safety lancets and heparinized disposal pipets. This procedure can be done without medical assistance and at specific time intervals.

The studies available in the literature for determining PAHs in blood are focused on DNA-PAH adducts [75–77] and unmetabolized PAHs [73,74,78,90]. PAHs have also been determined in serum [91–102] and plasma (fraction with serum and fibrinogen) [78,103–105] samples.

After blood collection, an additional centrifugation step before the extraction was required to isolate serum and plasma fractions of the sample. Only one of these works, analysing serum samples, reported the determination of parent and metabolized compounds [91], while the others only considered unmetabolized PAHs.

2.2.1. Extraction procedures

LLE has been the technique most frequently used to analyse blood samples [73,78–86]. The main advantages and disadvantages of the technique have been discussed in section 2.1.1 concerning urine samples. This technique usually requires centrifugation steps to separate organic and aqueous phases and, in some cases, further concentration by evaporating the solvent. In addition to this, Pleil et al. [78] incorporated an additional freezing step into the method. After LLE, the samples were centrifuged and frozen. The organic extract was poured off into another vial and the frozen blood or plasma layer was discarded, improving sample handling and avoiding the use of pipettes or other configurations to separate the different phases.

For serum and plasma samples, some studies required a saponification step previous to extraction [82,84–86]. Like blood, these samples were extracted by LLE [78,91–95,103] and multiple LLE steps combined with incubation, in which the sample was maintained at a constant temperature (50°C) thus increasing the extraction time by 50 min [96]. Wang et al. [93] incorporated a microwave extraction system (MES) to the procedure. After extraction, most of the studies required a clean-up step to remove co-extracted lipids and impurities that could interfere with the PAHs determination [73,79–82,84,86,92–95,97,103].

A technique that included solid-liquid extraction (SLE) and a further clean-up step has been employed to extract the compounds from serum samples [97]. The samples were initially freeze-dried (lyophilised) and then extracted with the corresponding organic solvent. No information regarding the extraction time and the volume of solvent used was provided.

An alternative method for extracting PAHs from blood was based on SPE and required an elevated extraction time (above 60 min) [87]. However, this technique was improved when it was miniaturized as pipette-tip solid phase extraction (PT-SPE) [88]. In this approach, the sorbent used was placed into a cut pipette tip and immobilized with cotton and hollow propylene fibre, which also filtered the blood sample. As compared to the traditional SPE, this novel approach used less solvent, each pipette could be reused at least ten times, and the amount of blood sample required was drastically reduced (from 2 mL to 200 μL).

Rajabi et al. [104] modified the conventional SPE, generating the air-assisted dispersive micro-solid phase extraction (AA-dμ-SPE) technique by preparing and using magnetic graphitic carbon nitride nanocomposites as adsorbents. The main advantage was the low-level consumption of adsorbent without the use of toxic organic solvents. The limitations when adsorbents are prepared in the laboratory have been previously discussed (section 2.1.1).

Several studies have also reported on the use of SPE in plasma [105]and serum samples [98–101].

Finally, different strategies using high temperature have been employed to extract analytes such as HS [89], HS-SPME [90] and thermal extraction (TE) followed by a cryo-trap [74]. Since undigested lipoproteins in serum samples could interfere with SPME and reduce the amount of PAHs adsorbed in the fibre, Poon et al. [102] included a proteolytic digestion step before the extraction. Although this improved the recoveries of PAHs by avoiding protein coating of the SPME fibre, this step also prolonged the extraction time (60 min). The inconvenience of using these techniques when determining PAHs with high molecular weight has been discussed in section 2.1.1.

In some of the studies previously described [73,82,83,86,94–96,98,100], in addition to determining PAHs in blood, these compounds were also determined in serum obtained from umbilical cords with the aim of studying the possible transference of these compounds from mothers to foetuses.

2.2.2. Separation and detection methods

GC coupled to qMS has been the analytical method most frequently used to measure PAHs in blood [73,74,78,82–88], serum [91–93,95,99,100] and plasma [78,103,105] samples. The use of GC coupled to QTrap-MS for the analysis of blood samples [90] and coupled to QqQ-MS for the analysis of serum samples [94,97,101] has also been reported. The LODs found varied depending on each study and ranged between 0.5 and 2950 ng L^{-1}. This can be observed in Table 2, where the most relevant studies for the determination of PAHs in blood, serum and plasma samples are summarized.

When HS-GC-FID [89] was employed to analyse blood samples, the sensitivity clearly worsened for the only compound determined (naphthalene, 50 μg L^{-1}) in comparison with GC-MS and HPLC-F/UV methods. However, it is important to highlight the minimum sample handling required by the HS-GC-FID method, since blood is added in a vial with distilled water and an internal standard. Since only one PAH was determined, the analysis time (21 min) was equal or shorter than that reported for the GC-qMS (22 min) [85] and HPLC-F/UV (35 min) [79–81] analysis. GC-FID was also used by Poon et al. [102] for the analysis of serum samples. In general, the LODs obtained increased between 1 and 4 orders of magnitude and the analysis time almost doubled when compared to those obtained with GC-qMS and HPLC-F, as can be observed in Table 2. Rajabi et al. [104] analysed plasma samples with a method based on AA-dμ-SPE extraction and GC-FID analysis. However, in contrast to serum samples, the worst limits of detection for plasma samples were found using the GC-qMS analysis (one order of magnitude, see Table 2) [103].

It has been stated elsewhere [82] that the sensitivity of GC-MS for PAHs is worse compared with HPLC-F. However, the studies that reported the use of HPLC-F to determine PAHs in blood samples [79–81] also required an additional analysis using HPLC-UV for the independent determination of acenaphthylene, because of inability to get response by fluorescence. This extended the time required to determine all the analytes under study. In view of the results available (Table 2), it can be observed that there is no significant difference with respect to sensitivity when GC and HPLC methods have been employed.

HPLC-F was also employed for the analysis of serum samples after extraction by LLE [96] and SPE [98], obtaining the best results in terms of sensitivity when SPE was used.

Table 2
Relevant studies for the determination of PAHs in blood, serum and plasma samples.

Reference	nº PAHs	Sample preparation procedure	Separation and detection method	Detection limits (ng L^{-1})
Blood				
[73]	55	LLE- clean up	GC-qMS	580–2900
[74]	1	TE	GC-qMS	1300
[78]	22	LLE-freezing	GC-qMS	5–121
[79–81]	9	LLE-clean up	HPLC-F/UV	8–50
[82]	7	Saponification-LLE-clean up	GC-qMS	30–90
[85]	16	Saponification-LLE-clean up	GC-qMS	10–80
[86]	16	Saponification-LLE-clean up	GC-qMS	1000
[87][a]	16	SPE	GC-qMS	82–114 ng g^{-1}
[88]	16	PT-SPE	GC-qMS	2–4
[89]	1	HS	GC-FID	50000
[90]	3	HS-SPME	GC-QTrapMS	0.5
Serum				
[91][b,c]	27	LLE	GC-qMS	2–117 pg g^{-1}
[92]	5	LLE-clean up	GC-qMS	5
[93]	27	MES-LLE-clean up	GC-qMS	50–100
[96]	5	LLE-incubation	HPLC-F	250–2500
[97][d]	16	SLE-clean up	GC-MS/MS	20–50
[98]	9	SPE	HPLC-F	50–100
[100]	16	SPE-clean up	GC-qMS	–
[101][d]	16	SPE	GC-MS/MS	50
[102]	16	Proteolytic digestion-SPME	GC-FID	2700–30400
Plasma				
[78]	22	LLE-freezing	GC-qMS	5–121
[103][e]	60	LLE-clean up	GC-qMS	2950
[104]	5	AA-dμ-SPE	GC-FID	300–600
[105][f]	13	SPE-clean up	GC-qMS	1–5 ng g^{-1}

-Blood:
[a]Detection limits expressed as ng g^{-1}.
-Serum:
[b]Determination of 15 unmetabolized and 12 metabolized PAHs.
[c]Detection limits expressed as pg g^{-1} wet weight.
[d]Quantification limits.
-Information not available.
-Plasma:
[e]Referred to benzo(*a*)pyrene.
[f]Detection limits expressed as ng g^{-1} lipid.

Accuracy was evaluated using a similar process to the one used for urine samples including matrix spiking and prediction of PAH concentration. The percent values were in a range of ±30% of the spiked concentration.

2.3. *Saliva samples*

The use of saliva samples as an alternative to blood or urine for the evaluation of different compounds has become an increasing trend. Additionally, as a non-invasive sampling method it is a suitable medium for screening and determining PAHs, especially parent compounds before they undergo biotransformation processes. The collection and handling of saliva is quick and easy. However, the volume of saliva available for analysis can be limited depending on several physiological factors. Moreover, the concentration of the compounds of interest can be affected by food, drink and the techniques used to stimulate saliva production.

To date very few applications have been reported for the determination of unmetabolized [104,106,107] and metabolized [107,108] PAHs in saliva samples.

2.3.1. *Extraction procedures*

Few techniques have been employed to extract PAHs from saliva samples. Rajabi et al. [104] applied the same AA-dμ-SPE extraction technique described in the section regarding plasma samples to analyse saliva.

LLE was employed to extract unmetabolized [106] and metabolized [108] PAHs in saliva samples. In both studies, the time required for extraction was short (1 min) and volumes of organic solvent equal or lower than 1 mL were used.

Carrizo et al. [107,108] reported on two methods that did not require prior extraction techniques or sample treatment. They were based on direct ionization by atmospheric pressure solid analysis probe (ASAP) coupled to Q-TOF-MS and both types of compounds, unmetabolized [107] and metabolized [108] PAHs, were determined. In one of these works, the authors compared the results obtained with ASAP and those provided when LLE was used [108]. The main advantage of the ASAP method versus LLE was the absence of sample treatment or prior extraction, reducing the time of analysis and the errors usually associated with these steps in the analytical process. In addition, ASAP provided higher recovery values than LLE when the same sample was analysed using both methods.

2.3.2. *Separation and detection methods*

After extraction through AA-dμ-SPE, the samples were analysed using GC-FID [104]. In general, the LODs were similar or worse than those found for urine and blood samples, as can be seen in Table 3 where the available methods for the determination of PAHs in saliva samples are summarized.

A method that included preconcentration of the analytes in a PTV system using solvent vent injection mode and further analysis with GC-qMS has been used for the determination of saliva taken from firefighters [106]. In Fig. 3, the chromatograms of two saliva samples obtained in the SIM mode when using this method are shown. Fig. 3A corresponds to a sample taken from a firefighter immediately after firefighting activities, where seven PAHs were

Table 3
Relevant studies for the determination of PAHs in saliva, exhaled breath and hair samples.

Reference	nº PAHs	Sample preparation procedure	Separation and detection method	Detection limits (ng L^{-1})
Saliva				
[104]	5	AA-dμ-SPE	GC-FID	300–600
[106]	16	LLE	PTV-GC-qMS	2–57
[107][a]	31	ASAP	Q-TOF-MS	5000–20000
[108][b]	2	ASAP	Q-TOF-MS	100000–500000
		LLE	HPLC-MS/MS	10000–50000
Breath				
[42,109][c]	1	Thermal desorption-cryo focusing	GC-PID	0.5 μg $(m^3)^{-1}$
[110]	2	SPE	HPLC-F	10000–50000
[111][d]	20	SPE-clean up	GC-qMS	0.01–5.72 ng cig^{-1}
Hair				
Unmet.[h]				
[113]	15	Digestion-LLE	HPLC-F	80–1200
[115]	26	Digestion-LLE-clean up	GC-qMS	50–100
[116]	17	Digestion-in-tube-SPME	HPLC-F	0.5–20.4
[117][e]	16	Soxhlet-clean up	GC-qMS	0.01–1 ng g^{-1}
[118][e]	16	Soxhlet-clean up	GC-qMS	0.5–5 ng g^{-1}
[119]	15	Digestion-LLE	GC-qMS	–
Met.[h]				
[114][f]	12	Digestion-LLE-derivatization-LLE	GC-NCI-qMS	20–310 nmol (g hair)$^{-1}$
[120][g]	12	Digestion-LLE-derivatization-LLE	GC-NCI-qMS	5–100 pmol g^{-1}

-Saliva:
[a]Determination of 15 metabolized and 16 metabolized PAHs.
[b]Determination of 2 metabolized PAHs.
-Breath:
[c]Quantification limits expressed as μg $(m^3)^{-1}$.
[d]Detection limits expressed as ng cig^{-1}.
-Hair:
-Information not available.
[e]Detection limits expressed as ng g^{-1}.
[f]Detection limits expressed as nmol (g hair)$^{-1}$.
[g]Quantification limits expressed as pmol g^{-1}.
[h]Unmet.: unmetabolized PAHs; Met.: metabolized PAHs.

detected (naphthalene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene). Fig. 3B corresponds to a saliva sample collected from the same firefighter two days after fire exposure, and two PAHs (fluorene, phenanthrene) were detected at lower concentration level in comparison to the immediate sample. These two PAHs were usually found in saliva samples taken from a control group, probably entering the body through diet.

ASAP-Q-TOF-MS methods were mainly used for screening and semi-quantification purposes of unmetabolized, nitro and oxo PAHs [107], as well as monohydroxyl PAHs [108]. Although the methods were rapid (3 min), an additional method based on LLE-HPLC-MS/MS was employed to confirm the results obtained in the determination of monohydroxyl PAHs [108]. Since selected ions were monitored using the confirmatory method, the sensitivity was better than that achieved with the ASAP-Q-TOF-MS [108]. However, the use of screening methods has the benefit of reducing the number of samples that required confirmation. The LODs obtained with ASAP-Q-TOF-MS for the determination of unmetabolized, nitro and oxo compounds [107] improved up to two orders of magnitude in comparison with the values reported for monohydroxyl PAHs [108], as can be seen in Table 3.

The technique based on LLE-PTV-GC-qMS [106] provided the best detection limits reported in the literature for the analysis of PAHs in saliva samples (see Table 3) and a shorter runtime (8.26 min) than separative methods based on GC-FID (13.6 min) [104] and HPLC-MS/MS (10 min) [108].

Good results in terms of accuracy were obtained in all cases, with similar ranges between 79 and 120%.

2.4. Exhaled breath samples

Exhaled breath seems to be a useful sample for recent exposure to PAHs with low molecular weight. However, a limited number of approaches have been reported in the literature [42,109–111]. Despite non-invasive sample collection, there is a need for specific systems to ensure the integrity of the sample collected.

