Universidad Rey Juan Carlos

TESIS DOCTORAL

Desarrollo de métodos analíticos avanzados basados en novedosas etapas de preparación de muestra y análisis por cromatografía acoplada a espectrometría de masas para la determinación de alcaloides opiáceos en alimentos

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Escuela Internacional de Doctorado

2024

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CERTIFICAN:

Que el trabajo descrito en la presente memoria, titulado **"Desarrollo de métodos analíticos avanzados basados en novedosas etapas de preparación de muestra y análisis por cromatografía acoplada a espectrometría de masas para la determinación de alcaloides opiáceos en alimentos"**, ha sido realizado bajo su dirección por **Dña. Gema Casado Hidalgo** en el Área de Química Analítica y presentado en el Departamento de Tecnología Química y Ambiental para optar al Grado de Doctor Internacional por la Universidad Rey Juan Carlos.

Asimismo, autorizan su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firman el presente documento en Móstoles a 08/01/2024.

Fdo. María Isabel Sierra Alonso **Fdo. Sonia Morante Zarcero**

AGRADECIMIENTOS

Tras estos cuatro intensos años estoy poniendo fin a una etapa de mi vida dura, disciplinada, pero sin ninguna duda la más bonita y gratificante que he vivido. Hace cinco años terminé de estudiar Ciencia y Tecnología de los Alimentos en la Universidad de Zaragoza, ciudad en la que nací. Después me vine a Madrid a realizar un máster y sin pensarlo me quedé para hacer la Tesis en Química Analítica en la Universidad Rey Juan Carlos con el objetivo de seguir formándome y de vivir la experiencia de lo que realmente es investigar. Hacer una Tesis Doctoral es un proceso de aprendizaje y de superación. En ocasiones puede ser muy dura y la mayor parte del tiempo te encuentras esforzándote al máximo, puedes estar meses obteniendo malos resultados y enfrentándote a obstáculos inesperados. Pero todo eso me ha permitido ser capaz de resolver problemas de forma más autónoma, ser perseverante hasta obtener buenos resultados y tener mi propia autocrítica. Al final es un proceso que disfrutas, la ilusión de enfrentarte a un proyecto totalmente nuevo y desconocido, la alegría de obtener tu primer "recovery" válido en un trabajo después de tantas pruebas y la satisfacción de terminar un proyecto y poder dar resultados que sirvan para aportar un granito de arena a la sociedad. Cerrando esta etapa solo puedo sentirme agradecida, por la enseñanza y el apoyo de cada una de las personas que han formado y forman parte de mi vida.

En primer lugar, quiero dar las gracias a la Universidad Rey Juan Carlos, al Departamento de Tecnología Química y Ambiental y, sobre todo, a mis directoras, Dr. Isabel Sierra y Dr. Sonia Morante por haberme guiado todos estos años para realizar mi Tesis Doctoral.

Gracias Isabel por haber confiado en mi desde el principio y por haber valorado siempre mi trabajo. Por darme la oportunidad para dedicarme estos años a algo que me apasiona, la investigación y la docencia, y por todos los proyectos para los que has contado conmigo.

Y a Sonia, gracias por guiarme día a día en el laboratorio y ayudarme con muchos de los problemas que me han ido surgiendo a lo largo de la investigación. Sin duda eres una de las personas de las que más he aprendido en mi vida, me has enseñado tu criterio, tu forma de pensar y me has contagiado tu perseverancia y tu amor por la ciencia.

También quiero agradecer a Damián Pérez por todo, por enseñarme tanto sobre el mundo de los materiales, el trabajo ordenado, minucioso y constante. Siempre has estado dispuesto para explicarme cualquier duda y te has preocupado de mi bienestar.

Y a mis compañeros de grupo de investigación, por ser mi apoyo día a día y haber compartido tanto en estos años. A Leo, mi chismoso favorito, que te has convertido en un verdadero amigo y te tengo que agradecer toda la ayuda que me has prestado estos años y siempre con una sonrisa. A Lorena, desde el principio mano a mano con cromito. Por todo lo que hemos vivido juntas, los congresos, viajes, cenas y largas charlas, gracias de verdad. Y también a Jesús, por ayudarme en todo lo que he podido necesitar desde que llegué a Madrid y todos tus chistes, siempre tendré una palabra maña que enseñarte. A Isa, que desde que llegaste me transmitiste confianza, me ofreciste tu ayuda y me escuchaste, gracias por todo eso y por recordarme lo mucho que me gusta el deporte. A Begoña, gracias por tener buenas palabras cada día y por los buenos momentos que hemos pasado juntas. También quiero darle las gracias a Sergio, por todo el apoyo que me ha brindado, te deseo lo mejor. Y por último quiero agradecer a Judith, Natalia y Gon por todo lo que me han enseñado estos años y darme confianza y ánimos para afrontar lo que ellos ya lograron. Judith, gracias por animarme en muchos de los momentos más difíciles. Natalia, gracias por los consejos que me has dado. Gon, gracias por el positivismo y la alegría que transmites. Y gracias a todos los alumnos que nos han ido acompañando a lo largo de estos años en el laboratorio, realizando sus trabajos de fin de grado, becas de colaboración o estancias. Aarón, Rubén, Lisa, Nicos, María, Gabriela e Ivana porque cada uno de vosotros me habéis aportado y enseñado muchas cosas.

También quería dar las gracias a todos los que me acompañaron durante mi estancia pre-doctoral en el Centro de Química de Madeira. Gracias al profesor Câmara por la oportunidad de poder realizarla allí, el positivismo y todo el conocimiento. Gracias Rosa por la ayuda para sacar adelante el trabajo. Y gracias, Jorge Pereira, Mariangie, Patricia, Yonathan, Laura, Carolina y Cristina por vuestra amabilidad y ayuda. Sin duda esta experiencia me hizo aprender mucho en todos los aspectos de mi vida.

Y no quiero dejarme de agradecer a todos los que estuvieron conmigo a los inicios de mi actividad investigadora, a mis profesores de la Universidad de Zaragoza, a los que

recuerdo con grandes valores docentes en los que me ayudaron a buscar mi camino. Gracias Celia Domeño y Margarita Aznar, mis directoras de TFG, por enseñarme este apasionante mundo. Y a Pedro Marco con el que tuve el placer de estar en el Centro de Investigación y Tecnología de Aragón.

Gracias también a todos con los que compartí mi año de máster de Nuevos Alimentos en la Universidad Autónoma de Madrid. Ese año terminé de decidir que lo que realmente me apasionaba era la investigación y en gran parte fue gracias a Diana Martín, mi tutora de TFM, a la que recuerdo con gran estima por toda la ayuda que me prestó.

Además, quiero agradecer a mis amigas de la universidad, Natalia, Rocío, Lorena, María, Leire, Ada y Silvia. Cuanto he aprendido de cada una de vosotras y que bien nos lo hemos pasado. Cada reencuentro es único y a pesar de la distancia que muchas veces nos separa, siempre estáis ahí.

Gracias a mis amigas del colegio, Pilar, Raquel, Natalia C, Natalia M, Sofía y Paula, juntas desde que nacimos, gracias por animarme siempre que lo necesito, alegraros de todos mis triunfos y apoyarme para luchar por mis sueños, os quiero mucho a todas chicas.

También quiero agradecer a toda mi familia de Moros, mi pueblo. A todos mis amigos, que por tiempo que pase todo sigue igual. Gracias a mis primos. María, por todo el ánimo y todo el cariño que me das. Y, sobre todo, gracias a mis abuelos, a los que todavía tengo la suerte de poder estar con ellos y a los que ya no están. Gracias por enseñarme las cosas más importantes de la vida y por sentiros tan orgullosos de mí.

Gracias a mis padres, a mamá por apoyarme siempre y darme los mejores consejos y a papá por no dejar que nunca me desanime y ayudarme en todo. Pase lo que pase sé que estáis ahí, incluso a distancia, y sin duda, sois mi ejemplo a seguir. Y a mi hermano Alberto, gracias tato. Contigo sé que siempre podré contar y nunca estaremos solos, siempre nos cuidaremos el uno al otro. Os quiero mucho a los tres.

Y por último quería agradecerle a mi compañero de vida. Javier, que siempre estás conmigo. Gracias por quererme tan bien, ayudarme incondicionalmente en todo y apoyarme a que cumpla todos mis sueños. Esta Tesis sin duda también es gracias a ti. Te quiero mucho.

A mi familia

“Solo imagina lo precioso que
puede ser arriesgarse y que
todo salga bien”
Mario Benedetti

Actualmente garantizar la Seguridad Alimentaria es uno de los mayores retos a nivel mundial debido a los numerosos peligros que pueden encontrarse en los alimentos. No en vano, este reto se contempla en los Objetivos de Desarrollo Sostenible (ODS) establecidos por la Organización de las Naciones Unidas (ONU). Para abordar este reto es importante cumplir con la estrategia "de la granja a la mesa" controlando toda la cadena alimentaria, ya que en cualquier punto puede haber una contaminación que suponga un riesgo para la salud del consumidor.

Una de las contaminaciones que más interés ha causado en las autoridades sanitarias en los últimos años son las producidas por las toxinas naturales, concretamente los alcaloides del opio (OAs). Estos compuestos (mayormente morfina, codeína, tebaína, papaverina, noscapina y oripavina) se encuentran en el látex de la planta *Papaver somniferum* L., comúnmente conocida como adormidera o amapola real. Se ha utilizado el látex desde la antigüedad con fines farmacológicos. Sin embargo, en los últimos años ha aumentado el uso alimentario de las semillas de esta planta, ya sea para añadirlas como toppings a ensaladas, yogures, productos de panadería o como rellenos para pasteles, elaboración de infusiones y aceites. Aunque las semillas no contienen OAs de forma natural, éstos pueden contaminarse durante su recolección con este látex, dando lugar a concentraciones preocupantemente altas. Por este hecho, el consumo de semillas de amapola y de alimentos que las contienen ha dado lugar a numerosas intoxicaciones e incluso falsos positivos en test de drogas.

Actualmente, el Reglamento (UE) 2023/915 legisla el límite máximo de morfina equivalentes (morfina + 0,2 × codeína) en semillas de amapola o productos de panadería elaborados con las mismas. A pesar de este reglamento tan reciente, las autoridades sanitarias siguen reclamando más estudios donde se evalúen los niveles de todos los OAs mayoritarios, y no solo morfina y codeína, ya que también pueden encontrarse en concentraciones altas e incluso pueden llegar a ser más tóxicos y los resultados aún son escasos. Además, es necesario recopilar los datos del contenido de OAs en distintas matrices alimentarias para establecer la exposición real de los consumidores y determinar el efecto que pueden tener algunos procesados culinarios en las concentraciones finales

de OAs, para lo cual es necesario desarrollar metodologías de análisis aún inexistentes hasta el comienzo de esta tesis.

Por todo ello, el objetivo principal de esta Tesis Doctoral ha sido desarrollar y validar métodos analíticos lo más respetuosos posible con el medio ambiente para cuantificar los seis OAs mayoritarios en distintas matrices alimentarias para recopilar datos y evaluar el efecto de varios tipos de procesado culinario, cumpliendo con los objetivos de la Química Analítica Verde (GAC) y la Preparación de muestra Verde (GSP) y, contribuir al cumplimiento de los ODS.

Para conocer y recopilar toda la información sobre la situación actual de esta familia de toxinas, se realizó y publicó un trabajo de revisión. En éste, se incluyó la causa de su presencia en los alimentos, el riesgo de su consumo y las medidas para prevenir su exposición. Además, se resumieron las técnicas de tratamiento de muestra y análisis utilizadas para cuantificar OAs en diferentes muestras.

Debido a que las matrices alimentarias tienen una gran variedad de componentes que pueden interferir en el análisis, es importante realizar un adecuado tratamiento de la muestra además de emplear una técnica de análisis sensible y selectiva. Por todo ello, las metodologías desarrolladas en la Tesis Doctoral están formadas por una primera etapa de extracción, seguida de una etapa de purificación o clean-up antes del análisis por cromatografía de líquidos o de gases acoplada a espectrometría de masas. Además, en todas ellas se trató de optimizar protocolos de preparación de muestra más respetuosos con el medio ambiente, utilizando menores volúmenes de disolventes orgánicos y tiempos de extracción o utilizando técnicas de purificación miniaturizadas. Para ello, se sintetizaron y caracterizaron nuevos materiales adsorbentes tanto magnéticos como silíceos, para su utilización en extracción en fase sólida (SPE) o en extracción en fase sólida magnética (MSPE) que permitieron miniaturizar los procedimientos, empleando menores cantidades de material adsorbente (< 50 mg). Todas las metodologías fueron validadas y aplicadas al análisis de muestras alimentarias comerciales con el objetivo de establecer la exposición real de los consumidores a estos tóxicos. Las muestras comerciales estudiadas fueron semillas de amapola, productos de panadería (tales como palitos de pan, pan de molde, galletas y bizcochos), infusiones, yogures y pastas y harinas

de semillas trituradas de amapola. Además, las metodologías analíticas desarrolladas también se utilizaron para evaluar la influencia de algunos tipos de procesado tales como la fermentación ácido-láctica, la transferencia tras el infusionado y la molienda.

Los resultados de estos estudios se han publicado en forma de ocho artículos de investigación y una nota de aplicación para la empresa, que forman parte de la sección de resultados y discusión de la presente Tesis Doctoral organizados en función del orden cronológico en el que fueron desarrollados y publicados.

En primer lugar, se evaluó el contenido de OAs en las semillas de amapola (Artículo 1). Para ello, se desarrolló una metodología analítica basada en una extracción sólido-líquido (SLE), una posterior purificación mediante extracción en fase sólida magnética miniaturizada (µ-MSPE), seguido de un análisis por cromatografía de líquidos de ultra-alta resolución acoplada a detector de masas de tándem (UHPLC-MS/MS). Para ello, se aplicó un nuevo material magnético sintetizado con núcleo de Fe_3O_4 y recubierto con una capa de sílice amorfa (Fe_3O_4@SiO_2) y una segunda capa de sílice mesoestructurada (Fe_3O_4@SiO_2@mSiO_2), dando lugar a una etapa de purificación muy rápida y sencilla empleando tan solo 50 mg de material. En este trabajo se puso de manifiesto la peligrosidad de un consumo no controlado de semillas de amapola ya que se determinaron concentraciones muy altas.

Posteriormente, se evaluó el contenido de OAs en los productos de panadería con semillas de amapola. Por un lado, se estudiaron el pan de molde y los palitos de pan (Artículo 2) y, por otro lado, las galletas y los bizcochos (Artículo 3). La metodología analítica desarrollada para analizar las muestras de panadería saladas (Artículo 2) se basó en una SLE, seguida de una purificación con µ-MSPE y un posterior análisis por HPLC-MS/MS. En este caso, se llevó a cabo una funcionalización de las partículas de Fe_3O_4 con ácido tereftálico y cloruro de hierro (III) (Fe_3O_4@TPA-Fe). Este material mostró una alta eficiencia en la extracción ya que con solo 1 mg se obtuvieron buenos valores de porcentajes de recuperación. Las concentraciones de OAs encontradas en estos productos de panadería fueron generalmente bajas, aunque dos de las muestras analizadas mostraron concentraciones superiores al límite establecido por la legislación para este tipo de muestras. Por otro lado, en la metodología analítica desarrollada para analizar las

muestras de panadería dulces (Artículo 3) se utilizó una extracción asistida por ultrasonidos (UAE) con el objetivo de disminuir el volumen de disolvente orgánico y el tiempo de extracción. Posteriormente, se hizo una purificación mediante extracción en fase sólida miniaturizada (µ-SPE) para la cual se empleó como material adsorbente 50 mg de la sílice SBA-15 seguido del análisis por HPLC-MS/MS. En todas las muestras analizadas se cuantificaron OAs, aunque las concentraciones fueron inferiores a las del límite legislado. Por tanto, en general, los resultados de ambos trabajos mostraron que los productos de panadería no suponen un peligro potencial por el bajo porcentaje de semillas de amapola que contienen, pero es fundamental controlar el nivel de OAs en las semillas porque si no algunas muestras de panadería pueden tener unas concentraciones de OAs que superen los límites legislados.

Después, se desarrollaron varias metodologías de análisis para determinar la concentración de OAs en infusiones con semillas de amapola. En primer lugar, se llevó a cabo un estudio sobre la transferencia de los OAs con el infusionado de las semillas de amapola. Para ello, se aplicó la novedosa técnica de micro-extracción µ-SPEed® (Artículo 4). La principal ventaja de esta técnica es su alta capacidad de preconcentración, permitiendo el análisis en técnicas con menor sensibilidad para estos analitos como la cromatografía de gases acoplada a detector de masas (GC-MS). Como resultado se obtuvo que hay una transferencia prácticamente completa de los OAs con diferentes condiciones de infusionado. Por otro lado, debido a la alta capacidad de preconcentración (hasta 10 veces) que se obtuvo con la µ-SPEed® para los OAs, se realizó una nota de aplicación para la industria en la que se utilizó la extracción y preconcentración con µ-SPEed® al análisis de OAs mediante cromatografía de líquidos de alta eficacia con detector de diodo array (HPLC-DAD), un equipo más disponible en los laboratorios en general (Nota de Aplicación 1). Y, por último, en el siguiente trabajo se desarrolló otra metodología para analizar OAs en infusiones con semillas de amapola. Esta vez, el método se basó en un primer paso de µ-MSPE seguido del análisis por HPLC-MS/MS (Artículo 5). Para ello, las partículas de Fe_3O_4@SiO_2@mSiO_2 se funcionalizaron con moléculas de β-ciclodextrina (β-CD), las cuales presentan gran efectividad en medios acuosos para intentar mejorar el porcentaje de recuperación de los OAs. Se utilizó este ligando de origen natural por ser más respetuoso con el medio ambiente. De esta manera, se

emplearon 50 mg de material y se obtuvieron mejores valores de recuperación que sin la funcionalización. Respecto a los resultados de las muestras, se utilizaron las condiciones de infusionado que más tasa de transferencia dieron en el anterior trabajo con el fin de evaluar las concentraciones de OAs que se podían llegar a obtener mediante la infusión empleando semillas comerciales. Como resultado se encontraron en algunas infusiones concentraciones preocupantemente altas que pone de manifiesto la peligrosidad de realizar esta práctica.

