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Miniaturization of sample preparation techniques as an essential strategy for the analysis of low-volume and complex samples

Cristian Azorín Luis

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Supervisors:

Alberto Chisvert Sanía

Juan Luis Benedé Veiga

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Working hard is important, but there is something that matters even more, believing in yourself

– J. K. Rowling –

Yo solo sé hacerlo, yo no sé intentarlo

– Bad Gyal –

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LIST OF ABBREVIATIONS

μ	μEME	Micro-electromembrane extraction
	μSPE	Micro-solid-phase extraction
	μTAS	Micro-total analytical systems
A	AALLME	Air-assisted liquid-liquid microextraction
	AGREE	Analytical GREEnness Metric Approach
	AGREEprep	Analytical GREEnness Metric for Sample Preparation
	AIBN	2,2'-azobis(2-methylpropionitrile)
B	BAμE	Bar adsorptive microextraction
	BAGI	Blue Applicability Grade Index
C	CBD	Cannabidiol
	CFME	Continuous flow microextraction
	CNT	Carbon nanotube
	COF	Covalent organic framework
	CQD	Carbon quantum dot
D	DμSPE	Dispersive micro-solid-phase extraction
	DD-SDME	Drop-to-drop single drop microextraction
	DES	Deep eutectic solvent
	DI	Direct immersion
	DLLME	Dispersive liquid-liquid microextraction
	DPX	Dispersive-pipette extraction
	DSDME	Directly suspended droplet microextraction
	DSPE	Dispersive solid-phase extraction
	DVB	Divinylbenzene
E	EDS	Energy dispersive X-ray spectroscopy

E	EE	Extraction efficiency
	EF	Enrichment factor
	EME	Electromembrane extraction
	ESI	Electrospray ionization
F	FPSE	Fabric phase sorptive extraction
G	GAC	Green Analytical Chemistry
	GAPI	Green Analytical Procedure Index
	GC	Gas chromatography
	GSP	Green Sample Preparation
H	HF-LPME	Hollow-fiber liquid phase microextarction
	HS	Headspace
	HSSE	Headspace sorptive extraction
I	I.D.	Internal diameter
	IL	Ionic liquid
	ISFME	*In-situ* solvent formation microextraction
	IT-SPME	In-tube solid-phase microextraction
	IUPAC	International Union of Pure and Applied Chemistry
L	LC	Liquid chromatography
	LLE	Liquid-liquid extraction
	LOD	Limit of detection
	LOQ	Limit of quantification
	LPME	Liquid-phase microextraction
M	MALLME	Microwave-assisted liquid-liquid microextraction

	MEPS	Microextraction by packed sorbent
	M-HS-AME	Magnetic headspace adsorptive microextraction
	MIL	Magnetic ionic liquid
	MIP	Molecularly imprinted polymer
	MLOD	Method limit of detection
	MLOQ	Method limit of detection
M	MNP	Magnetic nanoparticle
	MOF	Metal-organic framework
	MRM	Multiple reaction monitoring
	MS	Mass spectrometry
	M-SA-DSPE	Magnetic-based solvent-assisted dispersive solid-phase extraction
	mSBSDME	Miniaturized stir bar sorptive dispersive microextraction
	MSPD	Matrix solid-phase dispersion
	NADES	Natural deep eutectic solvent
N	NQS	Need, Quality and Sustainable index
	NVP	N-Vinylpyrrolidone
	Pa-EME	Parallel electromembrane extraction
	Pa-DDE	Parallel-dispersive droplet extraction
P	PALME	Parallel artificial liquid membrane extraction
	PIL	Polymeric ionic liquid
	PT-µSPE	Pipette tip micro-solid-phase extraction
	RAM	Restricted access material
	RDSE	Rotating-disk sorptive extraction
R	RGB	Red-Green-Blue
	RR	Relative recovery

R	RSD	Relative standard deviation
	RSM	Response surface methodology
S	SBME	Solvent bar microextraction
	SBSDME	Stir bar sorptive dispersive microextraction
	SBSE	Stir bar sorptive extraction
	SCSE	Stir-cake sorptive extraction
	SDME	Single drop microextraction
	SFOD	Solidification of floating organic drop
	SHS	Switchable hydrophobicity solvent
	SIM	Selected ion monitoring
	SLM	Supported liquid membrane
	SME	Stir membrane extraction
	SPE	Solid-phase extraction
	SPME	Solid-phase microextraction
	SPMS	Sample Preparation Metric of Sustainability
	SRSE	Stir-rod sorptive extraction
	SUPRA	Supramolecular solvent
T	TFME	Thin film microextraction
	THC	Δ^9-Tetrahydrocannabinol
U	UAE	Ultrasound-assisted extraction
	USAEME	Ultrasound-assisted emulsification-microextraction
	UV	Ultraviolet spectroscopy
V	VAE	Vortex-assisted extraction
	VALLME	Vortex-assisted liquid-liquid microextraction

V	VOC	Volatile organic compound
W	WAC	White Analytical Chemistry

RESUMEN

Introducción

La concienciación y preocupación por los problemas ambientales se ha extendido durante las últimas décadas a todas las áreas, incluyendo la Química Analítica. En este sentido, una de las tendencias de esta área de conocimiento es, en términos generales, eliminar o reducir el uso de sustancias químicas, minimizar el consumo energético, llevar a cabo una gestión adecuada de los residuos, aumentar la seguridad para el operador y conseguir métodos más automatizados y portátiles, tal y como se recoge en los principios de la llamada "Química Analítica Verde". No obstante, estos aspectos deben estar en consonancia con unos parámetros analíticos adecuados (sensibilidad, exactitud, precisión, etc.) y con aspectos prácticos como el coste económico, la utilidad y la ergonomía.

La **miniaturización** en Química Analítica implica reducir el tamaño de los elementos implicados en cada etapa del proceso analítico, incluida la preparación de la muestra, las técnicas de separación, si las hubiera, y la detección. Como resultado, se han descrito enfoques miniaturizados con cantidades de muestra y disolvente reducidas hasta el nivel de nanolitros. Los métodos miniaturizados implican numerosas ventajas como el consumo reducido de reactivos, disolventes y muestra; la generación de menos residuos; una mayor sensibilidad, rapidez, facilidad de portabilidad y automatización; y un menor consumo energético.

A pesar de que el objetivo final de la miniaturización es reducir la escala de todo el proceso analítico, el concepto debe considerarse desde una perspectiva más amplia. En este sentido, cualquier avance en la miniaturización de etapas individuales en el proceso analítico, como la preparación de muestra, contribuye al avance hacia sistemas analíticos miniaturizados con las ventajas que estos implican.

Cabe destacar que, aunque la preparación de muestra se considera una de las etapas más críticas y lentas dentro del proceso analítico, en muchas ocasiones es inevitable. Por ejemplo, en el análisis de trazas, en la mayoría de los casos se requieren procedimientos de preparación de muestra para separar los analitos de las posibles sustancias interferentes, para concentrarlos y/o para acondicionar la muestra al instrumento analítico.

Las metodologías clásicas de extracción (es decir, la extracción en fase sólida (SPE, del inglés *solid-phase extraction*) y la extracción líquido-líquido (LLE, del inglés *liquid-liquid extraction*)) están siendo gradualmente reemplazadas por las **técnicas de microextracción** desde su aparición a principios de los años 90. Estas técnicas de microextracción, ya sean basadas en fases de extracción sólidas (sorbentes) o líquidas (disolventes), siguen los mismos principios fundamentales que la SPE y LLE convencionales, respectivamente, pero las

cantidades de sorbentes y disolventes han sido considerablemente reducidas. Desde su aparición, los esfuerzos se han centrado no solo en el desarrollo de nuevas técnicas de microextracción, sino también en el uso de nuevos materiales de extracción con el objetivo de obtener un mayor rendimiento. Además, las técnicas de microextracción han avanzado a lo largo de las décadas, adaptándose a las tendencias y necesidades del momento y dando lugar a una numerosa variedad de técnicas tanto en fase sólida como líquida.

Por un lado, las **técnicas de microextracción basadas en sorbentes** implican el uso de materiales sólidos como fase extractante, lo que ofrece varias ventajas sobre las técnicas basadas en disolventes, que se discuten más adelante. Una de las principales ventajas es la amplia gama de tipos de sorbentes disponibles, lo que permite una mayor versatilidad en al etapa de extracción en función de los compuestos a determinar. Entre los materiales disponibles, los polímeros son los más utilizados, pero existen numerosas alternativas como las redes metal-orgánicas (MOFs, del inglés *metal-organic frameworks*), las redes orgánicas covalentes (COFs, del inglés *covalent organic frameworks*) o varios alótropos de carbono (por ejemplo, grafito, grafeno, nanotubos de carbono y puntos cuánticos de carbono), entre otros. Entre todos los materiales sólidos, los de tamaño nanométrico han ganado un interés significativo debido a su gran área superficial, que permite una mayor eficacia y cinética de extracción, lo que conduce a tiempos de análisis más cortos. Además, la combinación de estos materiales con nanopartículas magnéticas (MNPs, del inglés *magnetic nanoparticles*) da lugar a (nano)materiales magnéticos, que han sido ampliamente utilizados en las técnicas de microextracción por su fácil separación de la muestra/disolvente mediante la aplicación de campos magnéticos proporcionados por imanes.

Las técnicas de microextracción basadas en sorbentes se pueden clasificar como variantes de cuatro técnicas principales: la microextracción en fase sólida (SPME, del inglés *solid-phase microextraction*), la extracción por sorción sobre barra agitadora (SBSE, del inglés *stir bar sorptive extraction*), la extracción en fase sólida dispersiva (DSPE, del inglés *dispersive solid-phase extraction*) y la micro-extracción en fase sólida* (μSPE, del inglés *micro-solid-phase extraction*).

Cabe destacar en el contexto de la presente Tesis Doctoral una técnica de microextracción híbrida basada en la dispersión de materiales magnéticos como fase de extracción, denominada microextracción dispersiva por sorción sobre barra agitadora (SBSDME, del inglés *stir bar sorptive dispersive microxtraction*), desarrollada hace unos años por el grupo de investigación en el que se ha desarrollado la presente Tesis Doctoral. En esta técnica, se añade un material

* No debe confundirse micro-extracción en fase sólida (μSPE) con microextracción en fase sólida (SPME). Debido a la traducción al español, la diferencia entre ambas técnicas no queda debidamente evidenciada. En el **Capítulo 1** se explican en mayor detalle y se evidencia su diferencia.

sorbente con propiedades magnéticas a un vial que contiene la disolución a extraer y una barra magnética de neodimio de tal manera que el material es atraído por el imán. A una velocidad de agitación suficientemente elevada, el material se libera y se dispersa en la disolución, aumentando así el área de contacto entre la disolución y el material. Al detener la agitación, la barra agitadora de neodimio recupera rápidamente el sorbente magnético que contiene los analitos extraídos. A continuación, la barra de agitación recubierta con el material magnético se retira de la solución de muestra y se sumerge en otro vial con el disolvente de desorción adecuado para proceder a la desorción de los analitos para su posterior media en el correspondiente instrumento analítico.

Por otro lado, las **técnicas de microextracción basadas en disolventes**, a menudo denominadas microextracción en fase líquida (LPME, del inglés *liquid-phase microextraction*), se han convertido en una de las áreas de investigación y desarrollo más importantes en el campo de la preparación de muestra debido a su compatibilidad habitual con la mayoría de los instrumentos analíticos. De manera análoga a las técnicas basadas en sorbentes, la selección del disolvente adecuado como fase de extracción es crucial y deben tenerse en cuenta numerosas características fisicoquímicas, como la densidad, la temperatura de ebullición, el coeficiente de reparto octanol/agua, la viscosidad y la tensión superficial. Los disolventes clásicos empleados en LLE (acetato de etilo, hexano, cloroformo o diclorometano, entre otros) han sido reemplazados durante los últimos años por alternativas más ecológicas con el fin de ser menos perjudiciales para el medio ambiente y el investigador. En este sentido, han surgido distintos disolventes alternativos, como los disolventes de hidrofobicidad conmutable (SHSs, del inglés *switchable hydrophobicity solvents*), los líquidos iónicos (ILs, del inglés *ionic liquids*), los disolventes eutécticos profundos (DESs, del inglés *deep eutectic solvents*) y los disolventes supramoleculares (SUPRAs).

Las técnicas de microextracción basadas en disolventes se pueden clasificar como variantes de tres técnicas principales: la microextracción en gota (SDME, del inglés *single drop microextraction*), la microextracción en fase líquida con fibra hueca (HF-LPME, del inglés *hollow-fiber liquid-phase microextraction*) y la microextracción líquido-líquido dispersiva (DLLME, del inglés *dispersive liquid-liquid microextraction*).

La simplificación y sostenibilidad de la etapa de preparación de muestra es uno de los ejes en los que se ha volcado la comunidad analítica en las últimas décadas. Es por esto que estas técnicas de microextracción, ya miniaturizadas de por sí, han evolucionado hacia enfoques todavía más miniaturizados que intentan explotar sus beneficios y, si es posible, mejorar el rendimiento analítico. Sin embargo, es cierto que, a lo largo de los años, se han realizado numerosas mejoras respecto a los primeros enfoques de microextracción, prestando especial atención a la introducción de nuevas fases de extracción y a la

reducción de sus cantidades, pero no tanto a la reducción de los volúmenes de muestra. Esto puede suponer serios problemas para muestras valiosas y/o escasas, donde no se dispone de grandes volúmenes como algunos fluidos biológicos, ya sea por la naturaleza de la muestra en sí (por ejemplo, saliva, semen, fluido folicular o fluido cefalorraquídeo), las características de la persona en estudio (por ejemplo, recién nacidos, pacientes inmunodeprimidos o anémicos) o el contexto en sí (por ejemplo, restos en análisis forense).

Entre las posibilidades para lograr métodos más ecológicos se han realizado grandes esfuerzos por utilizar alternativas menos nocivas que las fases de extracción convencionales (más tóxicas y contaminantes); introducir nuevos materiales (líquidos o sólidos) con características específicas para aumentar la selectividad del análisis; y/o reducir sus cantidades. En muchos casos, esta reducción de la cantidad de fase extractante es el resultado de la reducción de la escala del sistema. Además, resulta evidente que es más fácil y asequible reducir la fase de extracción cuando se trata de una fase líquida, como es el caso de las plataformas microfluídicas, que si se trata de una fase sólida. Aunque la mayoría de los dispositivos microfluídicos dedicados a LPME funcionan bajo flujo laminar, la transferencia de masa se acelera debido a la corta distancia de difusión y a la alta relación superficie/volumen de los microcanales. Por lo tanto, los dispositivos miniaturizados en chip proporcionan un alto rendimiento analítico, mientras que se consumen volúmenes de muestra y disolventes mucho menor. A diferencia de las técnicas de LPME no miniaturizadas, donde las altas proporciones de volumen entre la fase dadora y aceptora proporcionan altos factores de preconcentración, en los dispositivos microfluídicos, donde las relaciones de volumen son más pequeñas, se pueden lograr factores de preconcentración más altos aumentando la relación de flujo de la muestra respecto al flujo del aceptor.

Por otro lado, vale la pena mencionar aquellos casos en los que el volumen de la muestra se reduce drásticamente a unos pocos microlitros, lo que es especialmente ventajoso para muestras con baja disponibilidad. Hay que tener en cuenta que, si bien es cierto que el uso de grandes volúmenes de muestra generalmente permite mayores factores de preconcentración, en el caso de utilizar volúmenes de muestra pequeños similares a los utilizados para la fase de extracción, la microextracción jugaría un papel de limpieza más que de preconcentración. De todos modos, esta será una tendencia importante a seguir en los próximos años, marcando el comienzo de la próxima generación de enfoques de preparación de muestra miniaturizados.

Cabe mencionar que a lo largo de las décadas se han hecho grandes avances en el campo de la microextracción y muchos de los enfoques descritos hoy en día son versiones mucho más reducidas en comparación con las técnicas de microextracción primigenias. Por ello, recientemente y en el contexto de la presente Tesis Doctoral se ha propuesto acuñar como técnicas de

"ultramicroextracción" a todos aquellos procedimientos de nueva generación concebidos como enfoques miniaturizados de las técnicas de microextracción, ya miniaturizadas de por sí, que se centran no sólo en minimizar las cantidades de la fase de extracción, sino también en reducir la cantidad de muestra, y cumplir con otros propósitos de la "Química Analítica Verde", como la reducción de la generación de residuos, la portabilidad, etc. De esta manera, se evidencia adecuadamente la evolución de las técnicas de tratamiento de muestra, ya que no sería justo categorizar todos estos nuevos enfoques a escala reducida de la misma manera que los anteriores.

Como se ha indicado anteriormente, la principal fuerza impulsora de este esfuerzo de la comunidad analítica es comprometerse con los principios de la "Química Analítica Verde". En consonancia con esta tendencia, en las últimas dos décadas, se han descrito diferentes herramientas métricas para evaluar la ecología de los métodos analíticos. Algunos ejemplos son "Analytical Eco-Scale", "AGREE" o "GAPI", entre otros. Estas herramientas dan una idea general de cuán de acuerdo con los principios de la "Química Analítica Verde" es un método y, por tanto, proporcionan una estimación gráfica y/o numérica, de tal manera que pueden guiar a los investigadores hacia prácticas más ecológicas. Cada una de ellas adopta sus propias reglas y consideraciones para evaluar el impacto medioambiental de una metodología. En términos generales, cabe destacar que cada herramienta presenta ventajas e inconvenientes y es más apropiada que otra en ciertos contextos, pero ninguna es universal. En lo que todas coinciden es en la importancia de la miniaturización y sus ventajas asociadas.

En resumen, a pesar de que se han alcanzado grandes logros en este campo, como las técnicas de microextracción basadas en microfluídica y chips, algunos aspectos del proceso analítico aún pueden necesitar algunas mejoras. Especialmente, algunos pasos, como la recuperación de disolvente o sorbente en técnicas dispersivas, suelen ser las principales limitaciones para la automatización y los mayores grados de miniaturización, y requieren, por tanto, de mayor investigación.

Objetivos y estructura

Los **objetivos** de esta Tesis Doctoral, enfocada a superar los nuevos retos en la preparación de muestra, son:

- La explotación de las ventajas ofrecidas por las técnicas de microextracción para la determinación de sustancias de interés en matrices complejas.
- La miniaturización y adaptación de técnicas de microextracción para el análisis simultáneo de varias muestras, sean complejas y/o de bajo volumen.

La presente Tesis Doctoral se ha dividido en tres secciones, que contienen un total de seis capítulos y las conclusiones generales.

La **SECCIÓN 1** está constituida por los **Capítulos 1** y **2**. En el **Capítulo 1** se describen brevemente los conceptos de miniaturización en Química Analítica y se describen y clasifican las técnicas de microextracción más relevantes. A continuación, en el **Capítulo 2**, se presenta una visión general de los avances hacia un mayor grado de miniaturización de las técnicas de microextracción, con especial énfasis en aquellas en las que se utilizan volúmenes de muestra reducidos (generalmente ≤ 1 mL). Este capítulo no proporciona una revisión exhaustiva de todas las aplicaciones miniaturizadas, sino una descripción general de los desarrollos y tendencias en el campo.

La **SECCIÓN 2** está constituida por los **Capítulos 3** a **6**. Estos capítulos están dedicados a la presentación y discusión de la metodología y los resultados obtenidos durante el desarrollo de las estrategias analíticas presentadas en esta Tesis Doctoral. Así, el **Capítulo 3** hace uso de la SBSDME para la determinación de trazas de Δ^9-tetrahidrocannabinol (THC, componente psicoactivo del cannabis) en productos cosméticos que contienen ingredientes derivados de la planta del cáñamo. En los **Capítulos 4** y **5**, se diseñan versiones miniaturizadas y de alta productividad de la SBSDME para analizar un mayor número de muestras simultáneamente. Para demostrar la aplicabilidad de estas estrategias, se han aplicado al análisis de muestras biológicas. En concreto, el **Capítulo 4** está dedicado a la determinación de cortisona y cortisol en saliva como biomarcadores de diversas enfermedades, y en el **Capítulo 5** se determinan dos cannabinoides presentes en la saliva como indicativos del consumo de cannabis. A continuación, en el **Capítulo 6**, se adapta y miniaturiza una técnica para la determinación de compuestos volátiles como biomarcadores de cáncer de pulmón en muestras de saliva.

Por último, en la **SECCIÓN 3** se describen las conclusiones generales derivadas de esta Tesis Doctoral.

En las páginas finales se incluyen dos anexos en los que se describen los modelos de diseño experimental utilizados a lo largo de los capítulos (**Anexo I**), y las publicaciones científicas derivadas de esta Tesis Doctoral (**Anexo II**).

Metodología y resultados

Capítulo 3. Determinación de trazas de tetrahidrocannabinol en productos cosméticos mediante microextracción dispersiva por sorción sobre barra agitadora seguida de cromatografía de líquidos acoplada a espectrometría de masas en tándem.

En este capítulo se presenta un método analítico para la determinación de THC a nivel de trazas en productos cosméticos. Como compuesto psicoactivo, se debe evitar la presencia de THC en los productos de consumo. Sin embargo, podría estar presente involuntariamente en productos ricos en cannabidiol o a base de cáñamo por contaminación durante el proceso de fabricación o isomerización del cannabidiol.

Debido a las bajas concentraciones esperadas y a la complejidad de las matrices cosméticas, es necesario un método sensible y selectivo para el control analítico de estos productos. En este sentido, el método presentado se basa en SBSDME seguida de cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS). En este capítulo, se empleó un material magnético compuesto de MNPs de $CoFe_2O_4$ incrustadas en un polímero comercial de fase reversa (Strata™-X-RP) como sorbente aprovechando su afinidad con el analito.

En las condiciones optimizadas, el método fue validado y mostró buenas características analíticas en términos de linealidad (al menos hasta 10 ng mL^{-1}), límites de detección y cuantificación (2.2 y 7.2 ng g^{-1}, respectivamente) y precisión (desviación estándar relativa < 10%). Además, se obtuvieron coeficientes de recuperación relativa entre el 99 y el 109%, lo que demuestra que el efecto matriz fue corregido utilizando THC deuterado como patrón subrogado.

Este nuevo enfoque se aplicó con éxito a diez muestras cosméticas disponibles comercialmente de diferentes matrices, demostrando así que es adecuado para el control analítico del THC en productos cosméticos. La metodología propuesta supera algunos de los inconvenientes de los trabajos anteriores con el mismo propósito, como los límites de detección más altos, los procedimientos que consumen una gran cantidad de tiempo y el consumo de grandes volúmenes de disolventes orgánicos.

Capítulo 4. Microextracción dispersiva por sorción sobre barra agitadora miniaturizada como un enfoque factible y de alta productividad para la preparación de muestras de baja disponibilidad. Determinación de cortisona y cortisol en saliva como prueba de concepto.

En este capítulo se presenta la miniaturización de la SBSDME para el análisis de muestras de baja disponibilidad. Esta nueva metodología se basa en los principios de la SBSDME, pero la cantidad de sorbente y, lo que es más importante, la cantidad de muestra se reducen considerablemente a una pequeña cantidad y unos pocos microlitros, respectivamente. En este sentido, se utilizaron insertos de vidrio de base plana de 400 µL e imanes de neodimio en forma de barra (3 mm de longitud x 2 mm de diámetro) como dispositivos de

extracción en lugar de los viales de 40 mL e imanes de 10 mm de longitud y 3 mm de diámetro empleados en la SBSDME convencional. Estos se situaron en un montaje para extracción múltiple diseñado específicamente para ello, que consta de una placa de agitación de alta velocidad y un soporte impreso en 3D que posibilita tratar 15 muestras simultáneamente. Este nuevo enfoque permite un análisis rápido, asequible, portátil y de alta productividad de muestras de bajo volumen, ampliando el potencial de la técnica. Se utiliza el mismo dispositivo de extracción a lo largo de las diferentes etapas, evitando así las transferencias, lo que reduce la manipulación de las muestras. Además, la reducción de las cantidades de muestra, sorbente y disolventes orgánicos permite una disminución considerable de la generación de residuos, consiguiendo una preparación de muestra más ecológica para el bioanálisis.

Como prueba de concepto de esta nueva metodología, se determinó cortisona y cortisol en saliva humana utilizando un material compuesto por un polímero de fase reversa (Strata^{TM}-X-RP) y MNPs de $CoFe_2O_4$. Se utilizó LC-MS/MS para medir ambos analitos obteniendo buenas características analíticas en términos de linealidad ($R^2 > 0.997$), límites de detección y cuantificación del método (22.6 y 75.5 ng L^{-1} para cortisona, y 19.3 y 64.3 ng L^{-1} para cortisol, respectivamente), precisión (desviación estándar relativa ≤ 11%) y coeficientes de recuperación relativa (78-134%).

Además, el método propuesto fue comparado con otros métodos previos basados en extracción para la determinación de cortisona y cortisol en muestras de saliva. El método propuesto presenta características analíticas comparables o mejores en términos de factor de preconcentración y límites de detección, empleando un volumen de muestra considerablemente reducido y, al igual que el resto de las técnicas dispersivas, el tiempo de extracción es bajo. En cuanto a la evaluación del impacto ambiental ocasionado desde el punto de vista de la preparación de muestra, obtuvo mayores puntuaciones, principalmente debido al bajo consumo de disolventes y muestra.

Capítulo 5. Una plataforma miniaturizada de alta productividad de 96 posiciones para la microextracción dispersiva basada en sorbentes: aplicación a la determinación de tetrahidrocannabinol y cannabidiol en saliva de fumadores de cannabis

En este capítulo se ha tomado como punto de partida la microextracción dispersiva por sorción sobre barra agitadora miniaturizada (mSBSDME, del inglés *miniaturized stir bar sorptive dispersive microxtraction*) desarrollada en el **Capítulo 4**. Así, se ha desarrollado una plataforma de extracción de 96 posiciones utilizando una placa de agitación de 96 posiciones y un soporte impreso en 3D diseñado a medida para ubicar los dispositivos de extracción miniaturizados, logrando una estrategia de preparación de muestra

miniaturizada de alta productividad. Con el fin de demostrar la aplicabilidad de esta novedosa plataforma, se ha llevado a cabo la determinación de THC y cannabidiol (CBD) en saliva humana y se ha aplicado a muestras recogidas tras el consumo de marihuana y cannabis legal rico en CBD. Solo se necesitaron 100 µL de saliva para el análisis y se lograron buenas características analíticas en términos de linealidad (al menos hasta 500 ng mL^{-1}), límites de detección (0.7 y 2.8 ng mL^{-1} para THC y CBD, respectivamente) y precisión (desviación estándar relativa ≤ 14%).

La miniaturización del dispositivo permite el uso de pequeños volúmenes de muestra (unos pocos microlitros) y el tratamiento de 96 muestras en paralelo, siendo la primera propuesta para llevar a cabo la microextracción dispersiva basada en sorbentes bajo el concepto de 96 pocillos. Además, este nuevo flujo de trabajo contribuye al desarrollo de métodos analíticos que cumplen con los tres pilares de la sostenibilidad, es decir, la ecología, la economía y la aplicabilidad.

Capítulo 6. Determinación de hexanal y heptanal en muestras de saliva mediante microextracción magnética por sorción en espacio de cabeza adaptada para el diagnóstico de cáncer de pulmón.

En este capítulo se presenta por primera vez un método analítico para la determinación de dos aldehídos endógenos (hexanal y heptanal) como biomarcadores de cáncer de pulmón en muestras de saliva.

El método se basa en una modificación de la microextracción magnética por sorción en espacio de cabeza (M-HS-AME, del inglés *magnetic headspace adsorptive microextraction*) seguida de cromatografía de gases acoplada a espectrometría de masas (GC-MS). Para ello, se utiliza un campo magnético externo generado por un imán de neodimio para mantener el sorbente magnético (MNPs de $CoFe_2O_4$ incrustadas en un polímero de fase reversa (Strata™-X-RP)) en el espacio de cabeza de un microtubo de centrífuga para extraer los aldehídos volatilizados. Posteriormente, los analitos se desorben en el disolvente adecuado y el extracto se inyecta en el sistema GC-MS para su separación y determinación. La metodología propuesta fue comparada con estrategias previas basadas en M-HS-AME, obteniendo resultados comparables en términos de parámetros analíticos e impacto medioambiental del método, pero reduciendo considerablemente el volumen de muestra necesario.

En las condiciones optimizadas, el método fue validado y mostró buenas características analíticas en términos de linealidad (al menos hasta 50 ng mL^{-1}), límites de detección (0.22 y 0.26 ng mL^{-1} para hexanal y heptanal, respectivamente) y precisión (desviación estándar relativa ≤ 12%). Este nuevo enfoque se aplicó con éxito a muestras de saliva de voluntarios sanos y de

pacientes diagnosticados de cáncer de pulmón, obteniéndose diferencias notables entre ambos grupos.

Estos resultados revelan la perspectiva del método como una herramienta potencial para el diagnóstico del cáncer de pulmón mediante el análisis de saliva. Este trabajo contribuye al campo de la Química Analítica presentando una doble novedad: por un lado, se propone sin precedentes el uso de M-HS-AME en bioanálisis, ampliando así el potencial analítico de esta técnica y, por otro lado, se realiza por primera vez la determinación de hexanal y heptanal en muestras de saliva.

Conclusiones generales

Como eje principal de la presente Tesis Doctoral, la SBSDME ha sido modificada, partiendo de su configuración inicial en el **Capítulo 3** y evolucionando a través de los **Capítulos 4** y **5** hacia un enfoque más miniaturizado y de mayor productividad. En el **Capítulo 6**, otra técnica completamente distinta (M-HS-AME) se ha adaptado y miniaturizado para la determinación de analitos volátiles. En todos ellos se utilizan como material sorbente (nano)composites magnéticos compuestos por MNPs y un polímero de equilibrio hidrofílico-lipofílico (ya sea comercial o sintetizado en el laboratorio), aprovechando las interacciones hidrofóbicas, π-π o de enlaces de hidrógeno con los analitos para su extracción, y el magnetismo conferido por las MNPs. Con esta evolución, la aplicabilidad de la SBSDME y la M-HS-AME se ha ampliado, disponiendo de configuraciones alternativas a elegir en función de la aplicación a la que vayan destinadas. Si se dispone de grandes volúmenes de muestra y se necesitan factores de preconcentración muy altos, la configuración original es perfectamente adecuada. Por el contrario, si el volumen de muestra está, de alguna manera, limitado y/o es necesario tratar un gran número de muestras simultáneamente, las versiones miniaturizadas de alta productividad constituyen herramientas más adecuadas.

La miniaturización implica también una mejora en el impacto medioambiental del método, la cual se consigue a través de la reducción del consumo de reactivos tóxicos, muestra y energía, la reducción de los residuos generados y el aumento del número de muestras tratadas por unidad de tiempo, y de la portabilidad. Sin embargo, todavía es necesario mejorar aspectos importantes como la automatización, la aplicabilidad *in situ* o la reutilización de materiales, por lo que estos aspectos constituirán el foco de futuras investigaciones.

Como conclusión final, se demuestra a lo largo de esta Tesis Doctoral que las técnicas de microextracción son indispensables para el análisis de muestras complejas y que su miniaturización es una estrategia esencial para ampliar su

aplicabilidad a muestras de baja disponibilidad, así como para mejorar la sostenibilidad de los métodos analíticos.

ABSTRACT

Large volumes of biological samples are often not available either because of the nature of the sample itself (*e.g.,* saliva, semen, follicular fluid, cerebrospinal fluid), the characteristics of the person under study (*e.g.,* newborns, immunosuppressed or anemic patients) or the context itself (*e.g.*, remains in forensic analysis). Besides, target compounds are usually at trace level and biological fluids are really complex matrices.

As a result, these matrices often require time-consuming sample pretreatments, which conflict with the principles of Green Analytical Chemistry, that advocate for direct analysis. In this context, the principles of Green Sample Preparation, which assume the need for sample preparation, focus on reducing sample size, chemical use, and waste production, among other considerations. Additionally, bioanalysis requires fast and cost-effective methods that can process a large number of samples in clinical laboratories daily. Thus, developing sample treatment techniques that can extract and concentrate analytes while minimizing the required amount of sample is crucial and highly significant for bioanalysis.

Since their introduction in the 90s, microextraction techniques have evolved and adapted to the trends and needs of the moment. Nevertheless, much of the scientific focus has been on developing safer and more environmentally friendly extraction phases and/or reducing their quantities, with less emphasis on minimizing sample size.

Additionally, reducing both the sample size and the volume of the acceptor phase may facilitate the miniaturization of extraction devices, leading to a reduction in physical space. This permits the development of parallel extraction devices in which a higher number of samples can be treated simultaneously.

Thus, the **objectives** of this Doctoral Thesis, focused to overcome these new challenges in sample preparation, are:

- Exploiting the advantages offered by microextraction techniques for the determination of substances of interest in complex matrices.
- The miniaturization and adaptation of microextraction techniques for the simultaneous analysis of a large number of complex and low-volume samples.

The present Doctoral Thesis has been divided into three sections, containing a total of six chapters and the general conclusions.

SECTION 1 is constituted by **Chapters 1** and **2**. In **Chapter 1**, the concepts of miniaturization in Analytical Chemistry are briefly described and the most relevant microextraction techniques are described and classified. Next, in **Chapter 2**, an overview of the advances towards further miniaturization of the already miniaturized microextraction techniques, with special emphasis on those in which reduced sample volumes (generally ≤ 1 mL) are used, is presented.

This chapter does not provide an exhaustive revision of all the miniaturized applications, but a glimpse of the developments and trends in the field.

SECTION 2 is constituted by **Chapters 3** to **6**. These chapters are devoted to the presentation and discussion of the methodology and results obtained during the development of the analytical methodologies presented in this Doctoral Thesis. In line with the general objectives, **Chapter 3** makes use of stir bar sorptive dispersive microextraction (SBSDME) for the determination of trace amounts of tetrahydrocannabinol (psychoactive component of cannabis) in cosmetic products containing ingredients derived from the hemp plant. In **Chapters 4** and **5**, miniaturized and high-throughput versions of SBSDME are designed to analyze a larger number of samples simultaneously. To demonstrate the applicability of these strategies, they have been applied to the analysis of biological samples. Specifically, **Chapter 4** is devoted to the determination of salivary cortisone and cortisol as biomarkers of various diseases, and in **Chapter 5** two cannabinoids in saliva are determined as indicatives of cannabis consumption. Then, in **Chapter 6**, magnetic headspace adsorptive microextraction (M-HS-AME) is adapted and miniaturized for the determination of volatile compounds as biomarkers of lung cancer in saliva samples.

Finally, **SECTION 3** describes the general conclusions derived from this Doctoral Thesis.

In the final pages two annexes are included, which describe the models of experimental designs used throughout the chapters (**Annex I**) and the scientific publications derived from this Doctoral Thesis (**Annex II**).

SECTION 1

INTRODUCTION

Chapter 1

Microextraction techniques

Part of this chapter is a compilation of the following published review article and book chapter:

C. Azorín, J.L. Benedé, A. Chisvert, Ultramicroextraction as a miniaturization of the already miniaturized. A step toward nanoextraction and beyond, J. Sep. Sci. 46 (2023) 2300223

C. Azorín, J.L. Benedé, A. Chisvert, Ionic liquid-based liquid-phase microextraction techniques, in: S. Carda-Broch, M.J. Ruiz-Angel (Eds.), Ionic Liquids in Analytical Chemistry: New Insights and Recent Developments, Elsevier, Amsterdam, 2022: pp. 73–102

1.1. Miniaturization in Analytical Chemistry

During the last decades, environmental concern has fortunately increased and spread to every area of society through Sustainable Development Goals [1], including Analytical Chemistry. The latest trends in this field described as the principles of Green Analytical Chemistry (GAC) pursue, in general terms, the elimination or reduction of the use of chemical substances, the minimization of energy consumption, the proper management of analytical waste and increased safety for the operator [2]. More recently, principles of White Analytical Chemistry (WAC) considered essential to balance the greenness of an analytical method with its usefulness and functionality, not only in terms of analytical efficiency (accuracy, precision, sensitivity) but also in terms of practical and economic considerations such as the cost, speed of analysis or ergonomics [3].

Miniaturization in Analytical Chemistry implies downscaling every single step in the analytical process, including sample preparation, separation techniques, if any, and detection. As a result, miniaturized approaches have been described with sample and solvent amounts diminished to the micro- or nanoliter level [4]. Undoubtedly, all of them pursue the highest possible analytical performance, in a rapid and cost-effective way, with reduced amounts of reagents and waste generation and, as far as possible, automated and portable for its *in-situ* applicability.

All these outcomes are the main driving forces for miniaturization, involving several possible benefits [5,6]:

1. Reduced consumption of reagents and solvents. Diminution of the analytical process directly permits the use of reduced volumes (or even absence) of expensive chemicals and toxic and/or pollutant solvents [7,8].
2. Reduced sample consumption. This is especially valuable for precious and/or scarce samples, where large volumes are not available (*e.g.*, biological fluids).
3. Reduced waste generation. As a direct result of the previous advantages, waste generation per analysis is highly reduced. Reutilization of chemicals and materials is also usually favored.
4. Improved sensitivity. Reduction of the extractant amount can directly increase sample-to-extractant ratio, enhancing enrichment factors and lowering limits of detection. It should be noted that miniaturized detection systems, while beneficial in several aspects, can cause a significantly deteriorated sensitivity.
5. Rapidity. High sample throughput is vital in analytical laboratories. On the one hand, a small scale offers greater control of molecular interactions, reducing time in diffusion-controlled systems [9]. On the other hand, the reduction of the device implies a reduction in physical space, permitting the development of parallel devices in which a higher number of samples can be treated simultaneously.

6. Portability. The diminution of the device, also enhance its portability to the sampling, or even extraction, site, reducing the risk of sample decomposition and contamination as well as the environmental pollution associated with sample transportation and storage [10].
7. Power consumption. Reduced devices usually require reduced power consumption, favoring the utilization of batteries and also contributing to the portability of the system.

Even though the final objective of miniaturization is to downscale the whole analytical process at once, like the so-called micro-total analytical systems (μTAS) [11], lab-on-a-disc [12] and lab-on-a-chip [13] devices, the concept should be considered from a wider perspective. In this sense, any advance in miniaturization of single steps in the analytical process, such as sample preparation, contributes to the progress towards miniaturized analytical systems with the advantages they imply.

1.2. Introduction to microextraction techniques

Both GAC and WAC stand for direct analysis of samples, avoiding sample preparation as it is considered one of the most critical and time-consuming steps within the analytical process [14] which has led to the widely spread dogma that "the best sample preparation is no sample preparation". However, far from this dream, the reality is that many times a sample preparation step is unavoidable [15]. For example, in trace analysis, sample preparation procedures are required in most cases to separate the target analytes from potential interfering substances, to concentrate them, and/or to condition the measurement solution to the analytical instrument [16]. In this sense, the principles of Green Sample Preparation (GSP) [5] have been recently described, assuming the existence of this step but pursuing the reduction of sample size, chemical usage and waste generation, among other principles.

One of the most important types of sample preparation techniques is based on the selective partitioning of the analyte or interferent between two immiscible phases. The partition of a solute between two immiscible phases is an equilibrium process that is governed by the distribution law. If the compound A is allowed to distribute itself between both donor and acceptor (or extractant) phase, the resulting equilibrium may be written as:

$$A_{donor} \rightleftharpoons A_{acceptor}$$

Ideally, the ratio of activities for A in the two phases will be constant and independent of the total quantity of A, so that,

$$K_P = \frac{(a_A)_{acceptor}}{(a_A)_{donor}} \qquad \text{(Eq. 1.1)}$$

where K_P is known as the partition coefficient and $(a_A)_{acceptor}$ and $(a_A)_{donor}$ are the activities of A in each of the phases. As with many other equilibria, under many conditions, activities can be approximate to molar concentrations without serious error, so that,

$$K_P = \frac{(a_A)_{acceptor}}{(a_A)_{donor}} \approx \frac{[A]_{acceptor}}{[A]_{donor}} \quad \text{(Eq. 1.2)}$$

where $[A]_{acceptor}$ and $[A]_{donor}$ are molar concentrations of A in both phases. In this case, K_P is useful to calculate the concentration of an analyte remaining in a solution after a certain number of extractions [17].

Classical extraction methodologies (*i.e.,* solid-phase extraction (SPE) and liquid-liquid extraction (LLE)) are being gradually substituted by microextraction techniques since the early 90s. Microextraction techniques, either those based on solid extraction phases (sorbents) or those based on liquid extraction phases (solvents), follow the same fundamental principles as conventional SPE and LLE, respectively, but the sorbent and solvent amounts are extremely reduced. As defined by IUPAC, microextraction techniques are those in which the extraction phase (liquid or solid) is substantially smaller than the sample volume, typically employing extraction phases below 100 µL or 10 mg and sample volumes above 1 mL [18].

