ENANTIODETERMINACIÓN DE CATINONAS EN ORINA MEDIANTE ELECTROFORESIS CAPILAR

Alberto Pérez Alcaraz

UNIVERSITAT ROVIRA i VIRGILI

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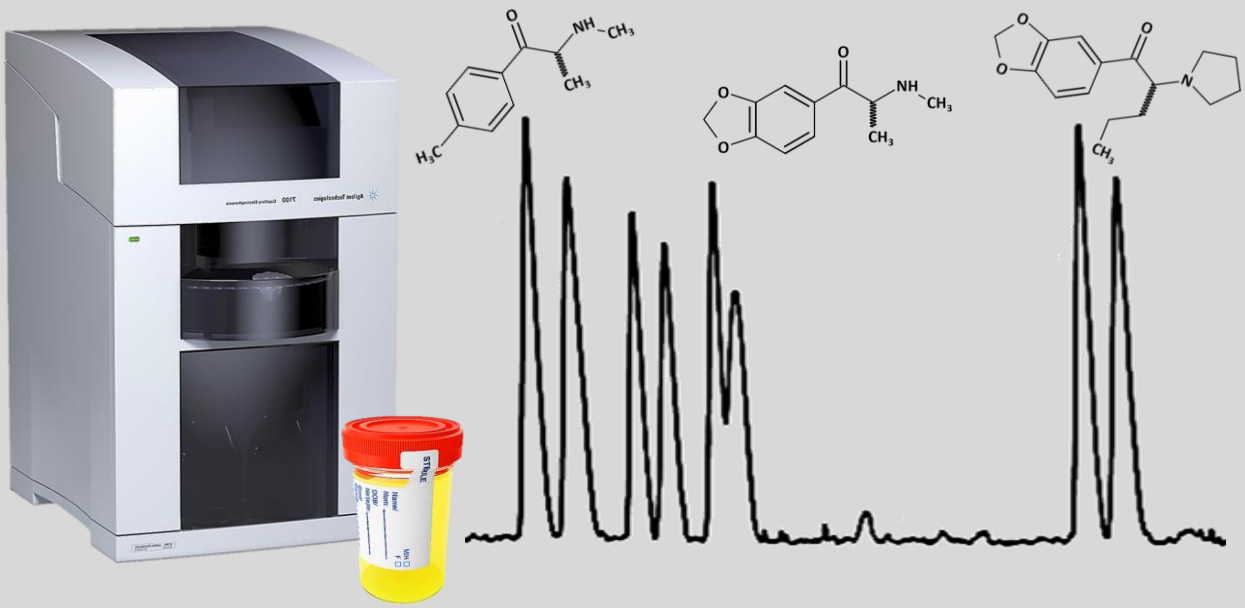

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TESIS DOCTORAL

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HACEMOS CONSTAR:

Que la presente Tesis Doctoral, titulada "ENANTIODETERMINACIÓN DE CATINONAS EN ORINA MEDIANTE ELECTROFORESIS CAPILAR", que presenta ALBERTO PÉREZ ALCARAZ para la obtención del título de Doctor por la *Universitat Rovira i Virgili*, ha sido realizada bajo nuestra dirección en el Departamento de Química Analítica y Química Orgánica de esta universidad y que todos los resultados presentados son fruto de experiencias realizadas por dicho doctorando.

Y para que conste firmamos este documento en Tarragona a 31 de mayo de 2021

Dra. Carme Aguilar Anguera

Dra. Marta Calull Blanch

Después de mucho esfuerzo, finalmente con la publicación de esta Tesis Doctoral llega el final de una etapa, aunque nunca imaginé que sería en medio de una pandemia. Ha sido un proceso duro, pero del que he aprendido mucho. No obstante, este camino no lo he recorrido solo. La Tesis me ha permitido conocer a grandes personas que han sido indispensables para que esta llegase a buen puerto. Durante este proceso también he contado con el inestimable apoyo de aquellos que ya formaban parte de mi vida antes de entrar en el doctorado. Las siguientes líneas son un agradecimiento y un reconocimiento a todas esas personas que han constituido parte esencial de esta etapa de mi vida.

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¡Muchas gracias a todos!

"Yo no tomo drogas. Yo soy una droga."

Salvador Dalí

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RESUMEN

[ESP] En los últimos años ha aumentado el consumo de derivados sintéticos de la catinona, un alcaloide que se encuentra de forma natural en las hojas del *khat*, puesto que representan una alternativa más asequible y accesible respecto a otras drogas ilícitas más conocidas como por ejemplo las anfetaminas. Después de ser consumidos, estos compuestos se pueden encontrar en el organismo, ya sea metabolizados o en su forma pura, a bajas concentraciones. Por lo tanto, suelen ser necesarios métodos altamente sensibles para determinar estas sustancias en muestras biológicas como sangre, cabello, saliva o, más comúnmente, orina, un tipo de matriz que presenta un muestreo no invasivo y que se puede obtener en grandes volúmenes, siendo su ventana de detección de entre 1 a 4 días desde el consumo.

Las catinonas presentan un centro quiral y, por tanto, se pueden encontrar en dos formas enantioméricas (*R* y *S*). Cada enantiómero puede presentar un comportamiento farmacocinético y farmacodinámico diferente y, por tanto, tener diferentes efectos en el organismo. Además, conocer la proporción de cada enantiómero puede aportar información útil sobre la ruta sintética llevada a cabo para la obtención de cada catinona y ayudar a rastrear su producción. Por estas razones, la enantioseparación de las catinonas sintéticas puede ser de gran utilidad. En este sentido, la electroforesis capilar es una técnica muy adecuada para este propósito, puesto que la enantioseparación se puede lograr simplemente agregando un selector quiral al electrolito de separación. Sin embargo, a pesar de esta y otras conocidas ventajas de esta técnica analítica, una de sus principales limitaciones es su baja sensibilidad, especialmente cuando se combina con la detección ultravioleta-visible. Para superar esta problemática se han desarrollado diferentes estrategias de preconcentración en línea. Estas técnicas se pueden dividir en dos grupos principales, aquellas basadas en principios cromatográficos y aquellas basadas en principios electroforéticos. Ambos tipos de técnicas de preconcentración han demostrado su eficacia a la hora de conseguir factores de preconcentración elevados y, por tanto, aumentar la limitada sensibilidad de la electroforesis capilar.

Ante lo expuesto anteriormente, el objetivo de la presente Tesis Doctoral ha sido desarrollar metodologías basadas en la técnica de electroforesis capilar para la enantiodeterminación de catinonas en muestras de orina. Además, para alcanzar los bajos niveles de concentración a los que generalmente se pueden encontrar estas sustancias en esta matriz, del orden de ng/mL, se han evaluado diferentes estrategias de preconcentración en línea, específicamente, el acoplamiento en línea entre la

extracción en fase sólida y la electroforesis capilar (*in-line* SPE-CE), ya sea en combinación con un detector de ultravioleta-visible o con un espectrómetro de masas, la técnica de *field-amplified sample injection* y la técnica de *electrokinetic supercharging*. Todas estas metodologías fueron validadas con éxito, demostrando así su potencial para aplicaciones toxicológicas y forenses.

[CAT] En els últims anys ha augmentat el consum de derivats sintètics de la catinona, un alcaloide que es troba de forma natural en els fulles del *khat*, degut a que representen una alternativa més assequible i accessible respecte a altres drogues il·lícites més conegudes com per exemple les amfetamines. Després de ser consumits, aquests compostos es poden trobar en l'organisme, ja sigui metabolitzats o en la seva forma pura, a baixes concentracions. Per tant, solen ser necessaris mètodes altament sensibles per determinar aquestes substàncies en mostres biològiques com sang, cabell, saliva o, més comunament, orina, un tipus de matriu que presenta un mostreig no invasiu i que es pot obtenir en grans volums, sent la seva finestra de detecció d'entre 1 a 4 dies des del consum.

Les catinones presenten un centre quiral i, per tant, es poden trobar en dues formes enantiomèriques (*R* i *S*). Cada enantiòmer pot presentar un comportament farmacocinètic i farmacodinàmic diferent i, per tant, tenir diferents efectes en l'organisme. A més, conèixer la proporció de cada enantiòmer pot aportar informació útil sobre la ruta sintètica que s'ha dut a terme per a l'obtenció de cada catinona i ajudar a rastrejar la seva producció. Per aquestes raons, l'enantioseparació de les catinones sintètiques pot ser de gran utilitat. En aquest sentit, l'electroforesi capil·lar és una tècnica molt adequada per a aquest propòsit, degut a que l'enantioseparació es pot aconseguir simplement afegint un selector quiral a l'electròlit de separació. Malgrat aquest i altres coneguts avantatges d'aquesta tècnica analítica, una de les seves principals limitacions és la seva baixa sensibilitat, especialment quan es combina amb la detecció ultraviolada-visible. Per tal de superar aquesta problemàtica s'han desenvolupat diferents estratègies de preconcentració en línia. Aquestes tècniques es poden dividir en dos grups principals, les basades en principis cromatogràfics i les basades en principis electroforètics. Tots dos tipus de tècniques de preconcentració han demostrat la seva eficàcia per tal d'aconseguir factors de preconcentració elevats i, per tant, augmentar la limitada sensibilitat de l'electroforesi capil·lar.

Davant l'exposat anteriorment, l'objectiu de la present Tesi Doctoral ha estat desenvolupar metodologies basades en la tècnica d'electroforesi capil·lar per a l'enantiodeterminació de catinones en mostres d'orina. A més, per assolir els baixos nivells de concentració als que generalment es poden trobar aquestes substàncies en aquesta matriu, de l'ordre de ng/ml, s'han avaluat diferents estratègies de preconcentració en línia, específicament, l'acoblament en línia entre l'extracció en fase sòlida i l'electroforesi capil·lar (*in-line* SPE-CE), ja sigui en combinació amb un detector d'ultraviolat-visible o amb un espectròmetre de masses, la tècnica de *field-amplified sample injection* i la tècnica de *electrokinetic supercharging*. Totes aquestes metodologies van ser validades amb èxit, demostrant així el seu potencial per a aplicacions toxicològiques i forenses.

[ENG] In the past few years the consumption of synthetic derivates of cathinone, an alkaloid naturally found in the leaves of the khat plant, has increased as they represent a more affordable and accessible alternative to other well-known illicit drugs such as amphetamines. After their consumption, these compounds can be found in the organism, either metabolized or in their pure form, at low concentrations. Therefore, highly sensitive methods are usually necessary to determine these substances in biological samples such as blood, hair, oral fluids, or, more commonly, urine, a type of matrix that presents a non-invasive sampling, can be collected in high volumes and has a detection window between 1 to 4 days from consumption.

Cathinones present a chiral centre, so they can be found in two enantiomeric forms (*R* and *S*). Each enantiomer can present different pharmacokinetic and pharmacodynamic behaviour and have different effects on the organism. Moreover, the enantiomers ratio can provide useful information about the synthetic route of each cathinone and help in tracking its production. For these reasons the enantioseparation of synthetic cathinones can be very useful. In this sense, capillary electrophoresis represents an excellent technique for this purpose, since enantioseparation can be achieved simply by adding a chiral selector to the background electrolyte. However, despite of this and other well-known advantages of this analytical technique, one of its main limitations is its poor sensitivity, especially when coupled with ultraviolet-visible detection. To overcome this, different in-line preconcentration strategies have been developed. These techniques can be divided into two main groups, those based on chromatographic principles and those based

on electrophoretic principles. Both types of preconcentration techniques have demonstrated their effectivity to achieve high preconcentration factors and therefore, increase the limited sensitivity of capillary electrophoresis.

In view of these facts, the aim of this Doctoral Thesis has been to develop methodologies based on capillary electrophoresis for the enantiodetermination of cathinones in urine samples. In addition, in order to achieve the low concentration levels at which these substances can generally be found in this matrix, in the order of ng/mL, different in-line preconcentration strategies have been evaluated, specifically, the in-line coupling between solid phase extraction and capillary electrophoresis (in-line SPE-CE), either in combination with an ultraviolet-visible detector or with a mass spectrometer, the field-amplified sample injection technique and the electrokinetic supercharging technique. All these methodologies were successfully validated, thus demonstrating their potential for toxicological and forensic applications.

1. INTRODUCCIÓN

La aparición de nuevas drogas en el mercado ilegal ha sido una constante en los últimos años, por un lado, con el objetivo de satisfacer la demanda de nuevos estupefacientes por parte de los consumidores de drogas tradicionales, y, por otro, para evitar las regulaciones existentes centradas en controlar tanto la producción como la venta de drogas de abuso. A estas sustancias se las ha denominado como Nuevas Sustancias Psicoactivas (*New Psychoactive Substances* - NPS). Este tipo de compuestos buscan replicar los efectos de otras sustancias psicoactivas, como los opioides, el cannabis o las anfetaminas y se caracterizan por su falta de regulación, lo que facilita su distribución y las convierte en una alternativa atractiva respecto a sus homólogas más tradicionales [1–3].

Entre los diferentes tipos de NPS hallamos los derivados sintéticos de la catinona, un alcaloide que se encuentra naturalmente en las hojas del *khat*. Este tipo de compuestos provocan estímulos similares a los de las anfetaminas, pero en comparación con estas drogas presentan un precio más bajo y una más fácil adquisición [2–10]. Después de su consumo, generalmente por vía nasal o ingeridas [3,4,6,9], las catinonas son absorbidas por el organismo produciendo de esta forma sus efectos estimulantes y, posteriormente, estos compuestos son excretados ya sea en su forma pura o metabolizada. Además, cabe señalar que el consumo prolongado de estas sustancias puede conllevar distintas amenazas para la salud humana, desde problemas psiquiátricos [11,12], hasta la muerte [13]. Por todo esto, el estudio de los efectos, las rutas metabólicas y otras características de este tipo de compuestos se ha convertido en una línea de investigación prioritaria tanto para las autoridades médicas como policiales, así como de distintos grupos científicos. Asimismo, con la finalidad de reducir su riesgo para la salud pública, es importante monitorizar el consumo de estas sustancias mediante la determinación de las mismas en diferentes matrices biológicas, como, por ejemplo, orina, sangre, cabello o saliva. En consecuencia, hay un creciente interés en el desarrollo de metodologías capaces de determinar estos compuestos y sus metabolitos en las diferentes matrices antes mencionadas, entre las que destaca especialmente la orina puesto que se puede obtener de forma no invasiva y en grandes volúmenes en comparación con otras matrices biológicas.

Es importante destacar que las catinonas tienen un centro quiral y esto implica la presencia de dos enantiómeros (*R* y *S*) que pueden presentar un comportamiento farmacocinético y farmacodinámico diferente [6,14]. Por esta razón, es de interés

para la comunidad científica el desarrollo de métodos analíticos que permitan la enantioseparación de estos compuestos [14,15]. En este sentido, en los últimos años se han explorado diferentes alternativas para conseguir la separación quiral de las catinonas. Estas van desde el uso de columnas quirales en metodologías basadas en la cromatografía de líquidos (*liquid chromatography* - LC) [15–17], a la derivatización de estos compuestos mediante el uso agentes derivatizantes quirales como paso previo a su separación por cromatografía de gases (*gas chromatography* - GC) [15,18]. Sin embargo, para este propósito destacan especialmente las metodologías basadas en la electroforesis capilar (*capillary electrophoresis* - CE) debido a que con esta técnica la enantioseparación se puede conseguir de manera sencilla, simplemente disolviendo un selector quiral en el electrolito de separación (*background electrolyte* - BGE) [19–26].

Además de la facilidad para conseguir una separación quiral, la CE presenta otras ventajas como su alta resolución o el bajo consumo de reactivos y muestra. Sin embargo, una de las principales limitaciones de esta técnica es su intrínseca baja sensibilidad sobre todo cuando se usa en combinación con un detector ultravioleta-visible (UV) [27]. Con el objetivo de superar este inconveniente, a lo largo de los últimos años, diversos autores han desarrollado y aplicado diferentes estrategias basadas en el uso de distintas técnicas de preconcentración dentro del propio capilar de separación [28–36]. Estas técnicas se pueden dividir en dos grupos principales en función del mecanismo de preconcentración, las técnicas basadas en principios cromatográficos y las técnicas basadas en principios electroforéticos. Dentro de las primeras, destaca la preconcentración de la muestra mediante el acoplamiento en línea de la extracción en fase sólida a la CE (*in-line* SPE-CE). En esta metodología, un sorbente de extracción en fase sólida (*solid phase extraction* - SPE) es colocado en el interior del capilar, próximo al extremo del capilar por donde se introduce la muestra, permitiendo la preconcentración de los analitos antes de su separación electroforética [28–32]. En cuanto a la segunda categoría de técnicas, destacan las técnicas de *stacking*, donde los analitos se concentran en una pequeña banda (*stack*) dentro del propio capilar debido a una reducción abrupta y temporal de su velocidad de migración [32–36]. Ambos tipos de técnicas han demostrado ser capaces de aumentar de manera considerable la sensibilidad de la CE [28–36].

La presente Tesis Doctoral se centra en el desarrollo de nuevos métodos analíticos basados en la combinación de la CE con diversas estrategias de preconcentración

dentro del propio capilar para la enantiodeterminación de catinonas en orina. En este sentido, la introducción se ha dividido en diferentes secciones. En primer lugar, se profundiza sobre las NPS, centrándose especialmente en su definición, sus mecanismos de distribución, sus hábitos de consumo y los problemas de salud derivados del mismo (sección 1.1). Posteriormente, en el apartado 1.2 se detallan aspectos relacionados con una familia concreta de NPS, las catinonas sintéticas, puesto que son los compuestos analizados a lo largo de la parte experimental de la presente Tesis Doctoral. Además, dentro de este apartado se incluye un artículo científico donde se revisa la bibliografía reciente correspondiente a la determinación de catinonas en muestras de orina. Seguidamente, se describen tanto la base teórica como las ventajas e inconvenientes de la CE (apartado 1.3). En la sección 1.3.1 se profundiza sobre los distintos aspectos relacionados con la separación quiral mediante CE y en la 1.3.2 se ahonda en el acoplamiento de la CE a la espectrometría de masas (*mass spectrometry* - MS). Finalmente, en la sección 1.3.3 se profundiza sobre las técnicas de preconcentración de muestra usualmente empleadas en CE, ya sean las técnicas basadas en principios cromatográficos o las basadas en principios electroforéticos (apartados 1.3.3.1 y 1.3.3.2 respectivamente).

1.1. Nuevas Sustancias Psicoactivas

El mundo de las drogas se encuentra sometido a una constante evolución, con el objetivo tanto de satisfacer las demandas de nuevas sustancias por parte de los consumidores, así como de evitar las regulaciones legislativas orientadas al control de la producción y venta de estupefacientes. Dentro de esta permanente evolución, destaca la continua aparición de Nuevas Sustancias Psicoactivas o NPS. Las NPS engloban a un grupo muy heterogéneo de sustancias cuya toxicidad en muchos casos no está perfectamente establecida. Además, es de destacar la falta de unanimidad en la definición de estos compuestos [1]. Por ejemplo, la *United Nations Office on Drugs and Crime* (UNODC) define a las NPS como "sustancias de abuso, ya sea en forma pura o en preparado, que no son controladas por la Convención Única de 1961 sobre Estupefacientes ni por el Convenio sobre Sustancias Psicotrópicas de 1971, pero que pueden suponer una amenaza para la salud pública" [2]. La definición del *European Monitoring Centre for Drugs and Drug Addiction* (EMCDDA) es muy similar a la de la UNODC. En particular, este organismo define a las NPS como "un nuevo estupefaciente o psicotrópico, en forma pura o en preparado, que no está controlado por la Convención Única de 1961 sobre Estupefacientes o el Convenio sobre Sustancias Psicotrópicas de 1971, pero que puede representar una amenaza para la salud pública comparable a la planteada por sustancias enumeradas en estos convenios" [37]. Por otro lado, según la Organización Mundial de la Salud (OMS) "las NPS son sustancias sintéticas desarrolladas para producir efectos sobre el sistema nervioso central similares a los de otras sustancias psicoactivas, como los opioides, el cannabis o las anfetaminas" [38]. Es importe destacar que la denominación de NPS no se refiere necesariamente a que estas sustancias sean de nueva creación, sino a que han emergido recientemente en el mercado y aún no están reguladas.

Bajo la denominación de NPS se incluyen una amplia variedad de sustancias que se pueden clasificar en diferentes familias o categorías de compuestos. Entre las más destacadas encontramos a los cannabinoides sintéticos [2,3,5,39,40], las piperazinas [2,3,5], los aminoindanos [2,5], las nuevas feniletilaminas [2,3,41], las triptaminas [2,42], los opioides sintéticos [43–45], las benzodiazepinas de diseño [46,47] y las catinonas sintéticas [2–10]. Esta variedad de familias comporta un gran dinamismo dentro del mercado de NPS lo cual dificulta todavía más su control. De entre todos estos grupos de sustancias, algunas familias presentan una mayor popularidad que otras. A modo de ejemplo el gráfico de la Fig. 1, obtenido a partir de los datos recogidos en el *European Drug Report* 2019 del EMCDDA [48], muestra la distribución de las 64.160 incautaciones de NPS analizadas en el año 2017 y a lo largo del

continente europeo por las respectivas agencias antidrogas de cada país. La mayor parte correspondían a muestras de cannabinoides sintéticos o de catinonas, representando un porcentaje de aproximadamente el 75%, mientras que el porcentaje restante correspondía a benzodiazepinas, opioides sintéticos y otros tipos de NPS. A partir de estos datos se puede concluir que los cannabinoides sintéticos y las catinonas son los tipos de NPS más ampliamente consumidos en la actualidad.

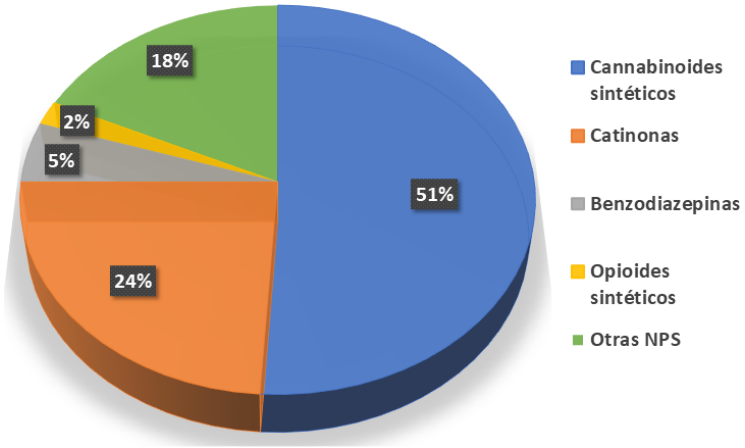

Figura 1. Porcentajes de las distintas familias de NPS detectadas en Europa respecto al número de incautaciones analizadas en el año 2017 según el *European Drug Report* 2019 del EMCDDA [48].

Cuando emergieron las primeras NPS, principalmente a principios de los años 2000, en muchos países fueron los puestos de venta de drogas más tradicionales los que desempeñaron un papel crucial en la distribución de estos compuestos. A partir de 2010 se empezó a observar un incremento exponencial en la distribución de esta clase de sustancias, tanto por los métodos tradicionales como por otras vías como internet. Actualmente, la globalización junto con el aumento de las capacidades tecnológicas han permitido que estos compuestos se propaguen rápidamente por todo el mundo una vez son introducidos en el mercado [1]. Las nuevas regulaciones legislativas aprobadas por los diferentes países han frenado la distribución de las NPS por las vías tradicionales, aunque en un mundo como el actual, donde la mayoría de gente tiene acceso a internet y a las redes sociales, no es de extrañar que estos compuestos se puedan hallar con facilidad en diversas webs [1,3,49] e incluso algunos traficantes han llegado a usar como plataforma de distribución redes sociales de uso común entre el público joven como son Facebook, Instagram o Twitter [49]. En

internet, las NPS se encuentran habitualmente etiquetadas como “no aptas para el consumo humano” y son vendidas como sales de baño, inciensos, fertilizantes o con otras denominaciones con el objetivo de evitar los posibles controles legislativos [1,3,49].

En los últimos años, la aparición de nuevas NPS en el mercado ha sido una constante. Por ejemplo, en el año 2018 según el *European Drug Report* 2019, el EMCDDA monitorizó más de 730 NPS, de las cuales 55 habían sido detectadas por primera vez en Europa ese mismo año [48]. En la Fig. 2 se muestra la evolución del número de NPS notificadas por primera vez en Europa del año 2005 al 2018 según ese mismo informe. Este gráfico muestra como a lo largo de estos años no han dejado de aparecer NPS de nuevo cuño, especialmente entre los años 2013 y 2015, siendo 2014 el año más prolífico en este sentido con 101 NPS notificadas por primera vez en Europa. No obstante, es importante señalar que a pesar de la constante aparición de NPS, este tipo de drogas son menos consumidas que las drogas tradicionales. Aun así, las NPS no dejan de representar un grave riesgo para la salud pública dado que generalmente se desconocen los efectos toxicológicos de este tipo de sustancias, sumado a que sus usuarios suelen ser personas jóvenes, con el peligro correspondiente que comporta el consumo de drogas a una temprana edad [1,11].

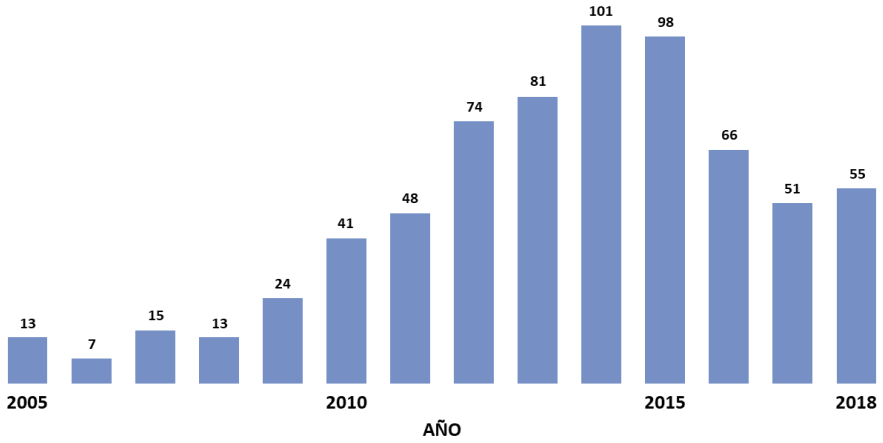

Figura 2. Número de NPS notificadas por primera vez en Europa del año 2005 al 2018 según el *European Drug Report* 2019 del EMCDDA [48].

Debido a la gran diversidad y a la temporalidad de las NPS existe una información limitada sobre los efectos farmacológicos y toxicológicos de estas sustancias. Los

efectos de este tipo de compuestos se estudian principalmente a través de las experiencias descritas por parte de sus consumidores en foros de internet o en encuestas de consumo. Las razones que argumentan los diferentes consumidores de NPS a la hora de decantarse por estas sustancias son diversas. Entre ellas podemos encontrar, al igual que para otras drogas, el placer producido por sus efectos estimulantes. Aunque en el caso de las NPS, algunos usuarios han afirmado que estos efectos son menores a los producidos por sus homólogas más tradicionales [1]. Asimismo, uno de los mayores atractivos de estos compuestos es su estatus legal, el cual permite su fácil adquisición, incluso, como previamente se ha comentado, entre los más jóvenes. De hecho, éstos encuentran en la facilidad de obtención de las NPS su principal atractivo [11,49]. Otro motivo directamente relacionado con el anterior es su menor precio en comparación con las drogas más tradicionales. No obstante, es habitual combinar el consumo de NPS con otros tipos de drogas, lo que aumenta su efecto y su riesgo. Incluso se ha documentado el uso de NPS como adulterantes de drogas tradicionales [1].

La proliferación de estas sustancias supone un reto tanto para los legisladores como para los sistemas de salud. Respecto a los primeros, es necesario desarrollar leyes no solo a nivel nacional, sino que también es imprescindible una legislación internacional capaz de regular la producción y venta de estos compuestos en todo el mundo. De hecho, por norma general, encontramos mayores restricciones en la normativa internacional que en la regional o nacional. Sin embargo, a cualquier nivel legislativo existe una disputa permanente entre el tiempo requerido en hallar evidencias de la necesidad de controlar una sustancia respecto a los daños producidos por la ausencia de control de la misma [1]. Además, el debate respecto a los beneficios que conlleva la prohibición de estos compuestos sigue abierto. Una regulación estricta puede dificultar la exploración del uso para fines terapéuticos de la sustancia en cuestión o incluso puede tener efectos contraproducentes [1,50]. Por ejemplo, en el Reino Unido la prohibición en el año 2010 de la mefedrona, un derivado sintético de la catinona, supuso un aumento del consumo de otras drogas más potentes y, en consecuencia, más perjudiciales para la salud como las anfetaminas o la cocaína, con el correspondiente incremento en el número de muertos a causa de estas sustancias [50]. Esto fue debido a que cuando la mefedrona era legal su precio era mucho menor al de la cocaína y las anfetaminas, hecho que permitió a esta catinona acaparar gran parte del mercado de estos compuestos. No obstante, cuando esta sustancia fue prohibida, su precio aumentó y sus usuarios

habituales no encontraron ninguna ventaja en su consumo, con lo que volvieron al consumo de drogas más tradicionales que además suponían un mayor riesgo para su salud.

La relevancia a nivel global que han adquirido las NPS ha supuesto la necesaria cooperación entre distintos países en ámbitos que van más allá del legislativo. Esto ha propiciado la creación de sistemas de alerta temprana que actúan como plataformas de intercambio de información sobre NPS de reciente aparición. Por ejemplo, a nivel europeo encontramos el *Early Warning System* (EWS), una red coordinada por el EMCDDA, en estrecha cooperación con la Europol, y que integra los sistemas de alerta de 30 países, como el Sistema Español de Alerta Temprana (SEAT) [51,52]. En concreto, la función del EWS es detectar de manera rápida las NPS, evaluar sus posibles riesgos y permitir la difusión de información entre los diferentes países miembros.

En cuanto a los sistemas sanitarios, el aumento del consumo de las NPS supone un grave problema de salud pública que se ve agravado por la habitual falta de conocimiento de sus posibles efectos toxicológicos. Estos compuestos pueden acarrear desde problemas psiquiátricos [11,12], hasta la muerte por causas que van desde la asfixia a una insuficiencia circulatoria aguda [13]. Además, también existe un riesgo sanitario asociado al potencial adictivo de varias de estas sustancias [12,53]. Respecto a la tipología de consumidores más vulnerables a este tipo de compuestos destacan los jóvenes en edad de escolarización, los sintecho, los consumidores de drogas inyectables, la comunidad gay o incluso, la población reclusa [1]. En el caso de los jóvenes, se estima que en Europa entre un 1 y un 8% ha probado, en algún momento, alguna de estas sustancias [1,11]. Respecto a los sintecho, es de destacar el alto consumo de estos compuestos en comparación con drogas más tradicionales. Esto es debido a que las NPS son por lo general más baratas que sus homólogas tradicionales. Este motivo también es argumentado por parte de las personas que se inyectan droga usualmente para la iniciación en el consumo de NPS por vía intravenosa. Esto, conjuntamente con la tendencia de estos usuarios a compartir las jeringuillas, puede acarrear graves consecuencias para la salud de los mismos. En cuanto a la comunidad gay, es cada vez más habitual el uso de NPS en el acto sexual, lo que generalmente va unido a comportamientos sexuales de alto riesgo que pueden conllevar la trasmisión de enfermedades infecciosas como el VIH o la hepatitis C [1]. Este tipo de compuestos también han sido detectados en prisiones de toda Europa,

especialmente en el norte y el centro del continente, pudiendo suponer una fuente de tensión en la convivencia entre internos, además de un riesgo para su salud [1,48].

Las NPS también constituyen un reto para los científicos centrados en el análisis de este tipo de compuestos. La facilidad con la que estas sustancias emergen en el mercado dificulta su identificación debido a la falta de disponibilidad de patrones de referencia. De hecho, la mayoría de las NPS no son detectables mediante los análisis de drogas rutinarios, lo que aumenta su atractivo entre los consumidores y, por tanto, su riesgo social [1]. Esto unido a la necesidad de monitorizar el consumo de estas sustancias mediante la determinación de las mismas en diferentes matrices biológicas o ambientales, conlleva un interés constante en el desarrollo de nuevos métodos analíticos para la determinación de estos compuestos en diferentes tipos de muestras. Estos métodos van desde la identificación de NPS y sus metabolitos en diversas matrices biológicas, como orina [10,45,47,54–57], sangre [10,45,47,55], saliva [10,55,58] o pelo [10,45,55,59], generalmente con el objetivo de monitorizar el consumo individual de estas sustancias, a la determinación de estos mismos compuestos en aguas residuales con la finalidad de estimar su consumo en grandes núcleos de población [10,57]. Entre las técnicas analíticas más utilizadas para la determinación de NPS y sus metabolitos destacan la LC [10,45,47,54,55,57–59], la GC [10,45,47,55,59] y la CE [10,55,56].

1.2. Catinonas sintéticas

En el Cuerno de África y en el sudoeste de la península Arábica, generalmente a una altura entre 1500 y 2500 m, podemos encontrar al *khat* (*Catha edulis*), un tipo de arbusto que puede llegar a alcanzar los 6 m de alto [2,8,60,61]. En estas regiones, las hojas del *khat* son cosechadas no por su valor nutritivo o sus propiedades medicinales, sino por sus efectos psicoactivos. Con el objetivo de experimentar estos efectos estimulantes, las hojas de este arbusto se consumen en forma de té o se mascan. De hecho, se estima que entre 5 y 10 millones de personas a nivel mundial mascan hojas de *khat* de manera habitual [60]. En la Fig. 3 se puede observar una imagen correspondiente a un arbusto de *khat*.

Figura 3. Arbusto de *khat* (*Catha edulis*).

Entre los más de cuarenta compuestos que contienen las hojas del *khat*, incluidos alcaloides, taninos, flavonoides, terpenoides, esteroles, glucósidos, aminoácidos, vitaminas y minerales, sus efectos estimulantes están asociados principalmente a la catinona, cuya estructura química se muestra en la Fig. 4A [8,60]. Esta sustancia es un alcaloide que puede provocar ansiedad, hiperactividad, euforia, excitabilidad o incluso una disminución del apetito [5,61]. Estos efectos están atribuidos al papel que ejerce la catinona como inhibidor de la recaptación de la dopamina, la norepinefrina y la serotonina, llevando a un incremento de concentraciones extracelulares del neurotransmisor y, por lo tanto, a un aumento en la neurotransmisión [61].

A pesar de que la catinona se puede encontrar de forma natural en las hojas del *khat*, también han surgido derivados sintéticos de la misma y concretamente, en las últimas décadas ha crecido de forma constante la obtención de estos derivados para fines recreativos [2–10]. Sin embargo, muchas de estas sustancias fueron diseñadas con objetivos medicinales mucho tiempo antes de su popularización en el ámbito recreativo, siendo este último relativamente reciente. A modo ilustrativo, se puede destacar que fue en el año 1928, cuando Hyde y colaboradores obtuvieron la primera

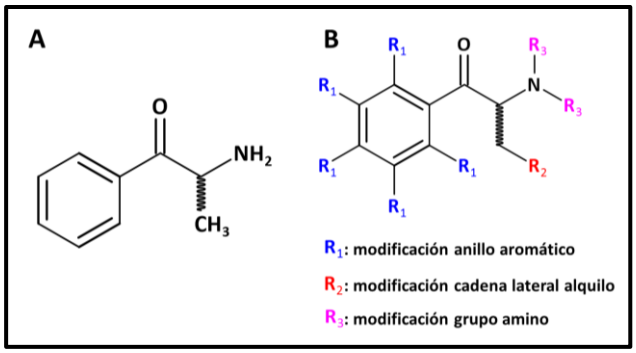

Figura 4. Estructura química de la catinona (A) y de sus derivados sintéticos (B).

catinona producida sintéticamente, la metcatinona [62] y un año más tarde se sintetizó la mefedrona [63], una catinona con especial trascendencia como sustancia de abuso casi un siglo después de su síntesis. La primera de estas sustancias puede servir como ejemplo paradigmático de la evolución que han tenido muchos de estos compuestos. Concretamente, la metcatinona fue en un principio utilizada en Rusia, en los años treinta, como antidepresivo [3–6]. Sin embargo, durante la década de los setenta su uso recreativo se estableció en varios países de la Unión Soviética y a principios de los años noventa el consumo abusivo de esta catinona se extendió por Estados Unidos, especialmente en el estado de Michigan [4,6,64]. No obstante, fue a principios de este siglo cuando el uso de este y otros compuestos similares se empezó a popularizar como alternativa legal a otras drogas tradicionales. En 2003 ya se había reportado el uso de catinonas sintéticas como alternativa a la 3,4-metilendioximetanfetamina (MDMA) y desde 2007 se podían encontrar referencias a estas sustancias en diferentes foros de Internet [6,7]. Particularmente, fue en el año 2008 cuando la mefedrona, cuya síntesis como se ha comentado se remonta 80 años atrás, entró con fuerza en el mercado europeo generando en poco tiempo una gran alarma social, especialmente en el Reino Unido e Irlanda [6–8,50]. En la actualidad, debido a la búsqueda continua de sustancias nuevas, legales, menos costosas y más potentes, por parte de los consumidores de drogas, la síntesis de nuevos derivados de la catinona se ha convertido en una industria fructífera, lo que ha llevado a la aparición de nuevas alternativas cada año. De hecho, según el *European Drug Report* 2019 del EMCDDA [48], las catinonas representaron un 24% de las incautaciones de NPS analizadas en Europa durante el año 2017, solo superadas por los cannabinoides sintéticos que representaron un 51% tal como se ha comentado anteriormente (Fig.

1). Además es importante destacar que en ese mismo año se detectaron 12 nuevas catinonas en suelo europeo [4].

Los derivados sintéticos de la catinona se pueden obtener a partir de las modificaciones estructurales de la propia catinona. En general y tal como se muestra en la Fig. 4B, estas modificaciones tienen lugar en tres regiones distintas de la molécula: el anillo aromático (R_1), la cadena lateral alquilo (R_2) y el grupo amino (R_3) [4]. Estas diferentes modificaciones dan como lugar a distintas catinonas sintéticas de las cuales algunos ejemplos se muestran en la Fig. 5. Los derivados más simples son los derivados N-alquilo o aquellos con un sustituyente alquilo o halógeno en cualquier posición del anillo aromático. La mayoría de catinonas sintéticas pertenecen a este grupo, e incluyen a la metcatinona, la etcatinona, la mefedrona, la 4-fluoro-metcatinona, la bufedrona, la pentedrona y la 3,4-dimetilmetcatinona entre otras. Otra estructura típica es la de aquellas catinonas que presentan un grupo metilendioxi en el anillo aromático. Es el caso de la metilona, la etilona, butilona y la pentilona. Otro grupo de catinonas sintéticas se caracteriza por la presencia de un grupo pirrolidino en el átomo de nitrógeno y dentro de este grupo destacan sustancias como la pirovalerona, la α-pirrolidinopentiofenona (α-PVP) o la 4-metil-α-pirrolidinobutiofenona (MPBP). De la combinación de los dos últimos grupos mencionados, aparece la familia de catinonas sintéticas que presenta tanto los grupos metilendioxi como pirrolidino. Son ejemplos de este conjunto de catinonas la 3,4-metilendioxi-α-pirrolidinopropiofenona (MDPPP), la 3,4-metilendioxi-α-pirrolidinobutirofenona (MDPBP) o la 3,4-metilendioxipirovalerona (MDPV).

Las catinonas sintéticas presentan una estructura química muy similar a la de las anfetaminas por lo que son referidas habitualmente como β-cetoanfetaminas. La principal diferencia de estos compuestos respecto a su anfetamina análoga es la presencia de un grupo cetona en el carbono β de la estructura química [2–10]. De hecho, la catinona es la β-cetona análoga de la anfetamina como muestra la Fig. 6. Esta semejanza estructural provoca también que ambos tipos de compuestos produzcan efectos parecidos en el organismo, aunque generalmente las catinonas sintéticas se consideran menos activas farmacológicamente en comparación a las anfetaminas [4]. Al igual que la catinona, los derivados de ésta actúan como inhibidores de la recaptación de la dopamina, la norepinefrina y la serotonina, lo que provoca sus efectos estimulantes [3–9]. Asimismo, es importante destacar que las catinonas presentan un centro quiral, lo que da lugar a dos enantiómeros que pueden

mostrar un comportamiento farmacocinético y farmacodinámico distinto y, por tanto, diferenciarse en su actividad biológica [6,14]. La propia catinona ya presenta esta diferenciación enantiomérica dado que la forma *S* es el componente más potente de las hojas de *khat* en la estimulación del sistema nervioso central [61]. Esta misma tendencia se ha corroborado para otras catinonas sintéticas, como la metcatinona [65], la α-PVP [66] o la MDPV [67], en diferentes estudios realizados en ratas que demostraron que el enantiómero que presenta mayor actividad es el *S*.

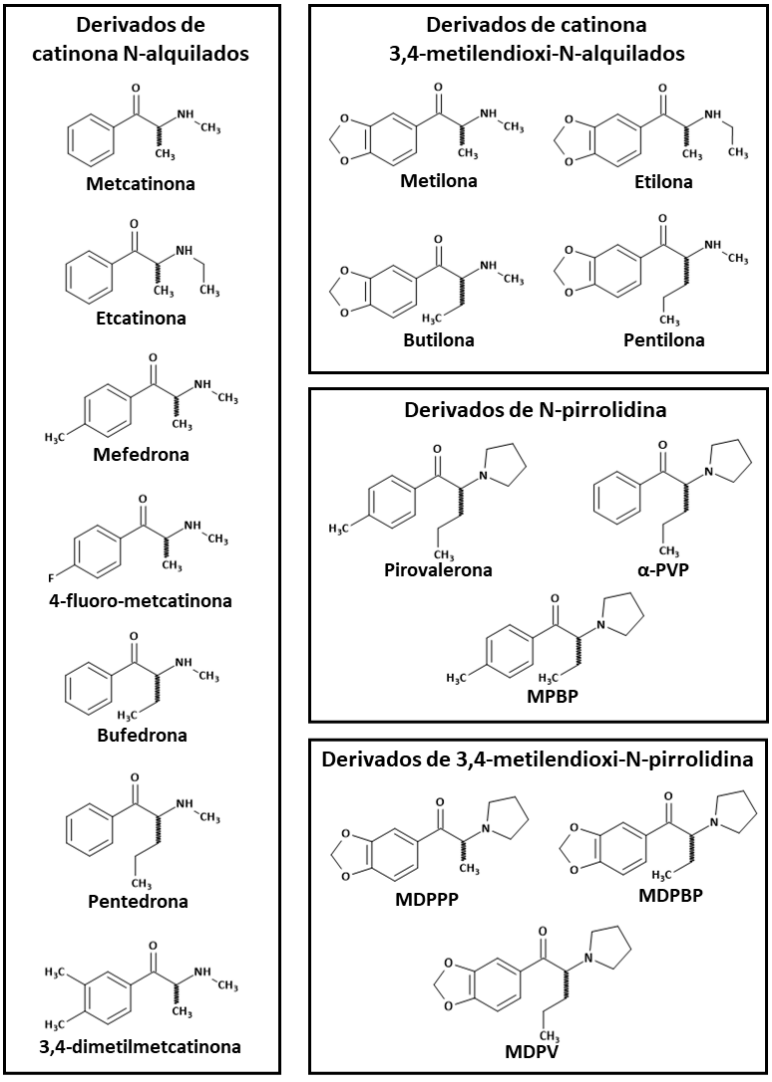

Figura 5. Estructura química de algunas catinonas sintéticas.

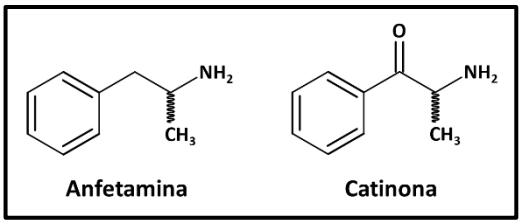

Figura 6. Estructuras químicas de la anfetamina y de la catinona.

En cuanto a su forma de adquisición, las catinonas no representan una excepción respecto a otras NPS. Actualmente estos compuestos se pueden adquirir principalmente en diferentes plataformas de internet donde son vendidos como "sales de baño", "alimento vegetal", "limpiador de alfombras" o "quitamanchas" en envases o bolsitas etiquetadas como "no aptas para el consumo humano" para evitar los controles legislativos [6,9]. Estas sustancias son generalmente compradas en forma de pastillas o de polvos blancos, amarillentos o marrones que pueden contener más de una catinona o también otros compuestos como cafeína o anestésicos locales [2,4–6]. Incluso se ha documentado el uso de catinonas como adulterantes de otras drogas clásicas [68]. Además, las catinonas principalmente son adquiridas por personas jóvenes y al igual que sucede con otras NPS, el motivo principal que argumentan sus consumidores a la hora de decantarse por ellas es su bajo coste y su fácil adquisición en comparación con otras drogas más tradicionales. Ambos aspectos son consecuencia del estado alegal en el que se encuentran muchos de estos derivados sintéticos [3,7,9].

Una vez adquiridas, las catinonas sintéticas son consumidas, de forma individual o en combinación con otras drogas, principalmente por vía nasal o directamente ingeridas. También ha sido descrita la administración rectal, la inhalación o la inyección intramuscular o intravenosa de estos compuestos [3,4,6,9]. Al ser consumidas, los efectos positivos descritos por los usuarios de las catinonas incluyen sobre todo un aumento de la energía, la simpatía, la sociabilidad y la líbido [5–7]. Sin embargo, también se pueden experimentar efectos adversos como sudoración, palpitaciones, náuseas y vómitos, dolor de cabeza, espasmos musculares, mareos, vértigo o pérdida de memoria a corto plazo y también del apetito [4,6,7]. Desde un punto de vista clínico, las catinonas presentan un amplio abanico de efectos negativos que incluyen afectaciones cardíacas, psiquiátricas o neurológicas, siendo la agitación el síntoma más común pudiendo ir desde una leve agitación hasta una psicosis severa.

Incluso, un consumo excesivo o prolongado de estas sustancias puede provocar la muerte [2–9]. Además, una vez consumidas, estas sustancias se pueden encontrar en diferentes matrices biológicas y en consecuencia su determinación en esta clase de muestras puede de ser de gran interés para la obtención de información de gran utilidad para las autoridades médicas y policiales.

Determinación de catinonas en muestras biológicas

Uno de los retos de la comunidad científica es el desarrollo de métodos que posibiliten la determinación de las nuevas sustancias psicoactivas, como son las catinonas, en distintos tipos de matrices biológicas con el objetivo de monitorizar el consumo de las mismas y también obtener información sobre posibles metabolitos. En este sentido, en la bibliografía se pueden encontrar numerosos trabajos centrados en la determinación de catinonas en muestras biológicas diversas como por ejemplo, orina [69–77], sangre [75–81], cabello [82–84], saliva [85–87], humor vítreo [81] o meconio [88]. A modo de comparación, en la Fig. 7 se muestran los porcentajes de artículos científicos publicados en los últimos 5 años que se han centrado en la determinación de catinonas en distintos tipos de matrices biológicas. En esta figura se puede observar que las muestras más comúnmente analizadas han sido orina y sangre. No obstante, hay otros tipos de matrices que también han sido objeto de estudio como por ejemplo pelo, saliva o, menos comúnmente, meconio o humor vítreo, entre otras. Los datos representados en la figura son el resultado de hacer una búsqueda bibliográfica centrada en el período 2015-2020 en la base de datos *Scopus* (palabras de búsqueda: catinonas y cada uno de los tipos de muestras biológicas, 1370 artículos en total).

Cada matriz biológica presenta diferentes ventajas e inconvenientes que se encuentran resumidos en la Tabla 1. Asimismo, cada matriz es más o menos adecuada en función del propósito del estudio realizado. Por ejemplo, si se pretende estudiar el consumo reciente de estupefacientes (durante las últimas 6-48 horas), las matrices más idóneas serán sangre o saliva. En cambio, si el objetivo es evaluar el consumo de drogas durante los últimos días, las muestras de orina son la mejor opción, y si lo que interesa es conocer el consumo durante los últimos meses, las muestras de cabello constituirán la opción preferible, ya que permiten llevar a cabo un análisis retrospectivo. También es importante destacar que un resultado negativo en el análisis de un matriz concreta solo implica la ausencia del analito en esa matriz y no

necesariamente prueba que no se haya consumido una determinada sustancia. Por tanto, por norma general, la mejor estrategia para monitorizar el consumo de catinonas suele ser su determinación simultánea en diversos tipos de muestras biológicas, ya que esto permite una mejor interpretación de los resultados analíticos obtenidos, puesto que cada matriz añade información complementaria al resultado global.

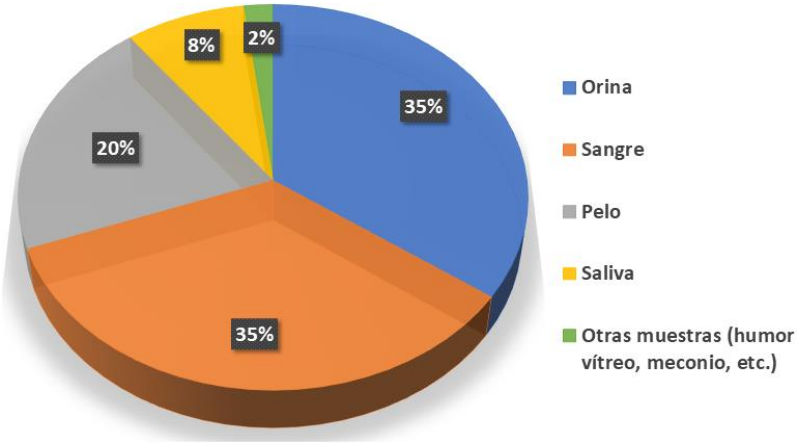

Figura 7. Porcentaje de los estudios publicados desde 2015 hasta 2020 centrados en la determinación de catinonas en distintos tipos de matrices biológicas (*Scopus*, diciembre 2020).

En el caso de la presente Tesis Doctoral, las metodologías desarrolladas para la determinación de catinonas se han centrado en una sola matriz biológica, la orina, debido a que su toma de muestra es no invasiva y se puede obtener en grandes cantidades en comparación con otras matrices. Con el objetivo de profundizar en el conocimiento de las estrategias utilizadas en la bibliografía para la determinación de catinonas en muestras de orina se realizó un artículo de revisión bibliográfica centrado en la discusión crítica de las publicaciones científicas de los últimos 5 años (2015-2020). Esta revisión, que está incluida en la siguiente sección, ha sido publicado en la revista científica *Trends in Analytical Chemistry 143 (2021) 116347* y en ella se hace especial énfasis en examinar los métodos analíticos empleados para la determinación de catinonas en orina, principalmente los centrados en técnicas cromatográficas y electroforéticas por ser los más comunes, así como en las principales estrategias de pretratamiento de muestra utilizadas. Además, puesto que las catinonas pueden ser inestables en determinadas condiciones, en este artículo se ha prestado especial atención en la discusión de los factores más importantes a

considerar para el correcto almacenamiento de esta matriz biológica, con el objetivo de prevenir la degradación de estos compuestos previamente a su análisis.

Tabla 1. Ventajas e inconvenientes en el análisis de diferentes matrices biológicas para la determinación de catinonas sintéticas.

Matriz biológica	Ventana de detección	Ventajas	Inconvenientes
Sangre	De minutos a horas desde el consumo	▪ Es posible una correlación entre la concentración hallada y la cantidad de droga consumida	▪ Método de extracción invasivo y se requiere personal especializado ▪ Volumen limitado ▪ Inestable sino se conserva en unas condiciones adecuadas
Orina	De 1 a 4 días desde el consumo	▪ Muestreo no invasivo ▪ Elevado volumen ▪ Concentraciones normalmente altas de los analitos y posibles metabolitos	▪ Fácilmente manipulable ▪ Inestable sino se conserva en unas condiciones adecuadas
Saliva	Desde horas a días desde el consumo	▪ Muestreo no invasivo ▪ Manipulación difícil ▪ Droga íntegra (no metabolizada)	▪ Volumen escaso ▪ Contaminación de la muestra por residuos de comida, etc.
Pelo	De semanas hasta meses desde el consumo	▪ Muestreo sencillo ▪ Difícilmente manipulable ▪ Muestreo no invasivo ▪ Permite la detección de las drogas consumidas y sus metabolitos	▪ Complejo tratamiento de muestra ▪ Posible contaminación de la muestra por el uso de productos cosméticos

1.2.1. Cathinones in urine samples: a review of recent advances for their determination by chromatographic and related techniques

CATHINONES IN URINE SAMPLES: A REVIEW OF RECENT ADVANCES FOR THEIR DETERMINATION BY CHROMATOGRAPHIC AND RELATED TECHNIQUES

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Abstract

In recent years, the consumption of synthetic cathinones has grown continuously as these drugs represent a more affordable and obtainable alternative to amphetamines. After consumption, cathinones have a stimulant effect and can be found in the human organism in their pure form or metabolized. As these compounds are difficult to monitor by routine drug screening it is very important to develop procedures capable of detecting and monitoring their consumption. Different strategies have been developed for the analysis of cathinones in biological matrices such as blood, hair or oral fluid. However, urine has been most widely used as the procedure is non-invasive and samples can be obtained in larger volumes than other biological matrices. The present review provides a critical discussion of the latest trends for the determination of cathinones in urine samples, focusing particularly on the use of chromatographic techniques, which is the approach most widely reported in the literature.

Keywords: *Capillary electrophoresis / Cathinones / Gas chromatography / Liquid chromatography / Mass spectrometry / Urine analysis /*

1 Introduction

Over the past decade, the constant emergence of legal alternatives to controlled drugs of abuse has been a major challenge for health and police authorities on a global scale. These compounds, referred to as new psychoactive substances (NPS), are defined by the United Nations Office on Drugs and Crime (UNODC) as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat" [1]. The denomination 'new' does not necessarily refer to newly invented substances but rather denotes substances that have recently come to popularity on the illicit market [2]. In fact, many NPS were first synthetized for medical or research purposes and only later repurposed as drugs of abuse. Although NPS affect the central nervous system in a similar manner to traditional drugs of abuse, they are usually cheaper and easy to obtain, making them an attractive alternative to conventional drugs, especially among young people [2–5].

There is a great variety of NPS which can be structurally classified in several groups of compounds such as aminonindanes, phenethylamines, piperazines, plant-based compounds, synthetic cannabinoids, synthetic cathinones or tryptamines [3,4]. Among these groups, synthetic cathinones, which include the compounds buphedrone, ethylone, methcathinone, mephedrone and methylone, are among the most frequently reported NPS in the literature [6,7]. Furthermore, according to the European Drug Report 2019 of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 24% of NPS seized in Europe in 2017 contained synthetic cathinones, only exceeded by synthetic cannabinoids (51%) [8].

Cathinones are synthetic derivatives of cathinone, the β-ketone analogue of amphetamine which can be naturally found in the leaves of khat (*Catha edulis*), a shrub native to East Africa and the Arabian Peninsula. The simplest cathinones are the N-alkyl derivatives or those with an alkyl or halogen substituent at any position on the aromatic ring. Most synthetic cathinones belong to this group, and include mephedrone, 4-fluoromethcathinone or buphedrone, among others. Another group of cathinones are the ones which present a methylenedioxy group on the

aromatic ring. This is the case of methylone or ethylone. There also some synthetic cathinones characterized by a pyrrolidinyl substitution at the nitrogen atom, and this is the case of substances such as pyrovalerone or α-PVP. Finally, from the combination of the last two groups mentioned, there are cathinones which have both methylenedioxy and pyrrolidine groups as MDPPP or MDPV [7]. The structure of some of the mentioned and other cathinones belonging to the different groups are shown in Fig. 1.

Cathinones are a growing and cheaper alternative to amphetamines that are usually sold on the internet or in "head shops" as "bath salts", "plant food", "carpet cleaner" or "stain remover" and are usually are labelled as "not for human consumption" to avoid regulatory controls [6,7,9–11]. Once purchased, mainly by young male abusers, synthetic cathinones are usually taken orally or nasally (snorted) and then produce their stimulating effects [7,9,12]. However, consumption of this kind of compounds can also produce diverse adverse effects related to cardiac, psychiatric and neurological affectations. Excessive or prolonged consumption of these drugs of abuse can even lead to death [9–11].

Cathinones have similar effects than amphetamines due to their similar structure, distinguished mainly by the presence of a carbonyl group at the β-position of the side chain of cathinones. As amphetamines, synthetic cathinones have an

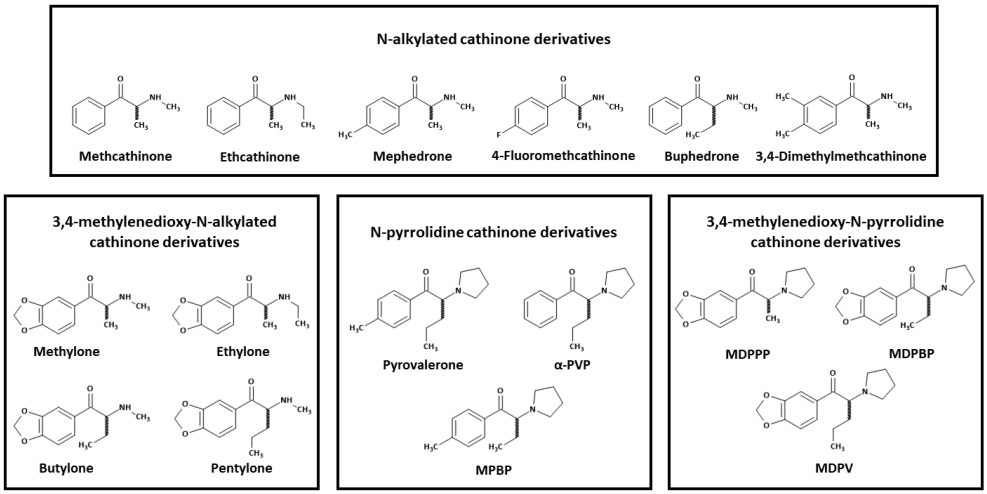

Figure 1. Chemical structures of some synthetic cathinones.

asymmetric carbon in their chemical structure, which implies the existence of two enantiomers. Each enantiomer can present different pharmacokinetic and pharmacodynamic behaviour, and the literature reports several cases in which the stimulant effects of cathinones are attributed mainly to the *S*(-) form [9,13,14]. Thus, the development of analytical methods capable of separating the two enantiomers will be crucial for further forensic and toxicological applications.

After their consumption, cathinones can be found in the body either metabolized or in their pure form in a high range of concentrations depending on the amount consumed, the time elapsed from consumption, and the way in which it is metabolized by the body. Several strategies, mainly based on high-sensitivity approaches such as GC-MS [15–35] or LC-MS [33,34,36–73], have been developed to analyse cathinones in different biological matrices, such as blood [17–24,35,47–56,66,68,74], hair [51] or oral fluids [55,66]. The preferred biological matrix for this purposes, however, is urine, because the procedure is non-invasive and samples are easy to collect and can be obtained in larger volumes than other matrices [75]. Urine has been used in multiple methodologies and for different objectives, including clinical and forensic applications, screening, stability studies, pharmacokinetic analysis and metabolomic studies [15–74,76–81]. Furthermore, as the simulating effects of cathinones can differ between *R* and *S* enantiomers, some of the studies in the literature use different strategies to achieve the chiral separation of these compounds [20,21,74,76–78].

This review offers a critical discussion of new trends and future directions of methodologies based on the analysis of cathinones in urine, focusing on articles published from January 2015 to December 2020. The review is divided into sections. The first section discusses recent studies that evaluate the appropriate storage conditions to avoid the degradation of cathinones in urine samples. The second section is a critical examination of the main pretreatment procedures that have been used in recent years to extract cathinones from urine. The final section reviews the main approaches developed for the analysis of cathinones in urine and includes comprehensive, up-to-date tables containing detailed information of different aspects of each approach. This review focuses primarily on approaches based on the use of

chromatographic techniques (GC or LC), since they are the most frequently used for cathinone analysis, but other approaches such as capillary electrophoresis (CE) are also discussed.

2 Urine sample storage

Due to the volume of work, clinical and forensic laboratories may not always analyse urine samples from cathinone abusers immediately upon arrival. The resulting delay can lead to the degradation of the studied compounds if they are not stable in the analysed matrix, and this can lead to errors when evaluating the results. In such cases, urine samples should be properly stored in the appropriate conditions to guarantee the stability of the analytes until analysis [82]. Several authors have performed studies to evaluate the most suitable storage conditions for cathinones in urine samples [28,32,46,48,57,68]. Most of the recent studies on this topic have been conducted by Glicksberg *et al.* [46,57,68], who submitted biological samples including urine to different storage conditions and analysed cathinone content by LC-MS/MS. Factors considered in the analysis were urine pH and temperature, storage time, and the concentration and type of cathinones. As a general trend, the results of these studies demonstrated that tertiary or pyrrolidine-type cathinones, such as pyrovalerone or MDPV, were the most stable, while ring-substituted secondary amines, such as 3-FMC or 4-FMC, were generally more unstable. The authors also concluded that the stability of synthetic cathinones in urine samples was influenced by pH and the temperature at which these samples were stored, observing that the degradation rate increased when one of these parameters rose. Alsenedi *et al.* [32] developed a GC-MS methodology to evaluate the long-term stability of cathinones and amphetamine-type stimulants in urine. They evaluated parameters including urine temperature and the concentration and type of cathinones. From their results, the authors concluded that urine samples should be frozen (-20°C) immediately on reception. In frozen samples, cathinones were stable even at the end of the study period of 201 days, but in refrigerated samples (4°C) and at room temperature, most cathinones were unstable after only three weeks or 24-48 h, respectively. The authors also observed that pyrrolidine-type cathinones were stable under all the evaluated conditions, which is consistent with the findings of Glicksberg *et al.*

[46,57,68], and were more stable than the other cathinone types.