Posteriormente, se evaluó el contenido de OAs en yogures comerciales y se estudió la influencia de distintas condiciones de fermentación y almacenamiento en refrigeración sobre el contenido de OAs en yogures caseros preparados con semillas de amapola (Artículo 6). Para ello, se desarrolló una metodología basada en una SLE con agua, una µ-MSPE con el material Fe_3O_4@SiO_2@mSiO_2@β-CD previamente sintetizado, seguido del análisis por HPLC-MS/MS. Como resultado se obtuvieron concentraciones muy bajas de OAs en yogures comerciales y se observó un efecto significativo de degradación de OAs en las primeras horas de fermentación.

Y, por último, se estudiaron los contenidos de OAs en pastas y harinas obtenidas a partir de semillas trituradas de amapola para posteriormente evaluar la influencia de distintos tipos y condiciones de molienda en el contenido de OAs final. Para analizar pastas y harinas de semillas trituradas de amapola, se optimizó una metodología (Artículo 7) basada en una extracción mediante UAE y una posterior µ-SPE con SBA-15 funcionalizada con grupos sulfónicos como material adsorbente de intercambio catiónico. Esto permitió tanto disminuir el volumen de disolvente y tiempo de extracción empleados durante la UAE como la cantidad de material adsorbente necesaria en la µ-SPE (25 mg). Los resultados mostraron que las pastas y harinas de semillas trituradas de amapola podían contener elevadas concentraciones de OAs, llegando a superar el contenido máximo legislado. Esta misma metodología fue empleada en el siguiente trabajo para evaluar si la molienda de las semillas puede considerarse como una buena práctica de reducción en el contenido de OAs final en pastas y harinas de semillas trituradas de amapola (Artículo 8). Para ello, se compararon las concentraciones iniciales obtenidas en las semillas enteras con las obtenidas tras molerlas con cinco diferentes tipos de molino y diferentes

condiciones (tiempo de molienda o número de moliendas consecutivas). Los resultados mostraron una notable degradación del 20% de la morfina, concretamente en los molinos en los que no se libera el contenido de aceite de las semillas, lo que apunta al papel protector de la grasa frente a la oxidación de la morfina. Por lo tanto, la elección del método de molturación resulta crucial. Sin embargo, aun reconociendo este hecho, basarse únicamente en este método como buena práctica para la reducción de los OAs puede ser insuficiente. Por lo tanto, es aconsejable combinar estos procesos con otros métodos para lograr una reducción eficaz de los OAs.

Como conclusión, se puede indicar que todas las metodologías analíticas desarrolladas en la presente Tesis Doctoral han permitido obtener información sobre el contenido de OAs en diferentes productos alimentarios comerciales y sobre la influencia de diferentes tipos de procesado culinario en este tipo de toxinas, dando respuesta a las peticiones de las agencias europeas de seguridad alimentaria. Además, se ha demostrado que el uso de nuevos materiales adsorbentes y la aplicación de novedosas etapas de preparación de muestra miniaturizadas pueden suponer un avance en la obtención de métodos más verdes y sostenibles y unido a distintas técnicas cromatográficas pueden contribuir a la mejora del control de OAs y a garantizar la seguridad alimentaria.

Nowadays, ensuring food safety is one of the greatest challenges worldwide due to the numerous hazards that can be found in food. Not surprisingly, this challenge is included in the Sustainable Development Goals (SDGs) established by the United Nations (UN). To approach this challenge, it is important to comply with the "farm-to-table" strategy by controlling the entire food chain, as contamination can occur at any point that poses a risk to consumer health.

One of the contaminations that have caused most interest among health authorities in recent years are those produced by natural toxins, specifically opium alkaloids (OAs). These compounds (mostly morphine, codeine, thebaine, papaverine, noscapine and oripavine) are found in the latex of the *Papaver somniferum* L. plant, commonly known as the opium poppy. Since ancient times, latex has been used for pharmacological purposes. However, in recent years the food use of the seeds of this plant has increased, either to add them as toppings to salads, yogurts, bakery products or as fillings for cakes, making infusions and oils. Although the seeds do not naturally contain OAs, they can become contaminated during harvesting with this latex, resulting in dangerously high concentrations. As a result, the consumption of poppy seeds and foodstuffs containing them has led to numerous intoxications and even false positive drug tests.

Currently, Regulation (EU) 2023/915 legislates the maximum limit of morphine equivalents (morphine + 0.2 × codeine) in poppy seeds or processed bakery products. Despite this very recent regulation, health authorities are still demanding more studies where the levels of all main OAs are evaluated, and not only morphine and codeine, as they can also be found in high concentrations and can even be more toxic and the results are still limited. In addition, it is necessary to collect data on the content of OAs in different food matrices to establish the real exposure of consumers and to determine the effect that some culinary processes may cause on the final concentrations of OAs, which requires the development of analytical methodologies that are still lacking at the beginning of this thesis.

Therefore, the principal aim of this Doctoral Thesis has been to develop and validate analytical methods as environmentally friendly as possible to quantify the six main OAs in different food matrices to collect data and evaluate the effect of various types of

culinary processing, in compliance with the objectives of Green Analytical Chemistry (GAC) and Green Sample Preparation (GSP) and, to contribute to the achievement of the SDGs.

To know and compile all the information on the current situation of this toxin family, a review was carried out and published. It included the cause of their presence in food, the risk of their consumption and the measures to prevent their exposure. In addition, the sample treatment and analysis techniques used to quantify OAs in different samples were summarized.

Since food matrices have a wide variety of components that can interfere with the analysis, it is important to perform an adequate sample treatment as well as to use a sensitive and selective analysis technique. For all these reasons, the methodologies developed in this Doctoral Thesis consist of a first extraction step, followed by a purification or clean-up step before analysis by liquid or gas chromatography coupled to mass spectrometry. In addition, all of them tried to optimize more environmentally friendly sample preparation protocols, using lower organic solvent volumes and extraction times or using miniaturized purification techniques. To this end, new adsorbent materials, both magnetic and silica, were synthesized and characterized for use in solid phase extraction (SPE) or magnetic solid phase extraction (MSPE), which allowed miniaturization of the procedures, using smaller adsorbent material amounts (< 50 mg). All methodologies were validated and applied to the analysis of commercial food samples in order to establish the actual exposure of consumers to these toxins. The commercial samples studied were poppy seeds, bakery products (such as breadsticks, sliced bread, biscuits and sponge cakes), infusions, yoghurts and pastas and ground poppy seed flours. Furthermore, the analytical methodologies developed were also used to evaluate the influence of some processing types such as lactic acid fermentation, transfer after infusion and grinding.

The results of these studies have been published as eight research articles and a company application note, which form part of the results and discussion section of this Doctoral Thesis organized according to the chronological order in which they were developed and published.

Firstly, the OAs content in poppy seeds was evaluated (Article 1). For this purpose, an analytical methodology was developed based on a solid-liquid extraction (SLE), a subsequent purification by miniaturized magnetic solid phase extraction (µ-MSPE), followed by analysis by ultra-high performance liquid chromatography coupled to tandem mass detector (UHPLC-MS/MS). For this purpose, a new magnetic material synthesized with Fe_3O_4 core and coated with a layer of amorphous silica (Fe_3O_4@SiO_2) and a second layer of mesostructured silica (Fe_3O_4@SiO_2@mSiO_2) was applied, resulting in a very fast and simple purification step using only 50 mg of material. This work revealed the danger of uncontrolled consumption of poppy seeds, as very high concentrations were determined.

Subsequently, the content of OAs in bakery products containing poppy seeds was evaluated. On the one hand, sliced bread and breadsticks (Article 2) and, on the other hand, biscuits, and sponge cakes (Article 3) were studied. The analytical methodology developed to analyze the salted bakery samples (Article 2) was based on a SLE, followed by purification with µ-MSPE and subsequent analysis by HPLC-MS/MS. In this case, a functionalization of Fe_3O_4 particles with terephthalic acid and iron (III) chloride (Fe_3O_4@TPA-Fe) was carried out. This material showed a high extraction efficiency since with only 1 mg good values of recovery percentages were obtained. The OAs concentrations found in these bakery products were generally low, although two of the samples analyzed showed concentrations higher than the limit established by legislation for this type of sample. On the other hand, in the analytical methodology developed to analyze the sweet bakery samples (Article 3), an ultrasound-assisted extraction (UAE) was used to reduce the organic solvent volume and the extraction time. Subsequently, purification was performed by miniaturized solid phase extraction (µ-SPE) for which 50 mg of silica SBA-15 was used as adsorbent material followed by HPLC-MS/MS analysis. In all the samples analyzed, OAs were quantified, although the concentrations were below the legislated limit. Therefore, in general, the results of both works showed that bakery products do not pose a potential hazard because of the low percentage of poppy seeds they contain, but it is essential to control the level of OAs in the seeds because otherwise some bakery samples may have OAs concentrations that exceed the legislated limits.

Afterwards, several analytical methodologies were developed to determine the concentration of OAs in poppy seed infusions. First, a study on the transfer of OAs with poppy seed infusion was carried out. For this purpose, the novel micro-extraction technique µ-SPEed® (Article 4) was applied. The main advantage of this technique is its high preconcentration capacity, allowing analysis in techniques with lower sensitivity for these analytes such as gas chromatography coupled to mass detector (GC-MS). As a result, it was obtained that there is a practically complete transfer of the OAs with different infusion conditions. On the other hand, due to the high preconcentration capacity (up to 10-fold) obtained with µ-SPEed® for OAs, a company application note was made in which µ-SPEed® extraction and preconcentration was used to analyze OAs by high-performance liquid chromatography with diode array detector (HPLC-DAD), a more widely available equipment in laboratories in general (Application Note 1). Finally, in the following work, another methodology was developed to analyze OAs in poppy seed infusions. This time, the method was based on a first µ-MSPE step followed by HPLC-MS/MS analysis (Article 5). For this purpose, Fe_3O_4@SiO_2@mSiO_2 particles were functionalized with β-cyclodextrin (β-CD) molecules, which present great effectiveness in aqueous media to improve the percentage recovery of OAs. This natural origin ligand was used because it is more environmentally friendly. Thus, 50 mg of material were used, and better recovery values were obtained than without functionalization. Regarding the results of the samples, the infusion conditions that gave the highest transfer rate in the previous work were used to evaluate the concentrations of OAs that could be obtained by infusion using commercial seeds. As a result, worryingly high concentrations were found in some infusions, which highlights the danger of this practice.

Subsequently, the OAs content in commercial yogurts was evaluated and the influence of different fermentation and refrigerated storage conditions on the OAs content in home-made yogurts prepared with poppy seeds was studied (Article 6). For this purpose, a methodology was developed based on an SLE with water, a µ-MSPE with the previously synthesized Fe_3O_4@SiO_2@mSiO_2@β-CD material, followed by HPLC-MS/MS analysis. As a result, very low OAs concentrations were obtained in commercial yogurts and a significant OAs degradation effect was observed in the first hours of fermentation.

Finally, the OAs contents in pasta and flours obtained from ground poppy seeds were studied to evaluate the influence of different types and conditions of grinding on the final OAs content. To analyze pastes and flours from ground poppy seeds, a methodology was optimized (Article 7) based on an UAE and a subsequent µ-SPE with SBA-15 functionalized with sulfonic groups as cation exchange adsorbent material. This allowed both to decrease the solvent volume and extraction time used during UAE and the adsorbent material amount needed in µ-SPE (25 mg). The results showed that ground poppy seed pastes and flours could contain high concentrations of OAs, even exceeding the maximum legislated content. This same methodology was used in the following work to evaluate whether seed grinding can be considered as a good practice to reduce the final OAs content in poppy ground poppy seed pastes and flours (Article 8).

For this purpose, the initial concentrations obtained in whole seeds were compared with those obtained after grinding them with five different types of grinders and different conditions (grinding time or consecutive grinding). The results showed a remarkable 20% degradation of morphine, specifically in grinders in which the oil content of the seeds is not liberated, which suggests the protective role of fat against morphine oxidation. Therefore, the choice of grinding method is crucial. However, even recognizing this fact, relying solely on this method as a good practice for OAs reduction may be insufficient. Therefore, it is advisable to combine these processes with other methods to achieve effective reduction of OAs.

In conclusion, it can be indicated that all the analytical methodologies developed in this Doctoral Thesis have allowed obtaining information on the content of OAs in different commercial food products and on the influence of different types of culinary processing on this toxin type, responding to the requests of the European food safety agencies. In addition, it has been demonstrated that the use of new adsorbent materials and the application of novel miniaturized sample preparation steps can represent an advance to obtain greener and more sustainable methods and, together with different chromatographic techniques, can contribute to the improvement of OAs control and to ensure food safety.

µ-dSPE: Extracción en fase sólida dispersiva miniaturizada

µ-SPE: Extracción en fase sólida miniaturizada

µ-SPEed®: Micro-extracción en fase sólida registrada por la empresa PREP Australia

AcN: Acetonitrilo

AE: Análisis Elemental

APCI: Ionización química a presión atmosférica

APS: WAX, intercambio aniónico débil

BET: Brunauer-Emmett-Teller

BfR: Instituto Federal Alemán de evaluación de riesgos

BJH: Modelo Barret-Joyner-Halenda

C_{18}: Octadecilsilano

C_4: Tetrilsilano

C_8: Octilsilano

CD: Ciclodextrina

CE: Comisión Europea

CI: Ionización química

CTAB: Bromuro de cetiltrimetilamonio

DAD: Detector de diodo-array

DRX: Difracción de Rayos-X

dSPE: Extracción en fase sólida dispersiva

ECD: Detector de captura de electrones

EFSA: Autoridad Europea de Seguridad Alimentaria

EI: Impacto de electrones

ESI: Ionización por electrospray

FAO: Organización de las Naciones unidas para la Agricultura y la Alimentación

Fe_3O_4: Magnetita

Fe_3O_4@SiO_2: Partículas magnéticas recubiertas con una capa de sílice amorfa

Fe_3O_4@SiO_2@mSiO_2: Partículas magnéticas recubiertas con una capa de sílice amorfa y una segunda capa de sílice mesoestructurada

Fe_3O_4@TPA-Fe: Partículas magnéticas funcionalizadas con ácido tereftálico y cloruro de hierro (III)

FID: Detector de ionización de llama

FT: Transformada de Fourier

FT-IR: Espectroscopía infrarroja de transformada de Fourier

GAC: Química Analítica Verde (del inglés *Green Analytical Chemistry*)

GC: Cromatografía de gases

Grupos CN: Grupos cianopropilos

ABREVIATURAS

Grupos NH_2: Grupos aminopropilos

GSP: Preparación de muestras verde (del inglés *Green Sample Preparation)*

HLB: Material adsorbente de balance hidrofílico-lipofílico

HMS: Hexagonal Mesoporous Silica

HPLC: Cromatografía de líquidos de alta eficacia

I.U.P.A.C.: Unión Internacional de Química Pura y Aplicada

IT: Trampa de iones

LC: Cromatografía de líquidos

LOD: Límite de detección instrumental

***m/z*:** Masa/nº de cargas del ion

MALDI: Desorción/ionización láser asistida por matriz

MCM: Mobil Composition of Matter

MDL: Límite de detección del método

MeOH: Metanol

MEPS: Técnica de micro-extracción por adsorbentes empaquetados

MFE-PAK® SCX: Material comercial de intercambio catiónico fuerte DEFINIRLO EN EL TEXTO

MNPs: Nanopartículas magnéticas (proviene del inglés *magnetic nanoparticles*)

MQL: Límite de cuantificación del método

MRM: Monitorización de reacción múltiple

MS: Detector de espectrometría de masas

MSPE: Extracción en fase sólida magnética

OAs: Alcaloides opiáceos

ODS: Objetivos de Desarrollo Sostenible

ONU: Organización de las Naciones Unidas

PFAs: 50% WAX, 50% C_{18}

PID: Detector de fotoionización

PS/DVB-RP: Material polimérico de fase reversa

PS/DVB-SAX: Material polimérico intercambio aniónico débil

PS/DVB-SCX: Material polimérico de intercambio catiónico fuerte

Q: Cuadrupolo

RASFF: Red de Alerta Rápida de Alimentos y Piensos

RSM: Metodología de superficie respuesta

SBA: Santa Barbara Amorphous

SBA-15: Santa Barbara Amorphous número 15

S_{BET}: Área superficial específica

SEM: Microscopía electrónica de barrido

SIM: Monitorización de iones seleccionados

SLE: Extracción sólido-líquido

SO_3^-: Grupos sulfónicos

SPE: Extracción en fase sólida

TCD: Detector de conductividad térmica

TEM: Microscopía electrónica de transmisión

TEOS: Tetraetilortosilicato

TOF: Tiempo de vuelo

TQ: Triple cuadrupolo

UAE: Extracción asistida por ultrasonidos

UHPLC: Cromatografía de líquidos de ultra-alta resolución

US: Ultrasonidos

UV-Vis: Detector de ultravioleta-visible

XRF: Rayos X de fluorescencia

α-Fe_2O_3: Hematita

γ-Fe_2O_3: Maghemita

µ-MSPE: Extracción en fase sólida magnética miniaturizada

INTRODUCCIÓN

1. INTRODUCCIÓN

Garantizar la seguridad alimentaria tiene hoy en día una importancia vital y es una de las mayores preocupaciones a nivel mundial. La seguridad alimentaria está definida por la Organización de las Naciones unidas para la Agricultura y la Alimentación (FAO) como la "situación que se da cuando todas las personas tienen acceso físico, social y económico a alimentos suficientes, inocuos y nutritivos que satisfagan sus necesidades dietéticas y preferencias alimentarias para una vida activa y saludable" [1], [2].

En el caso de los países desarrollados, el acceso a alimentos suficientes parece que está asegurado para todas las personas, pero garantizar la inocuidad de los alimentos, es decir, que los alimentos tengan niveles seguros y aceptables de peligro para que no puedan dañar la salud de los consumidores, juega un papel fundamental a la hora de garantizar la seguridad alimentaria. La falta de inocuidad en un alimento puede provocar efectos adversos o graves casos de intoxicaciones y ésta puede deberse a multitud de causas entre las que se encuentra la presencia de contaminantes [3]. Por este motivo, existen organismos a nivel mundial que se encargan de llevar a cabo una revisión permanente de la legislación alimentaria para garantizar la seguridad alimentaria, como es el caso de la Autoridad Europea de Seguridad Alimentaria (EFSA) a nivel europeo.