From the 90s to the present, many efforts have been focused within the area of sample preparation not only on the development of new microextraction approaches but also on the use of newer extraction materials with the aim to obtain better performance [19,20]. Microextraction techniques have also progressed over the decades, adapting to the trends and needs of the moment [21], and giving rise to a numerous variety of techniques in both solid and liquid phase [22–24]. **Figure 1.1** shows a broad classification of the most representative microextraction approaches, with special emphasis on derived techniques from one to another.

In the following subsections, a general description of the main microextraction techniques is provided.

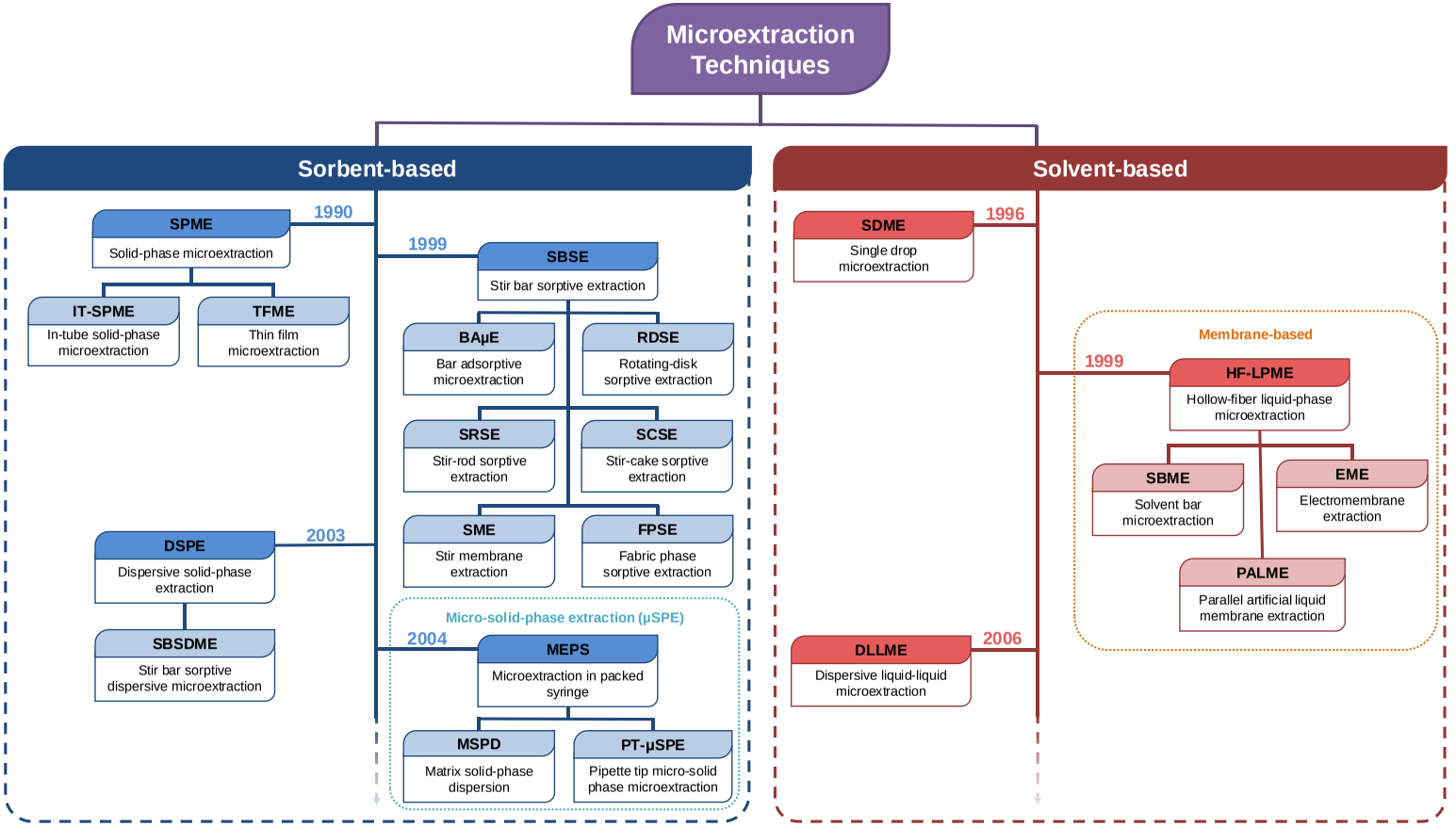


Figure 1.1. Broad classification of microextraction techniques. Reproduced with permission from [25].

1.3. Sorbent-based microextraction techniques

Before getting into the matter, a small reflection on terminology is in order. The first microextraction technique that emerged as an alternative to SPE was presented by Pawliszyn and co-workers [26] back in 1990 and it was called solid-phase microextraction (SPME). Since then, many others microextraction alternatives using solid phases for extraction in different configurations have been described. Therefore, this group of techniques should not be collected under the term “solid-phase microextraction”, as sometimes are coined, since it could cause confusion for readership. To this regard, a more appropriate general term to refer to this group is “sorbent-based microextraction”, thus avoiding this misleading classification.

Sorbent-based techniques involve using solid materials as the extractant phase, which offers several advantages over solvent-based techniques, described later in **Section 1.4**. One major benefit is the wide range of sorbent types available, allowing for greater flexibility in analysis planning. The choice of sorbent depends on the analyte being determined, providing researchers with a wide range of sorbents for various applications.

Among the available sorbent materials, polymers are the most commonly used either organic, inorganic or natural ones. They are microstructures with higher or lower porosity formed by the combination of monomers and have exceptional extraction capabilities. The ratio and nature of monomers and crosslinkers can be tuned to adapt the polymer to the required chemical and/or mechanical properties. Additionally, other crystalline frameworks such as metal-organic frameworks (MOFs) and covalent organic frameworks (COFs) are structured materials with intricate three-dimensional arrangements, incorporating pores and cavities and have been widely employed in analytical applications [27]. The choice of ligand allows customization of the crystalline framework based on the analytes to be detected. Besides, carbon-based materials have been considered as green sorbents and attracted much attention thanks to some of their advantages such as low toxicity, diverse structural morphology, or low synthesis cost. Thus, several carbon allotropes have been successfully used in the sample preparation field, including graphite, graphene, graphene oxide, carbon nanotubes (CNTs), and carbon quantum dots (CQDs), among others [28].

Even though the mentioned materials can be modified to adapt to different analytes, they are not specific and can extract various compounds to different extents. Therefore, a chromatographic step is usually required to separate the analytes from unwanted compounds and highly specific instrumental analysis techniques like mass spectrometry (MS) can be used to achieve selective determination. Another option for specificity is the use of sorbents that somehow exhibit enhance selectivity. For instance, antibodies in immunosorbents have binding centers that exclusively recognize a single compound, restricted access

materials (RAMs) are able to exclude the macromolecules present in the matrix and retain the small target analytes, and molecularly imprinted polymers (MIPs) can be synthesized to specifically recognize a singular analyte [28].

Among all the solid materials, nanometric-sized ones have gained significant interest due to their high surface area compared to larger materials. This high surface area enables higher extraction efficiency and extraction kinetics, leading to shorter analysis times [29].

Moreover, the combination of these sorbent materials with magnetic nanoparticles (MNPs), either by chemical bonding or just deposition onto the surface, leads to the synthesis of magnetic (nano)materials. Magnetic (nano)materials are nowadays widely used in microextraction techniques because the separation between the extraction phase and the sample/solvent, which is required in some microextraction techniques (as will be described in **Section 1.3.3**) is facilitated by a magnetic field. Thus, the use of highly energy and time-consuming stages, such as centrifugation or vacuum filtration are avoided [30]. In this sense, a huge variety of magnetic materials with more or less complex synthesis pathways has been described and applied to microextraction techniques [31].

1.3.1. Solid-phase microextraction

SPME is the most antique and most worldwide used sorbent-based microextraction technique. The original configuration of SPME, as presented in the 90s [26], consists of a silica fiber coated with a thin film of the appropriate sorbent in a syringe-like device for both direct-immersion (DI-SPME) and headspace extraction (HS-SPME) (see **Figure 1.2**).

After the extraction, desorption of analytes from the fiber can be made mainly by back-extraction in a small amount of organic solvent or by thermal desorption in the gas chromatography (GC) inlet [32]. As main limitation, silica fibers suffer from certain fragility and for this reason other supports like metallic ones have been employed [33]. Besides, in SPME, analytes diffuse from the sample to the surface of the fiber, being the extraction efficiency determined by the diffusion of analytes through the boundary layer around the coating [33]. Therefore, through the decades, different geometries and configurations have been presented beyond the classic fiber for SPME for an enormous variety of applications [34,35], giving rise, for example, to the thin film microextraction (TFME) or SPME blade. In this variant of SPME, the geometry of the support is changed from fiber to flat, so that the volume of the extraction phase is increased without increasing the thickness of the coating, thus enhancing extraction kinetics and analytical sensitivity [36,37]. Helin *et al.* also introduced the SPME arrow, which contains a larger volume of sorbent compared to a standard SPME fiber, demonstrating

its capacity to provide improved robustness and extraction efficiency [38]. **Figure 1.3** shows these main geometries for SPME.

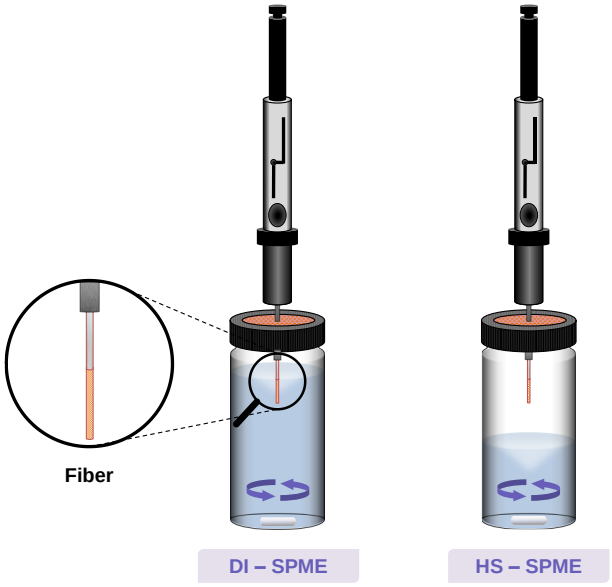


Figure 1.2. SPME assembly in direct immersion (DI) and headspace (HS) modes.

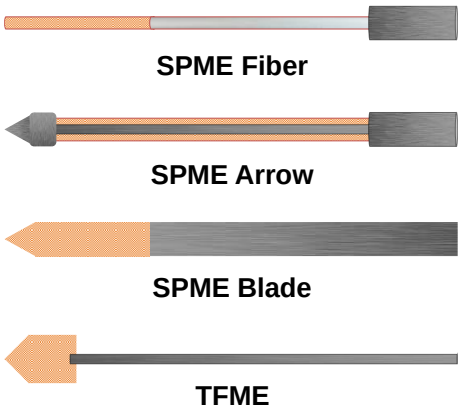


Figure 1.3. Main SPME sorbent support geometries.

The on-line coupling of SPME with liquid chromatography (LC) was proposed under the name of in-tube SPME (IT-SPME) [39], where the sorbent is not coating the outer surface of the extraction device but the inner wall of a capillary tube. The main advantages of this configuration are shorter analysis time, lower

sample and organic solvent volumes (in the order of a few microliters), higher precision of the method and system automation, leading to the development of different experimental set-ups [40].

1.3.2. Stir bar sorptive extraction and related techniques

Stir bar sorptive extraction (SBSE) appeared as an alternative to SPME in the late 90s, where an appropriate sorbent material covers a magnetic stir bar [41]. In this technique, the stir bar itself agitates the sample during the extraction stage, which enhance the mass transfer of the analytes from the donor phase to the extraction phase. The headspace mode, also known as headspace sorptive extraction (HSSE), is also possible by holding the bar in the headspace with different devices such as a magnetic clip. **Figure 1.4** shows these two possible configurations.

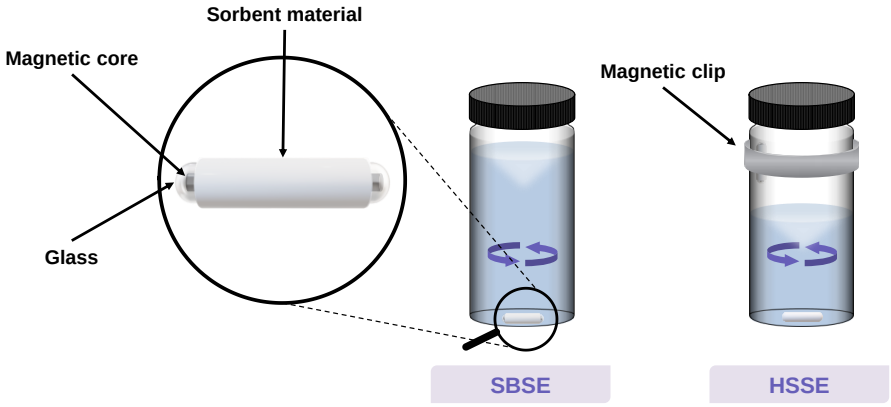


Figure 1.4. SBSE and HSSE assemblies.

The potential extraction capacity of SBSE depends on volume of both donor and acceptor phases as well as the partition coefficient of the analytes between both phases. In this sense, SBSE coatings are thicker than SPME fibers, which increases the potential extraction capacity. However, bearing in mind that extraction occurs under diffusion-controlled conditions, thicker coatings reduce extraction kinetics, which is not always fully compensated by solution stirring [42].

Thus, newer porous materials have been suggested to increase the contact area between the sorptive phase and the analytes. In most situations, it was necessary to restructure the extraction units in order to use the novel materials that led to new microextraction modalities. Besides, some alternatives were proposed to prevent the contact of the stirring bar with the vessel walls and thus

its damage, like bar adsorptive microextraction (BAμE) [43], rotating-disk sorptive extraction (RDSE) [44], stir-rod sorptive extraction (SRSE) [45], stir-cake sorptive extraction (SCSE) [46] and stir membrane extraction (SME) [47]. In these approaches, sorbent was placed onto a floating polypropylene device, a Teflon® disk, a stir rod, a stirring cake-shaped holder or a porous membrane hold in a rotating device, respectively.

Fabric phase sorptive extraction (FPSE), where the sorbent material is covering a fabric substrate, was described as an alternative sorbent-based approach [48]. This strategy is in close analogy to TFME but without the extraction device being fixed but suspended within the donor phase. The inherently porous structure of the sol-gel sorbent and the permeability of fabric significantly reduce the equilibrium extraction time, making FPSE a feasible sample preparation technique [49].

Recently, magnetic (nano)materials (*e.g.*, MNPs or composites made of MNPs and polymers) have been used also to expand the possibilities of headspace-based extraction techniques [30]. The main advantage of this approaches is the simplicity of its implementation in the sorption process due to the magnetic properties of the material, which allow to keep it in the headspace using an external magnet. Furthermore, the nanometric size of MNPs and the high porosity of polymers, provide these materials with large surface areas, which facilitates efficient adsorption of analytes and their miniaturization. In this sense, Nie *et al*. [50] were the first to use a magnetic material (*i.e.*, polystyrene-coated MNPs) in headspace extraction, specifically to extract volatile organic compounds (VOCs) from dried medicinal plants. Later, Vidal *et al*. [51] proposed a technique called magnetic headspace adsorptive extraction for environmental water analysis employing magnetic graphene oxide as extraction phase, and Timofeeva *et al*. [52] used a smaller version of this approach for food analysis, using Fe_3O_4@$Cr(OH)_3$ as sorbent material and renaming the technique as magnetic headspace adsorptive microextraction (M-HS-AME).

1.3.3. Dispersive micro-solid-phase extraction

Dispersive solid phase extraction (DSPE) (or DμSPE if the sorbent amount is in the micrograms range) was presented as an alternative to conventional SPE, where the sorbent is dispersed into the sample instead of percolating the sample through the packed sorbent [53]. The close contact that is achieved between the sorbent particles and the analytes combined with their large surface areas and extraction capacities favor kinetics of the sorption, thus increasing extraction efficiencies. Different strategies have been described to achieve the dispersion giving birth to ultrasound-, vortex-, air-, solvent- and effervescence-assisted DμSPE [54]. **Figure 1.5** shows the general procedure for DSPE.

The most time-consuming step of dispersive techniques is, however, the separation of the sorbent from the donor phase for the subsequent washing and desorption of analytes, usually performed by centrifugation or filtration. In this sense, magnetic (nano)materials, frequently combining MNPs and polymers or other sorbent materials, have been widely used in DμSPE due to their easy retrieval by applying an external magnetic field [30].

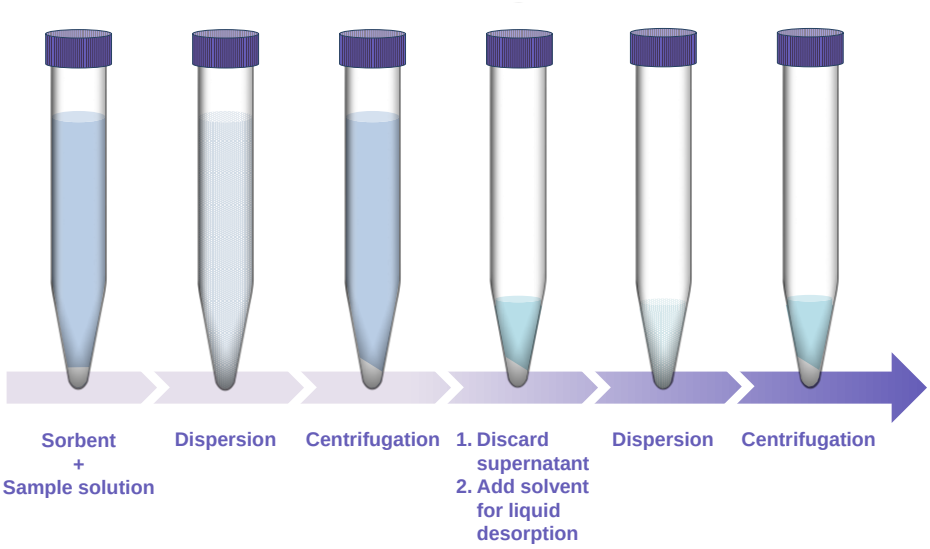


Figure 1.5. DSPE standard procedure.

In this context, a hybrid dispersive-based microextraction approach, which uses magnetic materials as extraction phase, termed stir bar sorptive-dispersive microextraction (SBSDME) was described in 2014 by the research group in which this Doctoral Thesis has been accomplished [55]. In this technique, which combines the operational modes of SBSE and DμSPE, a magnetic sorbent material is added to a vial containing the solution to be extracted and a neodymium magnetic bar (typically 10 mm length x 3 mm diameter), in such a way the material is attracted by the magnet. At high stirring rate, the material is released and dispersed into the donor solution, thus increasing the contact area between the sample and the extraction phase. When stirring is over, the neodymium stir bar rapidly retrieves the magnetic sorbent containing the extracted analytes. Then, the stir bar coated with the magnetic material is easily taken from the sample solution and the analytes can be either desorbed in an appropriate solvent for further injection into a chromatography instrument (mainly, but not exclusively), or thermally desorbed into a GC system. The fundamentals of this approach, and an overview of the published papers concerning analytical methods based on the use of the SBSDME approach were

compiled in a tutorial review [56]. **Figure 1.6** summarizes the SBSDME general procedure. This technique will be applied in **Chapter 3** for the analysis of cosmetic products.

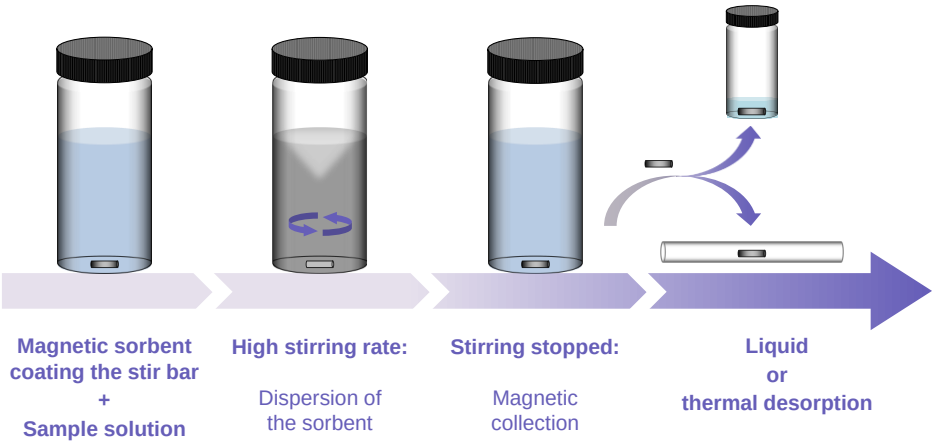


Figure 1.6. SBSDME standard procedure.

1.3.4. Micro-solid-phase extraction

Operational set-ups and sorbent disposition of micro-solid-phase extraction (μSPE) approaches follow the same principles than SPE, *i.e.*, the sample flow through the confined acceptor phase, but with reduced amounts of sorbent, sample, and solvents. In 2005, miniaturized conventional SPE in a 96-well plate format was described for high-throughput analysis of around 300 μL of biological sample volumes [57].

Other μSPE approaches consisted in confining a small amount of sorbent material at the end of a micro-syringe or a pipette tip, giving birth to the techniques known as microextraction by packed sorbent (MEPS) [58] and pipette tip μSPE (PT-μSPE) [59], respectively. The latter has been especially relevant as a result of the numerous varieties of commercial alternatives available [60] and the high-throughput automated PT-μSPE approaches that have been described [61]. **Figure 1.7** shows the standard procedures of MEPS and PT-μSPE.

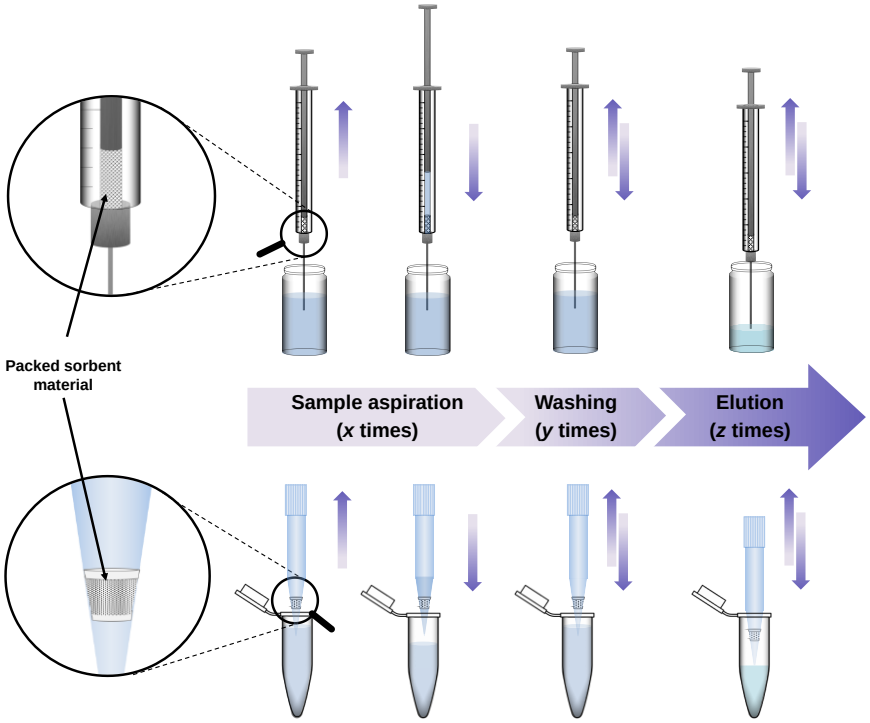


Figure 1.7. MEPS and PT-µSPE standard procedures.

The favored kinetics of dispersive approaches mentioned above have also permitted the dispersive modality of this technique by aspirating-dispensing cycles in dispersive-pipette extraction (DPX) [62]. In contrast to PT-µSPE, the sorbent in DPX is not packed between two frits but rather it is loose, causing the sorbent to disperse like in DSPE when the sample is aspirated. Besides improving the extraction kinetics, boosting sample throughput in comparison to PT-µSPE, the dispersive approach also helps avoiding clogging issues. Although many pipette-tip designs have been suggested, it has been found that the aspiration rate is not high enough to provide an effective dispersion. Based on this, it is possible to find commercially available tips that have an internal device designed to obstruct the flow and compel dispersion [63]. Moreover, more recently, the research group hosting this Doctoral Thesis incorporated the benefits of dispersion of magnetic (nano)materials into the pipette tip format [64]. Briefly, it involves quick dispersion of a magnetic sorbent material in the sample to entrap the target analytes, and its subsequent aspiration using a (semi)automatic pipette through a pipette tip with a small neodymium magnet inside, which retrieves the magnetic sorbent containing the analytes. After washing, the analytes are eluted by aspirating/dispensing an appropriate solvent. **Figure 1.8** shows the procedure of this magnetic-based pipette-tip microextraction.

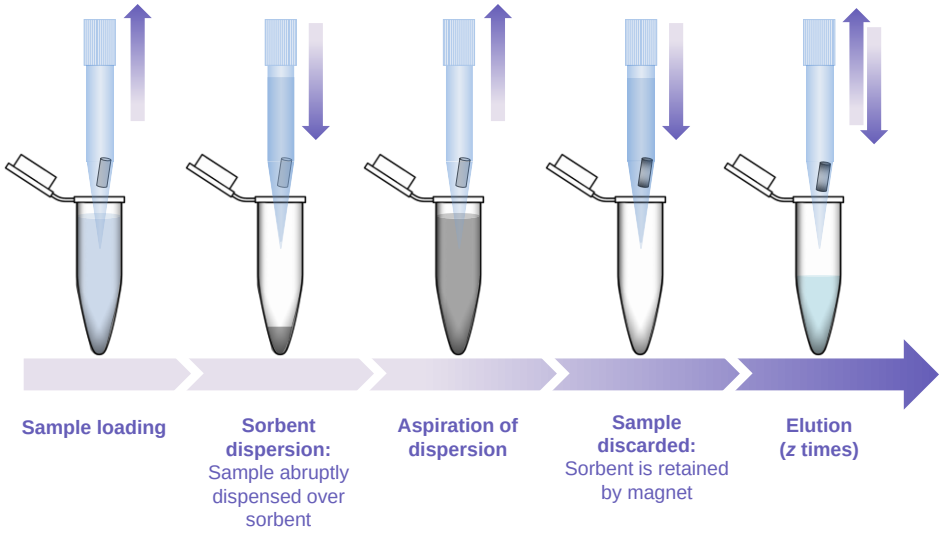


Figure 1.8. Magnetic-based PT-μSPE standard procedure.

From a slightly different point of view, matrix solid-phase dispersion (MSPD) was introduced to facilitate the chromatographic analysis of solid samples [65] as a simpler version of SPE. It consists in the direct mechanical blending of the sample with a solid abrasive material to produce a semi dry, homogeneous, and easy to handle material, which is then placed in a SPE cartridge, and a suitable solvent is percolated through the mixture, extracting analytes.

1.4. Solvent-based microextraction techniques

Solvent-based microextraction techniques, often referred to as liquid-phase microextraction (LPME), have become one of the most important research and development areas in the Analytical Chemistry field. One of their main advantages are the usual compatibility with most analytical instruments.

In analogy to sorbent-based techniques, the selection of the right solvent as extraction phase is crucial in LPME and may vastly influence the greenness of the method according to GAC and GSP. When selecting a solvent, numerous physicochemical characteristics should be taken into account such as density, boiling temperature, octanol/water partition coefficient, viscosity, and surface tension. Water insolubility is obviously also essential when extraction from water is needed. Additionally, other aspects like financial cost, energy and environmental costs associated with its production, recycling, pollution and toxicity are important aspects to consider. Moreover, solvent compatibility with the final analytical instrumentation must also be considered [66].

Classical LPME solvents are those that were used in LLE like diethyl ether, ethyl acetate, octanol, hexane, cyclohexane, dichloromethane or chloroform, among others. However, some of them present problems such as high volatility, toxicity or carcinogenicity. Nevertheless, some of them, such as dichloromethane or chloroform have been still widely employed as extraction solvents because there are only a limited number solvents whose density is higher than water, which is essential in some LPME modes.

Even though the employed volume of these solvents for LPME is in the microliter range, their use in routine analysis, where hundreds of analyses can be performed per day, should not be recommended due to their environmental and health concerns. For that reason, during the last years, greener alternatives have been proposed in order to be less harmful to the environment and the researcher following the trends of GAC and GSP [67].

In this sense, low-density solvents, such as alkanols, switchable hydrophobicity solvents (SHSs), and ionic liquids (ILs) were the first and most popular replacements for halogenated solvents, which provided similar or better results than these classical solvents. However, despite the good performances obtained and despite the improvements compared to halogenated solvents, some operational and/or environmental issues remained present. In this sense, researchers have continued investigating in order to obtain even greener solvents for LPME. Two of the most promising solvent alternatives are deep eutectic solvents (DESs) and supramolecular solvents (SUPRAs) [68].

SHSs are solvents consisting of amidines, secondary/tertiary amines or saturated fatty acids that show the capability of changing their hydrophilicity and, therefore, change from slightly miscible to completely miscible with water by means of a simple change in the aqueous medium. In general, a pH change enhances the formation of a water-soluble salt, hence solubilizing the SHS. After that, the reaction is forced backward producing the separation of the two phases [69]. However, due to the use of strong acids or bases for the pH change and the toxicity of some amines, they have not attracted the interest as much as other alternative solvents.

ILs are defined as substances composed of ions whose melting point is less than 100 ˚C, which result from the combination of an organic cation and an organic or inorganic anion. ILs present exceptional attributes to be employed as microextraction solvents, and moreover they possess high thermal stability and low vapor pressure. In addition, some of their properties such as viscosity, density and solubility can be tuned by combining properly the different cations and anions available [70,71]. In addition to these conventional ILs, there exist a peculiar subclass, known as magnetic ILs (MILs), which exhibit a strong susceptibility when exposed to an external magnetic field [72]. Another subclass is that composed by polymeric ILs (PILs) [73], *i.e.*, chain-shaped ILs where

monomeric units of ILs are repeated. Furthermore, they can be modified and functionalized to empower specific interactions with the analyte, promoting the extraction efficiency. In this sense, ILs provided a whole new world of possibilities for LPME and had been widely applied in the different modalities [74]. Nonetheless, one of their main drawbacks is their coupling with chromatographic devices and electrothermal atomic absorption spectroscopy instrumentation. Their lack of volatility is incompatible with GC, although different approaches were proposed to overcome this limitation [75–78]. Besides, as a result of their low volatility and high viscosity, they are also incompatible with LC coupled with MS, since the formation of the electrospray is highly affected, redounded in serious matrix effects and signal drift [79,80]. High viscosity may also difficult handling, diffusion and mass transfer. Furthermore, their high thermal stability should also be considered, because the elevated temperatures needed in electrothermal atomic absorption spectroscopy during the pyrolysis step can involve analyte loss [81]. Finally, the environmental aspects of most of these ILs should be discussed. ILs are usually called green solvents due their low vapor pressure, that avoids ILs to reach the atmosphere or to be inhaled by the operator. However, usually the components of the synthesis or the final products are toxic, and thus, they cannot be considered as green [82]. Therefore, in spite of the great properties of ILs, Deetlefs and Seddon [83] concluded that they are “green, but not green enough”.

SUPRAs are solvents generated after the coacervation of previously formed micelles in solution. First, amphiphiles are added to the solution, and when the critical aggregation concentration is achieved the amphiphiles groups are reorganized in micelles. Once micelles are formed, a change in the environment of the solution is produced in order to reduce the repulsion interaction between the amphiphiles forming the micelle. This action allows more amphiphile groups to be introduced into the micelles, forming bigger aggregates, that will interact with other aggregates forming the supramolecular solvent [84]. These SUPRAs possess exceptional extraction capabilities due to their great surface and their different polarity environments. Moreover, they can be synthesized *in situ* during the analysis and they do not need extra treatment to be introduced in LC systems [85], making them really attractive for microextraction approaches.

DESs are formed by two or three components combined by hydrogen bond interactions, creating a eutectic mixture with a melting point lower than the original parts. The formed DESs share some of the properties with ILs [86], such as thermal stability, low vapor pressure, and low flammability. Moreover, in analogy to ILs, DESs can be tailorable in function of the behavior of the analytes. In contrast, their obtention is usually easier, cheaper and safer for the environment and the operator due to their low toxicity and biodegradability [87], making DESs ideal solvents for microextraction approaches [88,89]. However, during the first years, the DESs employed cannot be catalogued as ‘green’. These were composed by hazardous components such as phenols and

halogenated phenols, pollutants as alkanols, or ILs which, at the same time, need a previous synthesis step. During the next years, safer components such as menthol [90], betaine [91], choline chloride and ethylene glycol [92] were employed for DES formation giving birth to the so-called 'natural DESs' (*i.e.,* NADESs).

1.4.1. Single drop microextraction

Single drop microextraction (SDME) was presented as the first downscaled version of classical LLE almost simultaneously by Jeannot and Cantwell [93] and He and Lee [94] in 1997. In this approach, in close analogy to SPME, a droplet of a few microliters (*ca.* 1-10 µL) of a water-immiscible organic solvent is hung for a defined time from the tip of a syringe needle being exposed either to the aqueous sample (*i.e.,* DI-SDME) or to the headspace (*i.e.,* HS-SDME) (see **Figure 1.9**). After extraction, the droplet is retracted back into the syringe needle and transferred to the analytical instrument, usually a GC or LC system.

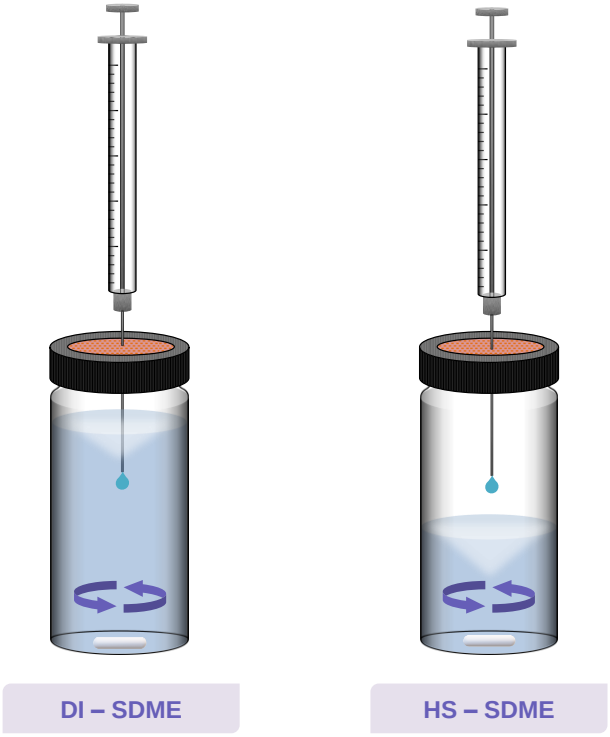


Figure 1.9. SDME assembly in direct immersion (DI) and headspace (HS) modes.

However, behind its apparent simplicity, it presents different drawbacks as a consequence of the low viscosity, high vapor pressure and low surface tension of most of the organic solvents typically employed. In practice, this means a low stability of the hanging droplet during sample stirring and the relatively rapid and changeful volatility when sample is heated as in HS-SDME mode. Aware of these problems, alternative solvents with better physicochemical characteristics were tested for the replacement of conventional solvents [67].

Mass transfer in SDME was enhanced by using dynamic versions such as continuous flow microextraction (CFME) [95] or the syringe-based approach proposed by He and Lee [94]. The first mode is based on a solvent droplet held in a flow of donor phase. In the latter, a small amount of organic solvent is withdrawn into a microsyringe followed by an aqueous sample. A very thin organic film is left and formed on the inner surface of the microsyringe barrel and needle. Repeated aspiration-dispensation of the sample ensures that both the organic film and the sample are periodically renewed. Cycle flow microextraction is a modification of CFME in which the sample is recirculated and returned to the sample reservoir, minimizing the sample size needed (*ca.* 1 mL) [96].

In 2006, another variation of SDME, consisting in placing a water-immiscible solvent (less dense than water) onto the surface of a stirred aqueous sample, was coined as directly suspended droplet microextraction (DSDME) [97]. Later, solidification of floating organic drop (SFOD) [98] was described to facilitate the retrieval of the acceptor phase after extraction. In this approach, after the extraction time, the sample vial is cooled in an ice bath and the solidified solvent drop is transferred to another vial and immediately melted for the subsequent analysis.

As important advance in SDME, Anderson and co-workers exploited the characteristics of a MIL, which does not hang from the tip of the syringe but from a rod magnet. This allows the use of larger volumes than in conventional SDME without compromising the droplet stability [99].

1.4.2. Hollow-fiber liquid-phase microextraction

Hollow-fiber liquid-phase microextraction (HF-LPME) was presented in 1999 by Pedersen-Bjergaard and Rasmussen [100]. It constitutes a LPME approach where the extraction solvent is filling a thin U-shaped porous hollow-fiber, usually made of polypropylene, immersed in the aqueous sample in such a way the ends are inserted into two syringe needles. The fiber mechanically protects the extraction solvent droplet and then avoids the droplet loss that often occurs in DI-SDME due to the stirring of the sample. Some years later, Lee and co-workers replaced the U-shaped fibers by lineal shorter ones with one of the ends sealed [101].

Later still, Jiang and Lee used two-end sealed fibers and allowed them to tumble freely in the sample solution during extraction under stirring with a magnetic bar [102], which increased the mass transfer between the organic phase in the fiber and the aqueous sample solution. This modality was coined as solvent bar microextraction (SBME). **Figure 1.10** shows the extraction assemblies for these operational modes. Development went even further when Xu *et al.* introduced a stainless steel wire in the lumen of the fiber combining the advantages of SBSE and HF-LPME, *i.e.,* this self-stirring device made extraction, clean-up and pre-enrichment into one-step [103]. As an additional advantage of HF-LPME, it should be said that the pores of the hollow-fiber contribute to the selectivity by preventing the extraction of high molecular weight substances.

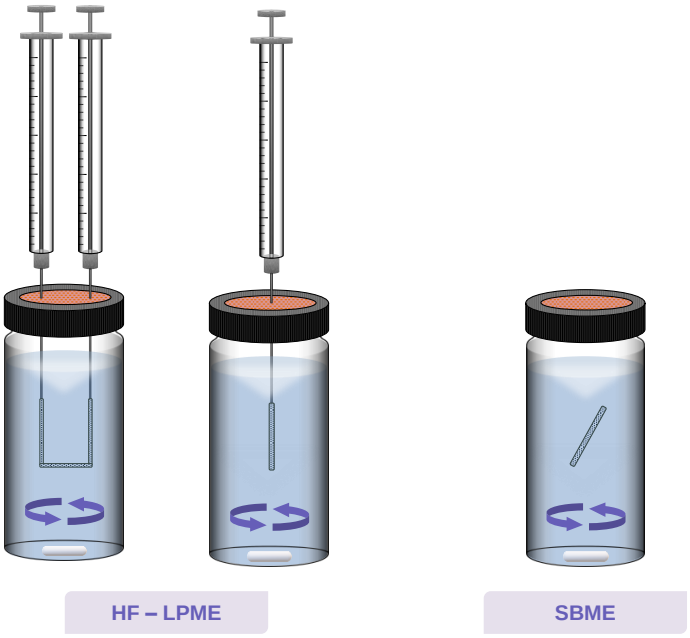


Figure 1.10. HF-LPME (two hollow-fiber configurations) and SBME assemblies.

These techniques can be carried out under two basic possible configurations, *i.e.,* two-phase and three-phase systems. In the two-phase system, a hollow-fiber of certain dimensions is clamped to a syringe needle filled with the extraction organic solvent. Then, it is soaked in the same organic solvent, which is immobilized in the pores of the fiber thus forming the so-called supported liquid membrane (SLM). Then, the fiber lumen is filled with the aid of the syringe, and finally it is exposed to the sample. The three-phase system is similar, but the extraction phase is an aqueous solution conveniently pH-adjusted, in such a way the SLM avoids the mixing between both donor and acceptor aqueous phases.