2.4.1. Extraction procedures

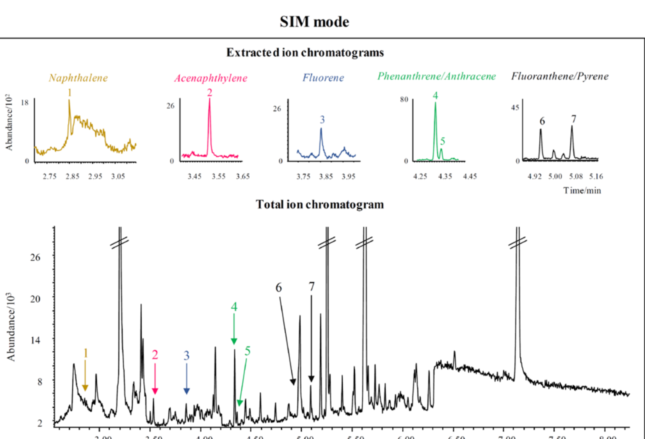
Reusable glass bulbs have been used for capturing breath samples (end-exhaled air) and further determination of naphthalene [42,109]. Before analysis, the samples were transferred to sorbent tubes that were further desorbed (2 min) with an automatic system by increasing temperature (225°C). The analytes were retained in a Tenax® cold trap (−30°C) that was quickly heated (0.3 min, 225°C) to transfer the compounds to the corresponding analytical column.

While only end-exhaled air was collected in those bulbs due to the higher pulmonary capacity compared to the volume of the containers, Brzeźnicki et al. [110] collected the samples by using a facial mask that was equipped with a larger volume container, with the capacity for sampling the exhaled air during a whole period of exposure of the study population. Once inside the container, the analytes were retained on glass fibre filters that were connected to a tube filled with resin. Whereas other studies [42,109] were based on desorption of the analytes by increasing temperature, these authors extracted PAHs by adding organic solvents.

Analysis of PAHs has also been carried out in exhaled cigarette smoke [111]. With the aid of a vacuum assisted system, the sample was aspirated from the mouth, collected on a Cambridge pad, and the analytes were further extracted by adding an organic solvent. The extract was cleaned up using SPE before the analysis.

Although extraction including solvents [110,111] usually provided better extraction than the technique requiring high temperature [42,109], due to the high boiling point of PAHs, the extraction time increased over 30 min as well as the volume of organic solvent (over 15 mL).

SIM mode



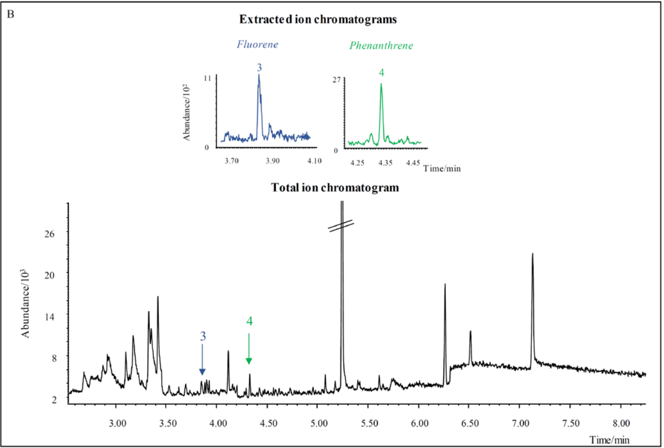


Fig. 3. Chromatograms obtained in the SIM mode for the determination of PAHs in saliva samples by LLE-PTV-GC-qMS. (A) Saliva taken from a firefighter immediately after firefighting activities. (B) Saliva taken from the same firefighter two days after firefighting activities.

2.4.2. Separation and detection methods

GC and HPLC, coupled to different detectors, were reported to be the methods of choice to determine PAHs in breath analysis. The available methods reported in the literature are summarized in Table 3. After thermally desorption of breath samples, cryo-focusing and further desorption, PAHs were determined by GC coupled to a photoionization detector (PID) [42,109]. When PAHs were extracted using organic solvents, the analysis was carried out using GC-qMS [111] and HPLC-F [110].

Since the study based on GC-qMS [111] reported the determination of a higher number of PAHs, the runtime was twice the time (66 min, approximately) required for GC-PID [42,109] and HPLC-F [110]. As can be observed in Table 3, the LODs vary, mainly due to the use of different extraction techniques and detection methods.

Since the results reported in these works were mainly related to the variation of PAH concentration due to exposure, no information about accuracy was provided.

2.5. Hair and follicular fluid samples

Human hair has also been considered for determining PAHs as indicators of long-term exposure since it is not affected by intra- or

inter-day extreme variations. Other matrices, on the other hand, present the advantage of providing information about recent exposure to PAHs.

The main advantage of this type of samples is that they are easy to collect (non-invasive and accessible) and transport before analysis.

In 2012 Appenzeller et al. [112] reviewed the literature available for the analysis of hair samples with the aim of biomonitoring exposure to organic pollutants. In this review, only two additional studies for the determination of unmetabolized PAHs [113] or their metabolites [114] were reported. Several approaches have since been developed for parent [115–119] and metabolized [120] compounds.

2.5.1. Extraction procedures

The main objective of these studies was the determination of PAHs inside the hair samples. However, the unmetabolized compounds are present in the environment and, consequently, in most of the studies the sample was washed previous extraction to eliminate the possible PAHs adsorbed on the surface. This step was generally considered for unmetabolized PAHs due to the fact that the metabolized compounds are produced inside the body. The presence or absence of this step was also considered depending on the environmental pollution [114]. Sample washing for the determination of unmetabolized PAHs has been carried out using hexane [113,115,116], water and shampoo [117,118] and Triton X-100 [119].

While in all of these studies the samples were cut in to pieces for their analysis, the studies that determined metabolized PAHs [114,120] included pulverization of the hair samples.

The extraction technique most frequently used was based on LLE after digestion [113–115,119,120]. Wang et al. [115] introduced a clean-up step after extraction. For metabolized PAHs [114,120], an additional derivatization step with (2S,4R)-*N*-heptafluorobutyryl-4-heptafluorobutoyloxy-propyl chloride ((S,R)-HFBOPCl) was required for the analysis, prolonging the procedure up to 70 min.

The use of Soxhlet extraction was reported by Wang et al. [117,118]. The procedure required 18 h to extract the analytes and clean-up and concentration steps, thus prolonging the time of analysis.

An alternative proposed by Yamamoto et al. [116] was an automatized method based on in-tube SPME using a capillary column as extraction device. After digestion, PAHs were automatically extracted in the column and further desorbed, reducing errors usually associated with the pretreatment processes. The time required for automatically extract and desorb the analytes (35 min) was reduced in comparison to Soxhlet technique, but was in the range of LLE (15–35 min).

2.5.2. Separation and detection methods

The most widely used method for the determination of PAHs in human hair was GC-qMS using electron ionization (EI) mode for parent compounds [115,117–119] and negative chemical ionization (NCI) mode for metabolized PAHs [114–120]. Schummer et al. [114] observed that the chromatograms obtained in the analysis of hair presented a significant background noise signal compared to other biological matrices. Since this effect was less important for high *m/z* ions, they were chosen for the analysis. As the authors stated, the use of EI produces more fragmentation than NCI, allowing the observation of molecular ions (high *m/z* ratios) with low values of noise. Despite the better sensitivity obtained by these authors when using the NCI mode, an additional derivatization step, as indicated in the extraction section, was required to obtain derivatives compatible with the detection method thus prolonging the time of sample pretreatment. The analysis using GC-qMS with NCI mode was also employed by Appenzeller et al. [120].

The use of HPLC-F was also reported for the determination of unmetabolized PAHs after LLE [113] or in-tube SPME [116] extraction, with runtimes (15–75 min) similar to those required for GC-MS methods (20–60 min). In Fig. 4, the typical chromatograms obtained for the determination of PAHs in hair samples by in-tube SPME-HPLC-F [116] of a non-smoker (Fig. 4A) and a smoker (Fig. 4B) are shown. An increase in the number of analytes detected as well as in the concentration level can be observed for smoker subjects.

Neal et al. [92] analysed follicular fluid samples using the same procedure previously described for serum samples based on LLE-GC-MS (see Table 2).

In Table 3, the most relevant studies for the determination of PAHs in human hair samples are shown. The methods used allowed to achieve low LODs (ppt-ppb range).

The same process reported in the previous matrices for accuracy was used in hair samples. The range value provided was similar in all cases, and ranged between 70 and 130%.

2.6. Other matrices

Whereas all previously considered matrices can be collected from the whole population, other approaches have been proposed using samples collected from a specific group of participants or with the aid of qualified specialists.

In recent years, several studies have been aimed at relating prenatal exposure to PAHs and pregnancy complications by determining concentration of these compounds in placenta. At the time of delivery, the sample was collected and stored appropriately until analysis. The extraction techniques included Soxhlet extraction, where different organic solvents were used [83,95], LLE [86,96,121] and selective pressurized liquid extraction (SPLE) as well [122]. In all cases, a clean-up step was required. The analysis of the samples was generally carried out using GC-qMS [83,86,95], GC-MS/MS [122] and HPLC-F [96].

Breast milk samples have also been analysed for the determination of PAHs. Not only is the foetus affected by prenatal exposure, but the infant can also be exposed to these analytes through diet after birth. In general, the extraction in human milk samples has been addressed through LLE [83,123–129]. The use of Soxhlet extraction has also been reported [100,130]. Most of the analytical methods employed were based on GC-MS, either with single quadrupole [83,100,123,126,130] or MS/MS [125] detection. A high resolution analytical method GC-HRMS (sector field MS) [124] has also been employed. Other studies reported the use of HPLC-F [127–129].

Finally, PAHs have also been determined in cerebrospinal fluid [131]. The work of two medical specialists was necessary to perform lumbar puncture in order to collect the sample. The procedure to determine PAHs included extraction by online-SPME followed by GC-qMS analysis.

3. Other relevant aspects

In the literature available before the period of time considered in this review, only studies mainly focused on the analysis of urine samples had been published. Since then, the use of different matrices such as blood, plasma, serum, saliva, exhaled breath and hair has been proposed to control exposure to PAHs. This has meant an advantage in the sampling procedure considering the current interest that non-invasive samples present, also facilitating sample treatment in the case of less complex matrices (saliva, exhaled breath, hair in comparison with urine and blood) that contain less analytes interfering in the determination of PAHs. One of the greatest advances in the field has been the development of non-separative methods, allowing the analysis of a great number of

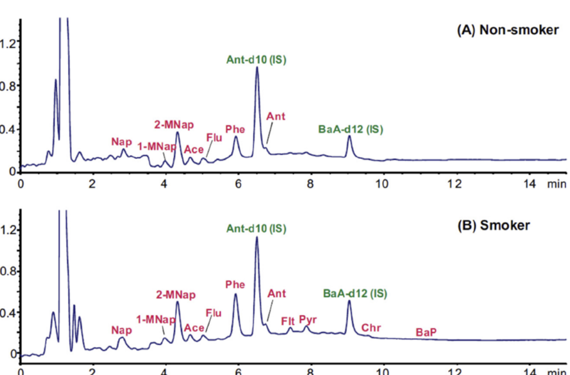


Fig. 4. Chromatograms obtained in the determination of hair samples by in-tube SPME-HPLC-F. (A) Hair sample taken from a non-smoker. (B) Hair sample taken from a smoker. Reprinted from Journal of Chromatography B, Vol. 1000, Yusuke Yamamoto, Atsushi Ishizaki, Hiroyuki Kataoka, Biomonitoring method for the determination of polycyclic aromatic hydrocarbons in hair by online in-tube solid-phase microextraction coupled with high performance liquid chromatography and fluorescence detection, 187–191, Copyright (2015), with permission from Elsevier.

samples in a shorter period of time, when compared with chromatographic methods, and providing useful information about each sample thanks to the use of chemometric techniques.

Green analytical chemistry (GAC) has gained great importance during the last years, which has led to practices more environmentally friendly in the laboratories all over the world. These practices can be observed when determining PAHs in the different samples considered. Prevention of waste has been attempted by using ASAP methods that did not require sample treatment [107,108]. Attempts to reduce the amount of sample required for analysis have also been described [88]. The use of organic and toxic solvents has been reduced in liquid extraction techniques until mL range [58,106,108] and it has also been avoided by using techniques based on adsorption and desorption processes such as HS-SPME [22], SPME [44], SBSE [56] and SPE [87]. Finally, reduction in the use of energy has been achieved by automatization of extraction and determination steps [40,54,116]. The development of non-separative methods has also minimized reagent and energy consumption since they are faster than those requiring chromatographic separation [48,59,69,70,107,108].

4. Conclusions

The determination of PAHs in different human samples has been reviewed, with special attention being focused on extraction procedures and the separation and detection methods used.

The samples considered in this review included urine, saliva, exhaled breath and hair samples (non-invasive matrices) as well as blood, serum and plasma (invasive matrices). Moreover, studies analysing placenta, breast milk and cerebrospinal fluid were also examined.

Clearly, non-invasive matrices are always preferred to those requiring specialists for collecting the sample. However, some considerations must be taken into account depending on the objective of the study. While urine samples could be more associated with metabolites due to biotransformation processes inside the body, matrices such as saliva, exhaled breath or blood samples are more related to recent exposure of unmetabolized PAHs. On the contrary, hair is the ideal matrix for long-term exposure studies.

Although several extraction procedures have been reported, the best results were obtained using techniques with direct contact between the analytes and the fibre or solvent due to the high boiling point of polycyclic aromatic hydrocarbons.

Finally, the analytical approaches reported were mostly based on separative methods. In general, the methods based on GC provided better results since they allowed a higher number of analytes to be determined in comparison with HPLC. Although there is a wide variety of GC detectors used, those studies with MS usually provided the best results in terms of sensitivity and selectivity.

In addition to the chromatographic methods, non-separative methods were also employed in the case of urine and saliva samples. They provided a fast response with less information than that obtained from a separative method, but in many cases the information obtained was enough to resolve the problem at hand. The implementation of screening methods that reduced the number of samples that needed to be subjected to the separative method is an interesting alternative.