En relación con esto, se encuentran los Objetivos de Desarrollo Sostenible (ODS) incluidos en la "Agenda 2030 sobre el desarrollo sostenible" aprobada por la Organización de las Naciones Unidas (ONU) en el año 2015 [4]. Los ODS que se relacionan con la obtención de la seguridad alimentaria de manera directa o indirecta y por tanto con esta tesis doctoral son los objetivos 2 "hambre cero", 3 "salud y bienestar" y 12 "consumo y producción responsables" (Figura 1), que tienen dentro de sus metas poner fin al hambre, lograr la seguridad alimentaria, mejorar la nutrición y promover una agricultura sostenible, garantizar una vida saludable y un estado de bienestar para todos y reducir la huella ecológica, es decir reducir las acciones que generan un impacto negativo en el medio ambiente.

Figura 1. Objetivos de Desarrollo Sostenible (ODS) incluidos en la "Agenda 2030 sobre el desarrollo sostenible" aprobados por la Organización de las Naciones Unidas (ONU) en el año 2015 (adaptado de ONU [4]). Con un mayor tamaño se destacan los ODS relacionados con esta Tesis Doctoral.

Por tanto, para asegurar la inocuidad de un alimento y con ello garantizar la seguridad alimentaria y los ODS que marca la ONU es necesario controlar todas las etapas de la cadena de suministro de alimentos, ya que todas son determinantes de la calidad del producto final. Para ello, se desarrolló la estrategia europea llamada "de la granja a la mesa" que contempla el control en las etapas que van desde que se obtienen los productos alimenticios hasta que le llegan al consumidor final. Gracias a esta estrategia, la seguridad alimentaria ha mejorado considerablemente en los últimos años al permitir prevenir o disminuir la contaminación de los alimentos [5].

La pérdida de la inocuidad de un alimento puede estar provocada por contaminantes de diferente naturaleza, tantos biológicos, como físicos o químicos [6]. Los contaminantes químicos son compuestos orgánicos o inorgánicos que pueden estar presentes en los alimentos accidentalmente produciendo daños a los consumidores tanto a corto plazo mediante intoxicaciones agudas como a largo plazo con intoxicaciones crónicas [7]. Tal y como se muestra en la Figura 2, dentro de este tipo de contaminantes se incluyen una gran variedad de compuestos de distintas procedencias.

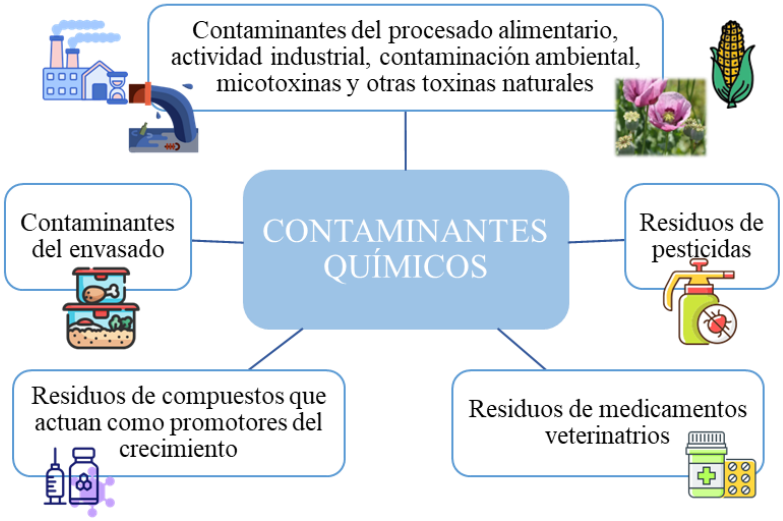


Figura 2. Clasificación de los distintos tipos de contaminantes químicos y su procedencia.

Uno de los grupos de contaminantes químicos que ha aumentado más la atención de las autoridades sanitarias son las toxinas naturales, considerándose en los últimos años como contaminantes prioritarios. Las toxinas naturales son metabolitos secundarios producidos como mecanismo de defensa natural por algunos organismos vivos, por lo que no son perjudiciales para los organismos que las producen, pero sí que pueden ser tóxicos para los seres humanos o animales que las consumen a través de los alimentos. Las principales toxinas naturales son las biotoxinas acuáticas (ciguatoxina, brevetoxina, etc.), glucósidos cianogénicos, furocumarinas (bergaptol, psoraleno, etc.), lectinas, micotoxinas (aflatoxinas, ocratoxina A zearalenona, etc.), glicoalcaloides (solanina y chaconina), hongos venenosos (muscimol y ácido iboténico) y alcaloides (pirrolizidínicos, opiáceos y tropánicos). Estas toxinas presentan diferentes estructuras químicas, función biológica y grado de toxicidad [8], [9].

Concretamente, los alcaloides son una de las familias de toxinas que más importancia están cobrando en los últimos años debido a su presencia en una gran cantidad de alimentos y al poco conocimiento que se tenía de ellos [10]. Dentro de los alcaloides, los alcaloides opiáceos (OAs) han mostrado recientemente especial interés debido a su presencia en concentraciones inesperadamente altas en alimentos [11], [12], y a que no fue hasta diciembre de 2021 cuando la Comisión Europea (CE) publicó una legislación armonizada entre todos los Estados Miembros de la Unión Europea para controlar esta

familia de toxinas [13]. Como consecuencia, de estos dos hechos, en los últimos años se han notificado numerosas alertas alimentarias por la contaminación de las semillas de amapola destinadas a uso alimentario con altas concentraciones de OAs, (principalmente morfina y codeína) [14], lo que podría provocar casos de intoxicaciones, efectos adversos e incluso falsos positivos en test de drogas [11], [15]. Además, los estudios se han centrado principalmente en morfina y codeína. Sin embargo, se ha visto que pueden estar presentes otros OAs en las semillas de amapola en concentraciones considerablemente altas y que incluso pueden llegar a ser más tóxicos [16], [17]. Por estos motivos, en los últimos años las autoridades sanitarias han prestado más atención a este tipo de toxinas y han reclamado un mayor número de estudios con el objetivo de establecer una exposición real de estas sustancias en los consumidores a través de los alimentos y con ello, evaluar el riesgo y hacer una legislación más acorde con ello [11], [18]. Por otro lado, dado que las semillas presentan altas concentraciones es importante evaluar tratamientos que permitan reducir este contenido [19], además de evaluar el efecto que pueden tener los distintos procesados culinarios a que son sometidos los alimentos que los contiene como el tratamiento térmico, la molienda o la fermentación en el contenido de OAs en productos finales o modificar los procesos de elaboración. Para abordar todo esto, es necesario desarrollar y validar de forma adecuada metodologías analíticas eficaces para cuantificar a esta familia de alcaloides en distintas matrices alimentarias [11], [12].

Este hecho, debe ir obligatoriamente ligado a los principios de la Química Verde, concretamente de la Química Analítica Verde (GAC, del inglés *Green Analytical Chemistry*), que indican que las metodologías tiendan a ser más respetuosas con el medio ambiente y la salud humana y eviten posibles daños colaterales para lograr una sociedad más sostenible y cumplir con los ODS [20]. La GAC fomenta la reducción del uso de productos/reactivos químicos tóxicos, el uso de equipos de bajo consumo y la generación mínima de residuos. Por este motivo, las tendencias actuales en el desarrollo de metodologías analíticas se centran en la miniaturización de los dispositivos de preparación de muestras, el desarrollo de técnicas de extracción minimizadas y el empleo de disolventes y materiales menos tóxicos [21], [22].

1.1 Alcaloides opiáceos en alimentos

Los OAs son compuestos que forman parte de la salvia de látex lechosa (opio) de la planta *Papaver somniferum* L., comúnmente conocida como adormidera o amapola real (Figura 3a). Esta planta es similar a la amapola común *Papaver rhoeas* L., la cual no contiene OAs, pero con los pétalos azules, blancos o morados, en vez de rojos. Gracias a la presencia de estos compuestos en el látex obtenido de las cápsulas de esta tradicional planta (Figura 3b), se ha utilizado desde la antigüedad con fines medicinales por sus propiedades farmacológicas, concretamente analgésicas, antitusivas y vasodilatadoras [11], [23].

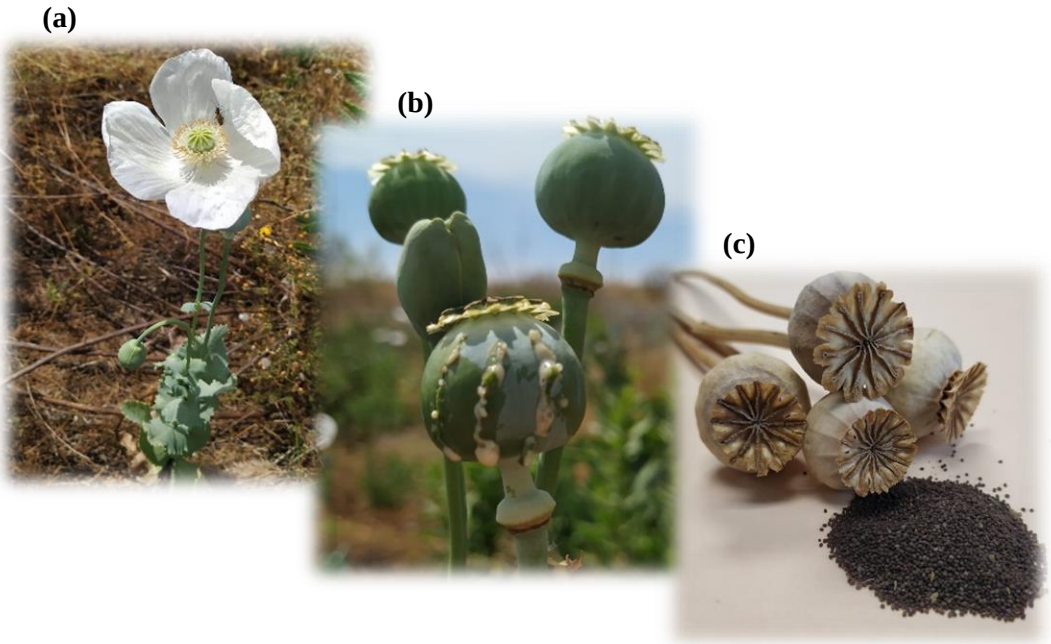
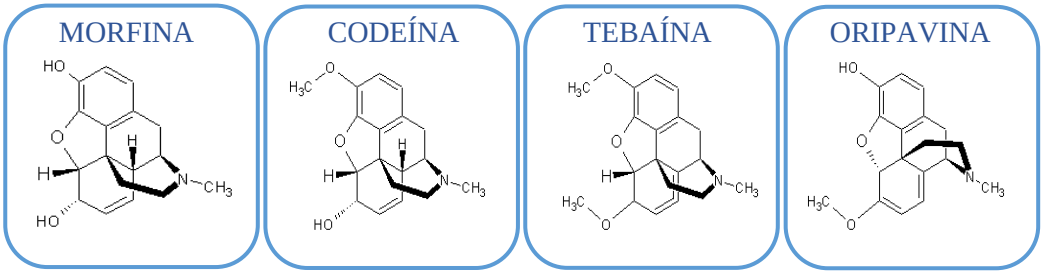


Figura 3. Imágenes realizadas de la amapola (a), su látex en el interior de las cápsulas (b) y de las semillas del interior de las cápsulas (c).

El látex puede contener mezclas de hasta 80 OAs diferentes, siendo los principales: cuatro fenantrenos (morfina, codeína, tebaína y oripavina) y dos becilisoquinolinas (papaverina y noscapina) [11], [18] (Figura 4).

FENANTRENOS:



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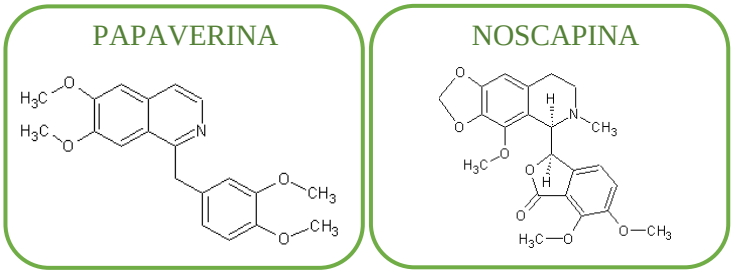

Figura 4. Principales OAs presentes en el látex de la planta *Papaver somniferum* L. clasificados según su estructura química.

Por otro lado, las semillas de amapola (Figura 3c) se han utilizado de forma tradicional en muchos países del este de Europa principalmente para la decoración de productos de panadería (como en panes, bizcochos, galletas, etc.) o como *toppings* en yogures, pasta o ensaladas, e incluso para la elaboración de tés y aceites [16], [17], [24]. Esto se atribuye a sus buenas propiedades nutricionales, ya que son ricas en ácidos grasos esenciales como el ácido linoleico y compuestos antioxidantes como la vitamina E [25], [26]. Es por ello por lo que en los últimos años se ha mostrado un aumento de la tendencia de su consumo a nivel mundial.

Sin embargo, aunque las semillas de amapola no contienen OAs de forma natural, se ha visto que pueden estar contaminadas con el látex de la propia planta. Esto puede deberse a las prácticas de cosechado, ya que actualmente en los países desarrollados se utilizan métodos automatizados que hacen que las semillas se impregnen de látex o también puede deberse a daños realizados por insectos [11]. El consumo de semillas de amapola contaminadas con altas concentraciones de OAs puede causar graves

intoxicaciones, efectos adversos en la salud, como náuseas y vómitos, somnolencia, problemas respiratorios y dependencia, especialmente en las personas más vulnerables e incluso falsos positivos en test de drogas [27]–[29].

1.1.1 Antecedentes bibliográficos: Trabajo de revisión

Por todo ello, para poner de manifiesto la situación actual con relación a la presencia de OAs en alimentos y establecer una serie de retos a resolver para mejorar el control de esta familia de toxinas y plantear los objetivos de esta Tesis Doctoral se realizó un trabajo de revisión bibliográfica (Trabajo de revisión 1), que se muestra a continuación. En él se explicaron las causas de su presencia en alimentos, el riesgo de su consumo y las acciones para prevenir su exposición. Además, se recopilaron las metodologías de análisis para cuantificar OAs desarrolladas hasta la fecha, contemplando tanto las técnicas de tratamiento de muestras como las técnicas de análisis.

Trabajo de revisión 1:

Opium alkaloids in food products: Current and future perspectives

Trends in Food Science & Technology (2021) 92-102

Trends in Food Science & Technology 108 (2021) 92–102

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Opium alkaloids in food products: Current and future perspectives

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PUBLICATION INFORMATION

Journal	Trends in Food Science & Technology
Publisher	Elsevier
Impact factor	16.002 (JCR, 2021)
Category	Food Science & Technology (2/144, Q1)
DOI	https://doi.org/10.1016/j.tifs.2020.12.013

Highlights

- Occurrence of opium alkaloid in poppy seeds and food elaborated with poppy seeds.
- Problems caused by consumption of food products contaminated with opioids.
- Actions to avoid opioids in food products.
- Analytical methods for the determination of opioids in poppy seeds and food products.

ABSTRACT

Background: In recent years, there has been increasing interest from health authorities in avoiding consumer exposure to opium alkaloids in food. Thus, recent cases of intoxication and false positive drug tests, from the consumption of poppy seeds and food, have been detected. In order to know more certainly the concentration of these substances in food and to establish more reliably the consumption of these toxics in the population, data on their presence in food should be further collected. These compounds are found at ultra-trace levels in complex matrices, so it is important to develop efficient analytical methods based on selective analytical techniques and adequate sample treatment, which is key to avoid matrix effects.

Scope and approach: This review summarizes the actual situation of opioids in food products. It establishes the cause of their presence in food, the risk of consumption and actions to prevent their exposure. In addition, it sums the techniques of sample treatment and analysis of all available articles on opioids in different samples.

Key findings and conclusions: The studies that have been made of opioids are mainly about morphine. For this reason, there is a need to do more studies with all of them. Besides, most of the studies are in biological samples, following consumption of poppy seeds or foods. Therefore, there is to develop and validate new methods that are effective for complex matrices such are foods, to know exactly the actual exposure to consumers and how to decrease it.

KEYWORDS: Opium alkaloids; Food products; Poppy seeds; Papaver plant; Analysis technique; Sample treatment.

1. Introduction

Opium poppy seeds are quite popular in many countries of the world as a food ingredient or to make tea. These seeds do not contain opium alkaloids, but poor harvesting practices or insect damage can contaminate them with latex, which is rich in opium alkaloids (AESAN, Spanish Food Safety and Nutrition Agency, 2016). Consumption of these alkaloids may involve several risks, especially for the most vulnerable people. Furthermore, some cases of intoxication and false positive drug tests have also been reported (Lachenmeier, Sproll & Musshoff, 2010; Sproll, Perz, & Lachenmeier, 2006).

Hence, many countries have taken measures, such as establishing for food use papaver plant varieties with a low level of alkaloids, setting maximum limits or, even, prohibiting their use for food (BfR, German Federal Institute for Risk Assessment, 2006; EFSA, European Food Safety Authority, 2011). However, there is no common legislation, which reduces their control and makes foreign commerce more difficult (AESAN, 2016). For this reason, the health authorities want to establish harmonised legislation but, to do this, it is needed to know the real exposure of all these toxins in population (EFSA, 2018). So far, studies are mainly focused on morphine, but all the opium alkaloids that can be found in poppy seeds should be considered. Therefore, it is important to carry out studies about the content of opium alkaloids in poppy seeds and food, which are being commercialized nowadays.