The three-phase system is used for the extraction of potentially ionizable compounds, which pass from the aqueous donor solution to the SLM and then are back-extracted into the acceptor aqueous solution located at the lumen of the hollow-fiber. In both cases, the acceptor phases are withdrawn with the syringe after extraction and transferred to the analytical instrument. In case of two-end sealed fibers, it is opened at one end and the acceptor phase is taken by means of a syringe.

Pedersen-Bjergaard and Rasmussen [104] also proposed in 2006 the application of an electric field as driving force for the electrokinetic migration of charged solutes between two aqueous compartments throughout a SLM. This approach, similar to the three-system HF-LPME but applying an electric field between two platinum wires (one dipped in the donor phase and the other dipped in the hollow-fiber lumen), is known as electromembrane extraction (EME). It was shown that the applied electric field significantly accelerated the extraction process when compared to conventional HF-LPME based on passive diffusion [105].

Later on, parallel artificial liquid membrane extraction (PALME) [106] or its electronically enhanced version (coined parallel EME, Pa-EME) [107], where the sample and acceptor phase are separated by a flat membrane impregnated with the organic solvent, were presented. These systems have been described in a 96-well plate format and have great advantages such as low sample (up to 250 µL) and solvent (*ca*. 25-100 µL) consumption, as well as the possibility of simultaneous extraction of multiple samples and nice prospects of automation.

1.4.3. Dispersive liquid-liquid microextraction

Dispersive liquid-liquid microextraction (DLLME), proposed by Rezaee *et al.* in 2006 [108], is by far the most popular LPME approach, due to its simplicity and rapidity. The original workflow is based on a solvent ternary system, *i.e.,* a water-immiscible extraction organic solvent denser than water is mixed with a water-miscible organic solvent, named “disperser solvent”, and subsequently, this mixture is rapidly injected into the aqueous sample. The disperser solvent causes the total dispersion of the water-immiscible extraction solvent within the bosom of the aqueous donor phase, forming what is known as “cloudy solution”, where the extraction solvent forms thousands of fine droplets. This makes the contact area between the extraction solvent and the sample to be extremely large, so the equilibrium state is quickly achieved. Then, phase separation is achieved by centrifugation, which causes the extraction organic solvent containing the analytes to be settled at the bottom. Finally, it is retrieved with the aid of a syringe and transferred to the analytical instrument. **Figure 1.11** describes the general procedure for DLLME.

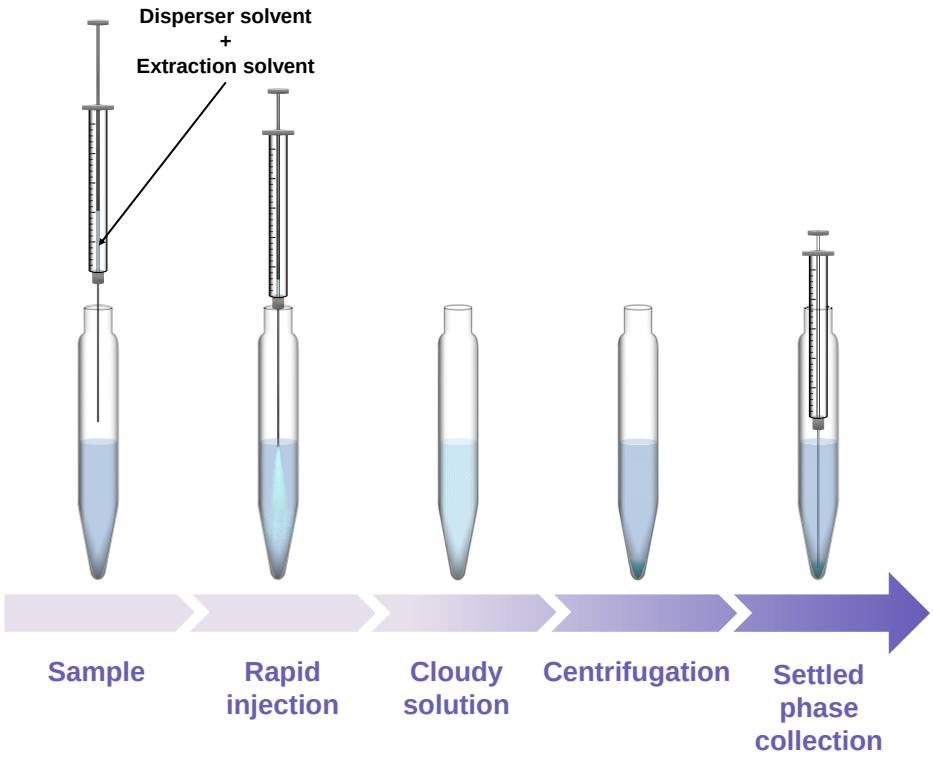


Figure 1.11. DLLME standard procedure.

It should be said that different modifications of the original DLLME appeared later, with different names and acronyms that is some cases confuse the most experienced reader [109]. Consequently, a large list of coined names, and their corresponding abbreviations, flooded the literature adding confusion to the readers [71]. In fact, the reader, in some cases, can find two names for referring to the same approach, whereas in other cases it can find different approaches named almost the same. In summary, in order to expedite the DLLME process, it can be assisted by an external energy source, such as ultrasound, microwaves or vortex (very important for labile compounds) resulting on the ultrasound assisted- [110] microwave assisted- [111] or vortex assisted-DLLME [112], respectively. However, the disperser solvent can negatively affect the partition coefficient of the target analytes between the aqueous donor phase and the organic extraction solvent, and then just ultrasound, microwaves or vortex have been applied, knowing them as ultrasound-assisted emulsification-microextraction (USAEME) [113], microwave-assisted liquid-liquid microextraction (MALLME) [114] or vortex-assisted liquid-liquid microextraction (VALLME) [115], respectively. In other cases, repeated aspiration/expulsion cycles of the mixture with the aid of a syringe are applied to obtain the cloudy

solution and this operational procedure is called air-assisted liquid-liquid microextraction (AALLME) [116].

As in SDME and HF-LPME, the first studies on DLLME were performed with conventional organic solvents and few years later alternative solvents such as ILs started to appear seeking to exploit their advantages [68]. The implementation of ILs in DLLME allowed to develop a new operational mode called *in-situ* solvent formation microextraction (ISFME), proposed by Baghdadi and Shemirani [117] in 2009. This is a homogeneous LPME technique, where a hydrophilic IL is solubilized into the aqueous sample forming a homogeneous solution, and then, an ion-exchange reagent is added thus forming the corresponding hydrophobic IL via a metathesis reaction, which is immiscible with water, so a cloudy solution is formed. In this approach, the IL-based extraction phase is formed *in situ* while simultaneously extracting analytes from the aqueous solution, greatly decreasing the extraction time. In addition, ISFME does not require the use of a heating/cooling steps, and/or disperser solvent or other external energy source to disperse the extraction solvent.

In all modes of DLLME, the centrifugation step is the most time-consuming procedure and also hinders automation of the whole process. In this sense, Cruz-Vera *et al.* [118] used a 10-mL plastic syringe as extraction vessel. After extraction, the plunger was slowly depressed allowing the retrieval of the extractant solvent (*i.e.,* an IL) from the wall. Besides, different alternatives to break the emulsion were proposed like solvent-terminated DLLME [119], salt-induced demulsification-DLLME [120], or solvent retrieval by a magnetic sorbent [121].

Chapter 2

Further miniaturization of microextraction techniques

The content of this chapter has been published in the following review article:

C. Azorín, J.L. Benedé, A. Chisvert, Ultramicroextraction as a miniaturization of the already miniaturized. A step toward nanoextraction and beyond, J. Sep. Sci. 46 (2023) 2300223

The simplification and sustainability of sample preparation is one of the axes in which the analytical scientific community has turned in recent decades. More specifically, extraction techniques have evolved into microextraction techniques (as described in **Chapter 1**). As said before, and according to IUPAC, microextraction techniques are those in which the extraction phase (liquid or solid) is substantially smaller than the sample volume, typically employing extraction phases below 100 µL or 10 mg and sample volumes above 1 mL [18]. Since their introduction in the 90s, as already discussed before, microextraction techniques have progressed over the decades, adapting to the trends and needs of the moment [21], and giving rise to a numerous variety of techniques in both solid and liquid phase [22–24] (see **Figure 1.1**).

These already miniaturized extraction techniques have evolved into more miniaturized approaches trying to exploit their benefits and, if possible, to enhance analytical performance. However, although it is true that over the years, and more intensely in the last decade, many improvements have been made compared to the first microextraction approaches to fulfill most of the outcomes demanded by GSP, special attention has been placed on introducing new extraction phases and reducing their amounts but not so much on reducing the sample volumes, and many of them still use volumes in the order of tens of milliliters.

It is worthy to consider that sample amount may be a challenge when the analysis of biological matrices is conducted. Large volumes of these samples are often not available either because of the nature of the sample itself (*e.g.,* saliva, semen, follicular fluid, cerebrospinal fluid), the characteristics of the person under study (*e.g.,* newborns, immunosuppressed or anemic patients) or the context itself (*e.g.*, remains in forensic analysis). This drawback gets worse if the target compounds are at trace level. Furthermore, biological matrices usually comprise many compounds with very diverse natures that make them really complex samples to deal with. As a consequence, these matrices require more or less time-consuming sample treatment steps. Besides, bioanalysis requires rapid and affordable methods that enable the analysis of a vast number of samples in clinical laboratories every day. Therefore, the development of sample treatment procedures that minimize the amount of sample needed for the analysis is interesting and of paramount importance in the context of bioanalysis.

Furthermore, the reduction of both the sample and the acceptor phase could lead to an effective miniaturization of the extraction device, which implies a reduction in physical space. This permits the development of parallel extraction devices in which a higher number of samples can be treated simultaneously.

The aim of this chapter is to give the reader an overview of the advances towards further miniaturization of the already miniaturized microextraction techniques,

with special emphasis on those in which reduced sample volumes (generally ≤ 1 mL) are used. Besides, when appropriate, a discussion of the impact of miniaturization on greenness is included. This chapter does not provide an exhaustive revision of all the miniaturized applications, but a glimpse of the developments and trends in the field.

2.1. Miniaturization of sorbent-based approaches

2.1.1. Solid-phase microextraction

SPME is the most used sorbent-based microextraction technique worldwide. The original configuration of SPME (as described in **Section 1.3.1**) consists of a silica fiber coated with a thin film of the appropriate sorbent in a syringe-like device [32].

About miniaturization of on-fiber SPME, there is not much space for further reduction of the sorbent amount, since it is already a thin coating on a small silica fiber (*e.g.,* 1 cm length, 100 µm thickness). However, the main opportunity of miniaturization is the reduction in the sample volume, although it compromises the enrichment factors to be achieved. In contrast with original configurations, where sample is placed in vessels of tens of milliliters, in 2010, Pacenti *et al.* placed the aqueous sample in 2-mL autosampler vials, reducing the diffusion distance and achieving the equilibrium state in sorter times [122]. This alternative was also applied for honey analysis, requiring just 200 mg of sample [123], and even further miniaturized using a conical insert for biological applications from 10 µL of sample [124]. In the latter, fibers as small as 4-mm length were used for smaller sample volumes (see **Figure 2.1.a**).

Among the non-fiber-based configurations, TFME has been useful for the analysis of reduced sample volumes (*i.e.,* 100 µL) by using blade-geometry supports [125]. Alternatively, Pawliszyn and co-workers presented in 2016 the so-called coated-tip SPME [126], in which a tip of an acupuncture needle was coated with the extraction phase (about 100-500 µm length, 5 µm thickness) allowing the analysis of sample volumes of microliters (*i.e.,* ≤ 10 µL). This approach is relevant as they downscaled both sample and extraction phase and permitted even *in vivo* analysis.

On the other hand, as previously discussed, the main advantages of IT-SPME are shorter analysis time, lower sample and organic solvent volumes, higher precision of the method and system automation. In addition, it allows coupling with new miniaturized chromatographic systems such as capillary LC and nano LC [127]. Additionally, Pawliszyn and co-workers presented another geometrical configuration of SPME toward miniaturization and greener and faster analysis [128]. In this work, a thin layer of sorbent was electrochemically deposited inside

a medical grade spinal needle for the analysis of very low volumes of untreated complex matrices (*i.e.*, single drop of whole blood, ≤ 2 µL). Desorption was carried out either offline or by direct coupling of the IT-SPME device.

A very smart approach was recently proposed by Cárdenas and Lucena's research group [129]. The sorbent material is deposited in the inner walls of a needle and sample is repeatedly aspirated and dispensed through the needle. After washing, the eluent desorbs the analytes from the coating, and it is directly electrosprayed in the tip of the needle, allowing direct coupling of desorption with ambient mass spectrometry (see **Figure 2.1.b**).

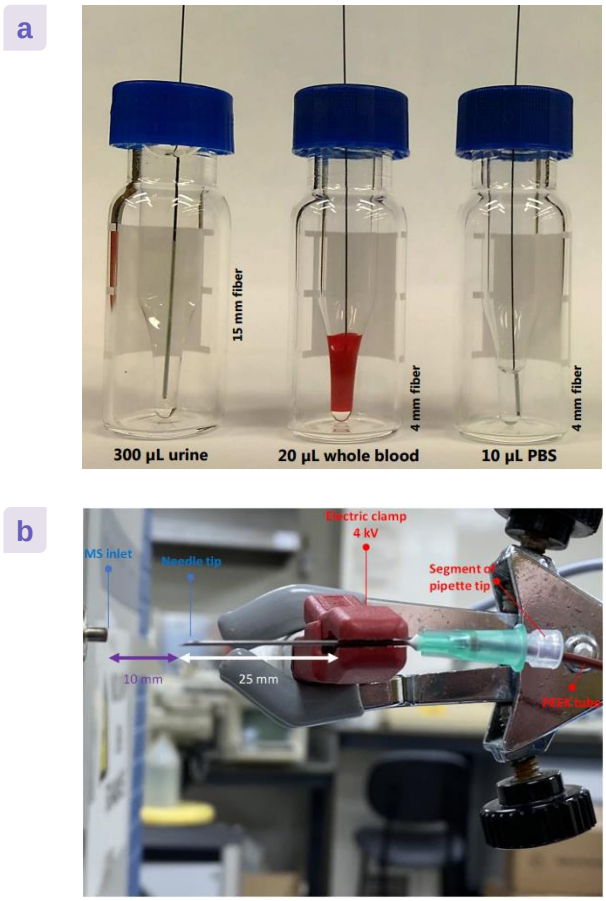


Figure 2.1. (a) Miniaturized extraction devices for SPME. (b) Manifold used for the direct ambient mass spectrometry analysis using a needle as extraction device. Reproduced with permission from [124] and [129], respectively.

2.1.2. Stir bar sorptive extraction and related techniques

In SBSE-based techniques, the extraction process is kinetically controlled being influenced by the sample volume, sorbent dimensions and stirring speed, and therefore they usually need relatively high volumes of sample or at least a minimal volume to cover the extraction device [130]. Thus, their miniaturization is challenging and they have been widely applied in environmental and food analysis, where sample volume may not be an issue [131,132]. Nonetheless, their applications in biological analysis are much scarcer [133,134]. Some methods have been described in which the desorption step was solvent-minimized by downsizing the microextraction device and introducing it in glass vial inserts for desorption. Moreover, they may use small amounts of sample, although samples were diluted prior to extraction, resulting in decreased sensitivity [135,136].

A bit more of advancement can be observed for FPSE, for which several methods have been described employing sample volumes as low as 50 µL [137] and desorption solvent volumes around 100 µL [138].

A new miniaturized adaptation of M-HS-AME has been developed in the context of this Doctoral Thesis and will be presented in detail in **Chapter 6**. In this approach, the extraction vials are replaced by centrifuge microtubes to adapt the technique to low sample volumes and expand its applicability for bioanalysis. Besides, the use of a thermo-shaker permitted the treatment of several samples at the same time [139].

2.1.3. Dispersive micro-solid-phase extraction

Miniaturized versions of DµSPE (usually employing centrifuge microtubes) of the different alternatives have been proposed for sample volumes below 1 mL, using either ultrasound- [140], vortex- [141,142] or solvent-assisted dispersion [143].

Following the development of SBSDME (as described in **Section 1.3.3**), a miniaturized version of SBSDME has been recently described in the context of this Doctoral Thesis [144]. Instead of using 40-mL vials and 10 mm length x 3 mm diameter magnets as in SBSDME, affordable flat-base glass inserts and minute bar-shape neodymium magnets (3 mm length x 2 mm diameter) were used here as extraction devices. They were held by a specifically designed multiextraction assembly, composed of a high-rate stirring plate and a tailor-designed 3D-printed support. It was successfully applied to treat up to 15 saliva samples of 350 µL simultaneously. This new miniaturized approach will be presented in **Chapter 4**. Later on, in **Chapter 5** the sample throughput will be expanded to the analysis of 96 samples simultaneously by using a 96-position stirring plate.

2.1.4. Micro-solid-phase extraction

In 2005, the conventional SPE set-up was miniaturized into a 96-well plate format for high-throughput analysis of around 300 µL of biological sample volumes [57].

Other µSPE configurations such as MEPS and PT-µSPE have permitted the analysis of small volumes of sample (*ca.* 50 µL) [145,146] and the development of high-throughput automated approaches [61]. The advantages of dispersive modalities and magnetic materials have also been exploited in miniaturized versions. For instance, the magnetic-based PT-µSPE (see **Figure 1.8**), described by the research group hosting this Doctoral Thesis, has been recently redesigned to allow the analysis of microsamples, needing just 10 µL of serum in its most miniaturized version [147]. This adaptation consists of a 1-mm cubic neodymium magnet stuck into a 200-µL pipette tip in such a way there is enough space for the solution to flow between the magnet and the tip walls as seen in **Figure 2.2**.

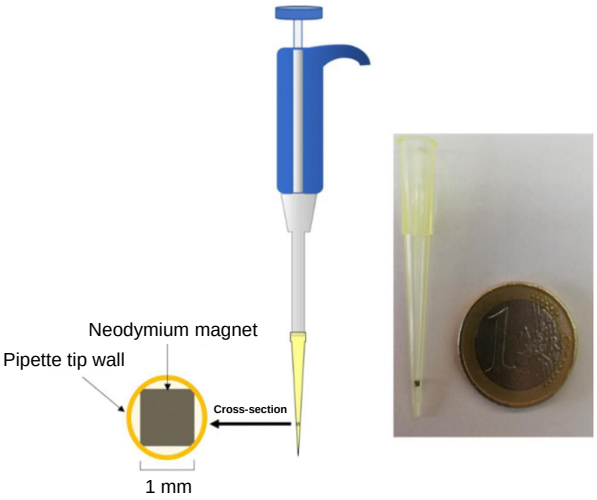


Figure 2.2. Extraction device of miniaturized magnetic-based pipette tip microextraction. Adapted with permission from [147].

µSPE has also followed the path of automation leading to different configurations and techniques based on on-line coupling with analytical techniques [148]. It is worthy to mention those methodologies based in lab-on-valve [149], 3D-printed microfluidic platforms [150] and even microchips [151,152] as miniaturized and automated applications of µSPE, joining the current trends in Analytical Chemistry.

Among the miniaturized extraction devices for MSPD, the usage of a Pasteur pipette or pipette tips as mixture holders [153,154] and the use of a precolumn for in-line coupling with LC [155] should be pointed out.

2.2. Miniaturization of solvent-based approaches

Talking about miniaturization of liquid-phase systems, the concept of microfluidics comes into play unavoidably. Since their introduction in the early 90s, microfluidic devices have gained important relevance in the Analytical Chemistry and sample preparation fields [156]. In LPME, miniaturized systems on a chip can provide high efficiency in short analysis time, increasing portability and reducing operational costs. Although most of the LPME-dedicate microfluidic devices operates under laminar flow, mass transfer gets faster due to short diffusion distance and a high surface/volume ratio of the microchannels. Thus, miniaturized on-chip devices provide high analytical throughput, while much lower sample and solvents volumes are consumed [14].

Unlike non-miniaturized LPME, where high volume ratios between donor and acceptor phase provide high enrichment factors, in microfluidic devices, where volume ratios are smaller, higher enrichment factors can be achieved by increasing the sample flow rate respective to the acceptor flow [157].

Some reviews can be found in the literature about the different fabrication techniques, designs, materials, operational modes and applications of microfluidic devices [158–162]. In broad terms, Xu and Xie [158] classified the microfluidic extraction approaches into three major groups:

1. Stop-flow: the acceptor phase remains static into a stream of the donor phase (**Figure 2.3.a**).
2. Co-current microfluidic: both donor and acceptor solutions flow continuously and simultaneously in the same direction. Parallel flow, droplet flow and slug flow-based are the main options (**Figure 2.3.b-c**).
3. Countercurrent microfluidics: both donor and acceptor solutions flow in opposite directions. The use of porous membranes is necessary to generate stable mass transfer interphases (**Figure 2.3.d**).

For the development of these devices contributing to miniaturization and automation of analytical procedure, 3D-printing is a remarkable modern trend. It is useful for the fabrication of sophisticated microfluidic devices [163] even though critical barriers such as resolution, throughput, and chemical and biological compatibility may still need some improvement [164].

Aside from these microfluidic systems, other miniaturized solvent-based technologies have been presented over the past decades, some of them based on the concepts of microfluidic systems, as will be seen later.

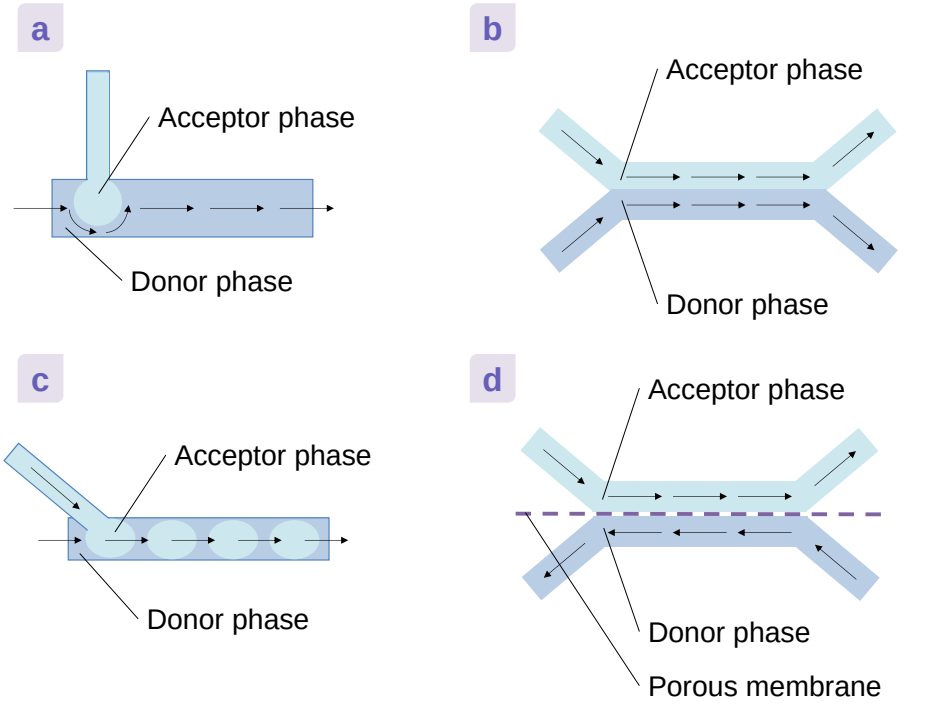


Figure 2.3. Schematic representation of four operating modes of microfluidics LPME: (a) Stop flow; (b) parallel flow; (c) slug flow; (d) membrane assisted. Adapted with permission from [14].

2.2.1. Single drop microextraction

As a result of the already minute amount of extraction solvent employed in SDME, there have been few variations since the 2000s, and novel solutions in the field of this technique are mainly focused on automation, reduction of sample volume or enhancement of extraction performance.

The first downscaled configuration was the so-called drop-to-drop SDME (DD-SDME), where only 7 µL of sample were employed and analytes were extracted into 0.5 µL of solvent [165]. Because of the reduced sample and solvent volume, the equilibrium is rapidly reached, however, enrichment factors are very restricted. This mode is very useful for the analysis of samples whose volume is

somehow limited, and thus it has been applied to biological fluids [166,167]. In 2020, Sun *et al.* went even further and described two different configurations of drop-to-drop SDME in the nanoliter level, employing 100 nL of sample and 10 nL of acceptor phase [168].

Recently, an important progression to enable high-throughput analysis was the use of a 96-well plate system for the simultaneous analysis of up to 96 samples in parallel, reducing substantially the extraction time required in the sample preparation step [169]. In this work, the magnetic properties of MILs were again exploited and even though the sample volume was 1.5 mL, this configuration can allow the use of smaller volumes for limited-availability sample preparation.

2.2.2. Membrane-based liquid phase microextraction

Different operational configurations resulted from the miniaturization of HF-LPME. In 2010, the first miniaturized configuration of membrane-based LPME in a microfluidic format was described under the name of droplet-membrane-droplet LPME [170]. In this technique, analytes are extracted from a small drop of sample (*ca.* 10-15 µL) to 10 µL of acceptor phase through a SLM (see **Figure 2.4.a**).

Pedersen-Bjergaard and co-workers applied LPME and EME into a chip, providing very high extraction efficiencies thanks to the short distances for analyte diffusion within the microfluidic chip. In addition, flowing instead of static phases and electrical field across the SLM improved extraction efficiencies [171,172]. These configurations have been used for diverse analysis of samples of limited availability such as biological fluids [173–179]. An alternative driving force has been proposed without the application of an electrical potential. Instead, the extraction of target analytes across the SLM is driven by a strong pH gradient. This setup allowed the online coupling to a mass spectrometer or LC system [180]. Coupling with capillary electrophoresis, using a crack in the capillary for SLM was also reported and coined nano-EME, allowing the extraction of analytes from 200 µL of sample into 8 nL of acceptor phase [181]. The main advantages of microchip membrane-based LPME and EME include minimal organic solvent consumption, the ability to handle a wide range of sample volumes, ease to use, potentially high enrichment factors from small sample volumes, and the ability to provide selective extraction of analytes depending on their polarity and charge.

Micro-electromembrane extraction (µEME) was proposed as an alternative miniaturized set-up in which the organic phase is formed as a plug of free liquid membrane between donor and acceptor solution inside of a capillary [182,183] (see **Figure 2.4.b**). The electrodes are inserted through both ends of the capillary

into the donor and acceptor solution, and the extraction is then carried out. This approach has also been fully automated and coupled to LC [184].

PALME and Pa-EME, as previously described, have been described in a 96-well plate format (see **Figure 2.4.c**) with low sample (up to 250 µL) and solvent (*ca*. 25-100 µL) consumption, high throughput and easy automation. These features make this technique a reliable approach for bioanalysis, where sample volume is often limited, and, consequently, has been recently employed for it [185–187].

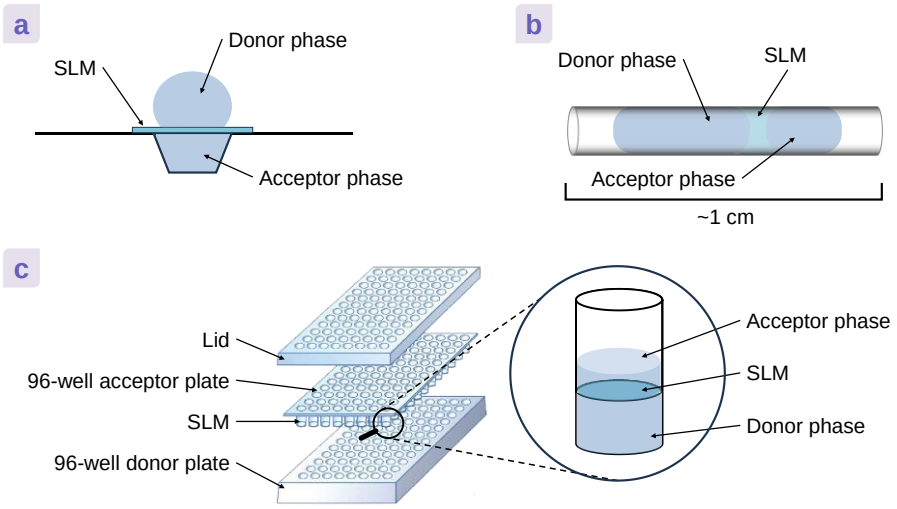


Figure 2.4. Schematic representation of (a) droplet-membrane-droplet LPME; (b) µEME; (c) PALME. Adapted with permission from [170], [182] and [106], respectively.

2.2.3. Dispersive liquid-liquid microextraction

Very few analytical methods employing miniaturized versions of DLLME can be found in literature, probably because the separation of the cloudy solution may be tricky at such small scale. However, some applications employing sample volumes between 50 µL and 1 mL, either air- [188,189] or vortex-assisted [190,191] have been described. More recently, Karami *et al.* adapted DLLME to the chip format [192]. The chip was designed exploiting the advantages of centrifugal microfluidic devices including bubble-free liquid handling, no need for any interface to external pumps, scalable centrifugal forces for sedimentation, no dead volume, and capability to be fully automated. This downscaling significantly decreased the amount of sample, reagent, and energy consumption,

shortened the analysis time, and provided good prospects for portability and high throughput.

Finally, it is worth mentioning the approach presented by Carasek and co-workers coined parallel-dispersive droplet extraction (Pa-DDE) [193]. In this technique, a MIL is formed *in situ*, achieving the dispersion of the acceptor phase and thus a rapid extraction of the analytes, which in combination with the 96-well format, provided excellent sample throughput.

2.2.4. Other solvent-based miniaturized approaches

Recently, You *et al.* [194] developed the so-called surface nanodroplet-based nanoextraction, where nanodrops of acceptor phase are formed onto the inner walls of a capillary tube. Due to the narrow diameter of the capillary, reliable extraction is achieved with a sample volume as low as 50 µL. Besides, this technique has emerged as an important advance for sample preparation thank to its high extraction efficiency, simplicity of operation, low cost and high environment benignity [195].

Wells and Kennedy described slug-flow nanoextraction (SFNE), based on droplet microfluidics, that allows multiple liquid−liquid extractions to be performed simultaneously in a capillary tube, using only 5 nL of both sample and extraction solvent per extraction [196].

2.3. Discussion

As discussed at the beginning of this chapter, one of the main objectives of Analytical Chemistry for the last decades has been to miniaturize the whole analytical process to be in accordance with the principles of GAC. Many researchers have turned to downscaling of separation and detection instruments, and the sample preparation stage has not been exempted from this trend. The common issues have been mainly to reduce the sample and solvent consumption, the waste generation, the execution costs, and the analysis time, all this to prioritize the development of sustainable and environmentally friendly methodologies. Additional inputs for new sample preparation methods are prospective portability and automation, reduced energy consumption, and analyst ergonomics. The microextraction techniques have played a very important role in this context since many analytical researchers have focused on them by developing new approaches or improving existing ones to adjust them to these requirements. In fact, the difference in these terms between the first techniques that emerged in the 90s and 2000s, and their subsequent evolutions, is already evident, as demonstrated during this chapter.

Among the possibilities to achieve more sustainable and feasible methods, great efforts have been done to use greener alternatives to the toxic and pollutant conventional extraction phases [68]; to introduce new materials, either liquid or solid, with specific characteristics to increase the selectivity of the analysis [21]; and/or to reduce their amounts [197]. In many cases, this reduction of extractant amount is a result of the system downscaling, as a means to achieve greener strategies, but also to solve specific problems and respond for the need of specialized research areas. Until today, it seems evident that it is easier and more affordable to reduce the extraction phase when it is a liquid phase, such as microfluidic platforms, than if it is a solid phase. In fact, using semi-automated systems that allow working with volumes of a few nanoliters is already a reality. On the contrary, using a few micrograms of sorbent material usually requires its previous dispersion in a liquid carrier, thus making it possible to manipulate such reduced amounts.

However, as also previously mentioned, the scientific community has traditionally not focused as much on reducing the volume of the sample used. Thus, in the literature we find numerous works in which a few microliters of solvent or a few milligrams of sorbent are used as extractant, but the sample volumes are still of the order of several milliliters, even tens.

On the other hand, it is worthy to mention those cases where the sample volume is drastically reduced to a few microliters as have been complied in this chapter. This is especially advantageous for samples with low availability, such as biological fluids. It should be noted that, although it is true that using high sample volumes generally allows higher enrichment factors (by a high donor/extraction phase ratio), in the case of using small sample volumes similar to those amounts used for the extraction phase, the microextraction would play a role of cleaning rather than preconcentration. Anyway, this is going to be a major trend to follow in the coming years, ushering in the next generation of miniaturized sample preparation approaches.

At this point it is important to make a discussion about terminology in the sample preparation field. The term “miniaturization” must be understood correctly. As it is clearly associated with a reduction in size and amounts, it should be conceived as a relative term, considering what is the “normal scale” [9]. From a practical approach, volumes of hundreds of microliters have been widely employed as extraction phase, but should these techniques not be considered as “microextraction” techniques because they employ amounts above 100 µL or 10 mg? How should they be termed then if they are clearly not LLE or SPE, respectively? It should be much simpler, and the term “microextraction” should be understood as reduced versions of classical SPE and LLE but not strictly restricted to the use of tens of µL of solvents or units of mg of sorbents.

It is true, however, that great advances have been made in the microextraction field over the decades and many approaches described nowadays are much more downscaled versions when compared with the primal microextraction techniques. Therefore, it has been recently proposed by the research group hosting this Doctoral Thesis to coin as “ultramicroextraction” techniques all those new generation procedures conceived as further miniaturized approaches of the already miniaturized microextraction techniques, which are focused not only on minimizing extraction phase amounts but also on reducing sample amount, and meeting other purposes of sustainable chemistry, such as reduced waste generation, portability, etc [25]. In analogy to “ultraviolet” or “ultracentrifugation”, this term widens the microextraction field with the prefix “ultra-” which means “beyond the ordinary level” or “to a greater extent”. Expanding IUPAC’s definition of “microextraction” [18], “ultramicroextraction” could be defined as those microextraction techniques employing sample volumes below 100 μL and acceptor phases below 10 μL or 1 mg as tentative threshold. However, as previously discussed, some degrees of miniaturization are limited by the ability of manipulating such small amounts. In fact, in many cases, donor and acceptor phase volumes may be similar but they still play an essential cleaning role in analytical processes. Likewise, they should be considered “ultramicroextraction” techniques if the sample volume is drastically reduced, even though there are no great differences between donor and acceptor phase volumes. In this way, the evolution of microextraction techniques is properly evidenced, as it would not be fair to categorize all these new downscaled approaches in the same way as those earlier ones.

From this perspective, why not using the term “nanoextraction” as downscaled microextraction? To this regard, it should also be noted that during the last decade, some authors have already coined the term “nanoextraction” to their approaches with apparently different criteria, based on the physical size of the material used (*i.e.,* nanomaterials *vs.* micromaterials). Nonetheless, nanomaterials have been extensively used in microextraction approaches for quite some time, so changing this terminology at this point is difficult, if not impossible, and not correct since the use of nanostructured materials does not guarantee the use of low amounts of them. Therefore, following the previous statements, the term “nanoextraction” could be reserved for the next generation, already on the way, of miniaturized extraction techniques with further reduced dimensions beyond ultramicroextraction, using both extraction phases and sample volumes in the nanoliter/microgram level, as properly employed by authors of some of those contributions described in **Section 2.2.4**.

As previously stated, the main driving force for these efforts of the analytical community is to commit to principles of GAC and GSP. Along with this trend, over the last two decades, different metric tools have been described to assess the greenness of analytical methods. They give a general idea of how in accordance with the principles of Green Analytical Chemistry a method is. They provide

estimations with graphical and/or numerical results, in such a way they can guide researchers to greener practices. Analytical Eco-Scale [198], Green Analytical Procedure Index (GAPI) [199], Analytical GREEnness Metric Approach (AGREE) [200], Analytical GREEnness Metric for Sample Preparation (AGREEprep) [201] or Sample Preparation Metric of Sustainability (SPMS) [202] are just a few examples of the several existing green metric tools. Some other metric tools evaluate other parameters related with WAC, such as analytical performance and applicability. Some examples are the hexagon-CALIFICAMET [203], the Red-Green-Blue (RGB) metric [204], the Need, Quality and Sustainable index (NQS) [205], and the Blue Applicability Grade Index (BAGI) [206].

Within this wide catalogue of evaluating tools, each one adopts its own rules and considerations to evaluate the sustainability of a methodology. For instance, some of them are focused on the entire analytical process and others on a specific step. The aim of this chapter is not to describe the criteria and fundamentals of these metrics, but the reader is encouraged to revisit any of the very interesting reviews that have been recently published about the most commonly used metric tools [207–210]. In these reviews, a wide description and discussion of each metric tool has been done applying them to different case studies, and also analyzing their advantages and limitations. In general terms, each tool presents its advantages and disadvantages and is appropriate for certain applications, but none is a universal one. Hence, the improvement of the existing ones or the development of the definitive sustainability assessment tool is highly encouraged.

Recently, a review article from the research group in which this Doctoral Thesis has been conducted was published with the aim of evidencing the importance of miniaturization in the development of greener methodologies. In this review, the influence of miniaturization in the evolution of sample preparation approaches in terms of greenness, comparing conventional extraction techniques, microextraction techniques and further-downscaled strategies for certain applications is evaluated [211], showing the beneficial impact of miniaturization.

2.4. Conclusions and future trends

In summary, miniaturization has undoubtedly become a powerful trend in Analytical Chemistry. Downscaled methods coupled in many cases to a greater or lower degree of automation offer important advantages, which are highly valued in analytical laboratories. High reduction of solvents and sorbents leads to reduced waste generation, downscaled extraction devices and thus lower analysis time and cost. Furthermore, an effective reduction of the analytical system increases the chances of portability and diminishes energy consumption and environmental pollution.

Even though high accomplishments have been achieved in this field, such as microfluidic- and chip-based microextraction techniques, some aspects of the analytical process may still need some improvement. Some steps like solvent/sorbent recovery in dispersive techniques are usually the main limitations for automation and higher degrees of miniaturization. Future research should be focused on finding alternatives to these procedures as well as expanding the variety of techniques to be used with volumes as low as a few nanoliters of sample, which is essential for low-availability samples. In this sense, ultramicroextraction is not the end of the road to miniaturization of sample preparation in Analytical Chemistry but only an intermediate step on the way to actual nanoextraction and beyond.

SECTION 2

EXPERIMENTAL, RESULTS AND DISCUSSION

Chapter 3

Trace determination of tetrahydrocannabinol in cosmetic products by stir bar sorptive dispersive microextraction followed by liquid chromatography-tandem mass spectrometry

The content of this chapter has been published in the following journal article:

C. Azorín, J.L. Benedé, A. Chisvert, A. Salvador, Trace determination of tetrahydrocannabinol (THC) in cosmetic products by stir bar sorptive dispersive microextraction followed by liquid chromatography-tandem mass spectrometry, Talanta 253 (2023) 123934

Abstract

An analytical method for the determination of Δ^9-tetrahydrocannabinol (THC) at trace level in cosmetics is presented. As a psychoactive compound, the presence of THC in consumer products should be avoided. However, it might be unintentionally present in cannabidiol-rich or hemp-based products by contamination or isomerization of cannabidiol (CBD). Due to the low concentrations expected, a sensitive and selective method is necessary for the analytical control of these products. In this sense, the presented method is based on stir bar sorptive dispersive microextraction (SBSDME) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In this chapter, a magnetic composite made of $CoFe_2O_4$ magnetic nanoparticles (MNPs) embedded in a commercial reverse-phase polymer (*i.e.*, Strata™-X-RP) was employed as magnetic sorbent material taking advantage of its affinity to the target analyte. Under the optimized conditions, the method was validated and showed good analytical features in terms of linearity (at least up to 10 ng mL^{-1}), limits of detection and quantification (2.2 and 7.2 ng g^{-1}, respectively) and precision (relative standard deviation < 10%). Moreover, relative recoveries between 99 and 109% were obtained, showing that matrix effects were negligible using deuterated THC (THC-D_3) as surrogate. This new approach was successfully applied to ten commercially available cosmetic samples of different matrices, thus showing it is suitable for the analytical control of THC in cosmetic products. The proposed methodology overcomes some of the drawbacks of the previous works with the same purpose, such as the higher limits of detection, time-consuming procedures, and consumption of large volumes of organic solvents.