In conclusion, depending on whether the authors wish to focus on the study of long-term or recent exposure to PAHs, and according to the desired results in terms of sensitivity and time of analysis, among others, the appropriate sample, extraction technique and separation and detection method must be selected.

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CAPÍTULO III

Determinación de aminoácidos en muestras de saliva

III.1 Artículo de investigación

Método no separativo basado en un espectrómetro de masas cuadrupolar para la determinación semi-cuantitativa de aminoácidos en muestras de saliva. Un estudio preliminar

Resumen

La búsqueda de biomarcadores en diferentes muestras biológicas se ha incrementado en los últimos años. Entre ellos, se ha prestado atención a la determinación de aminoácidos para obtener información acerca del estado del organismo. Los aminoácidos constituyen las unidades básicas estructurales de las proteínas y son importantes intermediarios metabólicos en diferentes procesos en el cuerpo humano. Entre las diferentes enfermedades descritas en las que se alteran los niveles habituales de aminoácidos se encuentran la esquizofrenia, la diabetes y diferentes tipos de cáncer, entre otras. En particular, en este último caso el perfil de aminoácidos se ve alterado en los estados tempranos de la enfermedad, aparentemente sin síntomas, mientras que otros biomarcadores de cáncer no se ven modificados en esa etapa o no son específicos para un determinado tipo de cáncer.

La determinación de estos compuestos se ha llevado a cabo en diferentes matrices biológicas, siendo las más habituales la sangre, el plasma y el suero. Sin embargo, debido al carácter invasivo de la toma de muestra de estas matrices se ha incrementado el número de estudios enfocados a la determinación de aminoácidos en muestras de orina y saliva. Algunos autores han descrito la utilidad de la saliva como fuente de información metabólica, ya que los niveles de diferentes sustancias en esta matriz se encuentran correlacionados con aquellos encontrados en suero y plasma, siendo así una excelente alternativa a la sangre. Estos compuestos se han analizado también en otro tipo de muestras biológicas incluyendo pelo, piel y heces, entre otras.

Hasta la fecha, los métodos desarrollados para la determinación de aminoácidos en muestras biológicas han incluido etapas de extracción, derivatización y posterior separación y detección de los analitos. El análisis se ha llevado a cabo mediante cromatografía líquida de alta resolución (HPLC) acoplada a diferentes tipos de detectores incluyendo espectrometría de masas (MS), fluorescencia (F) y detección ultravioleta (UV). Como alternativa, también se ha utilizado la cromatografía de gases (GC) utilizando la detección mediante

MS e ionización en llama (FID). La electroforesis capilar (*capillary electrophoresis*, CE) se ha empleado utilizando detección de conductividad sin contacto (*contactless conductivity detection*, CCD) y fluorescencia inducida por láser (*laser induced fluorescence*, LIF).

En la actualidad, se está produciendo un incremento en el desarrollo de métodos no separativos para determinar de manera simultánea tantos analitos como sea posible. Estos métodos se basan principalmente en MS y en resonancia magnética nuclear (*nuclear magnetic resonance*, NMR). En los métodos basados en espectrometría de masas se ha propuesto la utilización de distintos tipos de analizadores, principalmente de alta resolución. En la mayoría de los casos se aplicaron técnicas quimiométricas sobre la señal de perfil obtenida para estudiar la información contenida en los datos.

El objetivo de este trabajo fue la puesta a punto de un método sencillo y accesible para la mayoría de los laboratorios que permita la semicuantificación simultánea de dieciséis aminoácidos en muestras de saliva. Para ello, se propuso el acoplamiento de un inyector de temperatura programada (PTV) y un espectrómetro de masas cuadrupolar simple (qMS), con un posterior análisis multivariante de la información obtenida. La columna cromatográfica que une el inyector y el detector se mantuvo a alta temperatura (300 ºC) para eliminar la capacidad de separación de la misma. Cabe destacar que el uso de las rampas cromatográficas de temperatura habituales en esta misma configuración instrumental permitiría confirmar los resultados obtenidos con el método no separativo. Según nuestra información es la primera vez que ambas configuraciones (PTV-qMS y PTV-GC-qMS) se proponen para la determinación de aminoácidos en saliva.

En una primera etapa, se llevó a cabo una reacción de derivatización con el fin de obtener los derivados adecuados de los aminoácidos de estudio para el análisis mediante cromatografía de gases. La reacción se llevó a cabo utilizando

etil cloroformiato, piridina y etanol. Estos compuestos se añadieron sobre 650 µL de saliva que contenía el patrón interno. La muestra se agitó en vórtex para facilitar el contacto con los reactivos y el tubo que la contenía se abrió para eliminar el CO_2 formado en la reacción. Fue necesaria una etapa posterior de desgasificación en ultrasonidos para asegurar una adecuada repetibilidad.

Una vez obtenidos los derivados correspondientes se procedió a su extracción mediante LLE añadiendo 800 µL de acetato de etilo y agitando de nuevo en vórtex. Tras la centrifugación de la muestra, el extracto orgánico se recogió para su posterior análisis.

La inyección del extracto orgánico se llevó a cabo utilizando el PTV en el modo de venteo de disolvente. En el método con separación cromatográfica se utilizó un programa de temperatura adecuado en el GC para la separación de los compuestos de interés. Se estableció una temperatura inicial de 60 ºC (2 minutos) y, a continuación, esta se aumentó mediante dos rampas de temperatura, ambas a 60 ºC min^{-1}, hasta 145 ºC (3 minutos) y hasta una temperatura final de 300 ºC (1 minuto). El tiempo total del análisis fue de 10 minutos. A pesar de que los analitos se separaron en 9.60 minutos fueron necesarios 9 minutos adicionales para que la columna cromatográfica alcanzase las condiciones iniciales para el siguiente análisis. Por ello, el tiempo entre muestras fue de aproximadamente 19 minutos. En el método no separativo la columna cromatográfica se mantuvo a 300 ºC durante el tiempo total del análisis, que fue de 4.5 minutos, eliminando así su capacidad de separación. El método separativo se desarrolló fundamentalmente con el objetivo de confirmar los resultados obtenidos con el método no separativo. La detección en el MS se llevó a cabo en el modo SIM/*scan* combinado en el método separativo y solamente en *scan* cuando se utilizó el método no separativo.

Para evaluar la posible existencia de efecto matriz se prepararon curvas de calibración en agua ultrapura y en 8 muestras de saliva con 3 niveles de

concentración (nivel endógeno y dos muestras dopadas). Se encontraron diferencias cuando se compararon las pendientes de dichos calibrados en agua y salivas, así como entre salivas, concluyendo que existía efecto matriz. Por ello, la cuantificación de aminoácidos se llevó a cabo utilizando el método de adición estándar. Además, se utilizó un patrón interno (4-clorofenilalanina) para compensar las variaciones del proceso completo de análisis.

El método separativo se aplicó a la determinación de aminoácidos en 8 muestras de saliva. Para ello, se utilizó el área de pico correspondiente al ion de cuantificación extraído en el modo SIM para cada analito normalizado al área correspondiente del patrón interno. Las curvas de calibración se obtuvieron analizando cada saliva, diluida de manera adecuada, con 3 niveles de concentración (nivel endógeno y dos muestras dopadas). Todas ellas tuvieron coeficientes de determinación satisfactorios. Los aminoácidos encontrados en mayor concentración fueron los siguientes: alanina (0.269-16.2 mg L^{-1}), glicina (1.60-25.7 mg L^{-1}), prolina (0.319-40.4 mg L^{-1}), glutamina (0-133.6 mg L^{-1}), ornitina (1.19-10.7 mg L^{-1}), lisina (1.11-13.5 mg L^{-1}) y tirosina (0.858-11.8 mg L^{-1}). Los analitos restantes se encontraron en niveles más bajos de concentración (0.037-6.78 mg L^{-1}). La asparagina no se encontró en ninguna de las muestras analizadas.

Los límites de detección y cuantificación dependieron de cada muestra de saliva, pero se encontraron en todos los casos en el rango de ppb mostrando una elevada sensibilidad.

Se llevó a cabo el estudio de repetibilidad y reproducibilidad a dos niveles diferentes de concentración (nivel endógeno con adición de asparagina y un nivel de concentración más elevado). Los valores obtenidos en términos de RSD oscilaron entre 1-11 % y 2-14 %, respectivamente.

La exactitud del método se evaluó mediante la comparación de la concentración predicha por el método de adición estándar y aquella añadida en

muestras dopadas. Los valores obtenidos fueron satisfactorios y oscilaron entre el 80 y el 115 %.

Con el objetivo de explorar las posibilidades del método no separativo (PTV-qMS) se analizaron las mismas 8 muestras de saliva utilizadas con el método separativo con 3 niveles de concentración cada una de ellas. La cuantificación de aminoácidos se llevó a cabo utilizando las señales de perfil obtenidas. En este caso, la columna cromatográfica se mantuvo a alta temperatura durante todo el tiempo del análisis (4.50 minutos), por lo que el tiempo entre muestras (5 minutos) se redujo con respecto al método separativo (19 minutos).

La cuantificación se llevó a cabo mediante la técnica de calibración multivariante PLS1. Las muestras de saliva analizadas se dividieron en 2 grupos. El primero de ellos, utilizado para construir los modelos de calibración, estaba formado por 7 muestras de saliva y sus correspondientes muestras dopadas. Las muestras se seleccionaron de acuerdo con las concentraciones previamente determinadas mediante el método cromatográfico de modo que todo el rango de concentraciones estuviese cubierto en cada calibrado. El uso de diferentes salivas en la construcción de los modelos hizo posible la cuantificación a pesar del efecto matriz observado previamente al englobar la variabilidad entre diferentes muestras de saliva. El segundo grupo, utilizado como grupo de validación externa, estaba formado por la restante muestra de saliva y sus correspondientes muestras dopadas. En este caso no se utilizó patrón interno ya que sus relaciones m/z estaban interferidas con las de otros analitos presentes en la muestra al no llevarse a cabo la separación cromatográfica.

Las variables independientes utilizadas en los modelos PLS1 fueron la suma de las intensidades de todos los iones analizados (m/z) durante el tiempo de adquisición de datos, mientras que las variables dependientes fueron las concentraciones de aminoácidos predichas con el método separativo.

Inicialmente, se construyeron los modelos PLS1 utilizando todas las variables independientes. El proceso fue semejante al descrito previamente para la determinación de PAHs en orina (II.1.1). Se utilizó el proceso de validación cruzada con el objetivo de seleccionar el número de factores PLS óptimo que proporcionaba un menor error. A continuación, se utilizó el criterio de incertidumbre de Martens para la selección de las variables más significativas. Esto supuso una simplificación de los modelos y una reducción del número de factores PLS óptimo. Así mismo, se redujo el error de la etapa de validación cruzada. En el caso de la serina, metionina, fenilalanina e histidina todas las variables fueron significativas. Los errores medios relativos en la validación cruzada oscilaron entre 6 y 19 %.

Con el fin de calcular los límites de detección se utilizó una estrategia basada en la varianza de la concentración predicha por los modelos PLS1 desarrollada por Faber y Bro. Para ello, se utilizaron muestras de saliva no dopadas. Los límites de detección multivariantes (MDL) se encontraron, en todos los casos, en el rango de ppm. El método, por tanto, tuvo una sensibilidad adecuada teniendo en cuenta las concentraciones de aminoácidos encontradas en las muestras.

A continuación, se evaluaron la repetibilidad y reproducibilidad del método no separativo. Para ello y de manera semejante al método con separación cromatográfica, se utilizaron dos niveles diferentes de concentración. Los valores obtenidos en términos de desviación estándar relativa (RSD) oscilaron entre 1-11 % y 2-16 %, respectivamente, y fueron similares a aquellos obtenidos con el método separativo.

Por último, los modelos PLS1 óptimos se utilizaron para predecir la concentración de los 16 aminoácidos en el grupo de muestras externas. Los valores predichos fueron semejantes a aquellos encontrados con el método separativo. A pesar de que el intervalo de confianza del método no separativo fue

más amplio, las concentraciones obtenidas con el método de referencia se encontraron dentro de dicho rango. Los errores obtenidos cuando se compararon dichas concentraciones oscilaron entre el 2 y el 48 %. A pesar del bajo número de muestras predichas, estos resultados proporcionaron información acerca de la aplicabilidad del método no separativo como una primera aproximación para la semicuantificación de aminoácidos en muestras de saliva.

Cabe destacar que este trabajo no se enfocó a una enfermedad determinada, sino que se prefirió obtener información de un número mayor de compuestos en lugar de un conjunto limitado. Además, una vez construidos los modelos PLS1 el coste y tiempo del análisis es semejante independientemente del número de analitos que se desee determinar.

Inicialmente fue necesario utilizar el método separativo para determinar las concentraciones de aminoácidos en las muestras de saliva utilizadas. Posteriormente, las muestras se analizaron mediante el método no separativo para obtener las señales de perfil y poder generar los modelos PLS1. Una vez construidos los modelos, la propuesta metodológica es la siguiente. Las nuevas muestras se analizarían utilizando el método rápido sin separación, siendo éste utilizado como método de *screening*. Solo aquellas muestras cuyo rango de concentración fuese significativamente diferente de los valores tabulados para individuos sanos serían analizadas para confirmación con el método cromatográfico, reduciendo así el tiempo y el coste del análisis.

Entre los métodos revisados en bibliografía solo se ha encontrado un método no separativo para la determinación de aminoácidos en saliva. Si bien en este trabajo se describen límites de detección (LODs) inferiores a los del presente método reduciendo el tiempo de análisis, la instrumentación utilizada fue más compleja y no fue posible resolver aminoácidos isobáricos.

En conclusión, se han cumplido los objetivos establecidos para este trabajo. Se ha desarrollado un método no separativo rápido y simple para la

semicuantificación de aminoácidos en muestras de saliva. La sensibilidad del método fue suficiente dadas las concentraciones presentes de estos compuestos en las muestras de saliva, y se obtuvieron también buenos resultados en términos de precisión y exactitud.