From these studies it can be concluded that the storage conditions under which urine samples are kept can affect cathinones, especially if they are not of the pyrrolidine-type. The data reported in the different studies indicate that samples should be stored at a low pH (≈ 4), as cathinones are alkaline-labile drugs, and in the freezer (≈ -20°C) to prevent degradation that can lead to error when interpreting the results.

3 Urine samples pretreatment

To determine cathinones in urine samples, a pretreatment procedure is generally required to remove or to reduce the presence of matrix interferences or to preconcentrate the studied compounds. In recent studies, a variety of pretreatment strategies have been employed. In some cases, the reported approaches are very simple, such as urine dilution [45,47,58–60,65–67,69,80] or evaporation [26]. Olesti *et al.* [58] developed a LC-MS/MS method for the determination of 14 common NPS in urine samples. The sample pretreatment consisted of a sample dilution and direct injection strategy: 10 µL of urine samples were diluted in 170 µL of MeOH/water (15:85) and the obtained samples were analysed by the chromatographic technique. This fast sample pretreatment in combination with a high-sensitivity detector make the method very convenient for forensic purposes or doping control. In some studies, as a single pretreatment or as a complementary strategy for other pretreatment procedures, the urine samples were hydrolysed, usually with an enzyme such as β-glucuronidase [33,34,45,59,70–73] or by using an acidic solution [17,18,69]. Another used and simple approach consists of deproteinization by precipitation with an organic solvent such as acetone [52,53] or by using an acidic solution [79].

However, the most common pretreatment procedures in the analysis of cathinones in urine samples are liquid-liquid extraction (LLE) [16–19,24,27,29,30,34–37,48–50,54,56,62–64,76–78] and solid-phase extraction (SPE) [15,20–22,28,31–33,38–43,46,51,57,68,70,73,74]. The main advantages of LLE are its simplicity and generally acceptable recoveries for cathinones in urine samples. In recent studies, different organic solvents have been used in the LLE procedure to extract these new drugs from urine, in some cases in

their pure form such as example ethyl acetate [36,50,54,63,64] or n-butyl chloride [48,49] or as a combination of solvents such as ethyl acetate/2-propanol (4:1) [76] or ethyl acetate/dichloromethane/2-propanol (3:1:1) [17,18]. In some LLE procedures, prior to extraction, the biological samples were saturated with salt to reduce the solubility of the analytes and facilitate their extraction (salting-out) [34,62,77]. This salt addition increased the recovery of the LLE procedure in all cases. For instance, in a recent study in which cathinones were extracted from urine by LLE, the recovery values were increased by an average of 10% for all tested analytes by the addition of 0.05 g/mL of NaCl to the urine samples [77]. The use of commercial Toxi-Tubes to perform LLE of different analytes in biological samples is an alternative approach in clinical and forensic drug analysis. These commercial tubes simplify the pretreatment procedure and reduce the total analysis time needed [83–85], as the tubes contain the solvents and salts to perform LLE and it is only necessary to add the sample, mix and centrifuge. This approach has been used as pretreatment for the analysis of cathinones in urine samples [16]. Specifically, type A Toxi-Tubes were used in LLE of urine samples for the study of a suicide attempt in which several plastic bags containing different powders and labelled with common code names for synthetic cannabinoids and cathinones were seized from the patient. For the analytical procedure, urine samples were added to the Toxi-Tube and each sample was mixed and centrifuged. Next, the organic layers were collected into vials and evaporated to dryness under a nitrogen stream, then the final residue was dissolved in 50 mL of methanol and injected into a GC-MS system. Urine samples collected from the patient tested positive for two synthetic cathinones, α-PHP and α-PVP, which was consistent with the content of the plastic bags. Another recent trend based on LLE for the determination of organic compounds is to use liquid microextraction techniques to minimise the volume of the extraction solvent, which reduces environmental impact and lowers pretreatment costs [86]. Mercieca *et al.* [23], for example, developed a GC-MS method for the determination of NPS in urine and blood in which samples were pretreated by dispersive liquid-liquid microextraction (DLLME). The urine samples were first alkalinized with sodium hydroxide, after which the studied compounds were derivatized and DLLME was performed. The

cloudy solution was obtained by adding 350 μL of a mixture of chloroform (extractant)/MeOH (disperser) (1:2.5) to the urine sample. Then, the sample was centrifuged to deposit the fine droplets of the extractant phase at the bottom of the extraction tube. Finally, the infranatant phase was collected and injected into the GC-MS instrument. This extraction procedure gave recoveries of over 95% for the studied cathinones, demonstrating the suitability of the DLLME methodology for extracting these compounds from the biological matrix.

SPE is the other common strategy for extracting and, in some cases, preconcentrating cathinones before analysis. As cathinones are weak bases, cationic exchange cartridges are usually the preferred choice. Specifically, most SPE procedures for the extraction of cathinones from urine use sorbents based on strong cation exchange (SCX) [20–22,28,31–33,39,41–43,46,51,57,68,74]. For example, Pascual-Caro *et al.* [39] compared two different cation-exchange sorbents – a weak one (Oasis WCX) and a strong one (Oasis MCX) – to extract cathinones from urine samples. Although both sorbents achieved recoveries higher than 80% for the studied cathinones when the urine samples were analysed by LC-UV, a higher matrix effect was obtained with Oasis WCX when the same samples were analysed by LC-HRMS. So, in these conditions the SCX sorbent proved to be a more suitable option for sample pretreatment. Reversed-phase sorbents have also been successfully used in recent studies [15,40]. However, this is a less common approach than the use of cationic exchange sorbents, since, as stated above, cathinones are weak bases and generally show a stronger interaction with this kind of sorbents. Despite of this, reversed phase sorbents have been used for some authors in automated SPE pretreatments, in an attempt to drastically reduce the total analysis time and the handling time of the human operator [15,73]. For instance, Oasis HLB cartridges were employed in an automated SPE pretreatment procedure for the determination of α-PVP and its metabolite (OH-α-PVP) in forensic toxicology samples, specifically urine [15]. After extraction, the sample was derivatized before injection into a GC-MS system. The recoveries for this methodology were over 88%. Another interesting sample pretreatment approach was developed by Strickland *et al.* [73] for the determination of NPS in urine samples from “high-risk”

patients. For the pretreatment procedure, first the urine samples were hydrolysed with β-glucuronidase. Next, the target compounds were extracted using an automated liquid workstation (Automated Liquid Dispenser III) with narrow bore extraction plates (CEREX PSAX 96-Well NBE) and then the samples were analysed using a LC-MS/MS system. In this case, the sorbent consisted of microparticles of a strong anion exchange copolymer (PSAX) with extra small flow paths that ensure greater retention of the analyte molecules as they pass through the sorbent bed. The methodology achieved recoveries higher than 70% for all of the studied compounds. After its development, this LC-MS/MS procedure was applied effectively in routine analysis, demonstrating a greater potential for identification purposes than other previous methodologies.

Some authors have focused their research on the use of sorbents that increase the selectivity of the SPE procedure. In some recent approaches, for example, the SPE procedure is based on the use of molecularly imprinted polymers (MIPs) [38,44]. Murakami *et al.* [38] evaluated the efficiency of a commercial MIP (AFFINILUTE MIP-Amphetamine) for the extraction of a group of synthetic cathinones from urine and blood, prior to their analysis by LC-MS/MS. This commercial MIP was selected for of its high affinity for compounds with a similar structure to cathinones, such as amphetamines. With this sorbent, recoveries of over 60% were achieved for all the studied cathinones in urine samples. The authors also compared their MIP-based strategy with two other pretreatment approaches: LLE using chloroform/2-propanol (3:1) as the solvent, and SPE using Oasis HLB as the sorbent. The highest recoveries and matrix removal capability were achieved with the MIP sorbent. This could be attributed to the high affinity and selectivity of the commercial MIP for synthetic cathinones. Therefore, this MIP approach could represent a good alternative for the determination of cathinones in urine samples for clinical and toxicological purposes.

In an attempt to develop environmentally friendly approaches, some authors have applied strategies based on the use of solid-phase microextraction (SPME) [25,44]. For example, Alsenedi *et al.* [25] developed a GC-MS methodology for the determination of amphetamine-type stimulants and synthetic cathinones in urine after SPME

pretreatment. Three SPME fibre tips were evaluated, C18, C18-SCX (mixed mode) and PDMS-DVB, and in all cases the analytes were derivatized before analysis by GC-MS. The best results were achieved with the PDMS/DVB fibre tips, with recovery values in the range 2–80%, which were higher than those obtained for the other two tested materials, in the range 0.1–10%. In a recent study, Sánchez-González *et al.* [44] combined the selectivity of a MIP sorbent with a SPME approach by evaluating two in-house synthetized MIPs, an ethylone-based MIP and a 3-MMC-based MIP, in the SPME procedure for extracting cathinones from urine samples. After extraction, the cathinones were analysed by LC-MS/MS. The best results were obtained with the ethylone-based MIP, which gave recoveries in the range 65–90%.

The literature also reports novel strategies in which hybrid procedures between sampling and pretreatment are applied. An interesting approach was developed by Mercolini *et al.* [55], who analysed a group of cathinones by LC-MS/MS in urine, plasma and oral fluid samples from self-reported users after volumetric absorptive micro-sampling (VAMS). The VAMS approach is a recent microsampling technique in which a simple procedure is used to obtain dried specimens of biological fluids such as urine. It provides low sample volumes at high accuracy, and after extraction, the dried samples do not require frozen storage or refrigerated transport. To apply the technique, a small volume of the studied matrices (10 µL) was absorbed in the VAMS device by dipping their tips between in the biological sample for 2–4 seconds. The samples were then dried for 1 hour at room temperature. Next, the dried samples were extracted with methanol by solid-liquid extraction and then dried under vacuum. Finally, the extracts were reconstituted with a mixture of acetonitrile and water (50/50) both with a percentage of 0.1% of formic acid and analysed by LC-MS/MS. This strategy achieved recoveries higher than 75% for all the cathinone analogues in the three dried matrices, using a reduced sample volume.

4 Strategies based on GC-MS

In recent years, different strategies based on GC-MS have been developed for the analysis of cathinones in urine samples, which are summarized in Table 1. However, since cathinones are not volatile compounds, a sample derivatization step prior to injection into the GC-MS system is reported in the vast majority of these strategies.

Table 1. Strategies reported in the literature from 2015 to 2020 for the analysis of cathinones in urine based on GC-MS.

Compounds studied	Matrices	Urine pretreatment	LODs in urine[a]	Notes	Reference
α-PVP and its metabolites	Urine	Automatic SPE with Oasis HLB cartridges. Sample derivatization with pyridine and BSTFA	5-25 ng/mL	A real sample was analysed	[15]
Synthetic cannabinoids Synthetic cathinones	Powders Stomach content Urine	LLE with ToxiTubes		Samples were collected from an intoxication case	[16]
MDPV and its metabolites	Serum Urine	Hydrolysis of urine with hydrochloric acid. LLE with ethyl acetate/dichloromethane/2-propanol (3:1:1). Sample derivatization with pyridine and acetic anhydride		A metabolic pathway was proposed	[17]
α-PVP and its metabolites	Serum Urine	Hydrolysis of urine with hydrochloric acid. LLE with ethyl acetate/dichloromethane/2-propanol (3:1:1). Sample derivatization with pyridine and acetic anhydride		A metabolic pathway was proposed	[18]
Mephedrone	Blood Urine	LLE with methyl tert-butyl ether. Sample derivatization with MSTFA	4.5 ng/mL	A pharmacokinetic study was performed	[19]
Synthetic cathinones	Plasma Urine	SPE with a CSDAU203 cartridge (SCX). Sample derivatization with ethyl acetate and L-TPC	0.1-0.7 μg/mL	Chiral separation by indirect chiral derivatization with L-TPC	[20]
Synthetic cathinones	Plasma Urine	SPE with a CSDAU203 cartridge (SCX). Sample derivatization with ethyl acetate and L-TPC	0.26-0.76 ng/mL	Chiral separation by indirect chiral derivatization with L-TPC	[21]
NPS	Blood Urine	SPE with a ZSDAU020 cartridge (SCX). Sample derivatization with PFPA/ethyl acetate (2:1)	0.5-1 ng/mL		[22]
NPS	Blood Urine	Sample derivatization with hexyl chloroformate and simultaneous extraction by DLLME with chloroform/MeOH (1:2.5)	2-10 ng/mL	The method was used in urine samples which had previously tested positive for the studied substances	[23]
Amphetamine-derived synthetic drugs	Serum Urine	LLE with cyclohexane and simultaneous derivatization with PFBCl		Method for urine analysis was only partially validated	[24]
Synthetic cathinones Amphetamine-type stimulants	Urine	SPME with PDMS/DVB fibre tips. Sample derivatization with PFPA/ethyl acetate (2:1)	5-25 ng/mL	3 real samples were analysed	[25]

[a]For the cathinones under study

Table 1. (Continued).

Compounds studied	Matrices	Urine pretreatment	LODs in urine[a]	Notes	Reference
Synthetic cathinones	Urine	Evaporation to dryness. Sample derivatization by a two steps protocol: first with hydroxylamine hydrochloride and second with MSTFA	15-24 µg/mL (LOQ)	Different amounts of urine from a suspected drug user were analysed	[26]
Synthetic cathinones	Urine	LLE with n-butyl chloride. Sample derivatization with HFBA	25-50 ng/mL	The method was applied to 45 real samples from victims of sexual assault	[27]
Synthetic cathinones	Blood Urine	SPE with a CSDAU506 SPE cartridge (SCX). Sample derivatization with PFPA/ethyl acetate (2:1)	0.5-1 ng/mL	A stability study was performed over 30 days	[28]
2C Amines NBOMe compounds Synthetic cathinones	Urine	Protocol 1: LLE with ethyl acetate/isopropyl alcohol (90:1) Protocol 2: LLE with hexane. Sample derivatization with h PFPA or pyridine and acetic anhydride		Two GC-MS protocols are proposed	[29]
Synthetic cathinones Amphetamine-type stimulants	Urine	LLE with TBME. Sample derivatization with TFAA	10-30 ng/mL	The method was applied to 594 real samples	[30]
Synthetic cathinones	Urine	SPE with a SPEC DAU cartridge (SCX). Sample derivatization HFBA and ethyl acetate	5-20 ng/mL	The method was applied to 6 real urine samples from drug abusers	[31]
Synthetic cathinones Amphetamine-type stimulants	Urine	SPE with a ZSDAU20 cartridge (SCX). Sample derivatization with PFPA/ethyl acetate (2:1)	0.5-10 ng/mL	A stability study was performed over 201 days	[32]
Metabolites of 4-MEC	Urine	Hydrolysis of urine with β-glucuronidase and arylsulfatase. SPE with HCX cartridge (SCX). Sample derivatization with acetic anhydride and pyridine		A metabolic pathway was proposed	[33]
Metabolites of α-PBP	Urine	Salting-out assisted LLE with NaCl and chloroform/2-propanol (3:1). Sample derivatization with MSTFA		A metabolic pathway was proposed	[34]
Stimulant designer drugs	Blood Urine	LLE	20-100 ng/mL (cut-off)	Screening study of 2744 real samples collected in Budapest and 774 real samples collected in South-East Hungary from subjects prosecuted for illicit and/or designer drug use	[35]

[a]For the cathinones under study

For this, a derivatizing agent is employed to change a polar hydroxyl or amine group of the analysed cathinone to a less polar functional group [28]. Several derivatization agents have been used for this step, although pentafluoropropionic anhydride (PFPA) is most commonly reported [22,25,28,29,32]. Nisbet *et al.* [22] developed a GC-MS methodology for the determination of 23 NPS, including eight synthetic cathinones, in blood and urine. The analytes were extracted from both matrices with a pretreatment based on SPE (SCX), and derivatization with PFPA/ethyl acetate (2:1) was carried out before samples were injected into the GC-MS. This methodology yielded LODs of between 0.5 and 1 ng/mL for the studied cathinones in urine and was later used to study the stability of a group of cations in both blood and urine [28]. Other derivatizing agents have also been successfully used, including N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [15], heptafluorobutyric anhydride (HFBA) [27,31], pentafluorobenzoyl chloride (PFBCl) [24], N-methyl-N-(trimethylsilyl)-trifluoroacetamid (MSTFA) [19,34] and trifluoroacetic anhydride (TFAA) [30]. For example, in a recent study reporting the development of a GC-MS method for the determination of cathinones in urine from victims of sexual assault, cathinones were first extracted with a LLE procedure and then derivatized with HFBA [27]. The method was applied to 45 real samples and achieved LODs in the range 25–50 ng/mL. Six of the cases (13%) were confirmed as positive for methylone, which illustrates the emerging use of this drug in sexual assault. In another study, Ondra *et al.* [24] compared two derivatizing agents, HFBA and PFBCl, for the analysis of amphetamine-derived designer drugs in urine and serum samples from clinical patients by GC-MS. Derivatization with PFBCl yielded better purity, giving cleaner chromatograms than HFBA when blank urine samples were analysed. In most studies based on the use of GC for the analysis of cathinones in urine, these NPS are derivatized in one step, which is normally performed after sample extraction. However, in Molnár *et al.* [26], a two-step derivatization process was applied: the first step consisted of oximation with hydroxylamine hydrochloride in pyridine, the second was trimethylsilylation with MSTFA. Oximation was necessary because without this prior step, trimethylsilylation produced extremely unstable derivatives. The approach achieved LOQs between 15 and 24 μg/mL for the studied cathinones in urine. These relatively high LOQ values, compared

to those obtained with other GC-MS methodologies, can be mainly attributed to the sample pre-treatment, since in this case the urine samples were simply evaporated prior to the derivatization of the studied compounds. So, although the reported strategy was relatively simple, its applicability is limited to cases in which cathinones are present in urine at high concentration levels. It should also be noted that, in most cases, the derivatization step can be time consuming and this greatly lengthens the overall time required to analyse the target cathinones. To reduce this time, Mercieca *et al.* [23] developed a pretreatment procedure based on DLLME that allowed almost simultaneous derivatization and extraction of different NPS, including 12 cathinones, in urine and blood. In their study, the target analytes were derivatized with hexyl chloroformate and since this derivatization reaction occurs in aqueous conditions, the derivatization reagent was added directly to the sample. Next, DLLME was performed using a mixture of chloroform/MeOH to form the cloudy solution. Finally, the infranatant phase was collected and analysed by GC-MS. The obtained LODs ranged from 2 to 10 ng/mL. This procedure was applied to urine samples that had previously been confirmed as positive for the studied substances with a routine method based on ultra-high-performance liquid chromatography (UHPLC) coupled to MS/MS, and good correspondence was obtained between the results of the two methodologies. As stated above, in most cases this derivatization step is performed to convert cathinones into volatile compounds, but some authors have also used derivatization to indirectly achieve the chiral separation of cathinones [20,21]. Alremeithi *et al.* [20,21], for example, successfully used (S)-(-)-N-(trifluoro-acetyl)pyrrolidine-2-carbonyl chloride (L-TPC), a well-known commercial chiral derivatization agent [87], for the enantioseparation of cathinones in urine and plasma samples collected by the police. This chiral derivatization agent converts cathinone isomers to diastereomeric derivatives that can be separated on achiral stationary phases. In both studies, the cathinones were extracted from urine using a SPE (SCX) procedure and, the extracts were derivatized with L-TPC and analysed by GC-MS. The L-TPC derivatization procedure is summarized in Fig. 2. In both cases, the studied cathinones were at least partially enantioseparated, and in most cases, the enantiomers of the cathinones were baseline separated. The main difference between the two

studies was the ionization source: in one, the target compounds were ionized by electron impact (EI) [20], while in the other they were ionized by negative chemical ionization (NCI) [21]. NCI showed sensitivity that was two orders of magnitude higher than that of EI: the LODs for urine samples were between 0.26 and 0.76 ng/mL for GC-NCI-MS and between 0.1 and 0.7 µg/mL for GC-EI-MS. As these two studies show, the use of a chiral derivatization agent in the determination of cathinones by GC is of particular interest, since there is a need for analytical methodologies capable of enantioseparating these compounds.

5 Strategies based on LC

Due to the polar characteristics of cathinones and the aqueous nature of the urine matrix, LC is usually the preferred choice for analysing these compounds in urine samples [33,34,36–65,68–74]. The recent literature on this topic is summarized in Table 2. In most of the studies, LC is combined with MS detection, predominantly of two types: high-resolution mass spectrometry (HRMS) [39–43,52,53,59,61,70,72] and tandem mass spectrometry (MS/MS) [33,34,36–38,44–51,54–59,62–69,71–73]. The use of these high-sensitivity techniques achieved very low LODs.

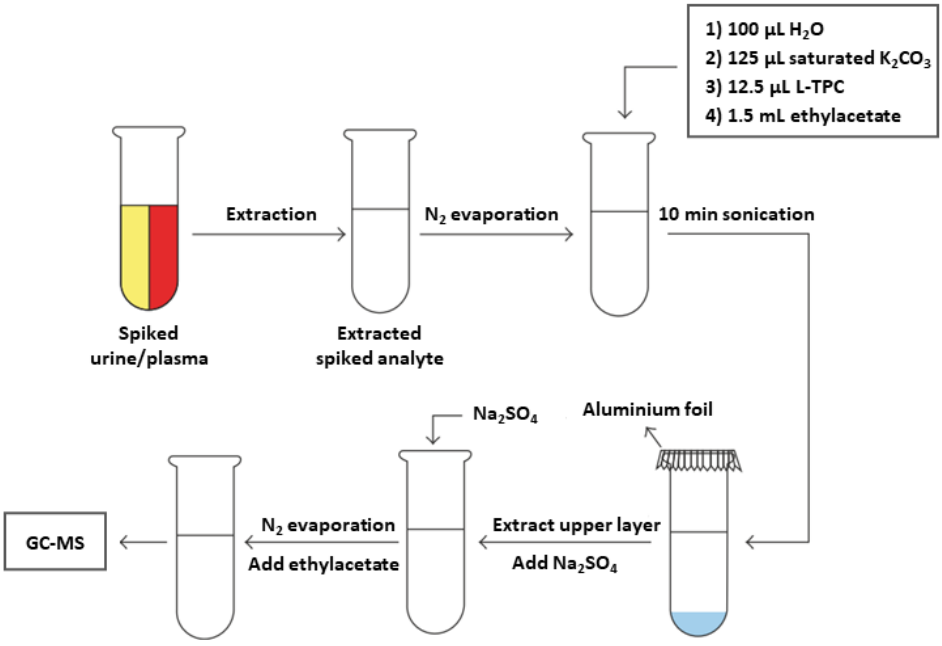

Figure 2. Scheme of a L-TPC derivatization process for synthetic cathinones prior to their injection in a GC-MS instrument. Reproduced with permission from Ref. [21].

Table 2. Strategies reported in the literature from 2015 to 2020 for the analysis of cathinones in urine based on LC.

Compounds studied	Matrices	Urine pretreatment	Analytical technique	LODs in urine[a]	Notes	Reference
Metabolites of 4-MEC	Urine	Hydrolysis of urine with β-glucuronidase and arylsulfatase. SPE with HCX cartridge (SCX)	LC-MS/MS		A metabolic pathway was proposed	[33]
Metabolites of α-PBP	Urine	Hydrolysis of urine with β-glucuronidase and sulfatase. LLE with MeOH	LC-MS/MS		A metabolic pathway was proposed	[34]
NPS	Blood Urine	LLE with ethyl acetate	LC-MS/MS	1-2.5 ng/mL (LOQ)	The method was applied to real samples provided by the police	[36]
NPS	Urine	LLE with acetonitrile	LC-MS/MS		Screening study of 20 pooled urine samples from London and a music festival in UK and 7 pooled urine samples from a music festival in Belgium	[37]
Synthetic cathinones	Blood Urine	SPE with an AFFINILUTE MIP-Amphetamine SPE cartridge	LC-MS/MS	0.011-0.13 ng/mL		[38]
Synthetic cathinones	Urine	SPE with an Oasis MCX cartridge (SCX)	LC-HRMS	0.04-0.16 ng/mL	Two extraction SPE procedures are compared	[39]
NPS	Urine	Hydrolysis of urine with β-glucuronidase. SPE	LC-HRMS		Screening study of 44 pooled urine samples collected from portable street urinals in London	[40]
NPS	Urine	SPE with SOLA CX cartridge (SCX)	LC-HRMS	1-5 ng/mL	Screening study of 62 urine samples from stimulant users	[41]
PV8 and its metabolites	Urine	SPE with SOLA CX cartridge (SCX)	LC-HRMS		A metabolic pathway was proposed	[42]
α-PVT and its metabolites	Urine	SPE with SOLA CX cartridge (SCX)	LC-HRMS		A metabolic pathway was proposed	[43]
Synthetic cathinones	Urine	SPME with an in-house synthesized MIP using EGDMA as functional monomer	LC-MS/MS	0.14-1.51 ng/mL		[44]
MDPHP and α-PBP and their metabolites	Urine	Two pretreatment techniques were applied: a dilution and a hydrolysis with β-glucuronidase	LC-MS/MS		A metabolic pathway was proposed for each cathinone	[45]

[a]**For the cathinones under study**

Table 2. (Continued).

Compounds studied	Matrices	Urine pretreatment	Analytical technique	LODs in urine[a]	Notes	Reference
Synthetic cathinones	Urine	SPE with a PolyChrom Clin II cartridge (SCX)	LC-MS/MS	0.25-5 ng/mL	A stability study was performed over 6 months	[46]
Mephedrone and its metabolites	Plasma Urine	Dilution in water	LC-MS/MS	0.4-4 ng/mL	A pharmacokinetic study was performed	[47]
Synthetic cathinones	Blood Urine	LLE with n-butyl chloride	LC-MS/MS		A stability study was performed over 6 months	[48]
α-PiHP	Bile Blood Internal organs tissues Urine	LLE with n-butyl chloride	LC-MS/MS	0.3 ng/mL	Samples were collected from a deceased person	[49]
α-PVP	Blood Internal organs tissues Urine	LLE with ethyl acetate	LC-MS/MS		Samples were collected from a deceased person	[50]
Synthetic cathinones	Bile Blood Gastric contents Hair Internal organs tissues Urine	SPE with a Bond Elut Certify cartridge (SCX)	LC-MS/MS	0.2 ng/mL	Samples were collected from a deceased person	[51]
4-MeOPBP	Blood Urine	Deproteinization with acetone	LC-HRMS	300 ng/mL	Samples were collected from a deceased person	[52]

[a]**For the cathinones under study**

Table 2. (Continued).

Compounds studied	Matrices	Urine pretreatment	Analytical technique	LODs in urine[a]	Notes	Reference
MPHP and one of its metabolites	Blood Liver tissue Urine	Deproteinization with acetone	LC-HRMS	0.5 ng/mL	Samples were collected from a deceased person	[53]
N-ethylpentylone	Blood Urine	LLE with ethyl acetate	UHPLC-MS/MS	1 ng/mL (LOQ)	Samples were collected from a deceased person	[54]
Synthetic cathinones	Oral fluid Plasma Urine	VAMS	LC-MS/MS	3 ng/mL	5 real samples from self-reported users were analysed	[55]
NPS	Blood Urine	LLE with n-butyl chloride/ethyl acetate (70:30)	LC-MS/MS	LOD < 2.5 ng/mL	Screening study of 110 real samples from deceased persons	[56]
Synthetic cathinones	Urine	SPE with a PolyChrom Clin II cartridge (SCX)	LC-MS/MS	1-2 ng/mL (LOQ)	A stability study was performed over 5-17 months by analysing 180 real samples	[57]
NPS	Urine	Dilution in MeOH/water (15:85)	LC-MS/MS	0.3-0.5 ng/mL	The method was applied to samples collected from volunteers after the self-administration of different drugs	[58]
α-PVP and its metabolites	Urine	Urine samples were prepared by three methods, dilution in acetonitrile, dilution in ice-cold acetonitrile and hydrolysis with β-glucuronidase	LC-HRMS LC-MS/MS		The structures of more than 60 α-PVP metabolites and their metabolic pathway was proposed	[59]
NPS	Urine	Dilution in methanol (UHPLC-MS) or 2-propanol (UHPSFSC-MS)	UHPLC-MS/MS UHPSFSC-MS/MS	0.02-5.15 ng/mL (UHPLC-MS) 0.01-1.04 ng/mL (UHPSFSC-MS)	Comparative between a UHPLC-MS method and a UHPSFSC-MS method	[60]

[a]**For the cathinones under study**

Table 2. (Continued).

Compounds studied	Matrices	Urine pretreatment	Analytical technique	LODs in urine[a]	Notes	Reference
Classical recreational drugs NPS	Urine		LC-HRMS	20 ng/mL	Screening study of 200 real samples from individuals undergoing an opioid maintenance treatment	[61]
5-PPDI and its metabolites	Urine	Salting-out assisted LLE with anhydrous sodium carbonate and ACN	LC-MS/MS		A metabolic pathway was proposed	[62]
Drugs of abuse	Urine	LLE with ethyl acetate	LC-MS/MS	1-2 ng/mL	Screening study of 769 real samples from suspected drug abusers	[63]
Drugs of abuse	Urine	LLE with ethyl acetate	LC-MS/MS	0.5 ng/mL	Screening study of 126 real samples from victims of sexual assault	[64]
Synthetic cathinones	Urine	Dilution in MeOH/water (50:50)	LC-MS/MS	0.1-0.5 ng/mL	Screening study of 67 real samples provided by the local law enforcement agencies	[65]
Classical recreational drugs NPS Related metabolites	Blood Oral fluid Urine	Dilution in M3® buffer solution	LC-MS/MS	0.07 ng/mL	Screening study of 56 real samples collected from antemortem and post-mortem cases	[66]
NPS	Urine	Dilution in water	LC-MS/MS	0.01-0.12 ng/mL	Screening study of 50 real samples obtained from a local drug testing laboratory	[67]
Synthetic cathinones	Blood Urine	SPE with a PolyChrom Clin II cartridge (SCX)	LC-MS/MS	0.25-5 ng/mL	A stability study was performed over 10 months	[68]

[a]For the cathinones under study

Table 2. (Continued).

Compounds studied	Matrices	Urine pretreatment	Analytical technique	LODs in urine[a]	Notes	Reference
Synthetic cathinones Tropane alkaloids Pharmaceutical narcotics	Urine	Two pretreatment techniques were compared: a dilution and an acid hydrolysis with HCl	LC-MS/MS	0.1 ng/mL	The procedure was tested on real samples	[69]
Classical recreational drugs NPS Anabolic steroids	Urine	Hydrolysis of urine with β-glucuronidase. SPE	LC-HRMS		Screening study of pooled urine samples collected from urinals in 9 UK cities	[70]
Classical recreational drugs NPS	Urine	Dilution in β-glucuronidase	UHPLC-MS/MS	6.3 ng/mL	A threshold accurate calibration technique was used to achieve matrix effect normalization	[71]
NPS	Urine Wastewater	Hydrolysis of urine with β-glucuronidase	LC-HRMS (qualitative analysis) LC-MS/MS (quantitative analysis)	0.04-0.46 ng/mL	Screening study of 56 pooled urine samples collected at music festivals across Europe	[72]
NPS	Urine	Hydrolysis of urine with β-glucuronidase. Automatic SPE with a CEREX PSAX 96-Well NBE plates	LC-MS/MS	15 ng/mL (cut-off)	The procedure was applied on real samples	[73]
Synthetic cathinones	Plasma Urine	SPE with a CSDAU203 cartridge (SCX)	LC-UV	1-1.47 µg/mL	2 chiral columns were compared in order to achieve the chiral separation	[74]

[a]**For the cathinones under study**

For example, Pascual-Caro *et al.* [39] obtained LODs of between 0.04 and 0.16 ng/mL for the determination of cathinones in urine by SPE LC with an Orbitrap analyser (HRMS). In a recent study, SPE-MIP pretreatment was combined with a LC-MS/MS methodology to obtain LODs of between 0.011 and 0.13 ng/mL [38]. These low LODs were achieved thanks to the high-sensitivity detector but also to the matrix effect reduction due to the high selectivity of the SPE-MIP procedure used in the sample pretreatment. In another study, Ambach *et al.* [36] developed a LLE LC-MS/MS methodology for the determination of NPS in urine and blood, obtaining LOQs of cathinones in urine in the range 1–2.5 ng/mL. Their methodology was applied to real samples provided by the police and some positive results were obtained, with cathinone the most detected compound. These high-sensitivity detectors have also been applied in different screening approaches for cathinones in urine [37,40,41,56,-61,63–67,70–72]. For instance, LC-HRMS was used to analyse the consumption trends of classical recreational drugs, NPS and anabolic steroids using pooled urine samples collected from street urinals in nine cities in the UK [40]. Mephedrone was the most commonly detected cathinone and the results indicated that classic drug use was more prevalent than the consumption of NPS. In another study, Kinyua *et al*. [37] used a LC-MS/MS methodology to determine NPS consumption in the UK and Belgium through the screening of pooled urine samples collected from a city centre in the UK and from music festivals in the UK and Belgium. The results showed that, as in the previous study, the most widely consumed cathinone was mephedrone. A LC-HRMS methodology has also been used to monitor NPS abuse by opioid-dependent patients undergoing opioid maintenance treatment [61]. Two hundred urine samples were analysed and a NPS was found in 26 of them, with α-PVP the most commonly detected cathinone. Some of the patients reported the consumption of intravenous amphetamine when they had in fact consumed a cathinone, which can be explained by the similar effects of these compounds. However, and due to the dynamic market of cathinones or other NPS available around the world, since new drugs are continuously appearing, these methods are limited because most of them are only focused to the determination of a certain number of drugs. In this regard, it is worth mentioning the research proposed for Kimble *et al.* who developed a method

for the screening of more than 800 NPS, including various cathinones, in urine [67]. In the proposed strategy from these authors, the method was qualitatively characterized by using 16 subsets of mixtures containing various non-coeluting NPS, and quantitatively validated for 80 representative NPS. By operating in this way, the authors could avoid validating each compound separately and consequently the time required for the validation process was considerably reduced. Finally, the developed method was successfully applied for the screening of 50 authentic urine specimens obtained from a local drug testing laboratory. To discriminate between compounds with similar retention times during the screening analysis, data acquisition was performed in dynamic MRM mode. This mode employed a minimum of two transitions for each analyte, thus increasing the selectivity of the method.

LC can be also a useful strategy for the chiral separation of cathinones in urine. Meetani *et al.* developed a method for the enantioseparation of 22 tertiary amine synthetic cathinones in urine and plasma samples by LC-UV [74]. Despite the relatively high cost, its common practice in LC to use columns with a chiral stationary phase to achieve the chiral separation of the studied analytes [88], as these columns reduce the total analysis time in comparison to indirect chiral derivatization. Therefore, the authors compared two commercial chiral columns, an Astec Cellulose DMP and an Amylose-based Chiralpak AS-H. The best results were achieved with the first column, which allowed the enantiomeric separation of 18 of the

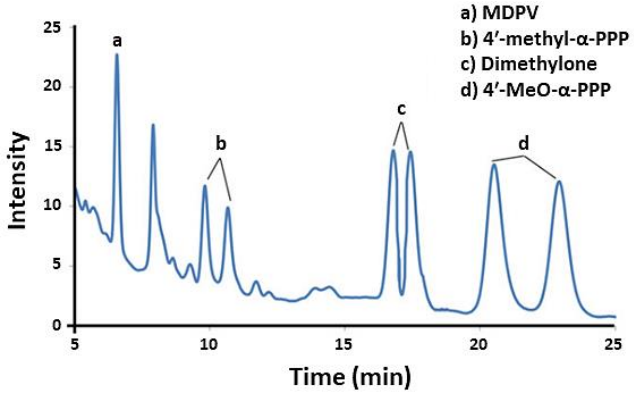

Figure 3. Chromatogram obtained for the simultaneous chiral separation of 4′-methyl-α-PPP, dimethylone and 4′-MeO-α-PPP spiked in a urine sample by LC-UV using a DMP cellulose column. MDPV is employed as internal standard. Reproduced with permission from Ref. [74].

22 compounds. Furthermore, the LC-UV methodology with the Cellulose DPM column was validated for the simultaneous enantiodetermination of three synthetic cathinones (4'-methyl-α-PPP, dimethylone and 4'-MeO-α-PPP) in urine and plasma. Fig. 3 shows the obtained chromatogram for a urine sample spiked with the studied cathinones and using MDPV as internal standard. However, the use of UV detection can impede the achievement of low LODs, as is observed in the LODs for the studied cathinones in urine, which range from 1 to 1.47 μg/mL, making them higher than those obtained in studies that used MS as detection system.

An alternative to conventional LC is the use of UHPLC, as the reduction of the column particle size and the application of high pressure generally increases separation efficiency over a shorter analysis time. Some authors have developed UHPLC-based methods for the analysis of cathinones in urine samples [54,60,71]. For example, Zawadzki *et al.* [54] developed a UHPLC-MS/MS method for the determination of N-ethyl-pentylone in blood and urine after LLE sample pretreatment with ethyl acetate. This methodology achieved a LOQ of 1 ng/mL for N-ethylpentylone in urine samples in less than five minutes. The UHPLC-MS/MS procedure was also used for the determination of this cathinone in a fatal intoxication case, and the compound was detected in both blood and urine samples obtained from the victim, demonstrating that this methodology is suitable for forensic applications. In view of these results, further studies should continue to explore UHPLC approaches for the determination of synthetic cathinones in biological samples such as urine.

6 Strategies based on other approaches

Although most of the studies that analysed cathinones in urine samples used methodologies based on the use of LC or GC, other approaches are also reported in the literature [60,76–80], as summarized in Table 3. The most notable of these approaches is CE [76–79], which has been used particularly in the enantiodetermination of cathinones, as the chiral separation of these compounds can be easily achieved by simply adding a chiral selector such as a cyclodextrin into the background electrolyte [89]. So, in CE, it is not necessary to use a specific chiral column as in LC, which can be expensive [74], nor do the studied compounds need to be derivatized before analysis as in GC [20,21]. Differ-

Table 3. Strategies reported in the literature from 2015 to 2020 for the analysis of cathinones in urine based on other methodologies.

Compounds studied	Matrices	Urine pretreatment	Analytical technique	LODs in urine[a]	Notes	Reference
NPS	Urine	Dilution in methanol (UHPLC-MS) or 2-propanol (UHPSFSC-MS)	UHPLC-MS/MS UHPSFSC-MS/MS	0.02-5.15 ng/mL (UHPLC-MS) 0.01-1.04 ng/mL (UHPSFSC-MS)	Comparative between a UHPLC-MS method and a UHPSFSC-MS method	[60]
Synthetic cathinones	Urine	LLE with ethyl acetate/2-propanol (4:1)	In-line SPE-CE-UV	3-8 ng/mL	Chiral separation by adding a cyclodextrin mixture to the BGE	[76]
Synthetic cathinones	Urine	Salting-out assisted LLE with NaCl and toluene	FASI-CE-UV	15-45 ng/mL	Chiral separation by adding a cyclodextrin mixture to the BGE	[77]
Synthetic cathinones	Urine	LLE with cyclohexane	EKS-CE-UV	4-8 ng/mL	Chiral separation by adding a cyclodextrin mixture to the BGE	[78]
Drugs of abuse	Urine	Deproteinization with HCl and a 100 mM sodium dodecyl sulphate micelles solution	MSS-CE-UV	75 ng/mL	On-line preconcentration employing a micellar solution	[79]
Synthetic cannabinoids Synthetic cathinones	Urine	Dilution in water:MeOH (50:50)	RapidFire-MS/MS	5 ng/mL	18,000 urine samples were analysed in 4 weeks	[80]
Mephedrone	Plasma Urine		MIP sensor	0.142 ng/mL		[81]

[a]**For the cathinones under study**

ent methodologies based on CE-UV for the enantiodetermination of cathinones in urine have recently been developed [76–78]. In these studies, the background electrolyte was a monosodium phosphate solution at pH 2.5 containing a mixture of cyclodextrins (2-hydroxypropyl β-cyclodextrin and β-cyclodextrin). However, as CE-UV cannot reach the low levels at which cathinones are usually found in urine samples, different preconcentration strategies combined in-line with the CE system have been evaluated, including chromatography-based preconcentration consisting of the in-line coupling of SPE with CE [76], which procedure is schematically represented in Fig. 4, or sample stacking strategies such as field-amplified sample injection (FASI) [77] or electrokinetic supercharging (EKS) [78]. The lowest LODs were achieved with in-line SPE-CE-UV [76], although similar values were achieved by the EKS-CE-UV-based strategy, which also had a shorter analysis time [78]. Another preconcentration approach for the determination of drugs of abuse, including mephedrone, in urine samples by CE-UV was developed by Aturki *et al.* [79], who used a micelle to solvent stacking approach to increase the method

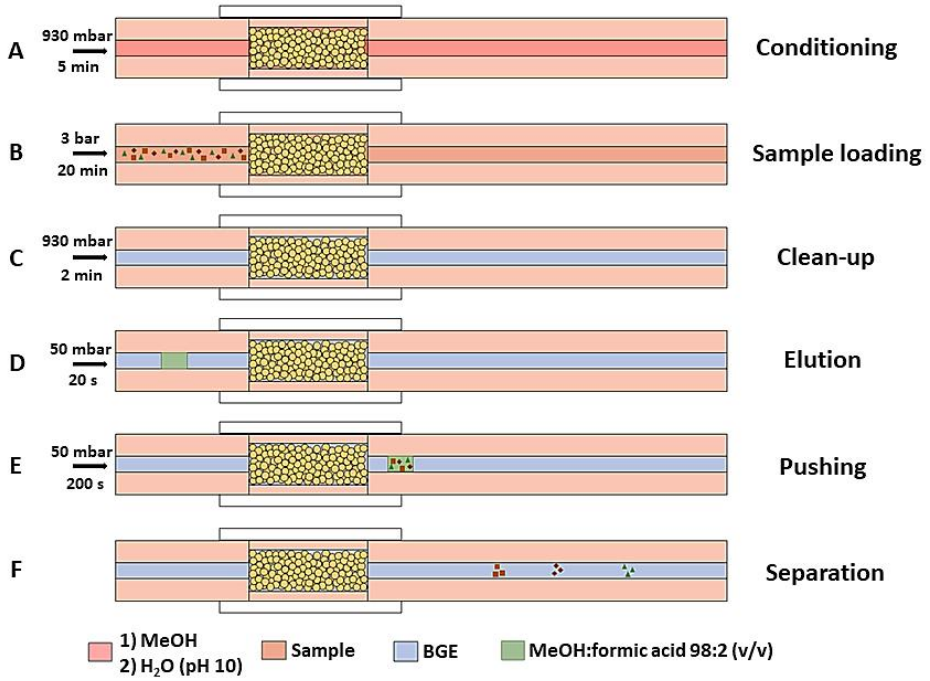

Figure 4. Scheme of an in-line SPE-CE procedure for the enantiodetermination of cathinones in urine samples. Reproduced with permission from Ref. [76].

sensitivity, obtaining a LOD of 75 ng/mL for mephedrone. However, unlike the other reported CE methodologies, the approach reported in this study did not include chiral separation.

An interesting screening approach was proposed by Neifeld *et al.* [80], who report a fast and selective screening method based on RapidFire-MS/MS for the detection of synthetic cathinones in urine. The RapidFire instrument (Agilent RapidFire 365) consists of an autosampler for ultrafast sample clean-up based on automated SPE and the subsequent introduction of the extract into a MS detector, allowing rapid screening of a large number of samples. The LOD obtained in the study (5.0 ng/mL) was higher than with a traditional LC-MS/MS but the analysis time was much lower. At optimum conditions, the analysis of synthetic cathinones in urine samples using RapidFire-MS/MS was completed in a total runtime of 9.1 seconds. This short analysis time enabled the authors to analyse 18,000 urine samples in four weeks, whereas in the case of LC-MS/MS, the screening took three months. The RapidFire-MS/MS approach is therefore a fast alternative to LC-MS for the screening of synthetic cathinones in urine. In another study, Razavipanah *et al.* [81] developed an electrochemical MIP sensor for the selective analysis of a single cathinone (mephedrone) in plasma and urine. The proposed electrode overcame some of the typical drawbacks of this type of sensor, such as long response times and difficult preparation. Furthermore, this sensor achieved a LOD of 0.142 ng/mL for the determination of mephedrone without sample pretreatment. In the future, novel combinations of electrochemical sensors and cathinones should be tested to expand the applicability of this strategy, which provides high selectivity and achieves high LOD without the need for pretreatment.

In the literature it is also possible to find strategies based on ultra-high-performance supercritical fluid chromatography (UHPSFC). This is the case of a study which compared two methodologies, one based on UHPLC and the other on UHPSFC, both coupled to a MS/MS system, for the determination of NPS in urine after simple dilution with methanol and 2-propanol, respectively [60]. Both methodologies allowed the studied compounds to be separated in less than three minutes. However, in UHPLC-MS/MS the LODs for cathinones ranged from 0.02 to 5.15

ng/mL, while in UHPSFC-MS/MS the values were slightly lower, from 0.01 to 1.04 ng/mL. Furthermore, UHPSFC gave lower RSD values, in terms of repeatability for both recovery and matrix effect. In fact, the average RSD values for recovery and matrix effects in UHPSFC were 4 and 6%, respectively, while in UHPLC a value of 12% was obtained in both cases. Therefore, UHPSFC is an interesting alternative to LC and to UHPLC for the determination of cathinones in urine.

7 Conclusions

The determination of cathinones in urine has been a matter of concern in recent years in fields such as forensic toxicology, clinical analysis or screening, stability, pharmacokinetics and metabolomics. This biological matrix presents a non-invasive sampling, is easy to collect and can be obtained in larger volumes than other biological matrices, so it is unsurprising that many procedures for the analysis of synthetic cathinones in urine samples have been developed. These new analytical procedures usually include a pretreatment step, which is commonly based on simple techniques like LLE or SPE, to remove or to reduce the presence of matrix interferences or to preconcentrate the studied compounds. Next, a high sensitive technique such as LC-HRMS, LC-MS/MS or GC-MS is used to reach the low usual levels at which cathinones can be present in urine samples (ng/mL). Since cathinones are non-volatile compounds, in GC-MS strategies is usually reported a sample derivatization step prior to the sample injection. On the other hand, due to the polar characteristics of cathinones and the aqueous nature of the urine matrix, the methodologies based on LC, especially in combination with a high sensitive detection system (as LC-HRMS or LC-MS/MS), represent a useful tool for the analysis of cathinones in urine. However, other approaches are also found in the literature, such as CE-based procedures applied particularly to the chiral separation of these compounds. Since new synthetic cathinones are continuously emerging, future studies should explore alternatives for the determination of these compounds and their metabolites in urine and other biological matrices.

Declaration of competing interest

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

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References

[1] United Nations Office on Drugs and Crime (UNODC), Early warning advisory of new psychoactive substances, (2019). https://www.unodc.org/LSS/Page/NPS (accessed July 5, 2020).

[2] A. Peacock, R. Bruno, N. Gisev, L. Degenhardt, W. Hall, R. Sedefov, J. White, K. V. Thomas, M. Farrell, P. Griffiths, New psychoactive substances: challenges for drug surveillance, control, and public health responses, Lancet. 394 (2019) 1668–1684. https://doi.org/10.1016/S0140-6736(19)32231-7.

[3] R. Rinaldi, G. Bersani, E. Marinelli, S. Zaami, The rise of new psychoactive substances and psychiatric implications: A wide-ranging, multifaceted challenge that needs far-reaching common legislative strategies, Hum. Psychopharmacol. 35 (2020). https://doi.org/10.1002/hup.2727.

[4] L. Orsolini, S. Chiappini, J.M. Corkery, A. Guirguis, D. Papanti, F. Schifano, The use of new psychoactive substances (NPS) in young people and their role in mental health care: a systematic review, Expert Rev. Neurother. 19 (2019) 1253–1264. https://doi.org/10.1080/14737175.2019.1666712.

[5] C. Miliano, G. Margiani, L. Fattore, M.A. De Luca, Sales and advertising channels of new psychoactive substances (NPS): Internet, social networks, and smartphone apps, Brain Sci. 8 (2018). https://doi.org/10.3390/brainsci8070123.

[6] R.A.S. Couto, L.M. Gonçalves, F. Carvalho, J.A. Rodrigues, C.M.P. Rodrigues, M.B. Quinaz, The analytical challenge in the determination of cathinones, key-players in the worldwide phenomenon of Novel Psychoactive Substances, Crit. Rev. Anal. Chem. 48 (2018) 372–390. https://doi.org/10.1080/10408347.2018.1439724.

[7] J.L. Gonçalves, V.L. Alves, J. Aguiar, H.M. Teixeira, J.S. Câmara, Synthetic cathinones: an evolving class of new psychoactive substances, Crit. Rev. Toxicol. 49 (2019) 549–566. https://doi.org/10.1080/10408444.2019.1679087.

[8] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), European drug report 2019, 2019. https://www.emcdda.europa.eu/publications/edr/trends-developments/2019 (accessed July 5, 2020).

[9] J.M. Prosser, L.S. Nelson, The toxicology of bath salts: A review of synthetic cathinones, J. Med. Toxicol. 8 (2012) 33–42. https://doi.org/10.1007/s13181-011-0193-z.

[10] A.L. Riley, K.H. Nelson, P. To, R. López-Arnau, P. Xu, D. Wang, Y. Wang, H. wei Shen, D.M. Kuhn, M. Angoa-Perez, J.H. Anneken, D. Muskiewicz, F.S. Hall, Abuse potential and toxicity of the synthetic cathinones (i.e., "Bath salts"), Neurosci. Biobehav. Rev. 110 (2020) 150–173. https://doi.org/10.1016/j.neubiorev.2018.07.015.

[11] C.L. German, A.E. Fleckenstein, G.R. Hanson, Bath salts and synthetic cathinones: An emerging designer drug phenomenon, Life Sci. 97 (2014) 2–8. https://doi.org/10.1016/j.lfs.2013.07.023.

[12] E. Pieprzyca, R. Skowronek, Ľ. Nižnanský, P. Czekaj, Synthetic cathinones – From natural plant

stimulant to new drug of abuse, Eur. J. Pharmacol. 875 (2020). https://doi.org/10.1016/j.ejphar.2020.173012.

[13] R. Kolanos, J.S. Partilla, M.H. Baumann, B.A. Hutsell, M.L. Banks, S.S. Negus, R.A. Glennon, Stereoselective actions of methylenedioxypyrovalerone (MDPV) to inhibit dopamine and norepinephrine transporters and facilitate intracranial self-stimulation in rats, ACS Chem. Neurosci. 6 (2015) 771–777. https://doi.org/10.1021/acschemneuro.5b00006.

[14] K.H. Nelson, R. López-Arnau, B.J. Hempel, P. To, H.N. Manke, M.E. Crissman, M.M. Clasen, K.C. Rice, A.L. Riley, Stereoselective effects of the second-generation synthetic cathinone α-pyrrolidinopentiophenone (α-PVP): assessments of conditioned taste avoidance in rats, Psychopharmacology (Berl). 236 (2019) 1067–1077. https://doi.org/10.1007/s00213-018-5070-x.

[15] K.W. Cheng, C.M. Hsieh, H.W. Chen, P.C. Chi, D.P. Yang, S.H. Chan, J.Y. Chen, H.L. Hwa, C.C. Fang, T.I. Weng, P.S. Chen, Determination of synthetic cathinone α-pyrrolidinovalero-phenone and its metabolite in urine using solid-phase extraction and gas chromatography–mass spectrometry, Rapid Commun. Mass Spectrom. 34 (2020) 1–6. https://doi.org/10.1002/rcm.8579.

[16] J. Klavž, M. Gorenjak, M. Marinšek, Suicide attempt with a mix of synthetic cannabinoids and synthetic cathinones: Case report of non-fatal intoxication with AB-CHMINACA, AB-FUBINACA, alpha-PHP, alpha-PVP and 4-CMC, Forensic Sci. Int. 265 (2016) 121–124. https://doi.org/10.1016/j.forsciint.2016.01.018.

[17] M. Grapp, C. Kaufmann, M. Ebbecke, Toxicological investigation of forensic cases related to the designer drug 3,4-methylenedioxypyrovalerone (MDPV): Detection, quantification and studies on human metabolism by GC-MS, Forensic Sci. Int. 273 (2017) 1–9. https://doi.org/10.1016/j.forsciint.2017.01.021.

[18] M. Grapp, C. Sauer, C. Vidal, D. Müller, GC-MS analysis of the designer drug α-pyrrolidinovalerophenone and its metabolites in urine and blood in an acute poisoning case, Forensic Sci. Int. 259 (2016) e14–e19. https://doi.org/10.1016/j.forsciint.2015.12.020.

[19] E. Olesti, M. Pujadas, E. Papaseit, C. Pérez-Mañá, Ó.J. Pozo, M. Farré, R. de la Torre, GC–MS Quantification Method for Mephedrone in Plasma and Urine: Application to Human Pharmacokinetics, J. Anal. Toxicol. (2017) 100–106. https://doi.org/10.1093/jat/bkw120.

[20] R.H. Alremeithi, M.A. Meetani, S.A. Khalil, A validated gas chromatography mass spectrometry method for simultaneous determination of cathinone related drug enantiomers in urine and plasma, RSC Adv. 6 (2016) 80576–80584. https://doi.org/10.1039/C6RA10583A.

[21] R.H. Alremeithi, M.A. Meetani, A.A. Alaidaros, A. Lanjawi, K. Alsumaiti, Simultaneous quantitative determination of synthetic cathinone enantiomers in urine and plasma using GC-NCI-MS, J. Anal. Methods Chem. (2018) 1–11. https://doi.org/10.1155/2018/4396043.

[22] L.A. Nisbet, F.M. Wylie, B.K. Logan, K.S. Scott, Gas chromatography-mass spectrometry method for the quantitative identification of 23 new psychoactive substances in blood and urine, J. Anal. Toxicol. 43 (2019) 346–352. https://doi.org/10.1093/jat/bky109.

[23] G. Mercieca, S. Odoardi, M. Cassar, S. Strano-Rossi, Rapid and simple procedure for the determination of cathinones, amphetamine-like stimulants and other new psychoactive substances in blood and urine by GC–MS, J. Pharm. Biomed. Anal. 149 (2018) 494–501. https://doi.org/10.1016/j.jpba.2017.1

1.024.

[24] P. Ondra, I. Válka, R. Knob, P. Ginterová, V. Maier, Analysis of amphetamine-derived designer drugs by gas chromatography with mass spectrometry, J. Anal. Toxicol. 40 (2016) 78–85. https://doi.org/10.1093/jat/bkv113.

[25] K.A. Alsenedi, C. Morrison, Determination of amphetamine-type stimulants (ATSs) and synthetic cathinones in urine using solid phase micro-extraction fibre tips and gas, Anal. Methods. 10 (2018) 1431–1440. https://doi.org/10.1039/c8ay00041g.

[26] B. Molnár, B. Fodor, A. Csámpai, E. Hidvégi, I. Molnár-Perl, Structure-related related new approach in the gas chromatography/mass spectrometry analysis of cathinone type synthetic drugs, J. Chromatogr. A. 1477 (2016) 70–75. https://doi.org/10.1016/j.chroma.2016.11.033.

[27] K.S. Hagan, L. Reidy, Detection of synthetic cathinones in victims of sexual assault, Forensic Sci. Int. 257 (2015) 71–75. https://doi.org/10.1016/j.forsciint.2015.07.040.

[28] H.L. Ciallella, L.R. Rutter, L.A. Nisbet, K.S. Scott, Extended stability evaluation of selected cathinones, Front. Chem. 8 (2020) 1–11. https://doi.org/10.3389/fchem.2020.597726.

[29] H. Ketha, M. Webb, L. Clayton, S. Li, Gas Chromatography Mass Spectrometry (GC-MS) for Identification of Designer Stimulants Including 2C Amines, NBOMe Compounds, and Cathinones in Urine, Curr. Protoc. Toxicol. 74 (2017) 4.43.1-4.43.10. https://doi.org/10.1002/cptx.33.

[30] E. Gerace, D. Caneparo, F. Borio, A. Salomone, M. Vincenti, Determination of several synthetic cathinones and an amphetamine-like compound in urine by gas chromatography with mass spectrometry. Method validation and application to real cases, J. Sep. Sci. 42 (2019) 1577–1584. https://doi.org/10.1002/jssc.2018012

49.

[31] W.Y. Hong, Y.C. Ko, M.C. Lin, P.Y. Wang, Y.P. Chen, L.C. Chiueh, D.Y.C. Shih, H.K. Chou, H.F. Cheng, Determination of synthetic cathinones in urine using gas chromatography-mass spectrometry techniques, J. Anal. Toxicol. 40 (2016) 12–16. https://doi.org/10.1093/jat/bkv108.

[32] K.A. Alsenedi, C. Morrison, Determination and long-term stability of twenty-nine cathinones and amphetamine-type stimulants (ATS) in urine using gas chromatography–mass spectrometry, J. Chromatogr. B. 1076 (2018) 91–102. https://doi.org/10.1016/j.jchromb.2018.01.027.

[33] A.G. Helfer, A. Turcant, D. Boels, S. Ferec, B. Lelièvre, J. Welter, M.R. Meyer, H.H. Maurer, Elucidation of the metabolites of the novel psychoactive substance 4-methyl-N-ethyl-cathinone (4-MEC) in human urine and pooled liver microsomes by GC-MS and LC-HR-MS/MS techniques and of its detectability by GC-MS or LC-MSn standard screening approaches, Drug Test. Anal. 7 (2015) 368–375. https://doi.org/10.1002/dta.1682.

[34] S. Matsuta, N. Shima, H. Kamata, H. Kakehashi, S. Nakano, K. Sasaki, T. Kamata, H. Nishioka, A. Miki, M. Katagi, K. Zaitsu, T. Sato, H. Tsuchihashi, K. Suzuki, Metabolism of the designer drug α-pyrrolidinobutiophenone (α-PBP) in humans: Identification and quantification of the phase I metabolites in urine, Forensic Sci. Int. 249 (2015) 181–188. https://doi.org/10.1016/j.forsciint.2015.02.004.

[35] L. Institóris, Z. Árok, K. Seprenyi, T. Varga, G. Sára-Klausz, É. Keller, R.A. Tóth, L. Sala, É. Kereszty, K. Róna, Frequency and structure of stimulant designer drug consumption among suspected drug users in Budapest and South-East Hungary in 2012-2013, Forensic Sci. Int. 248 (2015) 181–186. https://doi.org/10.1016/j.forsciint.2015.01.002.

[36] L. Ambach, A. Hernández Redondo, S.

König, V. Angerer, S. Schürch, W. Weinmann, Detection and quantification of 56 new psychoactive substances in whole blood and urine by LC-MS/MS, Bioanalysis. 7 (2015) 1119–1136. https://doi.org/10.4155/bio.15.48.

[37] J. Kinyua, N. Negreira, B. Miserez, A. Causanilles, E. Emke, L. Gremeaux, P. de Voogt, J. Ramsey, A. Covaci, A.L.N. van Nuijs, Qualitative screening of new psychoactive substances in pooled urine samples from Belgium and United Kingdom, Sci. Total Environ. 573 (2016) 1527–1535. https://doi.org/10.1016/j.scitotenv.2016.08.124.

[38] T. Murakami, Y. Iwamuro, R. Ishimaru, S. Chinaka, H. Hasegawa, Moleculary imprinted polymer solid-phase extraction of synthetic cathinones from urine and whole blood samples, J. Sep. Sci. 41 (2018) 4506–4514. https://doi.org/10.1002/jssc.201800874.

[39] S. Pascual-Caro, N. Fontanals, F. Borrull, C. Aguilar, M. Calull, Solid-phase extraction based on cation-exchange sorbents followed by liquid chromatography high-resolution mass spectrometry to determine synthetic cathinones in urine, Forensic Toxicol. 38 (2020) 185–194. https://doi.org/10.1007/s11419-019-00508-8.

[40] J.R.H. Archer, F. Mendes, S. Hudson, K. Layne, P.I. Dargan, D.M. Wood, Evaluation of long-term detection trends of new psychoactive substances in pooled urine from city street portable urinals (London, UK), Br. J. Clin. Pharmacol. 86 (2020) 517–527. https://doi.org/10.1111/bcp.14239.

[41] M. Concheiro, M. Castaneto, R. Kronstrand, M.A. Huestis, Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography-high resolution mass spectrometry and library matching, J. Chromatogr. A. 1397 (2015) 32–42. https://doi.org/10.1016/j.chroma.2015.04.002.

[42] M.J. Swortwood, K.N. Ellefsen, A. Wohlfarth, X. Diao, M. Concheiro-Guisan, R. Kronstrand, M.A. Huestis, First metabolic profile of PV8, a novel synthetic cathinone, in human hepatocytes and urine by high-resolution mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 4845–4856. https://doi.org/10.1007/s00216-016-9599-4.

[43] M.J. Swortwood, J. Carlier, K.N. Ellefsen, A. Wohlfarth, X. Diao, M. Concheiro-Guisan, R. Kronstrand, M.A. Huestis, In vitro, in vivo and in silico metabolic profiling of α-pyrrolidinopentiothiophenone, a novel thiophene stimulant, Bioanalysis. 8 (2016) 65–82. https://doi.org/10.4155/bio.15.237.

[44] J. Sánchez-González, S. Odoardi, A.M. Bermejo, P. Bermejo-Barrera, F.S. Romolo, A. Moreda-Piñeiro, S. Strano-Rossi, HPLC-MS/MS combined with membrane-protected molecularly imprinted polymer micro-solid-phase extraction for synthetic cathinones monitoring in urine, Drug Test. Anal. 11 (2019) 33–44. https://doi.org/10.1002/dta.2448.

[45] P. Kavanagh, M. Gofenberg, V. Shevyrin, O. Dvorskaya, G. Dowling, A. Grigoryev, Tentative identification of the phase I and II metabolites of two synthetic cathinones, MDPHP and α-PBP, in human urine, Drug Test. Anal. (2020) 1–10. https://doi.org/10.1002/dta.2891.

[46] L. Glicksberg, S. Kerrigan, Stability of synthetic cathinones in Urine, J. Anal. Toxicol. 42 (2018) 77–87. https://doi.org/10.1093/jat/bkx091.