3.1. Introduction

Cannabis sativa L. (hemp) is a plant species, mainly recognized for its use as recreational drug, which contains approximately 60 natural cannabinoids. These compounds can interact with the animal endocannabinoid system, which is involved in the regulation of appetite, pain sensitivity, mood and memory, and some of them exhibit pronounced psychoactive properties. Among all these compounds, its major constituents are CBD and THC, the latter widely known for its psychoactivity [212]. Over the last years, hemp extracts and cannabinoids (especially CBD) have been used as part of many consumer products such as food, medicines and cosmetics, seeking to exploit their anti-inflammatory, analgesic, hydrating, moisturizing and wrinkle-reducing properties [213].

European Regulation on cosmetic products [214] prohibits the presence of THC in cosmetic formulations as it is considered a psychotropic drug [215]. Additionally, hemp-derived raw materials must be obtained from specific parts of the plant (*i.e.*, seeds and leaves without the upper part of the plant) from hemp varieties with a THC content below 0.3% [216]. However, this limitation refers to the plant itself but not to the final product.

Even though this legislation pursues the absence of THC in the consumer product, different sources of impurities can hinder this goal. On the one hand, poor manipulation of the hemp plant can cause THC from upper parts of the plant to reach the extracts [217]. On the other hand, some studies manifest that, under certain conditions, cyclization of CBD to obtain THC can occur [218] and, even though the yield is low, it is still a subject of discussion [219]. In fact, THC has been previously found in cosmetics at levels up to 500 μg g^{-1} [220,221]. In this sense, analytical control to monitor the THC levels in those cosmetic products containing CBD and hemp extracts is of great interest.

As a result of their use as recreational drug, cannabinoids (including THC) have awakened interest in researchers and many methods for their determination in biological matrices, plant material and environment have been proposed [222–226]. In addition, the revolution of hemp-based products has led to an increase in awareness about safety of these products and different methods for the determination of cannabinoids in consumer products (including cosmetics) have been developed [220,221,227–231].

About sample treatment, direct determination of compounds at trace levels in complex matrices, such as cosmetics, is not often feasible. Therefore, it usually requires (micro)extraction techniques to separate the target analytes from the matrix, to concentrate them, and to condition the sample to the analytical instrument, in a just single step [232,233]. In this context, vortex- (VAE) and/or ultrasound-assisted extraction (UAE) [221,227,229–231], or a combination of liquid-liquid extraction (LLE) with solid-phase extraction (SPE) [220] have been

used to retrieve THC from the cosmetic matrix and, in the last case, to preconcentrate it. However, the limits of detection of some of these methods are relatively high (μg g^{-1}) to permit the quality control of a prohibited substance in cosmetic products such as THC. Many of them also consume large amounts of organic solvents, and/or they are time-consuming procedures, which does not meet the current trends within Analytical Chemistry.

The aim of this chapter is to develop a selective and sensitive analytical method based on SBSDME followed by LC-MS/MS analysis for the trace determination of THC in hemp-based cosmetic products. For this purpose, a magnetic composite made of $CoFe_2O_4$ MNPs embedded into a commercial pyrrolidone-modified styrene-divinylbenzene copolymer (*i.e.*, Strata™-X-RP) was used as sorbent material. The main parameters affecting the extraction step were optimized by a response surface methodology (RSM) [234], whereas a univariate approach was used to study the desorption parameters. LC-MS/MS was selected as analytical technique for its high selectivity and sensitivity, useful for trace analysis in cosmetic matrices.

3.2. Experimental

3.2.1. Reagents

All reagents and solvents were obtained from major suppliers. (-)-trans-THC solution 1.0 mg mL^{-1} in methanol (certified reference material) used as standard, and (-)-trans-THC-D_3 solution 100 μg mL^{-1} in methanol (used as surrogate) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For the synthesis of $CoFe_2O_4$ MNPs, cobalt(II) chloride hexahydrate and iron(III) chloride hexahydrate were purchased from Acros Organics (Fair Lawn, NJ, USA), and sodium hydroxide (reagent grade) was purchased from Scharlau (Barcelona, Spain). For the synthesis of $CoFe_2O_4$-Strata™-X-RP composite, Strata™-X 33-μm reversed phase polymer was taken from SPE cartridges (500 mg/6 mL) from Phenomenex (Torrance, CA, USA). Ethanol absolute was obtained from Panreac (Barcelona, Spain).

Ultrapure water (resistivity ≥ 18.2 MΩ·cm) was obtained from a Connect water purification system provided by Adrona (Riga, Latvia). LC-grade acetonitrile was obtained from Panreac. Sodium chloride used as ionic strength regulator was purchased from Scharlau. For the preparation of phosphate buffers, ortho-phosphoric acid and sodium dihydrogen phosphate monohydrate from Scharlau and sodium phosphate dodecahydrate from Sigma-Aldrich were used.

LC-MS grade methanol from Honeywell (Seelze, Germany), LC-MS grade water from Panreac and ammonium fluoride (98%) from Acros Organics were used to prepare the chromatographic mobile phase.

Nitrogen used as nebulizer and curtain gas in the MS/MS ion source was obtained by a NiGen LCMS 40-1 nitrogen generator from Claind S.R.L. (Lenno, Italy). Extra pure nitrogen (> 99.999%), used as collision gas in the MS/MS collision cell, was provided by Praxair (Madrid, Spain).

3.2.2. Samples

Ten commercial cosmetic samples with different composition, including creams, facial and hair masks, and shower and refreshing gels were purchased at local stores. All of them included the words “cannabis sativa seed oil” or “cannabidiol” as ingredients on their labels. Their brands are not mentioned for confidentiality reasons.

3.2.3. Apparatus

For the synthesis of the magnetic composite, a heating plate with magnetic stirrer from Stuart Scientific (Staffordshire, United Kingdom) and an oven from J.P. Selecta (Barcelona, Spain) were used.

For the LC-MS/MS analysis, an Agilent 1100 Series chromatography system comprised of a degasser, a binary pump, an autosampler and a thermostatic column oven, coupled to an Agilent 6410B Triple Quad MS/MS was employed.

A 10-position multiple stirring plate model MS-M-S10 from Labbox (Barcelona, Spain) and NdFeB magnets (54 MGO, 10 mm length x 3 mm diameter) from Supermagnete (Gottmadingen, Germany) were used for the dispersion of the magnetic composite in the extraction procedure.

An ultrasound bath (50 Hz, 360 W) from J.P. Selecta (Barcelona, Spain) was used for the dispersion of samples.

3.2.4. Synthesis of $CoFe_2O_4$-Strata™-X-RP magnetic composite

The synthesis of the $CoFe_2O_4$-Strata™-X-RP composite consisted of two steps:

1. Synthesis of the $CoFe_2O_4$ MNPs by wet chemical co-precipitation according to an adapted protocol [235].
2. Subsequent incrustation of MNPs on the polymeric surface.

First, 10.80 g of $FeCl_3 \cdot 6H_2O$ (40 mmol) and 4.76 g of $CoCl_2 \cdot 6H_2O$ (20 mmol) were dissolved in 200 mL of ultrapure water, and then 100 mL of a 3 M sodium hydroxide aqueous solution (300 mmol) were added dropwise under continuous stirring for one hour at 80 ºC. After this time, it was allowed to cool to room temperature to decant the formed solid using a magnet. Then, the solid was washed several times with ultrapure water and later with ethanol, dried in an oven at 80 ºC overnight, and grinded until obtaining a fine black powder.

For the preparation of the composite, where MNPs are embedded on the polymer surface, 0.15 g of the MNPs and 0.15 g of Strata™-X-RP were weighted, and 50 mL of ethanol were added. The mixture was stirred at room temperature for 72 h to ensure the MNPs were embedded on the polymer pores. Finally, the solid was decanted with the aid of a magnet and washed three times with ethanol to discard the remaining non-magnetic polymer. Afterwards, it was resuspended in ethanol and filtered under vacuum through a 10 µm-pore paper filter in order to discard the free MNPs and isolate the composite. It was dried in an oven at 80 ºC overnight and grinded into a fine powder.

3.2.5. Preparation of standard and sample solutions

The commercial 1.0 mg mL^{-1} THC solution was properly diluted to obtain a 200-µg mL^{-1} solution in methanol and was kept at -20 ºC protected from light. From this solution, an intermediate solution of 1 µg mL^{-1} in methanol was prepared weekly. A methanolic solution containing 250 ng mL^{-1} of THC-D_3 was also prepared weekly from the commercial solution and both were kept at -20 ºC protected from light. Subsequently, from these intermediate solutions, working standard solutions of THC (0.05 – 10 ng mL^{-1}), all of them containing 1 ng mL^{-1} of THC-D_3, were freshly prepared by proper dilution with a mixture of methanol:water 60:40 (v/v).

Regarding sample preparation, 0.05 g of homogenized sample were weighted into a 50-mL volumetric flask, 1 ng mL^{-1} of THC-D_3 was added and diluted up to the mark with a mixture of methanol:water 60:40 (v/v). Difficult-to-dissolve samples required sonication in an ultrasonic bath for *ca.* 5 to 10 min at room temperature for the correct leaching of the analyte from the sample, which was demonstrated that did not affect the analyte stability. Solutions were filtered under vacuum through a nylon filter with pore size of 0.45 µm to remove the non-soluble compounds.

3.2.6. SBSDME procedure

In dry vials, 5 mg of the $CoFe_2O_4$-Strata™-X-RP composite were weighted, a neodymium stir bar was inserted, and 2 mL of methanol were added to each of

them. Next, they were subjected to vigorous stirring (*ca.* 1000 rpm) for 1 min to ensure proper conditioning and washing of the magnetic material. Once this time elapsed, they were left to stand until the return of the composite to the stir bars was accomplished.

For the extraction procedure, the neodymium stirring bars covered with the previously conditioned material were inserted, with the help of plastic tweezers, into a series of 40-mL vials, which were placed on the multiple stirring plate. Then, 10 mL of previously prepared standard or sample solutions were introduced into each vial and vigorously stirred at *ca.* 1000 rpm for 15 min to achieve total dispersion of the composite within the solution, thus favoring the extraction of the analyte. Afterwards, the stirring was stopped, and the vials were left to rest so that the magnetic composite was retrieved by the magnetic field and recoated the stir bars again. Next, the stir bars were taken with plastic tweezers, and they were introduced into smaller vials, each of them containing 300 µL of acetonitrile to carry out the liquid desorption of the analyte. After 30 s of vigorous stirring (*ca.* 1000 rpm), the solvent was filtered through a 0.22-µm nylon syringe filter and transferred to injection vials to perform the LC-MS/MS analysis. **Figure 3.1** shows a schematic diagram of the proposed method.

3.2.7. LC-MS/MS analysis

Separations were carried out in a Zorbax SB-C18 (50 mm length, 2.1 mm I.D., 1.8 µm particle size) column from Agilent Technologies. Five microliters of each solution were injected into the chromatographic system. Mobile phase consisted of solvent A (water, 0.5 mM ammonium fluoride) and solvent B (methanol), by isocratic elution at a mixing ratio of 15:85% (v/v). The flow rate was 0.25 mL min^{-1} and the column temperature was kept constant at 35 ºC. The run time was 4 min.

The triple quadrupole MS detector operated in electrospray ionization mode (ESI), by multiple reaction monitoring (MRM). Specifically, positive polarity (ESI^+, capillary voltage at 6 kV) was used to measure the target analyte. The rest of conditions were: gas temperature at 310 °C, nebulizer gas flow rate at 12 L min^{-1}, nebulizer gas pressure at 35 psi and dwell time at 80 ms.

The m/z precursor → product ion transitions (collision energy, fragmentor) for quantification of THC and THC-D_3 were 315.2 → 193.1 (22 eV, 150 V) and 318.2 → 196.2 (26 eV, 140 V), respectively; and for identification they were 315.2 → 123.1 (34 eV, 150 V) and 315.2 → 93.1 (30 eV, 150 V) for THC, and 318.2 → 123.1 (38 eV, 140 V) and 318.2 → 93.1 (30 eV, 140 V) for THC-D_3.

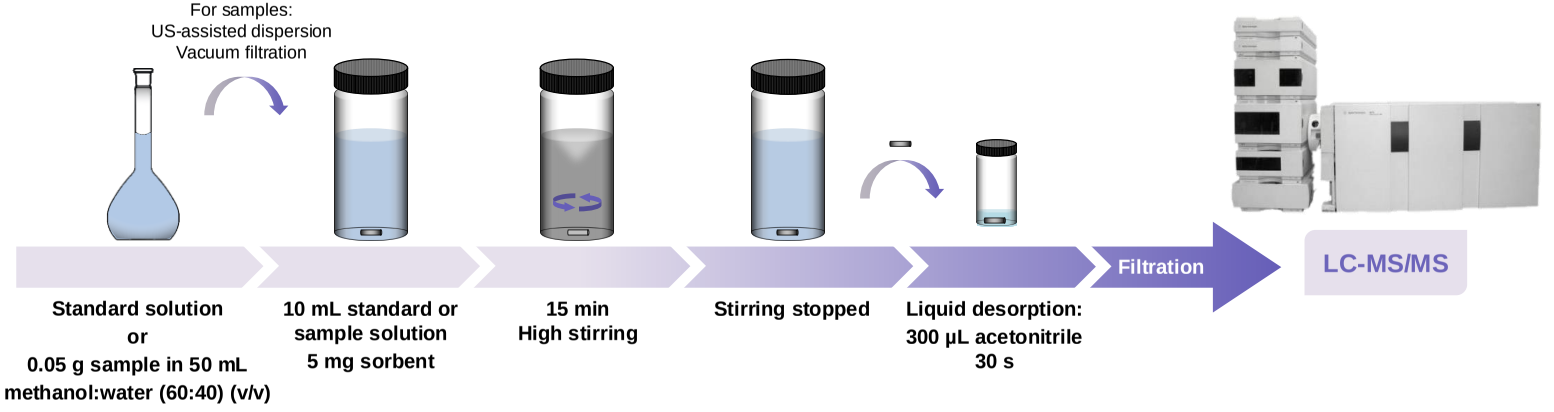


Figure 3.1. Schematic diagram of the proposed SBSDME – LC-MS/MS method.

The following figure shows a chromatogram of a standard solution after applying the proposed method.

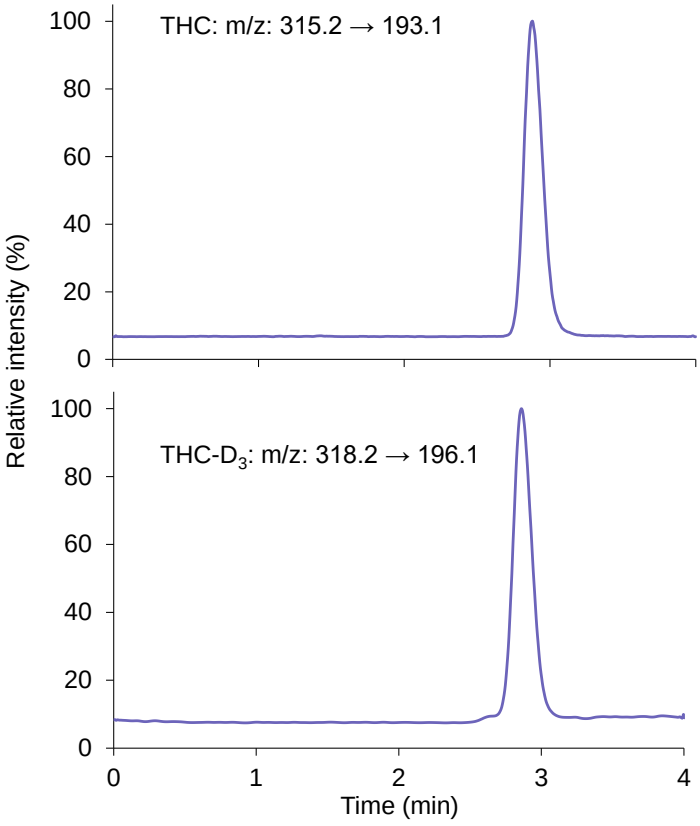


Figure 3.2. Chromatogram of a standard solution containing 1 ng mL^{-1} of THC and THC-D_3 (as surrogate) obtained by the proposed method.

3.3. Results and discussion

3.3.1. Preliminary experiments

During the selection of the chromatographic variables, some retention of THC on polypropylene material (*e.g.,* micropipette tips, syringes, filter holdings) was observed probably due to its high hydrophobicity (Log $K_{o/w}$ = 6.4). As shown in **Figure 3.3**, when a standard solution of THC was transferred using a plastic syringe, passing or not through a nylon filter, the signal decreased compared with the use of a glass Pasteur pipette. For this reason, working with this type of

material was avoided as far as possible throughout the work, using glass material instead. It should be noted that different filter materials (*i.e.*, nylon, cellulose acetate and polyvinylidene fluoride) were tested but comparable results were obtained.

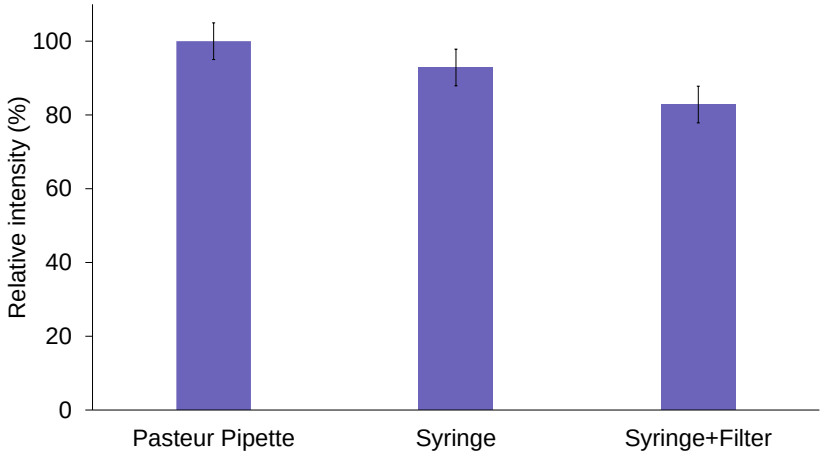


Figure 3.3. Effect of different ways of transferring a standard solution of 10 ng mL^{-1} on the THC signal.

In any case, one of the main advantages of using the deuterated analyte as surrogate is that these unavoidable and undesirable interactions are the same for both analyte and surrogate, hence they are corrected, and this burden can be easily overcome. In this sense, the presence of THC-D_3 in both standard and sample solutions allows them to be filtered through a syringe filter after the SBSDME procedure, avoiding more time-consuming steps like centrifugation.

3.3.2. Selection of the composite

As it was mentioned above, THC exhibits high hydrophobicity, hence the selection of $CoFe_2O_4$-Strata™-X-RP as sorbent material sought to exploit the ability of the pyrrolidone-modified styrene-divinylbenzene copolymer (*i.e.*, Strata™-X-RP) to interact with the analyte by hydrophobic interactions. Moreover, THC has an aromatic ring and a double bond able to interact by π-π stacking, and a phenolic moiety to stablish hydrogen bonding.

The $CoFe_2O_4$ MNPs confer to this sorbent the magnetism needed for an easy retrieval by means of the magnet in SBSDME. Cobalt ferrite ($CoFe_2O_4$) MNPs were preferred rather than usually-employed Fe_3O_4 MNPs due to their higher chemical stability [235]. The characterization of the material was described in a previous work of the research group [143]. Magnetization, particle size

distribution, morphology, specific surface area and pore size were established. In addition, energy dispersive X-ray spectroscopy (EDS) was performed for elemental analysis.

In order to study the extraction efficiency of $CoFe_2O_4$-Strata™-X-RP, different experiments were carried out employing bare MNPs, Strata™-X-RP copolymer and the magnetic composite. For Strata™-X-RP, as is not magnetic, the retrieval of the material was performed by centrifugation at 6000 rpm for 5 min. **Figure 3.4** shows that the extraction performance of MNPs was negligible, whereas both polymer and composite provided comparable results, thus showing that MNPs do not diminish the extraction capability of the polymer. As a result, it can be concluded that the responsible for the extraction of the analyte is the polymeric material.

Three different batches of composite synthesized as described in **Section 3.2.4** were used to extract a standard solution (under non-optimized conditions) and results were compared. The relative standard deviation (RSD) was below 7%, hence showing good repeatability for the synthesis and performance of different batches of the composite.

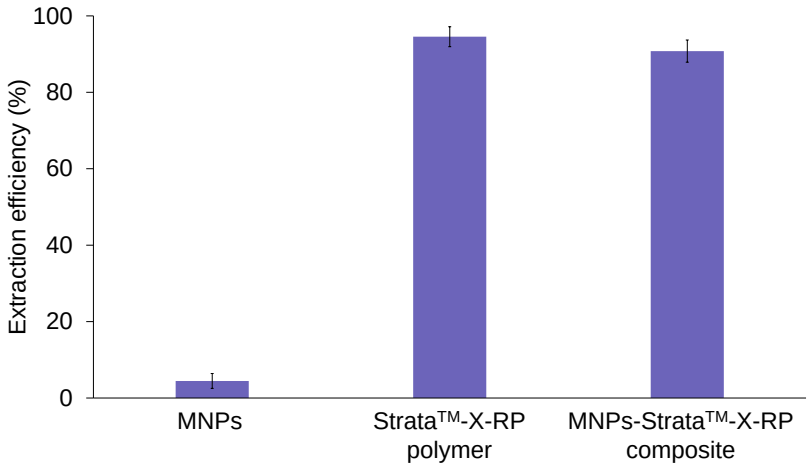


Figure 3.4. Comparison of the extraction efficiency between bare MNPs, Strata™-X-RP polymer and MNPs-Strata™-X-RP composite.

3.3.3. Optimization of the extraction variables in SBSDME

The donor phase is typically aqueous in most SBSDME applications [56], what benefits the interaction between analytes and sorbents when hydrophobic

interactions are relevant. Thus, the extraction of THC would be enhanced by the hydrophobic nature of the analyte, whose polarity is more similar to the hydrophobic sorbent than to the aqueous donor phase. However, THC is expected to be present within a cosmetic matrix containing a wide range of both polar and non-polar compounds (usually emulsions). The sample could be dispersed in water, but the hydrophobic analyte would be retained within the non-polar fraction of the matrix. A pure organic solvent such as methanol, where the cosmetic matrix can be easily dispersed and the analyte is soluble would be useful, but then, the extraction into the hydrophobic sorbent would be hindered. In this sense, a mixture of methanol and water balances both effects.

Different proportions of water were tested using a 10-ng mL^{-1} standard solution, in order to achieve the best results. **Figure 3.5** shows the effect of the proportion of water in the donor phase in terms of enrichment factor, which was defined as the ratio between the area of a standard solution after the extraction to the initial area. At low proportions of water, the organic nature of the donor phase hindered the extraction of the analyte because of the solvation of THC in methanol, worsening its transference to the sorbent. This effect was reduced as the aqueous content increased. However, at proportions of water higher than 60% (v/v) the enrichment factor decreased, probably due to the worse dispersion of the hydrophobic composite. Therefore, a mixture of methanol:water 60:40 (v/v) was selected as the optimal donor phase, allowing both good dispersion of the cosmetic sample and good extraction performance.

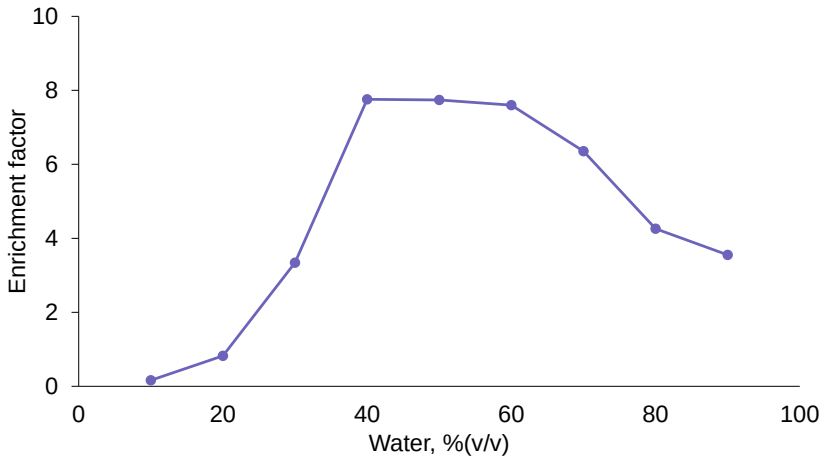


Figure 3.5. Effect of the proportion of water in the donor phase on the enrichment factor. (Extraction conditions: sample volume, 10 mL; sorbent amount, 10 mg; extraction time, 10 min; pH, not adjusted; ionic strength, not adjusted. Desorption conditions: solvent, methanol; solvent volume, 0.5 mL; desorption time, 5 min).

After the donor phase was studied, an RSM [234] based on a four-factor three-level Box-Behnken design was performed to stablish the optimal values of some other variables involved in the extraction procedure. The description of the statistics of the Box-Behnken design are included in **ANNEX I**. The studied variables and ranges were: (i) sorbent amount (5-25 mg), (ii) extraction time (5-30 min), (iii) pH of the aqueous portion of donor phase (2-10, 5 mM phosphate buffer) and (iv) ionic strength of the aqueous portion of the donor phase (0–20% (w/v) NaCl).

In this sense, according to **Eq. A-I.1**, considering 4 factors and 3 replicates of the central point, 27 experiments were required (**Table 3.1**).

10 mL of standard solution containing 10 ng mL^{-1} of THC in methanol:water 60:40 (v/v) were used to perform the experiments. After the extraction, the analyte was desorbed in 300 µL of methanol for 5 min. The selected analytical responses were the peak area of the analyte. A total of 54 experiments were performed, carrying out the design by duplicate. StatGraphics Centurion XVI (Stat Point Inc. Herndon, VA, USA) software was employed for the statistical analysis.

As shown in **Figure 3.6**, sorbent amount and ionic strength were the variables that most appreciably affected the extraction performance. The adequacy of the model was evaluated by the coefficient of determination (R^2), which was ≥ 0.89. This value indicates that the designed model was efficient for the prediction of response.

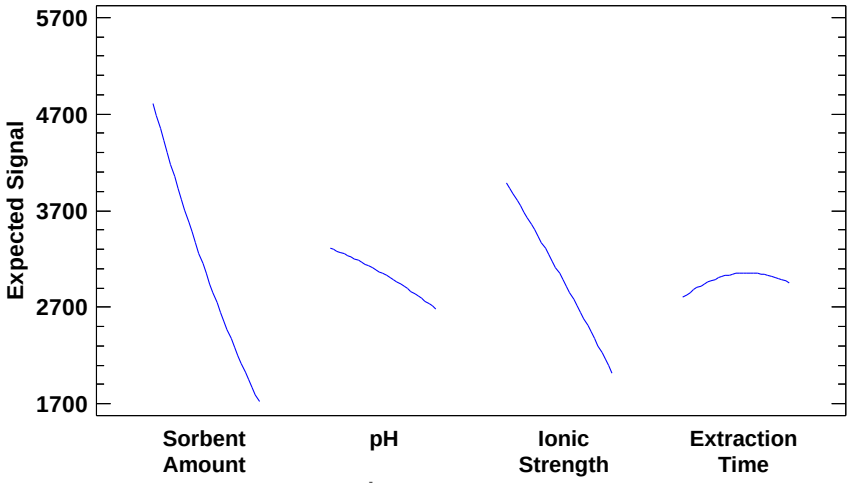


Figure 3.6. Correlation of the different variables that potentially affect the extraction of THC.

Table 3.1. Box-Behnken design for multivariate optimization of the critical variables

Step	Sorbent amount (mg)		pH		Ionic strength (% (w/v) NaCl)		Extraction time (min)	
	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded
1	5	-1	2	-1	10	0	17.5	0
2	25	1	2	-1	10	0	17.5	0
3	5	-1	10	1	10	0	17.5	0
4	25	1	10	1	10	0	17.5	0
5	15	0	6	0	0	-1	5	-1
6	15	0	6	0	20	1	5	-1
7	15	0	6	0	0	-1	30	1
8	15	0	6	0	20	1	30	1
9	5	-1	6	0	10	0	5	-1
10	25	1	6	0	10	0	5	-1
11	5	-1	6	0	10	0	30	1
12	25	1	6	0	10	0	30	1
13	15	0	2	-1	0	-1	17.5	0
14	15	0	10	1	0	-1	17.5	0
15	15	0	2	-1	20	1	17.5	0
16	15	0	10	1	20	1	17.5	0
17	5	-1	6	0	0	-1	17.5	0
18	25	1	6	0	0	-1	17.5	0
19	5	-1	6	0	20	1	17.5	0
20	25	1	6	0	20	1	17.5	0
21	15	0	2	-1	10	0	5	-1
22	15	0	10	1	10	0	5	-1
23	15	0	2	-1	10	0	30	1
24	15	0	10	1	10	0	30	1
25	15	0	6	0	10	0	17.5	0
26	15	0	6	0	10	0	17.5	0
27	15	0	6	0	10	0	17.5	0

Figure 3.7 shows response surfaces of desirability function (described in **ANNEX I**). As can be seen in **Figure 3.7.a**, higher sorbent amounts had a negative effect on desirability. It can be caused by the worse dispersion and caking of high amounts of magnetic composite in the elution solvent, producing less contact area between the sorbent and the elution solvent, therefore, the minimum amount of sorbent (*i.e.,* 5 mg) was selected. Regarding the extraction time, the best results were obtained at 25 min. However, as can be seen, no relevant differences were found from 15 min, so this value was selected to reduce time consumption.

As shown in **Figure 3.7.b**, pH did not substantially affect the extraction of THC, since its pK_a is 9.81, and therefore it is not charged at most of the pH range tested. However, at very alkaline pH, the analyte is negatively charged and hence the extraction based on hydrophobic interactions is hindered. As a result, pH was not adjusted since the sample solutions were expected to be neutral or slightly acidic. Finally, ionic strength showed a negative effect in extraction efficiency. High concentration of ions usually benefits the so-called “salting-out

effect", favoring the transfer of hydrophobic analytes from the aqueous solution to the hydrophobic sorbent. Nonetheless, in this case the donor phase is a mixture of methanol and water, so higher ionic strength may difficult the dispersion of the material within the donor phase. Therefore, no salt was added to sample solutions in order to minimize the ionic strength of the media.

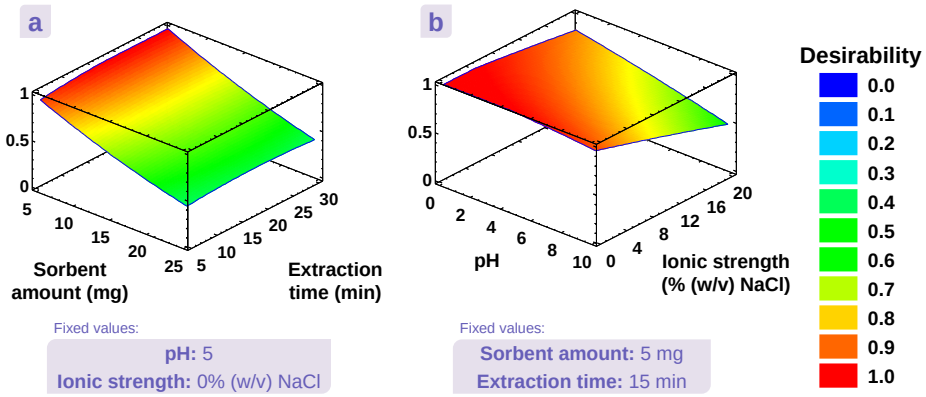


Figure 3.7. Response surface of the desirability function representing the relation between the studied variables: (a) sorbent amount *vs* extraction time, and (b) pH *vs* ionic strength.

In summary, the optimized method consisted of 5 mg of magnetic composite, 15 min of extraction time, and no adjustment of pH and ionic strength was required.

3.3.4. Optimization of the desorption variables in SBSDME

After the optimization of the extraction process, the variables involved in the desorption step were also studied. Different desorption solvents (*i.e.,* methanol, ethanol, isopropanol, and acetonitrile) and times (*i.e.,* 0.5, 1, 5 and 10 min) were tested.

For the study of the desorption solvent, 300 µL of the above-mentioned solvents were tested to desorb the analyte for 0.5 min after the previously optimized extraction of standard solutions. Results are shown in **Figure 3.8**. Ethanol and acetonitrile provided slightly higher signals than methanol and isopropanol. Acetonitrile was selected, since it provided better repeatability and chromatographic performance.

Once acetonitrile was selected as desorption solvent, the desorption time was tested. The composite recovered from the extraction vial was stirred at *ca.* 1000 rpm with 300 µL of acetonitrile for 0.5, 1, 5 and 10 min. It was shown that 0.5 min were enough for the desorption of the analyte.

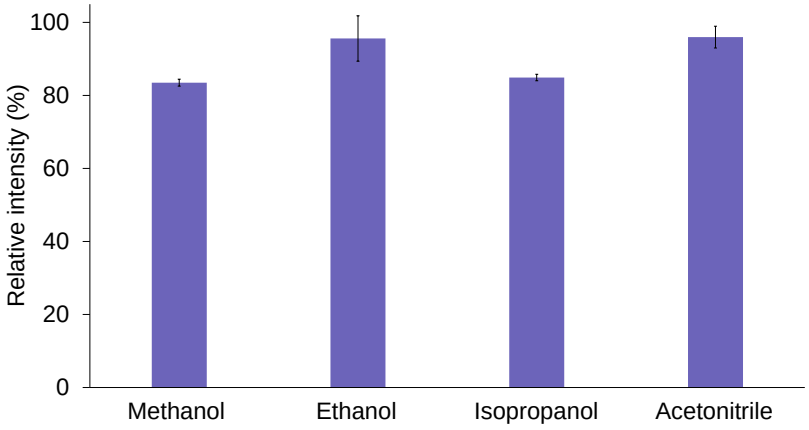


Figure 3.8. Relative intensity of THC obtained for different desorption solvents. (Extraction conditions: sample volume, 10 mL; sorbent amount, 5 mg; extraction time, 15 min; pH, not adjusted; ionic strength, not adjusted. Desorption conditions: solvent volume, 300 µL; desorption time, 0.5 min).

3.3.5. Analytical performance of the proposed method

The quality parameters of the proposed method, such linearity, enrichment factor (EF), extraction efficiency (EE), limits of detection (LOD) and quantification (LOQ), and intra- and inter-day precision, were evaluated under the proposed conditions.

The linearity was studied by measuring standard solutions at different concentrations after applying the proposed method, all of them containing 1 ng mL^{-1} of THC-D_3 as surrogate. Calibration curves were plotted using the ratio of the peak area of the target analyte to the surrogate (A_i/A_s) versus the analyte concentration. Linearity reached at least 10 ng mL^{-1} for THC with good determination coefficient ($R^2 > 0.9994$).

The EF was estimated as the ratio of the signal obtained after the extraction of a standard solution containing 1 ng mL^{-1} of THC and the signal of a standard solution of the same concentration prepared in acetonitrile without extraction. An EF of 19.3 ± 1.2 was achieved for THC, which corresponds to an EE of 58 ± 4%.

The LOD and LOQ were calculated as 3 times and 10 times, respectively, the signal-to-noise ratio of a 50 ng L^{-1} standard solution extracted by the proposed method. The LOD and LOQ values were 2.2 and 7.2 ng L^{-1}, respectively. The method limit of detection (MLOD) and quantification (MLOQ) were obtained from the previous limits considering the sample pretreatment (*i.e.,* the sample amount

weighted and dilution, according to **Section 3.2.5**), and were 2.2 and 7.2 ng g^{-1}, respectively, allowing the determination of THC in cosmetics at low trace level.

The precision of the method was evaluated applying the proposed method to five replicates of standard solutions containing the target analyte at three levels of concentration (*i.e.,* 0.05, 1 and 10 ng mL^{-1}). The results, expressed as RSD were 9.8, 3.4 and 0.7%, respectively, during the same working session (intra-day precision); and 9.5, 5.9 and 8.4%, respectively, in different working sessions (inter-day precision). These results revealed that good precision was achieved for the target analyte.

3.3.6. Analysis of cosmetic samples

In order to assess the applicability of the method, ten commercial samples with different matrices were analyzed. Samples were prepared as specified in the proposed method (see **Section 3.2.5**) prior to the SBSDME procedure and injected into the chromatographic system under the selected conditions. THC was below the MLOD in nine samples, whereas it was detected in one sample (cream) but below the MLOQ. A greater amount of this sample was weighted (*ca.* 0.6 g) to enable the quantification of THC. However, weighting large sample amounts is not always possible (depending on the sample) because filtration gets considerably complicated. This sample contained *ca.* 3.5 ± 0.4 ng g^{-1} of THC, most probably due to contamination during the manufacture process of raw materials ("cannabis sativa seed oil").

The matrix effects were evaluated by means of the relative recovery (RR) values, calculated as follows:

$$RR\ (\%) = \frac{Concentration_{spiked\ sample} - Concentration_{sample}}{Spiked\ amount} \cdot 100 \qquad \text{(Eq. 3.1)}$$

Three representative cosmetic samples of different matrices (*i.e*., a cream, a gel, and a face mask) were weighted and spiked with the appropriate volume of standard solution to get two concentration levels (0.5 and 5 µg g^{-1}), and then prepared according to the proposed method. The concentration of the analyte in both spiked and non-spiked samples was obtained from the proposed method. The obtained RR, calculated from **Eq. 3.1** for each sample are shown in **Table 3.2** and ranged from 99 to 109%, showing that matrix effects were negligible or, in other words, successfully corrected by the isotopically labelled surrogate.

Table 3.2. Relative recovery values obtained for the commercial samples

Sample [a]	Spiked amount (µg g^{-1})	RR (%) [b]
A	0.5	104 ± 7
	5.0	108 ± 2
B	0.5	99 ± 4
	5.0	109 ± 2
C	0.5	103 ± 6
	5.0	100 ± 1

[a] Sample A: gel; Sample B: cream; Sample C: face mask
[b] Mean of three replicates ± standard deviation

3.3.7. Comparison with previously reported methods

The proposed method was compared with other previously reported methods for the determination of THC in consumer products, including cosmetics. As can be seen in **Table 3.3**, the SBSDME approach described in this chapter presents comparable or even better analytical features in terms of precision and accuracy, but much lower MLOD than those methods without a preconcentration stage (*i.e.,* UAE and VAE) [221,227,229–231] and just the lixiviation of the analyte from the matrix to an organic solvent. However, a low MLOD is of utmost importance to carry out the analytical control of prohibited compounds such as THC.

On the other hand, when extraction and clean-up stages (*i.e.,* LLE and SPE) were performed [220], the MLOD was similar to the proposed method, but the consumption of several organic solvents, even organochlorines, was very high. Likewise, this methodology includes repetitive time-consuming steps (*e.g.,* centrifugation, evaporation and reconstitution) that considerably slow down the sample preparation procedure and diminishes the sample throughput.

Against, the proposed method needs to synthesize a sorbent material. However, in the same batch synthesis, enough material is obtained (*ca.* 300 mg) to carry out a high number of extractions, considering that only 5 mg of material are used per extraction.

Table 3.3. Comparison of the proposed method with other approaches for the determination of THC in cosmetic products

Sample Preparation	Instrumental Technique	MLOD (ng g^{-1})	RSD (%)	RR (%)	Ref.
LLE + SPE	GC-MS	2.0	< 20	70-130	[220]
VAE	LC-MS/MS	200	< 25	49-115	[221]
UAE	LC-MS/MS	50	< 10	84-114	[227]
UAE	LC-MS/MS	48	<6	97-111	[229]
UAE + VAE	GC-MS	1000	n.r. [a]	n.r.	[230]
UAE + VAE	LC-UV	10000	< 6	93-102	[231]
SBSDME	LC-MS/MS	2.2	< 10	99-109	This work

[a] n.r.: not reported

3.3.8. Evaluation of the greenness of the proposed method

The greenness of the proposed sample preparation method was evaluated by estimating its AGREEprep score [201], a metric tool for the assessment of analytical sample preparation. AGREEprep establishes a scale from 0 to 1 for each of the ten principles of Green Sample Preparation [5], considering environmental and health impact factors, such as the use of reagents, the energy consumption, sample size and generated waste. The obtained pictogram is showed in **Figure 3.9**.

As it can be observed, a low score of 0.37 was obtained, mainly due to the high consumption of toxic reagents (*i.e.*, methanol) and the generation of *ca.* 10 mL of waste per sample (*i.e.*, donor phase solution). Even though a great improvement on the greenness of the method can be achieved, very good analytical features were obtained, comparing with previously reported methods (see **Table 3.3**). Thus, outstanding analytical performance of the method is obtained in detriment of method greenness but achieving the determination of traces of THC in cosmetics.