III.1 Artículo de investigación

Método no separativo basado en un espectrómetro de masas cuadrupolar para la determinación semi-cuantitativa de aminoácidos en muestras de saliva. Un estudio preliminar

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Non-separative method based on a single quadrupole mass spectrometer for the semi-quantitative determination of amino acids in saliva samples. A preliminary study

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ABSTRACT

Amino acids have been of great interest in clinical studies since variation in their concentration may provide information about different disorders. For the first time, a non-separative method based on single quadrupole mass spectrometry (qMS) for the simultaneous semiquantitative determination of sixteen amino acids in saliva samples has been developed. The method includes derivatisation of amino acids with ethyl chloroformate-pyridine-ethanol to obtain volatile products, liquid-liquid extraction (LLE) and further analysis using a programmed temperature vaporizer (PTV) coupled to qMS. This method could be applied to the analysis of a great number of saliva samples, limiting the use of separative methods only when abnormal concentrations of amino acids were found, reducing analysis time and cost. The results obtained in the determination of amino acids using the non-separative method were compared to those obtained when a separative method based on gas chromatography (GC) was used, providing values of average relative predictive error (E %) ranging between 2 and 48%. Repeatability and reproducibility were tested, obtaining relative standard deviation (RSD) values equal to or lower than 11% and 16%, respectively. Detection limits were in the range of 0.076–8.747 mg L^{-1} for the non-separative method.

1. Introduction

The search for biomarkers in different biological samples has increased rapidly over the last years. With the aim of obtaining information about the state of the organism, diagnosing diseases as well as studying disease mechanisms, attention has been focused on the determination of amino acids. These compounds constitute the basic structural unit of proteins and are important metabolic intermediates in different processes in the human body, as a source of energy and precursors in the biosynthesis of neurotransmitters and polyamines, among other functions [1,2]. The different disorders reported affecting amino acid levels in the human body include phenylketonuria [3], maple syrup urine disease [3], homocystinuria [3], tyrosinemia [3], schizophrenia [4], diabetes [4] and different types of cancer such as breast [2,5], lung [2,6], and colorectal [2] cancer, showing different levels of amino acid profile.

Determination of amino acids has been performed in a number of biological matrices including blood [7], plasma [8–10] and serum [11,12] as the most commonly selected, since profiles of these compounds are significantly altered on early stages of disease apparently without symptoms while other commonly used cancer biomarkers are not altered in the initial stage and they are not specific for a type of cancer [2]. In this context, recent studies based on AminoIndex Cancer Screening (AICS) [2,13] have been reported for the early detection of cancer using the amino acid profile. This new approach is based on the analysis of plasma samples to obtain multiple amino acid concentrations. This, jointly with multivariate analysis, makes it possible to calculate risk for several types of cancer simultaneously with a high detection level and low false-positive rate [13].

Due to the invasive approach to collect blood, serum and plasma samples, urine [10,11,14–17] and saliva [5,17–27] samples have gained attention for non-invasive sampling in the determination of amino acids. The usefulness of saliva samples as a source of metabolic information has been demonstrated through different studies [28] that correlated serum and plasma levels of substances with those found in saliva, making this type of sample a possible alternative to blood. The use of other samples, such as hair [16], skin [29], faeces [11], cerebrospinal fluid [18] and bronchoalveolar lavage fluid [6] has also been

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reported.

For decades, the methods used to determine amino acids in biological samples included a separation step of the analytes, after extraction and derivatisation reaction to obtain adequate derivatives. High performance liquid chromatography (HPLC) has been used coupled to different detectors. Mass spectrometry detection (MS) has been employed using a single quadrupole [8], triple quadrupole (QqQ) [9,19] and time-of-flight (TOF) [5]. Fluorescence (F) [20,22], ultraviolet (UV) [21] and multiwavelength (MWL) [24] detectors have also been reported. GC coupled to different types of detectors has been an important alternative for the determination of amino acids in biological samples. Similar MS detectors, such as those used for HPLC, have been reported for GC methods, including single quadrupole [10,12,14,15], QqQ [16] and TOF [9,11], the latter analyser having been used both with one [9] and two-dimensional GC [9]. Additionally, quadrupole ion trap MS detector (QTrap) [6] has been employed. The possibilities of using GC with flame ionisation detection (FID) [32] have also been explored. Capillary electrophoresis (CE) has been employed both with contactless conductivity detection (CCD) [17] and laser induced fluorescence (LIF) [18,25].

Nowadays, there is a noticeable increase in the development of non-separative analytical methods for the simultaneous determination of as many analytes as possible. The methods reported have been mainly based on MS [30] and nuclear magnetic resonance (NMR) [31,32]. Methods reported for MS detection have included different sample injection modes, such as direct infusion (DI) [6,33–36] and flow injection analysis (FIA) [7,37–40]. The analyses have been performed using analysers such as QqQ [7,27,37], hybrid q-TOF [33], hybrid QqQ-TOF [6,38], Fourier transform ion cyclotron resonance (FT-ICR) [34,39], QTrap [6,40], hybrid QqQ/linear ion trap [35] and orbitrap [36]. In most cases, the desired information about the samples analysed required chemometric techniques applied to the profile signal obtained.

We report the possibilities of a programmed temperature vaporizer (PTV) coupled to a qMS followed by multivariate analysis for the simultaneous semiquantification of sixteen amino acids in saliva samples. In comparison with the methods previously mentioned, the proposal of using a single quadrupole mass spectrometer jointly with chemometrics makes the method cost-effective and accessible in all laboratories. To the best of our knowledge, there are no previous references about the use of this coupling for the non-separative analysis of amino acids in saliva. In addition, the use of the same configuration for further confirmatory analyses with a separative method has to be highlighted. In this case, instead of maintaining the chromatographic column at a high temperature to eliminate its separation capacity, temperature ramps were used. This is also the first time that GC is used to analyse amino acids in saliva.

2. Material and methods

2.1. Chemicals and standard solutions

The sixteen amino acids of the study (alanine, asparagine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, tryptophan, tyrosine and valine), the internal standard (IS, 4-chlorophenylalanine), ethyl chloroformate (ECF) as a derivatising reagent, pyridine (catalyst), and ethyl acetate as an extraction solvent were procured by Sigma-Aldrich (Steinheim, Germany). Ethanol absolute (EtOH) and hydrochloric acid (HCl) were supplied by Scharlab (Barcelona, Spain). Ultra-high quality water (UHQ) was obtained using a Wasserlab Ultramatic water purification system (Noain, Spain). The purity of all the compounds was at least 97%.

Stock solutions of each amino acid (1000 mg L^{-1}) in HCl were prepared and stored at 4 °C until subsequent dilution in UHQ water to prepare the corresponding working solutions.

2.2. Saliva samples

Unstimulated saliva samples were collected from nominally healthy adults (4 males and 4 females) in 10-mL glass vials with silicone septum cap and frozen at −26 °C until analysis. The participants were asked not to ingest food or beverages at least 2 h prior to sample collection. Before derivatisation and LLE extraction, the samples were thawed at room temperature and homogenized. Due to the high concentration of amino acids in the saliva samples, they were adequately diluted. The participants were informed about the objective of the study and written consent was obtained.

2.3. Derivatisation reaction (DER) and liquid-liquid extraction (LLE)

Ethyl chloroformate, pyridine and ethanol were used to convert amino acids into the corresponding volatile derivatives. The proportion between ethyl chloroformate, pyridine and ethanol was selected as described earlier [14]. Briefly, 33 µL of pyridine, 40 µL of EtOH and 40 µL of ECF were added to an 11.5-mL glass centrifuge tube with PTFE cap containing 650 µL of saliva sample and 58 µL of internal standard (final concentration, 0.4 mg L^{-1}). The mixture was vortexed for 0.5 min to facilitate contact between reagents and sample. The tube was opened to eliminate CO_2 from the aqueous matrix. Then, it was closed and vortexed again for 0.5 min.

After the reaction, an additional step of degassing in an ultrasonic bath (0.5 min) was necessary to ensure repeatability of the process before extraction.

Then, a volume of 800 µL of ethyl acetate was added to the mixture, the tube was vortexed again for 1 min and centrifuged at 2415 × *g* for an additional minute to separate phases. The organic phase was collected and placed in a GC vial for analysis.

2.4. PTV-GC-qMS conditions

The analyses were conducted using the instrumental configuration previously described [41]. 30-µL aliquots of the ethyl acetate phase were injected using a PTV inlet (CIS-4, Gerstel, Baltimore, MD) with a liner (71 mm × 2 mm) filled with deactivated glass wool. It was operated in the solvent vent mode, with an initial injector temperature of 75 °C for 1.25 min, using a vent flow of 75 mL min^{-1} at 5.00 psi. The solvent was eliminated and the liner was heated at the maximum rate permitted (12 °C s^{-1}) up to 270 °C, with an injection time of 1 min to desorb and transfer the derivatives of amino acids to the column. The final temperature was held for 5 min to clean the system. Purge flow was set at 150 mL min^{-1}. The PTV system was cooled with liquid CO_2.

For GC-qMS analysis, the device was equipped with a HP5-MS UI capillary column (30 m × 0.250 mm × 0.25 µm) from J&W Scientific (Folsom, CA, USA). The temperature gradient was as follows: 60 °C held for 2 min, then temperature was increased at a rate of 60 °C min^{-1} up to 145 °C (3 min) and further increased to 300 °C, held for 1 min. The total run time was 10 min. A solvent delay time of 4 min was set. A flow rate of 2 mL min^{-1} with helium N50 (99.999%, Air Liquide) as carrier gas was used. Electron-ionisation mode (EI, 70 eV) was employed. The set temperatures for ion source and quadrupole were 230 °C and 150 °C, respectively.

The analyses were performed in a synchronous SIM/scan mode. Full scan was used at the examined *m/z* range of 40–500 for target compound identification by comparison of the experimental spectra with those found in the NIST'08 database (NIST/EPA/NIH Mass Spectral Library, version 2.0). The three different scan groups with the corresponding data acquisition speed values used for compound identification can be seen in Table S1 (see Supplementary Information). In the SIM mode, four different groups including the four most abundant *m/z* ratios of each amino acid derivative were used with a dwell time value of 1 ms in all cases (see Table S1).

2.5. PTV-qMS conditions

The experimental conditions for PTV inlet were similar to those employed in the PTV-GC-qMS method. In this case, separation capacity of analytes in the column was removed by maintaining the chromatographic column at 300 °C throughout the signal recording time of 4.50 min. Emphasis has to be placed on the fact that it was not necessary to change the interface between the PTV injector and qMS detector when using the different methods.

The ionisation mode and temperatures for ion source and quadrupole were the same as those used in the GC method.

Full scan mode was used for data acquisition (0.83 scan s^{-1}), using a solvent delay time of 1.60 min. The *m/z* range was similar to the one used for separative measurements (40–500).

2.6. Data analysis

Enhanced ChemStation software [42] (Agilent Technologies) was used to collect data and The Unscrambler® 10.5 statistical package [43] (CAMO Software) was used to implement chemometric techniques.

3. Results and discussion

3.1. DER-LLE-PTV-GC-qMS method

A separative method based on DER-LLE-PTV-GC-qMS was developed to compare the results obtained with the non-separative method. The method was used for the quantification of sixteen amino acids in saliva samples. In Fig. 1a the extracted ion chromatogram in the SIM mode obtained for a saliva sample spiked with all the analytes is shown. The most abundant *m/z* for each amino acid derivative has been selected in the figure. As it can be seen in the chromatogram, the 16 amino acid derivatives were separated in 9.60 min (5 min when solvent delay time was not considered). Additionally, 9 min were required to achieve the initial conditions of the GC column, thus prolonging the total time between sample runs up to 19 min.

The existence of a matrix effect was confirmed when the slopes of the calibration curves obtained for ultrapure water and eight saliva samples containing the analytes at three different concentration levels (endogenous level and two spiked levels) were compared. This effect was evident between water and saliva samples as well as between the different saliva samples analysed, even when saliva samples were diluted. The differences between the slopes for each analyte in the saliva samples ranged between 40 and 83%. Consequently, quantification of amino acids was carried out by using a standard addition protocol. In addition to this, correction with 4-chlorophenylalanine as internal standard was necessary to compensate the variations of the whole process of analysis. All the samples were analysed in duplicate.

To perform the calibration models, the samples were diluted as necessary (up to 20 times) and spiked at two different concentration levels. The peak area for each analyte obtained by extracting the quantitation ion in the SIM mode (see Table S1) and normalized to the corresponding peak area for the internal standard was used for quantification. The method was applied to the determination of amino acids in eight saliva samples. Since asparagine was not found in any of the samples analysed, the samples were spiked with the analyte to obtain their analytical characteristics. The amino acids found in higher concentration level were alanine (0.269–16.2 mg L^{-1}), glycine (1.60–25.7 mg L^{-1}), proline (0.319–40.4 mg L^{-1}), glutamine (0–133.6 mg L^{-1}), ornithine (1.19–10.7 mg L^{-1}), lysine (1.11–13.5 mg L^{-1}) and tyrosine (0.858–11.8 mg L^{-1}). Lower concentration levels were found for the remaining analytes (0.037–6.78 mg L^{-1}). These results are in agreement with the concentration of amino acids described in the literature [25].

In Table 1 the figures of merit of the DER-LLE-PTV-GC-qMS method proposed for one of the studied saliva samples are shown. Similar results were obtained in all cases. All calibration curves (standard addition protocol) showed satisfactory coefficients of determination (R^2) for all the analytes in all samples.

Limits of detection (LOD) and quantification (LOQ) were determined for the sixteen amino acids in each of the samples and were based on the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The values obtained varied depending on the saliva sample, but they were in the same μg L^{-1} range as the levels shown for one of the saliva samples analysed in Table 1, thus showing high sensitivity.

Repeatability (n = 5 on the same day) and reproducibility (n = 5 per day on two consecutive days) of the method were evaluated by analysing a saliva sample containing all the analytes at two different concentration levels. The low level corresponded to the endogenous levels of amino acid with addition of asparagine (0.824 mg L^{-1}) and the high level corresponded to the same sample spiked with all the analytes at the highest calibration level (added concentrations ranging between 0.105 mg L^{-1} and 33.4 mg L^{-1} for isoleucine and glutamine, respectively). As it can be seen in Table 1, good results in terms of repeatability and reproducibility were obtained, with RSD values ranging between 0-11% and 2–14%, respectively.

In order to check the accuracy of the method, recovery was studied by predicting the concentration of amino acids in samples spiked with all the analytes using the standard addition method. The spiked samples were analysed in duplicate. The recovery values were calculated by comparison between the known concentration of each analyte in the spiked sample (endogenous level plus added concentration) and the predicted concentration by the standard addition method. The method showed good recovery of the spiked saliva samples with average values ranging from 80% to 115%.