[47] E. Olesti, M. Farré, E. Papaseit, A. Krotonoulas, M. Pujadas, R. de la Torre, Ó.J. Pozo, Pharmacokinetics of Mephedrone and Its Metabolites in Human by LC-MS/MS, AAPS J. 19 (2017) 1767–1778. https://doi.org/10.1208/s12248-017-0132-2.

[48] P. Adamowicz, A. Malczyk, Stability of synthetic cathinones in blood and urine, Forensic Sci. Int. 295 (2019) 36–45. https://doi.org/10.1016/j.forsciint.201

8.12.001.

[49] P. Adamowicz, A. Jurczyk, D. Gil, S. Szustowski, A case of intoxication with a new cathinone derivative α-PiHP – A presentation of concentrations in biological specimens, Leg. Med. 42 (2020) 101626. https://doi.org/10.1016/j.legalmed.2019.101626.

[50] B. Potocka-Banaś, T. Janus, S. Majdanik, T. Banaś, T. Dembińska, K. Borowiak, Fatal Intoxication with α-PVP, a Synthetic Cathinone Derivative, J. Forensic Sci. 62 (2017) 553–556. https://doi.org/10.1111/1556-4029.13326.

[51] C. Vignali, M. Moretti, A. Groppi, A.M.M. Osculati, L. Tajana, L. Morini, Distribution of the synthetic cathinone α-pyrrolidinohexiophenone in biological specimens, J. Anal. Toxicol. 43 (2019) E1–E6. https://doi.org/10.1093/jat/bky047.

[52] K. Shintani-Ishida, M. Nakamura, M. Tojo, N. Idota, H. Ikegaya, Identification and quantification of 4'-methoxy-α-pyrrolidinobutiophenone (4-MeOPBP) in human plasma and urine using LC–TOF-MS in an autopsy case, Forensic Toxicol. 33 (2015) 348–354. https://doi.org/10.1007/s11419-015-0281-x.

[53] K. Shintani-Ishida, Y. Kakiuchi, H. Ikegaya, Successful quantification of 4'-methyl-α-pyrrolidinohexanophenone (MPHP) in human urine using LC–TOF-MS in an autopsy case, Forensic Toxicol. 34 (2016) 398–402. https://doi.org/10.1007/s11419-016-0307-z.

[54] M. Zawadzki, K. Nowak, P. Szpot, Fatal intoxication with N-ethylpentylone: a case report and method for determining N-ethylpentylone in biological material, Forensic Toxicol. 38 (2020) 255–263. https://doi.org/10.1007/s11419-019-00483-0.

[55] L. Mercolini, M. Protti, M.C. Catapano, J. Rudge, A.E. Sberna, LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples, J. Pharm. Biomed. Anal. 123 (2016) 186–194. https://doi.org/10.1016/j.jpba.2016.02.015.

[56] M. Fagiola, T. Hahn, J. Avella, Screening of Novel Psychoactive Substances in Postmortem Matrices by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS), J. Anal. Toxicol. 42 (2018) 562–569. https://doi.org/10.1093/jat/bky050.

[57] L. Glicksberg, S. Rana, S. Kerrigan, Cathinone stability in authentic urine specimens, Forensic Sci. Int. 286 (2018) 54–60. https://doi.org/10.1016/j.forsciint.2018.02.016.

[58] E. Olesti, J.A. Pascual, M. Ventura, E. Papaseit, M. Farré, R. de la Torre, O.J. Pozo, LC-MS/MS method for the quantification of new psychoactive substances and evaluation of their urinary detection in humans for doping control analysis, Drug Test. Anal. (2020) 785–797. https://doi.org/10.1002/dta.2768.

[59] O.L. Zaikina, V. V. Shilov, A.N. Lodyagin, S.I. Glushkov, A.M. Grigoryev, Determination of the structures of free and glucuronidated metabolites of α-pyrrolidinovalerophenone in human urine by liquid Chromatography–Mass spectrometry with accurate mass measurement, J. Anal. Chem. 74 (2019) 489–504. https://doi.org/10.1134/S1061934819020138.

[60] L. Borovcová, V. Pauk, K. Lemr, Analysis of new psychoactive substances in human urine by ultra-high performance supercritical fluid and liquid chromatography: Validation and comparison, J. Sep. Sci. 41 (2018) 2288–2295. https://doi.org/10.1002/jssc.201800006.

[61] P. Heikman, M. Sundström, A. Pelander, I. Ojanperä, New psychoactive substances as part of polydrug abuse within opioid maintenance treatment revealed by comprehensive

high-resolution mass spectrometric urine drug screening, Hum. Psychopharmacol. 31 (2016) 44–52. https://doi.org/10.1002/hup.2512.

[62] A. Ishii, K. Kusakabe, N. Kato, S. ichi Sasaki, K. Tsujikawa, T. Wada, Studies on the phase I metabolites of the new designer drug 1-(2,3-dihydro-1H-inden-5-yl)-2-(pyrrolidine-1-yl)butan-1-one (5-PPDI) in human urine, Forensic Sci. Int. 310 (2020) 110214. https://doi.org/10.1016/j.forsciint.2020.110214.

[63] H.H. Lee, J.F. Lee, S.Y. Lin, B.H. Chen, Simultaneous identification of abused drugs, benzodiazepines, and new psychoactive substances in urine by liquid chromatography tandem mass spectrometry, Kaohsiung J. Med. Sci. 32 (2016) 118–127. https://doi.org/10.1016/j.kjms.2016.01.007.

[64] H.H. Lee, S.C. Chen, J.F. Lee, H.Y. Lin, B.H. Chen, Simultaneous drug identification in urine of sexual assault victims by using liquid chromatography tandem mass spectrometry, Forensic Sci. Int. 282 (2018) 35–40. https://doi.org/10.1016/j.forsciint.2017.11.006.

[65] S.Y. Fan, C.Z. Zang, P.H. Shih, Y.C. Ko, Y.H. Hsu, M.C. Lin, S.H. Tseng, D.Y. Wang, A LC-MS/MS method for determination of 73 synthetic cathinones and related metabolites in urine, Forensic Sci. Int. 315 (2020) 110429. https://doi.org/10.1016/j.forsciint.2020.110429.

[66] A. Di Trana, G. Mannocchi, F. Pirani, N. La Maida, M. Gottardi, S. Pichini, F.P. Busardò, A comprehensive HPLC–MS-MS screening method for 77 new psychoactive substances, 24 classic drugs and 18 related metabolites in blood, urine and oral fluid, J. Anal. Toxicol. 44 (2020) 769–783. https://doi.org/10.1093/jat/bkaa103.

[67] A.N. Kimble, A.P. DeCaprio, Systematic analysis of novel psychoactive substances. II. Development of a screening/confirmatory LC-QqQ-MS/MS method for 800+ compounds and metabolites in urine, Forensic Chem. 16 (2019) 100189. https://doi.org/10.1016/j.forc.2019.100189.

[68] L. Glicksberg, K. Bryand, S. Kerrigan, Identification and quantification of synthetic cathinones in blood and urine using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry, J. Chromatogr. B. 1035 (2016) 91–103. https://doi.org/10.1016/j.jchromb.2016.09.027.

[69] A.A. Azaryan, A.Z. Temerdashev, E. V Svetlichnaya, A.G. Kal, I.A. Balabaev, Determination of some cathinones, tropane alkaloids, and "pharmaceutical narcotics" in urine, J. Anal. Chem. 71 (2016) 995–1004. https://doi.org/10.1134/S1061934816090021.

[70] J.R.H. Archer, S. Hudson, O. Jackson, T. Yamamoto, C. Lovett, H.M. Lee, S. Rao, L. Hunter, P.I. Dargan, D.M. Wood, Analysis of anonymized pooled urine in nine UK cities: Variation in classical recreational drug, novel psychoactive substance and anabolic steroid use, Qjm. 108 (2015) 929–933. https://doi.org/10.1093/qjmed/hcv058.

[71] T.G. Rosano, P.Y. Ohouo, M. Wood, Screening with quantification for 64 drugs and metabolites in human urine using UPLC-MS-MS analysis and a threshold accurate calibration, J. Anal. Toxicol. 41 (2017) 536–546. https://doi.org/10.1093/jat/bkx035.

[72] L. Bijlsma, A. Celma, S. Castiglioni, N. Salgueiro-González, L. Bou-Iserte, J.A. Baz-Lomba, M.J. Reid, M.J. Dias, A. Lopes, J. Matias, L. Pastor-Alcañiz, J. Radonić, M. Turk Sekulic, T. Shine, A.L.N. van Nuijs, F. Hernandez, E. Zuccato, Monitoring psychoactive substance use at six European festivals through wastewater and pooled urine analysis, Sci. Total Environ. 725 (2020) 138376. https://doi.org/10.1016/j.scitotenv.2020.138376.

[73] E.C. Strickland, O.T. Cummings, A.L. Mellinger, G.L. McIntire, Development

and validation of a novel all-inclusive LC-MS-MS designer drug method, J. Anal. Toxicol. 43 (2019) 161–169. https://doi.org/10.1093/jat/bky087.

[74] M.A. Meetani, R.H. Alremeithi, M.K. Mousa, Enantioseparation of synthetic cathinones enantiomers with tertiary amine structure in urine and plasma, J. Chromatogr. Sci. 57 (2019) 361–368. https://doi.org/10.1093/chromsci/bmz008.

[75] F.A. Esteve-Turrillas, S. Armenta, M. de la Guardia, Sample preparation strategies for the determination of psychoactive substances in biological fluids, J. Chromatogr. A. 1633 (2020) 461615. https://doi.org/10.1016/j.chroma.2020.461615.

[76] A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE, Electrophoresis. 40 (2019) 1762–1770. https://doi.org/10.1002/elps.201900072.

[77] A. Pérez-Alcaraz, F. Borrull, M. Calull, C. Aguilar, Field-amplified sample injection combined with CE for the enantiodetermination of cathinones in urine samples, J. Sep. Sci. 43 (2020) 2914–2924. https://doi.org/https://doi.org/10.1002/jssc.201901067.

[78] A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis, Microchem. J. 158 (2020) 105300. https://doi.org/10.1016/j.microc.2020.105300.

[79] Z. Aturki, S. Fanali, A. Rocco, Online sample concentration and analysis of drugs of abuse in human urine by micelle to solvent stacking in capillary zone electrophoresis, Electrophoresis. 37 (2016) 2875–2881. https://doi.org/10.1002/elps.201600312.

[80] J.R. Neifeld, L.E. Regester, J.M. Holler, S.P. Vorce, J. Magluilo, G. Ramos, T.Z. Bosy, Ultrafast screening of synthetic cannabinoids and synthetic cathinones in urine by rapidfire-tandem mass spectrometry, J. Anal. Toxicol. 40 (2016) 379–387. https://doi.org/10.1093/jat/bkw025.

[81] I. Razavipanah, E. Alipour, B. Deiminiat, G.H. Rounaghi, A novel electrochemical imprinted sensor for ultrasensitive detection of the new psychoactive substance "Mephedrone," Biosens. Bioelectron. 119 (2018) 163–169. https://doi.org/10.1016/j.bios.2018.08.016.

[82] A.A. Aldubayyan, E. Castrignanò, S. Elliott, V. Abbate, Stability of synthetic cathinones in clinical and forensic toxicological analysis—Where are we now?, Drug Test. Anal. 13 (2021) 44–68. https://doi.org/10.1002/dta.2990.

[83] I.K. Zhurkovich, A.O. Rudenko, V. V. Chelovechkova, I.A. Merkusheva, N. V. Lugovkina, N.G. Kovrov, M. V. Pchelintsev, E. V. Verbitskaya, Zvartau, Determination of Buprenorphine and Naloxone in Patient Blood Plasma Using HPLC-MS, Pharm. Chem. J. 48 (2015) 690–695. https://doi.org/10.1007/s11094-015-1161-8.

[84] S. Zazoğlu, B. Anilanmert, M. Aydin, S. Cengiz, Fast confirmation for marijuana metabolite: THC-COOH, ultra-fast LC-MS/MS run time, and application to routine samples, Acta Chromatogr. 29 (2017) 253–265. https://doi.org/10.1556/1326.2017.29.2.09.

[85] C. Mazoyer, J. Carlier, A. Boucher, M. Péoc'h, C. Lemeur, Y. Gaillard, Fatal case of a 27-year-old male after taking iboga in withdrawal treatment: GC-MS/MS determination of ibogaine and ibogamine in iboga roots and postmortem biological material, J. Forensic Sci. 58 (2013) 1666–1672. https://doi.org/10.1111/1556-4029.12250.

[86] S.G. Dmitrienko, V. V. Apyari, V. V. Tolmacheva, M. V. Gorbunova, Dispersive liquid-liquid microextrac-

tion of organic compounds: An overview of reviews, J. Anal. Chem. 75 (2020) 1237–1251. https://doi.org/10.1134/S1061934820100056.

[87] J.M. Płotka, M. Biziuk, C. Morrison, Common methods for the chiral determination of amphetamine and related compounds I. Gas, liquid and thin-layer chromatography, TrAC - Trends Anal. Chem. 30 (2011) 1139–1158. https://doi.org/10.1016/j.trac.2011.03.013.

[88] R.B. Yu, J.P. Quirino, Chiral liquid chromatography and capillary electrochromatography: Trends from 2017 to 2018, TrAC - Trends Anal. Chem. 118 (2019) 779–792. https://doi.org/10.1016/j.trac.2019.07.011.

[89] M.G. Schmid, J.S. Hägele, Separation of enantiomers and positional isomers of novel psychoactive substances in solid samples by chromatographic and electrophoretic techniques - A selective review, J. Chromatogr. A. 1624 (2020) 461256. https://doi.org/10.1016/j.chroma.2020.461256.

1.3. Electroforesis capilar

La electroforesis es una técnica analítica de separación basada en las diferentes velocidades de migración de especies cargadas sometidas a un campo eléctrico. Esta técnica empezó a ganar importancia en los años treinta debido a los estudios llevados a cabo por el bioquímico Arne Tiselius para la separación de proteínas séricas. Posteriormente, a finales del siglo XX, esta técnica también tuvo un papel muy destacado en la secuenciación del ADN [27,89]. A pesar de que, como demuestra su uso a lo largo de la historia, la electroforesis ordinaria puede ser de gran utilidad, para la obtención de resultados cuantitativos de mayor precisión y también con el objetivo de facilitar la automatización de las metodologías empleadas, la mayor parte de los procedimientos electroforéticos de la actualidad se llevan a cabo en tubos capilares de sílice fundida, aplicación que es comúnmente conocida como electroforesis capilar o CE por sus siglas en inglés [27]. La Fig. 8 muestra un esquema de un sistema de CE. Como se puede observar, el capilar está situado entre dos viales (vial del *inlet* y vial del *outlet*) llenos de electrolito de separación o BGE. Asimismo, cada vial está conectado a un electrodo a través de los cuales se aplica el voltaje de separación.

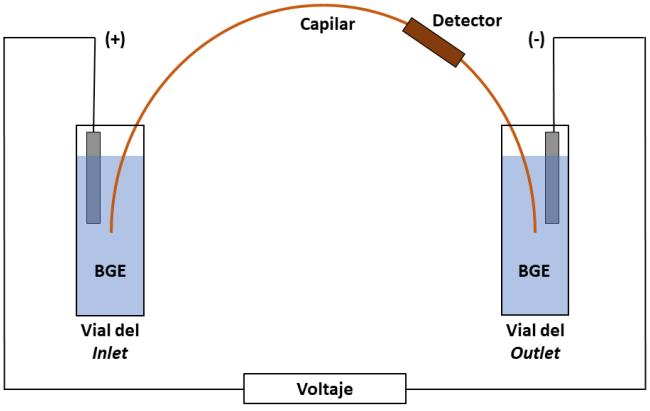

Figura 8. Esquema de un sistema de CE.

En CE la velocidad de migración de una especie cargada a través del capilar de separación (v) es directamente proporcional a su movilidad electroforética (μ_e) y a la fuerza del campo eléctrico aplicado entre los dos electrodos (E), es decir:

$$v = \mu_e E \qquad \text{(Ecuación 1)}$$

Al mismo tiempo la movilidad electroforética es directamente proporcional a la carga del compuesto analizado e inversamente proporcional a su tamaño. Esto

implica que para que dos especies se separen entre sí deben diferir en su carga o en el tamaño del ion. Cuando los compuestos analizados se separan exclusivamente debido a las diferencias entre sus movilidades electroforéticas nos encontramos ante la modalidad más simple de CE, la electroforesis capilar por zonas (*capillary zone electrophoresis* - CZE). Sin embargo, no es posible separar especies neutras mediante esta técnica analítica. En este sentido, cuando el objetivo es separar compuestos neutros por CE, generalmente se opta por aproximaciones basadas en el uso de micelas, concretamente por la técnica denominada cromatografía capilar micelar electrocinética (*micellar electrokinetic chromatography* - MEKC), a través de la cual estos compuestos neutros se separan debido a su reparto diferencial entre las micelas y el BGE [19,90].

El BGE, una solución salina amortiguadora, es de gran importancia en CE puesto que no solo afecta a la carga de los analitos, sino que también influye sobre una característica genuina de esta técnica, el flujo electroosmótico (*electroosmotic flow* - EOF). A valores de pH superiores a 3, los grupos silanol (Si-OH) de la superficie interna del capilar de sílice se encuentran desprotonados. La carga negativa de estos grupos silanol atrae a los cationes presentes en el BGE, lo que provoca que éstos se agrupen en la superficie del capilar. Mientras que los cationes más cercanos a los grupos silanoles quedan inmovilizados, el resto se mantiene móvil, generando una doble capa eléctrica adyacente a la superficie del capilar tal y como muestra la Fig. 9. Al aplicar una diferencia de potencial, los cationes móviles, situados en la parte exterior de la doble capa, son atraídos hacia el cátodo y puesto que estos cationes se encuentran solvatados, el solvente es arrastrado conjuntamente con éstos originando así el EOF [27].

Una vez los analitos son introducidos en el capilar de separación, concretamente en el extremo de entrada o *inlet*, éstos migran a lo largo del capilar en dirección al detector, situado cercano al *outlet,* obteniendo finalmente el electroferograma correspondiente. Dentro del capilar, la movilidad aparente o resultante de los analitos dependerá tanto de su movilidad electroforética como de la movilidad electroosmótica, siendo esta última una constante para todos los compuestos presentes en la muestra a unas condiciones determinadas de BGE. Así pues, el flujo electroosmótico acaba actuando de forma similar al mecanismo de bombeo de la fase móvil en LC. Como consecuencia de estas diferentes movilidades los analitos logran separarse. Como se muestra en la Fig. 9 el orden de elución en una separación

electroforética, en la que se aplica un voltaje positivo, es: en primer lugar, los cationes con una mayor carga seguidos por aquellos cationes con menor carga. Cuando la carga es idéntica, los cationes que presentan una mayor movilidad son aquellos de menor tamaño. Seguidamente aparecen las sustancias neutras, pero todas juntas ya que solo se desplazan debido al EOF. Finalmente, aparecen los aniones con menor carga seguidos por los aniones que presentan una mayor carga. Cuando la carga es idéntica, los aniones que presentan una mayor movilidad son aquellos de mayor tamaño.

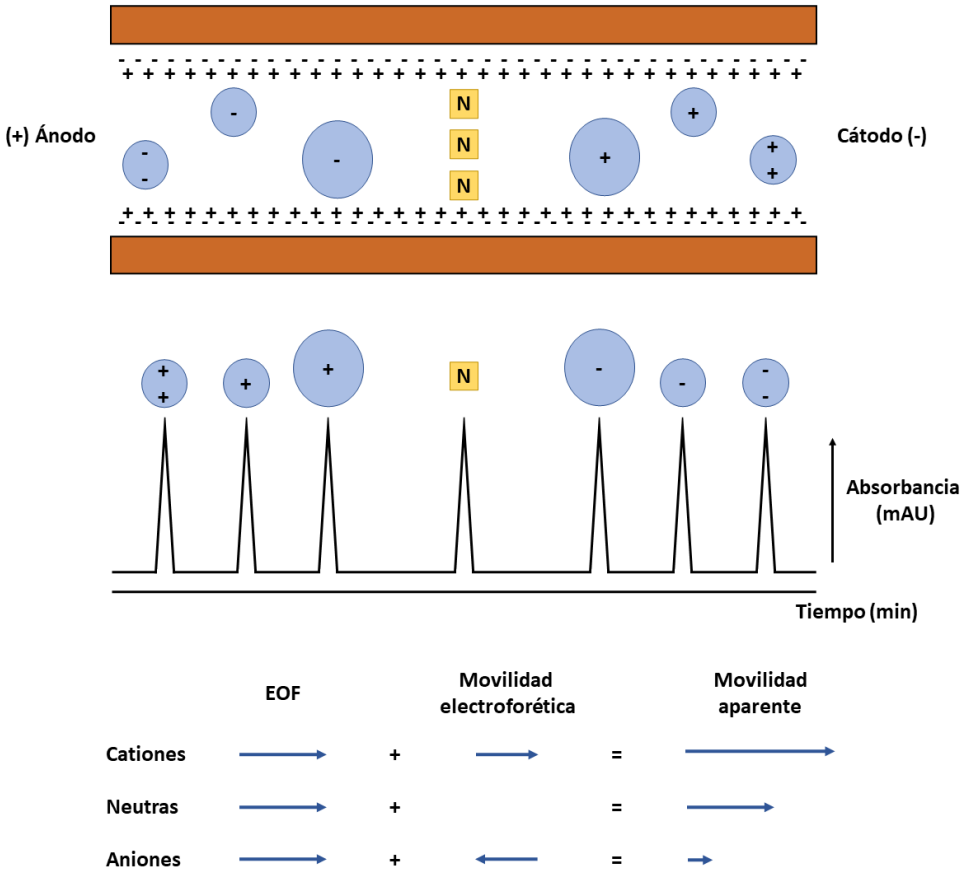

Figura 9. Esquema de una separación electroforética donde se aplica un voltaje positivo y el electroferograma resultante.

Entre las principales ventajas de la CE se encuentra su elevada capacidad de separación. Además el pequeño diámetro del capilar de separación permite inyectar

pequeños volúmenes tanto de reactivos como de muestra, con lo que se reduce el consumo de ambos, convirtiendo a esta técnica en ideal para el análisis de muestras de las cuales se dispone de muy poca cantidad [27]. Otra de las ventajas a destacar de esta técnica analítica es que permite la consecución de manera simple de una separación enantiomérica, debido a que ésta se puede lograr sencillamente adicionado un selector quiral en el BGE [23]. No obstante, la CE también presenta desventajas, de las cuales, destaca principalmente su intrínseca baja sensibilidad, especialmente cuando se utiliza junto a un detector UV, en comparación con otras técnicas analíticas como la GC o la LC. Por esta razón, es habitual encontrar estudios donde se intenta revertir esta baja de sensibilidad, siendo una de las opciones preferidas el uso de técnicas de preconcentración en línea en las que los analitos se preconcentran dentro del propio capilar previamente a su separación [28–36]. Tanto la separación quiral como las estrategias que tienen como fin mejorar la sensibilidad en CE son una parte fundamental del trabajo experimental realizado a lo largo de esta Tesis Doctoral y, por lo tanto, se comentarán en mayor profundidad en los siguientes apartados.

Separación de catinonas mediante CE

En los últimos años diversos autores han empleado la CE para diferentes aplicaciones relacionadas con la separación de catinonas. Generalmente, en estos procedimientos la separación electroforética se basa en la técnica de CZE [70,82,91–103], aunque en la bibliografía también encontramos algunos autores que han empleado estrategias basadas en MEKC para separar catinonas [69,104]. A modo de ejemplo, Švidrnoch y colaboradores emplearon una estrategia basada en MEKC-MS/MS para la determinación de un grupo de catinonas en orina [69]. En este tipo de estrategia la selección de un tensioactivo adecuado es un punto crítico puesto que éste podría entrar dentro del sistema de MS y, por tanto, contaminar el detector. Para evitar esta problemática los autores se decantaron por el uso de un tensioactivo volátil que fuese capaz de formar micelas también volátiles, en concreto, el ácido perfluorooctanoico. Con esta metodología se logró una separación satisfactoria para los compuestos estudiados, además se obtuvieron límites de detección *(limits of detection* - LODs) de entre 10 y 78 ng/mL. En otro estudio, se desarrolló una metodología basada en la técnica de MEKC con detección fluorescente para la determinación de catinonas sintéticas [104]. Debido a que estos compuestos no son fluorescentes, previamente a su análisis, fueron marcados con un isómero de

isotiocianato de fluoresceína I para posibilitar su detección. Esta metodología permitió separar satisfactoriamente la mayoría de los diferentes analitos y obtener LODs del orden de pg/mL en muestras estándar.

La gran mayoría de metodologías basadas en CE para la determinación de catinonas utilizan detección UV [82,91–93,96–103]. Por ejemplo Woźniakiewicz y colaboradores emplearon un procedimiento basado en CE-UV para determinar los valores de pK_a de un grupo de catinonas sintéticas [96,101]. Los valores obtenidos estuvieron entre 8,59 y 9,10, lo que se ajusta con la caracterización como bases débiles de estos compuestos. Estos mismos autores, también determinaron mediante datos obtenidos con CE-UV los valores de las entalpías y entropías estándar de disociación/desprotonación de estos compuestos [101]. Cabe destacar que en los últimos años diversos autores han desarrollado métodos para la determinación de catinonas en que se emplea la MS como sistema de detección [69,70,93–95] o la detección fluorescente [104,105]. Por ejemplo, Gottardo y colaboradores desarrollaron una metodología para el *screening* de múltiples NPS en orina, incluyendo varias catinonas, mediante CE-MS [70]. Esta metodología fue posteriormente aplicada de manera satisfactoria para el análisis de 10 muestras reales provenientes de consumidores habituales de NPS.

En la bibliografía también encontramos otras metodologías alternativas basadas en principios electroforéticos. Por ejemplo, Lloyd y colaboradores emplearon un dispositivo portátil de electroforesis con detección por fluorescencia inducida por láser (Agilent Bioanalyzer 2100) para el *screening* de catinonas en muestras incautadas de pastillas [105]. Este sistema demostró ser capaz de lograr resultados en un corto período de tiempo, con una especificidad mayor que otros procedimientos de testeo rápido utilizados en los laboratorios forenses como las pruebas de color, lo que permite el análisis de un gran número de muestras sin aumentar la carga de este tipo de laboratorios. Esto sumado a su bajo coste y su portabilidad, convierten a este sistema electroforético en una alternativa prometedora y práctica para el análisis forense de incautaciones de drogas.

Aunque la CE, tal como se ha comentado, es una técnica que se utilizado en los últimos años para diversas aplicaciones relacionadas con la separación de catinonas, cabe destacar que la mayor parte de metodologías donde se aplica esta técnica están vinculadas a la separación enantiomérica de estos compuestos [82,91–95,97–

100,103]. Es por ello que la próxima sección se centra en los diferentes aspectos asociados a la enantioseparación mediante CE.

1.3.1. Enantioseparación mediante electroforesis capilar

La separación quiral es una cuestión que está adquiriendo cada vez más importancia tanto en la industria farmacéutica como en los laboratorios forenses y toxicológicos [17–26]. Esto se debe a que los diferentes enantiómeros no suelen presentar la misma actividad biológica ya que la farmacocinética y la farmacodinámica puede diferir entre los enantiómeros de un mismo compuesto. De hecho, como ya se ha comentado en la sección 1.2, un ejemplo son las propias catinonas, de las cuales hay varios estudios que reflejan que en general el enantiómero *S* presenta una mayor actividad biológica que el *R* [65–67]. Además, al igual que con otras drogas, la concentración de un enantiómero respecto al otro en una muestra dada puede proporcionar información sobre su ruta sintética, puesto que en función de la ruta sintética seguida se generan diferentes cantidades de cada enantiómero. Así pues, conocer la relación entre los enantiómeros puede ser una herramienta de gran utilidad para rastrear la producción de estos compuestos [76,100].

Entre los diferentes procedimientos destinados a la obtención de una separación quiral destacan aquellos que se basan en técnicas cromatográficas (GC y LC) y en la CE. En el caso de las técnicas cromatográficas, las estrategias predominantes son, por una parte, la separación quiral indirecta mediante el uso de agentes derivatizantes quirales y por otra parte, la utilización de columnas quirales [17,18]. En el caso de la primera estrategia, especialmente utilizada en GC, los analitos se transforman en derivados diastereoméricos que pueden ser separados sin dificultad en una columna aquiral convencional [18]. Por ejemplo, Weiß y colaboradores utilizaron un método basado en GC-MS en combinación con un agente de derivatización quiral comercial, el (*S*)-(-)-N-(trifluoroacetil)pirrolidina-2-cloruro de carbonilo (L-TPC), para obtener la enantioseparación de varias NPS, incluidas 10 catinonas [106]. En el caso de las catinonas, mediante esta metodología se consiguió la enantioseparación de 4 de estos compuestos, con valores de resolución (*Rs*) de entre 0,95 y 2,69. Este mismo agente derivatizante quiral fue utilizado en dos metodologías basadas en GC-MS para la enantioseparación de catinonas en muestras de orina y plasma [76,77]. En ambos métodos se consiguió la separación quiral, ya sea de forma total o parcial, de todos los enantiómeros de las catinonas analizadas, con valores de *Rs* de entre 1,04 y 14,89 para el primer estudio [76] y de entre 0,80 y 17,07 para el segundo [77].

Respecto a la utilización de columnas quirales es importante destacar que existen distintos ejemplos básicamente empleando LC [16–18,75,107]. Por ejemplo, en un

estudio reciente se empleó de forma exitosa una columna *Lux AMP,* compuesta por una fase estacionaria a base de polisacáridos*,* para la enantioseparación mediante LC-MS de dos catinonas, la metilona y la etilona, en muestras de drogas incautadas [16]. En las condiciones óptimas de separación se consiguieron unos valores de *Rs* de 4,58 y 1,94 para la metilona y la etilona, respectivamente. En otros estudios publicados basados en esta misma estrategia se ha comparado la eficacia de distintas columnas quirales con el objetivo de lograr la separación quiral de diferentes catinonas sintéticas [75,107]. Por ejemplo, Meetani y colaboradores compararon dos columnas quirales, una *Astec Cellulose DMP* y una *Amylose-based Chiralpak AS-H*, con el objetivo de enantioseparar un grupo de catinonas sintéticas en muestras de plasma y orina mediante LC-UV [75]. En este caso, los mejores resultados se lograron con la primera columna que permitió la separación enantiomérica de 19 de los 22 compuestos con valores de *Rs* de entre 0,95 y 10,55, mientras que con la segunda se consiguió solamente la separación enantiomérica de 7 de los 22 compuestos con valores de *Rs* de entre 1,37 y 2,99.

No obstante, tanto la derivatización como el uso de columnas quirales presentan inconvenientes. Por un lado, la derivatización puede suponer un proceso largo con el consecuente incremento del tiempo de análisis. Por el otro, las columnas quirales pueden representar un alto coste añadido al método analítico puesto que, por norma general, este tipo de columnas son caras. En vista de estos inconvenientes varios autores han estudiado estrategias alternativas y, en este sentido, una técnica que ha destacado por su facilidad para lograr una separación enantiomérica es la CE. Esto es debido a que en este caso se puede obtener la separación quiral sencillamente agregando un selector quiral en el BGE [19–26]. Los enantiómeros tienen propiedades fisicoquímicas idénticas y, por lo tanto, su movilidad electroforética será exactamente la misma. No obstante, cuando estos enantiómeros interaccionan con el selector quiral que se ha adicionado al BGE, típicamente mediante interacciones no covalentes como fuerzas de Van der Waals o enlaces de hidrógeno, se origina la formación temporal de complejos diastereoisómeros que se pueden separar debido a sus diferentes movilidades electroforéticas [25]. Esta posibilidad de adicionar un selector quiral directamente en el medio de separación en CE ofrece una elevada flexibilidad, puesto que la naturaleza del selector quiral, así como su concentración, pueden ser fácilmente modificadas con el objetivo de favorecer la separación entre los dos enantiómeros.

En general, la separación quiral en CE se basa en dos mecanismos, uno cromatográfico y otro electroforético. El mecanismo cromatográfico surge de las diferencias entre las constantes de asociación de los enantiómeros con el selector quiral. El mecanismo electroforético se basa en las diferentes movilidades electroforéticas que podrían tener cada de uno de los dos complejos enantiómero-selector quiral debido a sutiles diferencias en el tamaño de estos complejos [25]. Estos dos mecanismos se pueden expresar matemáticamente mediante la siguiente ecuación, a través de la cual se puede calcular la diferencia de movilidad electroforética entre dos enantiómeros ($\Delta\mu$) [23]:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{\mu_e + \mu_{C1} K_1 [C]}{1 + K_1 [C]} - \frac{\mu_e + \mu_{C2} K_2 [C]}{1 + K_2 [C]} \qquad \text{(Ecuación 2)}$$

μ_1 es la movilidad electroforética del enantiómero que presenta un menor tiempo de migración y μ_2 la del enantiómero detectado a un mayor tiempo de migración. μ_e es la movilidad electroforética del analito en su forma libre. μ_{C1} y μ_{C2} son las movilidades electroforéticas de los complejos enantiómero-selector quiral. K_1 y K_2 son las constantes de asociación entre los enantiómeros y el selector quiral y $[C]$ es la concentración del selector quiral.

La Fig. 10 presenta un esquema de una enantioseparación en un sistema de CE-UV mediante el uso de un selector quiral y el electroferograma resultante, donde $K_2 > K_1$ y las movilidades electroforéticas de los complejos enantiómero-selector son iguales y menores que las del analito en forma libre. En este caso, la separación quiral se basa en el mecanismo cromatográfico, puesto que la diferencia en las constantes de asociación provoca una mayor tendencia a complejarse con el selector quiral por parte del E_2 respecto del E_1. Por tanto, la movilidad electroforética del E_2 se ve reducida en mayor medida que la del E_1 lo que permite la separación quiral de los dos enantiómeros. No obstante, cabe remarcar que debido al mecanismo electroforético también es posible la separación quiral de dos enantiómeros con la misma constante de asociación con el selector quiral, siempre y cuando las movilidades electroforéticas de los complejos enantiómero-selector sean diferentes.

Desde que se obtuvieron las primeras separaciones quirales mediante la técnica de CE a mediados de los años ochenta, la lista de compuestos empleados como selectores quirales no ha cesado de aumentar, hecho que contribuye notablemente

a incrementar la flexibilidad de la CE en la consecución de la enantioseparación de distintos analitos [23,25]. Estos selectores quirales se pueden agrupar en tres categorías: (a) macromoleculares, (b) supramoleculares, y (c) compuestos de bajo peso molecular. Las macromoléculas son moléculas de masa molecular relativamente elevada, cuya estructura comprende esencialmente una repetición múltiple de unidades de moléculas de masa molecular relativamente baja. Dentro de esta categoría encontramos polisacáridos como la maltodextrina, los glicosaminoglicanos o las ciclodextrinas (CDs) [24,26]. Las supramoléculas son sistemas formados por dos o más entidades moleculares que se mantienen juntas mediante interacciones no covalentes. Dentro de esta categoría podemos encontrar las micelas [19,24,26]. En cuanto a la tercera categoría podemos encontrar compuestos organometálicos capaces de formar un complejo con los analitos de interés o especialmente, los líquidos iónicos, un tipo de sales orgánicas con un punto de fusión muy bajo en comparación con otras sales [21,22,24,26].

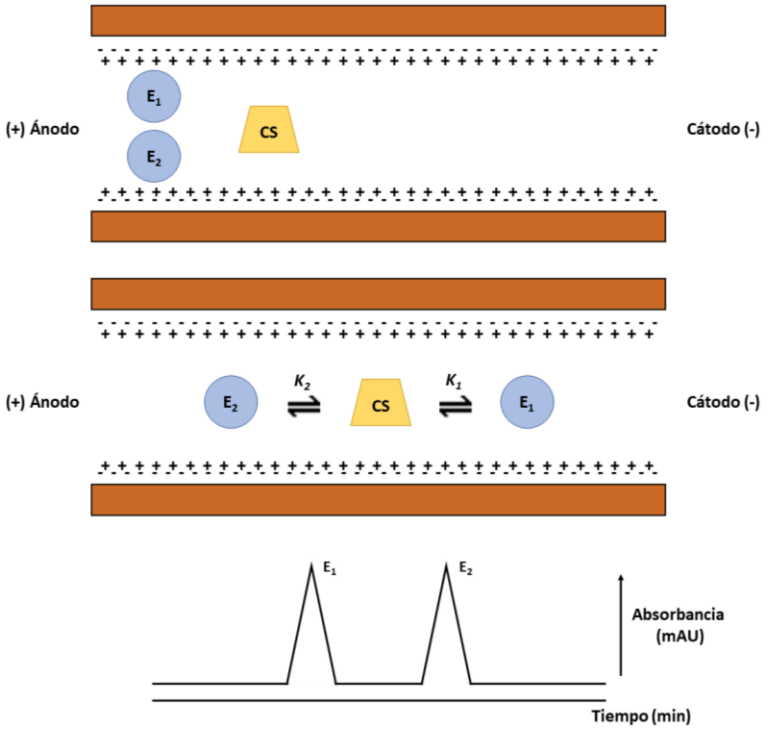

Figura 10. Esquema de una separación quiral mediante CE-UV en la cual se aplica un voltaje positivo y su electroferograma resultante. E_1 y E_2 representan a los enantiómeros del analito estudiado. CS es el selector quiral. K_2 y K_1 son las constantes de asociación entre los enantiómeros y el selector quiral, siendo $K_2 > K_1$.

Entre todos los selectores quirales empleados en CE, claramente destaca el uso de CDs. Esto es debido a su baja toxicidad, alta solubilidad en prácticamente todos los BGEs acuosos, transparencia en detección UV y alta disponibilidad comercial. Las CDs son oligosacáridos cíclicos formados por diferentes unidades de D-(+)-glucopiranosa unidas por enlaces α-1,4-glicosídicos. Estas macromoléculas presentan un exterior hidrófilo, por la presencia de grupos OH, mientras que la cavidad interior es hidrofóbica. Debido a esta dualidad hidrófila/hidrofóbica el enantiorreconocimiento se puede realizar tanto en el interior como en el exterior de la CD, a través de interacciones no covalentes, en función de las características de los analitos estudiados [25,26].

Las CDs se pueden clasificar según si son nativas o modificadas químicamente [20,24]. En particular, las CDs que conforman el grupo de las nativas son la α-CD, la β-CD y la γ-CD, y se caracterizan por el tamaño creciente de su cavidad hidrofóbica, siendo la α-CD (seis unidades de D-(+)-glucopiranosa) y la γ-CD (ocho unidades de D-(+)-glucopiranosa) la de menor y mayor tamaño, respectivamente. La estructura de estas CDs nativas se muestra en la Fig. 11. En cuanto a las CDs modificadas químicamente, estas son CDs nativas en las cuales los grupos OH han sido sustituidos por diferentes grupos funcionales para incrementar de este modo su interacción con las moléculas quirales o también para cargar positiva o negativamente la CD. Estos grupos funcionales pueden ser neutros (hidroxietil-, hidroxipropil-, metil-, etc.), catiónicos (monoamino-, 2-hidroxi-3-N,N,N-trimetilaminopropil-, etc.) o aniónicos (carboximetil-, sulfato-, etc.). De este modo podemos encontrar CDs como la α-CD sulfatada, la 2-hidroxipropil β-CD o la 2-hidroxi-3-N,N,N-trimetilaminopropil γ-CD, entre otras.

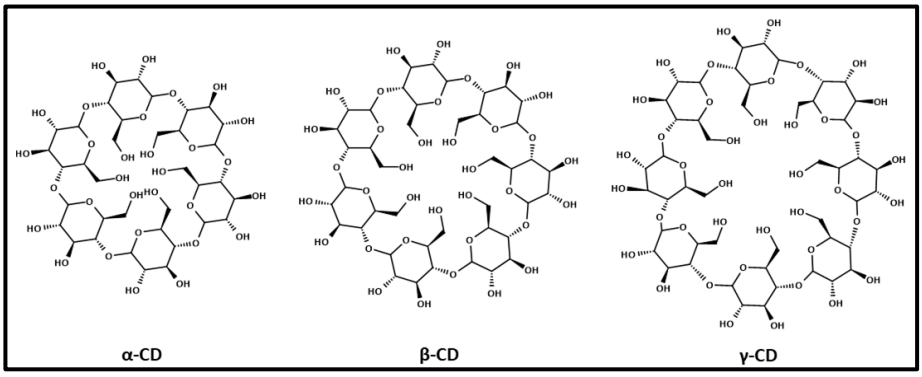

Figura 11. Estructura de las CDs nativas.

Enantioseparación de catinonas mediante CE

Aunque en la bibliografía es posible encontrar estrategias para la separación enantiomérica de catinonas sintéticas mediante GC [76,77,106] o LC [16,75,107], una gran parte de las metodologías diseñadas para tal fin están basadas en la técnica de CE. Como se ha comentado anteriormente, esto es debido a las amplias ventajas que ofrece la enantioseparación mediante CE. Con la finalidad de mostrar los estudios más recientes centrados en esta última técnica, la Tabla 2 incluye las características principales de las metodologías publicadas en los últimos años (2010-2020) para la enantioseparación de catinonas mediante CE.

Como se puede observar en la Tabla 2, los selectores quirales que han ofrecido mejores resultados y en consecuencia más utilizados son las ciclodextrinas (CDs) [14,15,82,91–94,97–100,103]. En este sentido, la variedad de CDs utilizadas es muy diversa y va desde CDs neutras [82,92,93,97,99], entre las que destaca la β-CD [82,93,97], a CDs aniónicas [91,94,98,100,103], ya sean carboximetiladas [98] o sulfatadas [91,94,100,103]. A modo de ejemplo, Nowak y colaboradores evaluaron diversas CDs neutras con el objetivo de enantioseparar tres catinonas, la metcatinona, la mefedrona y la 3-MMC mediante CE [99]. De entre todas las CDs testeadas, la 2-hidroxietil-β-CD fue la que proporcionó mejores resultados ya que permitió separar parcialmente los enantiómeros de las tres catinonas, con valores de *Rs* superiores a 0,5 para las tres. En otro estudio realizado por Hägele y colaboradores se compararon cuatro CDs, la β-CD, la acetil-β-CD, la 2-hidroxipropil-β-CD y la β-CD carboximetilada para evaluar su eficiencia en la consecución de la separación quiral de 61 catinonas [97]. En este caso, la CD que permitió la obtención de los mejores resultados fue la β-CD ya que con ésta se consiguió la separación parcial o total de los enantiómeros de 58 de los 61 analitos incluidos en el estudio. En un reciente estudio, con el uso de la β-CD carboximetilada se consiguió la separación enantiomérica de la mefedrona y sus metabolitos [98]. La separación quiral obtenida con esta CD fue mejor que la lograda con otras CDs neutras también evaluadas y también fue mejor que la obtenida utilizando la β-CD sulfatada. Los autores atribuyeron estos resultados a la gran capacidad del grupo carboxilo, presente en la β-CD carboximetilada, de interactuar con el grupo NH de los analitos y también al tamaño apropiado de la cavidad interior de esta CD.

A pesar del uso predominante de las CDs en la enantioseparación de catinonas, la baja volatilidad de estos compuestos limita su uso cuando el detector seleccionado es la MS y, por tanto, la mayoría de metodologías en este sentido están basadas en CE-UV [82,91–93,97–100,103]. No obstante, con el objetivo de emplear el acoplamiento CE-MS para la separación enantiomérica de catinonas, algunos autores han explorado la utilización de selectores quirales volátiles capaces de evitar la contaminación el sistema de MS. Por ejemplo, se pueden citar dos estudios llevados a cabo por Moini y colaboradores en los que se empleó satisfactoriamente un tipo de éter corona volátil, el (+)-ácido 18-corona-6-tetracarboxílico ((+)-18-C-6-TCA), como selector quiral para la separación enantiomérica de varias catinonas [94,95]. Sin embargo, en la bibliografía también se han reportado diferentes estrategias que permiten el uso de selectores quirales no volátiles en CE-MS. Estas estrategias se comentarán extensamente en la siguiente sección donde se profundiza en el uso de la MS como sistema de detección en CE y, especialmente, en su uso en procedimientos orientados a la separación quiral.

Tabla 2. Artículos relativos a la enantioseparación de catinonas mediante CE publicados en el periodo 2010-2020.

Compuestos estudiados	Catinonas estudiadas	Técnica	BGE	Selector quiral	Observaciones	Referencia
Catinonas sintéticas	Mefedrona; MDPV;	In-line SPE-CE-UV	Hidrogenofosfato de sodio 80 mM (pH 2,5)	β-CD 12 mg/mL	Enantiodeterminación mediante una técnica de preconcentración en línea	[82]
4-clorometcatinona	4-clorometcatinona	CE-UV	Acetato de amonio 50 mM (pH 4,5) con un 10% v/v de ACN	β-CD sulfatada 20 mg/mL	Caracterización analítica de la 4-clorometcatinona	[91]
NPS	MMC; MEC; EEC; FMC;	CE-UV	CElixir Reagent B	2-hidroxipropil-β-CD 80 mM	Comparativa LC-UV respecto CE-UV	[92]
Catinonas sintéticas	Bufedrona; Pentilona; Pentedrona; MDPV; Metedrona; Etcatinona; Dimetilcatinona; 4-FMC; 3,4-DMMC; Etilona; Metilona; Mefedrona;	CE-UV/CE-MS	CE-UV: Fosfato de sodio 100 mM (pH 2,5); CE-MS: Fosfato de sodio 50 mM (pH 2,5)	CE-UV: β-CD 10 mM; CE-MS: 0.6% γ-CD sulfatada	Adaptación de una separación quiral por CE-UV a CE-MS	[93]
Catinonas sintéticas y anfetaminas	3-FMC; 4-FMC; Pentedrona; 4-MEC; Metilona; Pentilona;	CE-MS	Ácido fórmico al 0,5%	0.125% γ-CD sulfatada en (+)-18-C-6-TCA 15 mM		[94]
Drogas y fármacos	Catinona; 4-metilcatinona; 3-FMC; Pentilona;	CE-MS	(+)-18-C-6-TCA 10 mM	(+)-18-C-6-TCA 10 mM	El BGE actúa al mismo tiempo de selector quiral	[95]
Catinonas sintéticas	4-MMC; 3-MMC; 2-MMC; 3,4-DMMC; Metedrona; Metilona; Dimetilona; Butilona; N, N-dimetilbutilona; Pentilona; 3-MeOMC; 2-CMC; 3-CMC; 4-CMC; 4-EMC; Mexedrona; 4-FMC; 3-FMC; 2-FMC; 4-BMC; Bufedrona; Etilona; 5-metiletilona; Bk-Etil-K; N-bencilnorbutilona; 5-metoximetilona; MDPV; MD-PHP; 5-DBFPV; 5-PPDi; bk-iVP; TH-PVP; 4-metilbufedrona; Pentedrona; 3-CEC; 4-CEC; N-etilbufedrona; N-etilhexedrona; Anfepramona; 4-MEC; 3-MEC; 4-metilcatinona; DL-4662; Bupropiona; 4-metilpentedrona; Benzedrona; α-PPP; M-PPP; 4-MPrC; 4-MeO-α-PVP; 4-Cl-PVP; 4-F-PVP; α-PVP; 4-F-PHP; PV8; 4-F-PV8; α-PIHP; Nafirona; DOMC; N-etilpentedrona;	CE-UV	Fosfato de sodio 10 mM (pH 2,5)	β-CD 10 mM	Comparación de 4 CDs como selectores quirales	[97]
Mefedrona y sus metabolitos	Mefedrona; 4-metilcatinona;	CE-UV	Fosfato de sodio 50 mM (pH 2,75)	β-CD carboximetilada 7,5 mM		[98]
Catinonas sintéticas	Efedrona; Mefedrona; Metafedrona;	CE-UV	Fosfato de sodio 50 mM (pH 3)	2-hidroxietil-β-CD 30 mM		[99]
Catinonas sintéticas	4-BMC; Bufedrona; Butilona; Catinona; 3,4-DMMC; Etcatinona; Etilbufedrona; Etilona; 3- FMC; 4-FMC; Metedrona; MDPV; 4-MEC; Mefedrona; Metcatinona; MDMC; Nafirona; Pentedrona; α-PPP;	CE-UV	Acetato de amonio 50 mM (pH 4,5) con un 10% v/v de ACN	β-CD sulfatada 20 mg/mL	Comparación de 5 CDs como selectores quirales	[100]
NPS	4F-PV8; Metedrona; 4-MeO-α-PVP; 3-MeOMC	CE-UV	Acetato de amonio 50 mM (pH 4,5) con un 10% v/v de ACN	Sulfobutiléter β-CD 16 mM		[103]

1.3.2. Espectrometría de masas como sistema de detección en electroforesis capilar

La absorbancia UV sigue siendo uno de los sistemas de detección más utilizados en CE, principalmente debido a su simplicidad y al hecho de que muchos compuestos presentan absorción UV. No obstante, con este tipo de detectores no es posible la identificación de los analitos de manera inequívoca. Esto unido al hecho que en los últimos años hayan aparecido diferentes interfases que han posibilitado el acoplamiento entre la CE y la MS ha supuesto un importante incremento en el uso de este tipo de detección [108–113]. A pesar de que existen una gran variedad de interfases capaces de posibilitar este acoplamiento, la más ampliamente empleada, especialmente para moléculas orgánicas cargadas como es el caso de las catinonas, es la ionización por electrospray (*electrospray ionization* - ESI) [26,108,110,112]. En este modo de ionización, tal como muestra la Fig. 12, la muestra se bombea a través de una aguja de acero inoxidable en la que se aplica un potencial generalmente entre 3 y 5 kV. La niebla resultante compuesta por finas gotas cargadas pasa por un proceso de desolvatación, con el principal objetivo de eliminar el solvente. Debido a este proceso, las gotas cada vez se vuelven más pequeñas, aumentando así su densidad de carga, hasta que la tensión superficial ya no puede soportar la carga en un punto conocido como límite de Rayleigh. Es entonces cuando se produce la llamada explosión de Coulomb provocando la división de cada gota en pequeñas gotitas. Este proceso se repite hasta que el solvente es eliminado del analito produciendo una molécula de analito que puede encontrarse múltiplemente cargada [27]. De esta forma el procedimiento de ESI permite que los compuestos separados mediante CE se puedan transferir de manera eficiente de la fase líquida a iones en fase gaseosa. Además, cabe remarcar que la ESI es un modo de ionización relativamente suave [108].

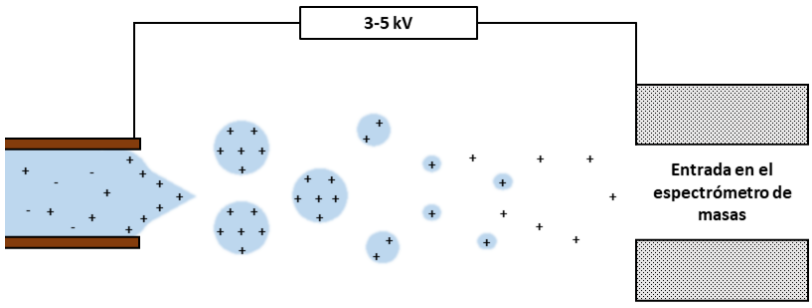

Figura 12. Mecanismo de la ionización por electrospray.

Una de las principales dificultades a solventar en CE-MS es asegurar el cierre del circuito eléctrico entre el instrumento de CE y la fuente de ionización. Esto es

necesario, puesto que si no se cierra el circuito se pueden producir ciertas reacciones electroquímicas indeseadas que afecten a la ionización y a la separación en términos de eficiencia, sensibilidad y estabilidad [108,110]. Para solucionar esta problemática, en una parte importante de la literatura nos encontramos instrumentación con interfases donde el capilar está conectado coaxialmente a un tubo desde el que se bombea un líquido auxiliar (*sheath liquid*) a un caudal entre 2 y 10 µL/min [108,110,112,114]. El objetivo de este líquido auxiliar es cerrar el circuito eléctrico entre el instrumento de CE y la fuente de ionización. Por lo tanto, en CE-ESI-MS se encuentran dos circuitos eléctricos, uno correspondiente al sistema de CE y el otro a la fuente de ionización, que pueden ser de diferente magnitud e, incluso, de diferente signo [110]. La Fig. 13 muestra un esquema de una interfase con líquido auxiliar coaxial.

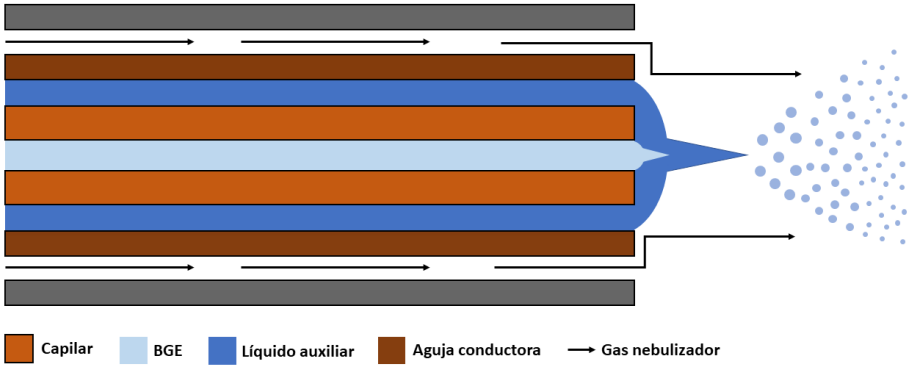

Figura 13. Esquema de una interfase con líquido auxiliar coaxial.

La composición y naturaleza del líquido auxiliar es obviamente un parámetro importante en CE-MS. Es importante remarcar que la composición de este líquido no tiene por qué ser estrictamente similar a la del BGE. Esto permite la posibilidad de utilizar líquidos auxiliares que contengan una proporción significativa de disolvente orgánico, por ejemplo, para mejorar la desolvatación o un ácido débil, con el fin de mejorar la protonación de los analitos, incluso si la separación se realiza utilizando un BGE alcalino [114]. De hecho, una mezcla hidroorgánica acostumbra a ser un buen compromiso en términos de conductividad y volatilidad para lograr una señal de MS óptima y estable, aunque esta composición puede variar ampliamente en función de los compuestos estudiados [108]. Un aspecto importante que cabe destacar en este tipo de interfases es que la adición del líquido auxiliar supone una dilución de la muestra y esto puede ocasionar una disminución de la sensibilidad del método

analítico [108,110,112,114]. Por ello, en los últimos años se han explorado diferentes interfases capaces de resolver esta problemática. Éstas se pueden clasificar en dos tipos, por un lado, encontramos las interfases que tienden a reducir al mínimo el caudal del líquido auxiliar (*nanoflow sheath liquid interfaces*) a valores del rango de 50 a 100 nL/min [108,112,114]. Por otro lado, están aquellas interfases capaces de funcionar sin líquido auxiliar (*sheathless interfaces*) [26,108,110,112,114]. La mayor dificultad en este tipo de interfases es cerrar correctamente el circuito eléctrico entre el instrumento de CE y la fuente de ionización. Por lo general, esto se logra recubriendo el *outlet* del capilar de separación de un material conductor, aunque hay procedimientos alternativos como la unión del capilar de separación a una funda metálica o a la introducción de un pequeño electrodo dentro del propio capilar [108]. El uso de ambos tipos de interfases pueden comportar un aumento en la sensibilidad de las metodologías basadas en CE-MS tal y como se ha documentado en diversos ejemplos de la bibliografía [108,110,112,114].

En los procedimientos de CE-MS también debe tenerse en cuenta que la efectividad de la fuente de ionización puede verse afectada cuando la corriente generada por el voltaje de separación aplicado en el instrumento de CE es demasiado elevada. Por este motivo en los métodos basados en este acoplamiento se tienden a emplear BGEs de menores concentraciones que en CE-UV, donde un valor de corriente elevado no constituye un problema. Además, la presencia de compuestos no volátiles en el BGE es incompatible con la fuente de ionización, ya que éstos pueden provocar tanto una supresión iónica como la contaminación de la fuente y esto podría producir una disminución importante de la sensibilidad [108,110,112,113]. Por ello, una de las principales consideraciones a tener en cuenta en el desarrollo de métodos basados en el acoplamiento CE-MS es precisamente evitar la presencia de compuestos no volátiles en el BGE. Estas restricciones limitan en gran medida la variedad de BGEs que se pueden utilizar en CE-MS. En este sentido, los BGEs más habitualmente empleados en esta técnica se basan en soluciones amortiguadoras de formiato de amonio, acetato de amonio, hidrogenocarbonato de amonio o carbonato de amonio [113].

Enantioseparación mediante CE-MS

Las restricciones propias en la selección de un BGE apropiado en las metodologías basadas en CE-MS también se aplican en la elección de un selector quiral. De hecho,

evitar la contaminación de la fuente de ionización puede representar todo un reto en la separación quiral mediante CE-MS, especialmente si se emplean selectores quirales no volátiles. La entrada de un selector quiral no volátil en la fuente de ionización, debido al EOF o por efecto de succión, puede suprimir significativamente la ionización de los analitos de interés, lo que se traduce en una pérdida en la sensibilidad [26]. Un ejemplo documentado en este sentido es el uso de las CDs como selectores quirales. Aunque, como se ha comentado anteriormente, las CDs son el tipo de selector quiral más empleado, en CE-MS este tipo de macromoléculas pueden causar una supresión significativa de la señal obtenida y un aumento del ruido debido a la contaminación de la fuente de ionización [26,113]. Con el objetivo de superar esta problemática, en la bibliografía se han propuesto diferentes alternativas.

Una alternativa es el uso de selectores quirales volátiles, evitando de este modo la contaminación de la fuente de ionización a pesar de que estos selectores quirales lleguen a introducirse en el sistema de MS [94,95,115]. En este sentido, en el caso concreto de las catinonas, algunos autores han optado por utilizar como selector quiral un éter corona volátil, el (+)-18-C-6-TCA, para la enantioseparación de estos compuestos [94,95]. Este es el caso de una metodología basada en CE-ESI-MS desarrollada por Rollman y colaboradores donde la pregabalina, la catinona y la normefedrona fueron enantioseparadas mediante el uso de ese éter corona, que actuaba al mismo tiempo de BGE y de selector quiral [95]. En las condiciones óptimas se consiguieron valores de *Rs* de 1.3, 3.7 y 3.8 para los enantiómeros de la pregabalina, la catinona y la normefedrona, respectivamente.

No obstante, cabe señalar que la variedad de selectores quirales volátiles disponibles es limitada. Es por este motivo que en los últimos años diferentes autores han explorado estrategias capaces de evitar la contaminación de la fuente de ionización en CE-MS en las que a su vez se puedan utilizar selectores quirales no volátiles. Entre las más utilizadas destacan dos, la *counter migration technique* (CMT) [26,93,94,113,116] y la *partial filling technique* (PFT) [26,93,94,113]. En la primera, se usan selectores quirales catiónicos o aniónicos en función de la polaridad aplicada en la separación electroforética. El objetivo es que el selector quiral cargado migre alejándose del sistema de detección, es decir, en dirección al *intlet* y de forma opuesta a los analitos. Así pues, si la polaridad aplicada es positiva se utilizarán selectores quirales aniónicos y viceversa. A modo de ejemplo, Mikuma y colaboradores desarrollaron un método basado en el acoplamiento CE-ESI-MS/MS

para la enantioseparación de anfetaminas empleando una CD aniónica, concretamente la γ-CD sulfatada, como selector quiral [116]. Al estar cargada negativamente, esta CD migra en dirección opuesta al espectrómetro de masas evitando así la contaminación del mismo. Este procedimiento fue capaz enantioseparar las 8 anfetaminas evaluadas, además fue aplicado de manera satisfactoria en la detección de anfetaminas a nivel traza en muestras incautadas de metanfetamina.

En la segunda estrategia, la PFT, solo se llena parcialmente el capilar con BGE al que previamente se le ha adicionado el selector quiral [26,113]. Como muestra la Fig. 14 el procedimiento en este tipo de estrategia es el siguiente: primero se realiza un *flush* con BGE sin selector quiral acondicionando de esta forma el capilar para la separación electroforética. Seguidamente, se inyecta una solución de BGE al que se le ha adicionado el selector quiral, pero este solo se introduce parcialmente dentro del capilar (por ejemplo, un 50%), lo que previene la entrada del selector quiral en el sistema de MS. Finalmente, se inyecta la muestra a analizar y se realiza la separación electroforética. Por lo general, cuanto menor es el volumen de BGE con selector quiral introducido en el capilar, menores son los valores de *Rs* obtenidos entre los enantiómeros de los diferentes analitos puesto que la cantidad total de selector quiral introducido en el capilar también es menor.

Un ejemplo de la aplicación tanto de la PFT como de la CMT lo podemos encontrar en una metodología basada en CE-ESI-MS desarrollada por Merola y colaboradores, derivada de un procedimiento previo basado en CE-UV que había logrado la enantioseparación de varias catinonas empleando β-CD como selector quiral [93]. Con el objetivo de evitar la contaminación de la fuente de ionización, la concentración del BGE empleado en la metodología de CE-UV, un tampón de fosfato 100 mM, se redujo a la mitad. Además, la β-CD fue sustituida por la γ-CD sulfatada, que como ya se ha indicado se trata de una CD aniónica que en condiciones de polaridad positiva migra en la dirección opuesta al detector de MS. Como medida complementaria en el procedimiento de CE-MS el capilar solo se llenó hasta un 70% de su capacidad total con el BGE que contenía el selector quiral. Esta metodología permitió la separación quiral de 8 de las 12 catinonas estudiadas y fue posteriormente aplicada para la enantiodeterminación de drogas procedentes de incautaciones policiales. En concreto se analizaron 8 muestras reales, siendo destacable que los resultados obtenidos con la metodología de CE-MS coincidieron con aquellos obtenidos

mediante el método de GC-MS empleado de forma rutinaria por las autoridades policiales para el análisis de este tipo de muestras.