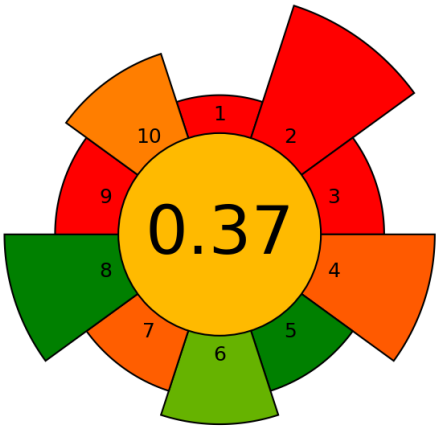


Figure 3.9. AGREEprep pictogram of the proposed method. (1-Sample preparation placement; 2-Hazardous materials; 3-Sustainability and renewability of materials; 4-Waste; 5-Size economy of the sample; 6-Sample throughput; 7-Integration and automation; 8-Energy consumption; 9-Post sample preparation configuration for analysis; 10-Operator's safety).

3.4. Conclusions

This chapter describes a new method based on SBSDME followed by LC-MS/MS analysis for the trace determination of prohibited THC in cosmetics. A magnetic composite material made of $CoFe_2O_4$ MNPs embedded into a commercial pyrrolidone-modified styrene-divinylbenzene copolymer (*i.e.*, Strata™-X-RP) interacts with the analyte through hydrophobic and π-π interactions and hydrogen bonding.

After the optimization of extraction and desorption conditions, and the overcoming of some inconveniences by using THC-D_3 as surrogate, this approach presents good analytical features, allowing a sensitive, simple and rapid determination of the analyte. This methodology has been successfully applied to real cosmetic samples of different matrix, thus proving its suitability for the analysis of cosmetic products and the monitoring of THC levels in hemp-based products, in order to guarantee the fulfillment with the legislation in force and the safety of users. The main advantages of the proposed method over previous works with same purpose are the higher sensitivity, the use of less organic solvents, and/or the shorter analysis time.

Chapter 4

Miniaturized stir bar sorptive dispersive microextraction as a high-throughput and feasible approach for low-availability samples. Determination of salivary cortisone and cortisol as a proof-of-concept

The content of this chapter has been published in the following journal article:

C. Azorín, J.L. Benedé, A. Chisvert, New challenges in sample preparation: Miniaturized stir bar sorptive dispersive microextraction as a high-throughput and feasible approach for low-availability sample preparation, Anal. Chim. Acta 1238 (2023) 340627

Abstract

The miniaturization of stir bar sorptive dispersive microextraction (SBDME) for the analysis of low-availability samples is presented. This new methodology is based on the principles of SBSDME, but the amount of sorbent and, most importantly, the amount of sample are considerably reduced to a tiny amount and a few microliters, respectively. Thus, affordable 400-µL flat-base glass inserts and minute bar-shape neodymium magnets (3 mm length x 2 mm diameter) were used as extraction devices hold by a specifically designed multiextraction assembly, which comprises a high-rate stirring plate and a 3D-printed support to treat 15 samples simultaneously. This new approach allows a fast, affordable, portable, and high-throughput analysis of low-volume samples, expanding the potential of the technique. The same extraction device is used along the different stages, thus avoiding transfers, which reduces sample handling. Besides, the reduction in the sample, sorbent and organic solvent amounts allows a considerable decrease of the waste generation, and thus pursues a green sample preparation for bioanalysis. As a proof-of-concept of this new methodology, cortisone and cortisol were determined in human saliva using a composite material made of a reversed-phase polymer (*i.e.*, Strata™-X-RP) and $CoFe_2O_4$ magnetic nanoparticles (MNPs). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure both analytes obtaining good analytical features in terms of linearity ($R^2 > 0.997$), method limits of detection and quantification (22.6 and 75.5 ng L^{-1} for cortisone, and 19.3 and 64.3 ng L^{-1} for cortisol, respectively), precision (relative standard deviation ≤ 11%) and relative recoveries (78-134%).

4.1. Introduction

Biomarkers or biological markers are those substances that, objectively measured in some part of the human body, report valuable information about some biological process, or on the contrary about some pathological process, or indicate the response of the body to a pharmacological treatment [236]. Biomarkers are also considered to be those substances that inform about the voluntary or involuntary exposure of human beings to certain exogenous agents, such as drugs of abuse or pesticides, respectively, and that can lead to pathological disorders.

It should be noted that some biomarkers are found at very low levels of concentration, which, together with the high complexity of biological fluids, makes their determination extremely difficult, thus, requiring the enrichment of the target compounds and/or the clean-up to separate potentially interfering compounds. In this sense, (micro)extraction techniques play an important role, since they allow both the enrichment and clean-up in a single step. However, as previously discussed, large volumes of biological samples are often not available, and routine bioanalysis requires rapid and affordable methods to treat a vast number of samples every day.

All these considerations led to the objective of this chapter: to contribute to the development of new feasible and high-throughput approaches that allow the routine sample treatment of low-availability biological samples and, moreover, simultaneously. This is in line with different microextraction workstations that have been previously developed, mainly based on the 96-well format: 96-blade solid-phase microextraction (SPME) [237,238], micro-solid-phase extraction (μSPE) [57,61], single drop microextraction (SDME) [169], hollow-fiber liquid-phase microextraction (HF-LPME) [239], parallel artificial liquid membrane extraction (PALME) [106] and electromembrane extraction (EME) [107,240]. However, although it is true that these systems allow to analyze many samples simultaneously, the extraction phase remains static, causing the extraction kinetics to be slow and, consequently, the extraction times are long, even hours. On the other hand, as previously demonstrated [241], the extraction efficiency increases when smaller particles of an extraction phase are used due to the large surface area and shorter diffusion route of the analytes, resulting not only in improved extraction efficiency but also in faster extraction kinetics. Additionally, the dispersion of the extraction phase also enables a higher contact area between the donor phase and the extractant, which accelerates the mass transfer between both phases [242]. For this reason, dispersive techniques boast of faster kinetics compared when non-dispersive ones [53].

From this perspective, a new multiextraction assembly has been tailor-designed in this chapter, consisting of a 3D-printed support mounted on a conventional high-rate stirring plate to hold 15 miniaturized extraction devices, each one

containing few microliters of sample (**Figure 4.1.a**). Each extraction device is based on a miniaturization of the SBSDME approach, developed few years ago by the research group in which this Doctoral Thesis has been carried out [55]. In this technique, a magnetic sorbent, initially attracted to the surface of a neodymium magnetic bar, is dispersed into the donor solution contained in a 40-mL extraction vial. When stirring is over, the magnetic bar rapidly retrieves the magnetic sorbent containing the extracted analytes for subsequent desorption and injection into a chromatographic system. The fundamentals of SBSDME, and an overview of the published papers using this technique have been recently compiled in a tutorial review [56]. As exposed in this review, SBSDME has been successfully applied to different matrices such as environmental samples, cosmetic products, urine or food, with very good analytical features. However, in all these applications, relatively high amounts of sample solution (*i.e.,* ≥ 5 mL, usually *ca.* 25 mL) were needed, thus not being possible to analyze low sample volumes as is required in those scenarios mentioned above. In the proposed miniaturized SBSDME (mSBSDME) the amount of sorbent and, most importantly, the amount of sample are considerably reduced. So, 40-mL vials and 10 mm length x 3 mm diameter magnets, typically used in SBSDME, have been replaced by 400-µL flat-base glass inserts and 3 mm length x 2 mm diameter magnets, respectively (**Figure 4.1.b**).

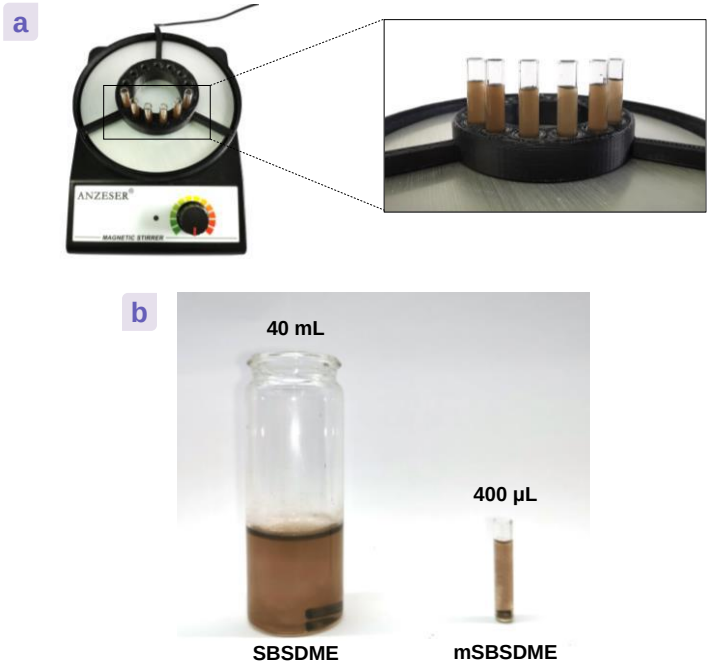


Figure 4.1. (a) Multiextraction assembly developed in this chapter. (b) Comparison of the extraction device between SBSDME and mSBSDME.

Herein, mSBSDME is presented for the first time, as a novel contribution to the miniaturization of sample preparation techniques for bioanalysis with important advantages over SBSDME as a consequence of this miniaturization:

1. The volume of sample is considerably reduced to only a few microliters.
2. Both the amount of sorbent and volume of elution solvent are substantially decreased and thus the generation of waste is diminished.
3. Up to 15 low-volume samples can be simultaneously treated, thus increasing the sample throughput and reducing analysis cost and time consumption.
4. The new multiextraction assembly is easily portable to any location to carry out on site sample treatment with the only requisite of a power supply, which could be solved with the use of low consumption batteries.

This multiextraction assembly can be used in any laboratory in an affordable and "ready-to-use" manner since it is easy to set up with glass inserts and magnets readily available in laboratories, beyond fabricating the support by the 3D-printing technology, highly affordable to anyone nowadays.

In order to present this approach and to show its potential, the determination of cortisone and cortisol in human saliva has been selected as a proof-of-concept. These salivary biomarkers are related to Cushing's syndrome [243], stress [244], and type 2 diabetes mellitus [245], among other diseases, and therefore, the determination of both compounds in human saliva is of great interest and different methods have been published [143,246–253]. For this purpose, a previously described magnetic composite, made of $CoFe_2O_4$ MNPs embedded into a commercial pyrrolidone-modified styrene-divinylbenzene copolymer (*i.e.*, Strata™-X-RP), was used as magnetic sorbent in mSBSDME prior to LC-MS/MS analysis. This composite material has shown good extraction performance for these analytes despite its low selectivity [143].

4.2. Experimental

4.2.1. Reagents

All reagents and solvents were obtained from major suppliers. Cortisone (99%) and prednisolone (≥ 99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Cortisol (98%) was provided by Acros Organics (Fair Lawn, NJ, USA).

For the synthesis of $CoFe_2O_4$ MNPs, cobalt(II) chloride hexahydrate and iron(III) chloride hexahydrate were purchased from Acros Organics, sodium hydroxide was purchased from Scharlau (Barcelona, Spain), and ethanol absolute was purchased from Panreac (Barcelona, Spain). For the synthesis of the composite, Strata™-X 33 µm polymeric reversed phase SPE cartridges (500 mg/6 mL) were obtained from Phenomenex (Torrance, CA, USA).

For the preparation of synthetic saliva, sodium chloride from Fisher Scientific (Loughborough, United Kingdom), potassium chloride, calcium chloride monohydrate, potassium thiocyanate and urea from Panreac, and sodium dihydrogen phosphate monohydrate from Scharlau were used.

Ultrapure water (resistivity ≥ 18.2 MΩ·cm) was obtained from a Connect water purification system provided by Adrona (Riga, Latvia). LC-MS grade methanol from Honeywell (Seelze, Germany), LC-MS grade water from Panreac, and ammonium fluoride (98%) from Acros Organics were used to prepare the chromatographic mobile phase.

Nitrogen used as nebulizer and curtain gas in the MS/MS ion source was obtained by a NiGen LCMS 40-1 nitrogen generator from Claind S.R.L. (Lenno, Italy). Extra pure nitrogen (> 99.999%), used as collision gas in the MS/MS collision cell, was provided by Praxair (Madrid, Spain).

4.2.2. Apparatus

For the synthesis of the magnetic composite, a heating plate with magnetic stirrer from Stuart Scientific (Staffordshire, United Kingdom) and an oven from J.P. Selecta (Barcelona, Spain) were used.

For the LC-MS/MS analysis, an Agilent 1100 Series chromatography system comprised of a degasser, a binary pump, an autosampler and a thermostatic column oven, coupled to an Agilent 6410B Triple Quad MS/MS was employed.

An EBA 21 centrifuge from Hettich (Tuttlingem, Germany), provided with a rotor of 15 cm radius for centrifuge tubes and a rotor of 9.7 cm radius for microcentrifuge tubes, was used.

4.2.3. Multiextraction assembly

Miniaturized extraction devices to carry out the mSBSDME consist of 400-µL flat-base glass inserts (31 mm height x 4 mm I.D.) from Labbox (Barcelona, Spain) and cylindrical neodymium magnets (3 mm length x 2 mm diameter, 45 MGO) from Supermagnete (Gottmadingen, Germany). For the design of the extraction assembly to hold the extraction devices, a magnetic stirrer MX-3K (18 W, 0-3000 rpm) from Anzeser (Frankfurt am Main, Germany) with a 3D-printed support for 15 flat-base glass inserts was used. The 3D-printed support was designed with FreeCAD software (version 0.19 from Jürgen Riegel, Werner Mayer and Yorik van Havre) and printed by *Impresión 3D LowCost* (Girona, Spain) with black poly(lactic acid) and 55% infill density.

4.2.4. Synthesis of $CoFe_2O_4$-Strata™-X-RP magnetic composite

The synthesis of the $CoFe_2O_4$-Strata™-X-RP composite consisted of two steps:

1. Synthesis of the $CoFe_2O_4$ MNPs by wet chemical co-precipitation according to an adapted protocol [235].
2. Subsequent incrustation of MNPs on the polymeric surface.

First, 10.80 g of $FeCl_3 \cdot 6H_2O$ (40 mmol) and 4.76 g of $CoCl_2 \cdot 6H_2O$ (20 mmol) were dissolved in 200 mL of ultrapure water, and then 100 mL of a 3 M sodium hydroxide aqueous solution (300 mmol) were added dropwise under continuous stirring for one hour at 80 ºC. After this time, it was allowed to cool to room temperature to decant the formed solid using a magnet. Then, the solid was washed several times with ultrapure water and later with ethanol, dried in an oven at 80 ºC overnight, and grinded until obtaining a fine black powder.

For the preparation of the composite, where MNPs are embedded on the polymer surface, 0.15 g of the MNPs and 0.15 g of Strata™-X-RP were weighted, and 50 mL of ethanol were added. The mixture was stirred at room temperature for 72 h to ensure the MNPs were embedded on the polymer pores. Finally, the solid was decanted with the aid of a magnet and washed three times with ethanol to discard the remaining non-magnetic polymer. Afterwards, it was resuspended in ethanol and filtered under vacuum through a 10 µm-pore paper filter in order to discard the free MNPs and isolate the composite. It was dried in an oven at 80 ºC overnight and grinded into a fine powder.

The characterization of the material was described in a previous work of the research group [143]. Magnetization, particle size distribution, morphology, specific surface area and pore size were established. In addition, energy dispersive X-ray spectroscopy (EDS) was performed for elemental analysis.

4.2.5. Preparation of synthetic saliva

Synthetic saliva was prepared according to an adapted protocol [254]. For that aim, 250 mL of an aqueous solution containing NaCl (400 mg L^{-1}), KCl (400 mg L^{-1}), $CaCl_2 \cdot H_2O$ (795 mg L^{-1}), $NaH_2PO_4 \cdot H_2O$ (690 mg L^{-1}), KSCN (300 mg L^{-1}) and urea (1000 mg L^{-1}) in ultrapure water were prepared.

4.2.6. Preparation of standard solutions

Three independent stock solutions containing 500 µg mL^{-1} of cortisone, 500 µg mL^{-1} of cortisol and 500 µg mL^{-1} of prednisolone (used as surrogate), respectively, in methanol were prepared and kept at 4 ºC protected from light. From these solutions, three intermediate solutions of 500 ng mL^{-1} in ultrapure

water were prepared weekly and kept at 4 ºC protected from light. From them, appropriate aliquots were diluted to obtain solutions of 35 ng mL^{-1} in synthetic saliva, which were prepared daily.

4.2.7. Sample collection and pretreatment

Saliva samples were collected in 15-mL centrifuge tubes from volunteers by the passive drooling method [255]. In contrast to swab collection methods (*e.g.*, Salivette®), that usually introduce variability and negatively affect results [256], passive drooling does not interfere with the salivary analytes being measured. After collection, samples were centrifuged at 6000 rpm for 10 min to separate saliva from leftovers and/or phlegm and the supernatant was transferred to a microcentrifuge tube. Then, samples were frozen at -20°C to precipitate mucins and thus reduce viscosity of saliva [257], thawed and centrifuged again at 18000 rpm for 5 min. The supernatant was kept at 4 °C until the analysis. Saliva can be stored up to 3 months at 4 °C [258].

Each volunteer gave written informed consent to participate in this study, which was conformed to the ethical guidelines of the Declaration of Helsinki and it was approved by the Ethical Committee of the University of Valencia (Spain).

4.2.8. mSBSDME procedure

First, to prepare the extraction devices, 0.5 mg of the $CoFe_2O_4$-Strata™-X-RP composite (prepared as described in **Section 4.2.4**) were weighted into a flat-base glass insert and a neodymium stir bar was introduced. Subsequently, 5 µL of methanol were added to assist dispersion of the material and facilitate the introduction of the aqueous standard and sample solutions in the narrow flat-base glass inserts by reducing the surface tension of water. To carry out the extraction stage, working standard solutions of cortisone (1 – 20 ng mL^{-1}) and cortisol (0.1 – 5 ng mL^{-1}) were directly prepared into the extraction device by adding the appropriate volume of the 35-ng mL^{-1} aqueous standard solutions of cortisol and cortisone, 50 µL of the 35-mg mL^{-1} aqueous standard solution of prednisolone and then diluted up to 350 µL with synthetic saliva. Regarding sample preparation, the same procedure of standards was followed, but introducing an appropriate aliquot of pretreated saliva (as described in **Section 4.2.7**) instead of cortisone and cortisol standard solutions. In any case, a minimum dilution of 1:1 v/v with synthetic saliva was necessary to reduce viscosity of saliva samples and ensure the dispersion of the extraction material (as discussed later in **Section 4.3.5**).

They were stirred for 1 min at *ca.* 3000 rpm to achieve total dispersion of the composite within the solution. Afterwards, the stirring was stopped, and the

extraction devices were left to rest so that the magnetic composite was retrieved by the magnetic field and recoated the stir bar again. Next, the solution was taken carefully with the aid of a gel loading pipette tip and discarded. Then, 350 µL of ultrapure water were added to the device to wash the sorbent, which were also discarded. In order to carry out the liquid desorption of the analytes, 20 µL of methanol were introduced into the extraction device. After 30 s of vigorous stirring, the sorbent was left to sediment on the magnet, and the methanolic extract containing the analytes was transferred to a conical glass insert inside of an injection vial. Then, 15 µL of ultrapure water were subsequently added to ensure good chromatographic performance. Finally, prior to LC-MS/MS analysis, the vial was centrifuged in a test tube at 6000 rpm for 5 min to ensure any particle that could be present was settled and prevent it to enter the chromatographic system. **Figure 4.2** shows a schematic diagram of the workflow of the proposed method.

4.2.9. LC-MS/MS analysis

Separations were carried out in a Zorbax SB-C18 (50 mm length, 2.1 mm I.D., 1.8 µm particle size) column from Agilent Technologies. Ten microliters of each solution were injected into the chromatographic system. Mobile phase consisted of solvent A (water, 0.5 mM ammonium fluoride) and solvent B (methanol), by isocratic elution at a mixing ratio of 50:50% (v/v). The flow rate was 0.25 mL min^{-1} and the column temperature was kept constant at 35 ºC. The run time was 5 min.

The triple quadrupole MS detector operated in positive electrospray ionization (ESI) mode (capillary voltage at 5 kV), by multiple reaction monitoring (MRM). The rest of conditions were: gas temperature at 350 °C, nebulizer gas flow rate at 11 L min^{-1} and nebulizer gas pressure at 50 psi.

The m/z precursor → product ion transitions (collision energy; fragmentor; dwell time) for quantification and for identification were, respectively, 361 → 163 (21 V; 140 V; 120 ms) and 361 → 105 (21 V; 140 V, 50 ms) for cortisone; 363 → 121 (26 V; 155 V; 120 ms) and 363 → 105 (26 V; 155 V; 50 ms) for cortisol; and 343 → 325 (0 V; 135 V, 120 ms) and 361→ 163 (0 V; 135 V, 50 ms) for prednisolone.

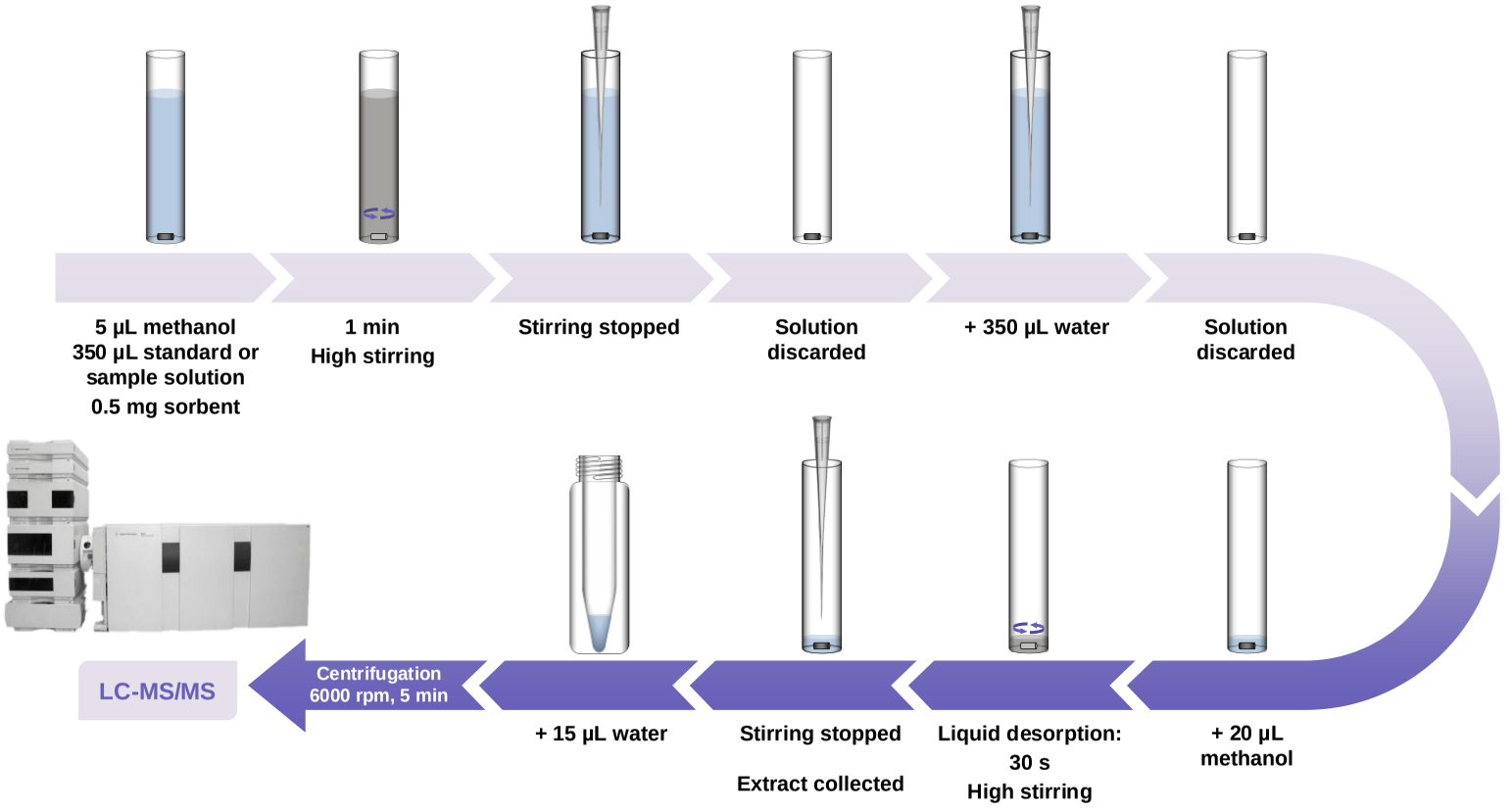


Figure 4.2. Schematic diagram of the proposed mSBSDME – LC-MS/MS method.

The following figure shows a chromatogram of a standard solution after applying the proposed method.

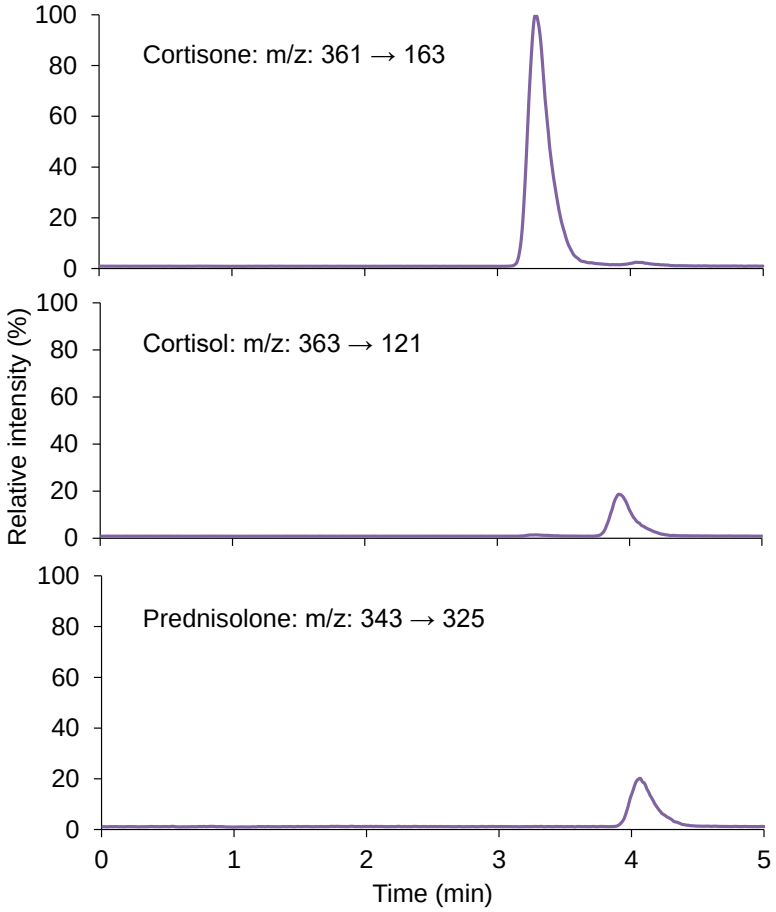


Figure 4.3. Chromatogram of a standard solution containing 10 ng mL^{-1} of cortisone, 2 ng mL^{-1} of cortisol, and 5 ng mL^{-1} of prednisolone (as surrogate) obtained by the proposed method.

4.3. Results and discussion

4.3.1. Concept and design of the multiextraction assembly

As previously mentioned, the objective of this chapter was to present a new feasible methodology to analyze low-availability samples, either for having a low amount/volume or for being hard to obtain. For this purpose, the SBSDME approach, developed by the research group hosting this Doctoral Thesis in 2014

[55], was considered as a starting point due to its great proven benefits [56], but it was necessary to make some important modifications in the methodology.

In this sense, the extraction was directly performed in low-volume inserts (up to *ca.* 400 µL capacity) to considerably reduce the sample volume, unlike the conventional SBSDME in which the extraction is typically carried out in 40-mL vials (see **Figure 4.1.b**). As a direct consequence of their reduced diameter, the size of the stir bars was also reduced from 10x3-mm magnets used in previous works to 3x2-mm magnets to fit the inserts. It is important to note that the inserts had to have a flat base, and not the classical conical ones, so that the stirring of the bar-shape magnets could be done correctly.

Once the new requirements of both the extraction vessel and stir bar were stablished, the new multiextraction assembly was designed. The main objective was to develop a methodology that could be applied in the routine analysis in a clinical laboratory, where a large number of samples need to be processed per day. Simultaneous analysis of several samples reduces energy and time consumption and, therefore, the analysis cost. Different configurations were explored in order to maximize the sample throughput, *i.e.*, to allow the extraction of the maximum number of samples at the same time.

Most stirring plates work based on a magnet rod attached to an engine to make it rotate. This magnet rod interacts with the bar-shape magnet, generating its rotation for stirring. In this case, the magnet rod of the stirring plate was able to interact with more than one of the small bar-shape magnets, therefore allowing to stir multiple samples at a time. To ensure equal stirring of all samples, they should be in a circular arrangement on the stirring plate, so that they were at the same distance from the center, hence, the magnetic field is equivalent at each position. As the perimeter of the circumference depends on its radius, a higher radius permits to place more extraction devices in a circular disposition. In this sense, different radius from the center of the stirring plate were tested at the maximum stirring rate (*i.e.*, 3000 rpm) by extracting 400 µL of a standard solution prepared in synthetic saliva containing 10 ng mL^{-1} of cortisone and cortisol. At low radius (*i.e.*, 1 cm), samples were vigorously stirred because the magnetic field from the magnet rod was strong, but only 6 extraction devices could be stirred simultaneously. At high radius (*i.e.*, 5.5 cm), up to 30 extraction devices could be stirred, but the magnetic field was not strong enough to completely disperse the material in the solution, thus causing a reduction in the extraction efficiency as shown in **Figure 4.4**. In this regard, there should be a trade-off between the maximum dispersion of material (and therefore maximum extraction efficiency) and the simultaneous extraction of a high number of samples. Thus, an intermediate radius (*i.e.*, 2.5 cm) was selected so that a maximum volume of 350 µL was correctly stirred. This arrangement allows the stirring of 15 extraction devices simultaneously.

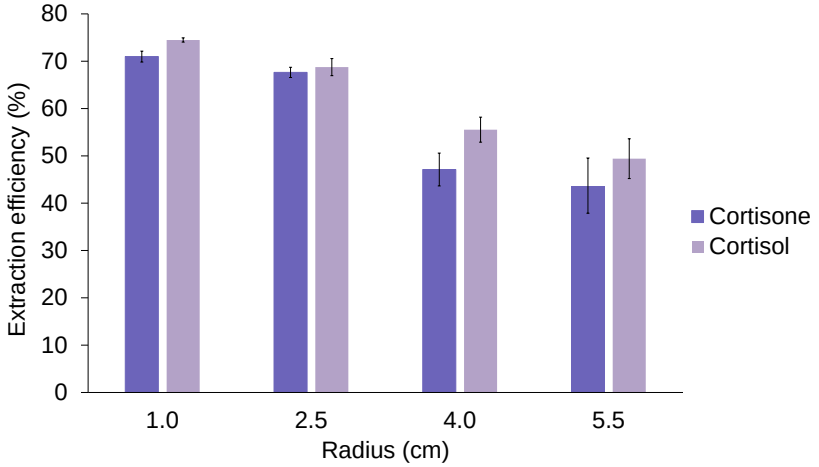


Figure 4.4. Influence of radius of the multiextraction assembly.

The influence of the radius of the multiextraction assembly in the dispersion of the sorbent material is illustratively shown in the following Supplementary Video:

Scan or click the code to access Supplementary Video

Finally, in order to hold the extraction devices during the whole extraction procedure, a specific support with an inner ring with capacity for 15 extraction devices was tailor-designed to perfectly attach it to the stirring plate, by using 3D-printing technology, as shown in **Figure 4.5**.

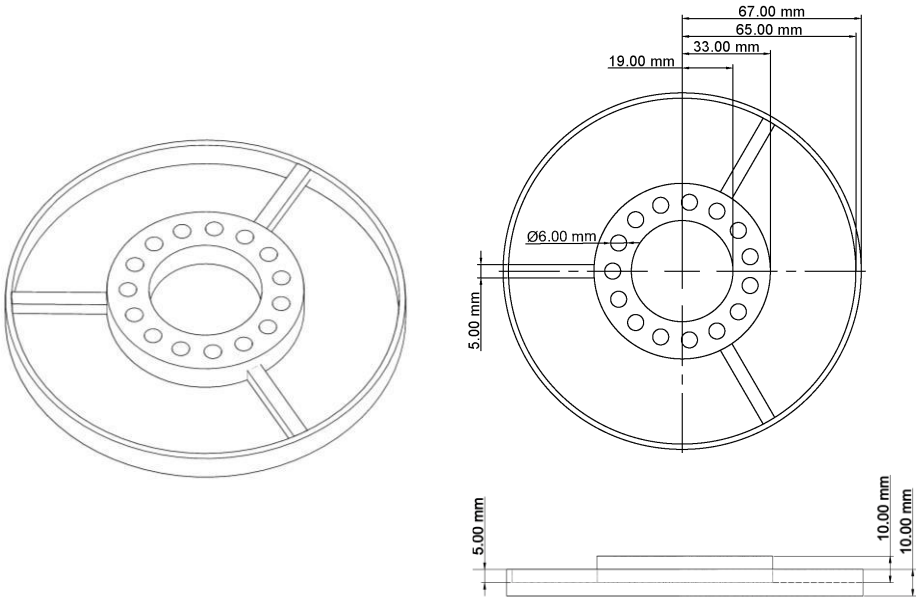


Figure 4.5. Tridimensional view and dimension sketches (top and lateral views) of the 3D-printed support.

In order to confirm the equal performance of all positions within the same ring, a same aqueous standard solution containing 10 ng mL^{-1} of cortisone, cortisol and prednisolone was extracted in the different positions. From a practical point of view, 0.5 mg of magnetic sorbent were weighted in each one of 15 extraction devices and 350 µL of the standard solution were extracted during 1 min in the 15 positions simultaneously. After removing the aqueous solutions and washing with 350 µL of water, the analytes were desorbed in 20 µL of methanol for 30 s. Subsequently, they were injected into the LC-MS/MS system. The relative standard deviation (RSD) of the 15 measures was below 7%, therefore proving the extraction is equivalent at any position of the assembly.

4.3.2. Optimization of the extraction variables in mSBSDME

A response surface methodology (RSM) [234] based on a three-factor three-level Box-Behnken design was performed to stablish the optimal values of the variables involved in the extraction procedure. The description of the statistics of the Box-Behnken design are included in **ANNEX I**. The studied variables and ranges were: (i) sorbent amount (0.5-2.5 mg), (ii) extraction time (1-10 min), and (iii) ionic strength (1–10% (w/v) NaCl). As analytes do not present ionizable groups, pH was not studied.

In this sense, according to **Equation A-I.1**, considering 3 factors and 3 replicates of the central point, 15 experiments were required (**Table 4.1**). Since human saliva is mainly composed of water (*ca.* 99%), 350 µL of aqueous standard solution containing 10 ng mL^{-1} of cortisone and cortisol were used to perform the experiments. After the extraction and washing, the analytes were desorbed in 20 µL of methanol for 30 s. The selected analytical responses were the peak area of the analyte. A total of 30 experiments were performed, carrying out the design by duplicate. StatGraphics Centurion XVI (Stat Point Inc. Herndon, VA, USA) software was employed for the statistical analysis.

Table 4.1. Box-Behnken design for multivariate optimization of the critical variables

Step	Ionic strength (%(w/v) NaCl)		Extraction time (min)		Sorbent amount (mg)	
	Uncoded	Uncoded	Coded	Coded	Uncoded	Coded
1	1	-1	1	-1	1.5	0
2	10	1	1	-1	1.5	0
3	1	-1	10	1	1.5	0
4	10	1	10	1	1.5	0
5	1	-1	5.5	0	0.5	-1
6	10	1	5.5	0	0.5	-1
7	1	-1	5.5	0	2.5	1
8	10	1	5.5	0	2.5	1
9	5.5	0	1	-1	0.5	-1
10	5.5	0	10	1	0.5	-1
11	5.5	0	1	-1	2.5	1
12	5.5	0	10	1	2.5	1
13	5.5	0	5.5	0	1.5	0
14	5.5	0	5.5	0	1.5	0
15	5.5	0	5.5	0	1.5	0

As shown in **Figure 4.6**, sorbent amount was the variable that appreciably affected the extraction performance. The adequacy of the model was evaluated by the coefficient of determination (R^2), which was ≥ 0.88. This value indicates that the designed model was efficient for the prediction of response.

Figure 4.7 shows response surfaces of desirability function (described in **ANNEX I**). As can be seen in **Figure 4.7.a**, higher sorbent amounts had a negative effect on extraction efficiency. It can be caused by the worse dispersion of high amounts of magnetic composite, producing less contact area between the sorbent and the solution, especially relevant in desorption step, where the volume is very small. Therefore, the minimum amount of sorbent (*i.e.,* 0.5 mg) was selected. Smaller quantities were not tested due to the inability to accurately weigh them. Regarding the extraction time and ionic strength, as can be seen in **Figure 4.7.b**, none of these variables significantly affected the extraction, and therefore, the ionic strength no needs to be adjusted and the minimum extraction time (*i.e.*, 1 min) was selected to reduce time consumption. Likewise, shorter

times were not tested to allow enough time for all the material to completely disperse within the solution.

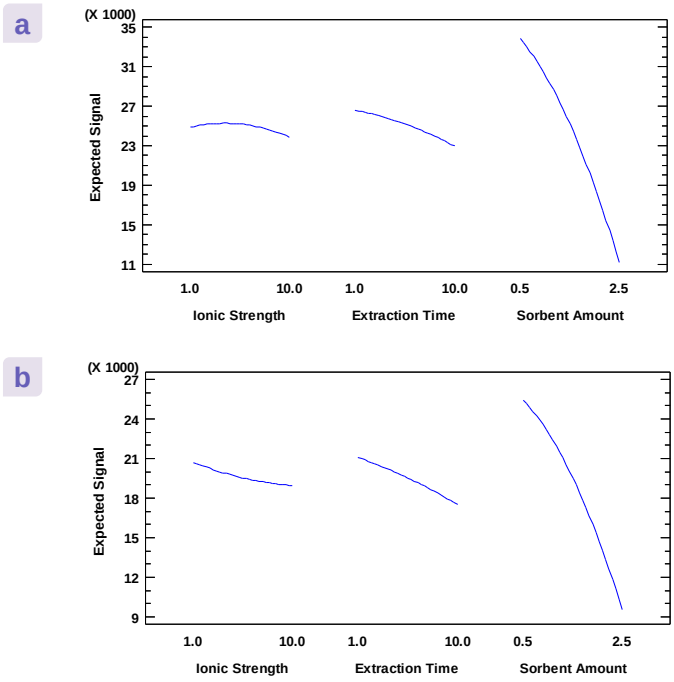


Figure 4.6. Correlation of the different variables that potentially affect the extraction of (a) cortisone and (b) cortisol.

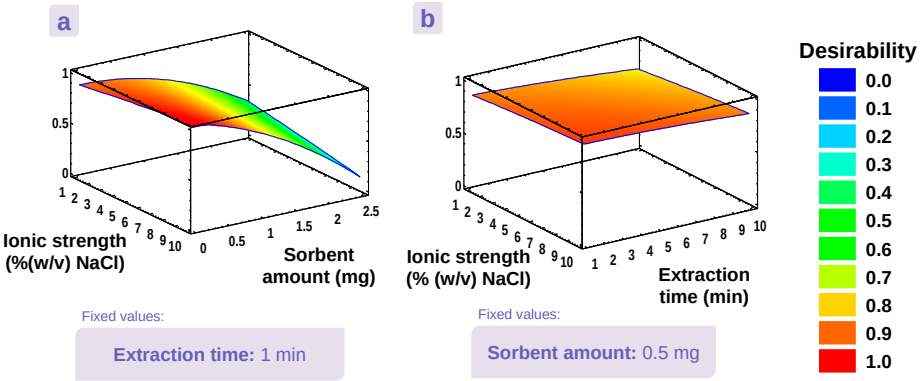


Figure 4.7. Response surface of the desirability function representing the relation between the studied variables in the extraction stage: (a) sorbent amount *vs* ionic strength, and (b) extraction time *vs* ionic strength.

4.3.3. Optimization of the desorption variables in mSBSDME

After the optimization of the extraction process, the variables involved in the desorption step were also studied using a univariate approach. Methanol was selected as desorption solvent because in previous studies no significant differences in signals were found among the tested solvents (*i.e.,* methanol, acetonitrile), but methanol provided the best chromatographic profiles. The volume was fixed at 20 µL to ensure the solvent covered the bar-shape magnet with the minimum possible amount to maximize the preconcentration of the analytes. The composite coating the surface of the magnet was stirred with 20 µL of methanol for 0.5, 1, 2 and 3 min. **Figure 4.8** shows negligible differences in extraction efficiency at different desorption times, so the minimum time (*i.e.*, 30 s) was selected.