3.2. DER-LLE-PTV-qMS method

With the aim of exploring the analytical possibilities of a non-separative method based on simple instrumentation, such as qMS, the same eight samples at three concentration levels (endogenous level and two spiked levels) analysed using the separative method were analysed maintaining the GC column temperature at 300 °C during the analysis time, thus eliminating the separation capacity of the column. Each sample was analysed in duplicate. The profile signals obtained with the non-separative method were used to perform targeted analyses with the aim of quantifying amino acids in saliva samples. As an example, in Fig. 1b the profile signal obtained when a spiked saliva sample was analysed using the non-separative method is shown. As can be seen in the figure, the time of analysis required was 4.50 min. Since the temperature of the GC column was constant during the whole time of analysis, the time required between sample runs was reduced to 5 min in comparison with the GC method (19 min).

Calibration was carried out by using Partial Least Squares (PLS1). The eight saliva samples were divided into two different groups. One of them, containing seven saliva samples and their corresponding spiked ones, were used to build the models for each analyte. They were selected according to the concentration values predicted with the separative method so that the whole concentration range was covered in the model. The other group is formed by the remaining non-spiked sample and the corresponding spiked ones and it was used as external validation set. Thereby, the matrix effect (see section corresponding to DER-LLE-PTV-GC-qMS method) was considered since the models included different saliva samples, thus covering the variability found between the different samples. No internal standard was used since their *m/z* ratios were interfered by the corresponding *m/z* ratios provided by other analytes present in the samples.

The independent variables used in the partial least squares regression (PLS1) were the sum of intensities of all the ions (*m/z* 40–500) monitored during data acquisition. The dependent variables were the concentration values predicted with the GC method.

Initially, the whole *m/z* range (40–500) was used to construct PLS1

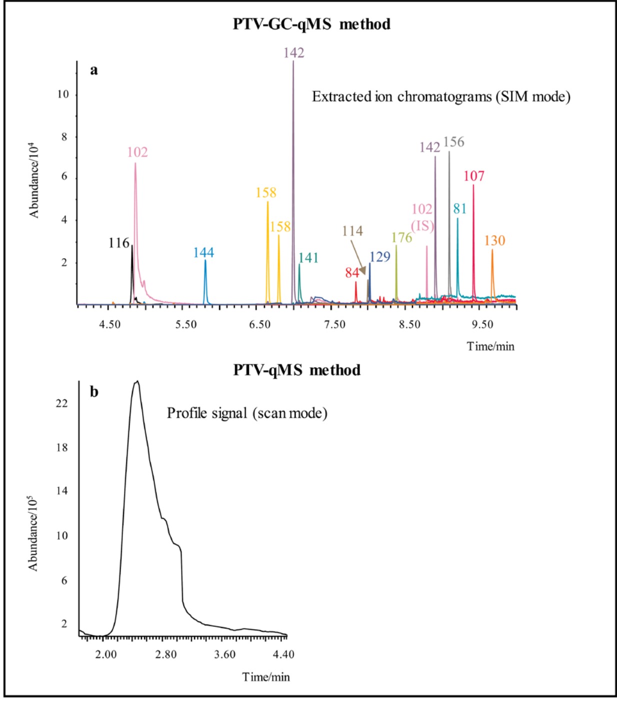

Fig. 1. (a) Extracted ion chromatograms (SIM mode) corresponding to the PTV-GC-qMS method and (b) profile signal (scan mode) corresponding to the PTV-qMS method for a spiked saliva sample (0.074–6.8 mg L^{-1}).

models. In order to select the optimum number of PLS factors that provided a minimum root mean standard error of validation (*RMSEV*), the process was carried out using cross-validation (leave one out). The average relative error (E %) with the selected PLS factors was calculated for each model as follows:

$$E\,(\%) = \frac{RMSEV}{\bar{c}} \cdot 100 \quad (1)$$

where $\bar{c}$ is the average concentration of the corresponding amino acid.

Table 1
Figures of merit of the DER-LLE-PTV-GC-qMS method for one of the studied saliva samples.

Analyte	t_R/min	R^2	Repeatability/RSD, %		Reproducibility/RSD, %		LOD/μg L^{-1}
			Low level	High level	Low level	High level	
Alanine	4.807	0.9969	6	8	8	6	2.58
Glycine	4.841	0.9963	2	2	9	8	25.2
Valine	5.772	0.9997	5	7	7	7	1.91
Leucine	6.613	0.9993	5	9	6	7	1.16
Isoleucine	6.771	0.9954	5	10	4	8	1.59
Proline	6.972	0.9985	8	11	6	7	1.02
Asparagine	7.065	0.9892	3	9	3	6	36.5
Glutamine	7.830	0.9837	2	6	11	7	183
Serine	8.001	0.9984	4	6	14	8	269
Methionine	8.017	0.9986	1	2	4	3	12.0
Phenylalanine	8.378	0.9933	1	2	3	2	1.12
Ornithine	8.913	0.9948	3	3	2	3	7.25
Lysine	9.104	0.9941	4	7	5	7	5.95
Histidine	9.202	0.9985	4	0	11	2	15.1
Tyrosine	9.434	0.9942	5	7	11	6	52.9
Tryptophan	9.660	0.9987	1	2	5	2	5.04

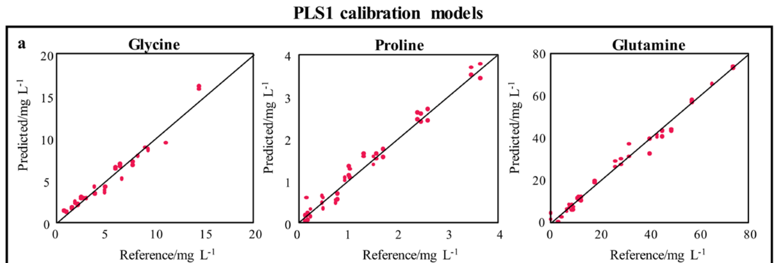

Fig. 2. Correlation plots of the predicted concentration versus reference concentration by GC method in the (a) calibration step (cross-validation leave one out) and in the (b) validation step when the models were used to predict an external set of samples.

In a second step, Martens uncertainty criterion was used to eliminate those *m/z* ratios whose regression coefficients provided uncertainty values higher than the corresponding absolute value from the model. In Table S2 (see Supplementary Information), the *m/z* variables selected for each analyte after applying Martens uncertainty criterion are shown. In the case of serine, methionine, phenylalanine and histidine, all the *m/z* variables were significant. The use of this criterion reduced the number of PLS factors in the models.

The PLS1 models for glycine, proline and glutamine are shown, as an example, in Fig. 2a. Table 2 shows the average relative error in the cross-validation step, ranging between 6% and 19%. The use of models with different saliva samples, partially, corrected the matrix effect.

Table 2
Characteristics of the PLS1 calibration models in the cross-validation step: average relative predictive error (E %) and number of optimum PLS factors after applying Martens uncertainty criterion.

Analyte	PLS1 calibration models	
	E %	PCs
Alanine	10	14
Glycine	14	3
Valine	13	5
Leucine	13	7
Isoleucine	19	13
Proline	11	11
Asparagine	6	19
Glutamine	9	12
Serine	17	12
Methionine	13	16
Phenylalanine	10	18
Ornithine	7	20
Lysine	9	16
Histidine	9	18
Tyrosine	11	12
Tryptophan	12	9

In order to calculate the multivariate detection limits (MDL) of the non-separative method, a strategy developed by Faber and Bro [44] and based on the variance of the concentration predicted by the PLS1 models was selected. The saliva samples without addition of amino acids (non-spiked samples) were used to obtain the corresponding MDLs. As can be seen in Table 3 the detection limits were in the range of μg L^{-1}, except for glycine, glutamine and serine, which were in the mg L^{-1} range. The values obtained were low enough considering the concentration of analytes found in the samples.

Repeatability (n = 5 on the same day) and reproducibility (n = 5 per day on two consecutive days) of the non-separative method were evaluated with the same samples used for the GC method at the two different concentration levels considered (endogenous levels of amino acid with addition of asparagine at 0.824 mg L^{-1} and the same sample spiked with all the analytes with concentrations ranging between 0.105 mg L^{-1} and 33.4 mg L^{-1} for isoleucine and glutamine, respectively). The *m/z* ratios used to evaluate these parameters were selected according to the most abundant *m/z* ratios in the spectrum of the corresponding derivatives of the amino acids. In Table 3 the *m/z* used as well as the results expressed as the RSD are shown. Good results were obtained in terms of repeatability and reproducibility for all the analytes, with RSD values ranging between 1-11% and 2–16%, respectively. These values were similar to the ones obtained when the chromatographic method previously described was employed.

The PLS1 models were used to predict the concentration of the sixteen amino acids under study in an external validation set of samples that did not participate in the building of the models. This set of samples included, as mentioned before, one saliva sample at three different concentration levels (endogenous level and two spiked levels) for each analyte. As an example, Fig. 2b shows the correlation plots of the predicted concentration versus GC concentration for glycine, proline

Table 3
Figures of merit of the DER-LLE-PTV-qMS method.

Analyte	m/z	Repeatability/RSD, %		Reproducibility/RSD, %		LOD/μg L^{-1}
		Low level	High level	Low level	High level	
Alanine	74	5	5	8	5	164
Glycine	84	4	4	6	16	2587
Valine	144	4	5	5	4	100
Leucine	158	7	7	5	6	111
Isoleucine	70	5	3	4	4	76.0
Proline	142	1	3	2	2	546
Asparagine	141	3	7	7	5	336
Glutamine	56	3	4	5	6	8747
Serine	86	3	2	7	8	1733
Methionine	129	3	2	4	5	83.8
Phenylalanine	176	5	2	9	7	88.2
Ornithine	258	10	11	12	10	241
Lysine	156	3	3	6	4	548
Histidine	81	4	3	3	4	536
Tyrosine	60	1	4	3	5	207
Tryptophan	130	2	1	13	8	87.6

Table 4
Amino acid concentration predicted by DER-LLE-PTV-qMS (non-separative method) and DER-LLE-PTV-GC-qMS (separative method) in a saliva sample.

Analyte	Predicted concentration/mg L^{-1}	
	DER-LLE-PTV-qMS	DER-LLE-PTV-GC-qMS
Alanine	5 ± 2	6 ± 1
Glycine	11 ± 4	10 ± 2
Valine	3.4 ± 0.6	3.3 ± 0.7
Leucine	3.2 ± 0.8	5 ± 1
Isoleucine	2.2 ± 0.8	2.1 ± 0.5
Proline	8 ± 4	9 ± 1
Asparagine	0 ± 6	0.0 ± 0.5
Glutamine	(10 ± 3)·10	(9 ± 1)·10
Serine	4 ± 2	3 ± 1
Methionine	0.8 ± 0.4	1.0 ± 0.2
Phenylalanine	5 ± 2	4.0 ± 0.7
Ornithine	4 ± 2	6 ± 1
Lysine	8 ± 6	11 ± 2
Histidine	3 ± 2	3 ± 1
Tyrosine	12 ± 4	14 ± 2
Tryptophan	0.7 ± 0.3	0.9 ± 0.2

and glutamine when the PLS1 models (Fig. 2a) were used. In Table 4, the concentration of amino acids predicted by the non-separative method (DER-LLE-PTV-qMS) for the non-spiked saliva sample included in the external validation set is shown. For comparison purposes, the concentration found when analysing this saliva sample with the separative method (DER-LLE-PTV-GC-qMS) is also shown. The values obtained with both methods were similar. As expected, the confidence interval for the predicted concentrations with the non-separative method is greater than that of the chromatographic method. The concentration obtained with the reference method is within the range of concentrations found with the proposed method.

The average relative predictive error (E %) and the average bias obtained in the prediction of amino acids in the external validation set are shown in Table S3 (see Supplementary Information). The first one, E %, was calculated as in expression (1), and average bias was obtained using the following expression:

$$\text{bias} = \frac{\sum_{i=1}^{m} (\hat{c}_i - c_i)}{\text{m}} \quad (2)$$

where c_i is the reference concentration provided by the GC method, $\hat{c}_i$ is the predicted concentration, and m is the number of samples. The values ranged between 2% and 48% and between −0.457 and 0.818 for E % and bias, respectively. The presence of positive and negative values observed in Table S3 indicated that there was not any trend in the data. Despite the low number of samples predicted, these results give an idea of the applicability of the proposed non-separative method as a first approach to semiquantify amino acids in saliva samples.

Since this work is not focused on any specific disease, it has been preferred to obtain information on a large number of compounds rather than focusing on a small group. On the other hand, once the PLS1 models have been developed, the cost and time of the analysis is identical regardless of the number of analytes to be determined.

3.3. Proposed methodological strategy

The development of the non-separative method based on PTV-qMS firstly required the use of the separative method based on PTV-GC-qMS to determine the concentration of amino acids in the group of samples used to calibrate and generate the PLS1 models. Once the models have been constructed, all the desired saliva samples whose amino acid content is to be determined would be analysed using the rapid non-separative method. Only those samples showing amino acid concentrations significantly different from those tabulated [45] for healthy subjects would be analysed using the separative method, thus confirming the previous result. Since the time required for the analysis of samples including chromatographic separation was higher (19 min) than the corresponding time for the non-separative method (5 min), the use of the second approach in the first screening of samples is an excellent alternative which reduces analysis time and cost.

As expected, the sensitivity achieved with the separative method was higher than the sensitivity of the non-separative method (between one and two orders of magnitude depending on the analyte). However, in both cases the values obtained were lower than the usual concentration found in saliva samples.

No significant differences were observed between the two methods in terms of precision. The average relative predictive error in the determination of amino acids in saliva samples using the non-separative method ranges between 2% and 48% when compared with the GC method. With the aim of reducing possible false negatives, all samples with concentration ranges coinciding with some abnormal value [45] should be analysed with the confirmatory method although this increases the number of false positives.

3.4. Method comparison: DER-LLE-PTV-qMS versus other non-separative methods published

The main limitation of separative methods lies in the time consumed for each analysis. However, the LODs provided are usually better when

separation is performed. Particularly, the best LODs for salivary amino acids have been reported for the CE-LIF method [18] (7–14 ppt).