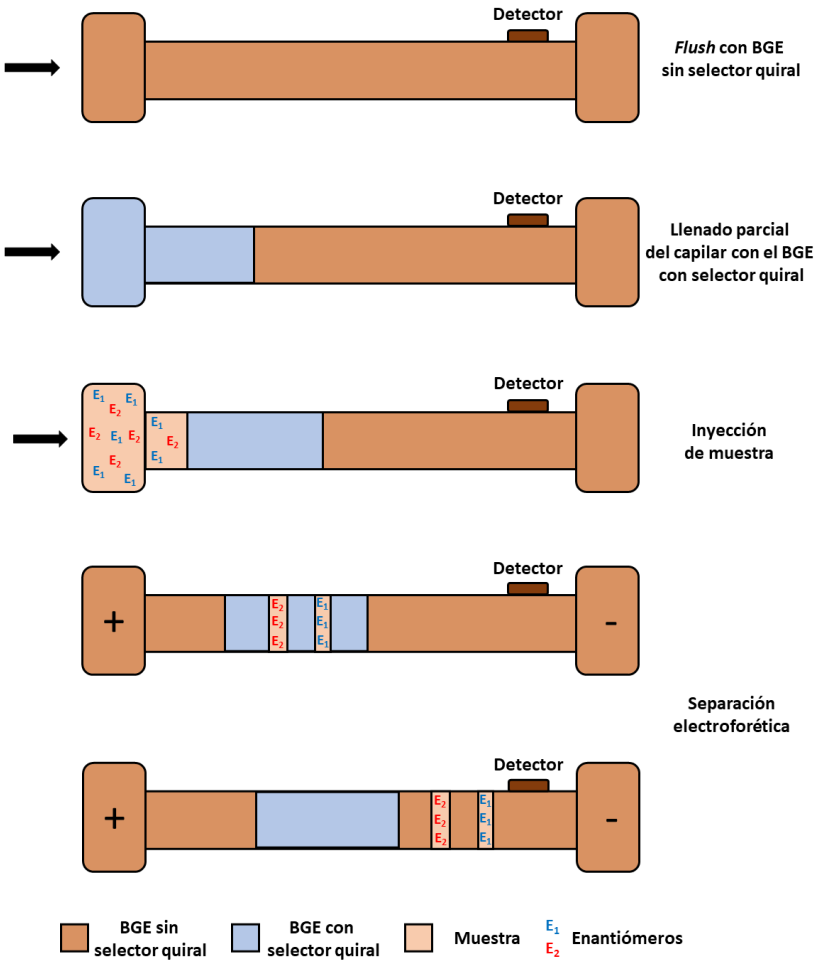

Figura 14. Esquema de las diferentes etapas durante un procedimiento de PFT.

1.3.3. Estrategias para la preconcentración de muestra en electroforesis capilar

La sensibilidad de las metodologías basadas en CE es por lo general bastante baja, especialmente cuando esta técnica se emplea junto a un detector de UV [27]. De hecho, cuando se usan métodos de detección óptica, la CE puede llegar a ser de hasta dos o tres órdenes de magnitud menos sensible que la LC [32]. Esta falta de sensibilidad está asociada a los pequeños volúmenes de muestra que normalmente son introducidos en los sistemas electroforéticos, generalmente del orden de pocos nL [28,30]. Además, en el caso específico de la CE-UV, al efecto que conlleva la inyección de pequeñas cantidades de muestra, se le añade la corta longitud del camino óptico presente en la sección del capilar de separación que actúa como celda de detección [28,34]. Esto supone una problemática para la determinación de analitos que se encuentran a bajos niveles de concentración en sus respectivas muestras. Es por ello que se han desarrollado diferentes estrategias de preconcentración capaces de aumentar la limitada sensibilidad de las metodologías basadas en CE [28–35].

Estas estrategias se pueden clasificar en función de la disposición entre el sistema de preconcentración y el instrumento de CE: *off-line*, *at-line*, *on-line* e *in-line*. En las técnicas *off-line* la preconcentración y la separación electroforética son dos etapas que no están conectadas directamente entre sí. En este tipo de estrategias la transferencia de la muestra desde el dispositivo de preconcentración al sistema de CE es realizada por un operador humano. Cuando los dos sistemas, tanto el preconcentrador como la separación electroforética, están totalmente diferenciados, pero la transferencia de la muestra desde el preconcentrador al instrumento de CE se realiza de forma automatizada, mediante un brazo robótico o algún método similar, estamos ante una técnica de preconcentración *at-line*. Las metodologías *on-line* corresponden a aquellos procedimientos en los que el preconcentrador y el sistema de CE son dos elementos distintos, pero están directamente conectados por algún tipo de interfase. Finalmente, las técnicas de preconcentración en línea (*in-line*) son aquellas donde la preconcentración y la separación electroforéticas conforman un único sistema, es decir, la preconcentración se realiza dentro del propio capilar de CE [28]. En el caso de la presente Tesis Doctoral se ha optado por emplear este último grupo de técnicas de preconcentración.

Dentro de las estrategias de preconcentración que se desarrollan dentro del propio capilar podemos encontrar dos grandes familias que se han aplicado en los últimos años en métodos basados en CE para la determinación de distintos tipos de

analitos en muestras diversas, las técnicas de preconcentración basadas en principios cromatográficos [28–32] y las técnicas de preconcentración basadas en principios electroforéticos [32–36]. En los siguientes subapartados se detallan algunas de las características de estos dos grupos de técnicas, haciendo especial énfasis en aquellas estrategias empleadas en la parte experimental de la presente Tesis Doctoral. Concretamente el acoplamiento en línea entre la SPE y la CE (*in-line* SPE-CE) y las técnicas de *stacking*, siendo la primera una técnica de preconcentración basada en principios cromatográficos y las segundas técnicas basadas en principios electroforéticos.

1.3.3.1. Técnicas de preconcentración basadas en principios cromatográficos (*in-line* SPE-CE)

Las técnicas de preconcentración en línea basadas en principios cromatográficos, son aquellas donde se aprovechan los mecanismos propios de la cromatografía para preconcentrar los analitos de interés. Dentro de este tipo de procedimientos destaca el acoplamiento en línea entre la SPE y la CE (*in-line* SPE-CE), referida en algunos estudios como *on-line* SPE-CE [117–121], puesto que esta estrategia ha demostrado una gran capacidad para lograr factores de preconcentración elevados [28–31]. En la técnica de *in-line* SPE-CE se introduce, cerca del *inlet* del capilar de separación, un microcartucho de extracción o preconcentrador que contiene un sorbente capaz de retener los analitos inyectados en el sistema de CE. En este tipo de sistema, al introducir grandes volúmenes de muestra mediante elevadas presiones de inyección y elevados tiempos de carga, es posible preconcentrar los analitos de interés, lo que permite un aumento considerable de la sensibilidad del método. Es importante remarcar que puesto que el preconcentrador forma parte íntegra del sistema de CE, todos los disolventes empleados para la preconcentración pasan directamente al capilar de separación, así como que durante la separación electroforética el voltaje de separación también se aplica a través del material de SPE [29,30].

A pesar de las ventajas de la técnica de *in-line* SPE-CE, no es posible encontrar comercialmente capilares donde el preconcentrador se encuentre incorporado de serie al capilar de separación y, en consecuencia, éste se tiene que preparar de forma manual. En este sentido, en la bibliografía se han descrito cuatro tipos de diseño para el preconcentrador: los capilares tubulares abiertos, las membranas, los sorbentes monolíticos y los *packed beds* [28,29,31]. Todos estos diseños están representados de forma esquemática en la Fig. 15. Por un lado, en los capilares tubulares abiertos el preconcentrador, que consiste en un trozo de capilar, tiene su superficie interior recubierta con partículas de sorbente. Para obtener el preconcentrador, es necesario modificar la pared interior del capilar mediante la aplicación de algún tipo de reactivo que permita su posterior recubrimiento con las partículas de sorbente. Por otro lado, las membranas o discos cargados con un sorbente de SPE son un tipo de diseño que consiste en impregnar una membrana con una fase estacionaria adecuada y posteriormente colocarla entre dos piezas de capilar. A pesar de que los dos diseños antes descritos han demostrado su eficacia para mejorar la sensibilidad de la CE, su capacidad de carga de muestra es limitada si la comparamos con otras estrategias de *in-line* SPE-CE y, en consecuencia, los factores de preconcentración obtenidos mediante este tipo de diseños no suelen ser elevados [28,31]. En cuanto a los sorbentes monolíticos, en los últimos años han surgido varios estudios donde se han

desarrollado estrategias basadas en *in-line* SPE-CE que emplean este tipo de preconcentradores. En este tipo de diseño, una pieza continua de una microestructura altamente porosa es preparada por polimerización *in situ* dentro del propio capilar de separación. A diferencia de los capilares tubulares abiertos o las membranas, este tipo de diseño si permite, generalmente, la obtención de elevados factores de preconcentración [28,31,122].

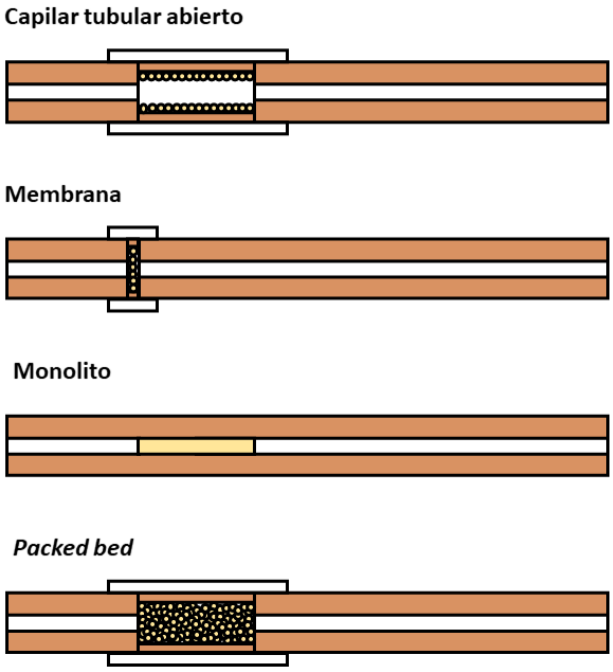

Figura 15. Representación esquemática de los diferentes diseños para *in-line* SPE-CE.

A pesar de que los diseños antes descritos se han empleado en múltiples aplicaciones [123–127], hasta la fecha, el tipo de diseño que se ha utilizado con mayor frecuencia es el de *packed bed* [28–31]. Este diseño generalmente presenta una mayor capacidad de retención que los otros diseños antes descritos, especialmente en comparación a los capilares tubulares abiertos y las membranas, debido a la mayor cantidad de partículas de sorbente presentes en el preconcentrador y, en consecuencia, los factores de preconcentración obtenidos suelen ser más elevados. Existen dos diseños distintos de *packed ped* en función de si las partículas de sorbente están retenidas mediante fritas o de si esta retención se logra sin necesidad de éstas (*fritless packed bed*).

En el diseño mediante fritas, las partículas de sorbente son introducidas en un pequeño trozo de capilar (4-7 mm) y éstas son retenidas en el interior del preconcentrador gracias a dos fritas, con lo que se evitan posibles fugas del sorbente durante los análisis. En este tipo de diseño, el tamaño de las partículas de sorbente no es un parámetro crítico. De hecho, se pueden utilizar partículas de sorbente de pequeño tamaño, permitiendo de esta forma una gran superficie activa para la preconcentración. Sin embargo, la presencia de fritas también puede causar la formación de burbujas, la interrupción de la corriente y afectar al EOF, lo que a su vez afecta a la reproducibilidad del tiempo de migración [28,29,31]. Además, en comparación con el *fritless packed bed*, en el diseño con fritas se puede producir un aumento del tiempo de análisis debido a un incremento de la contrapresión generada dentro del capilar de separación. No obstante, existen múltiples ejemplos en la bibliografía en que el diseño con fritas se ha utilizado para la preconcentración en línea de distintos tipos de analitos en diferentes aplicaciones [117–120]. Por ejemplo, Rossi y colaboradores utilizaron este diseño para la determinación de oxprenolol, un medicamento beta bloqueante, en muestras de orina mediante *in-line* SPE-CE-UV [117]. En este caso, gracias a la capacidad de preconcentración del *packed bed* se produjo una disminución de 400 veces en el LOD del oxprenolol en muestras estándar. Posteriormente, este método se aplicó de manera satisfactoria a muestras de orina enriquecidas con oxprenolol sencillamente añadiendo un pretratamiento de la muestra basado en una extracción líquido-líquido (*liquid-liquid extraction* - LLE). Otro ejemplo de aplicación de este tipo de diseño es la metodología desarrollada por Tascon y colaboradores para el análisis de un grupo de alcaloides de β-carbolina en un tipo de alga comestible, el wakame (*Undaria pinnatifida*), mediante *in-line* SPE-CE-MS [119]. En esta metodología también fue necesario un pretratamiento previo de la muestra, en este caso basado en una extracción sólido-líquido (*solid-liquid extraction* - SLE) seguida de varios procesos de filtración. La preconcentración en línea aumentó la sensibilidad del método en hasta 1000 veces respecto a un procedimiento basado en CE-MS. Los resultados obtenidos en este estudio mostraron que la técnica de *in-line* SPE-CE-MS puede considerarse como una estrategia atractiva para detectar niveles traza de alcaloides y otras moléculas bioactivas pequeñas en extractos de plantas complejas.

En el diseño sin fritas, el preconcentrador, un pequeño trozo de capilar (2-4 mm) lleno con sorbente de SPE, se coloca entre el extremo por donde se inyecta la muestra o *inlet* y el propio capilar de separación, ambos de un diámetro interno más estrecho

que el del preconcentrador. Las partículas de sorbente se retienen en el *packed bed* debido a que su tamaño es ligeramente mayor que el diámetro interno de los dos capilares entre los que está situado. La ausencia de fritas evita los problemas relacionados con la contrapresión, las perturbaciones del EOF, la formación de burbujas y la caída o interrupción de la corriente. No obstante, en este caso el tamaño de las partículas de sorbente es un factor clave a tener en cuenta. De hecho, al no retener las partículas de sorbente mediante fritas, éstas acostumbran a ser de mayor tamaño en comparación a las empleadas en el diseño con fritas, lo que limita la superficie activa de preconcentración. Cabe destacar que el diseño de *fritless packed bed* se ha utilizado también para múltiples aplicaciones [82,121,128–138]. Por ejemplo, Jooß y colaboradores evaluaron diferentes parámetros propios del diseño de *fritless packed bed*, en concreto, el tamaño de las partículas de sorbente y los diámetros internos del preconcentrador y del capilar de separación con el objetivo de desarrollar un método para el análisis de glicanos marcados con ácido 8-aminopireno-1,3,6-trisulfónico mediante *in-line* SPE-CE-ESI-MS [138]. En este caso, el tamaño óptimo para las partículas de sorbente era de 90 µm, mientras que el diámetro interno óptimo del preconcentrador y el capilar de separación era de 100 y 50 µm, respectivamente. En estas condiciones la construcción del preconcentrador era sencilla y reproducible. Con esta metodología se lograron factores de enriquecimiento de entre 450 y 1000 en comparación con el método CE-MS sin preconcentración, lo que permitió obtener LODs de unos pocos ng/mL, concretamente de entre 0.3 y 2 ng/mL.

Dentro del grupo de investigación en el que se ha desarrollado la parte experimental de esta Tesis Doctoral se cuenta con una amplia experiencia en el uso de preconcentradores basados en el diseño de *fritless packed bed* para la determinación de diferentes analitos en muestras biológicas o ambientales [82,131–137]. Por ejemplo, este tipo de diseño se utilizó para la determinación de barbitúricos en muestras de orina mediante *in-line* SPE-CE-UV posteriormente a un pretratamiento de muestra basado en una LLE, obteniendo *sensitivity enhancement factors* (SEFs) de entre 173 a 1840 y LODs en el rango de 5 a 60 ng/mL [133]. Otro ejemplo es la utilización de este diseño para la enantiodeterminación de catinonas en muestras de cabello [82]. En este caso se obtuvieron LODs de entre 0,02 y 0,10 ng/mg, gracias a la combinación de un procedimiento de *in-line* SPE-CE-UV junto con un pretratamiento de muestra basado en la extracción con líquidos presurizados (*pressurized liquid extraction* - PLE).

A pesar de los numerosos ejemplos en los que el diseño de *packed bed* ha demostrado su capacidad en la obtención de elevados factores de preconcentración, como ya se ha comentado, uno de sus principales inconvenientes es la necesidad de construir manualmente el preconcentrador. En este sentido, en la bibliografía encontramos soluciones alternativas para facilitar su construcción. Una de estas estrategias es la utilización de sorbentes magnetizados. Un ejemplo de la aplicación de este tipo de sorbentes desarrollado en nuestro grupo de investigación es un método basado en *in-line* SPE-CE en el cual se emplearon partículas magnéticas, específicamente partículas magnetizadas de C_{18} (C_{18}-FS-Fe_3O_4), para la determinación de distintas drogas, como la cocaína, en orina [131]. El uso de este tipo de partículas magnetizadas simplifica la construcción del sistema de preconcentración puesto que, gracias a sus propiedades magnéticas, estos materiales se pueden manipular fácilmente dentro de un capilar de CE al aplicar un campo magnético. Para este propósito y tomando como referencia un dispositivo diseñado por Morales-Cid y colaboradores [139], los autores construyeron un soporte de metacrilato que contenía dos imanes, tal como se muestra en la Fig. 16, capaz de inmovilizar localmente las partículas magnetizadas dentro del capilar. Los resultados obtenidos mediante esta estrategia fueron satisfactorios, mejorando la sensibilidad de detección de 125 a 700 veces en comparación con el procedimiento de CE sin preconcentración, lo que permitió obtener LODs de entre 20 a 50 ng/mL.

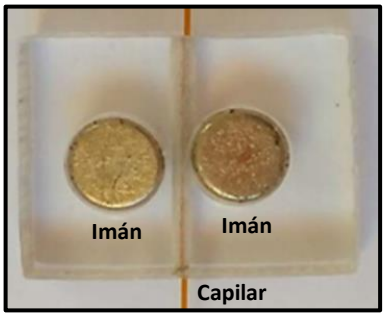

Figura 16. Dispositivo *in-line* SPE-CE para sorbentes magnéticos (adaptada de [131]).

El modo de operar en una metodología de *in-line* SPE-CE con el diseño de *packed bed* es relativamente simple y se divide en varias etapas que se encuentran representadas en la Fig. 17. En primer lugar, se realiza un acondicionamiento del sorbente mediante un *flush* (930 mbar) con una o varias soluciones adecuadas para el sorbente seleccionado (A). Posteriormente, se introudce la muestra a analizar en

las condiciones óptimas para que los analitos queden retenidos en el preconcentrador (B). Este proceso de carga de muestra generalmente requiere de varios minutos y de la aplicación de una presión relativamente elevada. Después de la inyección, se efectúa un *clean-up* con el propio BGE mediante un *flush* con el objetivo de acondicionar el capilar antes de llevar a cabo la separación electroforética (C). La etapa de elución se lleva a cabo inyectando un pequeño *plug* de un eluyente adecuado (D). Seguidamente, aunque no en todos los casos, se realiza un *push* con BGE, mediante la aplicación de una pequeña presión, para desplazar el *plug* de elución más allá del preconcentrador (E). Finalmente, se aplica el voltaje de separación (F). Hay que tener en cuenta que seleccionar unas condiciones óptimas en un procedimiento de *in-line* SPE-CE puede resultar complejo. Esto es debido a que es necesaria la compatibilidad entre la preconcentración (alta recuperación y selectividad), la separación (alta resolución y corto tiempo de análisis) y la detección (alta selectividad y sensibilidad) [31].

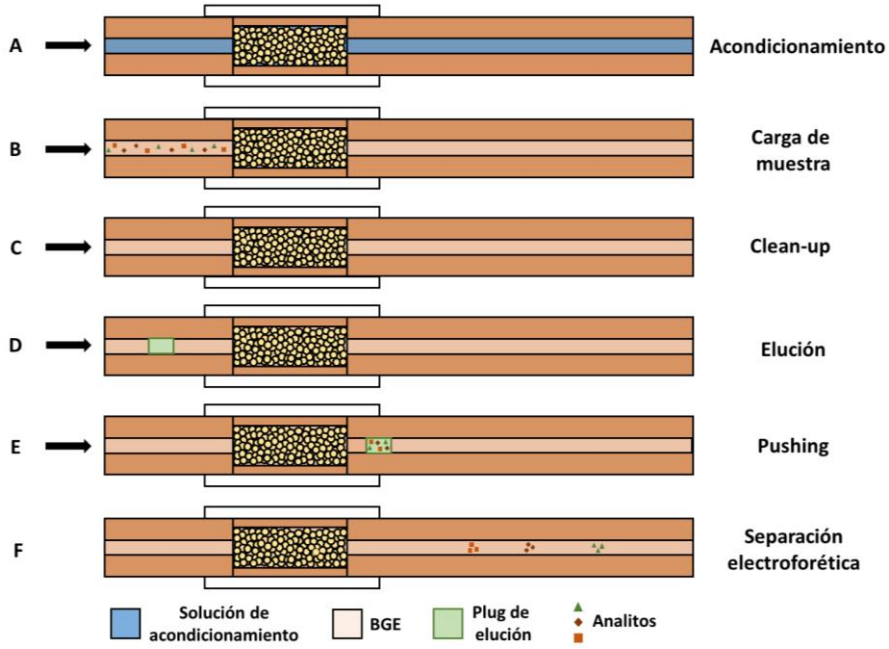

Figura 17. Esquema de las etapas durante un procedimiento de *in-line* SPE-CE donde el preconcentrador es un *packed bed*.

En el desarrollo de las metodologías basadas en el acoplamiento en línea de la SPE con la CE hay diferentes factores a considerar. Por un lado, hay que controlar que la

cantidad de muestra eluida durante el procedimiento de SPE no exceda el volumen de inyección aceptable para un procedimiento de CE, lo que puede dar lugar a un ensanchamiento de los picos de los analitos. Por otro lado, hay que tener en cuenta que la presencia del preconcentrador dentro del capilar de CE puede influir negativamente en la separación electroforética. Esto es debido a que el propio relleno del preconcentrador puede causar perturbaciones en el perfil de flujo laminar propio de la CE y también en el EOF. Asimismo, hay que considerar la posible degradación del sorbente debido a la presencia de ciertos componentes en las soluciones y en el BGE o por los valores extremos de pH a los que a veces se trabaja en CE [31]. Por tanto, en cualquier aplicación basada en este acoplamiento uno de los factores clave es la selección del sorbente. En este sentido, la variedad de sorbentes utilizados es muy amplia, desde los sorbentes cromatográficos convencionales de baja selectividad (C8, C18, HLB, intercambio catiónico o aniónico, etc.) hasta sorbentes más selectivos que contienen metales, polímeros de impresión molecular (*molecularly imprinted polymers* - MIPs), lectinas, anticuerpos, fragmentos de anticuerpos, aptámeros, etc. [29–31]. A modo de ejemplo, en un estudio reciente se desarrolló un método basado en *in-line* SPE-CE-MS para la determinación de α-sinucleína, una proteína neuronal, en sangre donde la preconcentración estaba asistida por aptámeros, un tipo de oligonucleótidos monocatenarios que pueden unirse a una molécula diana con alta afinidad y selectividad [121]. Esta metodología permitió obtener un LOD de 0,2 µg /mL, 100 veces menor que el obtenido mediante CE-ESI-MS. El método se aplicó posteriormente al análisis de α-sinucleína endógena en los fluidos resultantes de la lisis celular de los glóbulos rojos pertenecientes tanto a individuos sanos (muestra control) como a enfermos de Parkinson. En otro estudio se compararon tres tipos de sorbentes, uno mixto (Oasis HLB), uno de intercambio catiónico fuerte (Oasis MCX) y uno de intercambio aniónico fuerte (Oasis MAX) en una metodología basada en CE-ESI-MS para la determinación de filtros UV en muestras de agua de río [132]. El sorbente que mostró el mejor rendimiento y que se utilizó posteriormente para validar la metodología fue el Oasis MCX. Los LODs alcanzados para muestras estándar estuvieron en el rango de 0,01 a 0,05 ng/mL y los factores de enriquecimiento fueron de entre 3400 y 34000. Otro ejemplo de la variedad de sorbentes utilizados en *in-line* SPE-CE lo encontramos en un estudio reciente desarrollado por Moreno-González y colaboradores, en el que se estableció una metodología basada en *in-line* SPE-CE-ESI-MS/MS capaz de determinar patulina, una micotoxina, a niveles de 1 µg/kg en productos a base de manzana [128]. En esta metodología el sorbente utilizado en el preconcentrador consistía en un MIP

obtenido a partir de una columna de SPE comercial especialmente diseñada para la extracción de micotoxinas (AFFINIMIP) y, por tanto, era un tipo de sorbente muy selectivo. Debido a la introducción de este sorbente en el preconcentrador se pudo obtener un SEF de 1200 para la patulina. El método fue posteriormente aplicado a muestras reales (zumos de manzana y sidra) y se detectó la presencia de esta micotoxina en 9 de las 16 muestras analizadas, aunque siempre con valores por debajo del máximo establecido legalmente por la Unión Europea (50 μg/kg).

1.3.3.2. Técnicas de preconcentración basadas en principios electroforéticos

Otra de las alternativas para la preconcentración de muestra dentro del propio capilar de CE son aquellas estrategias donde esta preconcentración se logra a partir de procedimientos electroforéticos. Dentro de este tipo de procedimientos se encuentran las técnicas de apilamiento o *stacking*. Estas técnicas permiten concentrar los analitos de interés en una banda estrecha (*stack*) dentro del propio capilar de CE debido a una reducción abrupta y temporal de la velocidad de migración de estos analitos [32–36]. Estas técnicas se pueden agrupar en tres grandes grupos en función de los factores que inducen a la variación de la velocidad electroforética de los analitos [32]. Por un lado, encontramos aquellas técnicas donde estos cambios son debidos a alteraciones en el campo eléctrico. Al variar el campo eléctrico también lo hace la velocidad electroforética como indica la ecuación 1 (página 83) y por tanto estas variaciones pueden forzar a los analitos a apilarse en una banda estrecha. Por otro lado, están aquellas estrategias donde los analitos se apilan en la interfase muestra/BGE a través de un cambio en la velocidad electroforética producida por variaciones químicas entre la zona de la muestra y la del BGE. Estas variaciones pueden ser debidas a cambios en el pH o por la presencia de micelas u otros aditivos capaces de generar equilibrios de asociación con los compuestos estudiados. Finalmente, encontramos las metodologías donde se induce un cambio en la velocidad electroforética de los analitos al restringir físicamente su movimiento, generalmente, mediante uso de membranas nanoporosas. Al pasar a través de la membrana los analitos ven reducida su velocidad electroforética lo que permite concentrarlos en una banda estrecha.

En el caso específico de las catinonas, en el momento en el que se planteó esta Tesis Doctoral en bibliografía no se habían reportado estudios en relación a la determinación de estos compuestos mediante CE conjuntamente con técnicas de preconcentración electroforéticas. No obstante, dentro de nuestro grupo de investigación se cuenta con una amplia experiencia en el uso de estrategias de preconcentración electroforéticas basadas en la alteración del campo eléctrico para el análisis de parabenos [134], medicamentos antiinflamatorios [140,141] o barbitúricos [142]. Es por ello que en la presente Tesis Doctoral y con el objetivo de mejorar la sensibilidad de la CE para determinación de catinonas se decidió emplear este tipo de estrategias de preconcentración electroforéticas. Con el objetivo de profundizar en este tipo de metodologías, en los siguientes apartados se describen de forma extensa aquellas técnicas de preconcentración electroforéticas en que el proceso de *stacking* es debido a las variaciones del campo eléctrico. Concretamente,

se describen las siguientes técnicas: *field-amplified sample stacking*, *field-amplified sample injection*, *large-volume sample stacking, transient-isotachophoresis* y *electrokinetic supercharging*.

Field-amplified sample stacking* y *field-amplified sample injection

La *field-amplified sample stacking* (FASS) es una de las técnicas de preconcentración en línea basadas en principios electroforéticos más utilizada y, por tanto, más conocida. Como muestra el esquema de la Fig. 18A, en FASS la muestra se diluye en una solución de baja conductividad y se inyecta hidrodinámicamente dentro del capilar que, previamente, se ha llenado con un BGE de conductividad más elevada que la de la muestra. En estas condiciones, la aplicación de un elevado voltaje a través del capilar da como resultado una mayor intensidad del campo eléctrico en la zona de la muestra que en la zona del BGE. En consecuencia y, como se puede comprobar aplicando la ecuación 1 (página 83), los analitos en la zona de la muestra se desplazarán a una mayor velocidad electroforética que en la zona del BGE. Consecuentemente, los analitos se ralentizarán abruptamente al alcanzar la interfase muestra/BGE, lo que permite preconcentrarlos en la propia interfase [32,33].

Los SEFs obtenidos mediante esta técnica, habitualmente entre 10 o 20 [32], no son especialmente elevados en comparación con otras estrategias de preconcentración electroforéticas. En gran medida esto es debido a la limitada cantidad de muestra que se puede inyectar hidrodinámicamente mediante esta técnica, alrededor del 5% del volumen total del capilar, dado que volúmenes mayores pueden producir un importante ensanchamiento de banda de los picos de los analitos [143]. Sin embargo, debido a su simplicidad, que la hace compatible con diferentes tipos de BGE, esta estrategia de preconcentración se ha empleado en múltiples aplicaciones [35,144–147]. Por ejemplo, se ha utilizado para mejorar la sensibilidad de un método de CE, con un pretratamiento de muestra basado en una SPE con partículas magnéticas, para la determinación de varias drogas en orina [144]. Esta metodología permitió obtener LODs de entre 15 y 105 ng/mL para las diferentes drogas analizadas, lo que representó una mejora en la sensibilidad de alrededor 10 de veces respecto al procedimiento sin preconcentración. En otro estudio, Olędzka y colaboradores desarrollaron una metodología basada en FASS-MEKC para la determinación de benzodiazepinas, un tipo de medicamentos psicotrópicos, en muestras de orina [146]. Gracias a la etapa de preconcentración se consiguió un

aumento de 50 a 100 veces en la sensibilidad del método en comparación con el método MEKC sin FASS.

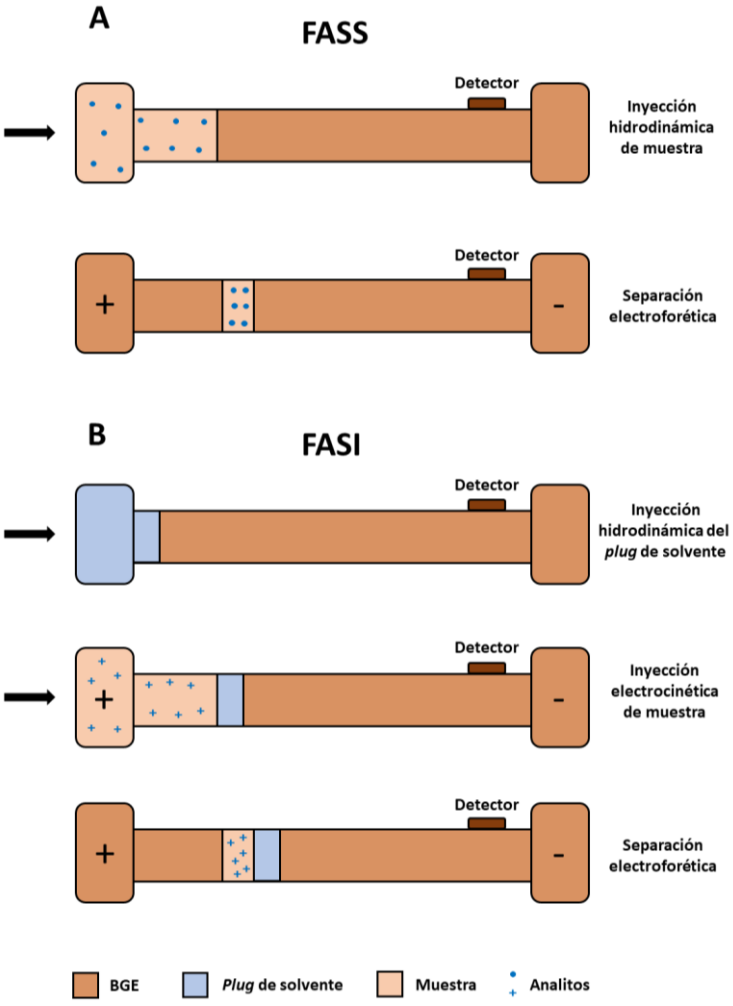

Figura 18. Esquema de las diferentes etapas durante un procedimiento de FASS (A) y un procedimiento de FASI (B).

La *field-amplified sample injection* (FASI) es otra técnica de gran popularidad dentro de las estrategias de *stacking,* principalmente, a causa de su sencillez. A diferencia de FASS, en esta técnica los analitos presentes en la muestra se inyectan electrocinéticamente, en lugar de hidrodinámicamente, tal como muestra el esquema de la Fig. 18B que representa de forma esquematizada las diferentes etapas

del proceso de FASI. Durante la etapa de inyección electrocinética (*electrokinetic injection* - EKI) los analitos cargados se introducen selectivamente en el sistema de CE debido su propia movilidad electroforética. Como ocurre en FASS, dentro del capilar los analitos son preconcentrados debido a la diferencia de conductividad entre la zona de la muestra, de baja conductividad, y la zona del BGE, de alta conductividad [32,33]. No obstante, debido a la selectividad de la EKI, en el extremo de entrada del capilar se puede acumular una gran concentración de compuestos cargados, aumentando de esta forma la conductividad en la zona de muestra y, por tanto, disminuyendo la intensidad del campo eléctrico de la misma. Esto puede afectar negativamente a la eficacia del proceso de *stacking* reduciendo los factores de preconcentración obtenido*s*. Por ello, previamente a la EKI de la muestra en FASI es habitual la inyección hidrodinámica de un pequeño *plug* de solvente de baja conductividad, generalmente un solvente orgánico, alguna solución hidroorgánica o incluso agua pura, con lo que se produce un aumento de la intensidad del campo eléctrico en la zona de inyección. Además, algunos autores han observado experimentalmente que la inyección de este *plug* permite aumentar la reproducibilidad del método [32].

Aunque con la técnica de FASI existe la posibilidad de mejorar la sensibilidad en hasta tres órdenes de magnitud, el hecho de que la muestra sea inyectada mediante una EKI solo permite concentrar analitos catiónicos o aniónicos en un mismo análisis. Además, al igual que en FASS, la inyección de grandes volúmenes de muestra en FASI puede producir un importante ensanchamiento de banda lo que limita la capacidad de preconcentración de esta técnica.

En la bibliografía es posible encontrar varias metodologías basadas en FASI para diferentes aplicaciones [35,134,148–154]. Por ejemplo, una estrategia de FASI-CE, en combinación con un pretratamiento basado en la microextracción líquido-líquido dispersiva (*dispersive liquid–liquid microextraction* - DLLME), consiguió SEFs de entre 40 y 130 para la determinación de tres drogas de abuso, MDMA, dietilamida de ácido lisérgico (LSD) y fenciclidina, en muestras de orina [149]. Mediante esta metodología se alcanzaron LODs de 1, 4,4 y 4,5 ng/mL para la MDMA, el LSD y la fenciclidina, respectivamente. En esta misma matriz biológica, la técnica de FASI también ha sido capaz de aumentar la sensibilidad de 5 a 10 veces en la determinación de la cocaína y sus metabolitos, con lo que se lograron LODs de entre 1,5 a 10 ng/mL, en una metodología de CE-ESI-MS con un pretratamiento de muestra basado en una SPE

[152]. En otro estudio, esta técnica ha permitido la obtención de SEFs de entre 78 y 94 y de LODs de entre 55,2 y 83,6 ng/mL en la determinación de tres anestésicos locales en orina sin necesidad de un pretratamiento previo, sencillamente diluyendo la muestra antes de inyectarla [153]. Cabe destacar, que en este estudio se observó una mejora en la repetibilidad y sensibilidad del método al inyectar un pequeño *plug* de metanol (MeOH) previamente a la inyección de la muestra.

Large-volume sample stacking

La principal característica de la técnica de *large-volume sample stacking* (LVSS) es la gran cantidad de muestra que se inyecta en el capilar. De hecho, en esta técnica se puede llegar a llenar hasta un 95% del volumen del capilar de muestra inyectada hidrodinámicamente. Posteriormente a esta inyección, la matriz de la muestra se elimina de forma continua. Durante el procedimiento de eliminación de la matriz los analitos se van apilando en la interfase muestra/BGE, debido a que su velocidad electroforética es mayor en la zona de la muestra que en la del BGE a causa de la diferencia de conductividad entre ambas zonas. Evidentemente, es esencial evitar que durante el proceso de eliminación de la matriz los analitos salgan por la entrada del capilar y, por ello, se debe controlar la transición entre el paso de eliminación de la matriz de la muestra y el inicio de la separación electroforética. Esta transición se realiza variando el EOF, ya sea mediante una conmutación de la polaridad o químicamente [32,33].

Cuando la LVSS se efectúa mediante un cambio de polaridad, la polaridad de los electrodos se invierte al final del proceso de eliminación de la matriz de la muestra tal y como muestra el esquema de la Fig. 19. Es importante remarcar que en este enfoque es fundamental que las movilidades electroforéticas de los analitos sean opuestas al EOF. Para asegurar que no se pierda parte de los analitos, la transición de la etapa de eliminación de la matriz a la etapa de separación electroforética se controla monitoreando la corriente. Cuando ésta alcanza el 90-95% de su valor respecto al capilar lleno con BGE, lo que indica que se ha eliminado la mayor parte de la matriz de la muestra, la polaridad se cambia para permitir la separación de los analitos preconcentrados [32,33,36]. Aunque la LVSS con conmutación de polaridad es muy popular, esta necesidad de monitorear meticulosamente la corriente hace de su automatización una tarea desafiante [32]. A pesar de ello, la técnica de LVSS con inversión de polaridad se ha utilizado para varias aplicaciones [35,134,155,156]. Por

ejemplo, para la determinación de barbitúricos en muestras biológicas obteniendo SEFs en el rango de 169,1 a 202,7 y límites de cuantificación (*limits of quantification* - LOQs) en el rango de 15 a 57 ng/mL [155]. Esta técnica de preconcentración también se ha empleado en el análisis de tetraciclinas en muestras de aguas de manantiales, de río y subterráneas [156]. En este caso, se obtuvieron SEFs de entre 303 y 428, y LODs de entre 20 y 50 ng/mL.

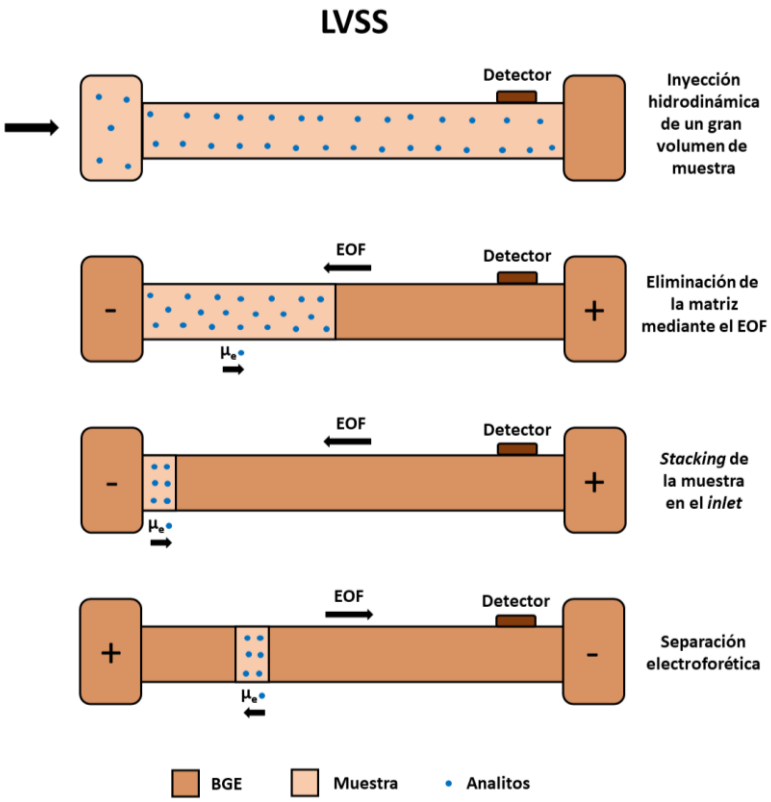

Figura 19. Esquema de las diferentes etapas durante un procedimiento de LVSS con variación del EOF mediante cambio de la polaridad.

Dentro del propio grupo de investigación, la técnica de LVSS se ha empleado para la preconcentración de parabenos, obteniendo un aumento de la sensibilidad de entre 53 a 77 veces con LODs de 3 a 4 ng/mL [134]. En este caso, el procedimiento de LVSS consistió primero en la inyección hidrodinámica de la muestra a 50 mbar durante 500 s. Seguidamente, para eliminar la matriz, se aplicó un voltaje negativo de -10 kV y cuando se alcanzó un 95% del valor de la corriente respecto al capilar

lleno con BGE, se procedió a aplicar un voltaje positivo de 20 kV para separar los compuestos.

Cuando la transición de la etapa de preconcentración a la de separación ocurre por medios químicos, la técnica se conoce como *large-volume sample stacking with an electroosmotic flow pump* (LVSEP) [32]. Como se muestra en el esquema de la Fig. 20 la clave de este enfoque es la progresiva reducción del EOF a medida que el capilar se llena de BGE [32,33]. Esto se consigue empleando BGEs ácidos o a los que se le ha adicionado un modificador del EOF, como la dietilentriamina o el óxido de polietileno. Una vez que la movilidad electroforética de los analitos es superior a la movilidad del EOF, éstos se dirigen hacia el detector. La ventaja de la LVSEP respecto a la LVSS es que la transición se controla químicamente y no manualmente y, por tanto, es más fácil de automatizar [32].

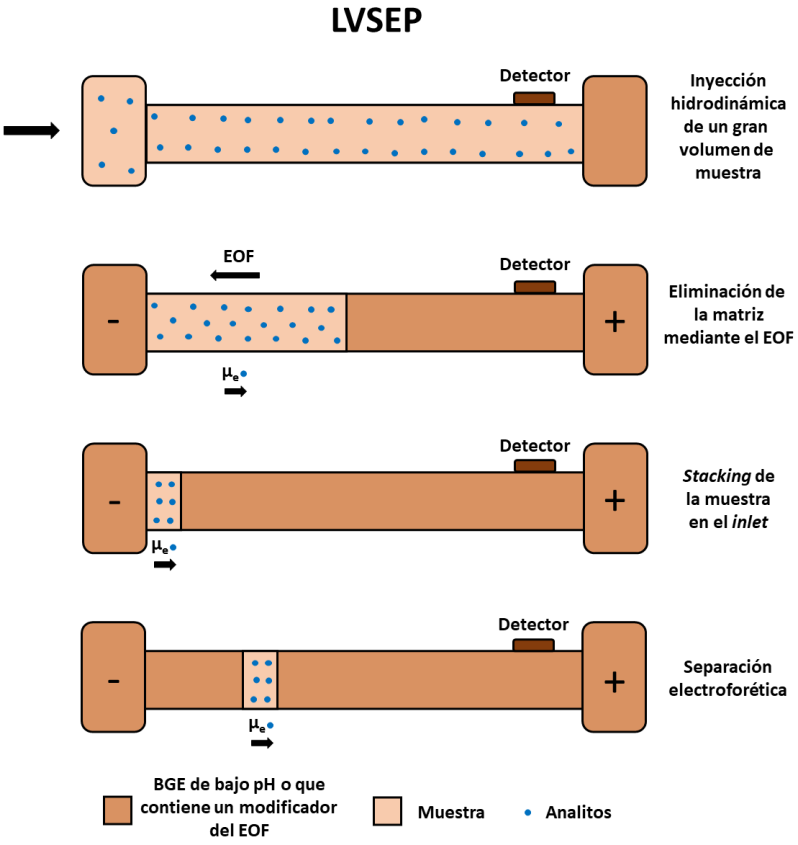

Figura 20. Esquema de las diferentes etapas durante un procedimiento de LVSEP.

La LVSEP se ha empleado para diferentes aplicaciones, por ejemplo, para la determinación de condroitín sulfato, dermatán sulfato y ácido hialurónico en muestras biológicas y cosméticas [157]. Con el objetivo de suprimir el EOF se empleó un BGE de bajo pH, concretamente una solución de dihidrogenofosfato de sodio 200 mM (pH 4). Esta metodología permitió obtener una respuesta 10 veces mayor que un procedimiento de CE sin preconcentración con lo que se consiguieron LODs de entre 100 a 500 ng/mL. En otro estudio, se desarrolló una metodología basada en LVSEP para la determinación simultánea de nitrato y nitrito en pescado [158]. En este caso, el EOF se suprimió mediante un BGE de ácido fórmico 30 mM (pH 4). La respuesta obtenida con esta estrategia de preconcentración fue 30 veces mayor que con una metodología de CE sin preconcentración lo que permitió obtener LODs en el rango de 0,55 a 0,82 µmol/L.

Transient-isotachophoresis

Cuando un procedimiento de isotacoforesis (*isotachophoresis* - ITP) se acopla de manera transitoria a la CZE, estamos ante una estrategia de preconcentración electroforética conocida como *transient-isotachophoresis* (*t*-ITP) [32]. Como muestra el esquema de la Fig. 21, en *t*-ITP los analitos se introducen hidrodinámicamente en el capilar entre un pequeño volumen de electrolito frontal (*leading electrolyte* - LE), que contiene iones con mayor movilidad que los analitos, y un pequeño volumen de electrolito terminal (*terminating electrolyte* - TE), que contiene iones con menor movilidad que los analitos. En estas condiciones, cuando se aplica un voltaje se crea un campo eléctrico no uniforme, siendo éste más intenso en la zona del LE que en la del TE. En consecuencia, y aplicando la ecuación 1 (página 83), los analitos migrarán más rápidamente en la zona del TE y más lentamente en la zona del LE, por lo que se preconcentran en bandas en función de su movilidad electroforética en la interfase LE/TE [27,32]. Posteriormente y a medida que avanza la separación electroforética, las bandas del LE y del TE se diluyen en el BGE y el sistema completa su transición hacia un procedimiento de CZE. Obviamente la composición del LE y del TE son factores determinantes para la correcta ejecución de esta técnica de preconcentración.

La *t*-ITP se ha utilizado satisfactoriamente en le preconcentración de diferentes tipos de analitos y en diversas aplicaciones [32,141,159–163]. Por ejemplo, Zheng y colaboradores combinaron esta técnica con un sistema de detección amperométrica

para el análisis de sustancias dopantes en muestras de orina [161]. El LE consistía en una solución de Tris-HCl mientras que como TE se empleó glicina. Mediante esta metodología se obtuvieron SEFs de entre 5115 y 5504. Estos factores de preconcentración permitieron obtener LODs de entre 0,042 a 0,95 pmol/L. En otro estudio se determinaron los niveles totales de diferentes aminotioles (cisteína, homocisteína y glutatión) en plasma sanguíneo mediante un pretratamiento basado en una derivatización y una LLE seguido de un procedimiento de *t*-ITP-CE [163]. En este caso se utilizó una solución de Cl^- como LE y ácido cítrico como TE. Con esta metodología se obtuvieron factores de enriquecimiento de entre 30 y 56 y LODs de 0,3 a 0,8 µmol/L.

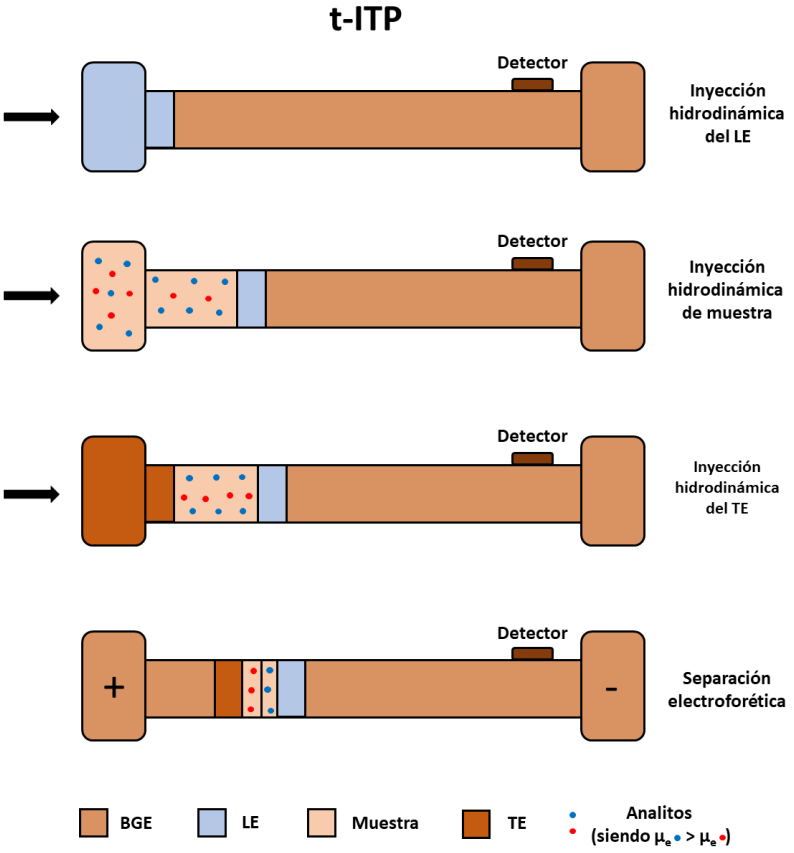

Figura 21. Esquema de las diferentes etapas durante un procedimiento de *t*-ITP.

La *t*-ITP incluso se ha combinado con otras técnicas de preconcentración en línea. Es el caso de un estudio llevado a cabo por Medina-Casanellas y colaboradores en el

que esta técnica se combinó con la técnica de *in-line* SPE-CE para el análisis de péptidos opioides en plasma humano mediante un sistema de detección de MS [159]. A pesar de las dificultades en la combinación de estas dos estrategias de preconcentración, es de destacar que la metodología de *in-line* SPE-*t*-ITP-CE-ESI-MS consiguió mejorar la sensibilidad en hasta 10 veces respecto al procedimiento de *in-line* SPE-CE-ESI-MS y en hasta 5000 veces respecto a *t*-ITP-CE-ESI-MS. No obstante, cabe remarcar que la repetibilidad del método combinado fue menor en comparación con la obtenida cuando ambas técnicas se aplican por separado.

Electrokinetic supercharging

En los últimos años, la combinación de dos o más estrategias de *stacking* para la preconcentración en CE ha supuesto una estrategia de gran interés para diversos autores. En este aspecto, la unión de FASI y *t*-ITP, representa una alternativa atractiva ya que ambas técnicas pueden complementarse y compensarse entre sí y, en consecuencia, es posible alcanzar unos menores LODs que aplicando cada una de estas metodologías por separado [32–34]. De hecho, esta combinación supera el principal inconveniente de FASI: la cantidad limitada de muestra que se puede inyectar antes de que el ensanchamiento excesivo de la banda afecte a la eficacia de la separación, puesto que el procedimiento adicional de *t*-ITP puede preconcentrar dichas bandas. La unión de estas dos estrategias fue introducida por primera vez por Hirokawa y colaboradores quienes la denominaron *electrokinetic supercharging* (EKS) [164].

Como muestra la Fig. 22 el procedimiento de esta técnica es similar al de la *t*-ITP. En concreto, la muestra se introduce entre un LE (compuesto por una solución de iones de alta movilidad) y un TE (compuesto por una solución de iones de baja movilidad). No obstante, a diferencia de *t*-ITP, donde la muestra se inyecta hidrodinámicamente, en EKS la muestra se introduce en el sistema de CE mediante una EKI. En estas condiciones, los compuestos estudiados se preconcentran en la interfase LE/TE y, a medida que avanza la separación electroforética, se produce la transición del sistema hacia una CZE [33,34].

La metodología de EKS ha sido empleada de manera altamente satisfactoria en los últimos años en varias aplicaciones [140,142,165–170]. Por ejemplo, para la determinación de sotalol y metoprolol, dos fármacos antihipertensivos, en muestras

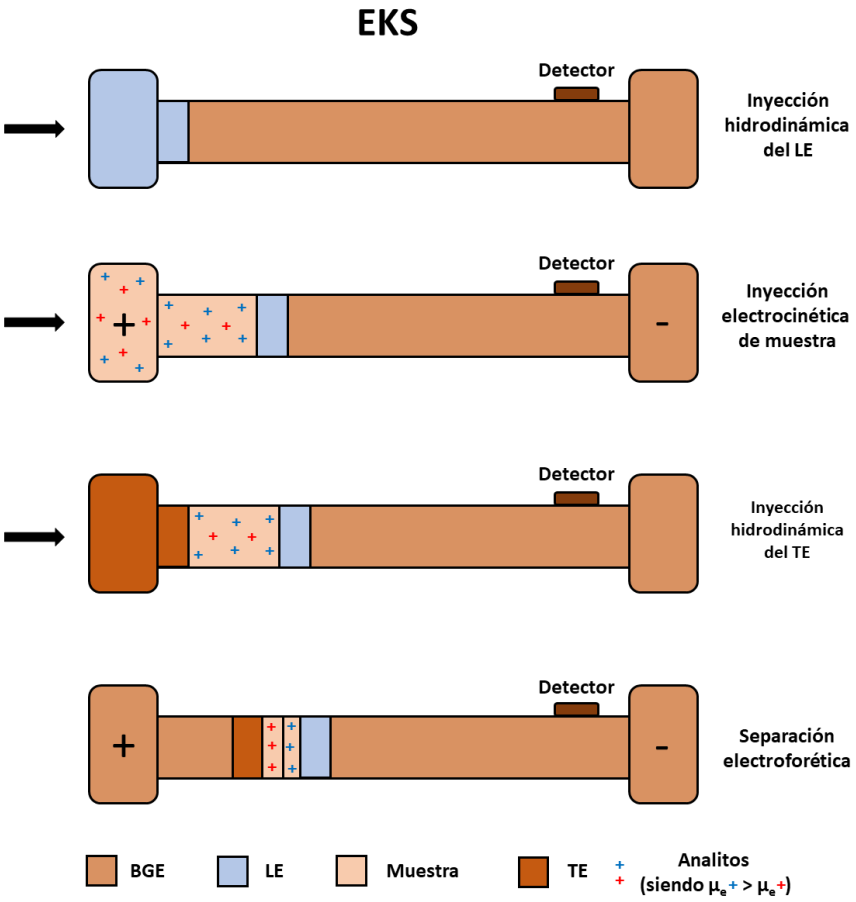

Figura 22. Esquema de las diferentes etapas durante un procedimiento de EKS.

de orina [168]. En este caso el LE era el propio BGE, un tampón de fosfato, mientras que el TE consistía en una solución de glicina. Los SEFs obtenidos en este estudio fueron de 1031 y 919 para el sotalol y el metoprolol, respectivamente. Estos altos factores de preconcentración permitieron obtener LODs de 5 ng/mL para el sotalol y de 10 ng/mL para el metoprolol. En otro estudio reciente, se desarrolló una metodología basada en EKS para la determinación de dos alcaloides, la berberina y la jatrorrizina [170]. En este estudio como LE se empleó una solución de KCl y como TE una solución de cloruro de dodecil trimetil amonio. Los resultados obtenidos mostraron un aumento en la sensibilidad respecto a una metodología de CE convencional de 2740 y 2928 veces para la berberina y la jatrorrizina, respectivamente, con LODs inferiores a 3 ng/mL en muestras estándar en ambos

casos. Posteriormente para demostrar la capacidad del método en el análisis de trazas de alcaloides en muestras biológicas complejas, éste fue aplicado a muestras fecales obtenidas de ratones a los que se les había administrado previamente los analitos estudiados. En este caso, previamente a sus análisis las muestras fecales fueron sometidas un pretratamiento basado en un SLE.

Dentro del propio grupo de investigación también se cuenta con experiencia en la aplicación de la técnica de EKS [140,142]. Por ejemplo, esta técnica fue empleada para la determinación de varios fármacos antiinflamatorios no esteroideos en muestras ambientales y biológicas, utilizando como LE una solución de NaCl y como TE una solución de ácido 2-(ciclohexilamino)etanosulfónico (CHES) [140]. Los SEFs conseguidos en este estudio fueron de entre 1615 y 2167. Estos altos factores de preconcentración permitieron la obtención de LODs del orden de pocos ng/mL, concretamente de 0,9 ng/mL para muestra de agua de río y de 2 ng/mL para muestras de plasma. En otro estudio se utilizó esta técnica de preconcentración para la determinación de barbitúricos en orina, empleando los mismos LE y TE que en el caso anterior [142]. En este caso, los SEFs obtenidos fueron de entre 875 y 1144 y los LODs de entre 1,5 y 2,1 ng/mL para muestras estándar.

1.4. Referencias

[1] Peacock, A., Bruno, R., Gisev, N., Degenhardt, L., Hall, W., Sedefov, R., White, J., Thomas, K. V., Farrell, M., Griffiths, P. Lancet 2019, 394, 1668–1684.

[2] United Nations Office on Drugs and Crime (UNODC) The challenge of new psychoactive substances, 2013.

[3] Nelson, M. E., Bryant, S. M., Aks, S. E. Emerg. Med. Clin. North Am. 2014, 32, 1–28.

[4] Gonçalves, J. L., Alves, V. L., Aguiar, J., Teixeira, H. M., Câmara, J. S. Crit. Rev. Toxicol. 2019, 49, 549–566.

[5] Larchenko, A. V., Suvorov, M. A., Andryukhin, V. I., Kaurov, Y. V., Suvorov, A. V. Sovrem. Tehnol. v Med. 2017, 9, 185–196.

[6] Prosser, J. M., Nelson, L. S. J. Med. Toxicol. 2012, 8, 33–42.

[7] German, C. L., Fleckenstein, A. E., Hanson, G. R. Life Sci. 2014, 97, 2–8.

[8] Valente, M. J., Guedes De Pinho, P., Lourdes Bastos, M., Carvalho, F., Carvalho, M. Arch. Toxicol. 2014, 88, 15–45.

[9] Riley, A. L., Nelson, K. H., To, P., López-Arnau, R., Xu, P., Wang, D., Wang, Y., Shen, H. wei, Kuhn, D. M., Angoa-Perez, M., Anneken, J. H., Muskiewicz, D., Hall, F. S. Neurosci. Biobehav. Rev. 2020, 110, 150–173.

[10] Couto, R. A. S., Gonçalves, L. M., Carvalho, F., Rodrigues, J. A., Rodrigues, C. M. P., Quinaz, M. B. Crit. Rev. Anal. Chem. 2018, 48, 372–390.

[11] Orsolini, L., Chiappini, S., Corkery, J. M., Guirguis, A., Papanti, D., Schifano, F. Expert Rev. Neurother. 2019, 19, 1253–1264.

[12] Rinaldi, R., Bersani, G., Marinelli, E., Zaami, S. Hum. Psychopharmacol. 2020, 35, e2727.

[13] Kraemer, M., Boehmer, A., Madea, B., Maas, A. Forensic Sci. Int. 2019, 298, 186–267.

[14] Silva, B., Fernandes, C., de Pinho, P. G., Remião, F. J. Anal. Toxicol. 2018, 42, 17–24.

[15] Schmid, M. G., Hägele, J. S. J. Chromatogr. A 2020, 1624, 461256.

[16] Lin, H. R., Kuo, F. W. Forensic Sci. Int. 2020, 317, 110528.

[17] Yu, R. B., Quirino, J. P. TrAC - Trends Anal. Chem. 2019, 118, 779–792.

[18] Ribeiro, C., Gonçalves, R., Tiritan, M. E. Crit. Rev. Anal. Chem. 2020.

[19] Modi, V. S., Patel, D. R., Bhai Prajapati, P. R., Basuri, T., Siddique, S. S. Int. J. Pharm. Sci. Rev. Res. 2016, 39, 156–162.

[20] Stavrou, I. J., Agathokleous, E. A., Kapnissi-Christodoulou, C. P. Electrophoresis 2017, 38, 786–819.

[21] Zhang, Q. TrAC - Trends Anal. Chem. 2018, 100, 145–154.

[22] Greño, M., Marina, M. L., Castro-Puyana, M. Crit. Rev. Anal. Chem. 2018, 48, 429–446.

[23] Fanali, S., Chankvetadze, B. Electrophoresis 2019, 40, 2420–2437.

[24] Yu, R. B., Quirino, J. P. Molecules 2019, 24, 1135–1153.

[25] Bernardo-Bermejo, S., Sánchez-López, E., Castro-Puyana, M., Marina, M. L. TrAC - Trends Anal. Chem. 2020, 124.

[26] de Koster, N., Clark, C. P., Kohler, I. Electrophoresis 2021, 42, 38–57.

[27] Skoog, D. A., James Holler, F., Crouch, S. R. Principios de análisis instrumental, Cengage Learning, Ciudad de México, 2008.

[28] Puig, P., Borrull, F., Calull, M., Aguilar, C. Anal. Chim. Acta 2008, 616, 1–18.

[29] Ramautar, R., Somsen, G. W., de Jong, G. J. Electrophoresis 2014, 35, 128–137.

[30] Ramautar, R., Somsen, G. W., de Jong, G. J. Electrophoresis 2016, 37, 35–44.

[31] Pont, L., Pero-Gascon, R., Gimenez, E., Sanz-Nebot, V., Benavente, F. Anal. Chim. Acta 2019, 1079, 1–19.

[32] Breadmore, M. C., Grochocki, W., Kalsoom, U., Phung, S. C., Rokh, M. T., Cabot, J. M., Ghiasvand, A., Li, F., Shallan, A. I., Keyon, A. S. A., Wuethrich, A. Electrophoresis 2019, 40, 17–39.

[33] Lian, D. S., Zhao, S. J., Li, J., Li, B. L. Anal. Bioanal. Chem. 2014, 406, 6129–6150.