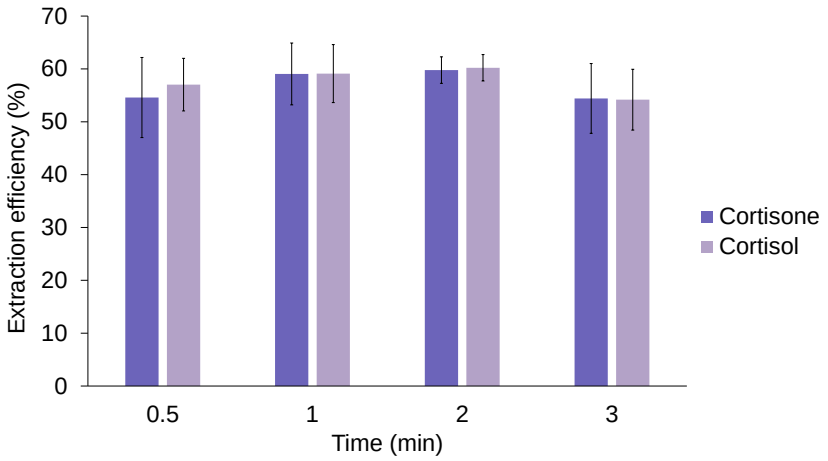


Figure 4.8. Optimization of the desorption time. (Extraction conditions: sample volume, 350 µL; sorbent amount, 0.5 mg; extraction time, 1 min; ionic strength, not adjusted. Desorption conditions: 20 µL of methanol).

4.3.4. Analytical performance of the proposed method

The quality parameters of the proposed method, such as linearity, enrichment factors (EF), extraction efficiency (EE), limit of detection (LOD), limit of quantification (LOQ), and intra- and inter-day precision were evaluated under the optimized conditions.

The linearity was studied by applying the proposed method to standard solutions at different concentrations prepared in synthetic saliva, all of them containing 5 ng mL^{-1} of prednisolone as surrogate. Calibration plots were built using the ratio of the peak area of each target analyte to the surrogate (A_i/A_s) versus the analyte concentration. Regarding the levels expected in real samples, working ranges

were fixed from 1 to 20 ng mL^{-1} for cortisone and from 0.1 to 5 ng mL^{-1} for cortisol although the linearity reached at least 20 ng mL^{-1} for both analytes. The determination coefficients reveal good linearity ($R^2 > 0.997$).

The EFs were estimated as the ratio of the signal obtained after the extraction of a standard solution containing 10 ng mL^{-1} of cortisone and 2 ng mL^{-1} of cortisol prepared in synthetic saliva and the signal of a standard solution of the same concentration prepared in methanol without extraction. They were 5.9 ± 0.3 for cortisone and 6.2 ± 0.3 for cortisol. The EEs were calculated considering a maximum EF of 10. In this sense, the EE values were 59 ± 3 and 62 ± 3% for cortisone and cortisol, respectively.

The LOD and LOQ were calculated as 3 times and 10 times, respectively, the signal-to-noise ratio of a standard solution prepared in synthetic saliva and subjected to the proposed method. The LOD and LOQ values were 22.6 and 75.5 ng L^{-1} for cortisone, and 19.3 and 64.3 ng L^{-1} for cortisol, respectively, allowing the determination of both analytes in human saliva.

The precision of the method was evaluated applying the proposed method to different standard solutions prepared in synthetic saliva containing the target analytes at three levels of concentration (*i.e.,* 1, 10 and 20 ng mL^{-1} for cortisone and 0.1, 2 and 5 ng mL^{-1} for cortisol), all of them containing 5 ng mL^{-1} of prednisolone (as surrogate). The results, expressed as RSD of the concentration obtained for five replicates during the same working session (intra-day precision) or in different working sessions (inter-day precision), are presented in **Table 4.2** and revealed that good precision was achieved for the target analytes.

Table 4.2. Precision of the proposed method

Analyte	Concentration (ng mL^{-1})	Intra-day precision (RSD, %) [a]	Inter-day precision (RSD, %) [a]
Cortisone	1	7.5	6.1
	10	3.4	11.0
	20	1.7	7.4
Cortisol	0.1	3.1	6.8
	2	1.4	7.6
	5	3.1	7.1

[a] n=5

4.3.5. Analysis of human saliva samples

Four saliva samples from volunteers (two male and two female) were collected as described in **Section 4.2.7**. In a first attempt, it was tried to extract 350 µL of a saliva sample directly, without any dilution. However, due to the slight viscosity of pretreated saliva, it was found that not all the material was dispersed

homogeneously between different saliva samples from different volunteers, which could hinder the reproducibility and accuracy of the extraction. Therefore, the effect of dilution was first tested. For this purpose, the saliva samples were spiked at two concentration levels (*i.e.*, 1, and 10 ng mL^{-1} for cortisone, and 0.1 and 2 ng mL^{-1} for cortisol) and diluted to two ratios (*i.e.*, 1:1 and 1:3 v/v) with synthetic saliva to evaluate the matrix effects by means of the relative recovery (RR) values, calculated as follows:

$$RR\ (\%) = \frac{Concentration_{spiked\ sample} - Concentration_{sample}}{Spiked\ amount} \cdot 100 \qquad \text{(Eq. 4.1)}$$

These results are presented in **Table 4.3**. Both dilution levels showed similar recovery values between 78 and 134%, thus proving a 1:1 dilution was enough to avoid matrix effects and then external calibration was suitable for quantification of cortisone and cortisol in saliva samples.

Table 4.3. Relative recoveries obtained from real samples at different saliva dilutions

Sample [a]	Dilution ratio	Spiked amount (ng mL^{-1})		RR (%) [b]	
		Cortisone	Cortisol	Cortisone	Cortisol
A	1:1	1	0.1	95 ± 4	95 ± 7
		10	2	85 ± 1	91 ± 7
	1:3	1	0.1	96 ± 3	89 ± 8
		10	2	91 ± 3	93 ± 2
B	1:1	1	0.1	95 ± 3	91 ± 8
		10	2	88 ± 1	92 ± 3
	1:3	1	0.1	94 ± 4	92 ± 6
		10	2	94 ± 6	96 ± 3
C	1:1	1	0.1	129 ± 5	78 ± 2
		10	2	134 ± 4	92 ± 2
	1:3	1	0.1	117 ± 9	98 ± 8
		10	2	124 ± 1	103 ± 3
D	1:1	1	0.1	125 ± 5	81 ± 6
		10	2	110 ± 4	87 ± 1
	1:3	1	0.1	93 ± 5	84 ± 2
		10	2	100 ± 2	89 ± 3

[a] Sample A and B: female; Sample C and D: male
[b] Mean of three replicates ± standard deviation

Then, these saliva samples were treated by the proposed mSBSDME approach and measured by LC-MS/MS. The nominal concentration of cortisone and cortisol are presented in **Table 4.4**, showing the suitability of the method to measure salivary levels of these two biomarkers. As expected, cortisone levels were higher than cortisol since the latter is rapidly converted to cortisone as the

salivary glands exhibit high levels of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) [259].

Table 4.4. Concentration of analytes in real saliva samples

Sample [a]	Found amount (ng mL^{-1}) [b]	
	Cortisone	**Cortisol**
A	1.31 ± 0.06	0.092 ± 0.003
B	3.27 ± 0.16	0.248 ± 0.010
C	3.82 ± 0.16	0.28 ± 0.04
D	5.90 ± 0.08	0.61 ± 0.02

[a] Sample A and B: female; Sample C and D: male
[b] Mean of three replicates ± standard deviation

4.3.6. Comparison of mSBSDME with previously reported approaches

Being the miniaturization of a microextraction technique, the volume of sample, sorbent material and waste are considerably reduced in mSBSDME. Furthermore, the possibility to extract up to 15 samples simultaneously with the designed extraction assembly is also one of its main virtues, since after the extraction time (*i.e.,* 1 min), a sample throughput lower than 7 s per sample is achieved. Even though it is true that 96-well approaches previously described [57,61,106,107,169,237–240] can extract many more samples simultaneously, extraction times are considerably longer due to their static nature, thus resulting in higher times per sample. Next step would be focused on increasing the number of devices using the 96-well format (as described later in **Chapter 5**) and the possibility of partial automation. The portability of the assembly, only requiring an external power supply, allows its use in any laboratory in an affordable and “ready-to-use” manner. To do this, it would only be necessary to previously weigh the material inside the inserts of the extraction device.

Compared to conventional SBSDME, the mSBSDME provides a series of advantages. On the one hand, the handling is easier since all the stages (*i.e.,* extraction, clean up, and desorption) are carried out in the same vessel (*i.e.,* the flat-base insert). Moreover, the amount of sorbent material obtained in the same synthesis allows a greater number of extractions to be performed, since less than one milligram is used per extraction. In contrast to its advantages, the tiny amount of material used in the approach (*i.e.,* 0.5 mg) is difficult to recover from the magnet after the process and, therefore, its reusage is not feasible. In any case, it is important to note that mSBSDME is proposed to analyze samples with

a low availability, but not for those in which the availability is greater (*e.g.,* environmental samples, urine, or extracts of other types of samples such as cosmetics). In fact, by using a larger volume of sample, higher preconcentration factors may be obtained, especially relevant when determining traces at the ng L^{-1} level. Therefore, conventional SBSDME is recommended for larger volumes of sample.

Additionally, the proposed approach has been compared with previous reported extraction-based approaches for the determination of cortisone and cortisol in saliva samples (see **Table 4.5**). The proposed method presents comparable or better analytical features in terms of EF and LODs than other reported methods. Besides, a considerably reduced volume of sample is used and, as well as the rest of dispersive-based techniques, the extraction time is low. The greenness of these methods was evaluated by estimating their AGREEprep score [201], a metric tool for the assessment of analytical sample preparation. AGREEprep establishes a scale from 0 to 1 for each of the ten principles of Green Sample Preparation [5], considering environmental and health impact factors, such as the use of reagents, the energy consumption, sample size and generated wastes. The obtained pictogram for the proposed method is showed in **Figure 4.9**.

As can be seen in **Table 4.5**, classical liquid-liquid extraction (LLE) provided the lowest score largely due to the use of dichloromethane as extraction solvent. Solid-phase extraction (SPE) and dispersive liquid-liquid microextraction (DLLME) provided similar score. Although the latter is a miniaturized technique, the fact of using a larger sample volume (up to 1.5 mL) harmed the result. The two DSPE-based methods (*i.e.*, magnetic-based solvent-assisted-DSPE (M-SA-DSPE), and the proposed mSBSDME) presented higher scores, mainly due to the low consumption of solvents as mentioned above. The methods based on on-line SPE present good AGREEprep scores because of the high degree of automatization and the low consumption of solvents and waste generation. Nonetheless, one of the reported methods includes other previous steps (*i.e.*, LLE and derivatization) that, despite achieving very low LODs, significantly reduced the greenness of the method.

Table 4.5. Comparison of mSBSDME with other extraction-based approaches for the determination of cortisone and cortisol in saliva

Extraction technique	Analytical technique	Sample volume (µL)	Extraction time (min)	EF	LOD (ng L^{-1})	Handling	Throughput	Waste	AGREEprep Score	Ref.
On-line SPE	LC-MS/MS	100	n.r. [a]	n.r.	141.4-281.2	+++	+++	+++	0.79	[246]
SPE	LC-MS/MS	300	n.r.	n.r.	72.5-108.1	+++	++	++	0.49	[247]
LLE	LC-MS/MS	50	5	n.r.	39.1	++	+	+	0.4	[248]
LLE + derivatization + on-line SPE	LC-MS/MS	250	4	n.r.	2-5	++	+	+	0.54	[249]
DLLME	LC-UV	1000-1500	2	5.0-6.3	110-160	++	+	++	0.48	[253]
M-SA-DSPE	LC-MS/MS	1000	1	5.2-5.6	18-29	++	+++	++	0.56	[143]
mSBSDME	LC-MS/MS	175	1	5.9-6.2	19.3-22.6	++	+++	+++	0.6	This work

[a] n.r.: not reported

Figure 4.9. AGREEprep pictogram of the proposed method. (1-Sample preparation placement; 2-Hazardous materials; 3-Sustainability and renewability of materials; 4-Waste; 5-Size economy of the sample; 6-Sample throughput; 7-Integration and automation; 8-Energy consumption; 9-Post sample preparation configuration for analysis; 10-Operator's safety).

4.4. Conclusions

In this chapter, a miniaturization of stir bar sorptive dispersive microextraction (mSBSDME) is presented for the first time for the feasible high-throughput analysis of low-availability samples in bioanalysis. The dispersion of the extraction material allows a high extraction kinetics that, together with the possibility of simultaneously analyzing of up to 15 samples, offers promising enhancements with respect to previously presented multi-extraction workstations.

This new methodology allows a rapid determination of target analytes employing few microliters of sample, organic solvents and sorbent, permitting the analysis of barely available samples, broadening the applications of the technique and getting closer to the current trends in Analytical Chemistry and the principles of GSP.

As a proof-of-concept of this new methodology, the determination of salivary cortisone and cortisol was selected. Good analytical features were obtained for both analytes. The successful application to saliva samples from different volunteers proves its potential as a new sample preparation technique for the analysis of biomarkers in saliva.

Despite the achievement of the simultaneous extraction of several samples, it is still below the previous (semi)automated approaches based on 96-well plates. Next step would be focused on expanding the possibilities of this new miniaturized methodology to treat a larger number of samples by adapting it to the 96-well format (as described later in **Chapter 5**) and by attaining a higher degree of (semi)automation.

Chapter 5

A 96-position high-throughput miniaturized platform for dispersive sorbent-based microextraction: application to the determination of tetrahydrocannabinol and cannabidiol in saliva of cannabis smokers

The content of this chapter has been submitted for publication in Analytica Chimica Acta.

Abstract

In order to obtain sustainable analytical methods, it is essential to develop kinetically efficient sample preparation strategies in which equilibria are reached faster, as dispersive extraction techniques. In addition, the higher the reduction in physical space (*i.e.*, microextraction techniques) the higher number of extraction devices can be located and thus the higher number of samples can be simultaneously treated. All this increases sample throughput, and contributes to the reduction of chemical waste, and energy and sample consumption. In this sense, multiposition extraction platforms are smart strategies to achieve these goals, but they are scarcely developed for dispersive microextraction techniques.

Taking miniaturized stir bar sorptive dispersive microextraction (mSBSDME) as a starting point, a 96-position extraction platform has been developed using a 96-position stirring plate and a tailor-designed 3D-printed support for locating the miniaturized extraction devices, achieving a high-throughput miniaturized sample preparation strategy. In order to show the applicability of this novel platform, the determination of Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in human saliva has been carried out and applied to samples collected after the consumption of marijuana and legal CBD-rich cannabis. Only 100 µL of saliva were needed for the analysis and good analytical features in terms of linearity (at least up to 500 ng mL^{-1}), limits of detection (0.7 and 2.8 ng mL^{-1} for THC and CBD, respectively), and precision (relative standard deviation ≤ 14%) were achieved.

The miniaturization of the device allows the use of small volumes of sample (*i.e.*, a few microliters) and the treatment of 96 samples in parallel, being the first proposal for carrying out dispersive sorbent-based microextraction under the concept of 96-well format. Additionally, this new workflow contributes to the development of analytical methods that meet the three pillars of sustainability, *i.e.*, greenness and easily affordable in terms of economics and applicability.

5.1. Introduction

As previously discussed, one way to improve method sustainability is miniaturization, as it is a powerful strategy to reduce reagents, sample consumption and waste generation. Additionally, reducing both the sample amount and the volume of the acceptor phase in extraction techniques may facilitate the miniaturization of extraction devices, leading to a reduction in physical space. This permits the development of parallel extraction devices in which a higher number of samples can be treated simultaneously in a short amount of time, resulting in decreased energy consumption, exposure hazards, and analytical expenses [25].

To this regard, it is worthy to mention those workstations based on 96-well format for solid-phase microextraction (SPME) [237,238,260], thin-film microextraction (TFME) [261], bar adsorptive microextraction (BAμE) [262], micro-solid-phase extraction (μSPE) [57,61], single-drop microextraction (SDME) [169], hollow-fiber liquid-phase microextraction (HF-LPME) [239], parallel artificial liquid membrane extraction (PALME) [106], electromembrane extraction (EME) [107,240], pipette-tip solid-phase extraction (PT-SPE) [263] and in situ Parallel-Dispersive Droplet Extraction (Pa-DDE) [193].

These 96-well workstations were developed to carry out simultaneous extractions in an attempt to counteract the long extraction times (even hours) of those classical microextraction techniques where the extracting phase remains static. On the other hand, dispersive techniques boast of short extraction times since the dispersion of the acceptor phase within the sample increases the contact area between both phases, favoring kinetics [53]. In this sense, it is worthy to draw attention to the Pa-DDE workstation, since it is the only 96-well-based platform for a dispersive extraction technique. Nonetheless, the acceptor phase is a liquid phase and, to the best of our knowledge, no dispersive sorbent-based applications in 96-well configurations have been described so far.

The aim of this chapter is to expand even further the possibilities of mSBDSME (developed in **Chapter 4**) and increase the number of samples to be treated simultaneously based on the concept of 96-well plate. To this regard, it should be emphasized that this is the first time that a 96-position platform is described for a dispersive sorbent-based microextraction technique. Besides, the extraction assembly is intended to be readily available for any laboratory, portable, and very cost-effective. In this sense, many analytical laboratories can adopt this approach, seeking to exploit its advantages such as low extraction times and resources consumption.

As a proof-of-concept of this methodology, the determination of THC and CBD in saliva from cannabis smokers is presented. According to the European Drug Report 2023 [264], cannabis is still the most consumed illicit drug in Europe.

Hence, there is a need to monitor THC levels in society to promote public safety, prevent accidents and control cannabis use. Apart from THC, other cannabinoids such as CBD have come into the focus of research and legislation, because it is thought to modulate the psychoactivity of THC and has shown promising therapeutic activity such as anti-inflammatory, anticonvulsive, anxiolytic, analgesic, neuroprotective, anticancer, and antioxidant effects [265]. In fact, in the last years a wide variety of CBD-rich products have been released into market including plants with a THC content below 0.3%, as permitted by the European legislation [216,266]. Due to the presence of traces of THC, the consumption of CBD-rich marijuana can also lead to detectable THC concentrations, exceeding the tolerable limits for driving, for example [267]. In this sense, the determination of these two main cannabinoids in saliva as non-invasive fluid is of great interest. A composite magnetic material comprised of $CoFe_2O_4$ magnetic nanoparticles (MNPs) entrapped into a divinylbenzene (DVB) and N-vinylpyrrolidone (NVP) copolymer ($CoFe_2O_4$@p(DVB-co-NVP)) is employed as sorbent material. After extraction, the analytes are measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

5.2. Experimental

5.2.1. Reagents

(-)-trans-THC and CBD solutions, both 1.0 mg mL^{-1} in methanol used as standards; and (-)-trans-THC-D_3 and CBD-D_3 solutions, both 100 µg mL^{-1} in methanol (used as surrogates) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For the synthesis of $CoFe_2O_4$ MNPs, cobalt(II) chloride hexahydrate and iron(III) chloride hexahydrate were purchased from Acros Organics (Fair Lawn, NJ, USA), and sodium hydroxide (reagent grade) was purchased from Scharlau (Barcelona, Spain). For the entrapment of MNPs, a polymer made of DVB 80%, NVP 99%, and 2,2'-azobis(2-methylpropionitrile) (AIBN) 98%, all from Sigma-Aldrich, was synthetized.

For the preparation of synthetic saliva, sodium chloride from Fisher Scientific (Loughborough, United Kingdom), potassium chloride, calcium chloride monohydrate, potassium thiocyanate and urea from Panreac (Barcelona, Spain), and sodium dihydrogen phosphate monohydrate from Scharlau were used.

Ultrapure water (resistivity ≥ 18.2 MΩ·cm) was obtained from a Connect water purification system provided by Adrona (Riga, Latvia). LC-grade acetonitrile was obtained from Panreac. LC-MS grade methanol from Honeywell (Seelze, Germany), LC-MS grade water from Panreac and ammonium fluoride (98%) from Acros Organics were used to prepare the chromatographic mobile phase.

Nitrogen used as nebulizer and curtain gas in the MS/MS ion source was obtained by a NiGen LCMS 40-1 nitrogen generator from Claind S.R.L. (Lenno, Italy). Extra pure nitrogen (> 99.999%), used as collision gas in the MS/MS collision cell, was provided by Praxair (Madrid, Spain).

5.2.2. Apparatus and materials

For the synthesis of the magnetic composite, a heating plate with magnetic stirrer from Stuart Scientific (Staffordshire, United Kingdom) and an oven from J.P. Selecta (Barcelona, Spain) were used.

For the LC-MS/MS analysis, an Agilent 1100 Series chromatography system comprised of a degasser, a binary pump, an autosampler and a thermostatic column oven, coupled to an Agilent 6410B Triple Quad MS/MS was employed.

Miniaturized extraction devices to carry out the mSBSDME consisted of 400-µL flat-base glass inserts (31 mm heigh x 4 mm internal diameter) from Labbox (Barcelona, Spain) and cylindrical neodymium magnets (3 mm length x 2 mm diameter, 45 MGO) from Supermagnete (Gottmadingen, Germany). The extraction assembly was comprised of a 96-position magnetic stirrer MIXdrive 96 MTP and a magnetic stirrer controller MIXcontrol MTP, both from 2MAG (Munich, Germany), and a tailor-designed 3D-printed support for 96 flat-base glass inserts. The 3D-printed support was designed with AutoCAD software (version 2024.1.3 from Autodesk, San Francisco, CA, USA) and printed by Imprimakers (La Pobla de Vallbona, Spain) with black poly(lactic acid) and 20-30% infill density. To speed up the extraction procedure, a 12-channel micropipette (10-100 µL) provided with gel loading pipette tips, from Fisher Scientific, was used.

Due to high hydrophobicity of the target cannabinoids, glass material such as vials, Hamilton syringes and glass centrifuge tubes were employed as far as possible throughout the work (as discussed later in **Section 5.3.1**).

An EBA 21 centrifuge from Hettich (Tuttlingem, Germany), provided with a rotor of 15 cm radius for centrifuge tubes was used.

5.2.3. Sample collection and pretreatment

Saliva samples were collected in 40-mL glass vials from volunteers by the passive drooling method [255]. In contrast to swab collection methods (*e.g.*, Salivette®), that usually introduce variability and negatively affect results [256], passive drooling does not interfere with the salivary analytes being measured. After collection, samples were kept at 4 ºC and protected from light until their

analysis. Before the analysis, samples were centrifuged in glass centrifuge tubes at 6000 rpm for 10 min to separate saliva from leftovers and/or phlegm.

Each volunteer gave written informed consent to participate in this study, which was conformed to the ethical guidelines of the Declaration of Helsinki, and it was approved by the Ethical Committee of the University of Valencia (Spain).

5.2.4. Synthesis of $CoFe_2O_4$@p(DVB-co-NVP) magnetic composite

The synthesis of the $CoFe_2O_4$@p(DVB-co-NVP) composite was performed as previously described [268] and consisted of two main steps:

1. Synthesis of the $CoFe_2O_4$ MNPs by wet chemical co-precipitation according to an adapted protocol [235].
2. Subsequent entrapment of MNPs into the polymeric network.

First, 10.80 g of $FeCl_3 \cdot 6H_2O$ (40 mmol) and 4.76 g of $CoCl_2 \cdot 6H_2O$ (20 mmol) were dissolved in 200 mL of ultrapure water, and then 100 mL of a 3 M sodium hydroxide aqueous solution (300 mmol) were added dropwise under continuous stirring for one hour at 80 ºC. After this time, it was allowed to cool to room temperature to decant the formed solid using a magnet. Then, the solid was washed several times with water and ethanol, dried in an oven at 80 ºC overnight, and grinded until obtaining a fine black powder.

For the entrapment of the MNPs into the DVB-co-NVP copolymer, 0.5 g of $CoFe_2O_4$ MNPs and 0.1 g of AIBN (0.6 mmol) were weighed into a round bottom flask. Then, 250 mL of acetonitrile, 4.3 mL of DVB (25 mmol), and 2.7 mL of NVP (25 mmol) were added. The mixture was dispersed in an ultrasound bath for 90 min kept at *ca.* 30 ºC and then it was sealed and stirred for 24 h at 60 °C in an oil bath. Finally, the solid formed was washed with acetonitrile and dried overnight at 80 °C, obtaining a dark grey fine powder.

5.2.5. Preparation of synthetic saliva

Synthetic saliva was prepared according to an adapted protocol [254]. For that aim, 250 mL of an aqueous solution containing NaCl (400 mg L^{-1}), KCl (400 mg L^{-1}), $CaCl_2 \cdot H_2O$ (795 mg L^{-1}), $NaH_2PO_4 \cdot H_2O$ (690 mg L^{-1}), KSCN (300 mg L^{-1}) and urea (1000 mg L^{-1}) in ultrapure water were prepared.

5.2.6. Preparation of standard solutions

The commercial 1.0 mg mL^{-1} solutions were properly diluted to obtain 200-µg mL^{-1} solutions in methanol and were kept at -20 ºC protected from light. From these solutions, an intermediate multicomponent solution of 1 µg mL^{-1} in synthetic saliva was prepared daily. A methanolic solution containing 500 ng mL^{-1} of THC-D_3 and CBD-D_3 (surrogates solution) was also prepared weekly from the commercial solutions and kept at -20 ºC protected from light. For the calibration curve, working standard solutions of THC and CBD (10-500 ng mL^{-1}) were directly prepared into the extraction device by adding the appropriate volume of the 1 µg mL^{-1} solution in synthetic saliva and diluted up to 100 µL with this same solvent. For samples, 100 µL of pretreated saliva were added to the extraction device. Next, 10 µL of the surrogates solution were added and stirred for a few seconds to allow the interaction of surrogates with the matrix.

5.2.7. mSBSDME procedure

First, a dispersion of the sorbent material in acetonitrile (10 mg mL^{-1}) was prepared and sonicated for 5 min. Nevertheless, the dispersion was resuspended by manual shaking before pipetting. Then, 40 µL of NaCl 17.5% (w/v) and 50 µL of the sorbent dispersion were subsequently added to the extraction vials and they were stirred for 1 min at *ca*. 2000 rpm to achieve total dispersion of the composite within the solution. Afterwards, the stirring was stopped, and the extraction devices were left to rest so that the magnetic composite was retrieved by the magnetic stir bar. Next, the solution was taken carefully and discarded. Then, 200 µL of ultrapure water were added to the device to wash the sorbent, stirred for 1 min, and also discarded. In order to carry out the liquid desorption of the analytes, 25 µL of acetonitrile were introduced into the extraction device. After 1 min of vigorous stirring, the sorbent was left to sediment on the magnet, and the extract containing the analytes was transferred to a conical glass insert inside of an injection vial. **Figure 5.1** shows a schematic diagram of the proposed method.

5.2.8. LC-MS/MS analysis

Separations were carried out in a Kinetex C18 (150 mm length, 2.1 mm I.D., 2.6 µm particle size) column from Phenomenex (Torrance, CA, USA). Five microliters of each solution were injected into the chromatographic system. Mobile phase consisted of solvent A (water, 0.5 mM ammonium fluoride) and solvent B (methanol), by isocratic elution at a mixing ratio of 10:90% (v/v). The flow rate was 0.25 mL min^{-1} and the column temperature was kept constant at 55 ºC. The run time was 4 min.

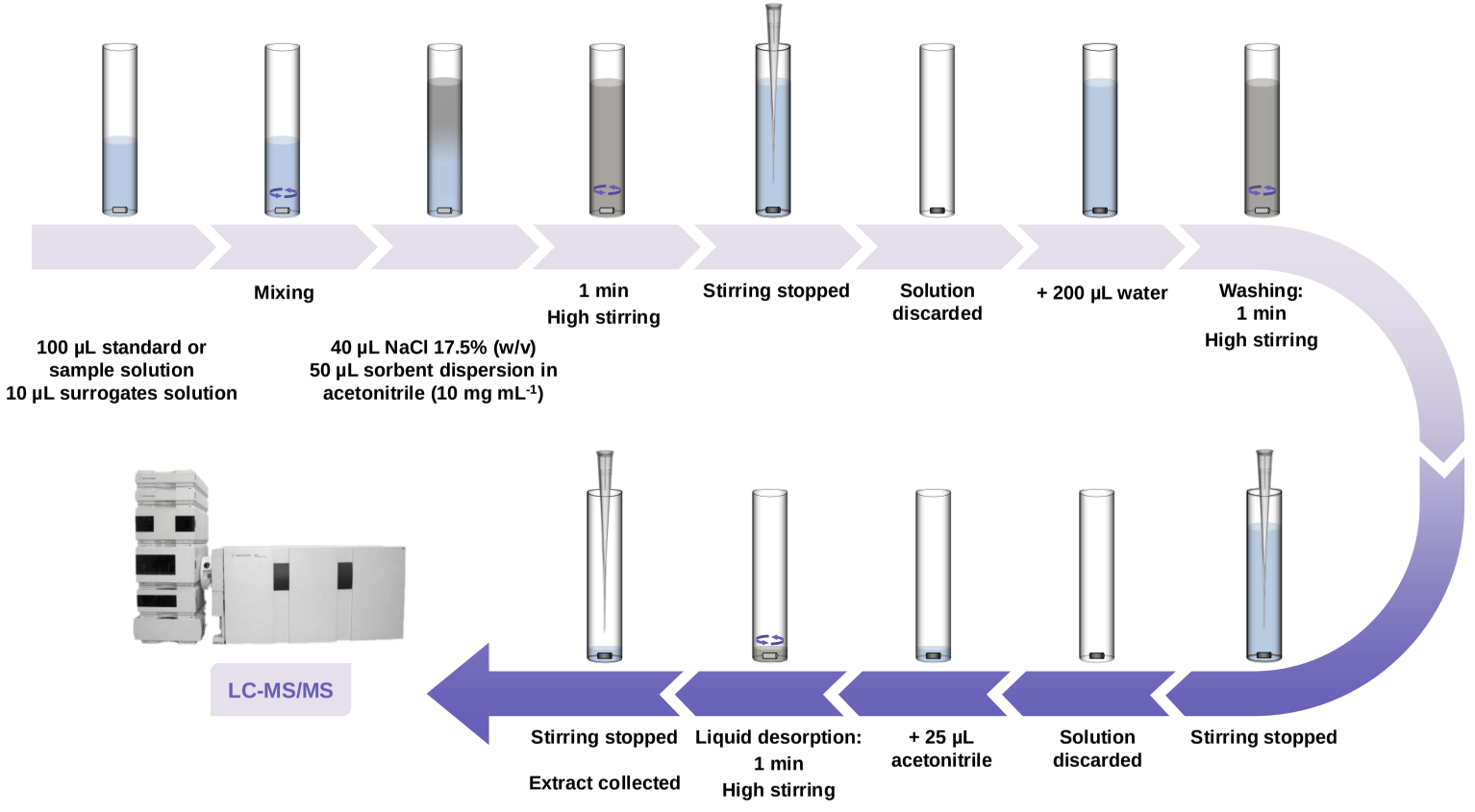


Figure 5.1. Schematic diagram of the proposed mSBSDME – LC-MS/MS method.

The triple quadrupole MS detector operated in electrospray ionization mode (ESI), by multiple reaction monitoring (MRM). Specifically, positive polarity (ESI+, capillary voltage at 6 kV) was used to measure the target analytes. The rest of conditions were: gas temperature at 310 °C, nebulizer gas flow rate at 12 L min^{-1}, nebulizer gas pressure at 35 psi and dwell time at 80 ms.

As both analytes are isomers, the m/z precursor → product ion transitions were equal for both. The m/z transition (collision energy, fragmentor) for quantification of THC and CBD was 315.2 → 193.1 (22 eV, 150 V); and for THC-D3 and CBD-D3 was 318.2 → 196.2 (26 eV, 140 V). For identification, they were 315.2 → 123.1 (34 eV, 150 V) and 315.2 → 93.1 (30 eV, 150 V) for THC and CBD; and 318.2 → 123.1 (38 eV, 140 V) and 318.2 → 93.1 (30 eV, 140 V) for THC-D_3 and CBD-D_3.

The following figure shows a chromatogram of a standard solution after applying the proposed method.

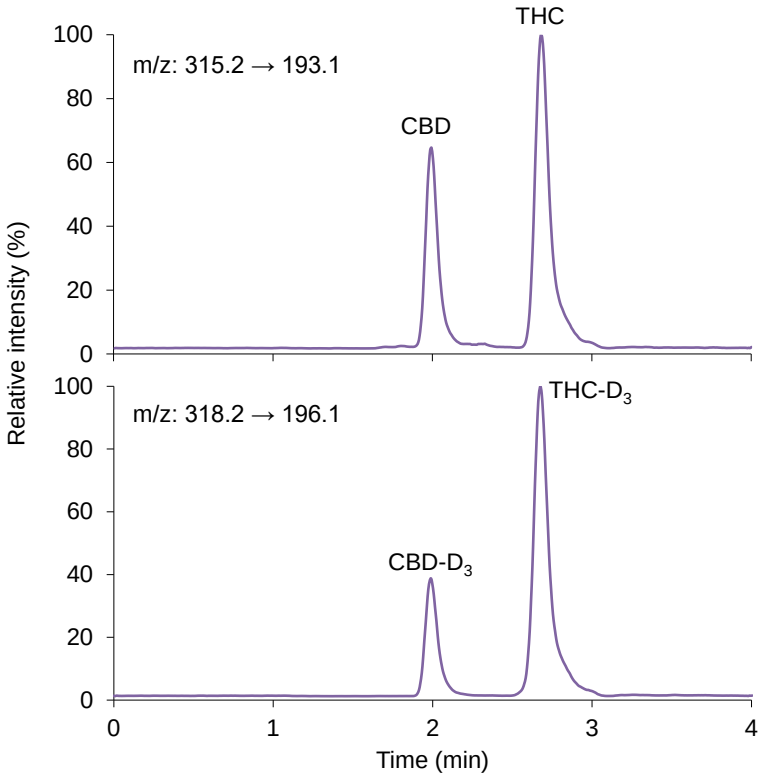


Figure 5.2. Chromatogram of a standard solution containing THC, CBD, THC-D_3 and CBD-D_3 at 50 ng mL^{-1} obtained after applying the proposed method.

5.3. Results and discussion

5.3.1. Conceptualization of the multiextraction platform

The design of the 96-position multiextraction platform was conceived taking as a starting point the miniaturized version of SBSDME presented in **Chapter 4**, where 15 samples can be extracted simultaneously [144]. In this new design, instead of using one stirring plate to stir several positions in a circular arrangement, a multi-stirring plate with 96 electromagnets providing 96 independent stirring positions was employed (**Figure 5.3.a**).

This stirring plate is intended to be used with commercial 96-well microtitration plates. However, they are usually made from plastic materials (*e.g.,* polypropylene, polystyrene, etc.) and wells diameter is such that the walls of adjacent wells touch each other. These characteristics may suppose some drawbacks for this technique. On the one hand, highly hydrophobic compounds like THC (and then CBD) can be partially adsorbed onto plastic material when prepared in aqueous solution, as demonstrated from previous works [269–271]. Therefore, glass material is a proper choice for these applications. In any case, the presence of deuterated surrogates in both standards and samples corrected these undesirable interactions and plastic pipette tips could be employed for the extraction procedure. On the other hand, magnets can interact with adjacent ones if the distance between wells is not high enough, thus preventing them to stir the solution properly.

These two limitations were easily overcome by the proposed assembly based on flat-base glass inserts, which are narrower and therefore magnets are more separated, and a 3D-printed support to located them (**Figure 5.3.b** and **c**). Besides, the proposed assembly is *ca.* 3 times cheaper than commercial plates [272]. The use of a 12-channel pipette is recommended to facilitate and speed up the procedure for the operator.

Furthermore, as an additional feature, the proposed platform is technically very simple, which allows it to be compact and easily portable, unlike above-mentioned 96-position workstations. The portability of the extraction devices represents a cost-effective alternative because the transportation and maintenance of the sample from the sampling point to the laboratory is minimized, which results in lower economic and resource expenditure.

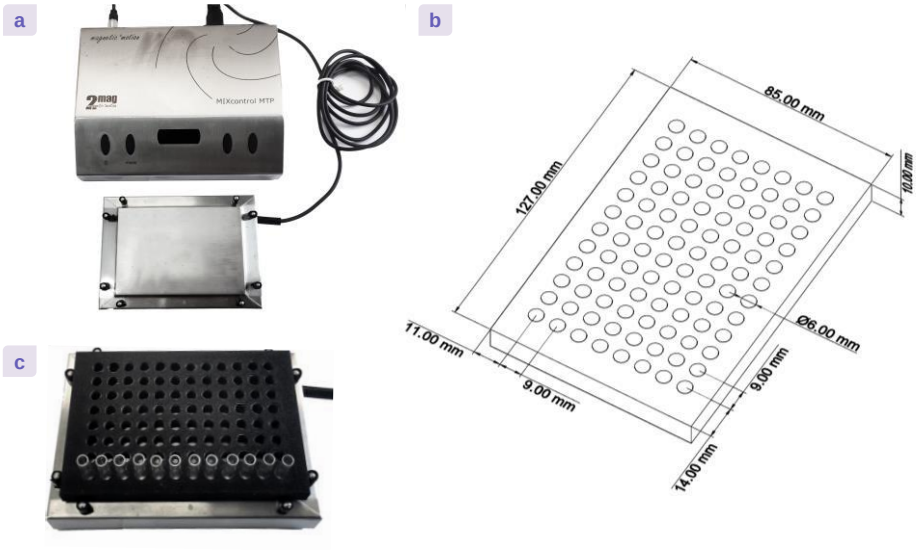


Figure 5.3. (a) Multi-stirring plate with 96 independent stirring positions; (b) sketch of the designed 3D-printed support; (c) 96-position extraction platform for mSBSDME.

5.3.2. Selection of the composite

As it was mentioned above, the target analytes exhibit high hydrophobicity and possess an aromatic ring and alcohol moieties, hence, the selection of $CoFe_2O_4$@p(DVB-co-NVP) as sorbent material sought to exploit the ability of the DVB and NVP copolymer to interact with the analyte by hydrophobic, π-π and hydrogen bond interactions.

The $CoFe_2O_4$ MNPs confer to this sorbent the magnetism needed for an easy retrieval by means of the magnet in mSBSDME. Cobalt ferrite ($CoFe_2O_4$) MNPs were preferred rather than usually-employed Fe_3O_4 MNPs due to their higher chemical stability [235]. Besides, the magnetization of the polymer through entrapment of MNPs provided fewer leaching problems compared to embedded materials, like those used previously [270]. The characterization of the material (*i.e.*, magnetization, morphology, adsorption features, and thermal stability) was described in a previous work of the research group in which this Doctoral Thesis has been carried out [268].

In order to study the extraction performance of $CoFe_2O_4$@p(DVB-co-NVP) towards THC and CBD, bare MNPs and three different batches of composite synthesized as described in **Section 5.2.4** were used to extract a standard solution (under non-optimized conditions) and results were compared (see

Figure 5.4). One-factor ANOVA tests revealed no significant differences between the three batches, hence showing good repeatability for the synthesis and performance of different batches of the composite. However, significant differences were found between the composite and bare MNPs, showing the extraction capacity of the material is mainly due to the copolymer.

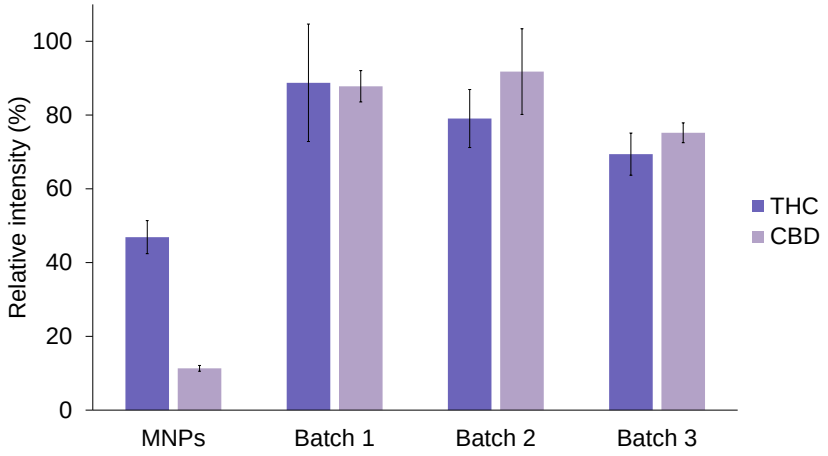


Figure 5.4. Comparison of the extraction performance between bare MNPs, and three batches of the $CoFe_2O_4$@p(DVB-co-NVP) composite.