The limitation of time consumption has been overcome using non-separative methods in the analysis of biological samples. Several methods based on NMR [31,32] and MS [6,7,27,33–40] have been proposed. NMR is very reproducible, but the sensitivity is lower than the one achieved with MS methods [1]. When using DI-MS [6,33–36] and FIA-MS [7,37–40], mass spectrometers based on QqQ [7,27,37], hybrid q-TOF [33] and QqQ-TOF [6,38], QTrap [35,40], orbitrap [36] and FT-ICR [34,39] have been reported, and the time required for the analysis is reduced by up to 2 min. However, they are not easily accessed in all laboratories.

To the best of our knowledge, only one non-separative method has been applied to the determination of amino acids in saliva samples [27]. The method was based on ESI-QqQ-MS. This method presents some advantages over the method proposed here. When using the ESI-QqQ-MS method, derivatisation was not required before the analysis. In addition, the instrumental LODs provided in UHQ water (0.001–0.018 mg L^{-1}) were lower than those achieved in saliva with the present method (0.076–8.747 mg L^{-1}) and the time required for the screening analysis was reduced approximately by half. However, it is necessary to highlight that the LODs achieved in this work were low enough to quantify amino acids in saliva samples due to the high levels of analytes found in the samples, which are within the usual concentration range. On the other hand, the drawbacks of ESI-QqQ-MS analysis lie in the inability, in most cases, to resolve isobaric amino acids due to the similar fragmentation pattern of the analytes as well as the limited accessibility of this equipment in common laboratories. These drawbacks have been partially overcome here by using a single quadrupole mass spectrometer with a hard ionisation source (EI), which generates a greater number of m/z fragments, and further application of chemometric techniques.

4. Conclusions

A novel non-separative method based on DER-LLE-PTV-qMS has led to satisfactory semiquantification of amino acids in saliva samples.

The LODs achieved ranged between 0.076 and 8.747 mg L^{-1} and they were low enough considering the high amino acid levels found in the samples.

This method is rapid and simple, also showing good results in terms of precision and accuracy. For these reasons, it should be considered as an excellent tool for the screening analysis of a great number of samples, allowing identification of saliva samples providing abnormal concentrations of amino acids. Since the separation capacity is eliminated by maintaining the GC column at an elevated temperature during the analysis time, the same instrumental configuration allows further confirmatory analysis by using the corresponding GC temperature gradient. Consequently, the number of samples requiring confirmatory analysis using the separative method is reduced, thus saving time and money.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120381.

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1 **Supplementary Material**

2

3 **Non-separative method based on a single quadrupole mass**
4 **spectrometer for the semi-quantitative determination of amino acids in**
5 **saliva samples. A preliminary study**

6 Patricia Martín Santos, Miguel del Nogal Sánchez*, José Luis Pérez Pavón, Bernardo
7 Moreno Cordero

8

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11

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13

14 Table S1. Scan and SIM groups used in the DER-LLE-PTV-GC-qMS method

Mode	Initial time/min		Characteristics	
scan (m/z 40-500)	4.00		scan s^{-1}	3.15
	6.30			5.92
	7.88			10.59
SIM	4.00		*m/z* selected* (dwell time 1 ms)	
		Alanine		**116**, 44, 72, 117
		Glycine		**102**, 74, 103, 130
		Valine		**144**, 55, 72, 116
	6.10			
		Leucine		**158**, 44, 101, 102
		Isoleucine		**158**, 102, 129, 130
		Proline		**142**, 70, 98, 114
		Asparagine		**141**, 42, 56, 69
	7.50			
		Glutamine		**84**, 56, 110, 156
		Serine		**114**, 60, 86, 102
		Methionine		**129**, 61, 101, 175
		Phenylalanine		**176**, 91, 102, 120
	8.65			
		4-chlorophenylalanine (IS)		**102**, 74, 125, 210
		Ornithine		**142**, 70, 98, 258
		Lysine		**156**, 56, 84, 128
		Histidine		**81**, 154, 238, 254
		Tyrosine		**107**, 102, 135, 192
		Tryptophan		**130**, 103, 131, 143

15

16 **m/z* in bold correspond to the ones selected for quantification purposes

17

18

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20

21

22 Table S2. *m/z* variables selected in the PLS1 models by the Martens uncertainty criterion
23 for the studied amino acids

Analyte	*m/z* variables selected by Martens uncertainty criterion
Alanine	42, 59, 60, 61, 63, 64, 67, 87, 97, 101, 102, 105, 107, 114, 116-118, 127, 130, 131, 142, 146, 156, 158, 159, 164, 173, 176, 180, 181, 190, 194, 201, 226, 228, 238, 239, 247, 272, 319
Glycine	40, 46, 49, 50-52, 54, 56, 62, 66, 79, 84-86, 101, 104, 106-108, 110, 113, 116, 117, 120, 123, 124, 128, 130, 132, 135, 136, 138, 139, 141, 142, 144, 149, 154, 155, 157, 158, 160, 166, 168-172, 179, 181, 182, 184, 186, 187, 191, 196, 199, 200, 213, 215, 227, 232, 238, 243, 247, 248, 252, 254, 305, 318, 320, 335, 336, 362-364, 379, 412
Valine	46, 69, 81, 100, 107, 114, 116, 126, 128, 129, 141, 148, 174, 185, 188, 191, 192, 194, 264, 288, 304, 342
Leucine	46, 47, 69, 86, 100, 107, 114, 115, 118, 120, 126, 128, 129, 141, 148, 174, 179, 180, 184, 191, 192, 194, 221, 249, 251, 264, 267, 268, 288, 304, 309, 324, 342, 393
Isoleucine	46, 71, 86, 90, 92, 100, 107, 115, 118, 120, 126, 128, 132, 148, 174, 176, 179, 180, 184, 191, 192, 194, 201, 209, 221, 248, 249, 251, 264, 267, 268, 287, 309, 323, 342, 393, 428, 429, 436
Proline	45, 59, 60, 61, 63, 70, 72, 84, 86, 87, 101, 103, 105, 107, 114, 115, 129, 131-134, 142, 146, 148, 155, 157-161, 173-177, 180, 189, 198, 200, 201, 205, 207-209, 224, 226, 243, 254, 256, 284, 287, 301, 302, 304, 377, 389
Asparagine	45, 46, 55, 56, 60, 61, 63, 69, 71, 79, 84, 86, 98, 99, 101, 126, 129, 132, 141, 149, 156-160, 168, 171-176, 180, 185, 188, 192, 203, 250, 258, 259, 272, 273, 288, 304
Glutamine	40-44, 46, 47, 54-57, 60-62, 64-66, 68-70, 74, 77, 81-86, 88-95, 97, 100, 101, 104-111, 113, 115-117, 120-125, 127, 128, 130-132, 134-142, 144, 145, 147, 149-151, 154, 155, 157, 158, 160, 161, 163-166, 168-173, 176, 178-187, 190, 191, 193, 196, 198-200, 203, 210, 213, 215, 217, 221, 222, 227, 228, 232, 234, 235, 238, 241, 243, 247, 251, 252, 254, 255, 259, 264, 277, 279, 280, 295, 296, 298, 303-305, 308, 318-321, 327, 328, 336, 348, 349, 351, 352, 355-358, 360, 362, 364, 365, 369, 370, 379, 388, 394, 399-402, 405, 412, 413, 415, 416, 427, 429, 430-433, 443, 446, 453, 460-463, 475, 476, 482, 487, 489, 490
Serine	40-500
Methionine	40-500
Phenylalanine	40-500
Ornithine	41, 42, 46, 53, 56, 59, 64-66, 70, 71, 72, 76, 79, 84, 86, 98, 100, 101, 103-105, 107-109, 112, 126, 129, 132, 136, 141, 142, 144, 149-151, 156, 157, 163, 172, 173, 175, 178, 193, 195, 198, 201, 207-210, 234, 235, 249-251, 253, 255, 258, 259, 267-269, 272, 282, 283, 293, 308-311, 325, 341-343, 367, 377, 405, 482
Lysine	56, 79, 85, 98, 101, 103-105, 108, 111, 116, 129, 132, 153, 156, 157, 173, 175, 178, 207, 209, 210, 226, 253, 258, 272
Histidine	40-500
Tyrosine	43, 47, 56, 62-64, 70, 74, 83, 84, 86, 98, 100, 101, 105-108, 111, 113, 114, 116, 126, 127, 129, 134, 139, 140, 144, 145, 152, 153, 155-157, 162, 164, 167, 171, 172, 174, 175, 185, 192, 212-214, 220, 226, 258, 259, 264, 272, 273, 280, 303, 352, 405, 407, 456
Tryptophan	40, 41, 42, 44, 49-52, 54, 55, 57, 61, 62, 78-80, 83, 84, 86, 88, 97, 98, 100, 109, 115, 116, 126, 128, 130, 132, 136, 144, 151, 157, 158, 171, 172, 176, 177, 181, 188, 210, 217, 226, 238, 239, 272, 304, 336, 364, 365, 383, 409, 411, 412, 425

24

25 Table S3. Average relative predictive error (E %) and bias when the concentration of
26 amino acids was predicted in the external validation set of saliva samples using the PLS1
27 models
28

	Prediction of the external validation set	
Analyte	**E %**	**Bias/mg L^{-1}**
Alanine	43	-0.162
Glycine	11	0.337
Valine	2	-0.002
Leucine	38	-0.119
Isoleucine	32	-0.040
Proline	10	-0.022
Asparagine	20	-0.021
Glutamine	5	0.818
Serine	28	0.171
Methionine	18	-0.030
Phenylalanine	22	0.039
Ornithine	48	-0.282
Lysine	31	-0.263
Histidine	24	0.278
Tyrosine	40	-0.457
Tryptophan	43	-0.139

29

CONCLUSIONES GENERALES

De forma general, puede concluirse que se ha alcanzado el objetivo principal propuesto en esta Tesis Doctoral, ya que se han desarrollado de manera satisfactoria nuevas metodologías analíticas basadas en espectrometría de masas. En ellas, el tratamiento de muestra es sencillo y, generalmente, rápido lo que reduce los errores asociados a esta etapa del proceso analítico. Además, se ha demostrado su aplicabilidad a través de la resolución de problemas de interés relacionados con el campo biológico.

En el primer capítulo de la Tesis, se ha puesto a punto con éxito una metodología basada en HS-PTV-GC-MS para la determinación de cetonas y acetato de etilo en muestras de orina de pacientes con cáncer de pulmón e individuos sanos. El uso de esta configuración instrumental permitió llevar a cabo el análisis de manera totalmente automatizada una vez que la muestra se depositó en el vial, reduciéndose los posibles errores asociados a la etapa de manipulación de muestra. Una vez determinadas las concentraciones en las muestras de orina de ambos grupos se llevó a cabo un estudio de comparación de las mismas, observándose diferencias significativas para tres de los analitos de estudio, acetato de etilo, 3-heptanona y 3-octanona, siendo el acetato de etilo el compuesto que proporcionó los mejores resultados.

En el segundo capítulo de la Tesis se han estudiado diferentes posibilidades para la determinación de hidrocarburos policíclicos aromáticos en muestras biológicas utilizando diversas metodologías. Debido a la naturaleza no volátil de estos compuestos se abordó la extracción y preconcentración de los mismos mediante extracción líquido-líquido y microextracción con sorbentes empaquetados. La extracción líquido-líquido, utilizada en el análisis de orina y saliva, permitió reducir el tiempo requerido en esta etapa del proceso. Por otro lado, el uso de la microextracción con sorbentes empaquetados permitió la completa automatización del proceso para el análisis de estos compuestos en saliva. El desarrollo de dos métodos utilizados en el análisis de muestras de saliva que difieren sólo en la técnica de extracción permitió la comparación de los

mismos, estableciendo ventajas e inconvenientes en cada uno de los casos. La metodología basada en LLE permitió la cuantificación de muestras de saliva mediante calibración externa gracias a la alta recuperación del proceso y a la ausencia de efecto matriz, mientras que en la basada en MEPS fue necesario utilizar el procedimiento de adición estándar aumentando así el volumen de saliva necesario. A pesar de la esperada mejora en los límites de detección en la extracción con MEPS debido al uso de bajos volúmenes de disolvente de elución, la baja retención de los PAHs en el cartucho hizo que los valores fuesen semejantes a los obtenidos con LLE. Sin embargo, como ya se ha descrito previamente, el proceso de MEPS se llevó a cabo de una manera totalmente automatizada.

En este segundo capítulo también se ha puesto a punto una metodología analítica no separativa basada en LLE-PTV-MS para la determinación de PAHs en muestras de orina. Las señales de perfil obtenidas se utilizaron con dos fines diferentes. Por un lado, con fines cuantitativos, utilizándose la calibración multivariante (PLS) para la obtención de los modelos de calibración y la consiguiente predicción de muestras externas al calibrado. Por otro lado, con fines cualitativos, se aplicaron diferentes técnicas de reconocimiento de pautas (PCA, SIMCA, LDA, PLS-DA y SVM) para la diferenciación de muestras en base a la presencia o ausencia de PAHs. Los resultados obtenidos en ambos casos fueron satisfactorios. Cabe destacar que la eliminación de la etapa de separación cromatográfica redujo de manera significativa el tiempo requerido para el análisis.

Finalmente, en este capítulo se incluyó un artículo de revisión enfocado a la determinación de PAHs en diferentes muestras biológicas. Se puede concluir que la metodología más adecuada será aquella que permita dicha determinación en función de los requisitos deseados, como el tipo de exposición, el tipo de analitos que se estudian, la sensibilidad y el tiempo de análisis, entre otros.

En el tercer capítulo de la Tesis se ha propuesto por primera la determinación de aminoácidos en saliva utilizando el acoplamiento PTV-MS, eliminando la etapa de separación previa a la detección. Los resultados obtenidos proporcionaron información semicuantitativa de estos compuestos en situaciones complejas, cuando existe una mezcla de ellos con solapamiento en sus espectros de masas.

En esta parte se ha aplicado de manera satisfactoria la conocida reacción de derivatización de aminoácidos con cloroformiato de etilo en medio acuoso (saliva). El uso de esta reacción hace que se pueda ampliar el grupo de compuestos analizados utilizando GC.

De manera general, las metodologías no separativas suponen una alternativa a los métodos habituales, pudiendo ser utilizadas como cribado en el análisis de un gran número de muestras. Posteriormente, para confirmar los resultados de aquellas muestras que hayan dado valores positivos se utilizarán metodologías confirmatorias separativas, que requieren un mayor tiempo de análisis.