[34] Lu, Y., Hou, X., Wang, D., Zhong, H. J. Liq. Chromatogr. Relat. Technol. 2017, 40, 528–535.

[35] Šlampova, A., Malá, Z., Gebauer, P. Electrophoresis 2019, 40, 40–54.

[36] Kitagawa, F., Kawai, T., Otsuka, K. Anal. Sci. 2013, 29, 1129–1139.

[37] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Legal approaches to controlling new psychoactive substances, 2016.

[38] World Health Organization (WHO) WHO recommends the most stringent level of international control for synthetic opioid carfentanil https://www.who.int/medicines/news/2017/WHO-recommends-most-stringent-level-int-control/en/ [accessed Sep 6, 2020].

[39] Alves, V. L., Gonçalves, J. L., Aguiar, J., Teixeira, H. M., Câmara, J. S. Crit. Rev. Toxicol. 2020, 50, 359–382.

[40] Abbate, V., Schwenk, M., Presley, B. C., Uchiyama, N. Pure Appl. Chem. 2018, 90, 1255–1282.

[41] King, L. A. Drug Test. Anal. 2014, 6, 808–818.

[42] Araújo, A. M., Carvalho, F., Bastos, M. de L., Guedes de Pinho, P., Carvalho, M. Arch. Toxicol. 2015, 89, 1151–1173.

[43] Salle, S., Bodeau, S., Dhersin, A., Ferdonnet, M., Goncalves, R., Lenski, M., Lima, B., Martin, M., Outreville, J., Vaucel, J., Fabresse, N. Toxicol. Anal. Clin. 2019, 31, 298–316.

[44] Karila, L., Marillier, M., Chaumette, B., Nicolas, F., Amine, B. Neurosci. Biobehav. Rev. 2019, 106, 133–140.

[45] Tabarra, I., Soares, S., Rosado, T., Gonçalves, J., Luís, Â., Malaca, S., Barroso, M., Keller, T., Restolho, J., Gallardo, E. Forensic Sci. Res. 2019, 4, 111–140.

[46] Zawilska, J. B., Wojcieszak, J. Neurotoxicology 2019, 73, 8–16.

[47] Manchester, K. R., Lomas, E. C., Waters, L., Dempsey, F. C., Maskell, P. D. Drug Test. Anal. 2018, 10, 37–53.

[48] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) European drug report 2019, 2019.

[49] Miliano, C., Margiani, G., Fattore, L., De Luca, M. A. Brain Sci. 2018, 8, 123.

[50] Nutt, D. Br. J. Clin. Pharmacol. 2020, 86, 445–451.

[51] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) EMCDDA operating guidelines for the European Union Early Warning System on new psychoactive substances, 2019.

[52] Sistema Español de Alerta Temprana (SEAT) Las Nuevas Sustancias Psicoactivas: un reto para la Salud Pública, 2014.

[53] Scherbaum, N., Schifano, F., Bonnet, U. Pharmacopsychiatry 2017, 50, 116–122.

[54] Peters, F. T. Bioanalysis 2014, 6, 2083–2107.

[55] Peters, F. T., Meyer, M. R. In Novel psychoactive substances: classification, pharmacology and toxicology, Dargan, P. I., Wood, D. M., Eds., Elsevier Inc., 2013, pp. 131–157.

[56] Baciu, T., Botello, I., Borrull, F., Calull, M., Aguilar, C. TrAC - Trends Anal. Chem. 2015, 74, 89–108.

[57] Bijlsma, L., Celma, A., López, F. J., Hernández, F. Curr. Opin. Environ. Sci. Heal. 2019, 9, 1–12.

[58] Øiestad, E. L., Øiestad, Å. M. L., Gjelstad, A., Karinen, R. Bioanalysis 2016, 8, 691–710.

[59] Kyriakou, C., Pellegrini, M., García-Algar, O., Marinelli, E., Zaami, S. Curr. Neuropharmacol. 2016, 15, 663–681.

[60] Engidawork, E. Phyther. Res. 2017, 31, 1019–1028.

[61] Al-Motarreb A, Baker K, B. K. Phyther. Res. 2002, 16, 403–413.

[62] Hyde, J. F., Browning, E., Adams, R. J. Am. Chem. Soc. 1928, 50, 2287–2292.

[63] Saem de Burnaga Sanchez, J. Bull. Soc. Chim. Fr. 1929, 45, 284–6.

[64] Emerson, T. S., Cisek, J. E. Ann. Emerg. Med. 1993, 22, 1897–1903.

[65] Davies, R. A., Baird, T. R., Nguyen, V. T., Ruiz, B., Sakloth, F., Eltit, J. M., Negus, S. S., Glennon, R. A. ACS Chem. Neurosci. 2020, 11, 1762–1769.

[66] Nelson, K. H., López-Arnau, R., Hempel, B. J., To, P., Manke, H. N., Crissman, M. E., Clasen, M. M., Rice, K. C., Riley, A. L. Psychopharmacology (Berl). 2019, 236, 1067–1077.

[67] Kolanos, R., Partilla, J. S., Baumann, M. H., Hutsell, B. A., Banks, M. L., Negus, S. S., Glennon, R. A. ACS Chem. Neurosci. 2015, 6, 771–777.

[68] Giné, C. V., Espinosa, I. F., Vilamala, M. V. Drug Test. Anal. 2014, 6, 819–824.

[69] Švidrnoch, M., Lněníčková, L., Válka, I., Ondra, P., Maier, V. J. Chromatogr. A 2014, 1356, 258–265.

[70] Gottardo, R., Sorio, D., Soldati, G., Ballotari, M., Porpiglia, N. M., Tagliaro, F. Electrophoresis 2021, 42, 450–459.

[71] Cheng, K. W., Hsieh, C. M., Chen, H. W., Chi, P. C., Yang, D. P., Chan, S. H., Chen, J. Y., Hwa, H. L., Fang, C. C., Weng, T. I., Chen, P. S. Rapid Commun. Mass Spectrom. 2020, 34, 1–6.

[72] Gerace, E., Caneparo, D., Borio, F., Salomone, A., Vincenti, M. J. Sep. Sci. 2019, 42, 1577–1584.

[73] Fan, S. Y., Zang, C. Z., Shih, P. H., Ko, Y. C., Hsu, Y. H., Lin, M. C., Tseng, S. H., Wang, D. Y. Forensic Sci. Int. 2020, 315, 110429.

[74] Pascual-Caro, S., Fontanals, N., Borrull, F., Aguilar, C., Calull, M. Forensic Toxicol. 2020, 38, 185–194.

[75] Meetani, M. A., Alremeithi, R. H., Mousa, M. K. J. Chromatogr. Sci. 2019, 57, 361–368.

[76] Alremeithi, R. H., Meetani, M. A., Khalil, S. A. RSC Adv. 2016, 6, 80576–80584.

[77] Alremeithi, R. H., Meetani, M. A., Alaidaros, A. A., Lanjawi, A., Alsumaiti, K. J. Anal. Methods Chem. 2018, 2018, 4396043.

[78] Bakdash, A. Open Chem. 2019, 17, 902–911.

[79] Woźniak, M. K., Banaszkiewicz, L., Wiergowski, M., Tomczak, E., Kata, M., Szpiech, B., Namieśnik, J., Biziuk, M. Forensic Toxicol. 2020, 38, 42–58.

[80] Margalho, C., Almeida, P., Rosado, T., Corte Real, F., Gallardo, E. J. Anal. Toxicol. 2020, 44, 92–102.

[81] Nowak, K., Szpot, P., Zawadzki, M. J. Forensic Sci. 2020, 65, 1784–1790.

[82] Baciu, T., Borrull, F., Calull, M., Aguilar, C. Electrophoresis 2016, 37, 2352–2362.

[83] Freni, F., Bianco, S., Vignali, C., Groppi, A., Moretti, M., Osculati, A. M. M., Morini, L. Forensic Sci. Int. 2019, 298, 115–120.

[84] Niebel, A., Krumbiegel, F., Hartwig, S., Parr, M. K., Tsokos, M. Forensic Sci. Med. Pathol. 2020, 16, 32–42.

[85] Sorribes-Soriano, A., Esteve-Turrillas, F. A., Armenta, S., Amorós, P., Herrero-Martínez, J. M. Anal. Chim. Acta 2019, 1052, 73–83.

[86] Fernández, P., Regenjo, M., Ares, A., Fernández, A. M., Lorenzo, R. A., Carro, A. M. Anal. Bioanal. Chem. 2019, 411, 193–203.

[87] Cooman, T., Santos, H., Cox, J., Filho, J. F. A., Borges, K. B., Romão, W., Arroyo-Mora, L. E. Forensic Chem. 2020, 19, 100231.

[88] López-Rabuñal, Á., Lendoiro, E., Concheiro, M., López-Rivadulla, M., Cruz, A., de-Castro-Ríos, A. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2019, 1124, 349–355.

[89] Vesterberg, O. Electrophoresis 1993, 14, 1243–1249.

[90] Silva, M. Electrophoresis 2013, 34, 141–158.

[91] Taschwer, M., Weiß, J. A., Kunert, O., Schmid, M. G. Forensic Sci. Int. 2014, 244, e56–e59.

[92] Li, L., Lurie, I. S. Forensic Sci. Int. 2015, 254, 148–157.

[93] Merola, G., Fu, H., Tagliaro, F., Macchia, T., McCord, B. R. Electrophoresis 2014, 35, 3231–3241.

[94] Moini, M., Rollman, C. M. Rapid Commun. Mass Spectrom. 2015, 29, 304–310.

[95] Rollman, C. M., Moini, M. Rapid Commun. Mass Spectrom. 2016, 30, 2070–2076.

[96] Woźniakiewicz, M., Nowak, P. M., Gołąb, M., Adamowicz, P., Kała, M., Kościelniak, P. Talanta 2018, 180, 193–198.

[97] Hägele, J. S., Hubner, E. M., Schmid, M. G. Electrophoresis 2019, 1787–1794.

[98] Řezanka, P., Macková, D., Jurok, R., Himl, M., Kuchař, M. Molecules 2020, 25, 2879.

[99] Nowak, P. M., Olesek, K., Woźniakiewicz, M., Kościelniak, P. Electrophoresis 2018, 39, 2406–2409.

[100] Mohr, S., Pilaj, S., Schmid, M. G. Electrophoresis 2012, 33, 1624–1630.

[101] Nowak, P. M., Woźniakiewicz, M., Mitoraj, M., Sagan, F., Kościelniak, P. J. Chromatogr. A 2018, 1539, 78–86.

[102] Aturki, Z., Fanali, S., Rocco, A. Electrophoresis 2016, 37, 2875–2881.

[103] Taschwer, M., Hofer, M. G., Schmid, M. G. Electrophoresis 2014, 35, 2793–2799.

[104] Emonts, P., Servais, A. C., Ziemons, E., Hubert, P., Fillet, M., Dispas, A. Electrophoresis 2021, 42, 1127–1134.

[105] Lloyd, A., Russell, M., Blanes, L., Somerville, R., Doble, P., Roux, C. Forensic Sci. Int. 2014, 242, 16–23.

[106] Weiß, J. A., Mohr, S., Schmid, M. G. Chirality 2015, 27, 211–215.

[107] Fu, Y., Borrull, F., Fontanals, N., Marcé, R. M. Chirality 2020, 32, 876–884.

[108] Bonvin, G., Schappler, J., Rudaz, S. J. Chromatogr. A 2012, 1267, 17–31.

[109] Zhong, X., Zhang, Z., Jiang, S., Li, L. Electrophoresis 2014, 35, 1214–1225.

[110] Lindenburg, P. W., Haselberg, R., Rozing, G., Ramautar, R. Chromatographia 2014, 78, 367–377.

[111] Cheng, H., Li, P., Liu, J., Ye, M. J. Anal. At. Spectrom. 2016, 31, 1780–1810.

[112] Stolz, A., Jooß, K., Höcker, O., Römer, J., Schlecht, J., Neusüß, C. Electrophoresis 2019, 40, 79–112.

[113] Castro-Puyana, M., Marina, M. L. In Chiral Separations: Methods and Protocols, Methods in Molecular Biology, Scriba, G. K. E., Ed., Springer Science+Business Media, New York, 2019, Vol. 1985, pp. 391–404.

[114] Gahoual, R., Leize-Wagner, E., Houzé, P., François, Y. N. Rapid Commun. Mass Spectrom. 2019, 33, 11–19.

[115] Hägele, J. S., Schmid, M. G. Chirality 2018, 30, 1019–1026.

[116] Mikuma, T., Iwata, Y. T., Miyaguchi, H., Kuwayama, K., Tsujikawa, K., Kanamori, T., Inoue, H. Forensic Sci. Int. 2015, 249, 59–65.

[117] Rossi, A. De, Desiderio, C. J. Chromatogr. B 2006, 839, 6–11.

[118] Pont, L., Benavente, F., Barbosa, J., Sanz-Nebot, V. Talanta 2017, 170, 224–232.

[119] Tascon, M., Gagliardi, L. G., Benavente, F. Anal. Chim. Acta 2017, 954, 60–67.

[120] Pont, L., Benavente, F., Jaumot, J., Sanz-nebot, V. Electrophoresis 2016, 37, 795–808.

[121] Pero-Gascon, R., Benavente, F., Minic, Z., Berezovski, M. V., Sanz-Nebot, V. Anal. Chem. 2020, 92, 1525−1533.

[122] Namera, A., Nakamoto, A., Saito, T., Miyazaki, S. J. Sep. Sci. 2011, 34, 901–924.

[123] Zhang, S., Miroslav, M., Haddad, P. R. Electrophoresis 2006, 27, 1069–1077.

[124] Janini, G. M., Zhou, M., Yu, L., Blonder, J., Gignac, M., Conrads, T. P., Issaq, H. J., Veenstra, T. D. Anal. Chem. 2003, 75, 5984–5993.

[125] Wu, Y., Zhang, W., Chen, Z. Electrophoresis 2012, 33, 2911–2919.

[126] Ortiz-villanueva, E., Benavente, F., Giménez, E., Yilmaz, F., Sanz-nebot, V. Anal. Chim. Acta 2014, 846, 51–59.

[127] Weng, Q., Fu, L., Li, X., An, R., Li, J. Anal. Chim. Acta 2015, 857, 46–52.

[128] Moreno-González, D., Jáč, P., Riasová, P., Nováková, L. Food Chem. 2021, 334, 127607.

[129] Wang, S., Xie, T., Dong, X., Hu, J., Men, L., Cao, J. Food Anal. Methods 2019, 12, 2139–2149.

[130] Pero-Gascon, R., Pont, L., Sanz-Nebot, V., Benavente, F. Methods Mol. Biol. 2019, 1972, 57–76.

[131] Baciu, T., Borrull, F., Neusüß, C., Aguilar, C., Calull, M. Electrophoresis 2016, 37, 1232–1244.

[132] Maijó, I., Fontanals, N., Borrull, F., Neusüß, C., Calull, M., Aguilar, C. Electrophoresis 2013, 34, 374–382.

[133] Botello, I., Borrull, F., Aguilar, C., Calull, M. Anal. Sci. 2014, 30, 971–977.

[134] Maijó, I., Borrull, F., Aguilar, C., Calull, M. Electrophoresis 2013, 34, 363–373.

[135] Maijó, I., Borrull, F., Calull, M., Aguilar, C. Electrophoresis 2011, 32, 2114–2122.

[136] Botello, I., Borrull, F., Aguilar, C., Calull, M. Electrophoresis 2012, 33, 528–535.

[137] Botello, I., Borrull, F., Calull, M., Aguilar, C., Somsen, G. W., De Jong, G. J. Anal. Bioanal. Chem. 2012, 403, 777–784.

[138] Jooß, K., Sommer, J., Bunz, S.-C., Neusüß, C. Electrophoresis 2014, 35, 1236–1243.

[139] Morales-Cid, G., Diez-Masa, J. C., de Frutos, M. Anal. Chim. Acta 2013, 773, 89–96.

[140] Botello, I., Borrull, F., Aguilar, C., Calull, M. Electrophoresis 2010, 31, 2964–2973.

[141] Botello, I., Borrull, F., Calull, M., Aguilar, C. Anal. Bioanal. Chem. 2011, 400, 527–534.

[142] Botello, I., Borrull, F., Calull, M., Aguilar, C. J. Sep. Sci. 2013, 36, 524–531.

[143] Chien, R. L., Dean, S. B. Anal. Chem. 1992, 64, 1046–1050.

[144] Chen, M. L., Suo, L. L., Gao, Q., Feng, Y. Q. Electrophoresis 2011, 32, 2099–2106.

[145] Huang, S. W., Hsieh, M. M., Chang, S. Y. Talanta 2012, 101, 460–464.

[146] Olędzka, I., Kulińska, Z., Prahl, A., Baczek, T. J. Anal. Toxicol. 2015, 39, 436–443.

[147] Olędzka, I., Kowalski, P., Plenis, A., Bączek, T. Electrophoresis 2017, 38, 1632–1643.

[148] Alshana, U., Göğer, N. G., Ertaş, N. J. Sep. Sci. 2012, 35, 2114–2121.

[149] Airado-Rodríguez, D., Cruces-Blanco, C., García-Campaña, A. M. J. Chromatogr. A 2012, 1267, 189–197.

[150] Chen, X., Tang, Y., Wang, S., Song, Y., Tang, F., Wu, X. Electrophoresis 2015, 36, 1953–1961.

[151] Fazeli-Bakhtiyari, R., Sorouraddin, M. H., Farajzadeh, M. A., Somi, M. H., Jouyban, A. Bioanalysis 2014, 6, 319–334.

[152] Hezinová, V., Aturki, Z., Klepárník, K., D'Orazio, G., Foret, F., Fanali, S. Electrophoresis 2012, 33, 653–660.

[153] López Guerrero, M. del M., Hernández-Mesa, M., Cruces-Blanco, C., García-Campaña, A. M. Electrophoresis 2015, 36, 2961–2967.

[154] Moreno-González, D., Krulišová, M., Gámiz-Gracia, L., García-Campaña, A. M. Electrophoresis 2018, 39, 608–615.

[155] Fan, L. Y., He, T., Tang, Y. Y., Zhang, W., Song, C. J., Zhao, X., Zhao, X. Y., Cao, C. X. J. Forensic Sci. 2012, 57, 813–819.

[156] Moreno-González, D., Lupión-Enríquez, I., García-Campaña, A. M. Electrophoresis 2016, 37, 1212–1219.

[157] Chindaphan, K., Wongravee, K., Nhujak, T., Dissayabutra, T., Srisa-Art, M. J. Sep. Sci. 2019, 42, 2743–2882.

[158] Kalaycıoğlu, Z., Erim, F. B. Food Anal. Methods 2016, 9, 706–711.

[159] Medina-Casanellas, S., Benavente, F., Barbosa, J., Sanz-Nebot, V. Electrophoresis 2011, 32, 1750–1759.

[160] Chen, Y., Zhang, L., Xu, L., Lin, J., Chen, G. Electrophoresis 2009, 30, 2300–2306.

[161] Zheng, L., Zhang, L., Tong, P., Zheng, X., Chi, Y., Chen, G. Talanta 2010, 81, 1288–1294.

[162] Fukushi, K., Fujita, Y., Nonogaki, J., Tsujimoto, J. ichi, Hattori, T., Inui, H., Beškoski, V. P., Hotta, H., Hayashi, M., Nakano, T. Anal. Bioanal. Chem. 2018, 410, 1825–1831.

[163] Ivanov, A. V., Nikiforova, K. A., Bulgakova, P. O., Virus, E. D., Kubatiev, A. A. Anal. Sci. 2018, 34, 505–508.

[164] Hirokawa, T., Okamoto, H., Gaš, B. Electrophoresis 2003, 24, 498–504.

[165] Dawod, M., Breadmore, M. C., Guijt, R. M., Haddad, P. R. Electrophoresis 2010, 31, 1184–1193.

[166] Abdul Karim, N. 'I, Wan Ibrahim, W. A., Sanagi, M. M., Abdul Keyon, A. S. Electrophoresis 2016, 37, 2649–2656.

[167] Thang, L. Y., Breadmore, M. C., See, H. H. J. Chromatogr. A 2016, 1461, 185–191.

[168] Xu, L., Meng, W., Lu, J., Cui, F., Gao, L., Chen, L., Xin, Y. J. Sep. Sci. 2020, 43, 2013–2248.

[169] Wells, S. S., Dawod, M., Kennedy, R. T. Electrophoresis 2019, 40, 2946–2953.

[170] Yan, J., Lian, L., Mao, H. Biomed. Chromatogr. 2020, 34, e4824.

2. OBJETIVOS

Teniendo en cuenta lo expuesto en la introducción y considerando el reto que supone para la comunidad científica el desarrollo de estrategias para la determinación de NPS como las catinonas, el objetivo principal de la presente Tesis Doctoral consiste en el desarrollo de metodologías analíticas capaces de lograr la enantiodeterminación de catinonas sintéticas en muestras de orina mediante CE. Además, con el propósito de alcanzar los bajos niveles de concentración a los cuales se pueden encontrar generalmente estos compuestos en esta matriz, del orden de ng/mL, se evaluarán diferentes estrategias de preconcentración en línea con la CE. Con la finalidad de lograr estos objetivos principales, se han establecido una serie de objetivos específicos que se indican a continuación:

- Evaluar diferentes selectores quirales para obtener la enantioseparación de las catinonas objeto de estudio.

- Estudiar las posibilidades ofrecidas por la técnica de *in-line* SPE-CE en la preconcentración de catinonas, poniendo especial énfasis en los factores que afectan a la carga de la muestra y al diseño del preconcentrador.

- Desarrollar un procedimiento *in-line* SPE-CE acoplado a la MS para la preconcentración y enantiodeterminación de catinonas en orina.

- Examinar y aplicar diferentes estrategias de preconcentración basadas en principios electroforéticos, concretamente FASI y EKS, en combinación con la CE para la preconcentración y enantiodeterminación de catinonas en orina.

3. PARTE EXPERIMENTAL, RESULTADOS Y DISCUSIÓN

Tal y como se ha comentado en la introducción, en los últimos años ha aumentado de forma considerable el consumo de los derivados sintéticos de la catinona y, por tanto, se requieren métodos analíticos capaces de monitorizar el consumo de estas sustancias. La separación enantiomérica de estos compuestos también resulta de gran interés puesto que cada enantiómero puede presentar diferente comportamiento farmacocinético y farmacodinámico. En este sentido y debido a las ventajas ofrecidas por parte de la CE para la obtención de una separación quiral, dentro del marco de la presente Tesis Doctoral se han desarrollado diversos estudios con el objetivo de establecer metodologías analíticas para la enantiodeterminación de catinonas sintéticas mediante esta técnica. Además, estas metodologías han buscado la combinación de la enantioseparación de catinonas con el uso de técnicas de preconcentración en línea con la finalidad de aumentar la sensibilidad de las metodologías desarrolladas. La mayoría de la parte experimental se ha realizado dentro del grupo de investigación de "Cromatografía, Aplicaciones Medioambientales (CROMA)" de la Universidad Rovira i Virgili que cuenta con una dilatada experiencia en el uso de técnicas de preconcentración en línea para el análisis de muestras biológicas mediante CE. Además, durante el desarrollo de la Tesis se realizó una estancia en el grupo de investigación "Bioanálisis" de la Universidad de Barcelona con el objetivo de crear sinergias y aprovechar su amplia experiencia en el acoplamiento entre la CE y la MS. Es también necesario remarcar que a lo largo de la esta Tesis Doctoral se ha contado con la financiación del Ministerio de Ciencia, Innovación y Universidades y del Fondo Europeo de Desarrollo Regional (FEDER) (Proyectos: CTQ2014-52617 y CTQ2017-88548-P).

Este capítulo contiene la parte experimental, los resultados y la discusión de los diferentes estudios de investigación que se han llevado a cabo en esta Tesis Doctoral. Puesto que estos estudios ya han sido publicados en diferentes revistas científicas de ámbito internacional, en las siguientes páginas se incluyen sus publicaciones derivadas en formato de artículo científico en inglés. Los estudios realizados se dividen en dos bloques, el primero referente a la aplicación de la técnica de *in-line* SPE-CE (sección 3.1) y el segundo relacionado con la aplicación de diferentes estrategias de preconcentración electroforéticas (sección 3.2), en ambos casos para la determinación de catinonas en orina. Además, cada uno de estos bloques incluye una breve introducción en la que se describe el contexto de la investigación y una discusión de los resultados obtenidos más relevantes. Para más

información, el anexo II presenta un listado con las publicaciones derivadas de la presente Tesis Doctoral.

3.1. Determinación de catinonas en orina mediante *in-line* SPE-CE

La baja sensibilidad de la CE, especialmente cuando se usa en combinación a la detección UV, puede suponer una desventaja en comparación con otras estrategias basadas en LC o GC. Una posible solución a esta limitación es el uso de técnicas de preconcentración en línea basadas en principios cromatográficos como la *in-line* SPE-CE [1–5].

En la estrategia de *in-line* SPE-CE basada en el diseño de *fritless packed bed* se llena un pequeño trozo de capilar de partículas de sorbente y se sitúa entre el extremo del capilar por donde se inyecta la muestra o *inlet* y el propio capilar de separación. Las partículas de sorbente quedan retenidas en el preconcentrador debido a que su tamaño es ligeramente mayor al diámetro interno de los dos capilares entre los que está situado el preconcentrador. Los altos factores de preconcentración obtenidos mediante esta técnica son debidos a la retención de los analitos de interés en el propio preconcentrador. No obstante, para obtener estos altos factores de preconcentración generalmente es necesario un elevado tiempo de carga de muestra lo que aumenta el tiempo de análisis total requerido en este tipo de estrategias. Es el caso de una metodología basada en el acoplamiento *in-line* SPE-CE-UV para la enantiodeterminación de diversas catinonas en muestras de cabello desarrollada dentro del propio grupo de investigación [6]. Concretamente, con el objetivo de obtener LODs adecuados para la determinación de estos compuestos en esta matriz biológica fue necesario introducir la muestra en el capilar a una presión de 930 mbar durante 40 min. Por tanto, a pesar de que con esta estrategia de preconcentración se aumentó de forma considerable la sensibilidad del método, el elevado tiempo asociado a la carga de muestra puede suponer un inconveniente importante para análisis rutinarios. Con el objetivo de superar esta desventaja, como parte de la línea de investigación de la presente Tesis Doctoral se planteó la optimización del procedimiento de carga de muestra, es decir de la presión aplicada durante la inyección, con la finalidad de reducir el tiempo necesario de introducción de muestra sin perder sensibilidad.

Las dimensiones del preconcentrador (longitud y diámetro interno) también pueden afectar a la sensibilidad del sistema de *in-line* SPE-CE [7]. Esto es debido a que estas tienen una influencia considerable en la cantidad de sorbente presente en el mismo y, por lo tanto, pueden influir en la cantidad de analitos retenidos. Por ello, también se planteó como objetivo de la parte experimental de la Tesis Doctoral evaluar como afectaba la longitud y el diámetro interno del preconcentrador en la

respuesta obtenida. Finalmente, otro de los retos planteados fue aplicar el método desarrollado al análisis de catinonas en muestras de orina.

Otro de los objetivos perseguidos en la presente Tesis Doctoral era utilizar como sistema de detección la MS en combinación con la enantiodeterminación y la preconcentración con *in-line* SPE-CE. Sin embargo, hay que tener en cuenta que el uso de la MS junto con la CE está sometido a ciertas restricciones, especialmente en la selección de un BGE adecuado. Estas restricciones se ven generalmente incrementadas si se pretende conseguir la separación quiral de los analitos. Esto es debido a que un selector quiral no volátil, como es el caso de las CDs, puede contaminar la fuente de ionización reduciendo de este modo la sensibilidad del método [8]. Sin embargo, no siempre es posible el uso de selectores quirales volátiles y es por ello que con la finalidad de evitar la contaminación de la fuente de ionización en la bibliografía se han empleado diversas estrategias. Entre todas ellas destacan dos, la CMT [8–11], en la que se emplea un selector quiral cargado que migra alejándose del espectrómetro de masas en la dirección opuesta a los analitos, y la PFT [8,9,12,13], en la que solo una parte del capilar se llena con el BGE que contiene el selector quiral evitando de esta manera su entrada en el sistema de detección. Es por ello que en el estudio que se incluye en la sección 3.1.2 se optó por evaluar ambas técnicas. Cabe remarcar que este último estudio fue realizado en colaboración con el grupo de investigación "Bioanálisis" de la Universidad de Barcelona debido a la amplia experiencia con la que contaban respecto al acoplamiento entre la CE y la MS y su utilización junto a estrategias de preconcentración en línea. Además, es de destacar que el método surgido de este estudio es el primero en el que se combinan estas dos estrategias (CE-MS y preconcentración en línea) con la enantiodeterminación.

A continuación, en los apartados 3.1.1. y 3.1.2 se incluyen los artículos científicos surgidos de ambas investigaciones. Concretamente, estos han sido publicados en las revistas científicas *Electrophoresis 40 (2019) 1762-1770* y *Talanta 225 (2021) 121994*, respectivamente.

Referencias

[1] Puig, P., Borrull, F., Calull, M., Aguilar, C., *Anal. Chim. Acta* 2008*, 616*, 1–18.

[2] Ramautar, R., Somsen, G. W., Jong, G. J. de, *Electrophoresis* 2014, *35*, 128–137.

[3] Ramautar, R., Somsen, G. W., Jong, G. J. de, *Electrophoresis* 2016, *37*, 35–44.

[4] Pont, L., Pero-Gascon, R., Gimenez, E., Sanz-Nebot, V., Benavente, F., *Anal. Chim. Acta* 2019, *1079*, 1–19.

[5] Breadmore, M. C., Grochocki, W., Kalsoom, U., Phung, S. C., Rokh, M. T., Cabot, J. M., Ghiasvand, A., Li, F., Shallan, A. I., Keyon, A. S. A., Wuethrich, A., *Electrophoresis* 2019, *40*, 17–39.

[6] Baciu, T., Borrull, F., Calull, M., Aguilar, C., *Electrophoresis* 2016, *37*, 2352–2362.

[7] Jooß, K., Sommer, J., Bunz, S.-C. C., Neusüß, C., *Electrophoresis* 2014, *35*, 1236–1243.

[8] Castro-Puyana, M., Marina, M. L., In *Chiral Separations: Methods and Protocols, Methods in Molecular Biology*, Scriba, G. K. E., Ed., Springer Science+Business Media, New York, 2019, *Vol*. 1985, pp. 391–404.

[9] Merola, G., Fu, H., Tagliaro, F., Macchia, T., McCord, B. R., *Electrophoresis* 2014, *35*, 3231–3241.

[10] Moini, M., Rollman, C. M., *Rapid Commun. Mass Spectrom.* 2015, *29*, 304–310.

[11] Mikuma, T., Iwata, Y. T., Miyaguchi, H., Kuwayama, K., Tsujikawa, K., Kanamori, T., Inoue, H., *Forensic Sci. Int.* 2015, *249*, 59–65.

[12] Xia, S., Zhang, L., Lu, M., Qiu, B., Chi, Y., Chen, G., *Electrophoresis* 2009, *30*, 2837–2844.

[13] Lee, S., Kim, S. J., Bang, E., Na, Y. C., *J. Chromatogr. A* 2019, *1586*, 128–138.

3.1.1. Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE

ENANTIOSELECTIVE DETERMINATION OF CATHINONES IN URINE BY HIGH PRESSURE IN-LINE SPE-CE

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Abstract

This work presents a strategy based on the in-line coupling of SPE and CE for the chiral determination of cathinones (*R,S*-mephedrone, *R,S*-4-methylephedrine and *R,S*-methylenedioxypyrovalerone) in urine samples, using a sample pretreatment based on liquid-liquid extraction. The chiral separation of the compounds is achieved by adding a mixture of 8 mM 2-hydroxypropil β-CD and 5 mM β-CD to the BGE, which consists of 70 mM of monosodium phosphate aqueous solution at pH 2.5. Oasis HLB was the selected sorbent for the in-line SPE device, and to reduce analysis time and LODs, several parameters affecting the in-line SPE system were evaluated, such as pressure and time of sample injection and dimensions of the SPE device. The highest preconcentration factors were achieved by using 3 bar of injection pressure for 20 min with an in-line SPE device of 2 mm length and 150 µm of i.d. The developed method was applied to determine the presence of the compounds in spiked urine samples. The LODs obtained were between 3 and 8 ng/mL, and these levels were below the usual concentrations at which these drugs are present in urine from cathinone abusers. Thus, the optimized method has the potential to be applied for toxicological and forensic purposes.

Keywords: *Cathinones / Chiral CE / In-line solid phase extraction / Oasis HLB / Urine analysis*

1 Introduction

In recent years, the consumption of synthetic derivatives of cathinone, an alkaloid naturally found in the leaves of the khat plant, has increased due to its stimulating properties and low price compared with amphetamines [1–3]. These synthetic cathinones can be found, for example, as "bath salts" on the internet with a label stating "not for human consumption". They are currently one of the most commonly abused drugs, hence the increased interest among of the police and health authorities in detecting and control these substances. This is reflected in the emergence of extensive literature on the detection and determination of cathinones [4–18].

Mephedrone and methylenedioxypyrovalerone (MDPV) are major components of these bath salts and both present chiral centres, which implies the presence of two enantiomers (*R* and *S*) [1,7,11,12,16,18–22]. It is important to highlight that drug enantiomers can follow different metabolic pathways in humans. Therefore, each enantiomer can present different pharmacokinetic and pharmacodynamic behaviour and have different effects on the organism, and in this regard some authors have suggested that the *S(-)* enantiomer is more biologically active than the *R*(-) form [1,7,19–22]. Moreover, the enantiomers ratio could provide useful information about the drug synthetic route and help in tracking its production [7,22]. For these reasons the enantioseparation of these compounds is important.

CE is currently proving to be an effective technique for performing chiral separation relatively easily, since it is possible to achieve enantioseparations by simply dissolving a chiral selector in the BGE [11,12,16,18,22–26]. Cyclodextrins are by far the most popular chiral selectors due, to their low toxicity, high solubility in virtually all aqueous background electrolytes, UV transparency and high commercial availability. These chiral selectors have been extensively used for a wide range of analytes and, in particular, β-CD or its derivates have been one of the most common choices used for the separation of cathinones [11,12,16,18,22]. For instance, β-CD has allowed to obtain the enantioseparation of the majority of a group of 12 cathinones [11] and a quite satisfactory enantioseparation of three cathinones in hair samples

[12], in both cases using a phosphate buffer. In relation to the derivates of β-CD, Mohr *et al.* [22] obtained resolution values between 0.9 and 2.9 using sulphated-β-CD as chiral selector and Nowak *et al.* [16] tested eight CDs in order to achieve the chiral separation of three cathinones being 2-hydroxyethyl-β-CD which presents the best results in terms of resolution.

Despite the well-known advantages of CE, one of its main limitations is its inherent poor sensitivity. To overcome this, different preconcentration strategies have been developed, and in recent years, there has been much interest in the in-line coupling of SPE with CE (in-line SPE-CE). In in-line SPE-CE a microcartridge (or preconcetrator) is inserted near the inlet end of the separation capillary, so it is an integral part of the separation capillary [12,27–35]. There are various designs used for the preconcentrator in in-line SPE-CE, the most popular being the fritless packed bed [12,29–35]. With this approach the preconcentrator, a small piece of capillary (2-4 mm) filled with SPE sorbent, is placed between an inlet (5-10 cm) and a separation capillary (50-100 cm) of a narrower i.d. than the preconcentrator. The sorbent particles are retained in the packed bed because of their size, which is slightly larger than the inner diameter of the separation capillary.

The main advantages of this preconcentration strategy are that it is easy to automate and all the solvent eluted from SPE is analysed by CE, which helps to increase sensitivity. However, to achieve low detection limits a large sample loading time (between 30-60 min) is generally needed at the pressure values normally used for injection (930 mbar) [12,32,34,35], and this in turn leads a long analysis time and can thus be a serious drawback for routine analysis. Even so, different methods have been developed with this strategy achieving very promising results. For example, several kinds of drugs in biological samples have been determined by in-line SPE-CE [12,32,34]. In all of them the usual sample loading times to reach the low concentration levels at which these compounds are usually present in those samples are higher than 30 min. For example, to achieve LODs at low ng/mg for cathinones in hair Baciu *et al.* applied 40 min of sample loading time [12]. Botello *et al.* applied 60 min to be able to reach ng/mL levels in the determination of barbiturates in urine samples [32]

and 40 min of sample injection time have been applied to achieve ng/mL levels for the determination of drugs of abuse in urine [34]. So, even the high sensitivity obtained the long sample loading times is a drawback of these strategies. To overcome this problem a possible alternative could be the increase of the sample injection pressure. For a same sample injection time an increase in injection pressure implies that a larger volume of sample can be introduced through the SPE device and, as long as the breakthrough volume is not exceeded, more analytes could be retained. Then, one way to reduce analysis time while maintaining or even increasing sensitivity may be by increasing the sample injection pressure. In addition, the dimensions of the SPE (length and diameter) can also have an effect on the sensitivity of the in-line SPE-CE system [33]. If there is an increase in the length, diameter or both, the amount of sorbent particles inside the in-line SPE device will consequently increase, and thus more analytes could be retained in the sorbent on condition that the breakthrough volume is not exceeded.

To document cathinone consumption, it is essential to analyse these compounds in biological samples such as urine, which can be obtained non-invasively and presents a detection window that can reach weeks. In recent years urine has been successfully used as a matrix for different applications [4–10,13–15] including the identification of cathinones in urine from victims of sexual assault [6,15] and screening studies for cathinone consumption [13,14]. These studies showed that cathinones can be found in urine at low concentration levels (ng/mL), so very sensitive methodologies are needed to determine these compounds. Due to these low levels, in previous studies the determination of cathinones in urine samples was mainly achieved by combining a chromatographic technique with MS detection such as LC-MS [4,5,8,9,13,15] or GC-MS [6,7,10,14] after an appropriate pretreatment, generally SPE [5,7–10] or LLE [6,13,14]. However, despite the benefits of CE in enantioseparation, as far as we know no studies have used this technique for the enantioseparation of cathinones in urine samples. In view of this, the main aim of our study is to develop a method for the enantioselective determination of *R,S*-MDPV, *R,S*-mephedrone and *R,S*-4-methyl-ephedrine in urine samples on the basis of in-line SPE-CE. Moreover, in

an attempt to improve in-line SPE methodology, the dimensions of the SPE device concentrator and the sample loading conditions were optimized in order to achieve high preconcentration factors by reducing the analysis time at the same time.

2 Materials and methods

2.1 Reagents and standards

The standards of *R,S*-mephedrone and *R,S*-MDPV were acquired as hydrochloride salts with a purity of 98% from LGC Standards (Teddington, UK). *R,S*-4-methyl-ephedrine was acquired as a solution in methanol (MeOH). Individual stock standard solutions of analytes (100 mg /L of *R,S*-4-methylephedrine, 1000 mg/L of *R,S*-mephedrone and 2000 mg/L of *R,S*-MDPV) were prepared by dissolving or diluting an appropriate amount of the standards in MeOH and keeping them in the freezer at -20°C. Working standard solutions of a mixture of all the compounds at a concentration of 10 mg/L were prepared weekly by diluting stock solutions in Milli-Q water and were stored at 4°C. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard solutions in Milli-Q water. Dichloromethane (DCM), ethyl acetate, isopropanol and MeOH, all of analytical-reagent grade, were purchased from J.T Baker (Deventer, Netherlands). Ammonium hydroxide 28%, formic acid 98%, hydrochloric acid 37%, monosodium phosphate 99%, phosphoric acid 85%, sodium hydroxide (NaOH) 97%, β-CD 97% and 2-hydroxypropil β-CD were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Milli-Q water was obtained with a water purification system from Veolia Water (Paris, France). Oasis HLB and Oasis major cation exchange cartridges (150 mg) with an average particle size of 60 µm were obtained from Waters Corp. (Milford, MA, USA).

2.2 Instrumentation

The electrophoretic system was a 7100 CE instrument equipped with a DAD from Agilent Technologies (Waldbronn, Germany). For all the experiments the capillary chamber was set at 25°C and 200 nm was used for the detection of the analytes. Bared fused-silica capillaries of different i.d. (50, 150 and 200 µm) were purchased from Polymicro Technologies (Phoenix, AZ, USA). The off-line SPE was carried out using a manifold system from Ashcroft (Stratford, CT, USA). The pH

measurements were performed with a GLP 21 pH-meter from Crison (Barcelona, Spain). A Universal 32 R centrifuge from Hettich (Kirch-lengern, Germany) was used.

2.3 Sample preparation

Urine samples were obtained from several non-addicted volun-teers. They were collected in polypropylene tubes and stored at -20°C until analysis. The method was validated using pooled urine, which was prepared by mixing the urine collected from the volunteers.

Before in-line SPE-CE analysis, the urine samples were alkalized to pH 10 with 28% ammonium hydroxide and then extracted in accordance with a LLE procedure. This LLE was as follows: 2 mL of a mixture of ethyl acetate/isopropanol (4:1, v/v) were added to a 2 mL alkalized urine sample. After vortex mixing for 1 min, samples were centrifuged for 10 min at 9000 rpm. The organic phase containing the analytes was transferred to a vial and a second extraction of the aqueous phase was performed by adding another 2 mL of the organic solvent mixture and repeating the same procedure. The two organic phases were then combined and 200 µL of isopropanol added. The obtained extract was dried under a gentle stream of N_2 until approximately 200 µL remained. The residue was then reconstituted to 2 mL with Milli-Q water (adjusted to pH 10 with 28% ammonium hydroxide). The sample was filtered through a 0.45 µm PTFE syringe filter, then transferred to a microvial for analysis and finally injected into the CE instrument.

2.4 CE separation

The CE separation was performed on a fused-silica capillary with a total length of 80 cm (72 cm effective length) and using an i.d. of 50 µm and an o.d. of 360 µm. The separation voltage was 30 kV (positive polarity). The BGE solution consisted of an aqueous solution of 70 mM of monosodium phosphate, adjusted to pH 2.5 with concentrated phosphoric acid, containing 8 mM 2-hydroxy-propil β-CD and 5 mM β-CD. Prior to its first use, the capillary was conditioned with NaOH 1 M for 40 min and with Milli-Q water for 10 min at 930 mbar. At the beginning of each working day, a conditioning step was performed with NaOH 0.1 M for 10 min, Milli-Q water for 5 min and BGE for 5 min, all of them at 930 mbar. Between runs, the conditioning step was performed with NaOH 0.1 M,

Milli-Q water and BGE, all of them at 930 mbar for 4 min. Standard samples were hydrodynamically injected at 50 mbar for 5 s.

2.5 In-line SPE procedure

The construction of the in-line SPE-CE device was based on the procedure described in [12]. Briefly, between the inlet (9 cm, 50 µm i.d.) and separation capillary (71 cm, 50 µm i.d.) a small piece of capillary (2 mm, 150 µm i.d.) filled with 60 µm Oasis HLB particles was placed. A PTFE tubing (250 µm i.d.), obtained from Saint Gobain (Courbevoie, France), was used for the connection between the capillaries and the SPE device.

The in-line SPE-CE procedure consisted of various steps. First, before injection the capillary with the preconcentrator was conditioned at 930 mbar with MeOH and Milli-Q water (adjusted to pH 10 with 28% ammonium hydroxide), both for 5 min. Then the urine sample extracts were injected at 3 bars for 20 min. After the injection, a clean-up step with BGE solution at 930 mbar for 2 min was performed. The elution stage was carried out by injecting 2% (v/v) of formic acid in MeOH at 50 mbar for 20 s. Then a pushing step with BGE at 50 mbar for 200 s was performed to displace the elution plug through the in-line SPE. Finally, 30 kV was applied for the CE separation of the analytes. A scheme of the in-line SPE-CE procedure can be found in Supporting Information Fig. S1.

3 Results and discussion

3.1 Optimization of the chiral separation by CE

As the drugs studied are weak bases with pK_a values between 8.83-9.13 [36], the selected BGE was a solution of 70 mM monosodium phosphate aqueous solution containing β-CD, at a concentration of 10.6 mM adjusted to pH 2.5. β-CD was selected as the chiral selector based on previous studies in which β-CD has been successfully used for the enantioseparation of different synthetic cathinones [11,12]. At these conditions the compounds were positively charged so that they would migrate towards the cathode. Standard solutions containing the analytes at a concentration of 25 µg/mL were injected into a capillary without the in-line SPE device at 50 mbar for 5 s, and the separation voltage applied was 30 kV. The electropherogram obtained under

these conditions is shown in Fi g. 1A. As it can be observed, the enantiomers of *R,S*-MDPV are baseline separated, but it was not possible to achieve the enantio-separation of the other cathinones. In attempt to find a suitable chiral selector to achieve the complete enantioseparation of the studied cathinones 2-hydroxypropil β-CD was evaluated. The use of this CD derivate at a concentration of 10.6 mM (Fig. 1B) instead of native β-CD improves the enantioseparation of *R,S*-mephedrone, but the other two pairs of enantiomers were not as well separated as when using β-CD. So, we tried different combinations of both CDs, specifically 5 mM of both, 5 mM of β-CD and 8 mM of 2-hydroxypropil β-CD and vice versa, and then 5 mM of β-CD and 10 mM of 2-hydroxy-propil β-CD and vice versa. The best separation was obtained with a BGE containing 2-hydroxypropil β-CD at a concentration of 8 mM and β-CD at a concentration of 5 mM (Fig. 1C) even that the enantiomers of *R,S*-4-methyl-

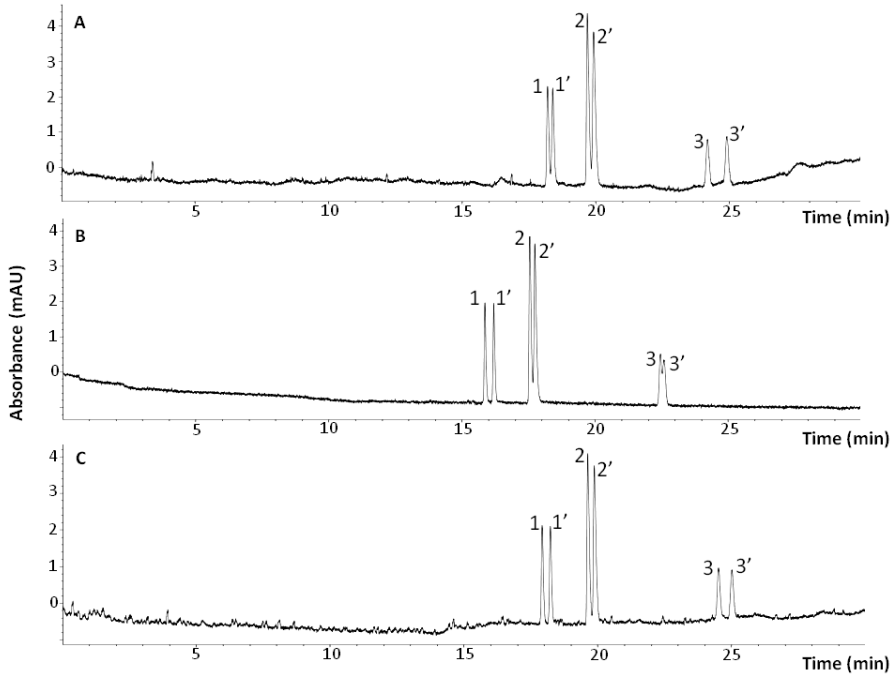

Figure 1. Electropherograms corresponding to the chiral separation optimization of the cathinones under study in a standard sample containing the analytes at a concentration of 25 µg/mL. The BGE consisted of a solution containing 70 mM of monosodium phosphate aqueous solution at pH 2.5 and (A) β-CD 10.6 mM, (B) 2-hydroxypropil β-CD 10.6 mM, and (C) 2-hydroxypropil β-CD 8 mM and β-CD 5 mM. Peak assignments: (1, 1') *R,S*-mephedrone, (2, 2') *R,S*-4-methylephedrine and (3, 3') *R,S*-MDPV.

ephedrine were not baseline separated. In view of these results, 70 mM of monosodium phosphate aqueous solution at pH 2.5 containing the dual system of CD previously mentioned was selected as BGE.

In these conditions the analysis time required for the enantio-separation of the target compounds was 25 min which was slightly higher than the required in previous studies for the enantiodetermination of the same compounds [11,12]. This fact can be attributed to the differences in the length of the separation capillary, differences of the BGE composition as the employed CD or also due to the use of a mixture of two CDs, instead of using one CD. However, unlike these previous studies in our case the baseline separation of the enantiomers of both mephedrone and MPDV has been achieved.

In order to assign correctly the *R(-)* and *S(-)* forms of the enantiomers it is necessary to acquire a pure enantiomer of each analyte. However, as far as we know, the target compounds are only sold as a racemic mixture, so it was not possible to purchase pure enantiomers for each compound. For this reason, it has been not possible the peak assignment of each enantiomer form.

3.2 In-line SPE optimization

As explained in the introduction, cathinones can be present in urine at low concentration levels. Therefore, when using CE a preconcentration step such as in-line SPE is required prior to their determination in order to reach these low levels. Oasis HLB, a polymeric sorbent with a polar group with lipophilic and hydrophilic retention characteristics, was selected because in previous studies high preconcentration factors were obtained for cathinones [12,17]. For example, LODs between 0.02-0.10 ng/mg were achieved for the determination of cathinones in hair by in-line SPE-CE, when 40 min of sample injection time at 930 bar were used [12]. Even the high sensitivity achieved with those conditions, an important drawback of that strategy was the long sample loading time. In an attempt to obtain high preconcentration enhancement factors by reducing the analysis time at the same time, the sample loading conditions have been optimized. In particular, we evaluated the pressure at which the sample is loaded into the system and its injection time.

Moreover, it has been also tested the i.d. of the preconcentrator and its length to increase the sensitivity of the method.

3.2.1 Pressure and sample loading time

The effect of increasing injection pressure on the response while simultaneously reducing the sample loading time was evaluated. When a high pressure value is used to load the sample it is important to ensure the integrity of the preconcentrator, because at high pressures it was observed that the mechanical friction could separate the PTFE tubing connection used between the separation capillary and the SPE device, thus reducing or interrupting the current.

For the present study, a standard sample containing the analytes at a concentration of 20 ng/mL was injected at different pressures: 930, 2000, 3000 and 4000 mbar for 10 min. Higher pressures were not evaluated because the PTFE tubing connection was unstable. Fig. 2A shows the results obtained in terms of peak area values. These were as expected because as the pressure increases, more sample volume is introduced into the SPE device and thus more analytes can be retained. It can be seen that the signal improved for each compound when the injection pressure was increased, with 4 bar providing the highest peak area. The resolution between enantiomers of each compound was also evaluated for the different sample injection pressure conditions. The results obtained are in the Supporting Information (Fig. S2A) where it can be observed that the resolution between the enantiomers peaks slightly decreases when the injection pressure increases from 930 mbar to 3 bars and is very similar when the injection pressure increases from 3 to 4 bars. For all the pressures, the resolution values between the enantiomers of mephedrone and MDPV were higher than 1.5 and in the case of the enantiomers of 4-methylephedrine all the resolution values were between 1.4 and 1.2. The standard deviations in terms of %RSD (n = 3) were also evaluated and showed a slight increase in their values when the pressure increased. However, the values obtained were below 8% even at the highest pressures and so no significant differences between results were observed.

Different sample loading times were also tested. Specifically, we

evaluated 5, 10, 20 and 30 min. Standard samples containing the analytes at a concentration of 10 ng/mL were injected at 3 bar, because at 4 bar we observed instability problems with the PTFE tubing connection at injection times higher than 10 min. As expected, an increase in the sample injection time means a signal improvement for each compound (Fig. 2B) because more sample volume is introduced. Furthermore, the resolution values between the enantiomers of the target compounds were very similar for the different evaluated injection times, as can be seen in Fig. S2B of the Supporting Information. However, when the injection time was 30 min, after several (more or less 10) consecutive analyses instability problems related to the PTFE tubing connection were observed. Considering all, 20 min was selected as the optimum injection time in order to avoid these problems and lengthen the life of the preconcentrator.

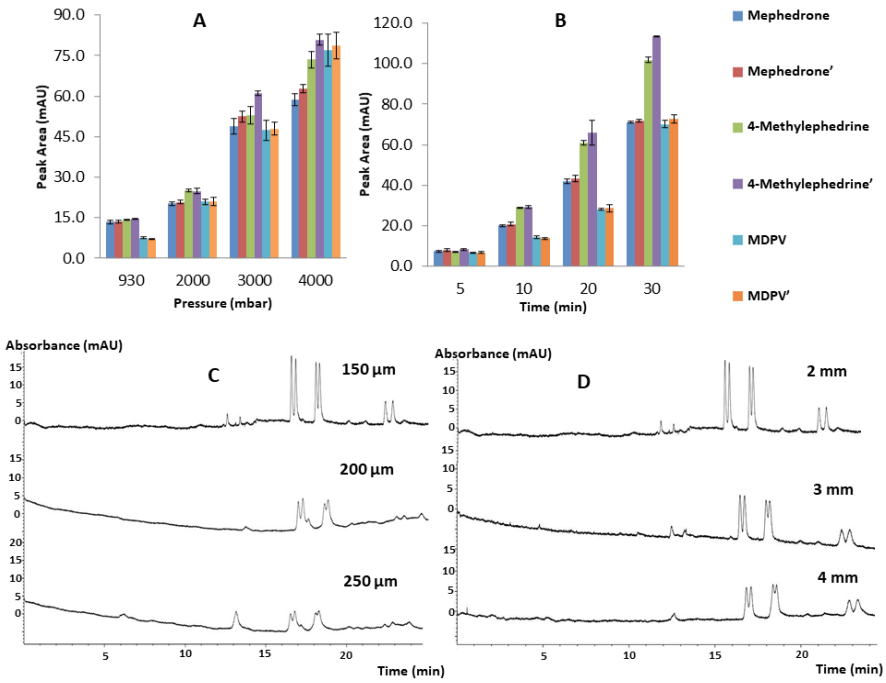

Figure 2. Optimization of the in-line SPE-CE, applying 30 kV as separation voltage and using a 70 mM phosphate aqueous solution, adjusted to pH 2.5, with a mixture of 2-hydroxypropil β-CD 8 mM and β-CD 5 mM as BGE: (A) pressure of injection (20 ng/mL, 10 min, in-line SPE: 2 mm length and 150 µm i.d.), (B) time of injection (10 ng/mL, 3 bar, in-line SPE: 2 mm length and 150 µm i.d.), (C) SPE i.d. (5 ng/mL, 3 bar, 20 min, in-line SPE: 2 mm length), and (D) SPE total length (5 ng/mL, 3 bar, 20 min, in-line SPE: 150 µm i.d.).

3.2.2 In-line SPE dimensions optimization

The dimensions of the preconcentrator, in particular its length and i.d., have a considerable influence on the amount of sorbent present in the SPE device and could therefore affect the amount of analytes retained. Hence, both parameters were evaluated. For the optimization of the in-line preconcentrator dimensions, standard samples containing the analytes at a concentration of 5 ng/mL were injected for 20 min at 3 bar. In the study of the preconcentrator i.d., capillaries of different i.d. – 150 µm, 200 µm and 250 µm – were tested. From the results obtained (Fig. 2C) we could see that the preconcentrator i.d. significantly influenced the separation of the analytes. In particular, the resolution was worse for capillaries with an i.d. higher than 150 µm. On the other hand, it is important to highlight that the response achieved for each compound decreases when the different tested i.d. increases. This could be attributed to an insufficient pushing or elution injection time. So, it was evaluated if an increase in pushing or elution injection time could have any effect in the achieved response. The pushing injection time was increased from 200 to 250 s. However, the same behaviour described above for all the analytes was observed. In the case of the elution time its injection time was increased from 10 to 30 s, but this increase caused current disturbances and it was no possible to evaluate this parameter. Taking into account these results and the resolution obtained for each i.d., 150 µm was the i.d. selected.

We also evaluated the influence of different preconcentrator capillary lengths (2 mm, 3 mm and 4 mm) on the response. As can be seen in the electropherograms obtained (Fig. 2D), the change in preconcentrator length also has a negative effect on the separation of the cathinones under study, since at greater length the resolution was worse. Therefore 2 mm was selected as the in-line SPE length.

3.2.3 Enrichment factor

To evaluate the extent of the improvement in CE sensitivity to the compounds under the optimized conditions using the in-line SPE preconcentration, we calculated the corresponding enrichment factors as the ratio of the LODs with and

without using in-line SPE. LODs were calculated as the concentration that gave a S/N of approximately 3. The results (see Table 1) were 8000, 7000 and 6000 for mephedrone, 4-methyl-ephedrine and MDPV respectively. In the literature, the injection pressures used for loading the sample into the in-line SPE preconcentrator are generally lower than 1 bar, with enrichment factors of between 450 and 1000 for organic sulfonates [33] and between 125 and 700 [34] or 2610 and 2930 [30] for drugs of abuse. In the present study, the application of 3 bar as the sample injection pressure led to higher enrichment factors, thus demon-strating the reported strategy's potential for the sensitive analysis of cathinones.

3.3 Urine extraction

The applicability of the method was demonstrated through the analysis of urine samples. To avoid interferences when urine was directly injected into the system, it was necessary to include a sample pretreatment step prior to loading the sample into the in-line SPE-CE. Two specific procedures were evaluated: off-line SPE and LLE. We based our selection on various studies that have reported a sample pretreatment prior to the analysis of cathinones in urine [5–10,13,14].

Different strategies based on the off-line SPE using strong cation exchange sorbents have been employed for the clean-up and extraction of cathinones from urine samples [5,7–10]. Glicksberg *et al.* [8], for example, obtained LODs of 1 ng/mL and 2 ng/mL for MDPV and mephedrone respectively in urine by LC-MS after pretreatment with a strong cation exchange sorbent. LLE has also been used by other authors to extract cathinones in urine [6,13,14]. For example, LODs between 10 and 50 ng/mL were obtained by GC-MS on urine samples after a sample pretreatment based

Table 1. LODs of standard solutions, with and without in-line SPE, and their corresponding enrichment factors.

Analyte	LOD without in-line SPE (ng/mL) (A)	LOD with in-line SPE (ng/mL) (B)	Enrichment factor (A/B)
Mephedrone	4000	0.5	8000
Mephedrone'	4000	0.5	8000
4-Methylephedrine	3500	0.5	7000
4-Methylephedrine'	3500	0.5	7000
MDPV	4500	0.75	6000
MDPV'	4500	0.75	6000

on LLE using *N*-butyl chloride as the extractant [4].

In the present study, an Oasis major cation exchange sorbent (150 mg cartridge, with a particle size of 60 µm) was selected for the off-line SPE. The procedure consisted of different steps: a conditioning step with 2 mL of MeOH, 2 mL of Milli-Q water and 2 mL of Milli-Q water adjusted at pH 5, followed by a loading step of 5 mL of sample at pH 5, then a washing step with 2 mL of 2% v/v of formic acid in MeOH and Milli-Q water, and finally an eluting step in which the compounds were eluted with 4 mL of MeOH/NH_4OH (95:5 v/v) and the extracts were dried under a N_2 current and injected into the CE system. Under these conditions the recoveries obtained were between 65-93% for standard samples containing the analytes at a concentration of 20 ng/mL. However, when this procedure was applied to spiked urine samples, the interferences overlapped the cathinone peaks even when the sample was diluted 1:10 (urine:water). We therefore focused on optimizing a procedure based on LLE. For that purpose, the LLE strategy used consisted of the following steps. The sample (2 mL) was alkalized to pH 10 to ensure that all the compounds were in their neutral form. Then 2 mL of an organic solvent were added and the mixture was vortex-mixed for 1 min then centrifuged at 9000 rpm for 10 min, and 200 µL of isopropanol were added. Finally, the organic extract was dried under a N_2 current to a volume of approximately 200 µL. This was injected into the CE system. Different organic phases were tested for the LLE procedure: dichloro-methane (DCM), hexane, ethyl acetate and isopropanol. The best recoveries were obtained with a mixture of ethyl acetate/isopropanol (4:1), with values from 71 to 108% for standard samples containing the analytes at a concentration of 20 ng/mL. These recoveries were calculated as the ratio between the response obtained for the standard sample at 20 ng/mL after performing the overall methodology including LLE, and the response obtained for the same standard sample without LLE.

When this strategy was applied to the analysis of urine samples, the electropherogram corresponding to a blank was free from interferences, although the recoveries obtained for this real sample were slightly lower than for the standards. Based on the results obtained in the pretreatment

step, LLE was chosen for further experiments. Fig. 3 shows the electropherogram obtained under the optimum conditions for LLE/in-line SPE-CE for a urine sample spiked with the analytes at a concentration of 40 ng/mL.

3.4 Method validation

The proposed LLE/in-line SPE-CE method was validated with pooled urine from non-addicted volunteers, which was spiked with the analytes, in terms of linearity, repeatability, reproducibility, LODs and LOQs. The values obtained are shown in Table 2.