Thus far, numerous studies have been done are on biological samples, following the consumption of seeds and poppy seed foods. Positive results have been obtained demonstrating the presence of considerable amounts of opioids in these samples (Moeller, Hammer & Engel, 2004; Newmeyer et al., 2015; Özbunar et al., 2019). However, there are few studies on food matrices, so research is required to develop and validate new analytical methods to quantify these compounds in food products. As they are found in very low concentrations in these complex matrices, it is necessary to use analytical methods involving selective techniques and adequate sample treatment. Concerning sample treatment, in recent years there has been increasing interest in exploring new techniques that extend beyond simple extraction of the target analytes with organic solvents. This is due to this technique is laborious and requires a high amount of

biologically toxic solvents. The use of more innovative and selective techniques has been increased, which allow the purification of the sample extract, thus avoiding matrix effects, necessary in food matrices. For these compounds, the most remarkable is the solid-phase extraction (SPE) with conventional commercial sorbents (Özbunar et al., 2019). However, current trends in sample preparation involve moving towards "greener" approaches by scaling down analytical operations and integrating new advanced materials as sorbents (Casado, Pérez-Quintanilla, Morante-Zarcero, & Sierra, 2017; Sierra & Morante-Zarcero, 2018). For other types of natural toxins, much progress has been made in the application of new materials in the purification stage and their integration in micro-extraction techniques (Casado, Gañán, Morante-Zarcero & Sierra, 2020). Unfortunately, as far as we know, only a recent study has been published for the analysis of opium alkaloids in foods, where miniaturization in sample preparation has been applied (Xu, Liu, Wang & Wei, 2019).

The aim of the present review is to compile all the information available on the presence of opioids in food products, to make a generalist analysis of the situation of this family of toxins today (problems involved in their consumption and solutions that are being carried out) including future research perspectives in this field (Fig. 1).

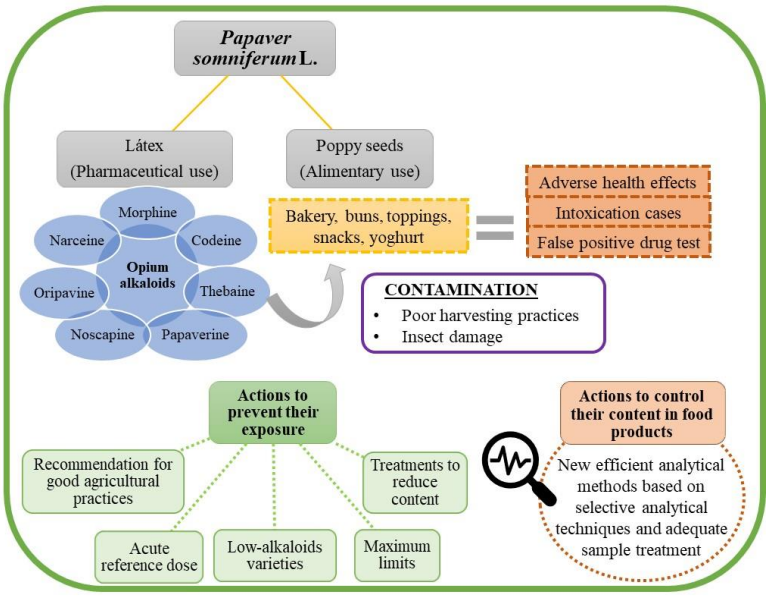

Fig. 1. Diagram showing the aspects to be included in this review.

2. Structure, properties and toxicity of opium alkaloids.

Opium poppy (*Papaver somniferum* L.) is a member of the Papaveraceae family, which has been known since ancient times as a medicinal and culinary plant for its pharmaceutical and nutritional properties. Its milky latex sap (opium) obtained from the capsules can contain up to 80 different opium alkaloids and morphine is present in the largest concentration. Fig. 2 shows chemical structures of the seven most common opioids in the latex of poppy plant, divided according to their chemical structure in two classes: phenanthrenes (morphine, codeine, thebaine and oripavine), which are also called morphinans and benzylisoquinolines (noscapine, papaverine and narceine). The phenantrenes have an aromatic A ring and a partially saturated B and C ring and with a nitrogen-containing D ring, spanning carbons 9 and 13 (Fig. 2). They have a common biosynthetic pathway; thebaine is the precursor of oripavine and of codeine, which are both precursors of morphine. The benzylisoquinolines, as their name suggests, consist of an isoquinolinic and a benzylic part. They have a methylated nitrogen atom and functional groups with oxygen. In this case, they are not closely related in the biosynthetic pathway and not show opiate-like behaviour because they do not show agonistic activity at the μ-receptor (EFSA, 2018).

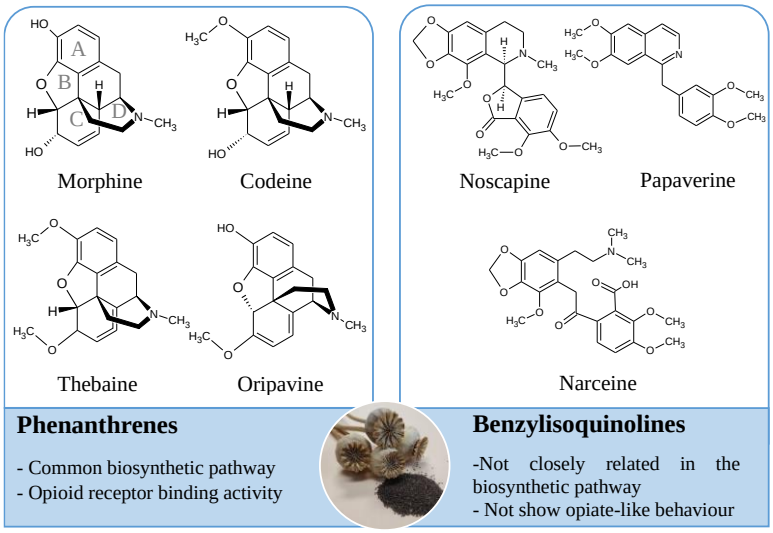

Fig. 2. Chemical structures of the seven most common opium alkaloids in the latex of poppy plant (*Papaver somniferum* L.) classified in their two families (phenanthrenes and benzylisoquinolines).

Thanks to the presence of these alkaloids in opium, this traditional plant is widely used for medicinal purpose, due to its pharmacological properties, to treat cramp, chronic lax and chronic cough (EFSA, 2018; Labanca, Ovesnà and Milella, 2018). Thus, morphine and codeine have analgesics properties, papaverine has coronary vasodilator function and noscapine is a cough suppressant and potentially anti-cancer drug (Demirkapu & Yananli, 2020), Other alkaloids, such as thebaine and oripavine, are not useful due to toxicity and low therapeutic index, but they are used as starting material for production of synthetic opiates (Table S1).

On the other hand, poppy seeds are used in food processing because of their good nutritional quality, since they are generally rich in fatty acids (Özcan & Atalay, 2006), predominantly linoleic, oleic and palmitic acids (Ghafoor, Özcan, AL-Juhaimi, Babiker & Fadimu, 2019). Besides, it is important to highlight that unlike latex, seeds hardly contain any opium alkaloids, so it was thought that they could be used freely for food processing. For this reason, they are increasingly used in Central Europe, such as in bakery products, in bean-jam buns, as toppings for dishes, in fillings of cakes and desserts and to produce edible oil (AESAN, 2018). Another widespread use of poppy seeds is in making tea that helps to relax and sleep (Haber, Pergolizzi & LeQuang, 2019; Powers, Swortwood & Erickson, 2018). In addition, these seeds are sold alone or mixed with other seeds, such as chia or flax, and are commonly added to salads or purees, and in ground form are used as flavouring ingredient of pasta. Other recent industrial uses of these seeds are the preparation of dairy products (yoghurts) and snacks (AESAN, 2016).

However, in recent years some studies have shown the presence of high amounts of opium alkaloids in poppy seeds. This may be due to contamination with the opium alkaloids present in the latex caused by poor harvesting practices or by insects. For this reason, the consumption of opium poppy seeds can be a health risk (EFSA, 2018). Table S1 summarizes either short-term effects, from over-consumption, and long-term effects, from prolonged exposure, to these alkaloids. This aspect may explain the cases of intoxication, false positive drug tests and dependencies that have appeared in recent years due to the consumption of poppy seeds. Hence, some scientists have tested biological samples, such as blood, urine or oral fluid after consumption of poppy seeds or poppy

seed foods. After that, they have shown that the consumption of these can give considerable amounts of opium alkaloids in biological samples. Bjerver, Jonsson, Nilsson, Schuberth and Schuberth already demonstrated this in a study in 1982 where healthy subjects that consumed one or two rations of poppy seed cake presented a significant content of morphine in urine. In another study, Hayes, Krasselt and Mueggler (1987) get considerable amounts of morphine and codeine in serum and urine following ingestion of poppy seeds.

In addition, there are poppy plants with different concentrations of opium alkaloids because alkaloid accumulation depends on several factors. The most influential are genetic factors and environmental conditions (EFSA, 2018). For example, Meos, Saks and Raal (2017) determined the morphine content in dried capsules of 34 different cultivars grown in Estonia, finding a broad range for this alkaloid between 0.57 and 6.76 g kg^{-1}. Hence, it is important to establish a classification according to the content of opioids, differentiating types with high opioid content from those with low concentrations. Theoretically, varieties that have high morphine levels (> 0.8%) are used in pharmacy and varieties with fewer morphine levels are used in food. However, some varieties have low morphine level, but a high level in other opium alkaloids, for example, papaverine (Stranska, Skalicky, Novak, Matyasova & Hejnak, 2013). For this reason, it is necessary to consider the quantity of all opium alkaloids. In addition, poppy seeds from some *P. somniferum* varieties with high alkaloid content, especially grown for pharmaceutical applications, are sometimes used as a sub-product for food use (EFSA, 2018). This is because no harmonised European legislation has yet been created on this family of toxins in poppy seeds for food purposes.

3. Opium alkaloid content in poppy seeds and food products

Table 1 collects the content of opium alkaloids (morphine, codeine, thebaine, papaverine, noscapine and narceine) that have been analysed in poppy seeds, poppy seed foods and poppy teas. In many of the studies showed in Table 1, the objective was to know the presence of these alkaloids in blood, oral fluid or urine after consumption of these products. Therefore, in order to carry out a controlled administration of these opioid

alkaloids to the subjects, its concentration in the poppy seeds, foods and teas were previously analysed. In some other works, the aim was to evaluated changes in the opium alkaloids concentration during food processing. For this reason, in parallel to establishing the concentration of these alkaloids in seeds, the levels of opioids in different food products prepared from poppy seeds were also analysed (Table 1).

In poppy seeds, significant amounts of opium alkaloids have reported in some studies (Table 1). For example, Bjerver et al. (1982) determined morphine in 5 poppy seeds (3 blue and 2 white seeds). They got considerable amounts of morphine, especially in two blue varieties (85.6 and 106.7 mg kg^{-1}). Hayes et al. (1987) found morphine and codeine in four samples of black poppy seeds in the range of 17 - 294 mg kg^{-1} and 3 - 14 mg kg^{-1}, respectively. In seeds from Australia, Casella et al. (1997) found 164 mg kg^{-1} of morphine, 31.8 mg kg^{-1} of codeine and 20.7 mg $kg^{-1}$of thebaine, whereas in seeds purchased in a shop in Manchester similar values were observed for morphine, but less than half for codeine and thebaine (Table 1). On the other hand, in some other works lower amounts of these compounds have been found. This is the case of Meadway, George and Braithwaite (1998), who reported 0.6 - 11.9 mg kg^{-1} for morphine and 0.3 - 0.7 mg kg^{-1} for codeine in 4 samples of blue and 1 sample of white poppy seeds from Australia, The Netherlands and Turkey. In some other studies, a high number of poppy seed samples have been analysed. Thus, Sproll et al. (2006) determined the opium alkaloid content of 83 different samples. Morphine was the main alkaloid in all of them (< 1 - 270 mg kg^{-1}), codeine was between < 0.3 - 56 mg kg^{-1}, and noscapine and papaverine were detected in isolated cases (mostly below the quantification limit).

Table 1. *Opium alkaloids content in poppy seeds, poppy seed foods and poppy teas that have been found in published works.*

Sample	Content (mg kg^{-1})						References
	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Narceine	
Poppy seeds							
Poppy seeds (3 blue, 2 white seeds)	2.6-106.7	-	-	-	-	-	Bjerver et al. (1982)
Black poppy seeds (4 samples of different origin)	17-294	3-14	-	-	-	-	Hayes et al. (1987)
Poppy seeds				-	-	-	
- Australian seeds	164	31.8	20.7	-	-	-	Casella et al. (1997)
- Seeds bought in Manchester	107	17.7	8.2	-	-	-	
Blue and white poppy seeds (5 samples of different origin)	0.6-11.9	0.3-0.7	-	-	-	-	Meadway et al. (1998)
Poppy seeds (83 different samples)	<1-270	<0.3-56	-	ND	2.1 (5 samples)	-	Sproll et al. (2006)
Poppy seeds (32 blue, 3 white and 3 ground blue poppy seeds)	0.2-241	<0.1-348	<0.1-106	<0.1-3.8	<0.1-6	<0.1-2.1	López et al. (2018)

Poppy seed foods							
Baking mixes containing poppy seeds (12 samples)	<0.3 -4	N.D.	-	N.D.	N.D.	-	Sproll et al. (2006)
Poppy seed filling (2 samples)	17.4-18.6	2.3-2.5	--	-	-	-	Pettitt et al. (1987)
Bean-jam buns decorated with poppy seeds	2.20 *	0.77 *	-	-	-	-	Yamaguchi et al. (2011)
Ready-to-use poppy seed filling for bakery (1 sample)	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	López et al. (2018)
Ready-to-eat bakery product with poppy seeds (2 samples)	0.5-0.6	N.D.	N.D.- 0.1	N.D.	N.D.	N.D.	
Poppy seed pastes (from white, yellow and blue-black poppy seeds from Turkey)	1.9-4	-	-	-	-	-	Özbunar et al. (2019)
Hot pot broth (29 samples)	0.022-0.029 (2 samples)	N.D.	N.D.	0.22×10^{-3} (1 sample)	0.16×10^{-3} (1 sample)	-	Guo et al. (2013)
Hot pot seasoning (30 samples)	N.D.	N.D.	N.D.	N.D.	N.D.	-	Xu et al. (2019)
Poppy teas							
Herbal teas (2 samples, containing parts of poppy plant)	10.4-31.5	-	-	-	-	-	Van Thuyne et al. (2003)
Poppy samples to make tea (19 bulk seeds, 1 seed powder, 1 seed tea bag, 1 liquid extract)	<1-2788	<1-247.6	<1-124	-	-	-	Powers et al. (2017)

N.D.: not detected; -: not studied. * µg per piece

More recently, López et al. (2018) determined the levels of opium alkaloids in samples acquired in the Netherlands, Germany, and Italy (33 blue, 3 white and 3 ground blue poppy seeds). All analysed samples contained morphine, in a range between 0.2 - 241 mg kg^{-1}. Codeine (< 0.1 - 348 mg kg^{-1}) and thebaine (< 0.1 - 106 mg kg^{-1}) were found in 78% of the samples. Lower amounts of noscapine (< 0.1 - 6 mg kg^{-1}), narceine (< 0.1 - 2.1 mg kg^{-1}) and papaverine (< 0.1 - 3.8 mg kg^{-1}) were also quantified in 85%, 46% and 37 % of the samples, respectively. In addition, in this study, some different opium alkaloid profiles could be identified depending on the origin of the samples. Thus, as can be seen from these works, the amount of opium alkaloids in poppy seeds is very variable. The poppy variety is an important influential factor, besides the geographical origin, the time of harvest and the external contaminations that can be produced during its recollection (López et al., 2018; Sproll et al., 2006).

The processing method for food containing poppy seeds is another important variable to consider. In this sense, as can be seen in Table S2, a significantly lower levels of opioids in poppy seed foods are observed. In baking mixes containing poppy seeds (Sproll et al., 2006), poppy seed filling for bakery (López et al., 2018; Pettitt, Dyszel & Hood, 1987) and pastes from white, yellow, and blue-black poppy seeds from Turkey (Özbunar et al., 2019) low levels of morphine and codeine were detected. This fact can be attributed to the production procedure of these mixtures where washing, soaking with water, heating, grinding and/or crushing can be applied. In the same way, Yamaguchi, Hayashida, Hayakawa, Nihira and Ohno (2011) determined opiates in bean-jam buns decorated with poppy seeds. The morphine and codeine concentrations obtained were 2.20 and 0.77 µg per one piece of bun, respectively. A similar situation was observed in two ready-to-eat bakery products containing poppy seeds, where 0.6 mg kg^{-1} morphine and 0.1 mg kg^{-1} thebaine, in one of them, and 0.5 mg kg^{-1} morphine and 0.2 mg kg^{-1} noscapine, in the other, was observed (López et al., 2018). For this reason, these authors concluded that there was a significant reduction in opium alkaloid content after heat processing.

In recent years, the addition of poppy husks (*pericarpium papaveris*) containing opium alkaloids to some food seasoning has been reported. This illegal use, motivated by economic interests because of the dependence that its taste can cause, has been applied

for the preparation of hot pots. For this reason, some works have studied the presence of opium alkaloids in these types of samples. One of them was Guo, Zhang, Zhao and Shao (2013) who analysed 29 hot pot broth samples and determined three positives: one contained with noscapine (0.22 µg kg^{-1}) and papaverine (0.16 µg kg^{-1}) and the two others with morphine (22.5 and 28.9 µg kg^{-1}). More recently, Xu et al. (2019) analysed 30 hot pot samples, but they showed concentrations below the detection limit (0.05-0.8 µg kg^{-1}) in all cases.

Finally, another popular use of poppy (seeds and plant) is in the preparation of teas. Van Thuyne, Van Eenoo and Delbeke (2003) analysed the morphine content in two herbal infusions, containing parts from the *papaver* plant among other herbals, obtaining 10.4 mg kg^{-1} in the first one, and 31.5 mg kg^{-1} in the second one. In the same way, Powers et al. (2017) studied opium alkaloids in teas prepared with bulk poppy seeds, poppy seed powder, poppy seed tea bag and liquid poppy extract. For this task, they prepared teas with four home-brewing methods (room temperature and heated water, with and without an acid modifier). Alkaloid yield varied between extractions, and under the best conditions concentrations in the range < 1 - 2788 mg kg^{-1} for morphine (heated neutral), < 1 - 247.6 mg kg^{-1} for codeine (heated acidic) and < 1 - 124 mg kg^{-1} for thebaine (heated acidic) were observed. Consequently, it was concluded that high levels of opium alkaloid could be found in these preparations and hence they could be potentially harmful.