5.3.3. Optimization of the mSBSDME variables

As known from previous works, pH is not expected to affect the extraction of the target analytes, as they are mainly neutral due to the high value of their pK_a (*i.e.*, $pK_a > 9$) [270,271]. Acetonitrile was selected as desorption solvent due to its demonstrated performance with a similar sorbent material and better chromatographic performance [270]. Besides, the ionic strength adjustment and acetonitrile content to precipitate proteins from saliva before extraction were adapted from a previous work [271]. As a difference, in the presented approach, the precipitation of proteins and extraction of the target analytes are carried out in one step, thus achieving an easier and faster workflow. Therefore, when the sorbent dispersion in acetonitrile is added to the insert, proteins precipitate, and as they are not recovered by the magnetic stir bar, they are discarded with the remaining turbid donor phase and the subsequent washing step. In preliminary experiments a washing time of 1 min was also stablished to achieve the total clean-up of proteins and salts.

The rest of variables involved in the mSBSDME (*i.e.,* sorbent amount, extraction time and desorption time) were evaluated with a screening study to determine which ones were critical. A Plackett-Burman design of was applied by to select

the variables that significantly affected the analytical response [273]. The studied variables and ranges were: (i) sorbent amount (as concentration of the dispersion in acetonitrile, 5-50 mg mL^{-1}), (ii) extraction time (1-10 min) and (iii) desorption time (1-5 min). 12 experiments were required (**Table 5.1)**. Aliquots of 100 µL of standard solution containing 100 ng mL^{-1} of the analytes prepared in synthetic saliva were used to perform the experiments. 40 µL of NaCl 17.5% (w/v) and 50 µL of sorbent dispersion were added. After the extraction, the analytes were desorbed in 25 µL of acetonitrile, as previously stablished. The selected analytical responses were the peak area of the analytes. A total of 24 experiments were performed, carrying out the design by duplicate and Minitab v.19.1 (Minitab, LLC. State College, PA, USA) software was employed for the statistical analysis.

Table 5.1. Plackett-Burman design for screening of critical variables

Step	Sorbent amount (mg)		Extraction time (min)		Desorption time (min)	
	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded
1	5	-1	10	1	1	-1
2	50	1	10	1	1	-1
3	5	-1	1	-1	5	1
4	50	1	1	-1	5	1
5	5	-1	10	1	5	1
6	50	1	10	1	1	-1
7	50	1	1	-1	1	-1
8	50	1	1	-1	5	1
9	5	-1	10	1	5	1
10	50	1	10	1	5	1
11	5	-1	1	-1	1	-1
12	5	-1	1	-1	1	-1

The results obtained were evaluated by ANOVA tests, obtaining adjusted $R^2 \geq 63\%$, and represented as Pareto charts of standardized effects, shown in **Figure 5.5**. As can be seen, only sorbent amount affected significantly to the extraction of both analytes, and, therefore, extraction and desorption times were set to the minimum (*i.e.,* 1 min) to achieve a faster method. Sorbent amount was optimized by a univariate approach (see **Figure 5.6**), selecting 10 mg mL^{-1} as concentration of the sorbent dispersion. Higher sorbent amounts were not completely recovered by the magnetic stir bar, thus, reducing extraction performance.

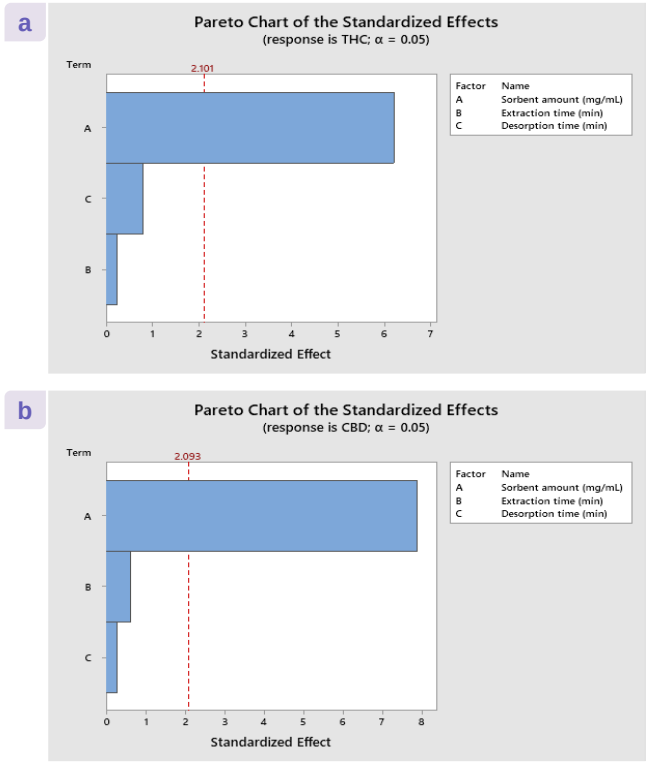


Figure 5.5. Pareto charts obtained in the Plackett-Burman design for: (a) THC and (b) CBD.

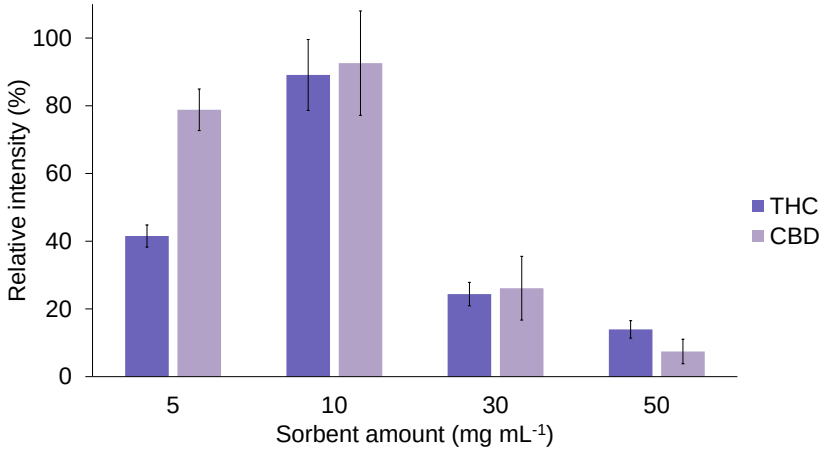


Figure 5.6. Effect of the sorbent amount (n = 3). (Extraction conditions: 100 µL standard solution; 40 µL NaCl 17.5 %(w/v); 50 µL sorbent dispersion; extraction time, 1 min. Desorption conditions: 25 µL acetonitrile; desorption time, 1 min).

5.3.4. Analytical performance of the proposed method

The quality parameters of the proposed method, such linearity, limits of detection (LOD) and quantification (LOQ), enrichment factors (EF), and intra- and inter-day precision, were evaluated under the optimized conditions.

The linearity was studied by measuring standard solutions prepared in synthetic saliva at different concentrations, all of them containing 50 ng mL^{-1} of surrogates. Calibration curves were plotted using the ratio of the peak area of each target analyte to its corresponding deuterated surrogate (A_i/A_s) versus the analyte concentration. The results indicated that the linearity reached at least 500 ng mL^{-1} for the target analytes. The determination coefficients revealed good linearity ($R^2 \geq 0.997$).

The LODs and LOQs were calculated as 3 times and 10 times, respectively, the signal-to-noise ratio of an analytes-free saliva sample spiked with 10 ng mL^{-1} after applying the proposed approach. The LOD and LOQ values were 0.7 and 2.4 ng mL^{-1}, respectively, for THC and 2.8 and 9.4 ng mL^{-1}, respectively, for CBD.

The EFs were estimated as the ratio of the slope of two calibration curves (10-500 ng mL^{-1}), one of them prepared in synthetic saliva and subjected to the proposed method, and the other one prepared in acetonitrile and directly injected into de LC-MS/MS without any extraction. EFs of 0.22 ± 0.02 and 1.12 ± 0.06 were achieved for THC and CBD, respectively. Even though these values are not very high (in fact, an actual enrichment is not achieved), the proposed method allows to reach very low LODs at the low ng mL^{-1} level. As the analytes are expected to be present at higher concentrations in saliva samples, the determination in saliva for the intended purpose of the method is accomplished. Besides, the clean-up step is necessary due to the high saline concentration of the saliva samples, which cannot be introduced directly in the MS instrument.

The precision of the method was evaluated by applying the proposed method to five independent replicates of standard solutions prepared in synthetic saliva containing the target analytes at three levels of concentration (*i.e.*, 10, 200 and 500 ng mL^{-1}). The results, expressed as the relative standard deviation (RSD, %) and presented in **Table 5.2** revealed that good precision was achieved for the target analytes.

Table 5.2. Precision of the proposed method

Analyte	Concentration (ng mL^{-1})	Intra-day precision (RSD, %) [a]	Inter-day precision (RSD, %) [a]
THC	10	13.9	12.4
	200	9.6	10.2
	500	2.8	12.2
CBD	10	9.6	7.9
	200	9.0	12.4
	500	3.4	8.5

[a] n=5

5.3.5. Analysis of real human saliva samples

Saliva samples from four non-smoker volunteers (samples A-D) collected as described in **Section 5.2.3** were spiked at two concentration levels (*i.e.,* 10, and 200 ng mL^{-1}) in order to evaluate the matrix effects by means of the relative recovery (RR) values, calculated as follows:

$$RR\ (\%) = \frac{Concentration_{spiked\ sample} - Concentration_{sample}}{Spiked\ amount} \cdot 100 \qquad \text{(Eq. 5.1)}$$

The results presented in **Table 5.3** proved that matrix effects were sufficiently corrected by the deuterated surrogates and internal standard calibration provided satisfactory results.

Table 5.3. Relative recoveries obtained from real samples at different spiked levels

Sample [a]	Spiked amount (ng mL^{-1})	Relative recovery (%) [b]	
		THC	CBD
A	10	110 ± 2	111 ± 2
	200	93 ± 3	96 ± 1
B	10	107 ± 4	106 ± 5
	200	93 ± 3	98 ± 1
C	10	103 ± 3	105 ± 8
	200	94 ± 2	97 ± 1
D	10	107 ± 8	111 ± 9
	200	97 ± 3	97 ± 6

[a] Sample A and B: female; Sample C and D: male
[b] Mean of three replicates ± standard deviation

Saliva samples from three illicit THC-rich cannabis smokers (samples E-G) and from three CBD-rich cannabis smokers (samples H-J) were collected after smoking as described in **Section 5.2.3** and treated by the proposed mSBSDME approach followed by LC-MS/MS. Results are shown in **Table 5.4**.

Table 5.4. Concentration of analytes in saliva samples from smokers

Sample [a]	Cannabis type	Found amount (ng mL^{-1}) [b]	
		THC	CBD
E	THC-rich	2680 ± 80	< LOD
F		24 ± 2	< LOQ
G		65 ± 7	< LOD
H	CBD-rich	10.6 ± 0.7	93 ± 10
I		9.2 ± 0.2	98 ± 7
J		53 ± 2	660 ± 60

[a] Sample E, F and H: female; Sample G, I and J: male
[b] Mean of three replicates ± standard deviation

As can be observed from **Table 5.4**, important differences have been found between both types of cannabis smokers. First, in THC-rich samples, CBD was below LOD or LOQ, as expected from the effort of marijuana croppers to selectively bred cannabis plants to produce highest THC and lowest CBD levels [265]. On the contrary, CBD-rich samples had higher levels of CBD and lower but still detectable levels of THC, exceeding tolerance limits for usual drugs tests.

5.3.6. Comparison of the proposed method with previously reported approaches

Compared with the previous configuration of mSBSDME (see **Chapter 4**) [144], the main advantage is the possibility to extract up to 96 samples simultaneously with the designed multiposition extraction platform. After the extraction, washing and desorption time (*i.e.*, 1 min each), a sample throughput lower than 2 s per sample is achieved.

The portability of the assembly, only requiring an external power supply, allows its use in any laboratory in an inexpensive and "ready-to-use" way. Additionally, it conserves the advances achieved with the miniaturization of the technique such as the easier handling (all stages in the same container), and the reduced consumption of sorbent, solvents and, very importantly, sample, which is essential for the analysis of those samples whose volume is limited.

However, it is true that in this particular application the previous centrifugation step of saliva samples is a limiting step, reducing the sample throughput.

Additionally, the proposed approach has been qualitatively compared with previous reported approaches for the determination of THC and CBD in saliva samples based on microextraction techniques (**Table 5.5**). As can be seen, the proposed workflow achieved comparable analytical performance (*i.e.*, LODs) when compared with previously reported approaches, and the sample volume is similar or smaller (even up to 10 times less) than other methods.

Having a look at the sample throughput values of these microextraction techniques, it can be clearly seen that higher sample throughput benefits the sustainability of the method. In those applications employing mSBSDME with multiposition extraction assemblies, the high number of samples being treated simultaneously considerably reduced analysis time and energy consumption. Moreover, it is aided by the dispersive nature of the technique, which increases the extraction kinetics. This effect can be also observed for the method based on microextraction by packed sorbent (MEPS), where the sample is percolated through the sorbent and the equilibrium is also reached faster than in static techniques (*i.e.*, SPME), achieving a higher throughput.

The greenness of these methods, as part of the degree of sustainability of a method, was evaluated by estimating their AGREEprep score [201], a metric tool that takes into account all the aspects raised in the ten principles of Green Sample Preparation [5]. This score considers environmental and health impact factors, such as the use of reagents, the energy consumption, sample size and generated waste. The obtained pictogram for the proposed method is showed in **Figure 5.7**.

Table 5.5. Comparison of the proposed method with other microextraction-based approaches for the determination of THC and CBD in saliva

Analytes [a]	Extraction technique	Analytical technique	Sample volume (µL)	LOD (ng mL^{-1})	Sample throughput (samples h^{-1}) [b]	AGREEprep Score [c]	Ref.
THC, **CBD**, CBN	SPME	GC-MS	100	0.5 (THC) 2 (CBD)	2	0.47	[274]
THC-COOH, THC-OH, **THC**, **CBD**, CBN	MEPS	LC-MS/MS	125	0.25 (THC) 0.30 (CBD)	24	0.47	[275]
THC	mSBSDME	MS/MS	250	2.25 (THC)	13	0.51	[271]
THC, **CBD**, CBN, synthetic cannabinoids	SPME	GC-MS	1000	1 (THC) 5 (CBD)	2	0.59	[276]
THC, **CBD**	mSBSDME	LC-MS/MS	100	0.7 (THC) 2.8 (CBD)	150	0.65	This work

[a] CBD: Cannabidiol; CBN: Cannabinol; THC: Δ^9-Tetrahydrocannabinol; THC-COOH: 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol; THC-OH: 11-Hydroxy- Δ^9-tetrahydrocannabinol

[b] Sample throughput of the extraction technique, estimated considering both the extraction and desorption steps, and the associated handling. The time spent on sample pretreatment and the instrumental analysis has not been considered

[c] Estimated value considering the whole sample preparation method

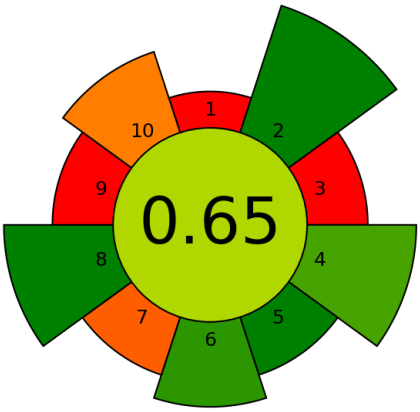


Figure 5.7. AGREEprep pictogram of the proposed method. (1-Sample preparation placement; 2-Hazardous materials; 3-Sustainability and renewability of materials; 4-Waste; 5-Size economy of the sample; 6-Sample throughput; 7-Integration and automation; 8-Energy consumption; 9-Post sample preparation configuration for analysis; 10-Operator's safety).

5.4. Conclusions

In this work, a 96-position multiextraction platform for mSBSDME is presented for the first time. The dispersion of the sorbent within the sample achieves high extraction kinetics that, together with the possibility of simultaneously extracting 96 samples, increase considerably sample throughput compared with previous approaches.

Additionally, the miniaturized configuration of the employed approach permits a rapid determination of target analytes employing few microliters of sample, organic solvents, and sorbent, thus being in accordance with current trends in Analytical Chemistry.

As a proof-of-concept of this new methodology, the determination of THC and CBD in saliva from cannabis smokers was selected. Good analytical features were obtained for both analytes, seeing differences between the consumption of marijuana and legal CBD-rich cannabis.

In short, the proposed approach offers a new high-throughput, feasible and cost-effective alternative for bioanalysis. It could be applicable in many areas such as forensic or clinical analysis, where many samples need to be treated every day and the volume of these samples is usually limited.

Chapter 6

Determination of hexanal and heptanal in saliva samples by an adapted magnetic headspace adsorptive microextraction for diagnosis of lung cancer

The content of this chapter has been published in the following journal article:

C. Azorín, A.L. López-Juan, F. Aparisi, J.L. Benedé, A. Chisvert, Determination of hexanal and heptanal in saliva samples by an adapted magnetic headspace adsorptive microextraction for diagnosis of lung cancer, Anal. Chim. Acta 1271 (2023) 341435

Abstract

In this chapter, an analytical method for the determination of two endogenous aldehydes (hexanal and heptanal) as lung cancer biomarkers in saliva samples is presented for the first time. The method is based on a modification of magnetic headspace adsorptive microextraction (M-HS-AME) followed by gas chromatography-mass spectrometry (GC-MS). For this purpose, an external magnetic field generated by a neodymium magnet is used to hold the magnetic sorbent (*i.e.*, $CoFe_2O_4$ magnetic nanoparticles (MNPs) embedded into a reversed-phase polymer) in the headspace of a microtube to extract the volatilized aldehydes. Subsequently, the analytes are desorbed in the appropriate solvent and the extract is injected into the GC-MS system for separation and determination. Under the optimized conditions, the method was validated and showed good analytical features in terms of linearity (at least up to 50 ng mL^{-1}), limits of detection (0.22 and 0.26 ng mL^{-1} for hexanal and heptanal, respectively), and precision (relative standard deviation ≤ 12%). This new approach was successfully applied to saliva samples from healthy volunteers and those with lung cancer, obtaining notably differences between both groups. These results reveal the prospect of the method as potential diagnostic tool for lung cancer by saliva analysis. This work contributes to the Analytical Chemistry field presenting a double novelty: on the one hand, the use of M-HS-AME in bioanalysis is unprecedentedly proposed, thus expanding the analytical potential of this technique, and, on the other hand, the determination of hexanal and heptanal is carried out in saliva samples for the first time.

6.1. Introduction

Lung cancer is the leading cause of cancer-related death in adults [277] being tobacco its main risk factor, related since 1954 by Doll *et al.* [278]. Worldwide, it is estimated that there are 1.6 million deaths due to lung cancer annually [279]. Clinical outcome for non-small cell lung cancer is directly related to stage at the time of diagnosis and, within early lung cancers (stage I), there is a relationship between tumor size and survival [280].

Many characteristics of lung cancer suggest that screening should be effective: high morbidity and mortality, significant prevalence (0.5 to 2.2%), identified risk factors allowing targeted screening for high-risk individuals, a lengthy preclinical phase for some types of lung cancer, and evidence that therapy is more effective in early-stage disease [281]. Prevention (by preventing smoking and promoting smoking cessation) will have far greater impact on reducing lung cancer mortality than screening. Nonetheless, lung cancer screening with low-dose computed tomography and treatment has been shown to significantly reduce the burden of lung cancer morbidity and mortality [282,283].

Therefore, it is crucial to identify, detect and quantify biomarkers that allow its early diagnosis. Endogenous aldehydes are by-products of cellular lipid oxidation caused by high levels of free radicals, which have been linked with some diseases, including cancer. Hence, aldehydes are considered potential biomarkers of oxidative activity [284,285]. To this regard, it has been reported that hexanal and heptanal are present in both breath and blood samples from cancer patients but not in control group [286,287], what makes them useful for the early diagnosis of lung cancer. Besides exhaled breath [288–290] and blood [287,291–299], they have been determined in other biological matrices such as urine [291,294,300–307], pleural effusion [308] and sweat [309].

Due to the correlation between numerous salivary and blood biomarkers and the non-invasive, painless, and straightforward nature of saliva collection, saliva has recently emerged as a potent and promising platform for bioanalysis [310]. However, to the best of our knowledge, there are no methods in the literature for the quantitative determination of hexanal and heptanal in saliva. Nonetheless, at this point, it should be mentioned the paper published by Shigeyama *et al.*, who identified them as potential cancer biomarkers in an untargeted study carried out in saliva [311].

It should be noted that bioanalysis usually comprises the determination of compounds at very low concentrations in complex biological matrices. Hence, selective and sensitive methods are necessary, typically combining (micro)extraction techniques with highly sensitive detectors. In this sense, chromatographic systems (both, liquid (LC) and GC) coupled to MS have been extensively used for the determination of hexanal and heptanal

[287,289,290,292,297–300,303,304,306,308,309]. Among the vast selection of (micro)extraction techniques available in literature, headspace solid-phase microextraction (HS-SPME) has been widely employed, exploiting the volatility of the analytes [291,298,299,301,306,308].

As a consequence of the physical and chemical characteristics of aldehydes, such as lack of intrinsic chromophores, high volatility and low chemical stability, the methods for their determination usually include a derivatization step. 2,4-Dinitrophenylhydrazine and O-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride are mainly proposed as derivatization reagents for both LC and GC analysis [289,290,292–299,302,307]. However, some approaches without derivatization have also been proposed for GC [287,288,291,300,301,304,306,308,309].

As previously stated, HS-SPME has become a benchmark for the separation and preconcentration of volatile analytes from a wide variety of matrices [312]. HS-SPME has clear advantages such as its simplicity and reproducibility, requiring minimal sample pretreatment and low (if not completely avoided) consumption of organic solvents, in consonance with the principles of Green Sample Preparation [5]. However, the fibers used in the HS-SPME method are usually expensive and the commercially-available sorbents are limited, thus reducing the possibilities for analytical laboratories.

In this chapter, a useful and reliable analytical method for the determination of hexanal and heptanal as lung cancer biomarkers in saliva samples is presented. For this purpose, the excellent performance that M-HS-AME presents has been exploited, thus expanding the possibilities of this technique. Nevertheless, it has been adapted by miniaturizing it and applied for the analysis of biological samples. A magnetic composite material made of $CoFe_2O_4$ MNPs embedded into a commercial reversed-phase polymer (*i.e.*, Strata™-X-RP) was used for the first time as sorbent material in M-HS-AME [143]. Cobalt ferrite ($CoFe_2O_4$) MNPs confer to this sorbent the magnetism needed and were preferred rather than usually-employed Fe_3O_4 MNPs due to their higher chemical stability [235].

The main parameters affecting the extraction and desorption stages were optimized by either a response surface methodology (RSM) or a univariate approach. After the extraction, samples were injected into a GC-MS system for quantification. This is the first time, to the best of our knowledge, that the determination of hexanal and heptanal in saliva and the application of M-HS-AME in biological matrices are reported.

6.2. Experimental

6.2.1. Reagents

Hexanal (98%) and heptanal (97%) were purchased from Fisher Scientific (Loughborough, United Kingdom). 2,2-Dimethyl-4-pentenal (90%) (used as surrogate) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

For the synthesis of $CoFe_2O_4$ MNPs, cobalt(II) chloride hexahydrate and iron(III) chloride hexahydrate were purchased from Acros Organics (Fair Lawn, NJ, USA), sodium hydroxide was purchased from Scharlau (Barcelona, Spain), and ethanol absolute was purchased from Panreac (Barcelona, Spain). For the synthesis of the composite, Strata™-X-RP 33 µm polymeric reversed phase SPE cartridges (500 mg/6 mL) were obtained from Phenomenex (Torrance, CA, USA). Octyl and octadecyl silica (C8 and C18) microparticles were obtained from Sigma-Aldrich.

Ultrapure water (resistivity ≥ 18.2 MΩ·cm) was provided by a Connect water purification system provided by Adrona (Riga, Latvia). Ethanol absolute and LC-grade acetonitrile were obtained from Panreac. Anhydrous sodium sulphate was purchased from Scharlau.

For the preparation of synthetic saliva, sodium chloride from Fisher Scientific, potassium chloride, calcium chloride monohydrate, potassium thiocyanate and urea from Panreac, and sodium dihydrogen phosphate monohydrate from Scharlau were used.

Extra pure helium (> 99.999%), used as carrier gas, was obtained from Carburos Metálicos S.A. (Paterna, Spain).

6.2.2. Apparatus and materials

For the synthesis of the magnetic composite, a heating plate with magnetic stirrer from Stuart Scientific (Staffordshire, United Kingdom) and an oven from J.P. Selecta (Barcelona, Spain) were used.

For the extraction stage, 1.5-mL centrifuge microtubes from Fisher Scientific, neodymium magnetic cubes (5 mm, 42 MGO) from Supermagnete (Gottmadingen, Germany), and a 24-position thermo-shaker model TS-100 SC-24N from BioSAN (Madrid, Spain) were used. For the dispersion of the magnetic composite in the desorption stage, a 10-position multiple stirring plate model MS-M-S10 from Labbox (Barcelona, Spain) was used. An EBA 21 centrifuge provided with a rotor of 15 cm radius from Hettich (Tuttlingen, Germany) was used to centrifuge the injection vials.

For the GC-MS analysis, an 8860 GC system coupled to a 5977B single quadrupole mass spectrometer, both from Agilent Technologies (Palo Alto, CA, USA), and provided with a PAL LSI 85 autosampler from CTC Analytics (Zwingen, Switzerland) was employed.

6.2.3. Synthesis of $CoFe_2O_4$-Strata™-X-RP magnetic composite

The synthesis of the $CoFe_2O_4$-Strata™-X-RP composite consisted of two steps:

1. Synthesis of the $CoFe_2O_4$ MNPs by wet chemical co-precipitation according to an adapted protocol [235].
2. Subsequent incrustation of MNPs on the polymeric surface.

First, 10.80 g of $FeCl_3 \cdot 6H_2O$ (40 mmol) and 4.76 g of $CoCl_2 \cdot 6H_2O$ (20 mmol) were dissolved in 200 mL of ultrapure water, and then 100 mL of a 3 M sodium hydroxide aqueous solution (300 mmol) were added dropwise under continuous stirring for one hour at 80 ºC. After this time, it was allowed to cool to room temperature to decant the formed solid using a magnet. Then, the solid was washed several times with ultrapure water and later with ethanol, dried in an oven at 80 ºC overnight, and grinded until obtaining a fine black powder.

For the preparation of the composite, where MNPs are embedded on the polymer surface, 0.15 g of the MNPs and 0.15 g of Strata™-X-RP were weighted, and 50 mL of ethanol were added. The mixture was stirred at room temperature for 72 h to ensure the MNPs were embedded on the polymer pores. Finally, the solid was decanted with the aid of a magnet and washed three times with ethanol to discard the remaining non-magnetic polymer. Afterwards, it was resuspended in ethanol and filtered under vacuum through a 10 µm-pore paper filter in order to discard the free MNPs and isolate the composite. It was dried in an oven at 80 ºC overnight and grinded into a fine powder.

6.2.4. Preparation of synthetic saliva

Synthetic saliva was prepared according to an adapted protocol [254]. For that aim, 250 mL of an aqueous solution containing NaCl (400 mg L^{-1}), KCl (400 mg L^{-1}), $CaCl_2 \cdot H_2O$ (795 mg L^{-1}), $NaH_2PO_4 \cdot H_2O$ (690 mg L^{-1}), KSCN (300 mg L^{-1}) and urea (1000 mg L^{-1}) in ultrapure water were prepared.

6.2.5. Sample collection and pretreatment

Saliva samples were collected in 15-mL centrifuge tubes from volunteers by the passive drooling method [255]. In contrast to swab collection methods (*e.g.*,

Salivette®), that usually introduce variability and negatively affect results [256], passive drooling does not interfere with the salivary analytes being measured. Following collection, samples were centrifuged for 10 min at 6000 rpm to separate saliva from other materials, such as phlegm or food leftovers, and the supernatant was then transferred to a microcentrifuge tube. Then, samples were frozen at -20°C to precipitate mucins and thus reduce viscosity of saliva [257], which was demonstrated to have no effect on the concentration of the analytes (see **Section 6.3.5**). Just before the analysis, they were thawed and centrifuged again at 18000 rpm for 5 min.

The samples included in this study were managed and provided by Biobanco La Fe (PT17/0015/0043) with the approval of both ethics and scientific committees and the ethical committee of the University of Valencia (Spain). The samples have been processed following standardized procedures conformed to the ethical guidelines of the Declaration of Helsinki.

6.2.6. Preparation of standard solutions

A multicomponent standard solution of the analytes (hexanal and heptanal) containing 1000 μg mL^{-1} in methanol was prepared. From this solution, an intermediate solution of 10 μg mL^{-1} in methanol was prepared. Similarly, 1000-μg mL^{-1} and 10-μg mL^{-1} solutions of 2,2-dimethyl-4-pentenal (surrogate) in methanol were prepared. These solutions were kept at -20 °C and protected from light.

A multicomponent solution of hexanal and heptanal of 100 ng mL^{-1} and a solution of 2,2-dimethyl-4-pentenal of 1 μg mL^{-1} were prepared daily from these solutions, both in synthetic saliva.

6.2.7. M-HS-AME procedure

The caps of 1.5-mL centrifuge microtubes were previously separated from them. Inside the caps, 1 mg of the $CoFe_2O_4$-Strata™-X-RP composite (prepared as described in **Section 6.2.3**) was weighed, and a piece of double-sided adhesive tape was attached on the outside face to hold a cubic neodymium magnet, used to hold the magnetic composite when they are flipped, as it will be seen later.

The working standard solutions of hexanal and heptanal (1-10 ng mL^{-1}) were prepared directly in the cap-less centrifuge microtubes by adding the appropriate volume of the multicomponent solution of 100 ng mL^{-1} and diluting up to 1 mL with synthetic saliva. Subsequently, 50 μL of the 1-μg mL^{-1} surrogate solution were added.

The sample solutions were similarly prepared by adding 50 µL of the 1-µg mL^{-1} surrogate solution to 1 mL of saliva, obtained as described in **Section 6.2.5**. If sample volume was limited (*i.e.,* < 1 mL), saliva samples were diluted up to 1 mL with synthetic saliva.

Caps containing the magnetic composite were turned upside down and attached to the tubes containing standard and sample solutions. Then, they were placed in the thermo-shaker at 30 ºC, and shaked at 1400 rpm for 40 min. After this time, the caps of the microtubes were removed and turned again placing the composite upwards. Then, the external magnets were removed.

To perform the liquid desorption of the analytes, a tiny amount of anhydrous Na_2SO_4 to remove the possible condensed water during extraction and 50 µL of acetonitrile were added. An empty microtube was placed onto the cap to facilitate handling. Afterwards, they were placed in the multiple-position magnetic stirrer and stirred for 30 s. Once this time had elapsed, the assembly was placed on a neodymium magnet to deposit the magnetic composite, then the microtube was removed and the extract was transferred with the aid of a pipette from the cap to an injection vial provided with a conical insert. Finally, they were centrifuged at 6000 rpm for 5 min to deposit the possible collected magnetic composite or desiccant at the bottom and prevent their injection into the GC-MS system. **Figure 6.1** shows a schematic diagram of the proposed method.

6.2.8. GC-MS analysis

Separations were carried out in a VF-WAXms capillary column (polyethylene glycol, 30 m length, 0.25 mm I.D., 0.25 µm film thickness) from Agilent Technologies.

One microliter of each extract was injected in split mode (split ratio 5:1) into the chromatographic system. Helium as carrier gas was set at a flow rate of 1 mL min^{-1}. The oven temperature program was set as follow: from 50 °C (3 min) to 100 °C at 15 °C min^{-1}, then to 230 °C at 50 °C min^{-1}. Injector, transfer line and source temperatures were set at 230 °C.

The chromatograms were recorded in selected ion monitoring (SIM) mode. The m/z values for quantification were 56, 70 and 55 for hexanal, heptanal and surrogate, respectively. For identification, they were 72 and 82 for hexanal, 55 and 86 for heptanal, and 41 and 43 for surrogate.

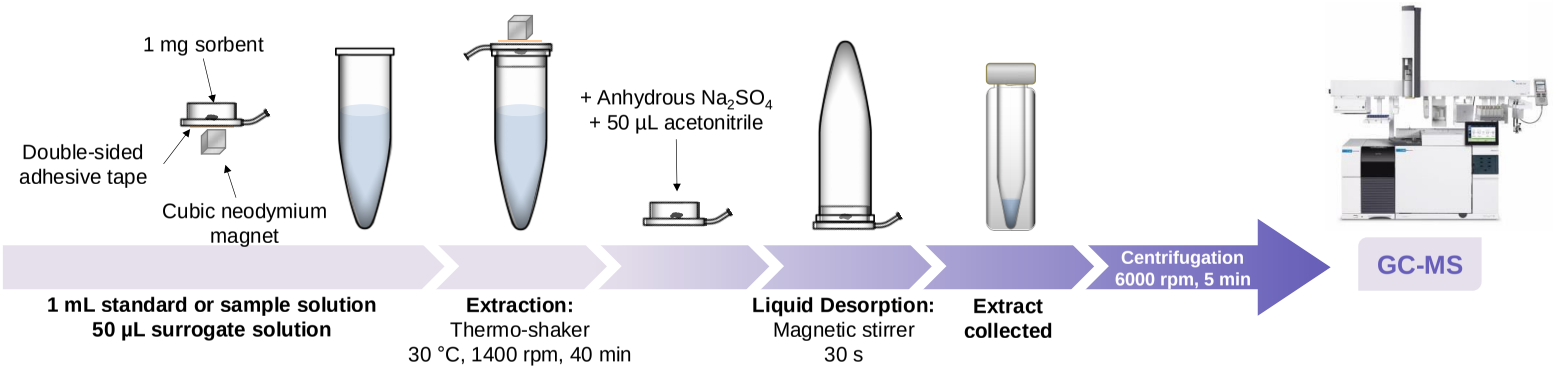


Figure 6.1. Schematic diagram of the proposed M-HS-AME–GC-MS method.

The following figure shows a chromatogram of a standard solution after applying the proposed method.

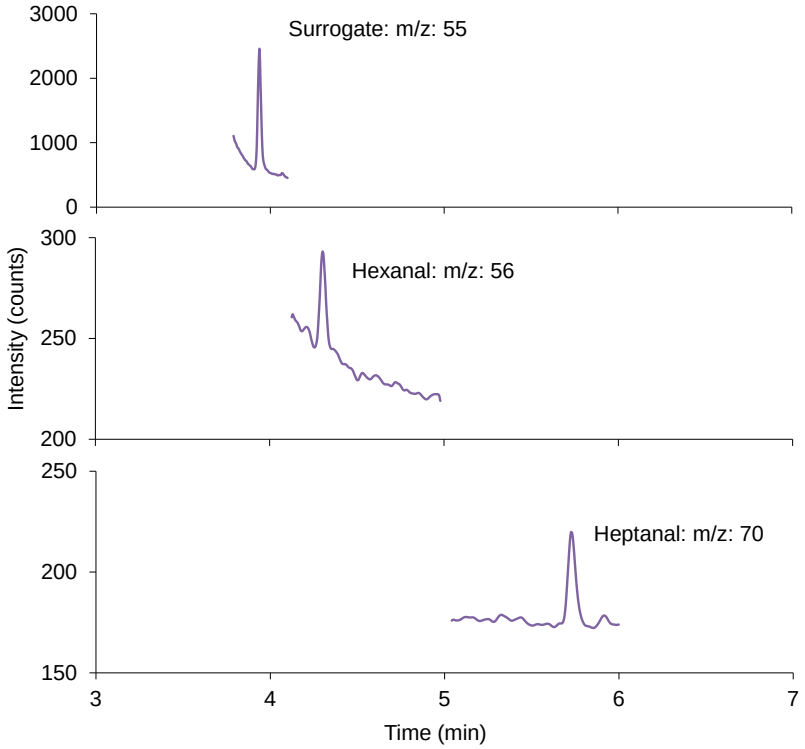


Figure 6.2. Chromatogram of a standard solution containing 0.75 ng mL^{-1} of hexanal, 0.85 ng mL^{-1} of heptanal, and 50 ng mL^{-1} of surrogate obtained by the proposed method.

6.3. Results and discussion

6.3.1. Adaptation of the extraction device

As previously described in **Section 1.3.2**, to the best of our knowledge, only three configurations have been described using magnetic (nano)materials as sorbent phase for headspace extraction.

In the first case [50] (**Figure 6.3.a**), the magnetic sorbent was placed in a 2-mL vial inside of a 10-mL vial containing the sample, both placed in an oven. The magnetism was only used to separate the sorbent from the extract after liquid desorption. Later, in the approach proposed by Vidal *et al.* [51] (**Figure 6.3.b**), the magnetic sorbent material was attached to the end of a small neodymium

magnet, located in the headspace of a 25-mL extraction vial. To fix this magnet in the headspace, another neodymium magnet was placed on the cap of the vial, without the need for any other device. The inner magnet was then eliminated by Timofeeva and co-workers [52] (**Figure 6.3.c**), placing the sorbent directly on the cap of the vial.

In this work, we proposed an adaptation of the latter employing 1.5-mL centrifuge microtubes, whose caps are separated from the body. The magnetic material is placed in the inner part of the cap and an external cubic magnet is used to keep the sorbent in place. A double-sided adhesive tape is used to easily attach and detach the external magnet to the cap (**Figure 6.3.d**).

An important advantage of this new approach is that a large number of samples can be treated simultaneously in a semi-automated way by using a thermo-shaker adapted for centrifuge microtubes. Additionally, the microtube cap can be easily turned upside down after the extraction, and liquid desorption can be performed in the same cap, avoiding the transfer of the magnetic composite. Moreover, the intrinsic magnetism of the composite is enough to be stirred by the magnet of the multi-position stirring plate during the desorption step, without the need to incorporate a stirring magnet, achieving an easy and effective dispersion of the sorbent within the desorption solvent.

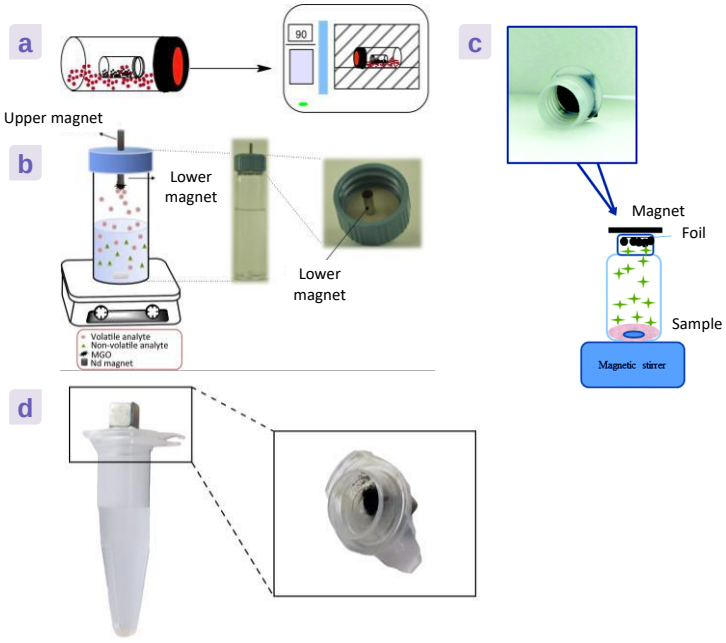


Figure 6.3. Different configurations of the extraction device for M-HS-AME: (a), (b) and (c) are reproduced with permission from [50], [51] and [52], respectively, and (d) is the proposed extraction device.

6.3.2. Selection of the composite

Hexanal and heptanal possess a hydrophobic carbon chain and a carbonyl group. Hence, different sorbent materials based in MNPs embedded into commercial hydrophobic sorbents were tested. MNPs were embedded into C8 and C18-coated silica and a commercial pyrrolidone-modified styrene-divinylbenzene copolymer (*i.e.,* Strata™-X-RP), following the procedure described in **Section 6.2.3**.