The linearity was evaluated using a matrix-matched calibration curve and pooled blank urine spiked with known amounts of each compound in a range between 5 and 500 ng/mL. As Table 2 shows, good linearity was obtained with regression coefficients (r^2) greater than 0.988. The intra-day and inter-day precision (in terms of peak area and migration time) was evaluated by analysing five replicates of urine spiked at two concentration levels (20 ng/mL and 100 ng/mL) on the same day and at five different days. The results, expressed as %RSD, under both intra and day-to-day conditions were mostly below 10% for the peak area and under 2% in the case of migration time. The resolution between the enantiomers of each compound was evaluated for triplicate at a concentration of 100 ng/mL. The obtained values for *R,S*-mephedrone and *R,S*-MPDV were above 1.5 and in the case of *R,S*-4-methylephedrine were above 1.2. The LODs were calculated as the concentration that gave a S/N of approximately 3, while the LOQs were set at the lowest point of the linear range. The LODs obtained for the cathinones studied were between

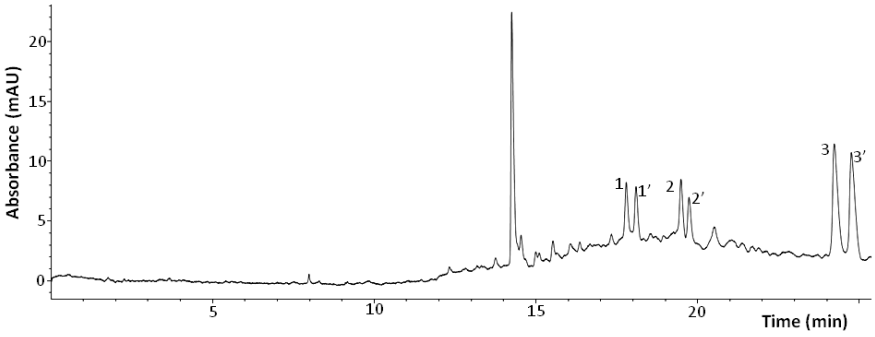

Figure 3. Electropherogram obtained under optimum conditions for LLE/in-line SPE-CE from pooled blank urine spiked with the studied compounds at a concentration of 40 ng/mL. Peak assignments: as in Figure 1.

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Table 2. Regression equations, repeatability and reproducibility values, LODs and resolution obtained for urine samples by LLE/in-line SPE-CE.

	Mephedrone	**Mephedrone'**	**4-Methylephedrine**	**4-Methylephedrine'**	**MDPV**	**MDPV'**
Linearity (ng/mL)	10-1000	10-1000	10-1000	10-1000	5-1000	5-1000
Calibration curve	y = 0.1008x + 28.1572	y = 0.0985x + 24.642	y = 0.2632x + 34.5367	y = 0.3358x + 22.7856	y = 1.8842x + 66.4233	y = 4.1442x - 63.6997
r^2	0.9930	0.9954	0.9981	0.9986	0.9878	0.9975
LOD (ng/mL)	8	8	7	7	3	3
Intraday RSD of peak area (%, n = 5)						
20 ng/mL	5	6.3	8.5	8.9	4.6	5.2
100 ng/mL	6.2	6.5	6.1	5.8	4.7	4.2
Intraday RSD of migration time (%, n = 5)						
20 ng/mL	1.3	1.2	0.7	0.6	1.2	1.2
100 ng/mL	0.8	1	1.1	0.9	0.9	0.9
Interday RSD of peak area (%, n = 5)						
20 ng/mL	5.8	9.5	11.2	12.4	7.3	7.6
100 ng/mL	6.8	7.1	7.1	8	6	5.9
Interday RSD of migration time (%, n = 5)						
20 ng/mL	1.7	1.6	1.3	1.4	1.5	1.5
100 ng/mL	1.2	1.5	1.6	1.4	1	1
Resolution (n = 3)	1.65		1.23		1.91	

3 and 8 ng/mL. These were comparable to those already published in previous studies. For example, various authors using a strategy based on LC-MS after a pretreatment with SPE using a strong cation exchange sorbent have reported LODs of between 1-2 ng/mL for MDPV and 1-2 ng/mL for mephedrone in urine [5,8]. LC-MS was also the strategy chosen by Paul *et al.* [4], although in this case a salting-out liquid-liquid extraction was used as the sample pretreatment and the LODs reported were of 2 ng/mL for MDPV and 2 ng/mL for mephedrone. GC-MS has also been used to determine cathinones in urine using SPE with a strong cation exchange sorbent as a sample pretreatment, and the reported LODs were of 5 ng/mL and 20 ng/mL for mephedrone and MDPV respectively [10].

To our knowledge this is the first strategy that has been developed for the chiral determination of cathinones in urine samples by capillary electrophoresis.

4 Concluding remarks

analysing cathinones in urine which allows the determination of these compounds at the usual levels at which they are present in this kind of biological sample from drug abusers.

Under the optimized conditions, the enantioseparation of synthetic cathinones in urine by adding CDs to the BGE was successfully achieved. The results demonstrate that using a binary CD system consisting of β-CD and 2-hydroxypropil β-CD is an effective strategy for the chiral separation of cathinones. High enrichment factors of between 6000 and 8000 were obtained for the three cathinones under study by using in-line SPE-CE. The study of the sample injection conditions and the dimensions of the preconcentrator in the in-line SPE process allows a reduction in analysis time while retaining sensitivity. The strategy developed enabled the analysis of urine samples by simply adding a LLE procedure prior to their introduction into the in-line SPE-CE system. The LODs obtained were between 3 and 8 ng/mL, which are similar to those reported by other authors using LC or GC.

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5 References

[1] Prosser, J. M., Nelson, L. S., *J. Med. Toxicol.* 2012, *8*, 33–42.

[2] Johnson, L. A., Johnson, R. L., Portier, R. B., *J. Emerg. Med.* 2013, *44*, 1108–115.

[3] German, C. L., Fleckenstein, A. E., Hanson, G. R., *Life Sci.* 2014, *97*, 2–8.

[4] Paul, M., Ippisch, J., Herrmann, C., Guber, S., Schultis, W., *Anal. Bioanal. Chem.* 2014, *406*, 4425–4441.

[5] Concheiro, M., Castaneto, M., Kronstrand, R., Huestis, M. A., *J. Chromatogr. A* 2015, *1397*, 32–42.

[6] Hagan, K. S., Reidy, L., *Forensic Sci. Int.* 2015, *257*, 71–75.

[7] Alremeithi, R. H., Meetani, M. A., Khalil, S. A., *RSC Adv.* 2016, *6*, 80576–80584.

[8] Glicksberg, L., Bryand, K., Kerrigan, S., *J. Chromatogr. B* 2016, *1035*, 91–103.

[9] Concheiro, M., Anizan, S., Ellefsen, K., Huestis, M. A., *Anal. Bioanal. Chem.* 2013, *405*, 9437–9448.

[10] Hong, W. Y., Ko, Y. C., Lin, M. C., Wang, P. Y., Chen, Y. P., Chiueh, L. C., Shih, D. Y. C., Chou, H. K., Cheng, H. F., *J. Anal. Toxicol.* 2016, *40*, 12–16.

[11] Merola, G., Fu, H., Tagliaro, F., Macchia, T., McCord, B. R., *Electrophoresis* 2014, *35*, 3231–3241.

[12] Baciu, T., Bo rrull, F., Calull, M., Aguilar, C., *Electrophoresis* 2016, *37*, 2352–2362.

[13] Kinyua, J., Negreira, N., Miserez, B., Causanilles, A., Emke, E., Gremeaux, L., Voogt, P. de, Ramsey, J., Covaci, A., van Nuijs, A. L. N., *Sci. Total Environ.* 2016, *573*, 1527–1535.

[14] Institóris, L., Árok, Z., Seprenyi, K., Varga, T., Sára-Klausz, G., Keller, É., Tóth, R. A., Sala, L., Kereszty, É., Róna, K., *Forensic Sci. Int.* 2015, *248*, 181–186.

[15] Lee, H. H., Chen, S. C., Lee, J. F., Lin, H. Y., Chen, B. H., *Forensic Sci. Int.* 2018, *282*, 35–40.

[16] Nowak, P. M., Olesek, K., Woźniakiewicz, M., Kościelniak, P., *Electrophoresis* 2018, *39*, 2406–2409.

[17] van Nuijs, A. L. N., Gheorghe, A., Jorens, P. G., Maudens, K., Neels, H., Covaci, A., *Drug Test. Anal.* 2014, *6*, 861–867.

[18] Li, L., Lurie, I. S., *Forensic Sci. Int.* 2015, *254*, 148–157.

[19] Meltzer, P. C., Butler, D., Deschamps, J. R., Madras, B. K., *J. Med. Chem.* 2006, *49*, 1420–1432.

[20] Schifano, F., Albanese, A., Fergus, S., Stair, J. L., Deluca, P., Corazza, O., Davey, Z., Corkery, J., Siemann, H., Scherbaum, N., Farre', M., Torrens, M., Demetrovics, Z., Ghodse, A. H., *Psychopharmacology (Berl).* 2011, *214*, 593–602.

[21] Kolanos, R., Partilla, J. S., Baumann, M. H., Hutsell, B. A., Banks, M. L., Negus, S. S., Glennon, R. A., *ACS Chem. Neurosci.* 2015, *6*, 771–777.

[22] Mohr, S., Pilaj, S., Schmid, M. G., *Electrophoresis* 2012, *33*, 1624–1630.

[23] Stavrou, I. J., Mavroudi, M. C., Kapnissi-Christodoulou, C. P., *Electrophoresis* 2015, *36*, 101–123.

[24] Řezanka, P., Navrátilová, K., Řezanka, M., Král, V., Sýkora, D., *Electrophoresis* 2014, *35*, 2701–2721.

[25] Tai, S., Morrison, C., *TrAC - Trends Anal. Chem.* 2017, *86*, 251–252.

[26] Stavrou, I. J., Agathokleous, E. A., Kapnissi-Christodoulou, C. P., *Electrophoresis* 2017, *38*, 786–819.

[27] Nozal, L., Arce, L., Simonet, B. M., Ríos, Á., Valcárcel, M., *Electrophoresis* 2006, *27*, 3075–3085.

[28] Almeda, S., Nozal, L., Arce, L., Valcárcel, M., *Anal. Chim. Acta* 2007, *587*, 97–103.

[29] Lara, F. J., García-Campaña, A. M., Neusüss, C., Alés-Barrero, F., *J. Chromatogr. A* 2009, *1216*, 3372–3379.

[30] Tak, Y. H., Toraño, J. S., Somsen, G. W., de Jong, G. J., *J. Chromatogr. A* 2012, *1267*, 138–143.

[31] Maijó, I., Fontanals, N., Borrull, F., Neusüß, C., Calull, M., Aguilar, C., *Electrophoresis* 2013, *34*, 374–382.

[32] Botello, I., Borrull, F., Aguilar, C., Calull, M., *Anal. Sci.* 2014, *30*, 971–977.

[33] Jooß, K., Sommer, J., Bunz, S.-C., Neusüß, C., *Electrophoresis* 2014, *35*, 1236–1243.

[34] Baciu, T., Borrull, F., Neusüß, C., Aguilar, C., Calull, M., *Electrophoresis* 2016, *37*, 1232–1244.

[35] Ramautar, R., Somsen, G. W., Jong, G. J. de, *Electrophoresis* 2016, *37*, 35–44.

[36] Nowak, P. M., Woźniakiewicz, M., Mitoraj, M., Sagan, F., Kościelniak, P., *J. Chromatogr. A* 2018, *1539*, 78–86.

Supporting Information

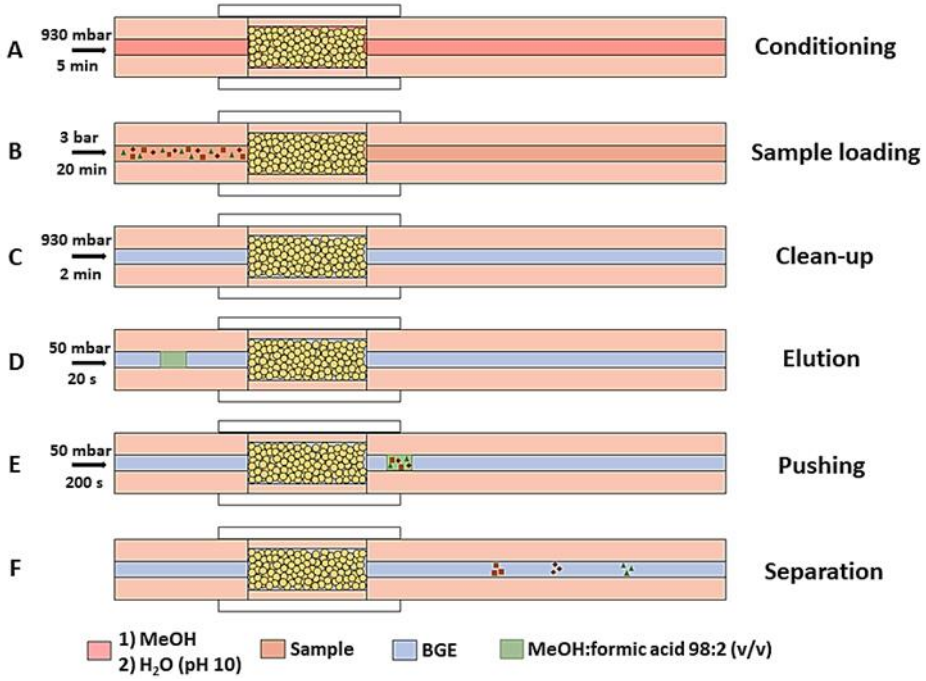

Figure S1. Scheme of the in-line SPE-CE procedure.

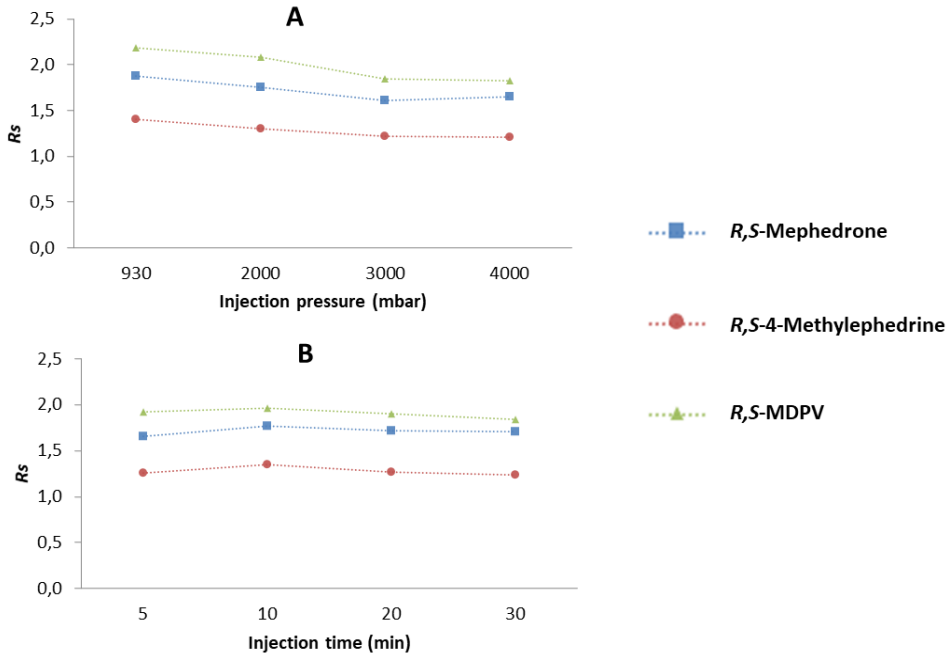

Figure S2. Optimization of the in-line SPE-CE in terms of resolution between the enantiomers of the target compounds, applying 30 kV as separation voltage and using a 70 mM phosphate aqueous solution, adjusted to pH 2.5, with a mixture of 2-hydroxypropil β-CD 8 mM and β-CD 5 mM as BGE: (A) pressure of sample loading (20 ng/mL, 10 min, in-line SPE: 2 mm length and 150 µm i.d.), (B) time of sample injection (10 ng/mL, 3 bar, in-line SPE: 2 mm length and 150 µm i.d.).

3.1.2. Enantiodetermination of R,S-3,4-methylenedioxypyrovalerone in urine samples by high pressure in-line solid-phase extraction capillary electrophoresis-mass spectrometry

ENANTIODETERMINATION OF *R,S*-3,4-METHYLENEDIOXYPYROVALERONE IN URINE SAMPLES BY HIGH PRESSURE IN-LINE SOLID-PHASE EXTRACTION CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

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Abstract

This study presents for the first time an in-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) method for the enantiodetermination of drugs of abuse in urine samples. The enantioseparation of *R,S*-3,4-methylenedioxypyrovalerone (*R,S*-MDPV) was achieved with a 10 mM ammonium acetate BGE (pH 7) that contained 0.5% (m/v) of sulphated-α-CD as chiral selector. At these pH conditions, this CD was negatively charged, which prevented its entrance into the mass spectrometer since it migrates in the opposite direction. To improve sensitivity, an in-line SPE-CE-MS method using high pressure for sample introduction (i.e. 20 min at 3 bars) was developed. Furthermore, the conditioning procedure and the first part of the electrophoretic separation were performed by switching off the nebulizer gas and the ionization source voltage to avoid non-volatile contaminant arrival into the mass spectrometer. The developed methodology was validated by analyzing urine samples, which required a very simple liquid-liquid extraction (LLE) sample pretreatment. Linearity ranged from 30 to 250 ng mL^{-1}, limit of detection (LOD) was 10 ng mL^{-1}, relative standard deviation (RSD) values were below 10.5% in terms of intra-day and inter-day precision and the relative error values were below 9% for peak areas accuracy

Keywords: *Capillary electrophoresis mass spectrometry / Enantiodetermination / In-line preconcentration / Cathinones / Sulphated cyclodextrins / Urine analysis*

1 Introduction

The production of alternatives to controlled drugs of abuse is a constant practice in the illicit marketplace. Among these alternatives, in recent years it has stood out the increase in consumption of synthetic derivatives of cathinone, an alkaloid naturally found in khat's leaves. This increase is mainly due to the similar euphoric effects of these cathinones, but lower prices and easier acquisition than amphetamines [1–3]. For this reason, the interest of the police and health authorities in the detection and determination of these substances has grown, as it is reflected in the emergence of extensive literature related to their analysis [4–18].

Synthetic cathinones are usually found in the illicit marketplace or at internet labelled as "bath salts" and *R,S*-3,4-methylenedioxypyrovalerone (*R,S*-MDPV) is one of their major ingredients [1,2,19]. This cathinone, which is a pyrrolidine derivative of pyrovalerone, acts as dopamine and norepinephrine reuptake inhibitor [3,19,20]. After producing the stimulating effect, the remaining compound is excreted from the body and can be found at low concentration levels in biological samples, such as urine, plasma or hair [6–18]. Therefore, the determination of MDPV in urine requires the use of high sensitive methods, and there are several examples in the literature that allow reaching the necessary levels, mainly based on gas chromatography (GC) [15–18] or liquid chromatography (LC) [10–13] with mass spectrometry (MS) or tandem MS detection.

MDPV presents an asymmetric carbon in its chemical structure. Consequently, it exists as two enantiomers that can have different biological activity. Indeed, it has been demonstrated that the *S*-form is a more potent reuptake inhibitor of dopamine and norepinephrine than the *R*-form [20]. Therefore, the enantioseparation of MDPV enantiomers can be of interest for clinical, toxicological and forensic purposes. However, this chiral separation is usually difficult or expensive to achieve by LC, which typically requires a specific chiral column [14], or by GC, which needs a derivatization step that increases the analysis time [18]. In this sense, capillary electrophoresis (CE) offers an interesting alternative, as the enantioseparation can be achieved by simply adding a chiral selector in the

BGE [21], being cyclodextrins (CDs) one of the preferred choices for the enantioseparation of cathinones by CE [4–9].

Despite the numerous advantages of CE, one of its major drawbacks is related with the relatively low sensitivity, as well as the limited selectivity with conventional UV detection. To solve these issues, different strategies have been proposed, including the hyphenation of CE with MS. CE-MS has been successfully used for the analysis of several kind of chiral compounds [4,5,22–27], including cathinones in standard solutions [4,5]. However, it requires the use of a volatile and low conductivity BGE with an appropriate chiral selector to obtain a good separation and stable electrospray, while preventing the mass spectrometer contamination [24]. It is well known that CDs can cause a significant signal suppression and an increase in the noise due to the contamination of the ionization source [24]. Therefore, it is important to develop strategies to prevent the entrance of incompatible chiral selectors into the mass spectrometer. Two strategies have been mainly highlighted in the literature to achieve chiral CE-MS: the counter migration technique [4,5,24–27] and the partial filling technique [4,5,22–24]. In the counter migration technique, charged chiral selectors that migrate in the opposite direction to the analytes and the mass spectrometer are employed. In the partial filling technique only a part of the capillary (e.g. 70-90%) is filled with the BGE containing the chiral selector avoiding its entrance into the mass spectrometer. Another complementary strategy is to switch off the ionization source during the conditioning step and/or part of the electrophoretic separation. Under these conditions, none of the compounds reaching the ionization source, including non-volatile chiral selectors, are ionized, hence they do not enter into the mass spectrometer [27].

The sensitivity in CE-MS may still be insufficient to reach the low levels at which MDPV is usually found in urine samples (ng mL^{-1}). Limits of detection (LOD) can be further decreased with the application of a sample preconcentration strategy, such as the in-line coupling of solid-phase extraction to CE (in-line SPE-CE) [6,7,28–30]. In this strategy, the in-line SPE microcartridge, which contains an appropriate sorbent, is an integral part of the separation capillary,

allowing the introduction of a large volume of sample to retain the target analytes. Then, after washing and filling the capillary with background electrolyte (BGE), the analytes are eluted in a small volume of an appropriate solution, resulting in sample clean-up and concentration enhancement before the electrophoretic separation and detection [6,7,28–30]. In-line SPE-CE-UV has been described for the enantiodetermination of cathinones in urine [6,7], but to the best or our knowledge in-line SPE-CE-MS has never been demonstrated for the enantiodetermination of cathinones or any other type of compounds in biological matrices.

The aim of this study was to develop an in-line SPE-CE-MS method for the sensitive enantioselective determination and unambiguous identification of chiral compounds in urine samples. The enantiodetermination of the drug of abuse *R,S*-MDPV was studied in urine samples. Different strategies were evaluated to ensure compatibility between the conditions required for an appropriate in-line preconcentration, enantioseparation and MS detection, demonstrating the feasibility of chiral in-line SPE-CE-MS for the first time.

2 Materials and methods

2.1 Reagents and standards

The standard of *R,S*-MDPV was provided as a hydrochloride salt with a purity of 98% by LGC Standards (Teddington, UK). An individual 2000 mg L^{-1} stock solution was prepared in MeOH and was kept in the freezer at -20 °C. This stock solution was stable for 6 months. Working standard solutions were prepared weekly by diluting the stock solution in water and were stored at 4 °C. The solutions with lower concentrations (≤1 mg L^{-1}) were prepared daily by diluting the working standard solutions in water.

Acetic acid (glacial), acetone 99.8%, ammonium acetate 98%, ammonium formate 97%, ammonium hydroxide 25%, dioxane 99.8%, formic acid 99%, propan-2-ol 99.9%, sodium hydroxide 98% and tetrahydrofuran (THF) 99,9% were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH) and water, all of them of LC-MS grade, were provided by PanReac Applichem (Barcelona, Spain). Sulphated-α-CD 98%, sulphated-β-CD 98% and sulphated-γ-CD 98% were supplied as sodium salts by Cyclolab (Budapest, Hungary).

2.2 Instrumentation

The pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52-03 from Crison Instruments (Barcelona, Spain). Centrifugal filtration was carried out in a 5417R centrifuge from Eppendorf Ibérica (Madrid, Spain). A Vortex Genius 3 from Ika (Staufen, Germany) was used for agitation.

2.3 BGE and sheath liquid solutions

The BGE consisted of a 10 mM ammonium acetate aqueous solution (pH 7) that contained 0.5% (m/v) of sulphated-α-CD. As sheath liquid solution for CE-MS was employed a hydroorganic mixture of 60:40 (v/v) propan-2-ol:water with 0.25% (v/v) of formic acid.

All solutions were degassed for 10 min by sonication and filtered through a 0.20 µm nylon filter from Micron Separations Inc (Westborough, MA, USA) before use.

2.4 CE-UV

CE-UV experiments were performed at 25 °C with a 7100 CE System from Agilent Technologies (Waldbronn, Germany) equipped with a spectrophotometric diode-array detector (DAD). Bare fused-silica capillary of 50 µm id and 80 cm of total length (72 cm effective length) were provided by Polymicro Technologies (Phoenix, AZ, USA).

All capillary rinses were done flushing at 930 mbar. Before the first use, the capillary was activated with NaOH 1 M (40 min) and water (10 min). At the beginning of each working day, the capillary was conditioned with NaOH 0.1 M (10 min), water (5 min) and BGE (5 min). Between each run, the capillary was rinsed with NaOH 0.1 M (5 min), water (5 min) and BGE (5 min). At the end of each run, the capillary was postcoditioned with water (5 min). The sample was injected at 50 mbar for 10 s and 25 kV (positive polarity, cathode in the outlet) were applied for the electrophoretic separation. The BGE voltage vial was refreshed after each analysis to ensure maximum repeatability. Instrument control, data acquisition and data processing were performed using ChemStation Software from Agilent Technologies.

2.5 CE-MS

CE-MS experiments were performed at 25 °C using a HP^{3D} CE

system coupled with an orthogonal G1603A sheath-flow interface to a LC/MSD Ion Trap SL mass spectrometer from Agilent Technologies. The sheath liquid was delivered at a flow rate of 3.3 µL/min by a KD Scientific 100 series infusion pump from KD Scientific (Holliston, MA, USA). Full scan mass spectra were acquired from 100 to 500 m/z in positive (ESI+) mode, and MDPV was detected as a singly charged molecular ion ($[M+H]^+$ 276.2). To avoid the unnecessary entrance of interfering compounds in the mass spectrometer, MS acquisition was split in two segments, each one with a different ESI voltage and nebulizer gas (N_2) pressure for the ionization source. Segment 1 conditions (0 V and 2 psi) were applied since the beginning of the capillary conditioning until minute 10 of the CE separation, when they were automatically switched to segment 2 conditions (4000 V and 7 psi). The rest of parameters were the same in both segments. The drying gas (N_2) flow rate and temperature were 2 L/min and 300°C, and capillary exit, skimmer, octopole 1, octopole 2, octopole radiofrequency, lens 1 and lens 2 voltages were set at 115.2 V, 48.4 V, 15.9 V, 0 V, 50 V, -9.1 V and -77.9 V respectively, with the trap drive at 41.9 (arbitrary units). This last group of parameters was automatically optimized infusing at 50 mbar through the separation capillary a 100 µg/mL MDPV standard solution. Instrument control, data acquisition and data processing were performed using CE/MSD Trap Software from Agilent Technologies.

A bare fused-silica capillary of 50 µm id and 80 cm of total length (Polymicro Technologies) was used for all the CE-MS separations. All capillary rinses were performed flushing at 930 mbar. New capillaries were activated with NaOH 1 M (40 min) and water (10 min) with the capillary outside of the CE-MS interface needle. Between days, the capillary was conditioned with ammonium hydroxide 0.1 M (10 min) and water (5 min). Between each run, the capillary was rinsed with ammonium hydroxide 0.1 M (5 min), water (5 min) and BGE (5 min). At the end of each run, the capillary was postconditioned with water (5 min). The sample was injected at 50 mbar for 10 s and 25 kV (positive polarity) were applied for the electrophoretic separation. The BGE voltage vial was refreshed after each analysis to ensure maximum repeatability.

2.6 In-line SPE procedure

The construction of the in-line SPE particle-packed fritless microcartridge was based on the procedure described in Ref. [7]. All bare fused-silica capillaries were provided by Polymicro Technologies. Briefly, a small piece of capillary (2 mm, 150 µm id) filled with 60 µm Oasis HLB sorbent particles from Waters Corp. (Milford, MA, USA) was placed between the inlet (8 cm, 50 µm id) and the separation capillary (72 cm, 50 µm id). As the id of the separation capillary was smaller than the sorbent particle size no frits were necessary to prevent sorbent bleeding. A PTFE tubing (250 µm id, Saint Gobain, Courbevoie, France) was used to connect the different capillary fragments.

The in-line SPE-CE procedure consisted of the following steps. First, the capillary was conditioned at 930 mbar with MeOH and 0.1 M ammonium hydroxide, both for 5 min. Then, the standard solutions or urine sample extracts were introduced at 3 bars for 20 min. Before the elution, the capillary was washed and filled with BGE at 930 mbar for 2 min. The water:MeOH:formic acid (68:30:2 v/v/v) eluent was injected at 50 mbar for 20 s and pushed through the capillary with BGE at 50 mbar for 150 s. Finally, 25 kV (positive polarity) were applied for the electrophoretic separation. The rest of conditions were as indicated for CE-MS.

2.7 Sample pretreatment

Urine samples were collected in polypropylene tubes from healthy volunteers, with the appropriate approval of the Ethical and Scientific Committees of the UB. A pool was prepared for method development. The pooled and the individual samples were fractionated and stored at -20 °C until analysis.

A liquid-liquid extraction (LLE) sample pretreatment, based on a LLE procedure described in Ref. [7], was applied before the in-line SPE-CE-MS analysis. First, the urine samples were alkalized to pH 10 with 25% ammonium hydroxide. Then, 0.5 mL of ethyl acetate/propan-2-ol (4:1 v/v) were added to 0.5 mL of alkalized urine sample. After vortex mixing for 1 min, samples were centrifuged for 10 min at 9000 rpm. The organic phase, containing the MPDV, was transferred to a polypropylene vial, and then a second extraction of the aqueous phase was performed by adding again 0.5 mL of ethyl

acetate/propan-2-ol (4:1 v/v). Then, both organic phases were combined and dried under a gentle stream of N_2. Finally, the residue was reconstituted with 0.5 mL water (adjusted to pH 10 with 25% ammonium hydroxide) and passed through a 0.20 µm nylon syringe filter before the analysis.

3 Results and discussion

3.1 BGE selection

The selection of an appropriate BGE is especially critical in CE-MS. The BGE must present low conductivity and be volatile to prevent salt precipitation in the ionization source, poor electrospray stability, ionization suppression and contamination of the mass spectrometer [24]. Moreover, the BGE must ensure an appropriate analyte ionization in solution to guarantee a proper separation and migration towards the detector. Since MDPV is a weak base, which presents a pK_a value of 9.13 [31], at acidic or neutral BGE conditions it is positively charged and migrates towards the detector in the cathodic end of a bare fused silica capillary. In our previous work, we used a BGE of 70 mM monosodium phosphate aqueous solution at pH 2.5 with a mixture of 8 mM 2-hydroxypropil β-CD and 5 mM β-CD for the enantiodetermination of a group of cathinones, including MDPV, by in-line SPE-CE-UV [7]. However, these BGE conditions were rapidly discarded due to the low compatibility of phosphate BGEs with CE-MS and the presence of neutral CDs that will continuously arrive to the mass spectrometer pushed by the electroosmotic flow. As an alternative to the acidic phosphate BGE, four different aqueous solutions were tested, in particular: 10 mM formic acid (pH 3), 10 mM acetic acid (pH 3.5), 10 mM ammonium formate (pH 6.5) and 10 mM ammonium acetate (pH 7). For all of these BGEs the electric current was below 50 µA that is mandatory to prevent electric arcing between the CE-MS interface needle and the mass spectrometer entrance [25]. When the acidic BGEs were used, the shape of the MDPV peak was distorted. This distortion was not observed with the ammonium salt solutions, but the acetate BGE allowed to detect MDPV in the shortest migration time and was selected for further experiments.

3.2 Chiral separation

In recent years, several studies based on CE-UV have demonstrated the chiral separation of cathinones by employing several CDs as chiral

selectors [4–9]. However, the selection of the chiral selector to add in the BGE can be a critical issue in CE-MS [4,5,22–27]. In this sense, it is important to find strategies to avoid the entrance of these non-volatile substances in the mass spectrometer. Among these strategies, partial filling [4,5,22–24] and counter migration techniques [4,5,24–27] are the main approaches.

To evaluate these strategies, we tested different conditions by CE-UV to avoid the unnecessary contamination of the mass spectrometer. For the partial filling technique different negatively charged CDs were tested as chiral sectors, namely sulphated-α-CD, sulphated-β-CD and sulphated-γ-CD. Anionic CDs are good candidates for the partial filling and the counter migration approaches because in positive polarity mode (cathode in the outlet) they would migrate in the opposite direction to the mass spectrometer. For this study, the capillary was first flushed with BGE without CD and then, prior to the sample injection (75 μg mL^{-1} MDPV, 10 s at 50 mbar) and voltage application (25 kV), 50, 60, 70, 80 or 90% of the total capillary length was filled with BGE containing 1% (m/v) of CD. However, under the studied conditions it was not possible to achieve the baseline enantio-separation of *R,S*-MDPV. Then, the partial filling approach was discarded. Alternatively, the same anionic CDs were evaluated for the counter migration approach. For this study, the conditions were the same as before, excepting for the capillary that was completely filled with BGE containing 1% of the studied sulphated-CDs before the sample injection. Sulphated-α-CD and sulphated-β-CD allowed the baseline separation of the enantiomers of *R,S*-MDPV, while sulphated-γ-CD was not able to do it. As the best resolution was achieved with sulphated-α-CD, this CD was the chosen chiral selector for further experiments with the counter migration approach.

As sulphated-CDs are ionic compounds, at high concentration they can negatively affect the CE-MS performance [25,27]. Therefore, lower concentrations of sulphated-α-CD in the BGE, namely 0.25, 0.5, 0.75 and 1% (m/v) were tested. With 0.25% (m/v) of this CD in the BGE we got only partial separation of both enantiomers, but with 0.5% (m/v) they were baseline resolved. In view of these results, 10 mM of ammonium acetate at pH 7

containing 0.5% (m/v) of sulphated-α-CD was selected as the optimized BGE. Interestingly, when 0.5% (m/v) of sulphated-α-CD was added to the acidic BGEs (i.e. 10 mM formic acid (pH 3) or 10 mM acetic acid (pH 3.5), the MDPV enantiomers were not detected, even with an analysis time of over an hour. This definitely discarded the use of acidic volatile BGEs in combination with negatively charged CDs for the enantioseparation of *R,S*-MDPV in the counter migration approach.

As it has been mentioned in the introduction section, another complementary strategy to avoid the entrance of contaminant compounds, including non-volatile chiral selectors, into the mass spectrometer is to switch off the ionization source during the conditioning step and part of the electrophoretic separation [27]. Therefore, the MS acquisition was split into two segments. First, the ionization was switched off since the beginning of the capillary conditioning until minute 10 of the CE separation, by setting the ESI voltage and the nebulizer gas pressure to the minimum possible values (i.e. 0 V and 2 psi, respectively). Then, ionization was switched on to detect the enantioseparation, by setting both parameters to the typical values in CE-MS (i.e. 4000 V and 7 psi, respectively). Under these conditions, the mass spectrometer could be operated for an extended period of time without implementing any specific maintenance procedure, apart from the typical weekly cleaning of the ionization source recommended in routine operation. Fig. 1 shows the extracted ion electropherogram (EIE) by (segmented) CE-MS for a 75 μg mL^{-1} MDPV standard solution with the optimized BGE. As can be seen, at these conditions the baseline enantioseparation of the *R,S*-MDPV was successfully achieved (resolution was 3.8).

3.3 In-line SPE-CE-MS optimization

The in-line SPE-CE-MS procedure was developed taking into account the (segmented) CE-MS method and an in-line SPE-CE-UV method for the enantiodetermination of a group of cathinones that we described in a previous work [7]. In that research, an in-line SPE fritless microcartridge of 2 mm length and 150 μm of id packed with Oasis HLB sorbent particles was used in combination with sample introduction at high pressure (3 bars) to achieve high enrichment factors, namely between 6000 and 8000.

The initial conditions for in-line SPE-CE-MS preconcentration were based on the optimum conditions found in that previous research [7]. These conditions were essentially the same as described for the final in-line SPE-CE-MS optimized conditions in the experimental section, except for the use of an eluent of 2% (v/v) of formic acid in MeOH. However, using this eluent composition, the chiral separation of the MDVP enantiomers was not achieved. This can be explained due to the different BGE conditions. The enantioseparation in CE is achieved due to the differences between the stability of the complexes formed by the CD and the enantiomers. Furthermore, it has been demonstrated that the presence of an organic modifier in the BGE can decrease the cathinone/CD binding constant, resulting in a decrease of the resolution between enantiomers [5]. Therefore, elution conditions required a careful optimization to ensure the highest enrichment factors while maintaining an appropriate enantioseparation.

The enantioseparation was studied using as eluent different hydroorganic mixtures compatible with MS detection. For this study, a 200 ng mL^{-1} MDPV standard solution was analyzed by in-line SPE-CE-MS with eluents containing from 10 to 40% (v/v) of acetone, ACN, dioxane, propan-2-ol, MeOH and THF, which were injected at 50 mbar for 20 s. The resolution values between both enantiomers obtained at the different tested conditions are summarized in Table 1. As can be observed, a high organic solvent content (40%) did not allow the enantioseparation and 30% (v/v) of MeOH was the highest

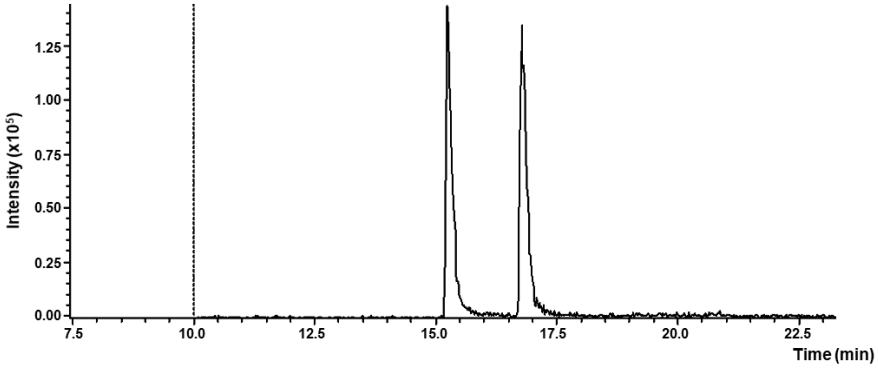

Figure 1. EIE ($[M+H]^+$ 276.2) by (segmented) CE-MS of a 75 µg mL^{-1} MDPV standard solution with the optimized BGE (10 mM ammonium acetate (pH 7) with 0.5% (m/v) of sulphated-α-CD). The ionization was switched off until minute 10.

percentage of organic solvent capable of maintaining the chiral separation. Moreover, this composition allowed the highest response for MDPV enantiomers. Taking as a reference these elution conditions (30% (v/v) MeOH at 50 mbar for 20 s), the influence of the eluent volume was also investigated, injecting the eluent at 50 mbar for 10 s or 30s. As it was expected, when the eluent volume was increased the enantioseparation was compromised, as it was also greater the amount of organic modifier in the capillary. On the other hand, when the eluent volume was reduced, the MDPV enantiomers response also decreased. Then, to evaluate if the acidification of the eluent could have a positive effect in the elution without compromising the enantioseparation, the addition of a 2% (v/v) of formic acid to the hydroorganic mixture was tested. This allowed increasing response more than 10 times without significantly compromising the chiral separation (resolution was 1.5). Therefore, the optimized eluent was selected as a mixture of water:MeOH:formic acid (68:30:2 v/v/v) injected at 50 mbar for 20 s.

To investigate the increase in the response for MDPV under the in-line SPE-CE-MS optimized conditions in comparison with CE-MS the enrichment factor was calculated. A value of 500 was obtained as the ratio between the LODs for the analysis of MDPV standards by CE-MS (4000 ng mL^{-1}) and in-line SPE-CE-MS (8 ng mL^{-1}). This confirmed the sensitivity enhancement potential of in-line SPE-CE-MS, while achieving the unambiguous identification of the separated enantiomers. However, this enrichment factor was lower than the value obtained for MDPV in our previous study by in-line SPE-CE-UV (i.e. 6,000) [7]. This can be explained due to the modifications needed in the BGE to make compatible the preconcentration and

Table 1. Resolution of the MDPV enantiomers by in-line SPE-CE-MS with different hydroorganic eluents. A 200 ng mL^{-1} MDPV standard solution was analyzed in all cases. Resolution was calculated from the EIE as: $Rs = 2 \times \frac{tm_2 - tm_1}{W_1 + W_2}$

	Percentage of the organic solvent in the hydroorganic solution (%, v/v)			
	10	**20**	**30**	**40**
Organic solvent	**Resolution between MDPV enantiomers**			
Acetone	1.1	n. e.	n. e.	n. e.
ACN	1.1	n. e.	n. e.	n. e.
Dioxane	0.6	n. e.	n. e.	n. e.
Propan-2-ol	0.6	n. e.	n. e.	n. e.
MeOH	2.0	1.6	1.5	n. e.
THF	n. e.	n. e.	n. e.	n. e.

n. e. = not enantioseparated

enantioseparation in in-line SPE-CE-MS, and especially due to the differences on the eluent composition. It is well known that an eluent of 2% v/v of formic acid in MeOH [7], presents a greater elution strength from an Oasis HLB sorbent than the water:MeOH:formic acid (68:30:2 v/v/v) solution optimized in the current study.

3.4 Urine sample pretreatment

The applicability of the in-line SPE-CE-MS method was tested by analyzing urine samples. However, before this could be possible it was necessary to develop a sample pretreatment to avoid the microcartridge saturation and poor ionization efficiency due to co-extraction of urine sample matrix components [7,10–13].

A LLE procedure based on alkalizing the urine to pH 10 and extracting MDPV with an ethyl acetate/propan-2-ol mixture was applied, as in our previous study by in-line SPE-CE-UV [7]. A recovery value of 87% was calculated as the ratio between the response by in-line SPE-CE-MS for a urine sample spiked with 100 ng mL^{-1} of MDPV and a 100 ng mL^{-1} standard solution without LLE. The matrix effect after performing the developed LLE procedure was calculated using the following expression:

$$\%\ \text{Matrix effect} = \frac{C_{spiked}}{C_{standard}} \times 100 - 100$$

Where C_{spiked} is the concentration of a urine extract spiked after the LLE procedure and $C_{standard}$ is the concentration of a standard at the same concentration as C_{spiked}, both analyzed by in-line SPE-CE-MS. Applying this equation at a concentration level of 100 ng mL^{-1} of MDPV, a value of -16% was obtained, hence signal suppression was observed when urine extracts were analyzed. This matrix effect value is within the range reported in the literature for cathinone analysis in urine by LC-MS in ESI+ mode (i.e. between 3.2 and -28%) [10–12]. From the obtained recovery and matrix effect values it was concluded that recovery of the LLE procedure was practically total, which agrees with the recovery value (i.e. 93%) obtained for MDPV when this LLE procedure was applied prior to in-line SPE-CE-UV [7].

Fig. 2 shows the total ion electropherogram (TIE) (A) and the EIE (B) by in-line SPE-CE-MS of a urine sample spiked with 100 ng mL^{-1} of

MDPV and pretreated by LLE, and the EIE (C) of a 100 ng mL^{-1} MDPV standard solution. As can be seen, the TIE does not present remarkable interferences in the scanned m/z range. Furthermore, the MDPV enantiomer peaks were wider and the number of theoretical plates ($N = 16 \times (t_m/w)^2$) lower by in-line SPE-CE-MS (Figure 2C, N = 14,796 and 8119 for MDPV enantiomers) than by CE-MS (Figure 1, N = 23,409 and 28,224, for MDPV enantiomers). As both the standard solution (Figure 2C) and the spiked urine sample (Figure 2B) present peak broadening by in-line SPE-CE-MS, this must be due to the in-line SPE microcartridge and not to a matrix effect. This kind of peak broadening has been described before in in-line SPE-CE [7,28–30], but good resolution between MDPV enantiomers was maintained (resolution was 1.5). It should not be either forgotten that a slight decrease in peak efficiency is always expected when moving from CE-UV to CE-MS due to the characteristics of the sheath-flow interface (N = 30,625 and 40,000 by CE-UV for MDPV enantiomers).

3.5 Method validation

The proposed LLE/in-line SPE-CE-MS methodology for the analysis of urine samples was validated, with spiked urine samples, in terms of selectivity, linearity, intra-day and inter-day precision, accuracy, LODs and LOQs following the guide published by the United Nations Office on Drugs and Crime (UNODC) [32].

To evaluate the selectivity 10 blank urine samples from different individuals were analyzed after the LLE pretreatment. At these conditions, no endogenous peaks were observed at the expected migration time for the MDPV enantiomers.

A matrix-matched calibration curve in the range between 30 and 250 ng mL^{-1} was used to evaluate the linearity. As it is shown in Table 2, good results were obtained in terms of linearity as the regression coefficients (r^2) were greater than 0.99.

The intra-day and inter-day precision were evaluated at 30 ng mL^{-1}, 100 ng mL^{-1} and 250 ng mL^{-1} (n = 5 at each concentration level on the same day or on five different days, respectively). As can be seen in Table2 the method provided good results in both cases as the obtained values, expressed as relative standard

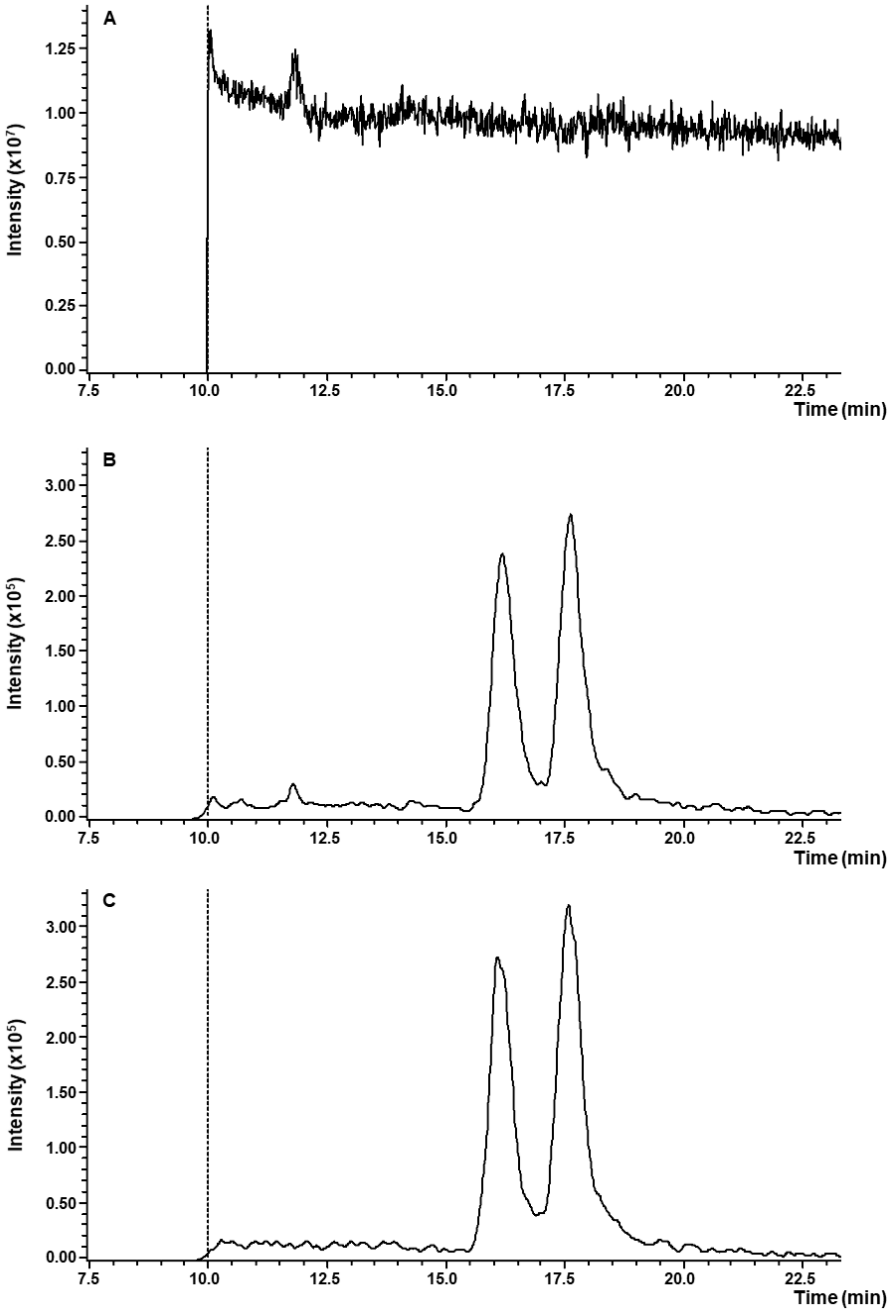

Figure 2. TIE (A) and EIE ($[M+H]^+$ 276.2) (B) of a urine sample spiked with 100 ng mL^{-1} of MDPV and pretreated by LLE, and EIE ($[M+H]^+$ 276.2) (C) of a 100 ng mL^{-1} MDPV standard solution. The ionization was switched off until minute 10. The rest of in-line SPE-CE-MS optimized conditions are indicated in the experimental section.

deviation (RSD), were below 9% and 10.5%, respectively. The accuracy was also investigated at the same concentration levels. For this purpose, the relative errors of peak areas (%RE) were calculated using the following expression:

$$\%RE = \frac{\left| \text{experimental response} - \text{theoretical response obtained in the calibration curve} \right|}{\text{theoretical response obtained in the calibration curve}} \cdot 100$$

As can be seen in Table 2 %RE values were good (below 9% for all concentration levels).

The LODs for the MDPV enantiomers were calculated by applying the signal-to-noise ratio (S/N) criterion of three, whereas the LOQ was set as the lowest concentration value of the linear range. The LOD and LOQ values were 10 ng mL^{-1} and 30 ng mL^{-1}, respectively. These LODs were suitable to reach the levels at which this cathinone is usually found in urine (ng mL^{-1}) and were similar or lower than those obtained using GC-MS for the analysis of MDPV in urine (i.e. between 5 and 30 ng mL^{-1}) [15–17]. Additionally, the obtained LODs were higher than those reported using LC-MS for the analysis of MDPV in urine (i.e. between 0.06 and 2 ng mL^{-1}) [10–13]. However, it is important to highlight that none of these alternative methods allowed the separation of MDPV enantiomers. Despite MS detection usually presents a higher sensitivity than UV detection, the LODs by in-line SPE-CE-MS were slightly higher than those obtained for the MDPV enantiomers in urine by in-line SPE-CE-UV in our recent work (i.e. 3 ng mL^{-1}) [7]. As indicated before, this can be explained due to the modifications needed to set an appropriate in-line SPE-CE-MS method for enantiomer analysis.

4 Concluding remarks

In this study we reported a successful methodology for the enantiodetermination of MDPV in urine samples by in-line SPE-CE-MS at the typical ng mL^{-1} levels at which this compound is present in this biological fluid. As far as we know, this is the first time that in-line SPE-CE has been demonstrated in combination with chiral CE-MS.

The enantioseparation of MDPV was achieved by adding 0.5% (m/v) of sulphated-α-CD to the BGE (10 mM ammonium acetate BGE (pH 7)). Due to the anionic character of this CD the chiral separation was conducted in

the counter migration approach without negatively affecting the mass spectrometer performance. The MS acquisition was also segmented to appropriately switch the ionization source and prevent the entrance of non-volatile contaminants into de mass spectrometer. For the elution a water:MeOH:formic acid (68:30:2 v/v/v) solution injected at 50 mbar for 20 s was providing enrichment factors of 500 times while maintaining an appropriate enantio-separation. The potential of the method for forensic, toxicological or clinical applications was demonstrated by validating the method for the analysis of urine samples. Remarkable figures of merit were obtained, including LODs of 10 ng mL^{-1} for both enantiomers.

In the future, novel combinations of sorbents, extraction conditions, separation capillaries, BGE compositions, separation approaches and detection conditions should be explored to further enhance sensitivity and expand the applicability of chiral in-line SPE-CE-MS that is able to provide a novel insight into enantiomer analysis at the low concentration level.

Novelty statement

We consider this work to be novel because this is the first time that in-

Table 2. Method validation in terms of linearity, intra-day and inter-day precision, accuracy of peak areas and LODs obtained for spiked urine samples by in-line SPE CE-MS.

	MDPV	**MDPV'**
Linearity (ng mL^{-1})	30-250	30-250
Calibration curve	y = 84071x + 218912	y = 104393x + 4988
r^2	0.991	0.995
LODs (ng mL^{-1})	10	10
Intra-day RSD of peak area (%, n = 5)		
30 ng mL^{-1}	8.7	8.7
100 ng mL^{-1}	7.5	7.7
250 ng mL^{-1}	7.7	7.9
Inter-day RSD of peak area (%, n = 5)		
30 ng mL^{-1}	10.3	9.2
100 ng mL^{-1}	8.7	8.4
250 ng mL^{-1}	9.2	8.8
Relative error of peak area (%, n = 5)		
30 ng mL^{-1}	8.7	8.4
100 ng mL^{-1}	7.9	8.0
250 ng mL^{-1}	8.1	8.3

line solid phase extraction capillary electrophoresis has been successfully combined with chiral capillary electrophoresis mass spectrometry.

Declaration of competing interest

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

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References

[1] C.L. German, A.E. Fleckenstein, G.R. Hanson, Bath salts and synthetic cathinones: An emerging designer drug phenomenon, Life Sci. 97 (2014) 2–8. https://doi.org/10.1016/j.lfs.2013.07.023.

[2] M.J. Valente, P. Guedes De Pinho, M. Lourdes Bastos, F. Carvalho, M. Carvalho, Khat and synthetic cathinones: a review, Arch. Toxicol. 88 (2014) 15–45. https://doi.org/10.1007/s00204-013-1163-9.

[3] M. Coppola, R. Mondola, 3,4-Methylenedioxypyrovalerone (MDPV): Chemistry, pharmacology and toxicology of a new designer drug of abuse marketed online, Toxicol. Lett. 208 (2012) 12–15. https://doi.org/10.1016/j.toxlet.2011.10.002.

[4] M. Moini, C.M. Rollman, Compatibility of highly sulfated cyclodextrin with electrospray ionization at low nanoliter/minute flow rates and its application to capillary electrophoresis/electrospray ionization mass spectrometric analysis of cathinone derivatives and their optical isomers, Rapid Commun. Mass Spectrom. 29 (2015) 304–310. https://doi.org/10.1002/rcm.7106.

[5] G. Merola, H. Fu, F. Tagliaro, T. Macchia, B.R. McCord, Chiral separation of 12 cathinone analogs by cyclodextrin-assisted capillary electrophoresis with UV and mass spectrometry detection, Electrophoresis. 35 (2014) 3231–3241. https://doi.org/10.1002/elps.201400077.

[6] T. Baciu, F. Borrull, M. Calull, C. Aguilar, Enantioselective determination of cathinone derivatives in human hair by capillary electrophoresis combined in-line with solid-phase extraction, Electrophoresis. 37 (2016) 2352–2362. https://doi.org/10.1002/elps.201600149.

[7] A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE, Electrophoresis. 40 (2019) 1762–1770. https://doi.org/10.1002/elps.201900072.

[8] A. Pérez-Alcaraz, F. Borrull, M. Calull, C. Aguilar, Field-amplified sample injection combined with CE for the enantiodetermination of cathinones in urine samples, J. Sep. Sci. 43 (2020) 2914–2924. https://doi.org/https://doi.org/10.100

2/jssc.201901067.

[9] A. Pérez-alcaraz, F. Borrull, C. Aguilar, M. Calull, An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis, Microchem. J. 158 (2020) 105300. https://doi.org/10.1016/j.microc.2020.105300.

[10] S. Pascual-Caro using, N. Fontanals, F. Borrull, C. Aguilar, M. Calull, Solid-phase extraction based on cation-exchange sorbents followed by liquid chromatography high-resolution mass spectrometry to determine synthetic cathinones in urine, Forensic Toxicol. 38 (2020) 185–194. https://doi.org/10.1007/s11419-019-00508-8.

[11] L. Glicksberg, K. Bryand, S. Kerrigan, Identification and quantification of synthetic cathinones in blood and urine using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry, J. Chromatogr. B. 1035 (2016) 91–103. https://doi.org/10.1016/j.jchromb.2016.09.027.

[12] M. Concheiro, M. Castaneto, R. Kronstrand, M.A. Huestis, Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography-high resolution mass spectrometry and library matching, J. Chromatogr. A. 1397 (2015) 32–42. https://doi.org/10.1016/j.chroma.2015.04.002.

[13] J. Sánchez-González, S. Odoardi, A.M. Bermejo, P. Bermejo-Barrera, F.S. Romolo, A. Moreda-Piñeiro, S. Strano-Rossi, HPLC-MS/MS combined with membrane-protected molecularly imprinted polymer micro-solid-phase extraction for synthetic cathinones monitoring in urine, Drug Test. Anal. 11 (2019) 33–44. https://doi.org/10.1002/dta.2448.

[14] M.A. Meetani, R.H. Alremeithi, M.K. Mousa, Enantioseparation of synthetic cathinones enantiomers with tertiary amine structure in urine and plasma, J. Chromatogr. Sci. 57 (2019) 361–368. https://doi.org/10.1093/chromsci/bmz008.

[15] E. Gerace, D. Caneparo, F. Borio, A. Salomone, M. Vincenti, Determination of several synthetic cathinones and an amphetamine-like compound in urine by gas chromatography with mass spectrometry. Method validation and application to real cases, J. Sep. Sci. 42 (2019) 1577–1584. https://doi.org/10.1002/jssc.201801249.

[16] W.Y. Hong, Y.C. Ko, M.C. Lin, P.Y. Wang, Y.P. Chen, L.C. Chiueh, D.Y.C. Shih, H.K. Chou, H.F. Cheng, Determination of synthetic cathinones in urine using gas chromatography-mass spectrometry techniques, J. Anal. Toxicol. 40 (2016) 12–16. https://doi.org/10.1093/jat/bkv108.

[17] K.A. Alsenedi, C. Morrison, Determination and long-term stability of twenty-nine cathinones and amphetamine-type stimulants (ATS) in urine using gas chromatography–mass spectrometry, J. Chromatogr. B. 1076 (2018) 91–102. https://doi.org/10.1016/j.jchromb.2018.01.027.

[18] R.H. Alremeithi, M.A. Meetani, A.A. Alaidaros, A. Lanjawi, K. Alsumaiti, Simultaneous quantitative determination of synthetic cathinone enantiomers in urine and plasma GC-NCI-MS, J. Anal. Methods Chem. (2018) 1–11. https://doi.org/10.1155/2018/4396043.

[19] K. Cameron, R. Kolanos, R. Verkariya, L. De Felice, R.A. Glennon, Mephedrone and methylenedioxypyrovalerone (MDPV), major constituents of “bath salts,” produce opposite effects at the human dopamine transporter, Psychopharmacology (Berl). 227 (2013) 493–499. https://doi.org/10.1007/s00213-013-2967-2.

[20] R. Kolanos, J.S. Partilla, M.H. Baumann, B.A. Hutsell, M.L. Banks,

S.S. Negus, R.A. Glennon, Stereoselective actions of methylenedioxypyrovalerone (MDPV) to inhibit dopamine and norepinephrine transporters and facilitate intracranial self-stimulation in rats, ACS Chem. Neurosci. 6 (2015) 771–777. https://doi.org/10.1021/acschemneuro.5b00006.

[21] R.B. Yu, J.P. Quirino, Chiral selectors in capillary electrophoresis: Trends during 2017–2018, Molecules. 24 (2019) 1135–1153. https://doi.org/10.3390/molecules24061135.

[22] S. Lee, S.J. Kim, E. Bang, Y.C. Na, Chiral separation of intact amino acids by capillary electrophoresis-mass spectrometry employing a partial filling technique with a crown ether carboxylic acid, J. Chromatogr. A. 1586 (2019) 128–138. https://doi.org/10.1016/j.chroma.2018.12.001.

[23] L. Sánchez-Hernández, E. Domínguez-Vega, C. Montealegre, M. Castro-Puyana, M.L. Marina, A.L. Crego, Potential of vancomycin for the enantiomeric resolution of FMOC-amino acids by capillary electrophoresis-ion-trap-mass spectrometry, Electrophoresis. 35 (2014) 1244–1250. https://doi.org/10.1002/elps.201300489.

[24] M. Castro-Puyana, M.L. Marina, Chiral capillary electrophoresis-mass spectometry, in: G.K.E. Scriba (Ed.), Chiral Sep. Methods Protoc. Methods Mol. Biol., Springer Science+Business Media, New York, 2019: pp. 391–404. https://doi.org/10.1007/978-1-4939-9438-0_23.

[25] T. Mikuma, Y.T. Iwata, H. Miyaguchi, K. Kuwayama, K. Tsujikawa, T. Kanamori, H. Inoue, The use of a sulfonated capillary on chiral capillary electrophoresis/mass spectrometry of amphetamine-type stimulants for methamphetamine impurity profiling, Forensic Sci. Int. 249 (2015) 59–65. https://doi.org/10.1016/j.forsciint.2015.01.015.

[26] X. Cui, C. Liang, F. Gong, R. Wang, C. Ni, Y. Wu, G. Chen, Y. Zhang, Simultaneous chiral analysis of amphetamine-type stimulants and ephedrine by capillary electrophoresis coupled to time-of-flight mass spectrometry, Chirality. 30 (2018) 1079–1087. https://doi.org/10.1002/chir.22987.

[27] J. Olsson, N.D. Marlin, L.G. Blomberg, Enantiomeric separation of omeperazole enantiomers by aqueous CE using UV and MS detection, Chromatographia. 66 (2007) 421–425. https://doi.org/10.1365/s10337-007-0317-4.

[28] R. Ramautar, G.W. Somsen, G.J. de Jong, Developments in coupled solid-phase extraction-capillary electrophoresis 2013-2015, Electrophoresis. 37 (2016) 35–44. https://doi.org/10.1002/elps.201500401.

[29] F. Benavente, S. Medina-Casanellas, E. Giménez, V. Sanz-Nebot, On-line solid-phase extraction capillary electrophoresismass spectrometry for preconcentration and clean-up of peptides and proteins, in: N. Thuy Tran, M. Taverna (Eds.), Capill. Electrophor. Proteins Pept. Methods Protoc. Methods Mol. Biol., Springer Science+Business Media, New York, 2016: pp. 67–84. https://doi.org/10.1007/978-1-4939-4014-1.

[30] L. Pont, R. Pero-Gascon, E. Gimenez, V. Sanz-Nebot, F. Benavente, A critical retrospective and prospective review of designs and materials in in-line solid-phase extraction capillary electrophoresis, Anal. Chim. Acta. 1079 (2019) 1–19. https://doi.org/10.1016/j.aca.2019.05.022.