According to these studies, in the evaluation of opioids in poppy seeds, it should be considered its posterior use in food. It is not the same to use the seeds to prepare bakery products, which involves a baking process, than to use them to make tea, or, as it is very common nowadays, to use them as a dressing in yoghurts or salads, which do not involve any previous treatment. According to Zentai, Sali, Szeitzné-Szabó, Szabó and Ambrus (2012) who evaluated the consumption pattern of poppy seeds in 2009 in Hungary, the consumption of raw and ground poppy seeds is higher (65%) than baked poppy seeds (35%). This is one more reason to control this family of contaminants and prevent them from the consumer. Unfortunately, there are few studies done in food samples, so it is difficult to establish the real exposure of opioids in the population from the consumption of food products containing poppy seeds.

4. Problems of their presence in food

According to EFSA, for every approximately 100 g of cake or bun the amount of seeds used varies between 3.8 and 41 g (near half of the product), with an average content of 14 g (EFSA, 2011). Despite being a low amount, in some cases, it is demonstrated that the consumption of poppy seeds contaminated with opium alkaloids can lead to adverse health effects, especially in babies, infants, the elderly and people with severe health issues. For example, their consumption can lead to light-headedness and enteroparesis (BfR, 2006).

Older articles from the 19th century already reported cases of child intoxication with opium poppy. However, in recent years there have been a few more cases. Thus, King, McDonough, Drummer & Berkovic (1997) reported the case of a 26-year-old baker who after drinking poppy seed tea went to the hospital for hallucinations and showed a high morphine content in his blood and urine. Agin, Calkavur, Özdemir and Bak (2003) published the case of a child intoxication in Turkey due to the ingestion of the boiled poppy plant. Another case of intoxication is the one published by Hahn et al. (2008), in which they reported a serious health impairment of a 6-week-old baby associated with the ingestion of the boiled poppy seeds.

Another point to note is that opiates are included in the federally mandated workplace and DUID (driving under the influence of drugs) testing programs because of their psychoactive properties and frequently abuse as illicit drugs, about 1.3 million approximately of consumers in Europe (Rosado, Barroso, Vieira & Gallardo, 2019). In addition, the universally accepted cut-off limit of 300 ng mL^{-1} for opiate testing, declared mandatory for all drug testing laboratories by the Substance Abuse and Mental Health Services Administration, has recently been questioned. This is due to the consumption of poppy seeds or products containing them without any illicit reason can cause false positive tests. For this reason, several studies measured opium alkaloids content in urine after ingestion of food products since the 1980s. An example is a study by Struempler (1987) that determined codeine and morphine concentrations in urine after ingestion of poppy seed bagels. Lo and Chua (1992) also determined these two opium alkaloids in urine after ingestion of curry meal. Meadway et al. (1998) and Yamaguchi et al. (2011) saw positive

results in urine after ingesting poppy seed products (cakes and bean-jam buns). Also, there are different works in which it has been shown that positive results are obtained in other biological matrices (blood, plasma or oral fluid) following ingestion of poppy seeds (Moeller et al., 2004; Newmeyer et al., 2015; Özbunar et al., 2019; Rohrig & Moore, 2003; Smith et al., 2014). Therefore, all these previous studies show that a positive result of any opium alkaloid does not necessarily mean an illicit drug use. For this reason, there is concern about interpreting the data produced when examining opiates. To avoid misunderstandings, professional athletes were advised not to take poppy seeds in their food or tea, as they are responsible for the appearance of doping substances in their biological samples (Van Thuyne et al., 2003). All this has attracted the interest of some researchers in determining biomarkers to differentiate whether the positive result is a result of illicit drug use or the ingestion unintentionally of food with poppy seeds. However, this is not an easy aspect and therefore there is some controversy. Cassella, Wu, Shaw and Hill (1997) and Meadway et al. (1998) suggested thebaine as a marker for culinary use because this type of opium alkaloid present in poppy seeds is not in drugs or in urine of real opiate drug users. Other authors argue that thebaine elimination varies significantly from one person to another, so its absence in a biological sample is not necessarily indicative of illicit drug use. Therefore, at present, some studies preferred to take more caution and choose to consider thebaine only as a supportive biomarker (Özbunar et al., 2019). On the other hand, Trafkowski, Madea and Musshoff (2006) published that noscapine and papaverine could be used cautiously as additional markers for illicit heroin abuse because these types of opium alkaloids have not been detected following oral ingestion of opium poppy seeds at normal doses. However, this is not completely true, as noscapine and papaverine could be present in high levels and thus give a positive result in the urine of abusers. Alternatively, Yamaguchi et al. (2011) published that, concentrations of free morphine and codeine in urine can become indicators to differentiate whether detected opiates are after the consumption of food with opium poppy seeds or are due to the abuse of opiates. In summary, more research is needed on the possible markers to be used because there are some ideas, but no evidence yet that can differentiate these two different uses.

5. Solutions

5.1 Legislation

Nowadays, there is no common legislation, and the situation in each country is different. Some countries have banned the use of poppy seeds for food uses. An example of this is China and some other Asian countries, where the government decided to ban the consumption of this plant. This is because some illegal traders were using *pericarpium papaveris* as a food additive to attract more customers, using it in the hot pot, which is one of the most popular dishes in Chinese restaurants (Guo et al., 2013). Another example is Belgium, where the use of poppy seed was banned in all foods, except bakery products (EFSA, 2011).

In general, the use of poppy seeds in food is not prohibited in Europe. This is because *P. somniferum* cultivars used for culinary purposes generally have low or moderate morphine concentrations. However, no common legislation has yet been established to differentiate between varieties with a high opium alkaloid content, and therefore only suitable for pharmaceutical use, and those which can be used in food because of their low content. In addition, another aspect to be consider is that poppy seeds with high opioid alkaloid content destined for gardeners as ornamental poppies are commonly found on the market, which leads to the uncertainty of whether the seeds of these plants are used for human consumption. For this reason, unlike the rest of Europe, according to BfR (2006), there are low-morphine varieties certified for cultivation in Germany ("Przemko") and Austria (“Edel-Weiß", "Edel-Rot", "Florian", "Josef", "Zero" and "Zero 2000"). Furthermore, some authors are researching in low-morphine content varieties, with the aim to establish cultivars for food consumption (Németh & Bernáth, 2009).

Despite the problems involved in the consumption of contaminated poppy seeds, as they are often consumed in small doses, it is not surprising that harmonised legislation has not yet been made in Europe, to establish the maximum limits for opium alkaloid in poppy seeds or food. However, the fact that poppy seed consumption is low may be questionable, as there are countries with a longer tradition than others. In Turkey, one of the main legal poppies producing countries in the world, they have a very widespread use of this product and can use more than 100 g of poppy seeds in one food (Özbunar et al.,

2019). In that respect, some countries have established maximum limits, and the BfR established a provisional reference value of 4 mg kg^{-1} of morphine in poppy seed for use in food, although this value is a limit of action that has no legislative purpose. Furthermore, Hungary has national maximum levels in poppy seeds of 30 mg kg^{-1} for morphine, 20 mg kg^{-1} for noscapine, 40 mg kg^{-1} for the sum of morphine and noscapine, 20 mg kg^{-1} for thebaine and 20 mg kg^{-1} for codeine (EFSA, 2011).

The fact that the situation is different in each country of European Union (EU) has generated numerous alert notifications in the RASFF (Rapid Alert System for Food and Feed), specifically 30 notifications since the first one in 2005 as shown in Table S3 (see supplementary material). All notifications were in seeds of different poppy varieties and origin, except one alert in 2019, which was in frozen bread. Besides, it should be noted that most of the notifications were based on the high morphine content. Only from 2015 notifications included other morphine alkaloids, but it was not specified which of them. As shown in Fig. 3, the high number of notifications generated since the beginning of 2019, compared to previous years is remarkable. This may be because other opium alkaloids started to be considered, as of the nine notifications that have been generated since 2019, seven include the other opium alkaloids. On the other hand, to prevent these products contaminated with high concentrations of opium alkaloids from reaching the consumer, several actions have been taken (Table S3). The action mostly carried out is the withdrawal of the product (57% of the cases). Other actions where prohibition to trade-sales ban (10%), official detention (7%), re-dispatch (7%) or destruction (3%). In short, as there is no common legislation, a large part of the alert notifications is created from the export of seeds from one country to another, which has more restrictive regulations. This situation jeopardises the viability of the single market as seed produced in one Member State cannot be commercialized in other (AESAN, 2016).

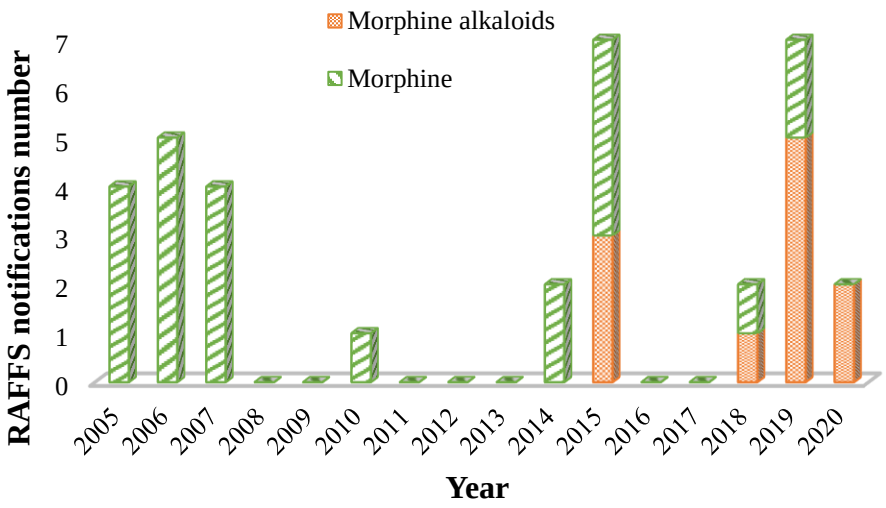

Fig. 3. Number of RASFF (Rapid Alert System for Food and Feed) notifications of morphine and morphine alkaloids per year from 2005 to 2020.

EFSA has published some scientific opinions on the public health risks from the presence of opium alkaloids in poppy seeds used for human consumption. The first was in 2011, prepared by the Expert Panel on Contaminants in the Food Chain (CONTAM Panel) which calculated an acute reference dose (ARfD) of 10 µg morphine per kg body weight (b.w.). This was an agreement between the different Member States of the EU in November 2016, which despite not being obligatory, involves their compromise and acceptation (AESAN, 2018). They considered that this amount represented the intake level above which foods with poppy seeds contaminated with opium alkaloid could be a health issue. It was established following a risk assessment, in which Germany, Hungary, Austria and the Netherlands indicated that morphine was the major alkaloid in poppy seed samples at concentrations up to 630 mg kg^{-1}. However, the conclusion published by EFSA was that more data needed to be collected on the presence of other opioids, such as codeine, papaverine, thebaine, noscapine and oripavine, in order to perform the risk assessment more accurately (EFSA, 2011). A second opinion of the CONTAM Panel (EFSA, 2018) confirmed the ARfD of 10 µg of morphine per kg b.w. and further established that the concentration of codeine in poppy seed samples should be calculated in morphine equivalents, using a factor of 0.2. Therefore, the ARfD is the sum of

morphine and codeine, expressed in morphine equivalents. Until 2018, risk assessments were mainly based on morphine and codeine, while other opium alkaloids remained unevaluated due to lack of data. It was suggested that these other alkaloids should not be underestimated, and considered less risky than morphine or codeine, since thebaine has been shown to have a higher acute lethality and the estimated exposure could present a health risk (Eisenreich et al., 2020). The situation since then has not yet been resolved and the absence of available data on other opium alkaloids means that further studies are still needed to carry out a successful hazard characterization.

5.1 Recommendations to prevent and to reduce opium alkaloids in poppy seeds

Another important aspect is that the problem of contamination of poppy seeds with latex opioid alkaloids could probably be solved by using less aggressive harvesting methods (BfR, 2006). For example, Moeller et al. (2004) reported that the harvesting method used in developing countries, where capsules are still opened manually and seeds are collected in a container, results in less contamination of the seeds with the milky sap. Consequently, the European Commission (2014) published recommendations for good agricultural practices to prevent and to reduce the presence of opioid alkaloids during cultivation, harvest and storage. For cultivation, it is recommended to choose varieties with lower content of opium alkaloids for food purpose, to control possible fungal diseases and pests and to use growth regulators to avoid lodging. During harvesting and storage, it is recommended to control the percentage of humidity.

On the other hand, it can be also recommended some processing practices to remove or reduce the presence of opium alkaloids in seeds and food with poppy seeds. Table S2, summarizes the research on this matter. The first efficient method published was to wash or soak with water the poppy seeds, that can reduce about 40-75% (Bjerver et al., 1982; Lo & Chua, 1992). Sproll, Perz, Buschmann and Lachenmeier (2007) found that using hot water can reduce about 100%. In addition, the grinding of the seeds can get a significantly morphine reduction (25-35%) (Sproll et al., 2007; Zenai et al., 2012). This is a very important concept for the elaboration food that contain crude poppy seeds as

toppings. Additionally, it has also been demonstrated that the combination of poppy seed pre-treatment (washing) with food preparation (baking) results in an overall reduction of 80-100% in the final food product (Lachenmeier et al., 2010). In summary, it seems that by adequate treatment of the seeds, most morphine content is eliminated. However, more studies are needed to know how these type of treatments affects the other opioid alkaloids.

6. Analysis methods

Table 2 collects different analytical methodologies that have been used in the literature (from 1982 until 2020) to determine opium alkaloids in poppy (seeds and plant), foods with poppy seeds and teas. As biological samples are out of the scope of this review, methods for opium alkaloids determination in these samples (blood, serum, urine, oral fluid) will not be discussed in this section. This information is presented in Table S4 (supplementary material).

Table 2. Summary of the different analytical methods used in the literature for the determination of opium alkaloids in poppy (seeds and plant), foods with poppy seeds and poppy teas.

Sample (amount)	Analyte	Analysis technique	Sample preparation		Recovery (%)	References
			Extraction conditions (solvent volume)	Purification (sorbent)		
Poppy seeds (5 g)	MOR, COD	GC-MS	Alkalinization to pH 9-9.5, isobutanol: dichloromethane, 10:90, v/v (5 - 10 mL) [a]	-	-	Struempler (1987)
Poppy filling (15 g)	MOR, COD	GC-MS	Alkalinization, isopropanol: chloroform, 10:90, v/v (50-75 mL) [a]	-		Pettitt et al. (1987)
Poppy seeds (2 g and 4 g)	MOR, COD, THEB, PAP	GC-MS	Methanol: dichloromethane, 10:90, v/v (10 mL) and isobutanol: dichloromethane, 10:90, v/v (5 mL) [a]	-	-	Paul et al. (1996)
Poppy seeds (0.5 g)	MOR, COD	GC-MS	Alkalinization to pH 9, isopropanol: chloroform 10:90, v/v (5 mL) [a]	-	-	Meadway et al. (1998)
Herbal teas (2 mL)	MOR	GC-MS	Methanol: dichloromethane, 10:90, v/v (5 mL) (after pH 9.5)	-	-	Van Thuyne et al. (2003)

Bean-jam buns, poppy seed (5 g)	MOR, COD	GC-MS	Alkalinization to pH 9-9.5, isobutanol: dichloromethane, 10:90, v/v (5 - 10 mL) [a]	-	-	Yamaguchi et al. (2011)
Poppy seeds (5 g)	MOR	GC-MS	0.1 M citric acid pH 4 (10 mL)	SPE (kieselgur, 15g)	-	Bjerver et al. (1982)
Poppy seeds (1 g)	MOR, COD	GC-MS	Citrate buffer, 0.1 M, pH 4.0 (5 mL)	SPE (Chem Elute)	-	Hayes et al. (1987)
Poppy sedes (0.5 g)	MOR, COD, THEB	GC-MS	Methanol (10 mL)	SPE (Clean Screen DAU, 200 mg)	-	Cassella et al. (1997)
Poppy seed pastes (0.2 g)	MOR, THEB	GC-MS	Methanol (5 mL)	SPE (Clean Screen DAU, 200 mg)	-	Özbunar et al. (2019)
Pericarpio papaveris (0.02- 0.06 g)	PAP	HPLC-DAD	HCl, 0.1 M (refluxed 1 h) NaOH, 1 M, pH 7 ATPS with 6 % PEG 4000 and 30% $(NH_4)_2SO_4$	-	97-106	Cao et al. (2007)
Poppy straw (0.1 g)	MOR, COD, THEB, NOS, PAP, ORIP	HPLC-DAD	Methanol (5 mL)	-	97 -100	Acevska et al. (2012a)
Poppy straw (0.1 g)	MOR, COD, THEB, NOS, PAP, ORIP	GC-FID/MS HPLC-DAD	Methanol (5 mL)	-	1) 99-101 2) 99-100	Acevska et al. (2012b)

Poppy capsules (0.05 g)	MOR, COD, THEB, PAP, NOS	HPLC-DAD	Water with 5% acetic acid, 0.1 M sodium citrate buffer (pH 6.0)	SPE (Oasis MCX)	100-112	Yoshimatsu et al. (2005)
Poppy capsules (0.5 g)	MOR, COD, PAP	HPLC-DAD CE-DAD	Ethanol:water, 50:50, v/v (20 mL)	SPE (kieselgur, 750 mg) only for HPLC	-	Meos et al. (2017)
Baking mixes with poppy seeds (10 g)	MOR, COD, PAP, NOS	HPLC-MS/MS	Methanol with 0.1% acetic acid (30 mL)	-	9,8 – 17,6[b]	Sproll et al. (2006)
Poppy seed (-)	MOR, COD, THEB, NOS	HPLC-MS/MS	Methanol with 1% HCl (-)	-	51-101	Zentai et al. (2012)
Poppy seed teas	MOR, COD, THEB	HPLC-MS/MS	-	-	-	Powers et al. (2017)
Poppy seeds, bakery products (10 g)	MOR, COD, THEB, NOS, PAP, NAR	UHPLC-MS/MS	Acetonitrile:water: formic acid, 80:19:1, v/v/v (100 mL)	-	77-172	López et al. (2018)
Hot pot broth (5 g)	MOR, COD, THEB, PAP, NOS	UHPLC-MS/MS	Hydrochloric acid (20 mL) Petroleum ether (10 mL)	SPE (Oasis MCX, 60 mg)	72-124	Guo et al. (2013)
Poppy straw	MOR, COD, THEB, PAP	HPLC-MS/MS	Water with 0.5% acetic acid	SPE (Oasis MCX)	-	Stranska et al. (2013)

Hot pot broth (2 g)	MOR, COD, THEB, PAP, NOS	HPLC-MS/MS	Water:acetonitrile, 50:50, v/v (20 mL)	MSPE (Fe_3O_4@SiO_2@ ADME, 50 mg)	80-115	Xu et al. (2019)
Crude extracts of *P. somniferum* (0.04 g)	MOR, COD, THEB, PAP, NOS, ORIP	LDI-MS/MS	Methanol (1.55 mL)	-	-	Skopikova et al. (2020)

[a] The organic layer was then back-extracted (BE) after acidifying/alkalinizing, see the papers for details of the protocol. [b] Relative error. – data not showed. MOR: morphine, COD: codeine, THEB: thebaine, PAP: papaverine, NOS: noscapine, NAR: narceine, ORIP: oripavine, ATPS: aqueous two-phase system, PEG: poly(ethylene glycol), SPE: solid phase extraction, MCX: mixed-mode, strong cation-exchange, HLB: hydrophilic-lipophilic balance, MSPE: magnetic solid phase extraction, GC: gas chromatography, HPLC: high-performance liquid chromatography, UHPLC: ultra-high performance liquid chromatography, MS/MS: tandem mass spectrometry, MS: mass detector, DAD: diode-array detector, FID: flame ionization detector, LDI: laser desorption ionization.