APÉNDICE I

Mención "Doctorado Internacional"

Con la elaboración y defensa de esta Tesis Doctoral se pretende optar a la obtención de la mención “Doctor Internacional” en el título de Doctor por la Universidad de Salamanca, al considerar que la doctoranda reúne los requisitos para optar a dicha mención:

- La doctoranda ha realizado una estancia de tres meses en el “Environmental and Industrial Toxicology Unit, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, en Milán (Italia), relacionada con esta Tesis Doctoral.
- En esta memoria se incluye un resumen en inglés que consta de una introducción, objetivos e hipótesis de trabajo, y finalmente, conclusiones generales de la Tesis Doctoral. Así mismo, para cada trabajo publicado, se detallan los objetivos y conclusiones específicos.
- La tesis cuenta con los informes favorables de dos doctores expertos pertenecientes a dos instituciones de educación superior no españolas.
- Uno de los miembros que ha de formar parte del tribunal evaluador de la tesis es un experto perteneciente a un centro de investigación de prestigio no español.

En este apéndice, se incluyen la certificación de la estancia breve, así como los pertinentes informes favorables.

PROGRAMA DE MOVILIDAD DE PERSONAL INVESTIGADOR: MODALIDAD IVb

CERTIFICADO DEL CENTRO RECEPTOR TRAS LA ESTANCIA BREVE O TRASLADO TEMPORAL
CERTIFICATE OF STAY IN A FOREING INSTITUTION

1. Beneficiario/ *Applicant:*
Nombre y apellidos/ *Name: Patricia Martín Santos*
D.N.I./ *National identity Card: 70903279Y*
Centro de adscripción de la beca/ *Home Institución: Universidad de Salamanca*
2. Centro en el que se ha realizado la estancia/ *Host institution:*
Nombre/ *Name: Università degli Studi di Milano*
Dirección/ *Adress: Department of Clinical Sciences and Comunity Health, Via S. Barnaba, 8 20122 Milano*
Localidad/ *Country: Italy*
3. Investigador responsable en el centro de la estancia/ *Responsable person in the Host*
Institución/ *Institution: Laboratory of Environmental and Industrial Toxicology,*
Nombre/ *Name: Silvia Fustinoni*
Cargo/ *Post: Professor*

CERTIFICO:
que el becario arriba mencionado ha realizado una estancia en este centro en las siguientes fechas: desde 30 / 04 / 2019 hasta 31 / 07/ 2019

THIS IS TO CERTIFY:
that the above mentioned person has performed a stay in this Institution in the following dates: From: 30 / 04 / 2019 To: 31 / 07 / 2019

Lugar y fecha:
City and date: Milano, 2 September 2019

Firma y Sello/ *Signature & Stamp*

Silvia Fustinoni

Universita' degli Studi di Milano
Dipartimento di Scienze Cliniche e di Comunita'

PROGRAMA DE MOVILIDAD DE PERSONAL INVESTIGADOR: MODALIDAD IVb-Universidad de Salamanca

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EDIFICIO MULTIUSOS I+D+I
C/ ESPEJO Nº 2 – 1ª PLANTA. 37007 SALAMANCA

MODELO DE INFORME DE MENCIÓN DOCTOR INTERNACIONAL

STANDARD FORM FOR THE INTERNATIONAL DOCTORATE MENTION

NOMBRE Y APELLIDOS / NAME OF THE EXAMINER

TOMASZ LIGOR

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DSc, CHEMISTRY

UNIVERSIDAD U ORGANISMO Y PAÍS / UNIVERSITY OR INSTITUTION AND COUNTRY

NICOLAUS COPERNICUS UNIVERSITY, TORUŃ, POLAND

TÍTULO DE LA TESIS OBJETO DE INFORME / TITLE OF THE TESIS

Rapid Methods based on Mass-Spectrometry for the Detection and Quantification of Biomarkers

NOMBRE Y APELLIDOS DEL DOCTORANDO / NAME OF THE CANDIDATE

Patricia Martín Santos

NOMBRE Y APELLIDOS DEL DIRECTOR/A(S) DE LA TESIS / NAME OF THE THESIS DIRECTOR(S)

José Luis Pérez Pavón
Miguel del Nogal Sánchez

Página 1 de 2

INFORME RAZONADO/REASONED REPORT

SOBRE LA CALIDAD CIENTÍFICA DE LA TESIS DOCTORAL (puede añadir las hojas que crea necesarias adjuntando a este informe)
ABOUT THE SCIENTIFIC QUALITY OF PhD (you can use all the pages you might need including this form)

The presented PhD thesis is focused on the development and validation of analytical methodologies for determination of biomarkers in different biological matrices. Moreover, an application of statistical methods for data recognition and exploration is essential part of investigations. The introduction presents the general objectives and methodologies as well as describes briefly goals and tasks.

The presented PhD thesis consists of three parts which are related to different set of biomarkers and analytical techniques applied for mentioned investigations.

The aim of the first part is the development of HS-PTV-GC-MS methodology for the analysis of C3 - C9 ketones and ethyl acetate in urine samples collected from patients suffering from lung cancer and controls. The developed method has been applied in the analyses of target compounds and allows to minimize sample manipulation and reducing errors associated with sample handling. Moreover, significant differences in the concentration of ethyl acetate, 3-heptanone and 3-octanone, in case of patients with lung cancer were observed.

The second chapter focuses on the development of a non-separative LLE-PTV-MS method for the analyses of polycyclic aromatic hydrocarbons in urine and saliva samples. In general, gas chromatography and mass spectrometry (GC-MS) or liquid chromatography (HPLC) with fluorescence detection are the most frequently used techniques for the determination of PAHs in various samples. However, direct sample injection into MS via PTV and short piece of fused silica tubing is innovative way. Such non-chromatographic methodology followed by chemometric tools was applied for the determination of 11 PAHs in urine samples. Therefore, important part of this work was application multivariate calibration, unsupervised (PCA) and supervised (SIMCA, LDA, PLS-DA, SVM) pattern recognition techniques for data treatment. Hence, the developed method is very rapid and reliable and could be used as screening tool for occupational measurements. Moreover, this part presents research work regarding fully automatic method based on microextraction by packed sorbents (MEPS) for the determination of PAHs in saliva samples. The developed methodology was applied for analyses of saliva samples collected from firefighters and non-exposed volunteers. This part of the work is very important because it deals with significant analytical aspects.

The third chapter presents method for determination of amino acids in saliva samples by derivatization with ethyl chloroformate and non-separative PTV-MS analysis. Developed methodology required chemometric techniques for MS signals treatment. The results were compared with those provided by chromatographic method (LLE-PTV-GC-MS).

Additionally, a review article is included as an annex. This review discusses the sampling, preconcentration, separation and detection methods for determination of PAHs in various biological samples (urine, blood, saliva, etc.).

The aims of the work were clearly specified, and three main topics were presented. The research work brings novel and interesting remarks. PhD thesis presents an original work of high scientific and social impact. Therefore, based on its novelty and significance, I consider and recommend PhD thesis to be defended in front of a committee and is worthy of being awarded the International Doctor Mention.

Informo que la calidad científica de esta Tesis es merecedora de obtener la Mención Doctor Internacional (marcar con una cruz una opción)
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FECHA / DATE : 14.02.2020

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SELLO DE LA INSTITUCIÓN DEL FIRMANTE / THE INSTITUTION'S STAMP OF THE PERSON WHO FIRMS

Página **2** de **2**

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MODELO DE INFORME DE MENCIÓN DOCTOR INTERNACIONAL

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Full Professor, Dean of the Faculty of Sciences

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Radboud University Nijmegen, The Netherlands

TÍTULO DE LA TESIS OBJETO DE INFORME / TITLE OF THE TESIS

Rapid methods based on MS for the detection and quantification of buiomarkers

NOMBRE Y APELLIDOS DEL DOCTORANDO / NAME OF THE CANDIDATE

Patricia Martin Santos

NOMBRE Y APELLIDOS DEL DIRECTOR/A(S) DE LA TESIS / NAME OF THE THESIS DIRECTOR(S)

José Luis Pérez Pavón
Miguel del Nogal Sánchez

Página 1 de 2

INFORME RAZONADO/REASONED REPORT

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ABOUT THE SCIENTIFIC QUALITY OF PhD (you can use all the pages you might need including this form)

Patricia Martín Santos developed, validated and compared analytical methods, based on MS for biomarker analysis in different biological matrices. She investigated the sample pre-treatment and the usefulness of a separation prior to the MS analysis. As analytes, she investigated VOCs in Urine; PAH in urine and saliva and amino acids in saliva
She applied her methods to discover or suggest biomarkers for diseases (Lung cancer) or exposure markers for fireworks or smokers.
She developed novel analytical methodologies and compared and validated her work with state of the art methods. She also va lid ate her work for real life aplications and to enhance the power and applicability she also applied state of the art chemometric techniques.
She published her work in 5 research papers in peer reviewed top analytical chemistry journals. Moreover she wrote a critical review paper on the PAH Analysis in TraC, the highest impact journal in analytical chemistry.
She has clearly proven to be an independent high quality researcher and therefore deserves certainly the doctorate titel.
The only thing I am missing is an overall (short) critical summary and future outlook. For the PAH analysis part this is done outstanding through the TrAC paper but an overall future outlook would have been a nice finishing touch

Informo que la calidad científica de esta Tesis es merecedora de obtener la Mención Doctor Internacional (marcar con una cruz una opción)
I inform that the scientific quality of this thesis is worthy of obtaining the International Doctor Mention (mark with across one option)

Favorable [X] FACULTY OF SCIENCES No favorable/Not favorable [ ]

FECHA / DATE : 23-03-2020

FIRMA / SIGNATURA original.

Radboud University Nijmegen
The Netherlands

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Página 2 de 2

VERSIÓN RESUMIDA EN INGLÉS

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1 Introduction

Introduction

This Doctoral Thesis is focused on the development of new analytical methodologies, separative and non-separative, based on the use of mass spectrometry (MS) in two different modalities. In the first one, it is coupled to gas chromatography technique (GC), which allows the separation of the analytes of study prior to their detection. Thus, information is obtained on the compounds individually. Thanks to the sensibility achieved with this methodology it is possible to detect, identify and quantify compounds that are present in the matrix in very low concentrations. In the second mode, the column of the gas chromatograph acts as a mere transfer line so that the prior separation of the compounds is not carried out. In this approach a profile signal of all the studied compounds is study. Information of interest is obtained through the use of chemometric techniques. Due to the absence of chromatographic separation step, this mode allows to perform analyses faster, being possible to use it for an initial screening of samples. The use of the methodology with separation step is applied mainly for confirmation purposes.

The Doctoral Thesis has been divided into three different parts depending on the set of biomarkers studied. The first one is focused on the determination of volatile organic compounds (VOCs) in urine samples, which have been associated with different diseases, including lung cancer. The second chapter focuses on the determination of polycyclic aromatic hydrocarbons (PAHs), some of which have been classified as possible carcinogens for the human species, in urine and saliva samples. It should be noted that although these compounds have been fundamentally described as exposure markers in certain work environments, exposure to them occurs regularly in everyday environments due to diet or smoking habits. In this chapter, a review article revising works focused on the determination of polycyclic aromatic hydrocarbons in different human biological matrices is also included. The last part of the Thesis is focused on the development of methodologies for the determination of amino acids in saliva

samples. The interest that this type of compounds receives is because their concentration levels vary in the presence of certain types of diseases, including cancer. Therefore, all the parts described are focused on the development of analytical methods for the analysis of biomarkers in biological samples, such as urine and saliva.

Due to the use of various biological matrices, as well as the different nature of the analytes studied, the sample treatment steps used in this Thesis (extraction, preconcentration and obtention of derivatives) are different.

In the first chapter, the extraction step of the VOCs under study, nine ketones and ethyl acetate, in urine samples is carried out by headspace sampling technique (HS). The use of this simple extraction technique, with minimal sample manipulation, allows the analysis of volatile compounds without interference from non-volatile compounds that may be present in the matrix. In addition, it allows the automation of the process, thus reducing the errors associated with this step of the analysis.

The second part of the Thesis is focused on the determination of PAHs in urine and saliva samples. Due to the non-volatile nature of these compounds, the extraction is carried out from a different perspective than previously described. Firstly, extraction of PAHs from urine (chapter II.1.1) and saliva (chapter II.2.1) samples is performed by using liquid-liquid extraction (LLE). This technique allows to extract the analytes quickly. Secondly, a fully automatic method based on microextraction by packed sorbents (MEPS) for the determination of PAHs in saliva samples (chapter II.2.2) is developed. This technique is based on a miniaturization of the conventional solid phase extraction technique (SPE) and allows the use of small volumes of sample and organic solvents. The cartridges used to extract the analytes can be reused a large number of times.

In the third part of the Thesis, a derivatization reaction is carried out for the determination of amino acids in saliva samples to improve their chromatographic characteristics. The use of ethyl chloroformate (ECF) as derivatizing reagent allows the reaction to be carried out in the aqueous matrix to be analysed. Once the corresponding derivatives compatible with the subsequent analysis technique have been obtained, the extraction is carried out using LLE extraction.

When the methodology used is based on the modality without chromatographic separation, it is necessary to apply chemometric techniques to the profile signals obtained to extract the maximum useful information. In this Thesis, these techniques are used for quantitative and qualitative purposes. In the first case, partial least squares regression (PLS1) is used, which allows the quantification of different compounds in complex mixtures. In the second case, the profile signals obtained are studied through the use of supervised and unsupervised pattern recognition techniques. The unsupervised technique used in this Thesis for data exploration is principal component analysis (PCA). The supervised techniques employed for the discrimination of samples are soft independent modelling of class analogy (SIMCA), linear discriminant analysis (LDA), partial least squares-discriminant analysis (PLS-DA) and support vector machines (SVM).

2 Aims and working hypothesis

Aims and working hypothesis

The general aim of this Doctoral Thesis is the development and validation of analytical methodologies focused on the identification and quantification of disease or exposure markers in different biological matrices. In addition, effort will put on simplifying sample treatment step and on reducing the total time of analysis whenever possible. The proposed methodologies for quantification may be separative, those including chromatographic separation prior to detection, and non-separative, applied in those situations where the analytes of interest are present in an enough concentration to be detected without carrying out a chromatographic separation. Additionally, in some of the cases the aim is also to discriminate samples based on criteria such as concentration or presence and absence of the studied markers.