[31] P.M. Nowak, M. Woźniakiewicz, M. Mitoraj, F. Sagan, P. Kościelniak, Thermodynamics of acid-base dissociation of several cathinones and 1-phenylethylamine, studied by an accurate capillary electrophoresis method free from the Joule heating impact, J. Chromatogr. A. 1539 (2018) 78–86.

https://doi.org/10.1016/j.chroma.2018.01.047.

[32] United Nations Office on Drugs and Crime (UNODC), Guidance for the validation of analytical methodology and calibration of equipment used for testing of illicit drugs in seized materials and biological specimens : a commitment to quality and continuous improvement, United Nations, New York, 2009. https://www.unodc.org/documents/scientific/validation_E.pdf (accessed February 1, 2020).

3.1.3. Discusión de resultados

Aunque los resultados obtenidos durante los estudios presentados en la sección anterior ya han sido comentados y discutidos en los artículos derivados de los mismos, en este apartado se resumen y discuten los aspectos más importantes. Por un lado, en el primero de estos estudios se optimizó y validó de manera satisfactoria una estrategia para la enantiodeterminación de catinonas (la *R,S*-mefedrona, uno de sus metabolitos, la *R,S*-4-metilefedrina, y la *R,S*-MDPV) en orina mediante *in-line* SPE CE-UV. Por otro lado, en el segundo estudio se adaptó el método previamente desarrollado de *in-line* SPE-CE con el objetivo de sustituir la detección UV por un sistema de MS. Este procedimiento de *in-line* SPE-CE-MS fue validado satisfactoriamente para la enantiodeterminación de la *R,S*-MDPV en orina.

En la metodología de *in-line* SPE-CE-UV la separación quiral de las catinonas analizadas se consiguió mediante la adición de una mezcla de dos CDs, concretamente la 2-hidroxipropil α-CD 8 mM y la α-CD 5 mM, al BGE (una solución acuosa de fosfato monosódico 70 mM a pH 2,5). En estas condiciones se obtuvieron valores de *Rs* entre 1,23 y 1,91 para los enantiómeros de los catinonas estudiadas. En el caso del procedimiento de *in-line* SPE-CE-MS se tuvo que variar tanto la composición del BGE como el tipo de selector quiral para evitar la contaminación de la fuente de ionización. Debido a que en CE-MS el BGE debe presentar una conductividad baja y ser volátil [1], se evaluaron diferentes BGE que cumplían estas características y el que mejor resultados ofreció fue una solución acuosa de acetato de amonio 10 mM (pH 7). En cuanto al selector quiral, se optó por emplear una CD aniónica, en concreto la α-CD sulfatada. En las condiciones de pH de trabajo, esta CD está cargada negativamente, lo que impede su entrada en el espectrómetro de masas ya que migra en sentido contrario a éste. Además, como medida complementaria, la etapa de acondicionamiento y la primera parte de la separación electroforética se realizaron manteniendo apagado el gas del nebulizador y el voltaje de la fuente de ionización para evitar la entrada de compuestos no volátiles en el espectrómetro de masas. En estas condiciones, este detector puede funcionar de forma prolongada sin la necesidad de implementar ningún procedimiento de mantenimiento específico, aparte de la limpieza semanal de la fuente de ionización recomendada por el fabricante.

En cuanto al preconcentrador, en los dos métodos se utilizó el mismo diseño. En concreto, un *fritless packed bed* formado por un capilar de 2 cm de largo y de diámetro interno de 150 µm y relleno de partículas de un sorbente polimérico

(Oasis HLB). Con el objetivo de reducir el tiempo de análisis, la muestra fue inyectada mediante la aplicación de una elevada presión externa (3 bares). Esta alta presión de inyección permitió utilizar un tiempo de carga de muestra de 20 min, que es un tiempo considerablemente menor que los 40 min previamente empleados en nuestro grupo de investigación para la determinación de catinonas en muestras de cabello [2].

La metodología de *in-line* SPE-CE permitió obtener elevados factores de preconcentración tanto mediante detección UV (de entre 6000 y 8000) como mediante MS (500). No obstante, cuando comparamos estos valores podemos constatar que el valor obtenido en la estrategia de *in-line* SPE-CE-MS es significativamente menor. Esto es debido a que las condiciones de las distintas etapas de preconcentración no son las mismas en ambos métodos. Concretamente, el eluyente utilizado en cada método es diferente, siendo el eluyente de la metodología con detección por MS el que contenía un menor volumen de solvente orgánico, lo que explica las diferencias en los factores de preconcentración. De hecho, para el procedimiento de *in-line* SPE-CE-MS se evaluaron diferentes mezclas hidroorgánicas con una baja concentración de solvente orgánico. Esto es debido a que las condiciones del BGE impedían el uso de un eluyente con un elevado volumen de solvente orgánico sin comprometer la separación enantiomérica. Sin embargo, en ambas metodologías se consiguieron LODs capaces de alcanzar los niveles de concentración a los cuales las catinonas se pueden encontrar habitualmente en orina (del orden de ng/mL) después de un pretratamiento de muestra basado en una LLE empleando una mezcla acetato de etilo/isopropanol (4:1) como solvente. En concreto estos LODs fueron de entre 3 y 8 ng/mL para el método con detección UV y de 10 ng/mL para el método con detección por MS. Por tanto, esta técnica de preconcentración demostró su aplicabilidad tanto para análisis clínicos como forenses y toxicológicos. En comparación con otros procedimientos analíticos, estos LODs son superiores a los obtenidos habitualmente en la determinación de estas mismas catinonas en muestras de orina mediante LC-MS (0,04-3 ng/mL [3–7]) y están dentro del rango de los obtenidos con GC-MS (2-30 ng/mL [8–12]), aunque es de destacar que en ninguno de esos estudios se llevó a cabo la separación enantiomérica de las catinonas analizadas.

Finalmente, es importante remarcar que el procedimiento de *in-line* SPE-CE-MS para la enantiodeterminación de catinonas es el primer método publicado en el que

se combina simultáneamente una estrategia de preconcentración en línea para CE, con la separación quiral y la detección por MS. Por tanto, abre el camino a este tipo de estrategias y puede servir como punto de partida para futuras investigaciones que traten de desarrollar metodologías similares.

Referencias

[1] Castro-Puyana, M., Marina, M. L., In *Chiral Separations: Methods and Protocols, Methods in Molecular Biology*, Scriba, G. K. E., Ed., Springer Science+Business Media, New York, 2019, *Vol*. 1985, pp. 391–404.

[2] Baciu, T., Borrull, F., Calull, M., Aguilar, C., *Electrophoresis* 2016, *37*, 2352–2362.

[3] Glicksberg, L., Bryand, K., Kerrigan, S. *J. Chromatogr. B* 2016, *1035*, 91–103.

[4] Mercolini, L., Protti, M., Catapano, M. C., Rudge, J., Sberna, A. E. *J. Pharm. Biomed. Anal.* 2016, *123*, 186–194.

[5] Sánchez-González, J., Odoardi, S., Bermejo, A. M., Bermejo-Barrera, P., Romolo, F. S., Moreda-Piñeiro, A., Strano-Rossi, S. *Drug Test. Anal.* 2019, *11*, 33–44.

[6] Pascual-Caro, S., Fontanals, N., Borrull, F., Aguilar, C., Calull, M. *Forensic Toxicol.* 2020, *38*, 185–194.

[7] Fan, S. Y., Zang, C. Z., Shih, P. H., Ko, Y. C., Hsu, Y. H., Lin, M. C., Tseng, S. H., Wang, D. Y. *Forensic Sci. Int.* 2020, *315*, 110429.

[8] Hagan, K. S., Reidy, L. *Forensic Sci. Int.* 2015, *257*, 71–75.

[9] Hong, W. Y., Ko, Y. C., Lin, M. C., Wang, P. Y., Chen, Y. P., Chiueh, L. C., Shih, D. Y. C., Chou, H. K., Cheng, H. F. *J. Anal. Toxicol.* 2016, *40*, 12–16.

[10] Alsenedi, K. A., Morrison, C. *Anal. Methods* 2018, *10*, 1431–1440.

[11] Mercieca, G., Odoardi, S., Cassar, M., Strano-Rossi, S. *J. Pharm. Biomed. Anal.* 2018, *149*, 494–501.

[12] Gerace, E., Caneparo, D., Borio, F., Salomone, A., Vincenti, M. *J. Sep. Sci.* 2019, *42*, 1577–1584.

3.2. Aplicación de diferentes estrategias de preconcentración electroforética para la determinación de catinonas en orina

Alternativamente a las técnicas basadas en principios cromatográficos, la preconcentración en línea en CE también se puede lograr mediante estrategias de preconcentración basadas en principios electroforéticos. Dentro de este tipo de metodologías destacan las llamadas técnicas de *stacking*, en las que los analitos se concentran en una banda estrecha (*stack*) dentro del propio capilar de separación debido a una reducción abrupta y temporal de su velocidad de migración [1–5].

En el momento de la realización de la presente Tesis Doctoral, no se tenía constancia de ninguna publicación centrada en el uso de técnicas de *stacking* para la determinación de catinonas. Por ello, se plantearon distintos estudios orientados a la aplicación de este tipo de estrategias de preconcentración en metodologías destinadas a la enantioseparación de catinonas en orina. Además, como las catinonas son bases débiles, en unas determinadas condiciones de pH estos compuestos se pueden encontrar cargados positivamente y, por tanto, se pueden beneficiar de la selectividad que ofrece una EKI. Por estos motivos, se estudiaron dos técnicas de preconcentración electroforética basadas en la inyección electrocinética de los compuestos analizados, FASI y EKS, con las que el grupo de investigación ya había trabajado anteriormente para la determinación de distintos tipos de compuestos en muestras ambientales y biológicas [6-8]. En la técnica de FASI, en primer lugar, se inyecta hidrodinámicamente un BGE de alta conductividad. Seguidamente, en algunas ocasiones, se inyecta hidrodinámicamente un pequeño *plug* de solvente de baja conductividad para mejorar la eficacia de la preconcentración y la reproducibilidad del método. Finalmente, los analitos, que se encuentran disueltos en un solvente de baja conductividad, se inyectan electrocinéticamente y se procede a la separación electroforética. Debido a la diferencia de conductividades entre la zona de muestra y la zona del BGE, los analitos se ralentizarán abruptamente al alcanzar la interfase muestra/BGE y, por tanto, se preconcentrarán en la propia interfase [1,2]. La técnica de EKS consiste en la unión de dos estrategias de preconcentración, FASI y *t*-ITP. En este caso la muestra es inyectada electrocinéticamente entre un LE, compuesto por iones de mayor movilidad que los analitos, y un TE, compuesto por iones de menor movilidad que los analitos. En estas condiciones, al aplicar el voltaje de separación electroforética, los compuestos analizados se concentrarán en la interfase LE/TE puesto que migrarán más rápidamente en la zona del TE que en la zona del LE [2,3].

El primero de estos estudios, en que se desarrolló la metodología de FASI-CE, fue publicado en la revista científica *Journal of Separation Science 43 (2020) 2914-2924* mientras que el segundo, donde se evalúa la estrategia de EKS-CE, fue publicado en la revista *Microchemical Journal 158 (2020) 105300.*

Referencias

[1] Breadmore, M. C., Grochocki, W., Kalsoom, U., Phung, S. C., Rokh, M. T., Cabot, J. M., Ghiasvand, A., Li, F., Shallan, A. I., Keyon, A. S. A., Wuethrich, A., *Electrophoresis* 2019, *40*, 17–39.

[2] Lian, D. S., Zhao, S. J., Li, J., Li, B. L., *Anal. Bioanal. Chem.* 2014, *406*, 6129–6150.

[3] Lu, Y., Hou, X., Wang, D., Zhong, H., *J. Liq. Chromatogr. Relat. Technol.* 2017, *40*, 528–535.

[4] Šlampova, A., Malá, Z., Gebauer, P., *Electrophoresis* 2019, *40*, 40–54.

[5] Kitagawa, F., Kawai, T., Otsuka, K., *Anal. Sci.* 2013, *29*, 1129–1139.

[6] Botello, I., Borrull, F., Aguilar, C., Calull, M. *Electrophoresis* 2010, *31*, 2964–2973.

[7] Botello, I., Borrull, F., Calull, M., Aguilar, C. *J. Sep. Sci.* 2013, *36*, 524–531.

[8] Maijó, I., Borrull, F., Aguilar, C., Calull, M. *Electrophoresis* 2013, *34*, 363–373.

3.2.1. Field-amplified sample injection combined with CE for the enantiodetermination of cathinones in urine samples

FIELD-AMPLIFIED SAMPLE INJECTION COMBINED WITH CE FOR THE ENANTIODETERMINATION OF CATHINONES IN URINE SAMPLES

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Abstract

This work presents a capillary electrophoresis methodology for the enantiodetermination of cathinones in urine employing a liquid-liquid extraction sample pretreatment. The cathinones were enantioseparated by adding a mixture of 8 mM 2-hydroxypropyl β-cyclodextrin and 5 mM β-cyclodextrin to the background electrolyte, which consists of 70 mM of monosodium phosphate aqueous solution at pH 2.5. Field-amplified sample injection was used as preconcentration strategy to improve the sensitivity. We studied various parameters that affect this stacking strategy, in particular, the sample solvent and its pH, the presence or absence of a low conductivity solvent plug introduced before the sample injection, the nature and volume of this plug, and the voltage and time of the electrokinetic injection of the sample. The optimum conditions were achieved by injecting a plug of isopropanol:H_2O 50/50 at 50 mbar for 5 s prior to the electrokinetic injection of the sample prepared in an aqueous solution of HCl 10^{-6} M. The sensitivity enhancement factors were from 562 to 601 in terms of peak area and from 444 to 472 in terms of peak height. The method was validated by analyzing spiked urine samples, obtaining a linear range of 25 to 1000 ng/mL and limits of detection ranging from 15 to 45 ng/mL.

Keywords: *Cathinones / Chiral capillary electrophoresis / Field-amplified sample injection / Urine analysis*

1 Introduction

In the last few years, the consumption of synthetic cathinones has significantly increased as they are a more affordable and easy to acquire alternative to conventional controlled substances [1,2]. These synthetic cathinones can be found on the Internet or at "smartshops" sold as "bath salts". Their main ingredients are mephedrone and methylenedioxypyrovalerone (MDPV) [1–4].

After their consumption, usually nasally insufflated or ingested [1], cathinones enter the organism where they produce their stimulating effects. Therefore, determining cathinones in different biological matrices, such as blood [5,6], hair [7,8] and oral fluids [9], can provide a useful tool for controlling their abuse. However, urine is the most common biological sample used to document drug consumption, because it is easy to collect, it is noninvasive and has a detection window which can reach weeks. Consequently, different studies have developed strategies that are capable of determining cathinones in urine samples at low concentration levels (ng/mL) [6,10–15]. In the literature the different approaches are mainly based on LC-MS [6,12–14] or GC-MS [10,11,15] in combination with a pretreatment strategy, for which the preferred choices are SPE [6,11–13] or LLE [10,14,15].

Cathinones present a chiral center and then they can be found in two enantiomeric forms (*R* and *S*) [1,3,4,7,16–18]. Unambiguous identification of these enantiomers in biological samples can be very useful in toxicological and forensic applications, since the two enantiomers can have different effects due to a different pharmacokinetic and pharmacodynamic behavior [1,3,4]. In fact, different studies confirm that the *S*(-) enantiomer is generally the most active form [1,3,16,18]. However, until now only a few authors have developed methods for the enantiodetermination of cathinones in biological samples as urine or blood [17,18]. Moreover, with LC or GC the enantioseparation of these compounds can be expensive and difficult since a chiral stationary phase is usually required [18] or in other cases time-consuming derivatization steps are needed [17]. Therefore, simpler alternatives that make it possible to enantiodetermine these compounds are of great interest.

In recent years, CE has proved to be an appropriate technique for enantiodetermining cathinones by simply adding an additive in the BGE, such as cyclodextrins (CDs) [7,19–22]. Moreover, this technique has other advantages over GC and LC, such as high efficiency, short analysis time and low consumption of the sample and reagents. These characteristics make the technique cheap and environmentally friendly.

Despite all the advantages of CE, one of its main drawbacks is the relatively low concentration sensitivity which can be achieved, especially when UV detection is used. To overcome this, a number of sample preconcentration strategies have been developed. Among them, stacking based methods are widely used [23,24]. These are based on introducing a large volume of sample that is concentrated into a small volume inside the capillary due to migration changes of the analytes between the sample zone and the BGE zone [23].

Field-amplified sample injection (FASI) is one of the most popular stacking techniques since it is quite simple. This technique is based on the electrokinetic injection of a large volume of a low conductivity sample into a capillary filled with a high conductivity BGE. In these conditions, when a high voltage is applied, the electric field strength is higher in the sample zone than in the BGE zone. Consequently, the analytes move quickly through the sample zone, but their mobility is drastically reduced when they arrive to the boundary between the sample zone and the BGE, which leads to the analytes being stacked. Moreover, the previous injection of a short plug of a low conductivity solvent generally enhances the sample electrokinetic injection because of the difference in conductivity between the sample and the solvent plug [25–34].

In recent studies, FASI has been successfully used for determining different analytes in urine, such as anesthetics [27], tetracyclines [28], beta(2)-agonists [31,32] or drugs [33,34]. The sensitivity enhancement factors (SEFs) obtained in these studies ranged between 41 and 1046 [27,28,31,33], which shows the preconcentration potential of the technique. An important feature for achieving high sensitivity when FASI is applied to complex matrices, such as urine, is that the sample needs to be free of inorganic salts because the presence of these salts can significantly disrupt the stacking of

the analytes. Therefore, a pretreatment step to clean up the urine sample before the FASI analysis is usually necessary [27,28,31–34].

The aim of this work was to develop a sensitive, simple and rapid method for enantiodetermining *R,S*-mephedrone, one of its metabolites *R,S*-4-methylephedrine, and *R,S*-MDPV in urine samples by CE. To achieve this, we developed an offline LLE procedure combined with FASI-CE. We studied the different parameters that affect FASI, such as sample solvent and its pH, injection of a solvent plug (composition and volume), sample injection voltage and time. To the best of our knowledge this is the first time that a FASI-based CE-UV procedure has been applied for enantiodetermining cathinones in urine samples.

2 Materials and methods

2.1 Reagents and standards

The standards of *R,S*-mephedrone and *R,S*-MDPV were purchased as hydrochloride salts with a purity of 98% from LGC Standards (Teddington, UK). *R,S*-4-methylephedrine was acquired as a solution of 1 mg/mL in methanol (MeOH) from LGC Standards (Teddington). Individual stock standard solutions of the cathinones (100 mg/L of *R,S*-4-methylephedrine, 1000 mg/L of *R,S*-mephedrone and 2000 mg/L of *R,S*-MDPV) were prepared by dissolving or diluting an appropriate amount of the standards in MeOH and were kept in the freezer at -20°C. Working standard solutions of a mixture of all the compounds were prepared weekly by diluting stock solutions in Milli-Q water and were stored at 4°C. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard solutions in Milli-Q water. Acetic acid, acetonitrile (ACN), dichloromethane (DCM), ethyl acetate, hexane, isopropanol, MeOH and toluene, all of analytical-reagent grade, were acquired from J.T Baker (Deventer, Netherlands). Ammonium hydroxide 28%, phosphoric acid 85%, monosodium phosphate 99%, sodium acetate 99%, sodium hydroxide (NaOH) 97%, β-CD 97%, and 2-hydroxypropyl β-CD were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Sodium chloride (NaCl) 99% was purchased from Fluka Honeywell (Morris Plains, NJ, USA). Milli-Q water was obtained with a water purification system from Veolia Water (Paris, France).

2.2 Instrumentation

The electrophoretic system was a 7100 CE System from Agilent Technologies (Waldbronn, Germany) equipped with a diode array detector (DAD). The pH measurements were performed with a GLP 21 pH-meter from Crison (Barcelona, Spain). A Universal 32 R centrifuge from Hettich (Kirchlengern, Germany) was used for the centrifugation processes.

2.3 CE conditions

Separations were performed in a bared fused-silica capillary of 50 µm id and total length of 80 cm (72 cm effective length) acquired from Polymicro Technologies (Phoenix, AZ, USA).

Before the first use, the capillaries were conditioned with NaOH 1 M for 40 min and 10 min of Milli-Q water at 930 mbar. At the beginning of each working day, a conditioning step was performed with NaOH 0.1 M for 10 min, Milli-Q water for 5 min and BGE for 5 min, all at 930 mbar. Between each run, the conditioning step was performed with NaOH 0.1 M, Milli-Q water and BGE, all at 930 mbar for 4 min. At the end of each run, a postconditioning step was carried out by injecting Milli-Q water for 5 min at 930 mbar.

The electrophoretic separation was achieved using a voltage of 30 kV (positive polarity). The BGE solution consisted of an aqueous solution of 70 mM of monosodium phosphate, adjusted to pH 2.5 (with concentrated phosphoric acid), containing 8 mM 2-hydroxypropyl β-CD and 5 mM β-CD. The BGE was prepared daily and filtered through a 0.45 µm filter prior to use. The temperature of the capillary chamber was set at 25°C and 200 nm was used for detecting the analytes.

2.4 Field-amplified sample injection procedure

The FASI-CE procedure consisted in the following steps: first, the capillary was filled with BGE. Then a plug of isopropanol:H_2O 50/50 was hydrodynamically injected at 50 mbar for 5 s. Analytes were solved in an aqueous solution of HCl 10^{-6} M and then electrokinetically injected by applying a voltage of 10 kV for 45 s. Finally, 30 kV was applied for the CE separation of the analytes.

2.5 Sample preparation

Urine samples were kindly provided by several nonaddicted volunteers in accordance with the national and institutional ethical committee and with the 1964 Helsinki Declaration. They were collected in polypropylene tubes and stored at -20°C until analysis. Pooled urine, prepared by mixing the urine collected from the volunteers, was used for validating the method.

A LLE was used to extract the analytes. First, 0.05 g/mL of NaCl were added to the spiked urine samples, and then alkalized to pH 10 with 28% ammonium hydroxide. Then, 2 mL of toluene was added to 2 mL of alkalized urine sample. The samples were vortex mixed for 1 min, then centrifuged for 10 min at 9000 rpm. The organic phase, containing the analytes, was transferred to a vial and a second extraction of the aqueous phase was performed by adding 2 mL of toluene and repeating the same procedure. Then, the two organic phases were combined and dried under a gentle stream of N_2 until they were completely dry. The residue was reconstituted with 2 mL of HCl 10^{-6} M. Finally, the samples were filtered through a 0.45 µm PTFE syringe filter, then transferred to a microvial and injected into the CE instrument to be analyzed with FASI-CE.

3 Results and discussion

3.1 Enantioseparation of the studied cathinones

In order to achieve the chiral separation of the target compounds, the selected BGE was based on a recent study by our research group in which the same group of cathinones were successfully enantioseparated [22]. This BGE consisted in an aqueous solution of monosodium phosphate at a concentration of 70 mM, adjusted to pH 2.5 (with concentrated phosphoric acid), containing 2-hydroxypropyl β-CD and β-CD at a concentration of 8 and 5 mM, respectively.

3.2 Field-amplified sample injection optimization

Cathinones can be present in urine samples at low concentration levels (ng/mL). Considering the low sensitivity of CE using UV detection, we considered FASI as an in-line preconcentration strategy. We optimized the parameters that may affect this technique to obtain

maximum signal enhancement. These parameters include the sample solvent and its pH, the presence or absence of a plug of low conductivity solvent introduced in the capillary before the sample injection, the nature and volume of this plug and the voltage and time for the electrokinetic sample injection.

3.2.1 Effect of the sample solvent and its pH

The sample solvent and its pH play an important role in the performance of FASI. The pH of the sample should be such that allows the cathinones to be in their cationic form because in this way they can be electrokinetically injected. The cathinones under study have pK_a values between 8.83 and 9.13 [35]. Therefore, for a sample pH below these pK_a values the compounds should be positively charged and thus they can be electrokinetically injected when a positive voltage is applied. The stacking phenomenon in FASI relies on the conductivity difference between the sample zone and the BGE zone, since in this way when a voltage is applied, analytes in the sample zone move rapidly toward the sample/BGE interface, where their velocity is reduced and they stack on the boundary into a very narrow zone prior to their CE separation. Therefore, the sample solvent must be less conductive than the BGE so that the electric field strength is much higher in the sample zone than in the BGE and consequently a field amplification effect can be generated. To investigate the effect of the sample solvent and its pH on the FASI efficiency, three different aqueous solutions were tested in order to obtain a maximum response: an aqueous solution of HCl 10^{-6} M (pH 6); an acetic acid (10^{-3} M)/sodium acetate (10^{-3} M) buffer (pH 4.75); and an aqueous solution of HCl 10^{-3} M (pH 3). In this study, a standard sample containing the analytes at a concentration of 5 µg/mL was electrokinetically injected by applying 10 kV for 10 s. The obtained results can be seen in Figure 1 and, as it can be observed, the peak areas were significantly higher when HCl 10^{-6} M was used as sample solvent in comparison with the other two tested conditions. This can be explained by a lower conductivity of the sample zone when this solvent was used and, consequently, the difference in conductivity with the BGE was higher, which improved the FASI signal [25,26]. Therefore, for further experiments, the compounds were solved in an aqueous solution of HCl 10^{-6} M.

3.2.2 Effect of the solvent plug

The introduction of a small plug of a low conductivity solvent before the sample injection can increase the sample stacking efficiency by providing an enhanced electric field [25–34]. Moreover, in some studies it has been demonstrated that by introducing this solvent plug better reproducibility is achieved than that obtained by performing FASI without a solvent plug [27,29]. Therefore, we analyzed how the presence of the solvent plug affects the response. First we focused on the nature of the solvent. In particular, we tested pure water and different water-organic solvent (ACN, MeOH and iso-propanol) mixtures at a proportion of 50/50. Pure organic solvent plugs were not tested to avoid current failures due to the formation of bubbles in the capillary [28]. For this study a standard sample containing the analytes at a concentration of 2 µg/mL, and dissolved in an aqueous solution of HCl 10^{-6} M, was electrokinetically injected by applying 10 kV for 10 s after the low conductivity solvent plug was hydrodynamically introduced for 10 s at 50 mbar. The results are shown in Figure 2A. It can be seen that the response obtained for all the compounds was significantly higher when a plug of isopropanol:H_2O 50/50 was used.

The effect of changing the ratio of isopropanol:H_2O in the solvent plug was also evaluated by comparing three different ratios (75/25, 50/50 and 25/75) and injecting a standard sample of 500 ng/mL and keeping the previous experimental conditions. Figure 2B shows that with the plug of isopropanol:H_2O 50/50 the sensitivity enhancement was higher for all the target compounds in comparison with the other two tested isopropanol:H_2O ratios.

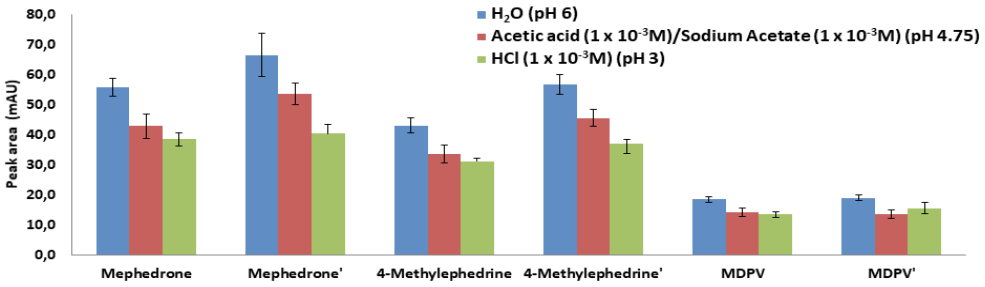

Figure 1. Sample solvent optimization for the FASI procedure with its standard deviations. For this optimization, standard samples containing the target compounds at a concentration of 5 µg/mL were dissolved in different solvents and were electrokinetically injected by applying 10 kV for 10 s.

The solvent plug length was also researched by varying its injection time in a range from 5 s to 20 s at 50 mbar (Figure 2C). Although a solvent plug can contribute to increasing the stacking efficiency as it has been demonstrated by the results previously reported, different authors have also demonstrated that this plug should be as short as possible. This is because the amount of analytes introduced into the capillary can decrease when the plug length increases since a longer plug would

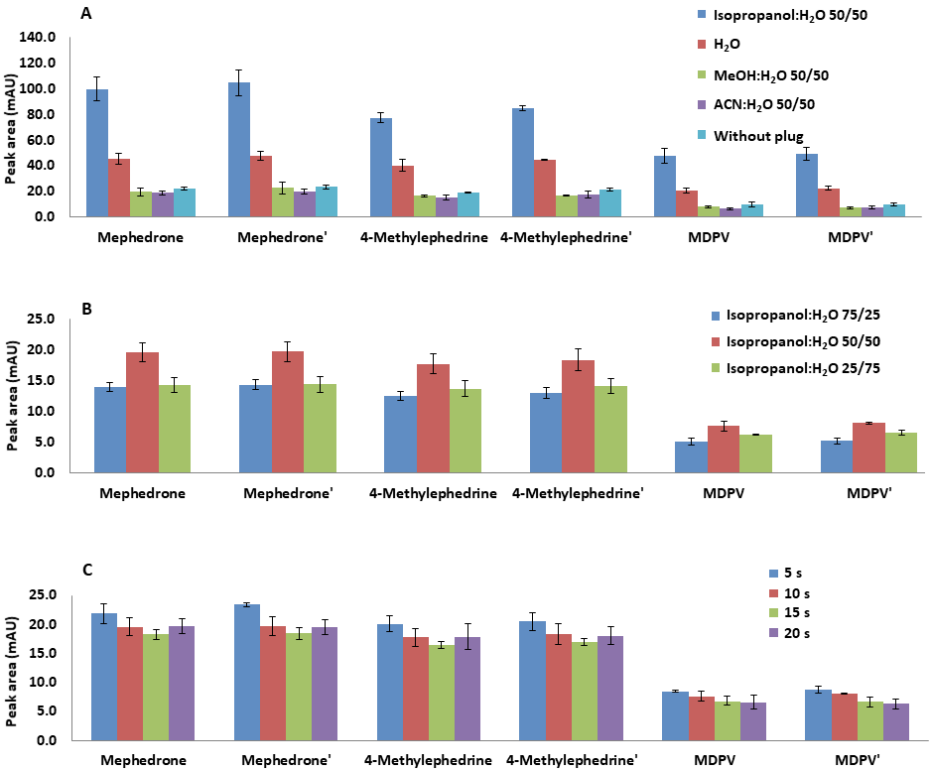

Figure 2. Optimization of the solvent plug conditions for the FASI procedure. (A) Optimization of the solvent plug composition with errors bars showing the standard deviation of the mean (n = 3). For this optimization, standard samples containing 2 µg/mL of the target compounds were dissolved in an aqueous solution of HCl 10^{-6} M and were electrokinetically injected by applying 10 kV for 10 s after the solvent plug was introduced for 10 s at 50 mbar. (B) Optimization of the isopropanol:H_2O ratio with errors bars showing the standard deviation of the mean (n = 3). For this optimization, standard samples containing the target compounds at a concentration of 500 ng/mL were dissolved in an aqueous solution of HCl 10^{-6} M and were electrokinetically injected after the isopropanol:H_2O plug was introduced using the same conditions as in Figure 2A. (C) Optimization of the solvent plug injection time with errors bars showing the standard deviation of the mean (n = 3). For this optimization, standard samples containing 500 ng/mL of the studied compounds were dissolved in an aqueous solution of HCl 10^{-6} M and were electrokinetically injected using the same conditions as in Fig 2A. Previously, a isopropanol:H_2O 50/50 plug was introduced at 50 mbar at different injection times.

result in a lower electric field strength [25,26,32,34]. Our results were in accordance with these findings because the higher sensitivity enhancement for all the analytes was obtained with the shortest plug length tested (5 s). Therefore, 5 s was the selected time.

3.2.3 Effect of sample injection voltage and time

The amount of analytes introduced in the capillary depends on the conditions of the electro-kinetic injection. It is therefore important to study the sample injection time and voltage. The effect of the sample injection time on the enrichment of the cathinones under study was investigated from 5 to 50 s while the injection voltage remained at 10 kV. Figure 3A shows that, as expected, peak areas gradually increase with the injection time. However, it is also necessary to consider the peak broadening that can produce a loss of resolution. Therefore, it is important to achieve a compromise between the sensitivity and resolution. For this reason, we evaluated the resolution values between the enantiomers of each compound with the further increase in the sample injection time, which are shown in Figure 3B. Based on these results, 45 s was selected as sample injection time since for higher times, even that the obtained peak areas were slightly higher, the loss of resolution was an important drawback. The sample injection voltage was also examined in the range between 5 and 10 kV. Voltages higher than 10 kV were not tested as current disturbances were observed. As the peak areas increased with increasing injection voltage and the obtained resolutions were acceptable in all the evaluated conditions, an injection voltage of 10 kV was selected to obtain efficient stacking.

3.3 Sensitivity enhancement factors

The SEFs in terms of peak areas (SEF_{area}) and peak heights (SEF_{height}) for the cathinones under study were calculated to determine the im-provement in CE sensitivity in the FASI-CE procedure applied. The SEF values were calculated based on the ratio of peak area or peak height obtained by FASI-CE to those obtained with conventional CE hydrodynamic injection (multiplied by the dilution factor). For this study, the hydrodynamic injection was performed by applying 50 mbar for 5 s. At these conditions, good results in terms of reproducibility were ob-

tained with RSD values below 6% (calculated according to peak area) for all the analytes.

The SEF_{area} values were 601, 569 and 562 and SEF_{height} values were 469, 444 and 472 for *R,S*-mephedrone, *R,S*-4-methylephedrine and *R,S*-MDPV, respectively. These SEFs emphasize the preconcentration potential of FASI as a stacking technique for determining cathinones.

3.4 Pretreatment of urine samples

Urine, as other biological samples, contains a high concentration of endogenous materials that can negatively affect the stacking efficiency of FASI since this technique is only capable of enriching low-ionic

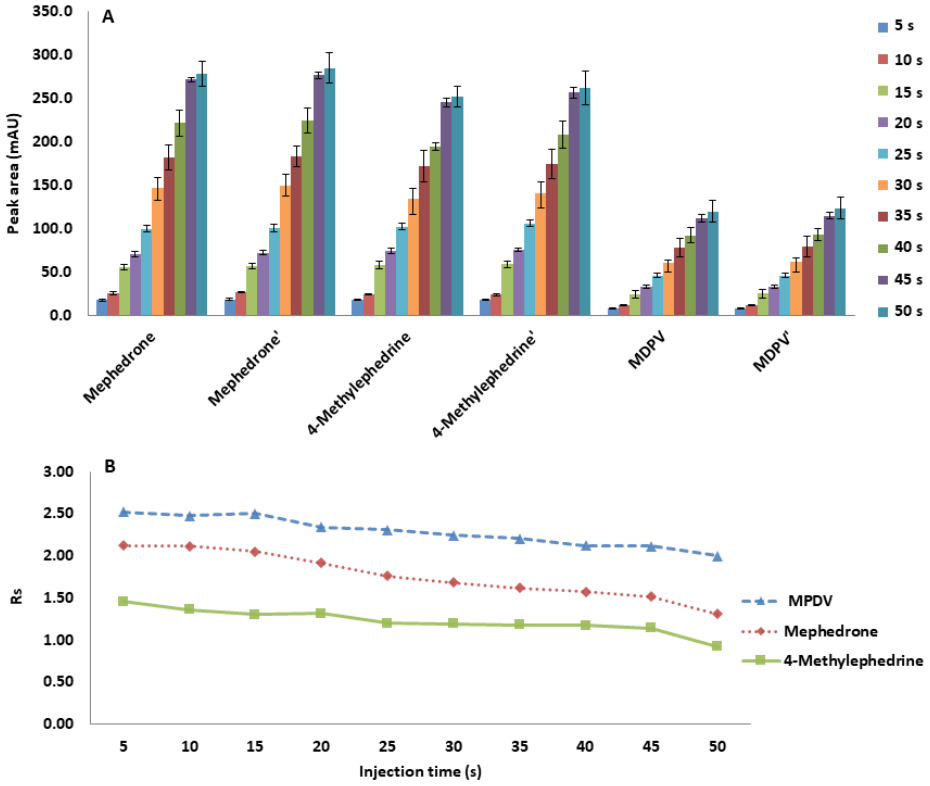

Figure 3. Sample time injection optimization for the FASI procedure in terms of: (A) peak area as a function of the electrokinetic injection time with errors bars showing the standard deviation of the mean (n = 3). (B) Resolution between the enantiomers of each compound as a function of the electrokinetic injection time. For this optimization, a plug of isopropanol:H_2O 50/50 was introduced at 50 mbar for 5 s prior to the electrokinetic injection of a standard sample, which contained 500 ng/mL of the studied compounds dissolved in an aqueous solution of HCl 10^{-6} M, by applying 10 kV for different injection times.

strength samples. Therefore, a previous extraction and clean up step is necessary in urine before its analysis by FASI-CE [33]. Different strategies have been used previously in the literature to reduce the urine matrix effect in FASI, such as a simple sample dilution [27,32] or a pretreatment based on dispersive liquid-liquid micro extraction [31,33]. In a previous work related with the determination of cathinones in urine by in-line SPE-CE a pretreatment based on LLE successfully extracted the analytes from urine samples [22]. Thus, in the present study we tested the same LLE methodology. Briefly, the procedure consisted in the following steps. First, urine samples were alkalized to pH 10 to ensure that analytes were in their neutral form and then a mixture of ethyl acetate/isopropanol (4:1) was used as the extraction solvent. The procedure was repeated twice and the mixed collected organic extracts were dried under a N_2 current. Finally, the extracted analytes were dissolved in an aqueous solution of HCl 10^{-6} M and injected into the CE for its FASI-CE procedure. However, and despite the good results obtained in our previous work, this LLE procedure was not an effective clean-up of the sample when FASI was applied. This was due to the presence of some high-intensity interferent peaks in the electropherogram that appeared before the cathinone peaks, as it can be seen in Figure 4A. In FASI, as the analytes are injected electrokinetically, this can lead to a possible prioritization in the injection of these interferents over the analytes. Consequently, this can have a great effect on the compound's response, as in the present case. This negative effect is reflected in the obtained recoveries for the overall process which were between 15 and 27%, as can be seen in Table 1. These recoveries include the sample extraction by LLE and the FASI injection and were calculated as the ratio between the response obtained for a urine sample spiked with the cathinones at a concentration of 200 ng/mL after performing the overall methodology, and the response obtained for a standard sample without using LLE.

The evaluation of other organic solvents such as dichloromethane (DCM), hexane and toluene was carried out. With both DCM and hexane, the target compounds were not recovered. Otherwise, the use of toluene significantly reduced the presence of the interferent peaks as it can be seen through Figure 4B, which shows an electropherogram

obtained for a blank urine sample using this solvent in the LLE procedure, and Figure 4C which shows an electropherogram from a urine sample spiked with the cathinones at a concentration of 200 ng/mL using the same conditions as for Figure 4B. The recovery values for the overall process when using this solvent are shown in Table 1 and were between 21 and 51%. Even the improvement in the recoveries with toluene due to a more effective clean-up of the urine interferents in comparison with the other tested solvents, they were still relatively low, but we chose that solvent as a compromise between the obtention of good sample clean-up and acceptable recoveries. In an attempt to improve them further, we evaluated the effect of adding NaCl to urine samples prior to the LLE process. The aim of this addition was to saturate the urine with salt to reduce the solubility of the compounds and facilitate their extraction (salting-out). Different concentrations of NaCl were tested, specifically 0.05, 0.1, 0.2 and 0.3 g/mL. The recoveries of cathinones were enhanced when NaCl was added to urine, but the best results were obtained using 0.05 g/mL, as

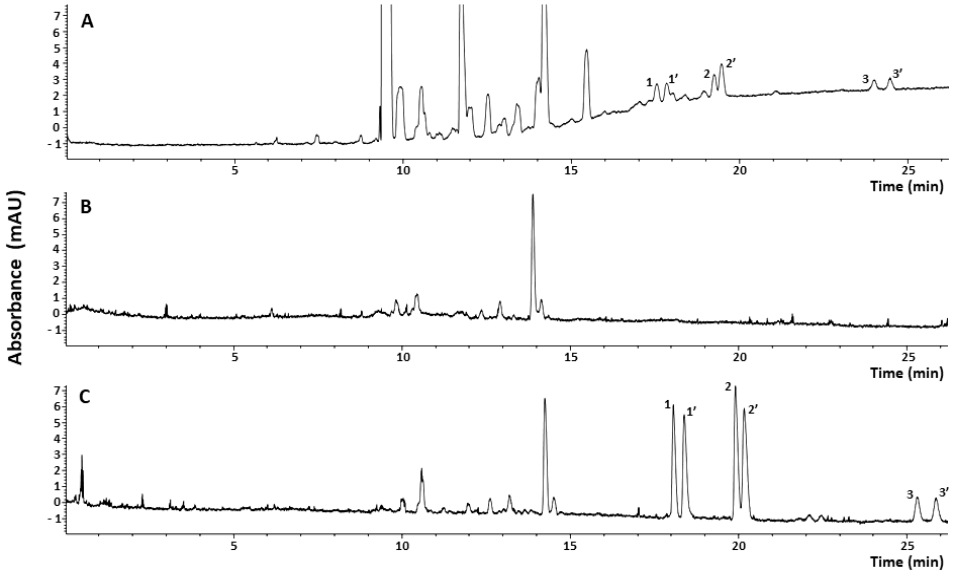

Figure 4. Electropherograms obtained by FASI-CE after a LLE procedure from: (A) urine sample spiked with the studied compounds at a concentration of 200 ng/mL and using ethyl acetate/isopropanol (4:1) as organic solvent (B) urine blank using toluene as organic solvent (C) urine sample spiked with the studied compounds at a concentration of 200 ng/mL using toluene as organic solvent. Peak assignments: (1, 1') *R,S*-mephedrone, (2, 2') *R,S*-4-methylephedrine and (3,3') *R,S*-MDPV.

shown in Table 1. The overall recovery values obtained in this case were between 33 and 65%. Although the recovery values were still low, the LLE procedure developed has an important benefit since it makes it possible to achieve an effective sample clean-up, which is essential to be able to perform FASI.

3.5 Method validation

The method was validated under the optimum conditions. Pooled urine samples collected from nonaddicted volunteers were spiked with a known amount of the studied analytes. The method was validated in terms of linearity, selectivity, repeatability, reproducibility, accuracy, LODs and LOQs. LODs were calculated using a S/N criterion of 3, whereas the LOQs were calculated using a S/N criterion of 10 and were set as the lowest point of the linear range. The values obtained are shown in Table 1.

A matrix match calibration curve was used to evaluate the linearity, using pooled blank urine spiked with known amounts of each compound in the ranges between 50 and 1000, 25 and 1000, and 100 and 1000 ng/mL, for mephedrone, 4-methylephedrine, and MDPV, respectively. As it can be seen in Table 1, the regression coefficients (r^2) were greater than 0.990 for all the compounds, so good linearity was obtained.

The selectivity test was performed for endogenous and exogenous interferents. Potential endogenous interferents were tested by analyzing 10 different urine blank samples. On the other hand, potential exogenous interferents were tested by analyzing urine blanks fortified with a group of amphetamines at 500 ng/mL, as these drugs are similar in structure and effects to cathinones [1,2]. In both cases, we did not observe any interferent peak which could interferer our target compounds signal.

Intraday (repeatability) and interday (reproducibility) precision were evaluated in terms of peak areas at three concentration levels, 100, 500 and 1000 ng/mL. The intraday study was performed by analyzing five replicates of urine spiked at each concentration level during the same day. The interday study was performed by analyzing five replicates of urine spiked at each concentration level during five different days. As is can be seen in Table 1, the RSD values for intra-day samples were below 9% for each

Table 1. Method validation in terms of regression equations, LODs, repeatability, reproducibility, accuracy, and recovery values obtained for spiked urine samples from nonaddicted volunteers by LLE/FASI -CE.

	Mephedrone	**Mephedrone'**	**4-Methylephedrine**	**4-Methylephedrine'**	**MDPV**	**MDPV'**
Linearity (ng/mL)	50-1000	50-1000	25-1000	25-1000	100-1000	100-1000
Calibration curve	y = 0.2424x + 1.073	y = 0.24435x + 3.112	y = 0.2477x + 21.065	y = 0.2589x + 21.754	y = 0.0932x + 1.5250	y = 0.0969x - 0.6250
r^2	0.9902	0.9911	0.9958	0.992	0.9993	0.9995
LOD (ng/mL)	20	20	15	15	45	45
Intraday RSD of peak area (%, n = 5)						
100 ng/mL	7.7	7.7	6.9	7.1	8.9	8.4
500 ng/mL	7.1	7.2	6.1	6.1	7.6	7.8
1000 ng/mL	7.8	7.6	7	7	8.3	8.4
Interday RSD of peak area (%, n = 5)						
100 ng/mL	8.5	8.8	7.7	7.8	10.3	10.2
500 ng/mL	8.1	8.3	7.7	6.6	9.5	9.1
1000 ng/mL	8.3	8.5	7.9	7.7	10	9.9
Accuracy in terms of relative error of peak area (%, n = 5)						
100 ng/mL	5.8	5.5	5.1	5.3	8.9	8.9
500 ng/mL	5.1	5.1	4.9	4.9	6.9	7.1
1000 ng/mL	5.5	5.4	5.2	5.3	8.3	8.5
Recoveries for 200 ng/mL (%, n = 3)						
Ethyl acetate/isopropanol (4:1)	15.9	16.8	18.7	27.3	18.3	18.3
Toluene	28.5	29	45.3	51	21.3	24.5
Toluene + 0.05 g/mL of NaCl	50.4	51.3	60.2	64.5	33.2	34.4

concentration level and were mostly below 10% in day-to-day conditions.

Due to the lack of availability of real urine samples and to prove the accuracy of the method, we evaluated the relative errors (%RE) for each compound in spiked urine samples in terms of peak areas at the same concentration levels as in intraday study. These values were calculated with the following equation using five replicates to calculate the average response:

$$\%RE = \frac{\left| \text{experimental response} - \text{theoretical response obtained in the calibration curve} \right|}{\text{theoretical response obtained in the calibration curve}} \cdot 100$$

The values obtained for relative errors were below 9% for each concentration level, as shown in Table 1.

The LODs obtained for the studied cathinones were 20, 15 and 45 ng/mL for mephedrone, 4-methylephedrine, and MDPV, respectively. These LODs were slightly higher than those obtained in a previous study by our research group, in which the values range between 3 and 8 ng/mL [22]. In that study, CE was used to enantiodetermine the same cathinones in urine samples with inline SPE as the preconcentration technique. However, it is important to highlight that when inline SPE-CE is used, a longer loading sample time (20 min) is required compared to that required by FASI-CE (45 s). Moreover, in this case the method is simpler as it is not necessary to manually construct the inline SPE device. In addition, the LODs obtained for this methodology are close to the values already published using other strategies reported in the literature. For instance, different strategies based on GC-MS established LODs between 5 and 50 ng/mL for cathinones in urine [10,11,15,17]. However, the lowest LODs for determining cathinones in urine have been achieved with methodologies based on LC-MS, which obtained values between 0.1 and 5 ng/mL [6,12,13]. Nevertheless, unlike the present study, none of these strategies based on LC-MS or GC-MS achieved the enantioseparation of the studied compounds; therefore, this is a clear advantage of the present methodology.

4 Concluding remarks

FASI-CE has been shown to be an effective and fast technique for preconcentrating cathinones in urine samples. Moreover, the target

compounds were enantioseparated using a binary CD system consisting of β-CD and 2-hydroxypropyl β-CD.

The proposed methodology provided SEFs between 562 to 601 in terms of peak area and between 444 to 472 in terms of peak height for standard samples without using an offline preconcentration procedure, which highlights the pre-concentration potential of the technique. Real samples can be analyzed by simply adding a LLE pretreatment prior to the FASI-CE procedure. The LODs obtained were between 15 and 45 ng/mL, which are close to the levels at which these compounds can be found in this kind of biological sample taken from drug abusers. Compared with other analytical methods for cathinones, this method has several advantages: it is simple, has quite high sensitivity, a low sample and reactive consumption, and it can enantioseparate the target compounds, and in this sense it could be routinely used for forensic applications.

Acknowledgments

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Conflict of interest

The authors have declared no conflict of interest

References

[1] Prosser, J. M., Nelson, L. S., The toxicology of bath salts: A review of synthetic cathinones. *J. Med. Toxicol.* 2012, 8, 33–42.

[2] German, C. L., Fleckenstein, A. E., Hanson, G. R., Bath salts and synthetic cathinones: An emerging designer drug phenomenon. *Life Sci.* 2014, 97, 2–8.

[3] Schifano, F., Albanese, A., Fergus, S., Stair, J. L., Deluca, P., Corazza, O., Davey, Z., Corkery, J., Siemann, H., Scherbaum, N., Farre', M., Torrens, M., Demetrovics, Z., Ghodse, A. H., Mephedrone (4-methylmethcathinone; 'meow meow'): Chemical, pharmacological and clinical issues. *Psychopharmacology (Berl).* 2011, 214, 593–602.

[4] Cameron, K., Kolanos, R., Verkariya, R., De Felice, L., Glennon, R. A., Mephedrone and methylenedioxypyrovalerone (MDPV), major constituents of "bath salts," produce opposite effects at the human dopamine transporter. *Psychopharmacology (Berl).* 2013, 227, 493–499.

[5] Fernández, P., González, M., Regenjo, M., Ares, A. M., Fernández, A. M., Lorenzo, R. A., Carro, A. M., Analysis of drugs of abuse in human plasma using microextraction by packed sorbents and ultra-high-performance liquid chromatography. *J. Chromatogr. A* 2017, 1485, 8–19.

[6] Murakami, T., Iwamuro, Y., Ishimaru, R., Chinaka, S., Hasegawa, H.,

Moleculary imprinted polymer solid-phase extraction of synthetic cathinones from urine and whole blood samples. *J. Sep. Sci.* 2018, 41, 4506–4514.

[7] Baciu, T., Borrull, F., Calull, M., Aguilar, C., Enantioselective determination of cathinone derivatives in human hair by capillary electrophoresis combined in-line with solid-phase extraction. *Electrophoresis* 2016, 37, 2352–2362.

[8] Montesano, C., Vannutelli, G., Massa, M., Simeoni, M. C., Gregori, A., Ripani, L., Compagnone, D., Curini, R., Sergi, M., Multi-class analysis of new psychoactive substances and metabolites in hair by pressurized liquid extraction coupled to HPLC-HRMS. *Drug Test. Anal.* 2017, 9, 798–807.

[9] Fernández, P., Regenjo, M., Ares, A., Fernández, A. M., Lorenzo, R. A., Carro, A. M., Simultaneous determination of 20 drugs of abuse in oral fluid using ultrasound-assisted dispersive liquid-liquid micro-extraction. *Anal. Bioanal. Chem.* 2019, 411, 193–203.

[10] Hagan, K. S., Reidy, L., Detection of synthetic cathinones in victims of sexual assault. *Forensic Sci. Int.* 2015, 257, 71–75.

[11] Hong, W. Y., Ko, Y. C., Lin, M. C., Wang, P. Y., Chen, Y. P., Chiueh, L. C., Shih, D. Y. C., Chou, H. K., Cheng, H. F., Determination of synthetic cathinones in urine using gas chromatography-mass spectrometry techniques. *J. Anal. Toxicol.* 2016, 40, 12–16.

[12] Glicksberg, L., Bryand, K., Kerrigan, S., Identification and quantification of synthetic cathinones in blood and urine using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *J. Chromatogr. B* 2016, 1035, 91–103.

[13] Concheiro, M., Castaneto, M., Kronstrand, R., Huestis, M. A., Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography-high resolution mass spectrometry and library matching. *J. Chromatogr. A* 2015, 1397, 32–42.

[14] Kinyua, J., Negreira, N., Miserez, B., Causanilles, A., Emke, E., Gremeaux, L., de Voogt, P., Ramsey, J., Covaci, A., van Nuijs, A. L. N., Qualitative screening of new psychoactive substances in pooled urine samples from Belgium and United Kingdom. *Sci. Total Environ.* 2016, 573, 1527–1535.

[15] Gerace, E., Caneparo, D., Borio, F., Salomone, A., Vincenti, M., Determination of several synthetic cathinones and an amphetamine-like compound in urine by gas chromatography with mass spectrometry. Method validation and application to real cases. *J. Sep. Sci.* 2019, 42, 1577–1584.

[16] Kolanos, R., Partilla, J. S., Baumann, M. H., Hutsell, B. A., Banks, M. L., Negus, S. S., Glennon, R. A., Stereoselective actions of methylenedioxypyrovalerone (MDPV) to inhibit dopamine and norepinephrine transporters and facilitate intracranial self-stimulation in rats. *ACS Chem. Neurosci.* 2015, 6, 771–777.

[17] Alremeithi, R. H., Meetani, M. A., Khalil, S. A., A validated gas chromatography mass spectrometry method for simultaneous determination of cathinone related drug enantiomers in urine and plasma. *RSC Adv.* 2016, 6, 80576–80584.

[18] Meetani, M. A., Alremeithi, R. H., Mousa, M. K., Enantioseparation of synthetic cathinones enantiomers with tertiary amine structure in urine and plasma. *J. Chromatogr. Sci.* 2019, 57, 361–368.

[19] Merola, G., Fu, H., Tagliaro, F., Macchia, T., McCord, B. R., Chiral separation of 12 cathinone analogs by cyclodextrin-assisted capillary electrophoresis with UV and mass spectrometry detection. *Electrophoresis* 2014, 35, 3231–3241.

[20] Nowak, P. M., Olesek, K., Woźniakiewicz, M., Kościelniak, P., Simultaneous enantioseparation of methcathinone and two isomeric

methylmethcathinones using capillary electrophoresis assisted by 2-hydroxyethyl-β-cyclodextrin. *Electrophoresis* 2018, 39, 2406–2409.

[21] Tai, S., Morrison, C., Chiral and stable isotope analysis of synthetic cathinones. *TrAC - Trends Anal. Chem.* 2017, 86, 251–252.

[22] Pérez-Alcaraz, A., Borrull, F., Aguilar, C., Calull, M., Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE. *Electrophoresis* 2019, 40, 1762–1770.

[23] Šlampova, A., Malá, Z., Gebauer, P., Recent progress of sample stacking in capillary electrophoresis (2016-2018). *Electrophoresis* 2019, 40, 40–54.

[24] Breadmore, M. C., Grochocki, W., Kalsoom, U., Phung, S. C., Rokh, M. T., Cabot, J. M., Ghiasvand, A., Li, F., Shallan, A. I., Keyon, A. S. A., Wuethrich, A., Recent advances in enhancing the sensitivity of electrophoresis and electro-chromatography in capillaries and microchips (2016-2018). *Electro-phoresis* 2019, 40, 17–39.

[25] Peng, X., Wang, H., Yang, B., Zhan, X., Wu, Y., Field-amplified sample injection-capillary electrophoresis for the determination of bisphenol A, α-Naphthol and β-naphthol in drinks and lake water. *Chromatographia* 2016, 79, 327–333.

[26] Zheng, Y., Peng, X., Wu, Y., Field-amplified sample injection and solid-phase extraction for sensitivity improvement of electrophoretic determination of indole-3-acetic acid and indole-3-butyric acid in food samples. *Food Anal. Methods* 2018, 11, 1155–1162.

[27] López Guerrero, M. del M., Hernández-Mesa, M., Cruces-Blanco, C., García-Campaña, A. M., On-line preconcentration strategy for the simultaneous quantification of three local anesthetics in human urine using CZE. *Electrophoresis* 2015, 36, 2961–2967.

[28] Moreno-González, D., Krulišová, M., Gámiz-Gracia, L., García-Campaña, A. M., Determination of tetracyclines in human urine samples by capillary electrophoresis in combination with field amplified sample injection. *Electrophoresis* 2018, 39, 608–615.

[29] Wang, Z., Guo, H., Chen, M., Zhang, G., Chang, R., Chen, A., Separation and determination of corynoxine and corynoxine B using chiral ionic liquid and hydroxypropyl-β-cyclodextrin as additives by field-amplified sample stacking in capillary electrophoresis. *Electrophoresis* 2018, 39, 2195–2201.

[30] Hamidi, S., Jouyban, A., Capillary electrophoresis with UV detection, on-line stacking and off-line dispersive liquid-liquid microextraction for determination of verapamil enantiomers in plasma enantiomers in plasma. *Anal. Methods* 2015, 7, 5820–5829.

[31] Us, M. F., Alshana, U., Lubbad, I., Göğer, N. G., Ertaş, N., Dispersive liquid-liquid microextraction based on solidification of floating organic drop combined with field-amplified sample injection in capillary electrophoresis for the determination of beta(2)-agonists in bovine urine. *Electrophoresis* 2013, 34, 854–861.

[32] Chen, C., Meng, L., Li, M., Zhu, Z., Simultaneous separation and sensitive detection of four β2-agonists in biological specimens by CE-UV using a field-enhanced sample injection method. *Anal. Methods* 2015, 7, 175–180.

[33] Airado-Rodríguez, D., Cruces-Blanco, C., García-Campaña, A. M., Dispersive liquid-liquid microextraction prior to field-amplified sample injection for the sensitive analysis of 3,4-methylenedioxymethamphetamine, phencyclidine and lysergic acid diethylamide by capillary electrophoresis in human urine. *J. Chromatogr. A* 2012, 1267, 189–197.

[34] Zhou, X., He, M., Chen, B., Yang, Q., Hu, B., Membrane supported liquid-liquid-liquid microextraction com-bined with field-amplified sample injection CE-UV for high-sensitivity analysis of six cardiovascular drugs in

human urine sample. *Electrophoresis* 2016, 37, 1201–1211.

[35] Nowak, P. M., Woźniakiewicz, M., Mitoraj, M., Sagan, F., Kościelniak, P., Thermodynamics of acid-base dissociation of several cathinones and 1-phenylethylamine, studied by an accurate capillary electrophoresis method free from the Joule heating impact. *J. Chromatogr. A* 2018, 1539, 78–86.

3.2.2. An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis

AN ELECTROKINETIC SUPERCHARGING APPROACH FOR THE ENANTIODETERMINATION OF CATHINONES IN URINE SAMPLES BY CAPILLARY ELECTROPHORESIS

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Abstract

This paper presents a strategy based on electrokinetic supercharging (EKS) in combination with capillary electrophoresis (CE) for the enantiodetermination of a group of cathinones in urine samples after sample pretreatment with liquid-liquid extraction (LLE). The background electrolyte (BGE) consisted of an aqueous solution of 70 mM of monosodium phosphate at pH 2.5 containing 8 mM 2-hydroxypropyl β-cyclodextrin (β-CD) and 5 mM β-CD, which acted as chiral selectors. To solve capillary electrophoresis's lack of sensitivity, we used EKS as the preconcentration strategy. We also tested several parameters affecting this strategy, i.e. the choice of suitable leading and terminating electrolytes, their injected volumes, and the sample injection times and voltages. The highest response enhancement was achieved by electrokinetically injecting the sample (10 kV, 120 s) between a leading electrolyte (LE) of 25 mM HCl and a terminating electrolyte (TE) of 75 mM tetrabutylammonium bromide, both of which were hydrodynamically injected at 50 mbar for 40 s. The method was validated by analysing spiked urine samples. The linear range went from 15 to 250 ng/mL, limits of detection (LODs) were between 4 and 8 ng/mL, and RSDs were below 11% in terms of intra-day and inter-day repeatability. This is therefore an efficient method for the enantiodetermination of cathinones in urine samples by forensic laboratories.

Keywords: *Cathinones / Enantiodetermination / Electrokinetic supercharging / Urine analysis / Capillary electrophoresis*

1 Introduction

Novel psychoactive substances (NPS) are a challenging problem worldwide. These compounds are widely available on various websites but, unlike classical synthetic drugs such as cocaine and amphetamines, knowledge of their health effects is limited. One of the most common types of NPS reported in the literature are cathinones [1]. These synthetic derivates from cathinone, a beta-ketone amphetamine naturally found in the leaves of the khat plant, currently represent a more affordable and accessible alternative to other well-known illicit drugs such as amphetamines [1,2].

Cathinones are usually ingested or nasally insufflated [3]. After their consumption, they can be found in the organism either metabolized or in their pure form at low concentrations. Highly sensitive methods generally based on liquid chromatography-mass spectrometry (LC-MS) [4–11] or gas chromatography-mass spectrometry (GC-MS) [12–15] have been developed to analyse these compounds in biological samples such as hair [8], oral fluids [9,11], blood [10,12] or, more commonly, urine [4–7,12–15], which is both easy to collect and non-invasive and has a detection window that can last weeks.

An important feature of cathinones is their chirality. The presence of an asymmetric carbon in their structure implies the presence of two enantiomers, only one of which displays biological activity in the human body while the other is inactive or may exhibit unwanted side effects [2]. In fact, the stimulant effects of cathinones are mainly attributed to the *S*(-) form [2,3,16,17]. There is therefore a need for analytical methods that allow the enantiodetermination of these compounds. In recent years, CE has been found to be an excellent technique for this purpose since enantioseparation can be achieved simply by adding a chiral selector to the background electrolyte [18,19]. Several compounds have been successfully used as chiral selectors in CE. With cathinones, the compounds usually used for that purpose are cyclodextrins [20–23]. However, despite all the advantages of CE (mainly simplicity and the low consumption of sample and reagents), CE's lack of sensitivity, especially when coupled with UV detection, limits its application when low detection limits are needed.