6.1 Analytical techniques used for detection and quantification of opium alkaloids

There are many suitable analytical techniques for detection and quantification of opium alkaloids (Fig. 4), such as gas chromatography (GC), high-performance or ultra-high performance liquid chromatography (HPLC, UHPLC) and capillary electrophoresis (CE).

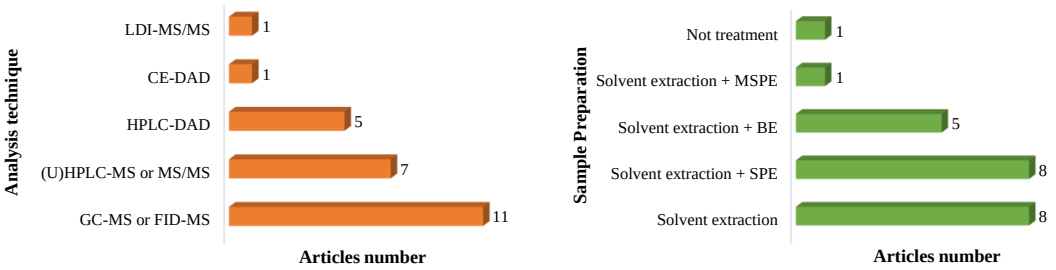

Fig. 4. Different (a) analytical techniques and (b) sample preparation treatment used for the determination of opium alkaloids in poppy (seeds and plant), foods with poppy seeds and poppy teas (from 1982 until 2020). For details, see Table 2.

As can be seen in Fig 4a, several studies have used GC coupled to mass spectrometric detection (GC-MS) for the analysis of opioids in this type of samples. However, analysis by GC requires a derivation step of the opium alkaloids, to make them volatile, and this step is complex, laborious and increases the costs of the method. Moreover, it is important to consider the thermolability of the compounds. For this reason, in general, articles using GC are the oldest of the studies collected for the analysis of opioids in these samples (Table 2). Different derivation conditions have been applied. For example, Bjerver et al. (1982) derivatized morphine with pentafluoropropionic anhydride (PFPA) at 65ºC for 30 min. Morphine and codeine were determined after derivatisation with TFA (trifluoroacetic anhydride) at 60ºC for 20 min (Hayes et al., 1987) and with 4-dimethylamino pyridine in acetic anhydride at 50ºC for 15 to 30 min (Struempler, 1987). Paul, Dreka, Knight and Smith (1996) quantified morphine, codeine, papaverine and thebaine after acetylation by acetic anhydride and pyridine. Morphine, codeine and thebaine were quantified after derivatisation with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) mixed with 1% TMCS (trimethylsilyl chloride)

(Casella et al., 1997; Meadway et al., 1998; Özbunar et al., 2019; Pettitt et al., 1987) or with pyridine (1:1, v/v) (Yamaguchi et al., 2011). Finally, Van Thuyne et al. (2003) analysed morphine after derivatisation with MSTFA (N-trimethylsilyl-N-methyl trifluoroacetamide) at 80ºC for 10 min. On the other hand, Acevska, Stefkov, Petkovska, Kulevanova and Dimitrovska (2012b) optimized a method based on GC with flame ionization detector (FID) coupled to MS spectrometry (GC-FID/MS), avoiding any derivation step before the analysis, which allowed to significantly reduce the total analysis time. This method was validated, and low detection limits (LD) were obtained between 0.91 and 1.95 µg mL^{-1} for morphine, codeine, thebaine, papaverine, noscapine and oripavine.

In these GC methods, chromatographic conditions were very similar. Columns containing non-polar stationary phases like Silyl-8 (Bjerver et al., 1982), DB-5 (Cassella et al., 1997; Paul et al., 1996; Struempler, 1987), DB-17 (Pettitt et al., 1987), HP5-MS (Acevska et al., 2012b; Meadway et al., 1998; Özbunar et al., 2019), HP-Ultra 2 (Yamaguchi et al., 2011) and HP-Ultra 1 (Van Thuyne et al., 2003) were used, with helium as the carrier gas. In some studies, the separation was performed under isothermal conditions at 220-250ºC (Bjerver et al., 1982; Hayes et al., 1987; Pettitt et al., 1987; Struempler, 1987), but all others work have used different temperature programs. The MS detector used was the quadrupole mass spectrometer (Hayes et al., 1987) or the ion trap mass spectrometer (Cassella et al., 1997). Electron impact (EI) ionization was the most used ionization method, and the mass spectrum was performed in selected ion monitoring (SIM) mode.

Although GC allows obtaining very low LD, the costly step of derivatization and the possible loss of the analytes has made the authors choose, in the most recent studies, alternative analysis techniques, such as HPLC (Fig. 4a). Because the large polarity range of the major opium alkaloids (Table S5), most applications used reversed-phase liquid chromatography with gradient elution and ion-pairing reagents. Different detectors have been used for the analysis of opioids by HPLC, as the diode-array detector (DAD) (Acevska et al., 2012a, 2012b; Cao et al., 2007; Meos et al., 2017; Yoshimatsu, Kiuchi, Shimomura & Makino, 2015). In these works, the most used stationary phases were non-

polar C18 (Acevska et al., 2012a, 2012b; Cao et al., 2007; Yoshimatsu et al., 2005) or C8 (Meos et al., 2017). Reversed-phase liquid chromatography (in isocratic or gradient elution mode) was used, with mobile phases composed by mixtures of methanol (MeOH) with aqueous solutions of trifluoroacetic acid (0.1%) or ammonium acetate (0.5%) and trimethylamine, to adjust the pH to 9.6 (Acevska et al., 2012a, 2012b; Cao et al., 2007). Ion pair chromatography was also used, as an effective reversed-phase technique for separation of ionized organic analytes. In this case, an ion pair reagent such us sodium heptanesulphonate is added to the acidic mobile phase (pH 3.2 – 3.5), that was composed by a mixture of acetonitrile and water (Meos et al., 2017; Yoshimatsu et al., 2005). However, these separation conditions are not compatible with MS detection due to the nonvolatile ion-pair reagents.

As can be seen in Table 2, recent published methods are performed with (U)HPLC with tandem mass spectrometry (MS/MS), and the most popular detector is the triple quadrupole (Q_qQ) mass spectrometer. The most widely used type of ionisation source is electrospray ionisation in positive mode (ESI^+). Almost all of them use multiple reaction monitoring (MRM) which provides even more reliable quantitative data of the analytes (Guo et al., 2013; López et al., 2018; Powers et al., 2017; Sproll et al., 2006; Stranska et al., 2013). In these works, the stationary phase was C18 (Guo et al., 2013; López et al., 2018; Powers et al., 2017; Sproll et al., 2006; Stranska et al., 2013; Zenai et al., 2012). Gradient elution was applied with a mobile phase that consisted in a mixture of MeOH:water (Guo et al., 2013) or MeOH:acetonitrile:water with a low percent of formic acid (Powers et al., 2017; Stranska et al., 2013). In some cases, ammonium carbonate was added to reach pH 9 (López et al., 2018; Sproll et al., 2006). On the other hand, in order to completely minimize the possibility of false positives in real samples, HPLC coupled to triple quadrupole-linear ion trap-tandem mass spectrometry (HPLC-Q_qQ_{LIT}-MS/MS) have been used recently by Xu et al. (2019). The precursors and product ions of the opiates were monitored by MRM and enhanced ion product (EIP) mode. A hydrophilic interaction column (HILIC) was used with ammonium formate:acetonitrile as mobile phase. The analytical column was set a 30º C and the separation of five opiates was carried out in 7 min.

In combination with chromatography, some authors have carried out studies with other techniques, such as CE-DAD and LDI-MS/MS (laser desorption ionization mass spectrometry), which were compared with HPLC. Thus, Meos et al. (2017) proposed CE-DAD as a strong alternative to HPLC-DAD for the analysis of this family of compounds, above all for its simplicity of sample preparation. However, they argued that this was true only at moderate concentrations, and for very low concentrations HPLC is needed because of its higher sensitivity. Furthermore, Skopikova, Hashimoto, Richomme and Schinkovitz (2020) determined opium alkaloids in crude extracts of *P. somniferum* with LDI-MS/MS and later, they compared to classical HPLC-MS/MS. They demonstrated that although HPLC-MS/MS analysis should be performed for precise quantification, rapid qualitative analysis of large sample batches was established, which is very interesting for industrial application.

6.2 Sample preparation

As can be seen in Fig. 4b, in several studies the target opiates are only extracted with solvents, but in others a clean-up step to eliminate matrix interferences is also applied (Table 2). Table S5 shows some physico-chemical parameters (water solubility, Log P and pKa) of opiates, in order to understand the different sample preparation strategies that have been evaluated until now. As can be seen, morphine is the compound with the highest water solubility (10.20 g L^{-1}) and lowest Log P (0.89), whereas the compound with the lowest water solubility (0.013 g L^{-1}) and highest Log P (3.08) is papaverine. Along with solubility, pKa values (between 8.07 and 8.38) are also very important properties to consider, as the extent of ionization (overall state of charge) for the opiates is a function of its intrinsic pKa value and the pH value of the solution. The extent of ionization can also control its solubility and the interaction with sorbents during the purification step. Based on these physico-chemical characteristics, various aqueous solutions (water, acetic acid, hydrochloric acid, and sodium citrate buffer), mixtures of these solutions with alcohols or pure alcohols have been evaluated for solid-liquid extraction (SLE). For the clean-up step, commercial SPE cartridges such as Oasis® HLB (a universal polymeric reversed-phase sorbent useful for a wide range of acidic, basic,

and neutral compounds), Oasis® MCX (a mixed-mode polymeric sorbent, useful to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups) and Clean Screen® DAU (a copolymerized sorbent, utilizing both a reverse (C8) phase and an ion exchange (benzenesulfonic acid) phase bonded to the same particle) have been evaluated, among others (Table 2).

Sample preparation for poppy seeds and food products analysis have been carried out mainly by conventional procedures, such as SLE. For this purpose, MeOH, either alone or with a low % of acid, has been the most widely extraction solvent used (Table 2). An example is a study of Sproll et al. (2006) that developed a method to analyse morphine and codeine in poppy seeds by HPLC-MS/MS. In this work, after optimization of the extraction parameters, 10 g of the seeds were extracted with 30 mL of MeOH with 0.1% acetic acid during 60 min in an automatic shaker at 250 rpm. The precision resulted in ranges between 7.4 and 9% (relative standard deviation, RSD) and the accuracy was between 9.8 and 17.6% (relative error). In the same way, Acevska et al. (2012a, 2012b) used MeOH for the extraction of morphine, codeine, thebaine, papaverine, noscapine and oripavine in poppy straw. In these works, 5 mL of extraction solvent was added to 0.1 g of sample, the mixture was sonicated for 20 min (40ºC) and then, it was centrifuged at 4000 rpm for 5 min. The extraction was performed twice, and the supernatants were mixed for further analysis by HPLC-DAD or GC-FID/MS. Good recovery values were obtained for the target analytes, near to 100% (*P* 95% ± 0.4 - 1.8%), in samples spiked at three concentration levels. Similarly, in a recent study of Skopikova et al. (2020), 0.04 g of the powdered plant was extracted with 1.55 mL of MeOH and the crude extracts were analysed by LDI-MS/MS for a rapid opiate detection.

Looking at these good recovery values, it seems that MeOH is a good extraction solvent for SLE. However, other extraction solvents have been used. For example, Cao, Li, He, Li & Liu (2007) optimised an extraction method based on aqueous two-phase system (ATP) containing 6% PEG (poly(ethylene glycol) with a molecular mass of 4000 and 30% $(NH_4)_2SO_4$ to analyse papaverine in pericarpium papaveris by HPLC-DAD. Results were compared with Soxhlet extraction, with 40 mL of MeOH during 4 h. Adequate recovery values (97.3%, RSD ≤ 1.8%) were achieved, with the advantage that

the developed method is more environmentally benign and cost effective because required less time. These good results could be based on hydrophobic interaction between papaverine and ATPS system (Table S5). In a more recent study, López et al. (2018) evaluated the effectiveness of different types of organic solvents for the analysis of opium alkaloids in poppy seeds and bakery products by UHPLC-MS/MS. A mixture of acetonitrile:water:formic acid (80:19:1, v/v/v) was selected for this purpose because it was more effective on ground poppy seeds. 10 g of the samples were mixed with 100 mL of the solvent for 30 min. Recovery values in the range of 70 - 120%, (RSD ≤ 20%), were obtained except for noscapine (150 - 170%). The presence of interferences from the matrix may be the reason for the non-satisfactory results in the case of noscapine. Finally, in other works, after alkalinisation to pH 9 – 9.5 (with carbonate or ammonium buffers), the opiates were extracted (in their neutral form) from poppy seeds with mixtures of chloroform:isopropanol, dichloromethane:isobutanol or dichloromethane:MeOH (90:10, v/v). Usually, to reduce interferences, the organic extract is then acidified (with sulphuric or hydrochloric acid), after this the pH of the aqueous phase is adjusted again to pH 9 - 9.5 and, finally, a re-extraction with the same mixture of solvents is carried out. This complex sample preparation protocol was coupled, in all cases, with analysis by GS-MS after derivatization of the opium alkaloids (Meadway et al., 1998; Paul et al., 1996; Pettit et al., 1987; Struempler, 1987; Yamaguchi et al., 2011).

For liquid samples (teas), liquid-liquid extraction (LLE) was applied by Van Thuyne et al. (2003) using methanol:dichloromethane, after adjusting the pH to 9.5, to analyse morphine by GC-MS. More recently, in homemade poppy seed teas, obtained under different conditions to simulate home brewing, Powers et al. (2017) quantified morphine, codeine and thebaine. In this work, any sample treatment was applied, and the tea was directly analysed by HPLC-MS/MS after appropriate dilutions. Overall accuracy ranged from 92.3 – 103.4% with minimal matrix effect.

As can be seen, in all the previously discussed studies only organic solvents were used for the extraction of the target analytes. However, to obtain a good clean extract avoiding the interference of the matrix components, and thus achieving good recovery values, a further purification step by solid-phase extraction (SPE), with different types of sorbents,

is included in some other works (Table 2). For example, in 1982, Bjerver et al. used 15 g of diatomaceous earth to purify the aqueous extract obtained from blue and white poppy seed (5 g of sample and 10 mL of 0.1 M citric acid, pH 4). More recently, Meos et al. (2017) determined morphine, codeine and papaverine in dry poppy capsules of 34 different cultivars used as ornamental plants. Samples (0.5 g) were mixed in ultrasound with 20 mL of ethanol:water (50:50, v/v). After dilution and adjustment to a pH of 9.5, 1 mL was passed through the SPE column (8 cm x 10 mm, filled with diatomaceous earth) and the analytes were eluted with 2-propanol:dichloromethane and analysed by HPLC-DAD. In the same way, Hayes et al. (1987) determined morphine and codeine in black poppy seeds from the USA. Samples (1g) were homogenized in 5 mL of 0.1 M citrate buffer and then passed through a Chem Elute column, which was filled with diatomaceous earth. After washing with water, the analytes were eluted with chloroform and analysed by GC-MS. In other works, Clean Screen® DAU extraction column (200 mg) have been used (Cassella et al., 1997; Özbunar et al., 2019). In both studies, firstly a solvent extraction with 5 - 10 mL of MeOH was carried out (0.2 and 0.5 g poppy seeds), the extracts were loaded in conditioned cartridges and analytes were eluted with ethyl acetate:isopropanol:ammonium hydroxide (84:12:4, v/v/v). After evaporation and derivatization, the analysis was carried out by GC-MS. Finally, in other works Oasis® MCX have been selected for the purification stage (Guo et al., 2013; Stranska et al., 2013; Yoshimatsu, et al., 2005), and the comparison with other cartridges such as Oasis® HLB was carried out. For example, Yoshimatsu et al. (2005) analysed morphine, codeine, thebaine, papaverine and noscapine by HPLC-DAD in powdered poppy capsules. For this purpose, 0.05 g of the sample was mixed with 5 mL of the extraction solvent (water, 5% acetic acid, 0.1 M sodium citrate buffer, pH 6.0) under sonication. An aliquot of the supernatant was purified by SPE (either Oasis® HLB or Oasis® MCX) and the influence of the extraction conditions was evaluated. Results obtained indicated that substances that affected morphine separation could not be removed with Oasis® HLB. For this reason, the Oasis® MCX cartridge was used and get good recoveries between 99.94 and 112.18% (RSD $\leq$ 1.35%). In a similar way, Guo et al. (2013) evaluated the use of both types of cartridges to analyse morphine, codeine, papaverine, noscapine and thebaine in hot pot broth by HPLC-MS/MS. First, 20 mL of HCl was mixed with 5 g of hot pot and an

ultrasonic extraction was carried out. After, to remove fats, a second extraction with petroleum ether was applied. Once the water phase-extracts were purified by SPE, good recovery values were obtained with both cartridges, except for morphine using Oasis® HLB (only a 10% recovery). Moreover, with Oasis® MCX better purification results (72 - 124%, RSD ≤ 25%) were obtained, because of its high specificity for basic compounds as opiates (Table S5).