Mass spectrometry allows to significantly simplify analytical procedures due to the enormous amount of information provided. This means that the sample treatment steps can be reduced considerably. In addition, the data provided by the technique when it is not coupled to chromatographic systems allow, in many cases, the obtaining of qualitative, semi-quantitative or quantitative information to solve the analytical problem under study.

The starting hypothesis of this Thesis is the feasibility of using the coupling of a programmed temperature vaporizer to a gas chromatograph-mass spectrometer (PTV-GC-MS), or directly the coupling of a programmed temperature vaporizer with a mass spectrometer (PTV-MS) for the determination of volatile and non-volatile compounds in urine and saliva samples.

The specific aim of the first part is the development of a method based on HS-PTV-GC-MS for the quantification of ketones and ethyl acetate in urine samples. Some applications using this instrumental configuration for the analysis of different VOCs in biological matrices have been previously reported in the literature. Taking this into account, the method is expected to be appropriate when

applied to these ten compounds in urine samples. Once the methodology has been developed, the possibility of finding significant differences in the concentration of any of the compounds of interest is considered when comparing samples belonging to healthy individuals and patients with lung cancer.

The second part of this Thesis focuses on the analysis of PAHs in urine and saliva samples. Different goals are raised within it. One of them is the development of a rapid method without chromatographic separation, based on LLE-PTV-MS, for the quantification of PAHs in urine samples. In this work it is hypothesized that this methodology is applicable to the determination of semi-volatile or non-volatile compounds. The sensitivity achieved should be enough to quantify these compounds in urine samples with a concentration range similar to that usually described for occupational exposed individuals. In view of the good results described in the literature when chemometric techniques are applied to the profile signals obtained with MS, their use is here proposed for the quantification of PAHs in urine and for the discrimination of samples based on the presence or absence of PAHs.

Another goal of this second part of the Thesis is the development of separative methodologies with different extraction techniques and the comparison of the results obtained. Specifically, two methodologies were compared: **LLE**-PTV-GC-MS and **MEPS**-PTV-GC-MS. Because of the nature of the compounds that are here determined (non-polar and with low volatility) and the satisfactory results that these techniques have previously provided in the determination of similar compounds in other matrices, the methodologies are expected to be appropriate when applied to the determination of PAHs in saliva samples.

The third and last part is focused on the determination of amino acids in saliva samples. In this case, the aim is the development of a rapid and non-separative methodology in which a derivatization reaction (DER) is carried out.

Subsequently, the derivatives are analysed by LLE-PTV-GC-MS. The effectiveness of the reaction of amino and acid groups with ECF in aqueous medium has been widely described to date. For this reason, it is expected to be suitable for the quantification of amino acids in saliva samples. With this goal, chemometric techniques (PLS) will be applied to the obtained profile signals. This multivariate calibration technique has previously shown its usefulness when applied to the quantification of compounds in complex situations in which there is an overlapping in the mass spectra.

CHAPTER I

Determination of volatile organic compounds in urine samples

I.1 Research paper

Determination of ketones and ethyl acetate-a preliminary study for the discrimination of patients with lung cancer

Analytical and Bioanalytical Chemistry

409 (2017) 5689-5696

Aim

The aim of this chapter is the development of a separative methodology based on HS-PTV-GC-MS to detect and quantify ketones (acetone, 2-butanone, 2-pentanone, 4-methyl-2-pentanone, 2-hexanone, 3-heptanone, 2- heptanone, 3-octanone, and 2-nonanone) and ethyl acetate in urine samples from healthy individuals and lung cancer patients.

In order to study the possibilities of these compounds as biomarkers of lung cancer disease, the existence or absence of significant differences in their concentrations in samples from controls and patients will be studied.

Conclusions

The proposed method has been successfully applied to the quantification of ketones and ethyl acetate in urine samples.

The HS-PTV-GC-MS coupling allowed the fully automation of the process, minimizing sample manipulation and reducing errors associated with this step of the analysis.

Significant differences in the concentration of three of the ten analytes studied were observed when comparing samples from healthy individuals and patients with lung cancer: ethyl acetate, 3-heptanone, and 3-octanone. These analytes could be considered useful tools for discerning samples, enhancing the possible group of helpful biomarkers of lung cancer disease.

CHAPTER II

Determination of polycyclic aromatic hydrocarbons in biological samples

II.1 Research paper

Quantitative and qualitative analysis of polycyclic aromatic hydrocarbons in urine samples using a non-separative method based on mass spectrometry

Talanta

181 (2018) 373-379

Aim

The aim of this chapter is the development of a non-separative method based on LLE-PTV-MS to detect and quantify polycyclic aromatic hydrocarbons in urine samples. These compounds are interesting since some of them have been classified as possible or probable carcinogens to humans. They are usually studied as exposure markers in different situations, such as work environment, diet or according to smoking habits.

The profile signals obtained will be subjected to chemometric treatment using multivariate calibration (PLS1) as well as unsupervised (PCA) and supervised (SIMCA, LDA, PLS-DA, SVM) pattern recognition techniques.

Additionally, a separative methodology based on LLE-PTV-GC-MS will be used to check the results obtained by the non-separative methodology.

Conclusions

The direct coupling of a programmed temperature vaporizer with a mass spectrometer (PTV-MS) is suitable to analyse non-volatile compounds in urine samples. Specifically, this coupling has allowed the obtaining of quantitative and qualitative information about polycyclic aromatic hydrocarbons in urine samples.

Multivariate calibration has permitted the simultaneous quantification of eleven PAHs in urine samples.

The results obtained when applying principal component analysis were satisfactory, allowing us to distinguish samples with presence and absence of PAHs.

Supervised pattern recognition techniques showed excellent discrimination between samples with and without PAHs. Neither false negatives nor false positives were found, while only one sample containing PAHs was detected as an outlier when using SIMCA.

The method was rapid, simple and allowed the obtaining of quantitative and qualitative information simultaneously in the same analysis.

II.2 Research paper

Liquid-liquid extraction-programmed temperature vaporizer-gas chromatography-mass spectrometry for the determination of polycyclic aromatic hydrocarbons in saliva samples. Application to the occupational exposure of firefighters

Talanta

192 (2019) 69-78

Aim

The aim of this chapter is the determination of polycyclic aromatic hydrocarbons in saliva samples using a separative methodology based on LLE-PTV-GC-MS.

The developed methodology will be used to analyse saliva samples collected from firefighters and non-exposed volunteers.

Initially, principal component analysis will be applied to the data to explore separation between the different groups of samples studied. Then, quantification of PAHs in the saliva samples will be carried out.

Conclusions

The principal component analysis revealed discrimination between the groups of samples considered (firefighters and non-exposed volunteers).

The developed methodology allowed to quantify the concentration of PAHs in all the saliva samples analysed. Fluorene was found in all the samples, phenanthrene was found in some samples belonging to both groups, and naphthalene, acenaphthylene, anthracene, fluoranthene and pyrene were only found in samples taken from the firefighters.

The analysis of unmetabolized PAHs instead of the corresponding derivatives after biotransformation makes this methodology simple, rapid, effective and very suitable to establish previous exposure to PAHs.

II.3 Research paper

A sensitive and automatic method based on microextraction by packed sorbents for the determination of polycyclic aromatic hydrocarbons in saliva samples

Microchemical Journal

152 (2020) 104274

Aim

The aim of this chapter is the development of a method based on MEPS-PTV-GC-MS for the determination of salivary polycyclic aromatic hydrocarbons. Once validated, the methodology will be applied to the analysis of PAHs in saliva samples collected from non-smoker volunteers.

The analytical characteristics of the method will be compared with those obtained by the LLE-PTV-GC-MS method, as well as with those previously reported in bibliography.

Conclusions

The use of MEPS as extraction technique allowed the development of a fully automated methodology, thus reducing errors associated to sample preparation step.

The methodology was satisfactory applied to the quantification of PAHs in nine saliva samples. Phenanthrene was detected and quantified in one of the samples analysed.

In comparison with the methodology based on LLE extraction, the proposed method reduced the amount of organic solvent used, maintaining the sensitivity reported in the previous method. However, because of the existence of matrix effect, quantification was to be performed using the standard addition method, thus requiring higher saliva sample volumes.

II.4 Review article

Determination of polycyclic aromatic hydrocarbons in human biological samples: A critical review

Trends in Analytical Chemistry

113 (2019) 194-209

Aim

A review focused on the determination of polycyclic aromatic hydrocarbons, both unmetabolized and metabolized, in human biological samples is conducted.

In this work, the matrices revised will be urine, blood, plasma, serum, saliva, exhaled breath, hair, follicular fluid, placenta, breast milk and cerebrospinal fluid.

From a critical perspective, advantages and disadvantages will be discussed regarding sampling methods, preconcentration procedures and different strategies of separation and detection of PAHs. In addition, works without a separation step of the analytes before detection will be reviewed.

Conclusions

The methodologies available in the literature for the determination of PAHs in human biological samples have been reviewed from a critical perspective, with special attention on extraction procedures and separation and detection methods.

Among the human biological matrices revised, there is a preference for the use of those corresponding to non-invasive sampling. However, the aim of the study must be established in advance, since it can lead to the selection of the appropriate matrix. Particularly, urine is frequently used when determining metabolites of PAHs, while blood, saliva and exhaled breath are more related to unmetabolized PAHs after recent exposure. For long-term exposure studies, the matrix commonly used is hair.

Regarding the extraction techniques reviewed, the best results were provided by those with direct contact between the analytes and a fiber or a solvent.

Finally, the analytical methodologies reviewed were based on separative methods in most cases. The methods based on gas chromatography allowed the analysis of a greater number of analytes simultaneously. On the contrary, some works based on liquid chromatography required the use of different detection techniques due to the lack of response of the PAHs of interest, thus increasing the analysis time. The best results in terms of sensitivity and selectivity were achieved by using mass spectrometry.

In the case of urine and saliva, methods without separation prior to detection have also been proposed. These methods provided a quick response with less information than separative methods. However, this information, generally subjected to chemometric techniques, was enough to solve the problem under study. These non-separative methods were generally used for screening

when analysing a large number of samples. Subsequently, only those samples with concentrations considered as anomalous were analysed using a separative methodology for confirmation purposes. This way, the number of samples that had to be analysed with the separative methods was reduced, and with that, the time of analysis.

In conclusion, when determining PAHs in human biological matrices, it is necessary to establish the type of exposure, the type of analytes, the desired sensitivity and the analysis time, among others, in order to select the most appropriate methodology to be used.

CHAPTER III

Determination of amino acids in saliva samples

III.1 Research paper

Non-separative method based on a single quadrupole mass spectrometer for the semi-quantitative determination of amino acids in saliva samples. A preliminary study

Talanta

208 (2020) 120381

Aim

In this chapter, the analytical possibilities of a non-separative methodology based on LLE-PTV-MS for the simultaneous quantification of amino acids in saliva samples are explored. The interest of these compounds lies in the fact that their concentration may vary in the presence of different diseases.

Derivatization in aqueous medium (saliva sample) will be addressed through the use of ethyl chloroformate as derivatizing reagent.

Quantification will be performed by multivariate calibration (PLS1) using the profile signals obtained in the analysis of saliva samples.

The results obtained will be checked by comparison with those provided by a separative methodology based on LLE-PTV-GC-MS.

Conclusions

It can be concluded that the DER-LLE-PTV-MS methodology proposed offers quantification possibilities for complex mixtures of amino acids without any separation step.

After constructing the PLS models in different saliva samples, thus compensating the possible existence of matrix effect, the methodology was applied to the quantification of sixteen amino acids in a group of saliva samples external to those included in the models.

The results obtained were good enough even though there is an important overlapping in the mass spectra of the corresponding derivatives of amino acids.

3 General conclusions

General conclusions

In general, it can be concluded that the main aim proposed in this Doctoral Thesis has been achieved, since new analytical methodologies based on mass spectrometry have been satisfactorily developed. In them, the sample treatment is simple and generally fast, which reduces the errors associated with this step of the analytical process. In addition, its applicability has been demonstrated through the resolution of problems of interest related to the biological field.

In the first chapter, the proposed methodology was satisfactory employed to the determination of ketones and ethyl acetate in urine samples. Comparison between concentrations found in urines from healthy volunteers and patients with lung cancer allowed to find significant differences for ethyl acetate, 3-heptanone and 3-octanone. These compounds may be useful biomarkers to classify future unknown samples.

In the second chapter, focused on polycyclic aromatic hydrocarbons, different methodologies were used to quantify these compounds in urine and saliva samples. Both separative and non-separative methodologies were developed and compared. While separative methods provided better results in terms of sensitivity, the time required for analysis was significantly reduced when eliminating the separation step in the non-separative approach.

The developed methods were satisfactory applied to the quantification of PAHs in urine and saliva samples.

The use of pattern recognition techniques and multivariate calibration for the treatment of the profile signals obtained by the non-separative method (PTV-MS) represents a simple and effective solution for the detection and quantification of PAHs in urine samples.

When analysing PAHs in saliva, two separative methodologies based on LLE-PTV-GC-MS and MEPS-PTV-GC-MS were developed and compared. MEPS methodology allowed the automation of the process, as well as a reduction in the volume of organic solvent used, maintaining the sensitivity obtained with the LLE methodology. However, in contrast to the LLE methodology, the time required for extraction was high and the existence of matrix effect forced the samples to be quantified by the standard addition procedure, requiring the use of larger volumes of saliva.

Finally, a review article focused on the determination of PAHs in different human biological samples was included in this chapter. It can be concluded that the most appropriate methodology will be that which allows an adequate determination meeting the desired requirements, such as the type of exposure, the type of analytes studied, the sensitivity and the time of analysis, among others.

In the third chapter, a non-separative method has been successfully applied to the rapid detection and quantification of amino acids in saliva samples. The results obtained provided information about the simultaneous semi-quantification of these compounds in complex situations, when there is overlapping in their mass spectra. The use of a derivatization reaction based on ethyl chloroformate as derivatizing reagent makes it possible to expand the group of compounds analysed using GC.

In general, non-separative methodologies are an alternative to the usual methodologies, being used as screening in the analysis of a large number of samples. Subsequently, separative confirmatory methodologies, which usually require a longer time for analysis, would be used only in those cases in which anomalous concentrations of the analytes studied were found.