The extraction performance of these magnetic composites and bare MNPs was evaluated and compared. As can be seen in **Figure 6.4**, the magnetic composite based on Strata™-X-RP showed greater performance for the extraction of hexanal and heptanal due to its hydrophobic and dipole-dipole interactions with the analytes, unlike the other two materials that only offered hydrophobic interactions. Therefore, the former was selected for the subsequent work. It was also proved that the polymer, but not the MNPs, was the responsible for the extraction of analytes.

The characterization of this material, including synthesis repeatability, can be revisited in a previous work of the research group in which this Doctoral Thesis has been carried out [143]. Magnetization, particle size distribution, morphology, specific surface area and pore size were established. In addition, energy dispersive X-ray spectroscopy (EDS) was performed for elemental analysis.

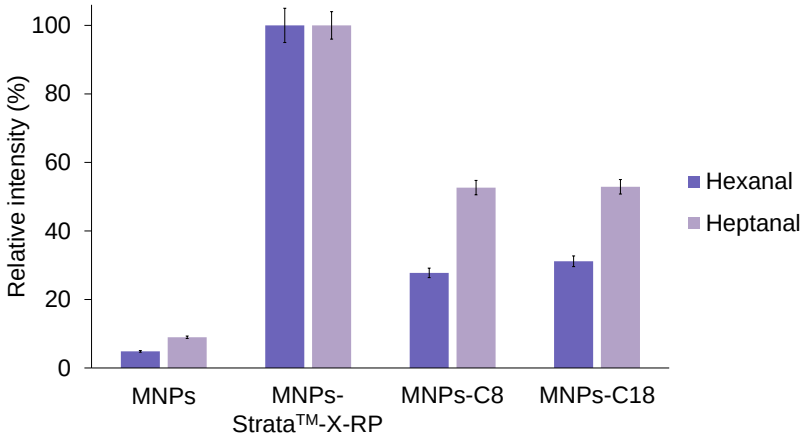


Figure 6.4. Comparison of extraction performance between different magnetic composites and bare MNPs.

6.3.3. Optimization of the desorption variables in M-HS-AME

In order to ensure maximum preconcentration of analytes, the minimum solvent volume (*i.e.,* 50 µL) was used for the desorption step in subsequent experiments. Lower volumes were not feasible because its retrieval from the microtube cap containing the sorbent and the desiccant was highly complicated from the practical point of view. Both solvent and desorption time were optimized to maximize the recovery of analytes from sorbent material after the extraction.

For the optimization of the desorption solvent, a Simplex-Centroid mixture design [313] was used. The description of the statistics of the Simplex-Centroid design are included in **ANNEX I**. Three GC-compatible solvents were selected (*i.e.,* methanol (X_1), acetonitrile (X_2) and acetone (X_3)). In this sense, according to **Equation A-I.5**, 7 experiments (**Table 6.1**) were required. Points 1, 2 and 3 (triangular vertices) correspond to the pure pseudo-components. Points 4, 5 and 6 were the binary mixtures of two, and point 7 (the center of the triangle) was the ternary mixture.

Table 6.1. Simplex-Centroid Design for optimization of the desorption solvent

Step	Acetonitrile	Methanol	Acetone
1	1	0	0
2	0	1	0
3	0	0	1
4	0.5	0.5	0
5	0.5	0	0.5
6	0	0.5	0.5
7	0.333	0.333	0.333

Aliquots of 1 mL of standard solution containing 50 ng mL^{-1} of the analytes were extracted under the following extraction conditions: 1 mg of sorbent material, 10 min, 40 °C, 1400 rpm and desorbed for 1 min in 50 µL of solvent. The selected analytical responses were the peak area of the analytes. StatGraphics Centurion XVI (Stat Point Inc. Herndon, VA, USA) software was employed for the statistical analysis. The triangular surface obtained with the proposed Simplex-Centroid design and a quadratic function with adjusted determination coefficients from 61 to 97% is shown in **Figure 6.5**. As it can be observed, the desorption was more efficient when acetonitrile was used. This is probably due to the better dispersion of the sorbent material in this solvent. Therefore, acetonitrile was selected as desorption solvent.

Then, the desorption time was tested by a univariate approach. The composite recovered from the extraction was stirred in 50 µL of acetonitrile for 0 (*i.e.,* placing the solvent on the microtube cap and retrieving it immediately without stirring), 0.5, 1, 3 and 5 min. **Figure 6.6** shows that 0.5 min provided the best results.

Higher desorption times might lead to analyte loss by volatilization, so 0.5 min was selected as desorption time.

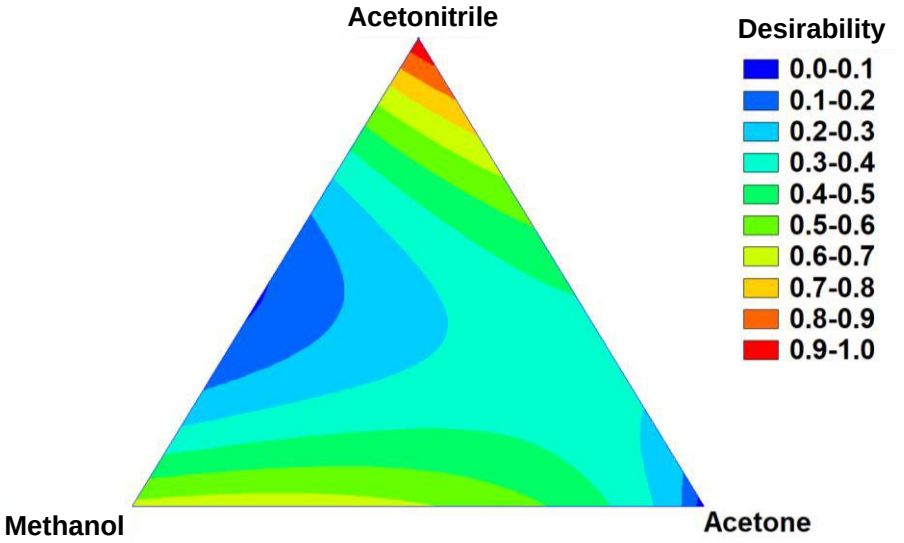


Figure 6.5. Triangular surface obtained through simplex-centroid design for the optimization of the desorption solvent. (Extraction conditions: sample volume, 1 mL; sorbent amount, 1 mg; extraction time, 10 min; 40 °C; 1400 rpm. Desorption conditions: solvent volume, 50 µL; desorption time, 1 min).

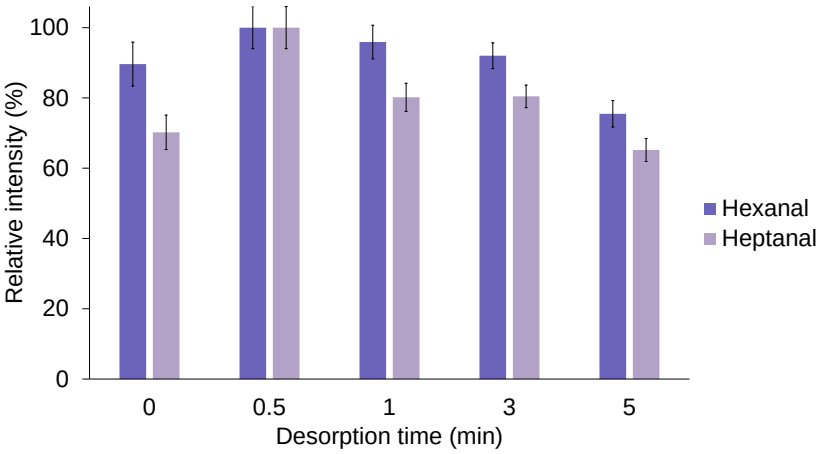


Figure 6.6. Optimization of the desorption time. (Extraction conditions: sample volume, 1 mL; sorbent amount, 1 mg; extraction time, 10 min; 40 °C; 1400 rpm. Desorption conditions: 50 µL acetonitrile).

6.3.4. Optimization of the extraction variables in M-HS-AME

An RSM [234] based on a four-factor three-level Box-Behnken design was performed to stablish the optimal values of the variables involved in the extraction procedure. The description of the statistics of the Box-Behnken design are included in **ANNEX I**. The studied variables and ranges were: (i) sorbent amount (0.5-2 mg), (ii) extraction time (5-60 min), (iii) temperature (30-60 ºC) and (iv) shaking rate (600-1400 rpm). The volume of the donor solution was set to 1 mL trying to increase the ratio between donor an acceptor phases but as a compromise between the capacity of the microtube (< 1.5 mL) where the microextraction was performed and the restrictions encountered for sample availability. On the other hand, since the analytes do not present ionizable moieties in their chemical structure, their partition coefficients are not affected by the pH, and thus this variable was not considered relevant and as consequence it was not considered in the optimization.

In this sense, according to **Equation A-I.1**, considering 4 factors and 3 replicates of the central point, 27 experiments were required (**Table 6.2**). Since human saliva is mainly composed of water (*ca.* 99%), aliquots of 1 mL of standard solution containing 50 ng mL^{-1} of the analytes prepared in synthetic saliva were used to perform the experiments. After the extraction, the analytes were desorbed in 50 µL of acetonitrile for 0.5 min, as previously stablished. The selected analytical responses were the peak area of the analytes. StatGraphics Centurion XVI (Stat Point Inc. Herndon, VA, USA) software was employed for the statistical analysis.

As shown in **Figure 6.7**, temperature was the variable that appreciably affected the extraction performance. The adequacy of the model was evaluated by the coefficient of determination (R^2), which was ≥ 0.78. This value indicates that the designed model was efficient for the prediction of response.

Figure 6.8 shows response surfaces of desirability function. As shown in **Figure 6.8.a**, the best responses were obtained when the mass of the sorbent was between *ca.* 0.8 and 1.1 mg. The mass transfer of analytes from the donor phase to the sorbent depends on the ratio of the sorbent area to the volume of the aqueous solution. Therefore, extraction performance increased from 0.5 to 1.1 mg of composite. However, for higher amounts, this value decreased because more sorbent within the same volume of elution solvent hindered its correct dispersion during the desorption step. Hence, 1 mg of sorbent material was used for extraction.

Table 6.2. Box-Behnken Design for multivariate optimization of the critical variables

Step	Sorbent amount (mg)		Extraction time (min)		Temperature (ºC)		Shaking rate (rpm)	
	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded
1	0.5	-1	5	-1	45	0	1000	0
2	2	1	5	-1	45	0	1000	0
3	0.5	-1	60	1	45	0	1000	0
4	2	1	60	1	45	0	1000	0
5	1.25	0	32.5	0	30	-1	600	-1
6	1.25	0	32.5	0	60	1	600	-1
7	1.25	0	32.5	0	30	-1	1400	1
8	1.25	0	32.5	0	60	1	1400	1
9	0.5	-1	32.5	0	45	0	600	-1
10	2	1	32.5	0	45	0	600	-1
11	0.5	-1	32.5	0	45	0	1400	1
12	2	1	32.5	0	45	0	1400	1
13	1.25	0	5	-1	30	-1	1000	0
14	1.25	0	60	1	30	-1	1000	0
15	1.25	0	5	-1	60	1	1000	0
16	1.25	0	60	1	60	1	1000	0
17	0.5	-1	32.5	0	30	-1	1000	0
18	2	1	32.5	0	30	-1	1000	0
19	0.5	-1	32.5	0	60	1	1000	0
20	2	1	32.5	0	60	1	1000	0
21	1.25	0	5	-1	45	0	600	-1
22	1.25	0	60	1	45	0	600	-1
23	1.25	0	5	-1	45	0	1400	1
24	1.25	0	60	1	45	0	1400	1
25	1.25	0	32.5	0	45	0	1000	0
26	1.25	0	32.5	0	45	0	1000	0
27	1.25	0	32.5	0	45	0	1000	0

Regarding the extraction time (**Figure 6.8.b**), it must be sufficient to reach the state of equilibrium. However, at very long times, an important amount of water condensed on the surface of the sorbent, hence, hindering the desorption of analytes and also broadening the chromatographic peaks. As a result, 40 min were selected as extraction time. Despite this prolonged time, it should be noted that up to 24 samples can be extracted simultaneously using the thermo-shaker.

Temperature had an inverse effect on the extraction performance, as shown in **Figure 6.7** and **Figure 6.8.b**. Even though higher temperatures favour the volatilization of analytes, adsorption is an exothermic process [314], so a trade-off between both effects was necessary. Moreover, similarly to long extraction times, at high temperatures water condensed on the surface of the sorbent, with the drawbacks already mentioned. Therefore, the selected working temperature was 30 ºC.

Regarding the shaking rate (**Figure 6.8.b**), an improvement was observed at higher rates because the movement of the solution improved the transfer of the analytes to the headspace. Therefore, the selected value was the maximum value allowed by the thermo-shaker, *i.e.*, 1400 rpm.

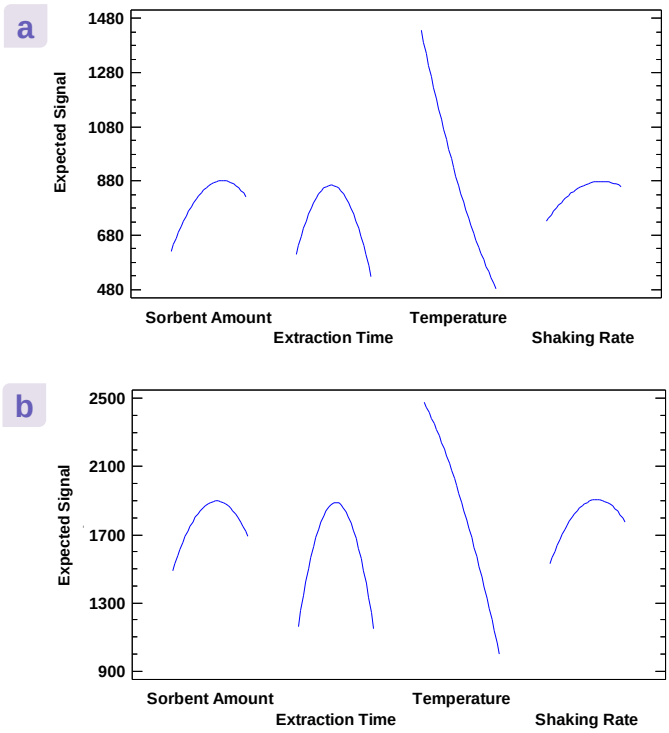


Figure 6.7. Correlation of the different variables that potentially affect the extraction of (a) hexanal and (b) heptanal.

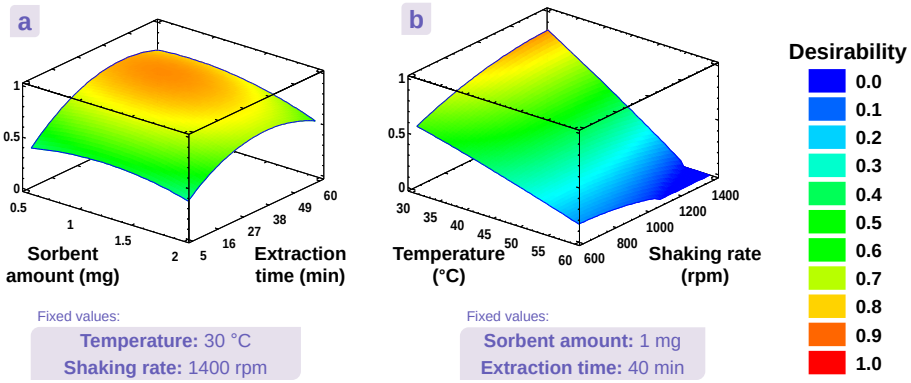


Figure 6.8. Response surface of the desirability function representing the relation between the studied variables: (a) sorbent amount *vs* extraction time, and (b) temperature *vs* shaking rate.

Saline content of synthetic saliva was measured by conductimetry being equivalent to 0.25% (w/v) NaCl. This value was then considered as average saline content of real saliva samples, which is consistent with reported values [143]. To this regard, saline content of donor phase was studied employing 10-ng mL^{-1} standard solutions prepared with different saline contents from 0 to 5% (w/v) NaCl (**Figure 6.9**). Extraction performance increased with the slightest presence of NaCl due to the salting-out effect (*i.e.,* 0.25% (w/v) NaCl was enough to improve sensitivity of the method). Signals slightly improved at higher salt concentrations, but worse precision was obtained, probably due to the higher viscosity of donor phase, what hindered reproducible extraction. Therefore, synthetic saliva was considered an appropriate solvent for standard solutions and saliva samples did not need any saline adjustment.

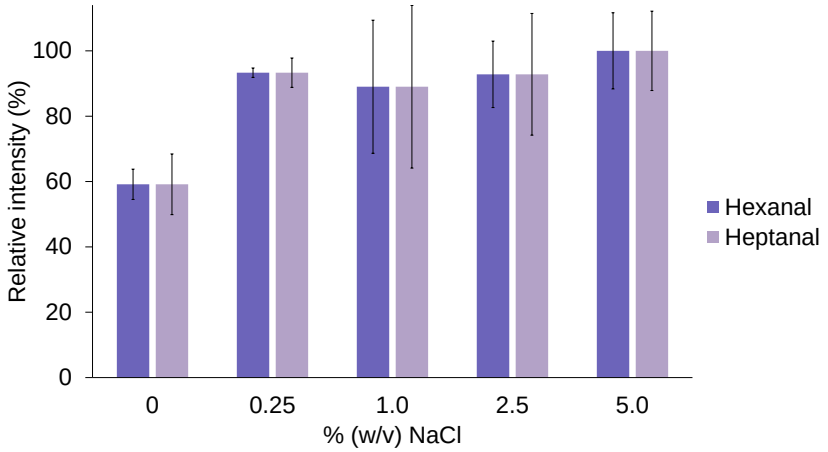


Figure 6.9. Optimization of saline content of donor phase. (Extraction conditions: sample volume, 1 mL; sorbent amount, 1 mg; extraction time, 40 min; 30 °C; 1400 rpm. Desorption conditions: 50 µL acetonitrile, 30 s).

6.3.5. Considerations about precipitation of mucins

In order to check that the freeze/thaw cycle and the precipitation of mucins does not interfere the results, a saliva sample from a volunteer was centrifuged for 10 min at 6000 rpm to remove phlegm or food leftovers, and then it was divided in two aliquots. One of them was spiked with the analytes at 5 ng mL^{-1} and both were frozen to precipitate mucins. Then, they were thawed and centrifuged again at 18000 rpm for 5 min. The spiked one was subjected to the proposed method. The unspiked one was spiked with the analytes at 5 ng mL^{-1}, and then it was subjected to the proposed method. Results (shown in **Figure 6.10**) revealed non-significant differences ($p > 0.05$) due to the precipitation of mucins, that means, mucins did not entrap the target analytes.

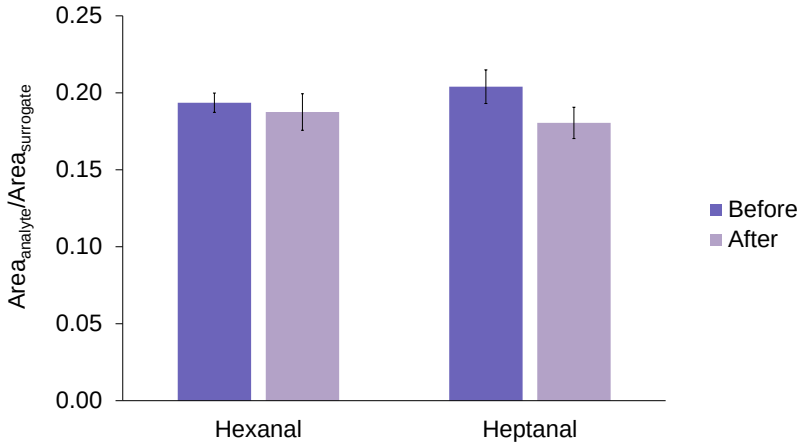


Figure 6.10. Results obtained for hexanal and heptanal in a saliva sample spiked before or after the freeze-thaw cycle carried out to precipitate mucins.

6.3.6. Analytical performance of the proposed method

Under the optimized conditions the analytical quality parameters of the proposed method, including linearity, enrichment factor (EF), extraction efficiency (EE), limit of detection (LOD), limit of quantification (LOQ), and intra- and inter-day precision, were evaluated.

The linearity was studied by measuring standard solutions prepared in synthetic saliva at different concentrations, all of them containing 50 ng mL^{-1} of surrogate. Calibration curves were plotted using the ratio of the peak area of each target analyte to the surrogate (A_i/A_s) versus the analyte concentration. The results indicated that the linearity reached at least 50 ng mL^{-1} for the target analytes. However, the working range was set from 1 to 10 ng mL^{-1} due to the low expected values. The determination coefficients reveal good linearity ($R^2 \geq 0.990$).

The EFs were estimated as the ratio of the signal obtained after the extraction of a standard solution prepared in synthetic saliva containing 10 ng mL^{-1} of the analytes and the signal of a standard solution of the same concentration prepared in acetonitrile without performing any extraction. An aliquot of surrogate was added to act as internal standard to both extracted and non-extracted solutions. EFs of 12 ± 2 and 15 ± 2 were achieved for hexanal and heptanal, respectively. The EEs were calculated considering a maximum EF of 20. In this sense, the EE values were 60 ± 9 and 74 ± 8% for hexanal and heptanal, respectively.

The LODs and LOQs were calculated as 3 times and 10 times, respectively, the signal-to-noise ratio of a standard solution prepared in synthetic saliva after

applying the proposed approach. The LOD and LOQ values were 0.22 and 0.75 ng mL^{-1}, respectively, for hexanal and 0.26 and 0.85 ng mL^{-1}, respectively, for heptanal.

The precision of the method was evaluated applying the proposed method to five replicates of standard solutions prepared in synthetic saliva containing the target analytes at three levels of concentration (*i.e.*, 1, 5 and 10 ng mL^{-1}). The results, presented in **Table 6.3**, expressed as relative standard deviation (RSD, %) revealed that good precision was achieved for the target analytes.

Table 6.3. Precision of the proposed method

Analyte	Concentration (ng mL^{-1})	Intra-day precision (RSD, %) [a]	Inter-day precision (RSD, %) [a]
Hexanal	1	10.4	12.0
	5	6.5	8.6
	10	9.8	4.7
Heptanal	1	5.6	8.7
	5	7.0	6.7
	10	4.0	8.9

[a] n=5

6.3.7. Analysis of human saliva samples

Three randomly selected saliva samples from healthy volunteers were spiked at two concentration levels (*i.e.,* 1, and 5 ng mL^{-1}) in order to evaluate the matrix effects by means of the relative recovery (RR) values, calculated as follows:

$$RR\ (\%) = \frac{Concentration_{spiked\ sample} - Concentration_{sample}}{Spiked\ amount} \cdot 100 \qquad \text{(Eq. 6.1)}$$

The results presented in **Table 6.4** proved matrix effects were low and external calibration was suitable for quantification of hexanal and heptanal in saliva samples by the proposed method.

Saliva samples from five healthy volunteers (three male and two female) and from five lung cancer patients at Stage IV (three male and two female) were collected as described in **Section 6.2.5**. The volumes of samples from lung cancer patients were variable and below 1 mL (0.2-0.85 mL), therefore, they were diluted up to 1 mL with synthetic saliva. Then, they were treated by the proposed M-HS-AME approach and measured by GC-MS. Results, besides other clinical parameters, are shown in **Table 6.5**.

Table 6.4. Relative recoveries obtained from real samples at different spiked amounts

Sample [a]	Spiked amount (ng mL^{-1})	RR (%) [b]	
		Hexanal	Heptanal
A	1	135 ± 5	133 ± 4
	5	94 ± 7	105 ± 2
B	1	131 ± 6	125 ± 4
	5	101 ± 6	96 ± 6
D	1	75 ± 9	82 ± 9
	5	101 ± 8	123 ± 7

[a] Sample A and B: male; Sample D: female
[b] Mean of three replicates ± standard deviation

Table 6.5. Relevant data from saliva samples and concentration of the analytes

Group	Sample [a]	Smoker [b]	Cancer type	Concentration (ng mL^{-1}) [c]	
				Hexanal	Heptanal
Healthy volunteer	A	n	-	< 0.22 [d]	< 0.85 [e]
	B	n	-	< 0.22 [d]	< 0.26 [d]
	C	a	-	< 0.75 [e]	< 0.85 [e]
	D	n	-	< 0.75 [e]	< 0.85 [e]
	E	n	-	< 0.75 [e]	< 0.85 [e]
Lung cancer patient	F	e	small-cell	3.3 ± 0.2	< 1.0 [e]
	G	e	adenocarcinoma	1.1 ± 0.1	< 1.1 [e]
	H	e	small-cell	6.6 ± 1.3	2.1 ± 0.3
	I	e	adenocarcinoma	9.5 ± 0.2	< 1.3 [d]
	J	n	adenocarcinoma	10.8 ± 0.3	2.0 ± 0.1

[a] Sample D, E, I and J: female; Sample A, B, C, F, G and H: male
[b] a: active smoker; e: ex-smoker; n: non-smoker.
[c] Mean of two replicates ± standard deviation.
[d] Estimated LOD, according to sample dilution, if any
[e] Estimated LOQ, according to sample dilution, if any

Analytes from all healthy volunteers were below the LOD or LOQ, whereas lung cancer patients showed higher values of the analytes. These results serve as a starting point for clinical research of this disease and show the diagnostic potential of the proposed method, which is the unique study where these biomarkers have been determined in saliva. These results will be extended by analyzing samples from a larger cohort of volunteers both healthy and unhealthy in different stages of the disease.

6.3.8. Comparison of the proposed method with previously reported approaches

The proposed approach has been compared with the previous M-HS-AME methodologies reported in terms of extraction conditions and greenness.

As evidenced in **Table 6.6**, the proposed approach requires a smaller amount of sample and considerably lower sorbent amount compared with previous approaches. It is true, however, that they are methods for different analytical applications and the availability of samples differs (*e.g.,* 20 mL of environmental water are more affordable than saliva).

On the other hand, extraction time is slightly higher in the proposed methodology, but it must be noted that the extraction time, and also the temperature, to carry out the headspace extraction depends on the nature of compounds under study, which are different in the compared methods.

In terms of analytical performance, none of the previously reported methods stated the EFs, therefore we were not able to compare the preconcentration capability of the techniques. However, it is expected that methods with high donor-acceptor phase ratio (even more avoiding liquid desorption such as Vidal *et al.* [51]) are able to achieve higher EFs and therefore lower LODs.

Table 6.6. Comparison of the proposed M-HS-AME approach with the previous configurations

Matrix	Analytes	Sample amount	Sorbent amount (mg)	Extraction time (min)	Extraction temperature (°C)	Desorption conditions	EF	AGREEprep Score	Ref.
Traditional Chinese Medicine	VOCs	1.0 g	10	15	90	500 µL ethyl acetate, 5 min	n.r. [b]	0.61	[50]
Environmental water	Chlorobenzenes	20 mL	10	30	RT [a]	Thermal desorption, 240 ºC, 5 min	n.r.	0.56	[51]
Baby food	Volatile phenols	0.50 g	10	10	90	100 µL 0.01M NaOH, 10 min	n.r.	0.57	[52]
Saliva	Linear aldehydes	1 mL	1	40	30	50 µL acetonitrile, 30 s	12-15	0.62	This work

[a] RT: room temperature

[b] n.r.: not reported

The greenness of these methods was evaluated by estimating their AGREEprep score [201], a metric tool for the greenness assessment of analytical sample preparation techniques. AGREEprep grades from 0 to 1 each one of the ten principles of GSP [5], considering environmental and health impact factors, such as the use of reagents, the energy consumption, sample size and throughput, and generated waste. The obtained pictogram for the proposed method is showed in **Figure 6.11**. As can be seen in **Table 6.6**, all the presented approaches were comparable in terms of greenness. Slight differences are due to higher sample size and/or higher energy consumption and lower sample throughput in refs. [51,52].

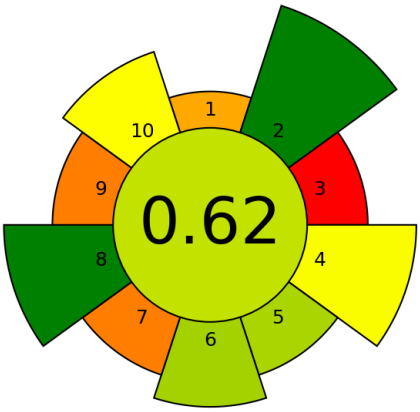


Figure 6.11. AGREEprep pictogram of the proposed method. (1-Sample preparation placement; 2-Hazardous materials; 3-Sustainability and renewability of materials; 4-Waste; 5-Size economy of the sample; 6-Sample throughput; 7-Integration and automation; 8-Energy consumption; 9-Post sample preparation configuration for analysis; 10-Operator's safety).

6.4. Conclusions

In this chapter, a method based on M-HS-AME followed by GC-MS for the determination of hexanal and heptanal as lung cancer biomarkers in human saliva samples is proposed. For this purpose, an adapted extraction device for M-HS-AME has been described for the first time for the analysis of biological samples, whose volume is usually limited. The presented work contributes to the development of simple methods for bioanalysis, being useful not only for the Analytical Chemistry field but also for medical science.

Good analytical features were obtained and it was successfully applied to saliva samples from healthy and ailing volunteers, showing notably differences

between them. Further research should be done in order to assist whether it is possible or not to detect the disease at earlier stages by means of this simple saliva analysis. This would allow the proposed methodology to be a promising tool for clinical research, focused on achieving earlier and easier cancer diagnosis through target screening of high-risk people.

SECTION 3

GENERAL CONCLUSIONS

The main **objective** of this Doctoral Thesis has been to overcome new challenges in sample preparation, such as those arising for the analysis of complex matrices or samples whose amount is limited. In this sense, through **Chapters 3** to **6**, the exploitation, miniaturization and adaptation of microextraction techniques for the simultaneous analysis of many samples has been conducted.

Specifically, stir bar sorptive dispersive microextraction (SBSDME) has been employed for the determination of traces of Δ^9-tetrahydrocannabinol in cosmetic products. Taking advantage of this microextraction techniques, high enrichment factors, and therefore, very low limits of detection were achieved in short extraction times. High donor/acceptor phase ratio and high extraction efficiencies contributed to achieve such high sensitivity in the order of ng g^{-1} in the complex cosmetic matrix. For its part, the dispersion of the sorbent material provided high extraction kinetics and thus, short analysis time as shown in **Chapter 3**.

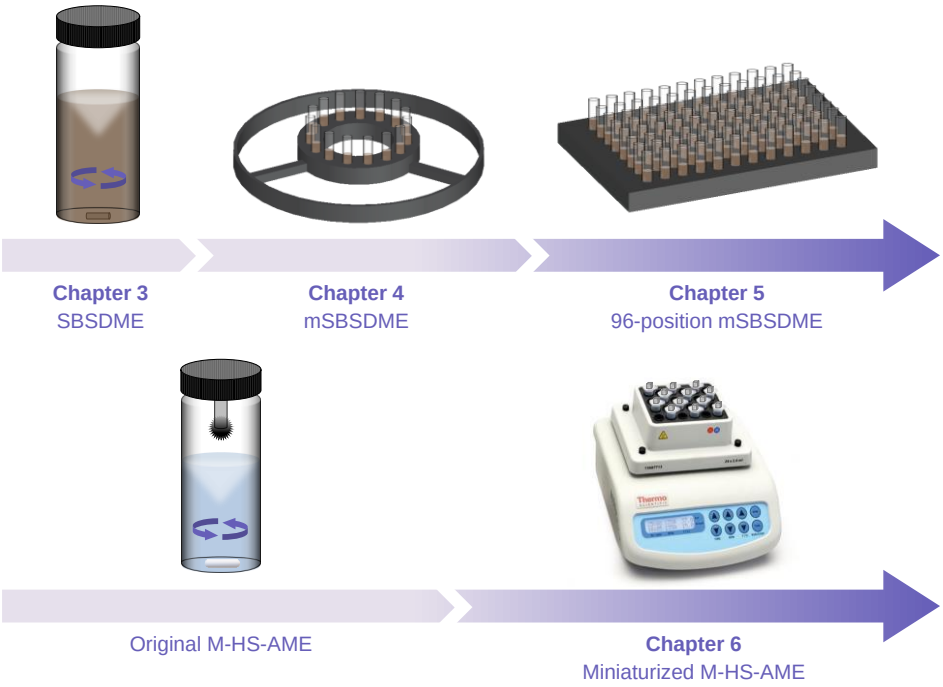
SBSDME has been successfully miniaturized, thus considerably reducing the amount of sorbent and, most importantly, the amount of sample. So, 40-mL vials and 10 mm length x 3 mm diameter magnets, typically used in SBSDME, have been replaced by 400-µL flat-base glass inserts and 3 x 2 mm magnets, respectively. This miniaturization allowed the design of a multiextraction assembly, permitting the treatment of 15 samples simultaneously. Moreover, the consumption of organic solvents, sample and energy are drastically reduced, significantly improving the greenness of the method in **Chapter 4**.

This effort to increase the throughput of the sample preparation step was continued with the design of a 96-position extraction assembly. The simultaneous treatment of 96 samples and the use of a multichannel pipette, significantly increased the number of samples to treat per unit of time and also the operator ergonomics, thus being useful for routine clinical laboratories, as shown in **Chapter 5.**

Finally, magnetic headspace adsorptive microextraction (M-HS-AME) has been miniaturized and adapted for the extraction of volatile analytes. The most important novelty has been the reduction of the sample volume, expanding the applicability of the technique to bioanalysis in **Chapter 6**.

The main axis of the present Doctoral Thesis has been the adaptation and miniaturization of two microextraction techniques to further miniaturized and high throughput approaches. SBSDME has been modified from its original configuration in **Chapter 3**, evolving through **Chapters 4** and **5**; and M-HS-AME has been adapted in **Chapter 6**. In all of the presented approaches, magnetic (nano)composites comprised of $CoFe_2O_4$ magnetic nanoparticles and a hydrophilic-lipophilic balance polymer (either commercial or lab-made) are used as sorbent material, exploiting the hydrophobic, π-π or hydrogen bond interactions with the analytes for their extraction and the magnetism conferred

by MNPs, as required by the operational mode of this techniques. With this evolution the applicability of the techniques has been widely expanded, having alternative configurations to choose depending on the application it is intended for. If large volumes of sample are available and very high enrichment factors are needed (*e.g.*, environmental, food or cosmetics analysis), then, the original configurations are perfectly suitable. On the contrary, if sample volume is somehow limited and/or a large number of samples need to be treated (*e.g.*, bioanalysis), the miniaturized high-throughput versions are a feasible tool.



Furthermore, as widely discussed during this Doctoral Thesis, miniaturization also implies an improvement in the greenness of the method. It is achieved through the reduction of toxic reagents, sample and consumption, the reduction of waste generation and the increase in sample throughput and chances of portability, and so, it has been demonstrated by the AGREEprep score of the different approaches presented in this thesis. However, important aspects need to be improved such as automation, *in-situ* applicability and sustainability or reuse of materials, and so, they constitute the focus of future research.

As final conclusion, it has been demonstrated throughout this Doctoral Thesis that microextraction techniques are indispensable for the analysis of complex samples and that their miniaturization is an essential strategy in order to widen their applicability to low-volume samples, to increase sample throughput and also to improve the greenness of analytical methods.

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ANNEX I

MULTIVARIATE ANALYSIS STATISTICS

Box-Behnken design

The Box-Behnken designs are a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. The number of experiments required for the development of the designs (N) is defined as follows:

$$N=2k(k-1)+C_P \qquad \text{(Eq. A-I.1)}$$

where k is the number of factors; and C_P is the replicates of the central point. The following quadratic polynomial equation was applied to evaluate the multiple linear regression for each response:

$$Y=\beta_o+\sum \beta_i X_i+\sum \beta_{ii} X_i^2+\sum \beta_{ij} X_i X_j+\varepsilon \qquad \text{(Eq. A-I.2)}$$

where Y is the analytical response; β_o is a constant term; β_i, β_j, and β_{ij} are the regression coefficients of the design; X_i and Xj are the different variables; and ε is the residual error.

The global desirability of the experiment (D) was applied to evaluate the different responses and to achieve the optimal extraction conditions to maximize the analytical responses of the analytes:

$$D=\left(d_1(Y_1)\cdot d_2(Y_2)\cdots d_n(Y_n)\right)^{1/n} \qquad \text{(Eq. A-I.3)}$$

where n is the number of responses in the optimization process, and $d_i(Y_i)$ is the individual desirability of each response in the experiment. The individual desirability is calculated as:

$$d_i=\frac{Y_i-Y_{min}}{Y_{max}-Y_{min}} \qquad \text{(Eq. A-I.4)}$$

Undesirable responses and fully desirable responses are ranged between 0 and 1, respectively, for each response.

Simplex-Centroid design

The adjustment for the simplex region established for the study was delimited into three pseudo-components (independent variables (X_1, X_2, X_3)), ranging from 0 to 1. The number of experiments required for the development of the designs (N) is defined as follows:

$$N=2^q-1 \qquad \text{(Eq. A-I.5)}$$

where *q* is the number of components of the mixture.

The design points in this statistical model are represented in triangular diagrams by polynomial equations defined by the q^{th}-order mixture. These designs usually fit three models: linear (Eq. A-I.6), quadratic (Eq. A-I.7) or special cubic (Eq. A-I.8):

$$Y=\sum_{i=1}^{q}\beta_i X_i+\varepsilon \qquad \text{(Eq. A-I.6)}$$

$$Y=\sum_{i=1}^{q}\beta_i X_i+\sum\sum_{i<j}^{q}\beta_{ij}X_iX_j+\varepsilon \qquad \text{(Eq. A-I.7)}$$

$$Y=\sum_{i=1}^{q}\beta_i X_i+\sum\sum_{i<j}^{q}\beta_{ij}X_iX_j+\sum\sum\sum_{i<j<k}^{q}\beta_{ijk}X_iX_jX_k+\varepsilon \qquad \text{(Eq. A-I.8)}$$

Interactions are evaluated in β_i, β_{ij} and β_{ijk}; X_i, X_j and X_k are the studied factors and Y is the experimental response to the factors.

The global desirability of the experiment (D) was applied to evaluate the different responses and to achieve the optimal extraction conditions to maximize the analytical responses of the analytes:

$$D=\left(d_1(Y_1)\cdot d_2(Y_2)\cdots d_n(Y_n)\right)^{1/n} \qquad \text{(Eq. A-I.9)}$$

where n is the number of responses in the optimization process, and $d_i(Y_i)$ is the individual desirability of each response in the experiment. The individual desirability is calculated as:

$$d_i=\frac{Y_i-Y_{min}}{Y_{max}-Y_{min}} \qquad \text{(Eq. A-I.10)}$$

Undesirable responses and fully desirable responses are ranged between 0 and 1, respectively, for each response.

ANNEX II

SCIENTIFIC PUBLICATIONS DERIVED FROM THIS DOCTORAL THESIS

Research articles

1. C. Azorín, J.L. Benedé, A. Chisvert, A. Salvador, Trace determination of tetrahydrocannabinol (THC) in cosmetic products by stir bar sorptive dispersive microextraction followed by liquid chromatography-tandem mass spectrometry, Talanta 253 (2023) 123934

2. C. Azorín, J.L. Benedé, A. Chisvert, New challenges in sample preparation: Miniaturized stir bar sorptive dispersive microextraction as a high-throughput and feasible approach for low-availability sample preparation, Anal. Chim. Acta 1238 (2023) 340627

3. C. Azorín, J.L. Benedé, A. Chisvert, A 96-position high-throughput miniaturized platform for dispersive sorbent-based microextraction: application to the determination of tetrahydrocannabinol and cannabidiol in saliva of cannabis smokers, Submitted to Anal. Chim. Acta

4. C. Azorín, A.L. López-Juan, F. Aparisi, J.L. Benedé, A. Chisvert, Determination of hexanal and heptanal in saliva samples by an adapted magnetic headspace adsorptive microextraction for diagnosis of lung cancer, Anal. Chim. Acta 1271 (2023) 341435

Review articles

5. C. Azorín, J.L. Benedé, A. Chisvert, Ultramicroextraction as a miniaturization of the already miniaturized. A step toward nanoextraction and beyond, J. Sep. Sci. 46 (2023) 2300223

Book chapters

6. C. Azorín, J.L. Benedé, A. Chisvert, *Ionic liquid-based liquid-phase microextraction techniques*, in: S. Carda-Broch, M.J. Ruiz-Angel (Eds.), *Ionic Liquids in Analytical Chemistry: New Insights and Recent Developments,* Elsevier, Amsterdam, 2022: pp. 73–102