One of the main drawbacks of SPE is the considerable consumption of organic solvents that presents problems with waste generation. Therefore, a current objective of researchers is to develop new sample preparation techniques that require less amounts of organic solvents and are more environmentally friendly simpler and faster. In addition, many research studies have focused on the development of nano-sized sorbent materials for the pre-concentration of target analytes and to reduce matrix effects. One example is the work of Xu et al. (2019) who synthesized a new sorbent with the aim of determine possible presence of opium alkaloid in different hot pot samples. This synthesis was based on the formation of magnetic Fe_3O_4 particles, coated with non-porous silica, functionalized with amantadine (Fe_3O_4@SiO_2@ADME material). Once the material was synthesized, the sample was extracted and purified by magnetic solid-phase extraction (MSPE). Firstly, 2 g of sample (diluted with water) was extracted with acetonitrile. The supernatant was evaporated, and water was added for the subsequent purification of this mixture by MSPE under optimized conditions. Good recovery values, between 80 and 115% (RSD 4.3 - 10.7%) for morphine, codeine, thebaine, papaverine and noscapine were observed. Therefore, the results obtained in this work demonstrated the advantages of MSPE compared to SPE, as less volumes of organic solvent and amount of sorbent (50 mg) was used.

7. Future perspectives and conclusions

Several actions that can be taken to control the levels of opium alkaloids in food products, as establishing: a) the maximum limits in seeds or food, b) a classification of different varieties of poppy plants with seeds for food purposes, c) good harvesting practices to minimize contamination and d) good processing practices to minimize the

concentration of opium alkaloids. Nowadays, there are few studies of opium alkaloids in food samples, there are mainly in biological samples. In addition, as can be seen in Fig. 5 studies of opium alkaloids are mainly about morphine, and the other alkaloids have been less studied, especially, noscapine, narceine and oripavine. Thus, in order to know the real exposure of consumers and to be able to make a hazard characterization of its consume, there is a need to carry out more studies of all opioids in poppy seeds and poppy seed food available on the market.

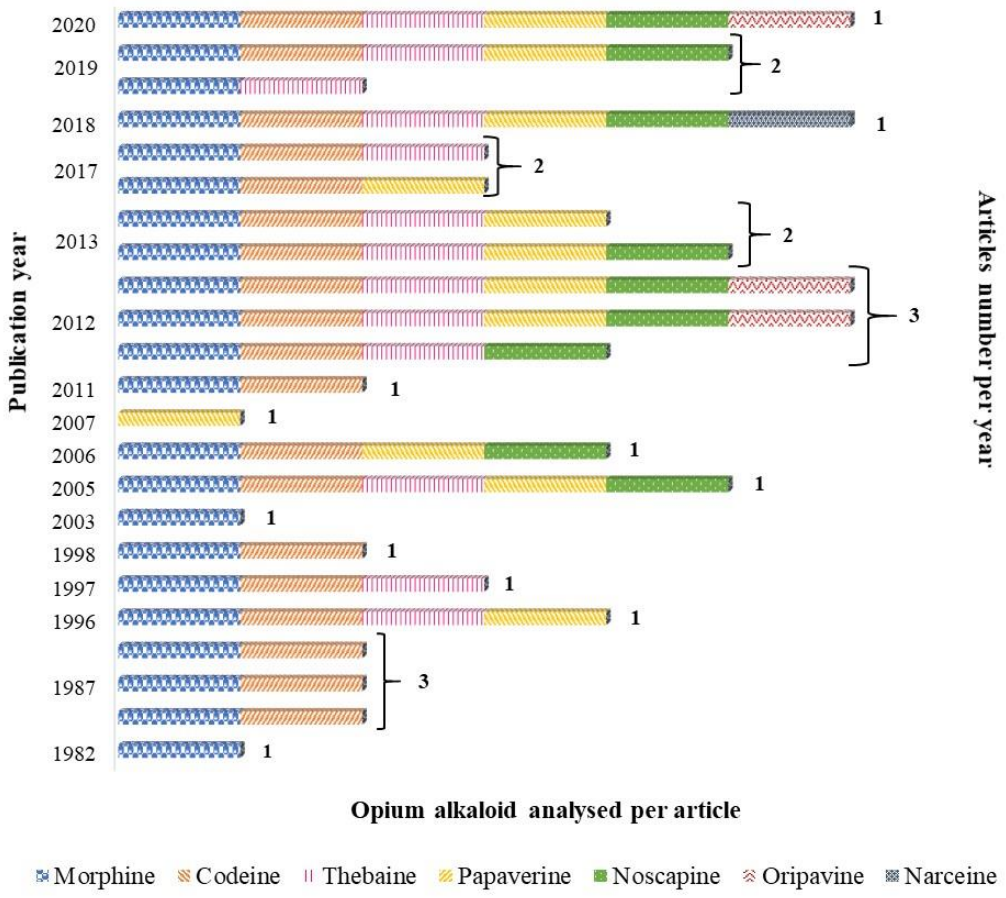

Fig. 5. Opium alkaloids that have studied in poppy seeds or poppy seed foods in articles published between 1982 and 2020.

To perform these studies, researchers must consider that the quantities of these compounds are very low and are found in complex matrices. Hence, it is essential to develop and validate new analytical methods based on adequate sample preparations and selective analytical techniques. Most studies use HPLC-MS/MS and conventional extraction techniques, such as solvent extraction and SPE. However, nowadays, sample preparation techniques are evolving towards more sophisticated and environmentally friendly modes. Nevertheless, the development of advanced analytical strategies for the determination of opium alkaloids in poppy seeds or foods, through the application of new materials and micro-extraction techniques, is still a great future challenge.

Acknowledgments. Authors thank financial support to MCIU/AEI/FEDER, UE (Proyecto RTI2018-094558-B-100, EVALKALIM).

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Supplementary material

Table S1. Pharmacological properties and toxicity of main opium alkaloids present in poppy seeds and food products.

Opium alkaloid	Properties	Toxicity	
		Short term	**Long term**
Morphine	Analgesic	Nausea, vomiting, constipation, drowsiness, euphoria, sedation and depression	No evidence of carcinogenicity Genotoxic only *in vivo* Reproductive and developmental effects Dependency and tolerance
Codeine	Analgesic	Constipation and nausea	No carcinogenicity, teratogenicity, genotoxicity or neurotoxic effects Dependency and tolerance
Thebaine	Not used therapeutically	No data were identified	No data were identified
Papaverine	Coronary vasodilator	Headache, gastrointestinal disorders, tachycardia, sweating, dizziness, seizures	Icterus, eosinophilia and liver enzyme changes (reversible)
Noscapine	Cough suppressant and potentially anti-cancer drug	Headache and dizziness	No data were identified
Oripavine	Not used therapeutically	No data were identified	No data were identified
Narceine	Not used therapeutically	No data were identified	No data were identified

Table S2. Summary of pre-treatments and processing methods that reduce the alkaloids content in poppy seeds and poppy seed food products.

Process	Morphine reduction (%)	Reference
Washing or soaking with cold water (seeds)	40-75	Bjerver et al. (1982) Lo and Chua (1992) Sproll et al. (2007)
Washing or soaking with hot water (seeds)	100	Sproll et al. (2007)
Grinding (seeds)	25-35	Sproll et al. (2007) Zenai et al. (2012)
Pre-treatment (seeds) and baking (food product)	80-100	Lachenmeier et al. (2010)

Table S3. List of RASFF notifications (from 2005 to 2020) of poppy seeds or poppy seed foods with high contents of morphine or morphine alkaloids.

Date	Subject	Notified by	Countries concerned	Action taken	Distribution status	Risk decision
27/10/2005	High content of morphine (228 mg kg^{-1}) in poppy seed from Australia	Germany	Australia (O), Germany (D)	Product recall or withdrawal	Distribution restricted to notifying country	Undecided
07/12/2005	High content of morphine (48 mg kg^{-1}) in ground poppy seeds from Austria	Hungary	Austria (O), Hungary (D)	Product recall or withdrawal	Distribution on the market (possible)	Undecided
21/12/2005	High content of morphine (200 mg kg^{-1}) in poppy seed from unknown origin	Germany	Germany (D), unknown origin (O)	Product recall or withdrawal	Distribution restricted to notifying country	Undecided
28/12/2005	High content of morphine (44.4 mg kg^{-1}) in poppy seed from Hungary	Hungary	Croatia (D), Hungary (D/O), Slovakia (D)	Product recall or withdrawal	Product past use-by date	Undecided
10/01/2006	High content of morphine (200 mg kg^{-1}) in poppy seed from the Netherlands	Germany	Austria (D), Germany (D), Netherlands (O)	Product recall or withdrawal	Distribution on the market (possible)	Undecided
13/01/2006	High content of morphine (44 mg kg^{-1}) in poppy seed from the Slovak Republic	Hungary	Hungary (D), Poland, Slovakia (O)	Destination of the product changed	Distribution on the market (possible)	Undecided
19/12/2006	High content of morphine (53 mg kg^{-1}) in poppy seed from the Czech Republic via the Slovak Republic	Hungary	Czech Republic (O), Hungary (D), Slovakia	Official detention	Distribution on the market (possible)	Undecided

19/12/2006	High content of morphine (40 mg kg^{-1}) in poppy seed from the Slovak Republic	Hungary	Hungary (D), Slovakia (O)	Official detention	Distribution on the market (possible)	Undecided
22/12/2006	High content of morphine (93 mg kg^{-1}) in poppy seed from the Czech Republic via the Netherlands and via Liechtenstein	Germany	Czech Republic (O), Germany (D), Liechtenstein (O), Netherlands	Withdrawal from the market	Distribution on the market (possible)	Undecided
22/01/2007	High content of morphine (60-80 mg kg^{-1}) in poppy seed from the Czech Republic via the Slovak Republic	Hungary	Austria (D), Czech Republic (O), Germany (D), Hungary (D), Poland (D), Romania (D), Slovakia (D)	Withdrawal from the market	Distribution on the market (possible)	Undecided
07/09/2007	High content of morphine (60 mg kg^{-1}) in poppy seed from Hungary	Hungary	Hungary (D/O), Russia (D)	Physical/chemical treatment	Distribution restricted to notifying country	Undecided
26/10/2007	High content of morphine (40 mg kg^{-1}) in poppy seeds in bulk from Hungary	Hungary	Hungary (D/O), Netherlands (D), Russia (D), Ukraine (D)	Prohibition to trade - sales ban	Distribution on the market (possible)	Undecided
26/10/2007	High content of morphine (80 mg kg^{-1}) in poppy seeds in bulk from Hungary	Hungary	Hungary (D/O), Slovakia (D)	Prohibition to trade - sales ban	Distribution on the market (possible)	Undecided
24/06/2010	High content of morphine (72 mg kg^{-1}) in poppy seeds from unknown origin, via the Netherlands	Germany	Germany (D), Hungary (D), Netherlands (O), unknown origin (O)	Destruction	Distribution on the market (possible)	Undecided

14/01/2014	High content of morphine (88.1 mg kg^{-1}) in poppy seeds from Hungary	Czech Republic	Czech Republic (D), Hungary (O), Poland (D), Slovakia (D)	Withdrawal from the market	Distribution to other member countries	Serious
16/05/2014	High content of morphine (56.2 mg kg^{-1}) in blue poppy seeds from France, packaged in Slovakia	Czech Republic	Commission Services, Czech Republic (D), France (O), Slovakia (O)	Withdrawal from the market	Distribution to other member countries	Serious
21/05/2015	High content of morphine (96.93 mg kg^{-1}) and of morphine alkaloids (142 mg kg^{-1}) in poppy seed from France, via Poland	Czech Republic	Czech Republic (D), France (O), Poland (D), Ukraine (D)	Informing recipient(s)	Distribution to other member countries	Serious
03/06/2015	High content of morphine (137 mg kg^{-1}) and of morphine alkaloids (150 mg kg^{-1}) in blue poppy seeds from Australia, via the Netherlands	Czech Republic	Australia (O), Czech Republic (D), Netherlands	Detained by operator	No distribution from notifying country	Serious
17/06/2015	High content of morphine (69.61 mg kg^{-1}) in grey poppy seeds from France, via Poland	Czech Republic	Czech Republic (D), France (O), Poland, Ukraine (D)		No distribution from notifying country	Serious
22/06/2015	High content of morphine (59.13 mg kg^{-1}) and of morphine alkaloids (102.82 mg kg^{-1}) in ground poppy seeds from the Czech Republic	Czech Republic	Czech Republic (D/O), Slovakia (D)	Withdrawal from the market	Distribution restricted to notifying country	Serious
06/09/2018	High content of morphine (67.7 mg kg^{-1}) and of morphine alkaloids (205 mg kg^{-1}) in poppy seeds from Poland	Czech Republic	Croatia (D), Czech Republic (D), Germany (D), Greece (D), Poland (D/O), Romania (D)	Withdrawal from recipient(s)	Distribution to other member countries	Undecided

09/01/2019	High content of morphine (15.5 mg kg^{-1}) in ground poppy seeds from Slovakia	Czech Republic	Commission Services, Czech Republic (D), Slovakia (D/O)	Withdrawal from the market	No distribution to other member countries	Not serious
21/03/2019	High content of morphine (2.43, 5.2, 4.8 mg kg^{-1}) in frozen bread with poppy seeds from France	France	Belgium (D), France (O), Greece (D), INFOSAN, Luxembourg (D), Poland (D), Portugal (D), Romania (D), Spain (D), United Kingdom (D), United States (D)	Withdrawal from recipient(s)	Distribution to other member countries	Serious
02/04/2019	High content of morphine alkaloids (88.5 mg kg^{-1}) in blue poppy seeds from Turkey	Czech Republic	Czech Republic (D), INFOSAN, Latvia (D), Turkey (O)	Re-dispatch	Distribution to other member countries	Serious
19/07/2019	High content of morphine alkaloids (172 mg kg^{-1}) in blue poppy seeds from the Czech Republic	Czech Republic	Croatia (D), Czech Republic (D/O), Estonia (D), Germany (D), Italy (D), Latvia (D), Lithuania (D), Poland (D), Slovakia (D), Sweden (D)	Re-dispatch	Distribution to other member countries	Serious
05/09/2019	High content of morphine alkaloids (369 mg kg^{-1}) in poppy seeds from Slovakia	Czech Republic	Czech Republic (D), INFOSAN, Romania (D), Russia (D), Slovakia (O), United Kingdom (D)	Detained by operator	Distribution to other member countries	Serious
09/09/2019	High content of morphine alkaloids (74.7 mg kg^{-1}) in ground blue poppy seeds from the Czech Republic	Slovakia	Czech Republic (D/O), Hungary, Slovakia (D)	Withdrawal from the market	Distribution to other member countries	Serious

13/11/2019	High content of morphine alkaloids (114 mg kg^{-1}) in poppy seeds from Poland, via Slovakia, packaged in the Czech Republic	Czech Republic	Czech Republic (O), INFOSAN, Poland (O), Russia (D), Slovakia	Informing recipient(s)	Distribution to non-member countries	Serious
09/01/2020	High content of morphine alkaloids (59.8 mg kg^{-1}) in blue poppy seeds from Slovakia	Czech Republic	Czech Republic (D), Slovakia (O)	Withdrawal from the market	Distribution restricted to notifying country	Serious
14/01/2020	High content of morphine alkaloids (50.2 mg kg^{-1}) in blue poppy seeds from Slovakia	Czech Republic	Czech Republic (D), Slovakia (O)	Informing recipient(s)	Distribution restricted to notifying country	Serious

D: distribution; O: origin.

Table S4. Summary of the different analytical methods used in the literature for the determination of opium alkaloids in biological samples (urine, serum, blood, plasma and oral fluid)

Sample (amount)	Analyte	Analysis technique	Sample preparation		References*
			Extraction conditions (volume)	Purification (sorbent)	
Urine (5 mL)	MOR, COD	EMIT+GC-MS	Isobutanol: methylene chloride, 10:90, v/v (5 to 10 mL) (after pH 9.5)[a]	-	Struempler (1987)
Urine	MOR, COD	ELISA+GC-MS	-	-	Pettitt et al. (1987)
Urine (1 mL)	MOR, COD	EMIT+GC-MS	Isopropanol:chloroform, 10:90, v/v (5 mL) (after pH 9)[a]	-	Meadway et al. (1998)
Blood and urine	MOR	ELISA+GC-MS	Solvent extraction (non-specified)	-	Moeller et al. (2004)
Urine (5 mL)	MOR	EMIT+GC-MS	[a]	SPE (diatomaceous earth, 15 g)	Bjerver et al. (1982)
Serum and urine (1 mL)	MOR, COD	EMIT+GC-MS	[a]	SPE (Chem Elute)	Hayes et al. (1987)
Urine (5 mL)	MOR, COD, THEB	EMIT+GC-MS	[a]	SPE (Clean Screen DAU, 0.2 g)	Cassella et al. (1997)
Urine and serum	MOR, COD, PAP, NOS	EMIT+HPLC-MS/MS	Not specified	SPE	Trafkowski et al. (2006)
Urine (2 mL)	MOR, COD	EMIT+GC-MS	[a]	SPE (Bond Elut Certify)	Yamaguchi et al. (2011)
Urine (3 mL)	MOR, COD	EMIT+GC-MS	[a]	SPE (Cerex Polycrom Clin II)	Smith et al. (2014)
Urine	MOR, THEB	EMIT+GC-MS	[a]	SPE (Clean Screen DAU)	Özbunar et al. (2019)

(2 mL)					
Urine	MOR	GC-MS	-	-	Narcessian et al. (1997)
Urine	MOR, COD	GC/MS	Not specified	-	Thevis et al. (2003)
Urine (3 mL)	MOR	GC/MS	Methanol:dichloromethane, 10:90, v/v (5 mL) [a]	-	Van Thuyne et al. (2003)
Urine and oral fluid (1 mL)	MOR	GC/MS	Urine[a], Oral fluid: Methanol (100 mL)[a]	SPE (CleanScreen ZSDAU)	Rohrig and Moore (2003)
Blood, urine (1 mL)	MOR, COD	HPLC-MS/MS	[a]	-	Bailey et al. (2010)
Blood and plasma (1 mL)	MOR, COD, PAP, NOS	HPLC-MS/MS	-	SPE (C_{18},200 mg)	Taylor and Elliott (2009)
Blood (1 mL)	MOR, COD	HPLC/MS	Water with 1% formic acid (2 mL)	SPE (Strata-XC, 60 mg)	Newmeyer et al. (2015)

[a] First, acid, basic or enzymatic hydrolysis of urine. See the papers for details of the protocol. MOR: morphine, COD: codeine, THEB: thebaine, PAP: papaverine, NOS: noscapine, SPE: solid phase extraction, HPLC: high performance liquid chromatography, MS/MS: tandem mass spectrometry, MS: mass detector, ELISA: enzyme linked immunosorbent assay, EMIT: enzyme multiplied immunoassay technique.