To improve sensitivity, authors have proposed various preconcentration strategies based on stacking principles. With these strategies, a large volume of sample is usually injected into the capillary and the analytes are then focused into a narrow zone [24,25]. Although sensitivity is increased by these approaches, it can sometimes be limited. To increase sensitivity, numerous authors have proposed combining two or more preconcentration techniques. One example of this is EKS, which combines field-amplified sample injection (FASI) with transient isotachophoresis (*t*-ITP) [26–33]. EKS overcomes the main drawback of FASI – the limited amount of sample that can be injected before excessive band broadening affects the separation quality – because the extra *t*-ITP step involved can preconcentrate such bands. To perform EKS, the analytes are electrokinetically introduced between a LE, which contains ions with a higher mobility than the analytes, and a TE, which contains ions with a lower mobility than the analytes. Under these conditions, the analytes can migrate more quickly in the TE zone and more slowly in the LE zone, so they become focused on the LE/TE interface. This produces an important gain in sensitivity and several authors have reported high sensitive enhancement factors (SEFs) in the determination of various kinds of analytes using this preconcentration strategy in combination with CE [26–33]. Some of these applications have used EKS-CE as the preconcentration method for analysing biological samples. For example, enhancement factors between 160 and 600 were obtained in the determination of tamoxifen and its metabolites in human plasma [30] and between 2193 and 2976 in the determination of biogenic amines in mice brain [31]. Both these studies based their pretreatment on LLE.

The aim of the present study is to develop a sensitive method based on EKS-CE for the chiral determination of four cathinones in urine samples. To the best of our knowledge, no study has yet been based on EKS for the enantiodetermination of this kind of compounds in biological samples.

2 Materials and methods

2.1 Reagents and standards

All reagents were of analytical reagent grade. Cyclohexane, methanol (MeOH), methyl tert-butyl

ether and toluene were purchased from J.T. Baker (Deventer, Netherlands). Ammonium hydroxide 28%, β-CD 97%, 2-hydroxypropyl β-CD, hydrochloric acid 37%, phosphoric acid 85%, potassium chloride 99%, monosodium phosphate 99%, sodium acetate 99%, NaOH 97%, tetrapropylammonium bromide 98%, tetrabutylammonium bromide 98% and tetrapentyl-ammonium bromide 99% were acquired from Sigma-Aldrich (Saint Louis, MO, USA). NaCl 99% was purchased from Fluka Honeywell (Morris Plains, NJ, USA). Milli-Q water was obtained with a water purification system from Veolia Water (Paris, France).

The standards of *R,S*-methylone, *R,S*-mephedrone and *R,S*-MDPV were purchased as hydrochloride salts with a purity of 98% from LGC Standards (Teddington, UK). *R,S*-4-methyl-ephedrine was acquired as a solution of 1 mg/mL in MeOH from LGC Standards (Teddington, UK). Standard stock solutions of the cathinones (100 mg/L of *R,S*-4-methylephedrine, 1000 mg/L of *R,S*-mephedrone and 2000 mg/L of *R,S*-methylone and *R,S*-MDPV) were prepared in MeOH and stored in the freezer at -20 °C. Working standard solutions of a mixture of all the compounds at a concentration of 20 μg/mL were prepared weekly by diluting the stock standard solutions in Milli-Q water and kept at 4 °C. The solutions with lower concentrations were prepared daily by diluting suitable volumes of the working standard solutions in Milli-Q water.

2.2 Instrumentation

For the electrophoretic separations we used a 7100 CE System from Agilent Technologies (Waldbronn, Germany) equipped with a UV DAD. All pH measurements were carried out with a GLP 21 pH-meter from Crison (Barcelona, Spain). A Universal 32 R centrifuge from Hettich (Kirchlengern, Germany) was also used.

2.3 CE conditions

The BGE, which consisted of 70 mM of monosodium phosphate, 8 mM of 2-hydroxypropyl β-CD and 5 mM of β-CD (adjusted to pH 2.5 with concentrated phosphoric acid), was prepared by dissolving the appropriate amount of each compound in Milli-Q water.

A bared fused-silica capillary of 50 μm id and 80 cm in total length (72 cm effective length), acquired from

Polymicro Technologies (Phoenix, AZ, USA), was used as separation capillary.

New capillaries were conditioned by subsequently flushing 1 M NaOH for 40 min and Milli-Q water for 10 min. At the start of each working day, the capillary was conditioned with 0.1 M NaOH for 10 min, Milli-Q water for 5 min and BGE for 5 min, all at 930 mbar. Between runs, a conditioning step was performed with 0.1 M NaOH, Milli-Q water and BGE, all at 930 mbar for 4 min. A postconditioning step was carried out at the end of each run by flushing Milli-Q water for 5 min at 930 mbar to ensure good reproducibility.

For all experiments, the capillary chamber was heated at 25 °C and the wavelength used to detect the cathinones was 200 nm.

2.4 EKS-CE procedure

EKS-CE was performed as follows. First, a plug of LE, consisting of an aqueous solution of 25 mM of HCl, was hydrodynamically injected at 50 mbar for 40 s. The samples were then electrokinetically injected by applying a voltage of 10 kV for 120 s. The next step was to hydrodynamically inject the TE, an aqueous solution of 75 mM of tetrabutylammonium bromide, at 50 mbar for 40 s. Finally, a separation voltage of 30 kV was applied, and separation took place.

2.5 Sample preparation

Drug-free urine samples were obtained from several non-addicted volunteers. These were collected in polypropylene tubes and kept in the freezer at -20 °C until analysis. Pooled urine, prepared by mixing the urine samples obtained from the volunteers, was used to validate the method. Before beginning the procedure, the analytes were added to the urine to simulate a real sample.

Before electrophoretic analysis, liquid-liquid extraction (LLE) was performed as sample pretreatment for extracting target compounds from the urine samples. The procedure was as follows: 2 mL of cyclohexane were added to a 2 mL urine sample alkalized to pH 10 with 28% ammonium hydroxide. After vortex mixing for 1 min, the samples were centrifuged for 10 min at 9000 rpm. The organic phase, containing the cathinones, was then transferred to a vial and immediately a second extraction was performed by adding

another 2 mL of cyclohexane to the remaining aqueous phase and repeating the same procedure. Next, the two organic phases were combined, and the final extract was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 2 mL of Milli-Q water (adjusted to pH 6 with concentrated HCl). Finally, a 0.45 µm PTFE syringe filter was used to filter the sample and the extract was transferred to a microvial for analysis. This pretreatment procedure took about 40 min to be completed.

3 Results and discussion

3.1 Enantioseparation by CE

The BGE consisted of an aqueous solution of 70 mM of monosodium phosphate, 8 mM of 2-hydroxypropyl β-CD and 5 mM of β-CD (adjusted to pH 2.5 with concentrated phosphoric acid). This was selected after a recent study by our research group in which the same group of cathinones were also enantioseparated [22]. For all experiments the cathinones were solved in an aqueous solution at pH 6. At this pH, the cathinones are positively charged, since they present pK_a values between 8.83 and 9.13 [34], which should support their electrokinetic injection in EKS.

3.2 EKS optimization

EKS is a powerful preconcentration strategy that combines FASI with *t*-ITP. It involves the electrokinetic injection of a long plug of sample between a high mobility LE and a low mobility TE. Its enormous potential for preconcentrating and determining different analytes in different matrixes has been demonstrated [26–33]. However, as far as we know, EKS has not yet been tested for analysing cathinones. We therefore investigated using EKS as an in-line preconcentration strategy for improving the detection sensitivity of the target cathinones. To do so, we studied the parameters that affect the preconcentration efficiency of EKS, including the choice of suitable LE and TE, their injected volumes, and the sample injection time and voltage.

3.2.1 Optimization of the LE

An important characteristic of an effective LE is that it must have faster mobility than the target analytes [27]. The cathinones studied present electrophoretic mobilities between 22.3 x 10^{-9} and 14.1 x 10^{-9} m^2 V^{-1} s^{-1} in their cationic form. We therefore evaluated as LE different salts that

contain a co-ion with high electrophoretic mobility. In particular, we evaluated the following co-ions: sodium, potassium and proton, which present the following electrophoretic mobilities 51.9 x 10^{-9}, 76.2 x 10^{-9} and 362.5 x 10^{-9} m^2 V^{-1} s^{-1}, respectively [29]. For this optimization, a standard sample containing the target compounds at a concentration of 1.25 μg/mL was electrokinetically injected by applying 10 kV for 20 s after hydrodynamic introduction of the LE (40 s, 50 mbar) at a concentration of 50 mM. The TE (40 s, 50 mbar), which consists of an aqueous solution of 50 mM of tetrabutylammonium bromide, was then added. Our results for the various LE tested are shown in Fig. 1A. For all the cathinones under study, the greater the difference between the electrophoretic mobilities of the analytes and the LE, the greater the response, being HCl which provided the best stacking efficiencies of all the LE. On the other hand, when NaCl and KCl were used as LE, the response was lower because the difference between the electrophoretic mobilities of sodium and potassium ions and the electrophoretic mobilities of the target compounds was not big enough to generate an efficient ITP state [27]. Moreover, the results were fairly similar for these two LE, probably because of the similarity between their electrophoretic mobilities. HCl was therefore selected as the most suitable LE for later experiments.

The concentration of LE can also influence the *t*-ITP process. On the one hand, the concentration of LE must be high enough to provide effective sample stacking (at least 50 times higher than the concentration of the target compounds). On the other hand, a highly concentrated LE can negatively affect the current during CE separation due to an increase in the Joule effect [27]. To find a proper LE concentration, different concentrations of HCl were tested (25, 50, 75 and 100 mM) under the same conditions as those used to study the nature of the LE, except that the concentration of the analytes was lower, i.e. 400 ng/mL in this case. Fig. 1B shows that the stacking efficiency decreased for all cathinones as the LE concentration increased. Despite that trend, when lower concentrations of LE were evaluated (below 25 mM), reproducibility problems were observed. The optimum LE concentration was therefore set at 25 mM for later experiments.

Finally, we also studied the effect of the amount of LE by evaluating its hydrodynamic injection for different times (10, 40, and 70 s). All experiments were performed under the same conditions as the previous optimization but with an aqueous solution of 25 mM of HCl as the LE. Fig. 1C shows that when LE injection time increased, the peak areas for all target compounds increased. However, note that a high LE volume can reduce the resolution between cathinones. This is due to a more prolonged *t*-ITP zone in the capillary before the destacking step, which reduces the capillary length available for CZE separation [26,30]. In our case, although there was not so much loss in resolution at high injection

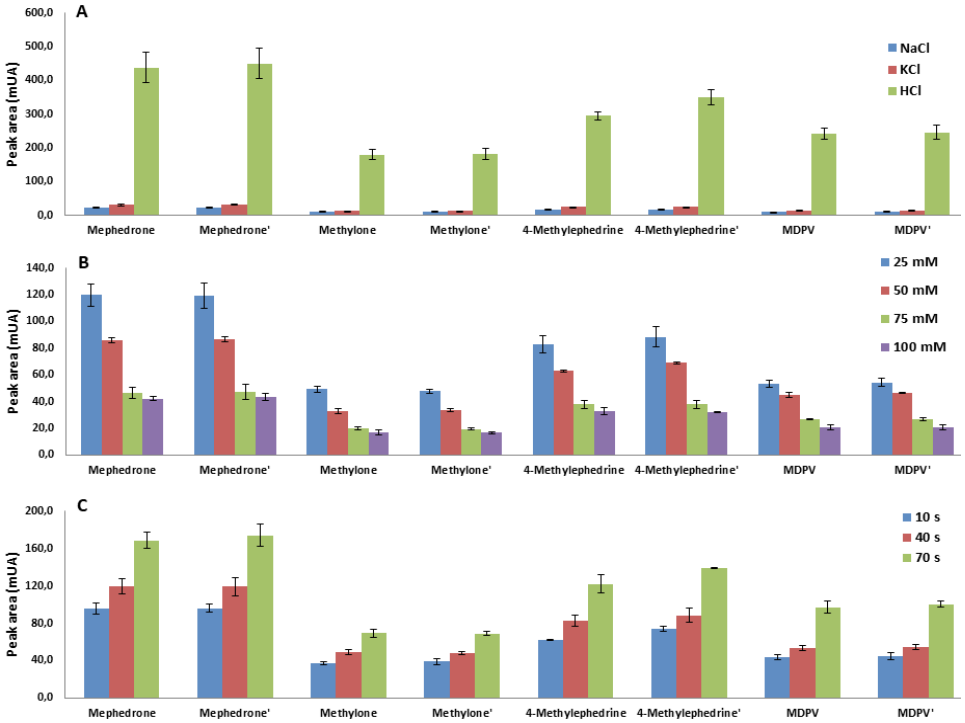

Figure 1. Optimization of the LE nature and volume. A) Optimization of LE nature. For this optimization, different LE at a concentration of 50 mM were injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 1.25 μg/mL for 20 s and applying 10 kV, 50 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s. B) Optimization of LE concentration. For this optimization, a solution of HCl at different concentrations was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 400 ng/mL for 20 s and applying 10 kV, a 50 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s. C) Optimization of LE time of injection. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for different injection times. After the electrokinetic injection of a standard sample of 400 ng/mL for 20 s and applying 10 kV, a 50 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s.

times, we observed current instability problems when using 70 s. To prevent further current disturbances, we therefore selected 40 s as the optimum value.

3.2.2 Optimization of the TE

The ideal TE has a lower electrophoretic mobility than the analytes. Tetra-substituted ammonium salts could therefore become optimal TE candidates since the tetra substitution increases the size of these salts and considerably reduces their electrophoretic mobility. For example, tetrabutylammonium bromide has been used successfully as TE for analysing melamine in milk powder and liquid milk by EKS-CE [29]. We therefore tested the applicability of three tetra-substituted ammonium salts as TE, i.e. tetrapropylammonium bromide, tetrabutylammonium bromide, and tetrapentylammonium bromide. For this optimization, a standard sample containing the analytes at a concentration of 200 ng/mL was electrokinetically injected after the hydrodynamic injection of LE under the above optimized conditions. The TE at a concentration of 50 mM was then injected at 50 mbar for 40 s. Our results are shown in Fig. 2A. With tetrapentylammonium bromide, the chiral separation of the cathinones was completely distorted, and with this TE the resolution between cathinones was negatively affected, and for some of them a peak overlap was observed, so this TE was discarded. For the other two TE, the best response for all compounds was obtained with tetrabutylammonium bromide. This can be attributed to the greater difference between the corresponding mobility of this salt and the mobilities of the analytes.

TE concentration can also affect the *t*-ITP process. As with LE, TE concentration must be high enough to provide effective sample stacking but low enough to avoid increasing the Joule heating effect [27]. We tested various concentrations of tetrabutylammonium bromide (25, 50, 75 and 100 mM) under the same experimental conditions as those in the previous study. As we can see in Fig. 2B, when the concentration increased from 25 to 75 mM the peak areas increased but at higher TE concentrations the response slightly decreased. We therefore selected 75 mM as the optimal TE concentration.

Finally, we also evaluated TE injection time. The TE zone must be

large enough to ensure efficient sample stacking [30]. However, if this zone is too large, it reduces the capillary length available for the separation of the stacked analytes and separation efficiency may be seriously affected [26,30]. The TE injection time was optimized by testing different times (10, 40, and 70 s). For this study, we used the same conditions as for the optimization of TE concentration. As expected, our results (see Fig. 2C) show that the response was highest when 70 s was used as the TE injection time. However, under these conditions some current disturbances were detected. To prevent these problems, we selected 40 s as the TE injection time.

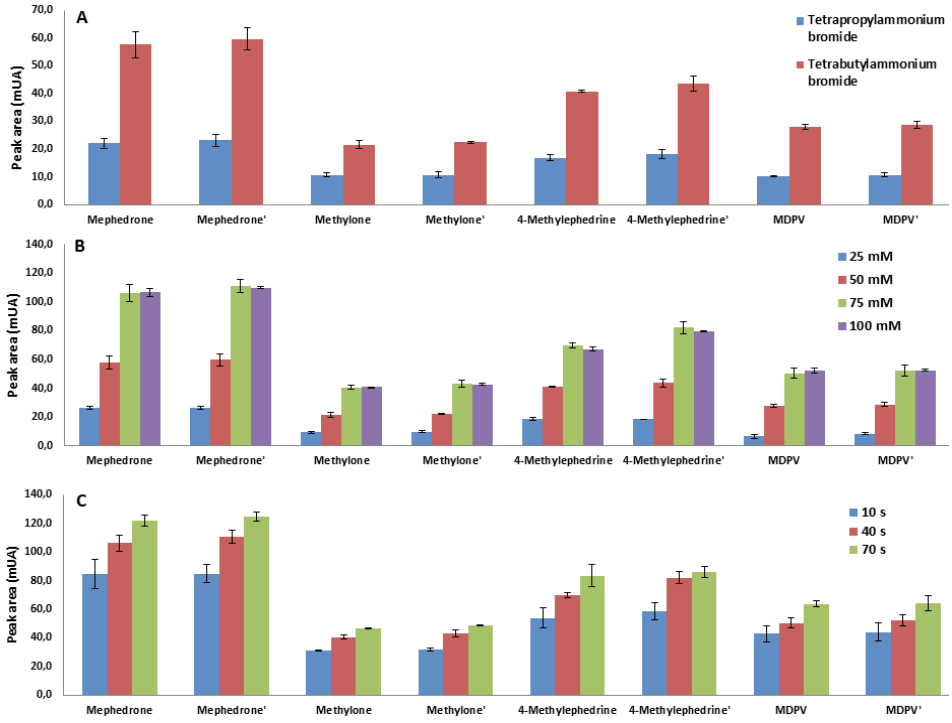

Figure 2. Optimization of the TE nature and volume. A) Optimization of TE nature. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 200 ng/mL for 20 s and applying 10 kV, different TE at a concentration of 50 mM were then injected at 50 mbar for 40 s. B) Optimization of TE concentration. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 200 ng/mL for 20 s and applying 10 kV, a tetrabutylammonium bromide solution at different concentrations was then injected at 50 mbar for 40 s. C) Optimization of TE time of injection. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 200 ng/mL for 20 s and applying 10 kV, a 75 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for different injection times.

3.2.3 Sample injection optimization

We also evaluated the effect of the sample injection conditions (voltage and time). In theory, a longer sample injection time means that a larger sample volume can be introduced into the capillary, thus increasing detection sensitivity. However, a longer injection time can also decrease the resolution between the analytes, thus hindering their enantioseparation. It is important, therefore, to consider both the gain in sensitivity and good separation efficiency. In this study, the LE was first injected at the previous optimized conditions and a standard sample containing the analytes at a concentration of 100 ng/mL was then injected by applying 10 kV between 20 and 140 s. Finally, the TE was hydrodynamically injected under the optimum conditions. The analytes responses are shown in Fig. 3A. As we can see, when the sample injection time increased, the response

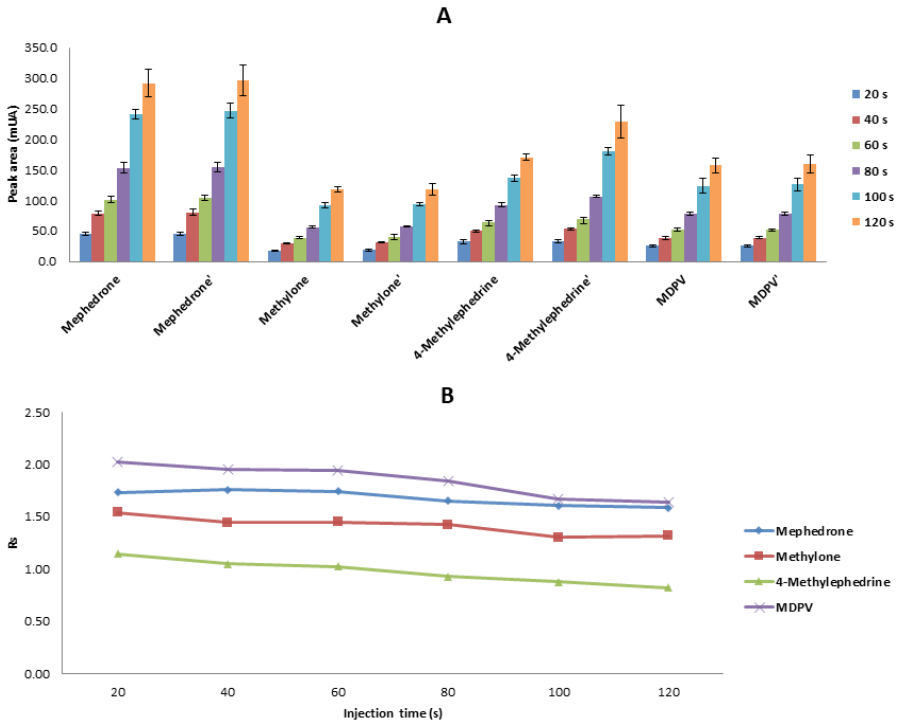

Figure 3. Electrokinetic sample injection time optimization for the EKS procedure in terms of: A) peak area; and B) resolution. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 100 ng/mL for different injection times and applying 10 kV, a 75 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s.

obtained for all compounds gradually increased. However, when the injection time exceeded 120 s, current disturbances were observed. It was therefore not possible to evaluate longer injection times. Fig. 3B shows the resolutions obtained in this study. Resolution slightly decreased as the injection time increased, but the values obtained remained practically constant. We therefore concluded that we could introduce a large amount of sample without losing much resolution. The effect of sample injection voltage on peak sensitivity was also tested. A decrease in the injection voltage could induce higher current stability, which could allow for longer electrokinetic injection times and a higher sample volume [27]. The effect of using a lower injection voltage was therefore also evaluated. To do so, an injection voltage of 2 kV was tested between 300 and 500 s (since the voltage was lower, the injection time was increased to obtain similar responses to those obtained by applying 10 kV). At sample injection times above 460 s, current disturbances occurred and the response for all analytes was lower than when 10 kV was applied for 120 s. The electrokinetic injection conditions selected to achieve efficient sample stacking and high detection sensitivity were an injection voltage of 10 kV and an injection time of 120 s.

3.3 Sensitivity enhancement factor

To determine the increase in the response of the compounds obtained with EKS-CE compared to CE, we calculated the sensitivity enhancement factors in terms of peak areas (SEF_{area}) and peak heights (SEF_{height}). To do so, we compared the peak areas and peak heights obtained under the optimized EKS conditions with those obtained by a CE procedure in which the sample was hydrodynamically injected at 50 mbar for 5 s.

The values of SEF_{area} were 1314, 1313, 722 and 940 and the values of SEF_{height} were 1213, 1310, 993 and 1161 for *R,S*-mephedrone, *R,S*-methylone, *R,S*-4-methylephedrine and *R,S*-MDPV, respectively, for standard solutions. Fig S1. of the Supplementary material presents the comparison of two electropherograms obtained for a standard sample containing the studied compounds at a concentration of 30 μg/mL hydrodynamically injected at 50 mbar for 5 s (A), and for a standard sample containing the studied compounds at a concen-

tration of 30 ng/mL, obtained by the optimized EKS-CE procedure (B). As it can be seen, despite the difference between the standards concentrations for both cases, the obtained responses for each cathinone were very similar. These results demonstrate the power of preconcentration with EKS as a stacking strategy and clearly show that this dual in-line focusing strategy significantly improved sensitivity for the cathinones under study.

3.4 Urine sample pretreatment

This method was used to analyse urines samples. Before doing so, however, we needed to develop a sample pretreatment because of the co-ions that may be present in the sample matrix and that can affect the EKS procedure [35].

LLE was tested as the sample pretreatment because in previous studies it was shown to be effective in extracting cathinones from urine samples [4–6,12–15,22]. Several organic solvents were evaluated to achieve optimum extraction. These were cyclohexane, dichloromethane (DCM), ethyl acetate/isopropanol (4:1), hexane, methyl tert-butyl ether and toluene. The LLE procedure comprised the following steps. First, the urine samples were alkalized to pH 10. Then, 2 mL of organic solvent were added to 2 mL of the urine sample. Next, samples were vortex mixed for 1 min and then centrifuged for 10 min at 9000 rpm. After separating the organic phase, the extraction procedure was repeated by adding another 2 mL of organic solvent. The organic extracts were then combined and evaporated to dryness under a gentle stream of N_2. The residue was then reconstituted with 2 mL of Milli-Q water at pH 6 and filtered before analysis using EKS-CE. Our results show that the only organic solvents that successfully extracted the target compounds were toluene and cyclohexane.

Recovery values were calculated as the ratio between the response obtained for a urine sample spiked with the analytes at a concentration of 100 ng/mL after all the procedure was performed and the response obtained for a standard sample containing the cathinones at a concentration of 100 ng/mL when the LLE step was not used. The recoveries ranged from 29 to 80% and from 50 to 98% for toluene and cyclohexane, respectively. Cyclohexane was therefore selected as the extraction solvent.

Fig. 4 shows the electropherogram obtained under the optimum conditions: of a urine blank (A) and of a urine sample spiked with the target compounds at a concentration of 100 ng/mL (B), both obtained by performing EKS-CE after an LLE procedure in which cyclohexane was used as organic solvent.

3.5 Method validation

The suitability of this EKS-CE procedure was tested by validating the method in terms of linearity, selectivity, intra-day and inter-day repeatability, accuracy, limits of detection (LODs) and limits of quantification (LOQs) in accordance with the *Guidance for the validation of analytical methodology and calibration of equipment used for testing of illicit drugs in seized materials and biological specimens* [36].

All validation studies were carried out by employing pooled urine samples collected from non-addicted volunteers spiked with a known amount of the target compounds. Table 1 shows the values obtained for the validation process.

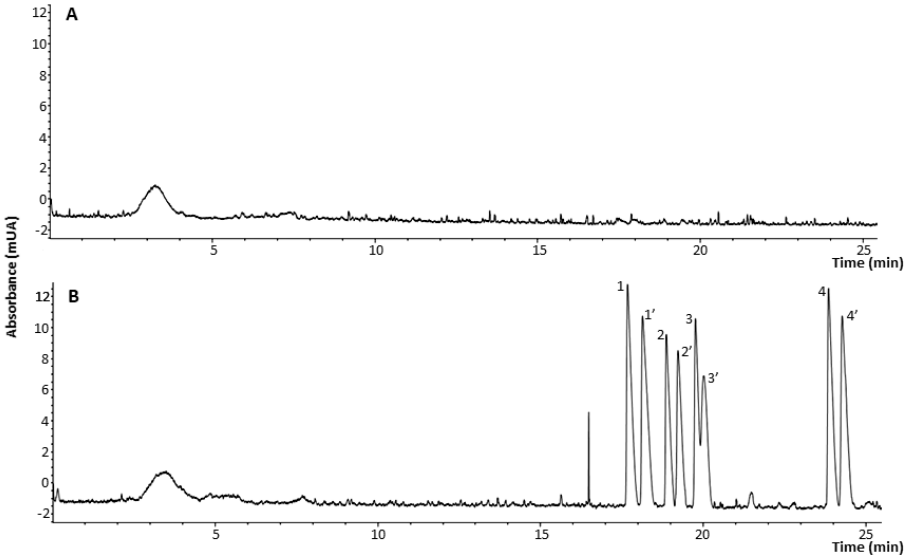

Figure 4. Electropherograms of A) a blank of a urine sample and B) a urine sample spiked with the studied compounds at a concentration of 100 ng/mL, both obtained by EKS-CE after an LLE procedure using cyclohexane as organic solvent. The EKS procedure was performed by first injecting the LE solution (25 mM of HCl) at 50 mbar for 40 s. After, the sample was electrokinetically injected at 10 V for 120 s, and then, the TE solution (75 mM of tetrabutylammonium bromide) was injected at 50 mbar for 40 s. Peak assignments: (1, 1') *R,S*-mephedrone, (2, 2') *R,S*-methylone (3,3') *R,S*-4-methylephedrine and (4, 4') *R,S*-MDPV.

Linearity was evaluated using a matrix match calibration curve in a concentration range between 15 and 250 ng/mL for mephedrone and between 20 and 250 ng/mL for the other cathinones under study. Under these conditions, correlation coefficients (r^2) were above 0.990 for all compounds.

Selectivity was tested by analysing 10 blank urine samples from different individuals. When these samples were evaluated using the method we have developed, compounds that could interfere with the target analytes were not observed, as can be seen in Fig. 4A.

The precision of the whole method was tested in terms of intra-day and inter-day repeatability at three concentration levels, i.e. 20 ng/mL, 100 ng/mL and 250 ng/mL. Intra-day repeatability was evaluated on the same day by analysing five replicates of urine spiked at each concentration level. Inter-day repeatability was evaluated on five different days by analysing five replicates of urine spiked at each concentration level. The results, expressed as relative standard deviation (RSD), are shown in Table 1. Since the method presented RSD values below 10% and 11% for intra-day and inter-day repeatability, respectively, the method proved to be satisfactory in terms of precision.

Because of the lack of availability of real urine samples from cathinone abusers, and to prove the accuracy of the method, the relative errors of the peak areas (%RE) were evaluated at three concentration levels, i.e. 20 ng/mL, 100 ng/mL and 250 ng/mL. The values were calculated by analysing five replicates of urine spiked at each concentration level and using the following equation:

$$\%RE = \frac{\left|\text{experimental response} - \text{theoretical response obtained in the calibration curve}\right|}{\text{theoretical response obtained in the calibration curve}} \cdot 100$$

Table 1 shows the relative errors. All values were below 10% for all three concentration levels.

LODs were calculated using a signal-to-noise criterion of 3, while the LOQs were established as the lowest point of the linear range. LODs for the target compounds were 4, 8, 7 and 6 ng/mL for mephedrone, methylone, 4-methylephedrine, and MDPV, respectively. The LODs were higher to those achieved when cathinones were determined in urine samples using methods based on LC-MS (LODs

Table 1. Method validation in terms of regression equations, LODs, intra-day and inter-day repeatability and relative errors obtained for urine-spiked samples from non-addicted volunteers by LLE/EKS-CE.

	Mephedrone	**Mephedrone'**	**Methylone**	**Methylone'**	**4-Methylephedrine**	**4-Methylephedrine'**	**MDPV**	**MDPV'**
Linearity (ng/mL)	15-250	15-250	20-250	20-250	20-250	20-250	20-250	20-250
Calibration curve	y = 1.6034x - 10.063	y = 1.6438x - 10.793	y = 0.912x - 5.5065	y = 0.9396x - 7.7627	y = 1.1102x - 9.3257	y = 1.1751x - 10.158	y = 1.3935x - 6.4722	y = 1.389x - 7.3039
r^2	0.9903	0.9930	0.9938	0.996	0.9925	0.9922	0.9955	0.9973
LOD (ng/mL)	4	4	8	8	7	7	6	6
Intra-day repeatability RSD in terms of peak area (%, n = 5)								
20 ng/mL	6.1	6.1	9.3	8.9	7.0	6.9	6.8	6.6
100 ng/mL	5.2	5.2	8.0	7.5	6.0	5.8	5.9	5.7
250 ng/mL	5.7	5.4	8.4	8.2	6.4	6.4	6.3	6.0
Inter-day repeatability RSD in terms of peak area (%, n = 5)								
20 ng/mL	7.4	8.0	10.3	9.7	8.1	8.5	8.7	9.3
100 ng/mL	6.7	6.5	9.2	8.9	7.8	7.4	7.6	6.7
250 ng/mL	7.3	7.4	9.9	9.9	7.8	7.7	8.1	8.5
Relative error in terms of peak area (%, n = 5)								
20 ng/mL	7.7	7.9	9.3	9.5	8.4	8.6	8.5	8.8
100 ng/mL	6.9	7.0	8.4	8.5	7.5	7.6	7.7	7.6
250 ng/mL	7.5	7.7	8.7	9.1	8.0	8.3	8.0	8.1

from 0.25 to 5 ng/mL [4,5,7]). In general, they were also slightly lower than those obtained with methods based on GC-MS (LODs between 1 and 50 ng/mL [12–14]). An important advantage of this strategy compared to many methods available in the literature is that the analytes could be enantioseparated. Our research group previously presented methods that were also based on CE for the enantiodetermination of cathinones using preconcentration techniques combined in-line with CE. With one of these methods we used a chromatographic preconcentration technique (in-line SPE) [22], while with the other we used an electrophoretic preconcentration technique (FASI) [37]. As we expected, the LODs obtained with the strategy developed in the present study were lower than with FASI. This is because EKS is a combination of FASI and *t*-ITP and greater sensitivity is normal with a dual preconcentration technique. On the other hand, the LODs were similar to those we obtained with in-line SPE. An important advantage of the EKS-based methodology, however, is the shorter analysis time involved. For the in-line SPE-CE method, sample loading time was high. Also, since the in-line SPE device was homemade, we also need to consider the time taken to construct it. In general, therefore, EKS is a powerful preconcentration strategy that combines the power of preconcentration of a large electrokinetic injection with *t*-ITP to achieve low LODs relatively simply and easily.

4 Concluding remarks

An effective EKS-CE method for the enantiodetermination of four cathinones in urine samples was developed and validated for the first time.

Our SEF_{area} and SEF_{height} values, which ranged from 940 to 1314 and from 993 to 1310, respectively, highlight the preconcentration potential of the EKS methodology. This technique can achieve the enantiodetermination of the studied analytes with high preconcentration factors and low LODs simply by performing an in-line preconcentration procedure after LLE extraction.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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References

[1] R.A.S. Couto, L.M. Gonçalves, F. Carvalho, J.A. Rodrigues, C.M.P. Rodrigues, M.B. Quinaz, The analytical challenge in the determination of cathinones, key-players in the worldwide phenomenon of Novel Psychoactive Substances, Crit. Rev. Anal. Chem. 48 (2018) 372–390. https://doi.org/10.1080/10408347.2018.1439724.

[2] B. Silva, C. Fernandes, P.G. de Pinho, F. Remião, Chiral resolution and enantioselectivity of synthetic cathinones: a brief review, J. Anal. Toxicol. 42 (2018) 17–24. https://doi.org/10.1093/jat/bkx074.

[3] J.M. Prosser, L.S. Nelson, The toxicology of bath salts: a review of synthetic cathinones, J. Med. Toxicol. 8 (2012) 33–42. https://doi.org/10.1007/s13181-011-0193-z.

[4] L. Glicksberg, K. Bryand, S. Kerrigan, Identification and quantification of synthetic cathinones in blood and urine using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1035 (2016) 91–103. https://doi.org/10.1016/j.jchromb.2016.09.027.

[5] M. Concheiro, S. Anizan, K. Ellefsen, M.A. Huestis, Simultaneous quantification of 28 synthetic cathinones and metabolites in urine by liquid chromatography-high resolution mass spectrometry, Anal. Bioanal. Chem. 405 (2013) 9437–9448. https://doi.org/10.1007/s00216-013-7386-z.

[6] J. Kinyua, N. Negreira, B. Miserez, A. Causanilles, E. Emke, L. Gremeaux, P. de Voogt, J. Ramsey, A. Covaci, A.L.N. van Nuijs, Qualitative screening of new psychoactive substances in pooled urine samples from Belgium and United Kingdom, Sci. Total Environ. 573 (2016) 1527–1535. https://doi.org/10.1016/j.scitotenv.2016.08.124.

[7] M. Paul, J. Ippisch, C. Herrmann, S. Guber, W. Schultis, Analysis of new designer drugs and common drugs of abuse in urine by a combined targeted and untargeted LC-HR-QTOFMS approach, Anal. Bioanal. Chem. 406 (2014) 4425–4441. https://doi.org/10.1007/s00216-014-7825-5.

[8] C. Montesano, G. Vannutelli, M. Massa, M.C. Simeoni, A. Gregori, L. Ripani, D. Compagnone, R. Curini, M. Sergi, Multi-class analysis of new psychoactive substances and metabolites in hair by pressurized liquid extraction coupled to HPLC-HRMS, Drug Test. Anal. 9 (2017) 798–807. https://doi.org/10.1002/dta.2043.

[9] A.M. Ares, P. Fernández, M. Regenjo, A.M. Fernández, A.M. Carro, R.A. Lorenzo, A fast bioanalytical method based on microextraction by packed sorbent and UPLC-MS/MS for determining new psychoactive substances in oral fluid, Talanta. 174 (2017) 454–461. https://doi.org/10.1016/j.talanta.2017.06.022.

[10] P. Fernández, M. González, M. Regenjo, A.M. Ares, A.M. Fernández, R.A. Lorenzo, A.M. Carro, Analysis of drugs of abuse in human plasma using microextraction by packed sorbents and ultra-high-performance liquid chromatography, J. Chromatogr. A.

1485 (2017) 8–19. https://doi.org/10.1016/j.chroma.2017.01.021.

[11] P. Fernández, M. Regenjo, A. Ares, A.M. Fernández, R.A. Lorenzo, A.M. Carro, Simultaneous determination of 20 drugs of abuse in oral fluid using ultrasound-assisted dispersive liquid-liquid microextraction, Anal. Bioanal. Chem. 411 (2019) 193–203. https://doi.org/10.1007/s00216-018-1428-5

[12] R.H. Alremeithi, M.A. Meetani, S.A. Khalil, A validated gas chromatography mass spectrometry method for simultaneous determination of cathinone related drug enantiomers in urine and plasma, RSC Adv. 6 (2016) 80576–80584. https://doi.org/10.1039/C6RA10583A.

[13] K.S. Hagan, L. Reidy, Detection of synthetic cathinones in victims of sexual assault, Forensic Sci. Int. 257 (2015) 71–75. https://doi.org/10.1016/j.forsciint.2015.07.040.

[14] W.Y. Hong, Y.C. Ko, M.C. Lin, P.Y. Wang, Y.P. Chen, L.C. Chiueh, D.Y.C. Shih, H.K. Chou, H.F. Cheng, Determination of synthetic cathinones in urine using gas chromatography-mass spectrometry techniques, J. Anal. Toxicol. 40 (2016) 12–16. https://doi.org/10.1093/jat/bkv108.

[15] L. Institóris, Z. Árok, K. Seprenyi, T. Varga, G. Sára-Klausz, É. Keller, R.A. Tóth, L. Sala, É. Kereszty, K. Róna, Frequency and structure of stimulant designer drug consumption among suspected drug users in Budapest and South-East Hungary in 2012-2013, Forensic Sci. Int. 248 (2015) 181–186. https://doi.org/10.1016/j.forsciint.2015.01.002.

[16] F. Schifano, A. Albanese, S. Fergus, J.L. Stair, P. Deluca, O. Corazza, Z. Davey, J. Corkery, H. Siemann, N. Scherbaum, M. Farre', M. Torrens, Z. Demetrovics, A.H. Ghodse, Mephedrone (4-methylmethcathinone; 'meow meow'): chemical, pharmacological and clinical issues, Psychopharmacology (Berl). 214 (2011) 593–602. https://doi.org/10.1007/s00213-010-2070-x.

[17] R. Kolanos, J.S. Partilla, M.H. Baumann, B. A. Hutsell, M.L. Banks, S.S. Negus, R. A. Glennon, Stereoselective actions of methylenedioxypyrovalerone (MDPV) to inhibit dopamine and norepinephrine transporters and facilitate intracranial self-stimulation in rats, ACS Chem. Neurosci. 6 (2015) 771–777. https://doi.org/10.1021/acschemneuro.5b00006.

[18] S. Fanali, B. Chankvetadze, Some thoughts about enantioseparations in capillary electrophoresis, Electrophoresis. 40 (2019) 2420–2437. https://doi.org/10.1002/elps.201900144.

[19] I.J. Stavrou, E. A. Agathokleous, C.P. Kapnissi-Christodoulou, Chiral selectors in CE: recent development and applications (mid-2014 to mid-2016), Electrophoresis. 38 (2017) 786–819. https://doi.org/10.1002/elps.201600322.

[20] G. Merola, H. Fu, F. Tagliaro, T. Macchia, B.R. McCord, Chiral separation of 12 cathinone analogs by cyclodextrin-assisted capillary electrophoresis with UV and mass spectrometry detection, Electrophoresis. 35 (2014) 3231–3241. https://doi.org/10.1002/elps.201400077.

[21] T. Baciu, F. Borrull, M. Calull, C. Aguilar, Enantioselective determination of cathinone derivatives in human hair by capillary electrophoresis combined in-line with solid-phase extraction, Electrophoresis. 37 (2016) 2352–2362. https://doi.org/10.1002/elps.201600149.

[22] A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE, Electrophoresis. 40 (2019) 1762–1770. https://doi.org/10.1002/elps.201900072.

[23] P.M. Nowak, K. Olesek, M. Woźniakiewicz, P. Kościelniak, Simultaneous enantioseparation of methcathinone and two isomeric methylmethcathinones using capillary electrophoresis assisted by 2-hydroxyethyl-β-cyclodextrin, Electrophoresis. 39 (2018) 2406–2409. https://doi.org/10.1002/elps.201800142.

[24] M.C. Breadmore, W. Grochocki, U. Kalsoom, S.C. Phung, M.T. Rokh, J.M. Cabot, A. Ghiasvand, F. Li, A.I. Shallan, A.S.A. Keyon, A. Wuethrich, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2016-2018), Electrophoresis. 40 (2019) 17–39. https://doi.org/10.1002/elps.201800384.

[25] A. Šlampova, Z. Malá, P. Gebauer, Recent progress of sample stacking in capillary electrophoresis (2016-2018), Electrophoresis. 40 (2019) 40–54. https://doi.org/10.1002/elps.201800261.

[26] N. 'I Abdul Karim, W. A. Wan Ibrahim, M.M. Sanagi, A.S. Abdul Keyon, Online preconcentration by electrokinetic supercharging for separation of endocrine disrupting chemical and phenolic pollutants in water samples, Electrophoresis. 37 (2016) 2649–2656. https://doi.org/10.1002/elps.201600207.

[27] I. Botello, F. Borrull, C. Aguilar, M. Calull, Electrokinetic supercharging focusing in capillary zone electrophoresis of weakly ionizable analytes in environmental and biological samples, Electrophoresis. 31 (2010) 2964–2973. https://doi.org/10.1002/elps.201000184.

[28] Z. Xu, K. Nakamura, A.R. Timerbaev, T. Hirokawa, Another approach toward over 100 000-fold sensitivity increase in capillary electrophoresis: electrokinetic supercharging with optimized sample injection, Anal. Chem. 83 (2011) 398–401. https://doi.org/10.1021/ac102661b.

[29] Y. Lu, D. Wang, C. Kong, C. Jia, M.C. Breadmore, Analysis of melamine in milk powder and liquid milk by capillary zone electrophoresis after electrokinetic supercharging, Food Anal. Methods. 8 (2015) 1356–1362. https://doi.org/10.1007/s12161-014-9987-3.

[30] L.Y. Thang, M.C. Breadmore, H.H. See, Electrokinetic supercharging in nonaqueous capillary electrophoresis for online preconcentration and determination of tamoxifen and its metabolites in human plasma, J. Chromatogr. A. 1461 (2016) 185–191. https://doi.org/10.1016/j.chroma.2016.07.067.

[31] W.F. Wang, F.R. Ju, Y.L. Ran, H.G. Zhang, X.G. Chen, Detection of biogenic amines in C57BL/6 mice brain by capillary electrophoresis electrokinetic supercharging, Analyst. 141 (2016) 956–962. https://doi.org/10.1039/c5an01642h.

[32] L. Nyssen, M. Fillet, E. Cavalier, A. Servais, Highly sensitive and selective separation of intact parathyroid hormone and variants by sheathless CE-ESI-MS/MS, Electrophoresis. (2019) 1–8. https://doi.org/10.1002/elps.201800507.

[33] M. Dawod, D.S. Chung, High-sensitivity capillary and microchip electrophoresis using electrokinetic supercharging, J. Sep. Sci. 34 (2011) 2790–2799. https://doi.org/10.1002/jssc.201100384.

[34] P.M. Nowak, M. Woźniakiewicz, M. Mitoraj, F. Sagan, P. Kościelniak, Thermodynamics of acid-base dissociation of several cathinones and 1-phenylethylamine, studied by an accurate capillary electrophoresis method free from the Joule heating impact, J. Chromatogr. A. 1539 (2018) 78–86. https://doi.org/10.1016/j.chroma.2018.01.047.

[35] Y. Lu, X. Hou, D. Wang, H. Zhong, Advances of a capillary electrophoretic on-line concentration

technique: electrokinetic super-charging, J. Liq. Chromatogr. Relat. Technol. 40 (2017) 528–535. https://doi.org/10.1080/10826076.2017.1330756.

[36] United Nations Office on Drugs and Crime, Guidance for the validation of analytical methodology and calibration of equipment used for testing of illicit drugs in seized materials and biological specimens : a commitment to quality and continuous improvement, United Nations, 2009.

[37] A. Pérez-Alcaraz, F. Borrull, M. Calull, C. Aguilar, Field-amplified sample injection combined with CE for the enantiodetermination of cathinones in urine samples, J. Sep. Sci. (2020) In press. https://doi.org/https://doi.org/10.1002/jssc.201901067.

Supplementary material

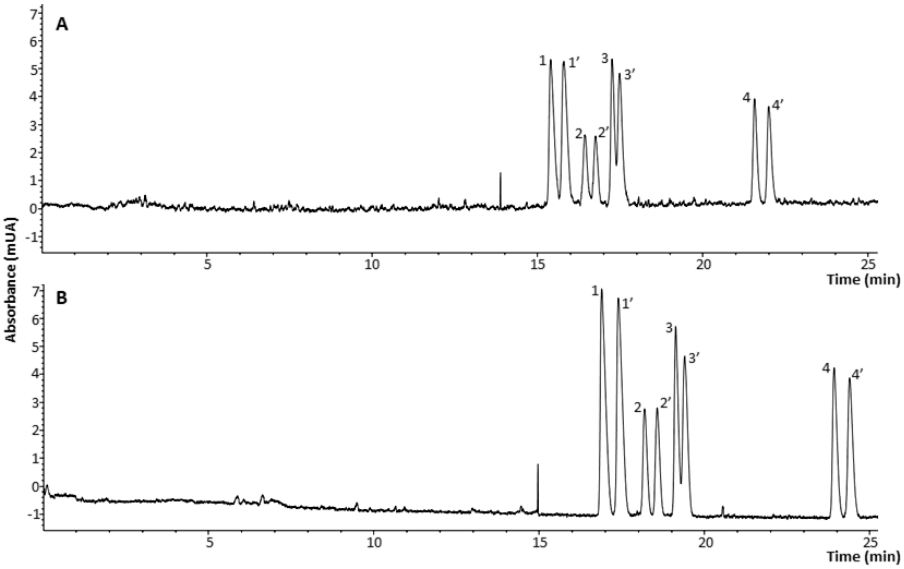

Figure S1. Comparative of the electropherograms obtained for A) a standard sample containing the studied compounds at a concentration of 30 µg/mL hydrodynamically injected at 50 mbar for 5 s and B) a standard sample containing the studied compounds at a concentration of 30 ng/mL, obtained by the optimized EKS-CE procedure. The EKS procedure was performed by first injecting the LE solution (25 mM of HCl) at 50 mbar for 40 s. After, the sample was electrokinetically injected for 120 s and applying 10 kV, and then, the TE solution (75 mM of tetrabutylammonium bromide) was injected at 50 mbar for 40 s. Peak assignments: (1, 1') *R,S*-mephedrone, (2, 2') *R,S*-methylone (3,3') R,S-4-methylephedrine and (4, 4') *R,S*-MDPV.

3.2.3. Discusión de resultados

A partir de los resultados presentados en los dos artículos anteriores se puede concluir que tanto la metodología de FASI-CE como la de EKS-CE demuestran un gran potencial para lograr la preconcentración electroforética de catinonas. No obstante, cada una de estas técnicas de *stacking* ofrece sus particularidades que serán discutidas a continuación.

La separación quiral de las catinonas se obtuvo en ambas técnicas mediante el mismo BGE con el que previamente se habían obtenido buenos resultados para el método de *in-line* SPE-CE-UV. Concretamente, el BGE consistía en una solución acuosa de fosfato monosódico 70 mM a pH 2,5 al que se le había adicionado una mezcla binaria de CDs (2-hidroxipropil α-CD 8 mM y α-CD 5 mM). Bajo estas condiciones, los valores de *Rs* obtenidos entre los enantiómeros de las diferentes catinonas incluidas en estos estudios son muy similares para ambas estrategias de preconcentración electroforética. No obstante, a medida que aumenta el tiempo de inyección electrocinética de la muestra se observa una disminución en la *Rs,* siendo ésta más pronunciada en FASI que en EKS. En ambos casos está pérdida de *Rs* es debida a un incremento de la cantidad de muestra inyectada dentro del capilar y que implica un ensanchamiento de los picos de los analitos. No obstante, en el caso de EKS el proceso de *t*-ITP evita la dispersión de la muestra y consecuentemente la pérdida de *Rs* no es significativa. De hecho, en EKS no se pudo incrementar el tiempo de inyección de la muestra por encima de 120 s, no por una pérdida de *Rs*, sino porque a tiempos mayores se observaron perturbaciones en la corriente eléctrica.

Es importante remarcar que la preconcentración en FASI y EKS está basada en diferentes mecanismos electroforéticos tal y como ya se ha comentado en el apartado de la introducción. Estos distintos mecanismos de preconcentración implican que las variables a optimizar en ambas técnicas sean diferentes. De hecho, la optimización es más sencilla en FASI, donde la principal variable a evaluar es la idoneidad de la inyección de un *plug* de solvente previamente a la inyección electrocinética de la muestra. En el caso de EKS se tienen que encontrar tanto un LE como un TE apropiados para que el procedimiento de preconcentración tenga el efecto deseado, por lo que la complejidad es mayor.

Los factores de preconcentración obtenidos con ambas técnicas ofrecen valores muy distintos. En concreto, tal como muestra la Tabla 1, los SEFs fueron de entre

562 a 601 en términos de área de pico y de 444 a 472 en términos de altura de pico para la metodología de FASI-CE, y del rango de 722 a 1314 en términos de área de pico y de 993 a 1310 en términos de altura de pico para el procedimiento de EKS-CE. Es decir, la estrategia de EKS-CE presenta una mayor sensibilidad que la de FASI-CE. Esto es debido a que con la primera técnica se puede inyectar electrocinéticamente la muestra durante un tiempo más prolongado, sin que ello afecte de forma significativa a la *Rs* entre los enantiómeros de la catinonas. Esta diferencia entre los valores SEFs también repercute en los LODs obtenidos para cada procedimiento, siendo éstos del rango de 15 a 45 ng/mL para el método de FASI-CE y de 4 a 8 ng/mL para la estrategia de EKS-CE. Esta mayor sensibilidad de EKS respecto de FASI es concordante con otros estudios previamente publicados [1–3]. Por ejemplo, en un estudio donde se compararon diferentes técnicas de *stacking*, incluidas FASI, t-ITP y EKS, para el análisis de arsénico en agua potable, Nyssen y colaboradores obtuvieron menores LODs con EKS [1].

Al comparar los LODs obtenidos con las estrategias de FASI-CE y EKS-CE con aquellos obtenidos mediante otras estrategias para la determinación de estas mismas catinonas en muestras de orina, estos son superiores a los obtenidos habitualmente mediante LC-MS (0,04-3 ng/mL [4–8]) y similares a los obtenidos con GC-MS (2-30 ng/mL [9–13]), aunque es de remarcar que en estos estudios no se llevó a cabo la separación enantiomérica de catinonas.

Tabla 1. SEFs obtenidos en las metodologías de FASI-CE y EKS-CE para el análisis de catinonas.

Analito	SEF FASI-CE		SEF EKS-CE	
	Área del pico	Altura del pico	Área del pico	Altura del pico
Mefedrona	601	469	1314	1213
Metilona	-	-	1313	1310
4-metilefedrina	569	444	722	993
MDPV	562	472	940	1161

A pesar de la diferencia en los LODs obtenidos, ambas técnicas son capaces de alcanzar las concentraciones a las cuales las catinonas pueden encontrarse habitualmente en orina, del orden de ng/mL, aunque es importante remarcar la necesidad de un pretratamiento previo de la muestra. En particular, para ambas estrategias se desarrolló un procedimiento de LLE. Este pretratamiento es necesario

dado que se emplea una inyección electrocinética, un tipo de inyección más selectiva que la inyección hidrodinámica. En nuestro caso la matriz era orina y esta pude contener interferentes con carga que podrían inyectarse de forma prioritaria sobre los analitos. La presencia de estos interferentes puede tener un efecto muy perjudicial al disminuir de forma considerable la señal obtenida para los analitos y, por tanto, la sensibilidad del método. En el caso de la metodología de FASI-CE esta problemática se evitó mediante una *salting-out assisted* LLE con NaCl y tolueno, obteniendo unos valores de recuperación global de entre el 33 y el 65%. Estas recuperaciones fueron mejoradas en el método de EKS-CE al emplear ciclohexano como solvente en una LLE convencional, concretamente obteniendo valores de entre un 50 y un 98%.

Referencias

[1] Nyssen, L., Fillet, M., Cavalier, E., Servais, A., *Electrophoresis* 2019, 1–8.

[2] Lee, H. G., Kwon, J. Y., Chung, D. S., *Talanta* 2018, *181*, 366–372.

[3] Chui, M. Q., Thang, L. Y., See, H. H., *J. Chromatogr. A* 2017, *1481*, 145–151.

[4] Glicksberg, L., Bryand, K., Kerrigan, S. *J. Chromatogr. B* 2016, *1035*, 91–103.

[5] Mercolini, L., Protti, M., Catapano, M. C., Rudge, J., Sberna, A. E. *J. Pharm. Biomed. Anal.* 2016, *123*, 186–194.

[6] Sánchez-González, J., Odoardi, S., Bermejo, A. M., Bermejo-Barrera, P., Romolo, F. S., Moreda-Piñeiro, A., Strano-Rossi, S. *Drug Test. Anal.* 2019, *11*, 33–44.

[7] Pascual-Caro, S., Fontanals, N., Borrull, F., Aguilar, C., Calull, M. *Forensic Toxicol.* 2020, *38*, 185–194.

[8] Fan, S. Y., Zang, C. Z., Shih, P. H., Ko, Y. C., Hsu, Y. H., Lin, M. C., Tseng, S. H., Wang, D. Y. *Forensic Sci. Int.* 2020, *315*, 110429.

[9] Hagan, K. S., Reidy, L. *Forensic Sci. Int.* 2015, *257*, 71–75.

[10] Hong, W. Y., Ko, Y. C., Lin, M. C., Wang, P. Y., Chen, Y. P., Chiueh, L. C., Shih, D. Y. C., Chou, H. K., Cheng, H. F. *J. Anal. Toxicol.* 2016, *40*, 12–16.

[11] Alsenedi, K. A., Morrison, C. *Anal. Methods* 2018, *10*, 1431–1440.

[12] Mercieca, G., Odoardi, S., Cassar, M., Strano-Rossi, S. *J. Pharm. Biomed. Anal.* 2018, *149*, 494–501.

[13] Gerace, E., Caneparo, D., Borio, F., Salomone, A., Vincenti, M. *J. Sep. Sci.* 2019, *42*, 1577–1584.

4. CONCLUSIONES

Las conclusiones más relevantes que se pueden extraer de los diferentes resultados obtenidos a lo largo de esta Tesis Doctoral se detallan a continuación:

- Las CDs nativas o neutras son selectores quirales adecuados para la enantioseparación de catinonas mediante CE-UV, especialmente si se emplean en forma de mezcla binaria. Cuando esta separación quiral se realiza mediante la detección por MS, el uso de CDs sulfatadas (aniónicas) junto con una estrategia de CMT permite evitar la entrada de las CDs en la fuente de ionización y, por tanto, prevenir la contaminación del espectrómetro de masas.

- La utilización de técnicas de preconcentración en línea, ya sean basadas en principios cromatográficos o principios electroforéticos, es una estrategia efectiva para aumentar la sensibilidad de la CE en la determinación de catinonas en muestras de orina.

- La aplicación de una elevada presión durante la carga de muestra en el acoplamiento *in-line* de la CE con la SPE permite reducir considerablemente el tiempo total de análisis del método obteniendo una elevada sensibilidad. De hecho, de todas las estrategias de preconcentración evaluadas en esta Tesis Doctoral la *in-line* SPE-CE es la que ha permitido obtener LODs más bajos para la enantiodeterminación de catinonas en orina.

- La combinación de la estrategia *in-line* SPE-CE con la detección con MS ha permitido la obtención de resultados muy prometedores, si bien se requiere de ciertas modificaciones respecto a la estrategia de *in-line* SPE-CE-UV encaminadas a evitar la contaminación de la fuente de ionización. Estas modificaciones se han centrado en sustituir o cambiar las sustancias empleadas en el proceso de separación y preconcentración por otras con características volátiles.

- Las técnicas de *stacking* evaluadas, FASI y EKS, han demostrado su eficacia para la preconcentración de catinonas en muestras de orina. Además, el tiempo de análisis se reduce en comparación con las

estrategias basadas en *in-line* SPE-CE ya que el tiempo necesario para el proceso de carga de muestra es menor.

- El uso de la técnica de EKS, que es una combinación de las técnicas de FASI y *t*-ITP, permite analizar una mayor cantidad de muestra que en FASI sin que se observe una pérdida significativa de la *Rs* entre los enantiómeros de las catinonas. Esta inyección más prolongada permite obtener elevados SEFs, lo que aumenta la sensibilidad del método.

- En el análisis de muestras de orina mediante los diferentes métodos analíticos desarrollados en la presente Tesis Doctoral es necesario realizar un pretratamiento de la muestra para evitar la entrada de interferentes en el capilar. En particular, se ha observado que una LLE es un pretratamiento efectivo a la vez que simple para conseguir este propósito. En el caso concreto de la aplicación de FASI y EKS se requiere de un *clean-up* de muestra muy exhaustivo, puesto que en la orina pueden encontrase interferentes cargados positivamente que se introduzcan prioritariamente durante la EKI, lo que afecta de forma negativa a la intensidad de la señal de las catinonas, así como a la reproducibilidad de la inyección electrocinética.

- Todas las estrategias empleadas a lo largo de la presente Tesis Doctoral permiten alcanzar los niveles de concentración habituales a los que se pueden encontrar las catinonas en muestras de orina (ng/mL). Por tanto, son metodologías adecuadas para análisis con finalidades clínicas, toxicológicas y forenses.

ANEXOS

Anexo I. Abreviaturas

(+)-18-C-6-TCA: (+)-ácido 18-corona-6-tetracarboxílico

ACN: acetonitrilo

BGE: electrolito de separación

CD: ciclodextrina

CE: electroforesis capilar

CHES: ácido 2-(ciclohexilamino)etanosulfónico

CMT: *counter migration technique*

CZE: electroforesis capilar por zonas

DCM: diclorometano

DLLME: microextracción líquido-líquido dispersiva

EI: impacto electrónico

EIE: *extracted ion electropherogram*

EKS: *electrokinetic supercharging*

EMCDDA: *European Monitoring Centre for Drugs and Drug Addiction*

EOF: flujo electroosmótico

ESI: ionización por electrospray

EWS: *Early Warning System*

FASI: *field-amplified sample injection*

FASS: *field-amplified sample stacking*

GC: cromatografía de gases

HRMS: espectrometría de masas de alta resolución

ICP: plasma acoplado inductivamente

LC: cromatografía de líquidos

LE: electrolito frontal

LLE: extracción líquido-líquido

LOD: límite de detección

LOQ: límite de cuantificación

LSD: dietilamida de ácido lisérgico

LVSEP: *large-volume sample stacking with an electroosmotic flow pump*

LVSS: *large-volume sample stacking*

L-TPC: (S)-(-)-N-(trifluoroacetil)pirrolidina-2-cloruro de carbonilo

MALDI: desorción/ionización laser asistida por matriz

MDMA: 3,4-metilendioximetanfetamina

MDPBP: 3,4-metilendioxi-α-pirrolidinobutirofenona

MDPPP: 3,4-metilendioxi-α-pirrolidinopropiofenona

MDPV: 3,4-metilendioxipirovalerona

MEKC: cromatografía capilar micelar electrocinética

MCX: intercambio catiónico fuerte

MeOH: metanol

MIP: polímero de impresión molecular

MPBP: 4-metil-α-pirrolidinobutiofenona

MS: espectrometría de masas

MS/MS: espectrometría de masas en tándem

NaCl: cloruro de sodio

NaOH: hidróxido de sodio

NCI: ionización química negativa

NPS: Nuevas Sustancias Psicoactivas

OMS: Organización Mundial de la Salud

PFT: *partial filling technique*

PLE: extracción con líquidos presurizados

α-PVP: α-pirrolidinopentiofenona

***Rs*:** resolución

SCX: intercambio catiónico fuerte

SEAT: Sistema Español de Alerta Temprana

SEF: *sensitivity enhancement factor*

SLE: extracción sólido-líquido

SPE: extracción en fase sólida

SPME: microextracción en fase sólida

TE: electrolito terminal

THF: tetrahidrofurano

TIE: *total ion electropherogram*

***t*-ITP:** isotacoforesis transitoria

UHPLC: *ultra-high-performance liquid chromatography*

UHPSFC: *ultra-high-performance supercritical fluid chromatography*

UNODC: *United Nations Office on Drugs and Crime*

UV: ultravioleta-visible

VAMS: *volumetric absorptive microsampling*

Anexo II. Publicaciones

Lista de publicaciones derivadas de la presente Tesis Doctoral:

- A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE, Electrophoresis. 40 (2019) 1762–1770. https://doi.org/10.1002/elps.201900072.

- A. Pérez-Alcaraz, F. Borrull, M. Calull, C. Aguilar, Field-amplified sample injection combined with CE for the enantiodetermination of cathinones in urine samples, J. Sep. Sci. 43 (2020) 2914–2924. https://doi.org/https://doi.org/10.1002/jssc.201901067.

- A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis, Microchem. J. 158 (2020) 105300. https://doi.org/10.1016/j.microc.2020.105300.

- A. Pérez-Alcaraz, F. Borrull, C. Aguilar, M. Calull, F. Benavente, Enantiodetermination of *R,S*-3,4-methylenedioxypyrovalerone in urine samples by high pressure in-line solid-phase extraction capillary electrophoresis-mass spectrometry, Talanta. 225 (2021) 121994. https://doi.org/10.1016/j.talanta.2020.121994.

- A. Pérez-Alcaraz, F. Borrull, M. Calull, C. Aguilar, Cathinones in urine samples: a review of recent advances for their determination by chromatographic and related techniques, TrAC - Trends Anal. Chem. 143 (2021) 116347. https://doi.org/10.1016/j.trac.2021.116347

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