* Bailey K., Richards-Waugh, L., Clay, D., Gebhardt, M., Mahmoud, H., & Kraner, J. C. (2010). Fatality involving the ingestion of phenazepam and poppy seed tea. *Journal of Analytical Toxicology*, 34, 527-532. Taylor, K., & Elliott, S. (2009). A validated hybrid quadrupole linear ion-trap LC-MS method for the analysis of morphine and morphine glucuronides applied to opiates deaths. *Forensic Science International*, 187, 34-41.Thevis, M., Opfermann, G., & Schänzer, W. (2003). Urinary concentrations of morphine and codeine after consumption of poppy seeds. *Journal of Analytical Toxicology*, 27, 53-56. See de manuscript for other reference.

Table S5. Physical-chemical properties of the seven most common opium alkaloids in the latex of poppy plant (*Papaver somniferum* L.).

Opium alkaloid	pK_a	LogP	Water solubility (g L^{-1})
Morphine	8.21	0.89	10.20
Codeine	8.21	1.19	0.580
Thebaine	8.38	1.53	0.700
Oripavine	8.38	1.46	0.870
Noscapine	8.38	1.46	0.180
Papaverine	8.07	3.08	0.013
Narceine	8.33	2.06	0.045

1.1.2 Actualización legislativa

Como se ha puesto de manifiesto en el trabajo de revisión, este tema era bastante desconocido para la población en general y no existía una legislación armonizada entre los Estados Miembros de la Unión Europea. Como medidas de control, cada país ha tenido una legislación y situación diferente, algunos países han prohibido el uso de semillas de amapola para usos alimentarios y otros optaron por poner sus propios límites máximos [30]. Además, la CE en 2014 publicó una serie de recomendaciones de buenas prácticas para prevenir y disminuir el contenido de OAs, donde se recomendó utilizar para uso alimentario las semillas de variedades con bajos contenidos de OAs, controlar posibles enfermedades y plagas, entre otros. Por otro lado, también se establecieron buenas prácticas de procesamiento como el lavado, el tratamiento térmico o la molienda de las semillas [19]. Sin embargo, la falta de una legislación y un control armonizado ha derivado en numerosas alertas sanitarias en la Red de Alerta Rápida de Alimentos y Piensos (RASFF). Concretamente, se han dado hasta 50 notificaciones desde 2005 de altos niveles de morfina en semillas de amapola llegando hasta casi 400 ppm, lo que ha supuesto la retirada inmediata de los productos alimentarios [14].

Por ello, finalmente en diciembre de 2021 se publicó por primera vez una legislación a nivel europeo que regula el límite máximo permitido de equivalentes de morfina en semillas de amapola y productos de panadería. Siendo 20 mg/kg de equivalentes de morfina (morfina + 0,2 × codeína) para semillas de amapola destinadas al consumo humano directo y 1,5 mg/kg para los productos de panadería [13]. Recientemente, se ha publicado una nueva actualización y se encuentra en vigor el Reglamento (UE) 2023/915 de la Comisión de 25 de abril de 2023 relativo a los límites máximos de estos contaminantes en los alimentos y en el que ya incluyen a los OAs, pero sin realizar ninguna modificación respecto al primer reglamento publicado [31]. Además, hay que tener en cuenta que la transformación alimentaria puede reducir un 25-100% del contenido de OAs. Por lo que es conveniente que el fabricante de los productos de panadería disponga de información detallada de las semillas de amapola utilizadas como ingredientes en tales productos, incluido la concentración de equivalentes de morfina, y que el proveedor de semillas informe al productor de productos de panadería.

La publicación de esta legislación armonizada ha supuesto un avance a la seguridad alimentaria en cuanto al control de OAs en alimentos. Sin embargo, las autoridades sanitarias como la EFSA y el Instituto Federal Alemán de evaluación de riesgos (BfR), reclaman más estudios para establecer de forma más precisa la exposición real a los consumidores de todos los OAs y no solo a la morfina y codeína, ya que el resto pueden llegar a ser incluso más tóxicos [11], [18], [32]. De esta forma, se podrá establecer una ARfD y legislación acorde con ello. Por tanto, para ello es necesario desarrollar nuevas metodologías analíticas con el fin de cuantificar de forma eficaz los seis principales OAs que puede estar presentes en las semillas de amapola y distintas matrices alimentarias.

1.1.3 Importancia del estudio del efecto del procesado en el contenido de OAs en los alimentos

Como se ha puesto de manifiesto en el trabajo de revisión, algunos procesados pueden disminuir el contenido de OAs en los alimentos, lo que el estudio del efecto de estos procesados podría ser muy útil para diseñar estrategias que permitan prevenir y reducir altas concentraciones de estos tóxicos en los alimentos y, de esta forma, asegurar la seguridad alimentaria.

El procesado culinario que ha sido evaluado por la comunidad científica durante el desarrollo de esta Tesis Doctoral es el tratamiento térmico. Esto se debe a que las semillas de amapola cada vez se están utilizando más en productos de panadería y por ello, se está evaluando si al añadir las semillas antes del horneado puede disminuir el contenido de OAs. Sin embargo, este es un tema controvertido ya que hay investigadores que dicen que son compuestos con una elevada estabilidad térmica y otros trabajos que afirman que hay un considerable efecto de degradación de los OAs con el horneado en productos de panadería [10], [33]. Por un lado, Shetge y col. (2020) realizaron un estudio sobre la influencia del tratamiento térmico y de lavado en las concentraciones de morfina, codeína y tebaína en semillas de amapola. En este trabajo se estudió la cinética de degradación del tratamiento térmico, obteniendo aparentemente cinéticas de degradación de primer orden dentro de las temperaturas ensayadas (120-200 ºC), mostrando que los OAs eran relativamente estables al tratamiento térmico. A 120 ºC se observó una degradación

mínima y si la hubo fue a partir de los 120 min. Por otro lado, a 200 ºC, la estabilidad de morfina y codeína se mantuvo durante aproximadamente 32 a 39 min, mientras que la tebaína solo se mantuvo 3 min. Por último, el lavado con agua redujo las concentraciones de OAs en las semillas en aproximadamente un 50-80%, mientras que el tratamiento con vapor resultó en una reducción de la morfina en solo una muestra. Además, la cocción no tuvo un efecto significativo en las concentraciones de OAs. Sin embargo, en el trabajo de Carlin y col. (2020) se demostró una considerable degradación de cinco OAs (morfina, codeína, tebaína, papaverina y noscapina) con el tratamiento térmico. Para evaluarlo, realizaron una comparación entre las concentraciones encontradas en las semillas de amapola y las encontradas en productos de panadería (magdalenas y panecillos) preparados con las semillas previamente analizadas. Como resultado obtuvieron concentraciones más bajas después del horneado, con una reducción cercana al 100% para todos los OAs en los productos de panadería, especialmente en las magdalenas horneadas a 180 ºC durante 15 min. Además, observaron que cuando las semillas de amapola se colocaron en la superficie de las magdalenas no se detectaron concentraciones de OAs, lo que podría deberse a un potenciado efecto de las altas temperaturas del horneado [24].

Estos resultados controvertidos han sido posteriormente discutidos en la bibliografía [34], [35]. Como por ejemplo en el trabajo de Kuntz y col. (2021) donde mostraron que el valor de recuperación más alto de morfina en tortas decoradas con semillas de amapola sin moler fue del 50% a 180 ºC durante 20 min y el valor más bajo para tortas molidas fue solo del 16%. Estos autores afirmaron que la estabilidad de los OAs evaluada en otros trabajos podría ser el resultado de una exposición al calor insuficiente, ya que la mayoría de los estudios realizados sobre este tema no habían considerado la temperatura alcanzada dentro del producto, por lo que la matriz podría proteger a los OAs de la degradación. Por todo ello, concluyeron que se debe tener especial cuidado en futuros diseños experimentales y en el seguimiento de los parámetros críticos del proceso de horneado, como la distribución del calor, para evitar resultados inciertos en la degradación de los OAs [34]. Además, el contenido altamente heterogéneo de OAs en las semillas de amapola también es un aspecto que dificulta el abordaje de estos estudios. Esta contaminación tan heterogénea que puede suponer altas variaciones de la concentración incluso en el mismo lote de semillas puede deberse a que la presencia de OAs en las

semillas es una contaminación accidental y, además, a que las concentraciones de OAs presentes en el látex están influenciadas por diversos factores tales como la variedad de la planta, el clima, el tipo de suelo, el tiempo de recolección, entre otros.

Posteriormente, Shetge y col. (2022) volvieron a realizar estudios sobre el efecto del tratamiento térmico con noscapina, que era uno de los OAs que no había estudiado en su trabajo anterior. En este caso, determinaron con los experimentos de tratamiento con calor seco que la noscapina se degradaba a 160-200 ºC, observándose una pérdida del 50% de noscapina después de 3,4 min a 200 ºC. Además, se observó un aumento de su estabilidad cuando estuvo incorporada en una magdalena, ya que su concentración disminuyó tras los 16 min a 200 ºC. Otro trabajo posteriormente publicado fue el de Vera-Baquero y col. (2022) en el que se investigó la degradación térmica de morfina, codeína, tebaína, papaverina y noscapina en muestras de palitos de pan elaborados con harina de maíz y semillas de *Papaver somniferum* L. Los estudios mostraron una disminución del contenido de OAs durante el horneado (180 ºC durante 20 min) entre un 14 y 58 % para tebaína, noscapina y papaverina y hasta un 100% para codeína y morfina. Además, los resultados evidenciaron que la degradación térmica de la morfina y la codeína fue mayor cuando se agregaron semillas de amapola a los palitos de pan como *topping*, lo que coincidió con los hallazgos encontrados por Carlin y col. (2020). Por tanto, confirmaron que además de las condiciones de calentamiento, también es importante considerar la ubicación de las semillas de amapola en el producto al aplicar el tratamiento térmico [36].

En definitiva, tal y como afirmaron Casado y col. (2023), de manera general el tratamiento térmico influye en la degradación de los OAs ya que el contenido de OAs es menor en las muestras tratadas que en las semillas sin tratar. Sin embargo, todavía queda mucho por explorar para extraer conclusiones sobre la estabilidad térmica de los OAs [10]. No obstante, además de conocer la estabilidad térmica de estos compuestos, es importante saber si se pueden formar compuestos de degradación a las temperaturas y tiempos a los que se somete el alimento. Si este es el caso, es importante identificarlos y evaluar su toxicidad, ya que, si se forman compuestos potencialmente peligrosos, se deben considerar para que se elabore una legislación que evite el consumo de alimentos que puedan causar efectos adversos al consumidor [10], [33].

Además, como se ha hecho mención anteriormente, las autoridades sanitarias tienen en cuenta que el tratamiento térmico puede disminuir el contenido final de OAs en el producto final. Sin embargo, no se tiene en cuenta otros tratamientos a los que son sometidos las semillas y no se han evaluado por la comunidad científica. Es por ello por lo que en esta Tesis Doctoral se ha determinado la influencia de la fermentación ácido-láctica, el infusionado y la molienda. De tal forma que sea posible determinar si pueden llegar a ser considerados este tipo de procesados culinarios buenas prácticas para reducir el contenido de OAs en los alimentos.

1.2 Metodologías de análisis empleadas en esta tesis doctoral

El desarrollo de metodologías eficaces y sostenibles para analizar contaminantes en alimentos es un reto para la comunidad científica debido a que son analitos que se encuentran a bajas concentraciones en los alimentos, los cuales son matrices muy complejas formadas por una gran variedad de compuestos que pueden interferir en el análisis tales como proteínas, azúcares, lípidos, vitaminas, entre otros. Por este motivo, es necesario contar con técnicas de análisis sensibles y selectivas, y, además de esto, realizar un tratamiento de muestra adecuado que incluya en la mayoría de los casos una etapa de purificación o limpieza.

1.2.1 Técnicas de extracción y/o purificación empleadas en la etapa de preparación de muestra

Las primeras metodologías de preparación de muestra para analizar contaminantes en alimentos eran tediosas, requerían mucho tiempo y, lo que es más importante, consumían grandes cantidades de recursos que generaban residuos de laboratorio peligrosos. Por ello, la etapa de preparación de muestra siempre se ha considerado como la etapa más costosa del método ya que es la que requiere más tiempo y puede afectar tanto a la exactitud como a la precisión de los resultados [37], [38]. Por ello, la preparación de muestras representaba una fuente importante del impacto negativo total de las metodologías analíticas en el medio ambiente, y, en ocasiones impedía lograr los principios de la GAC. De esta manera, Galuszka y col. en 2013 propusieron 12 principios como directrices generales para métodos analíticos ecológicos. En este enfoque, el primer principio sugería aplicar técnicas analíticas directas para evitar la preparación de muestras [39]. Sin embargo, se ha demostrado que la etapa de preparación de la muestra desempeña un papel muy importante en una metodología de análisis de alimentos, siendo una etapa analítica de la que no se puede prescindir, ya que en el análisis de los alimentos es fundamental y se necesita la conversión a un formato adecuado para el análisis, siendo esencial para (1) preconcentrar los analitos objetivo hasta un nivel superior al límite de detección (LOD) del instrumento analítico; (2) aislar los analitos objetivo de la matriz original de la muestra

y eliminar interferentes de la matriz que pueden afectar al modo de ionización de la muestra aumentando o disminuyendo la señal de los analitos en el caso de emplear espectrometría de masas; (3) lograr una limpieza de la muestra antes del análisis instrumental que permita aumentar la vida útil del equipamiento (columnas y detectores) al obtener extractos más limpios y (4) obtener un extracto compatible con la técnica analítica que se va a utilizar [40].

Por todo ello, uno de los retos más importantes para la comunidad científica en la actualidad es desarrollar etapas de preparación de muestra más sostenibles. De esta manera, surgió el enfoque de la preparación de muestras verde (GSP) establecido por López-Lorente y col. en el 2022, donde se establecieron 10 principios, tal y como se muestra en la Figura 5, que han sido tenidos en cuenta en las metodologías desarrolladas en esta tesis en la medida de lo posible según la naturaleza de los analitos y la matriz a analizar en cada caso.

Principio 1. Favorecer la preparación de muestras *in situ*

Principio 2. Utilizar disolventes y reactivos más seguros,

Principio 3. Emplear materiales sostenibles, reutilizables y renovables

Principio 4. Minimizar el desperdicio

Principio 5. Minimizar las cantidades de muestras, productos químicos y materiales

Principio 6. Maximizar el rendimiento de la muestra

Principio 7. Integrar pasos y promover la automatización

Principio 8. Minimizar el consumo de energía

Principio 9. Elegir la configuración de preparación posterior a la muestra más ecológica posible para el análisis

Principio 10. Garantizar procedimientos seguros para el operador

Figura 5. Los 10 principios de la preparación de muestras verde.

1.2.1.1 Extracción sólido-líquido (SLE) y extracción asistida por ultrasonidos (UAE)

La técnica de preparación de muestra más antigua y comúnmente utilizada para extraer los analitos de muestras sólidas es la extracción sólido-líquido (SLE). Esta técnica se basa en el uso de una fase líquida (disolvente) seguida de una agitación tras la cual se extraen los analitos gracias a su solubilidad [41]. Posteriormente, para recuperar la fase líquida donde están disueltos los analitos se suele hacer una centrifugación, decantación o filtración para eliminar los restos de fase sólida. Sin embargo, esta técnica puede consumir cantidades de disolventes orgánicos altas y por ello, actualmente se están buscando otras alternativas que sean más respetuosas con el medio ambiente basadas en los principios GSP. Al ser indispensable el empleo de disolventes para las muestras sólidas, las tendencias actuales son reducir las cantidades al máximo optimizando todo lo posible la relación muestra-disolvente y empleando disolventes lo más verdes posibles. Además, para intentar reducir más la cantidad de disolvente y los tiempos de extracción se han desarrollado algunas técnicas entre las que destaca la extracción asistida por ultrasonidos (UAE) que permite obtener metodologías más sostenibles de una forma simple, económica, y eficiente (Figura 6) [42].

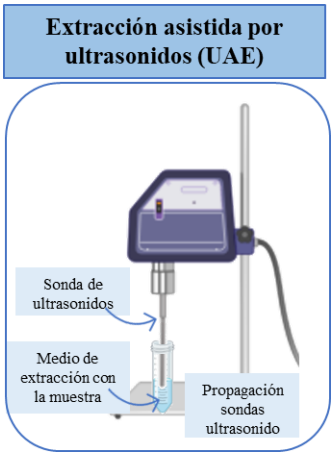

Figura 6. Diagrama de las distintas partes que componen la extracción asistida por ultrasonidos (UAE).

Esto se debe a que permite reducir el tiempo de extracción y los volúmenes de disolvente utilizados de forma considerable. Por este hecho, la UAE es una de las técnicas modernas de extracción más explotadas en los últimos años por sus múltiples ventajas,

siendo empleada para compuestos antioxidantes, compuestos fenólicos e incluso para distintos tipos de contaminantes orgánicos [43]–[45]. La mejora en la extracción se debe al efecto de cavitación que las ondas de los ultrasonidos mejoran la penetración del disolvente en la matriz con el mínimo daño a las propiedades estructurales y moleculares de los compuestos de interés, permitiendo el uso de disolventes y asegura una mejor penetración en la matriz [46], [47]. Además, se puede trabajar en dos modos diferentes de sonicación, en continua o pulsada. Trabajar en modo pulsado puede tener más ventajas de cara a que al aplicar menor energía, además de ser más respetuoso con el medio ambiente, facilita que el extracto no se caliente demasiado y reduce la degradación de compuestos lábiles térmicamente [43].

Tanto en la SLE convencional como en la UAE es importante optimizar diversas variables para que sean además de eficaces, lo más respetuosas con el medio ambiente posible, entre las que se encuentra la selección de un disolvente adecuado en el que sean solubles los analitos diana, su volumen, el pH del medio, la cantidad de muestra, el tiempo y el modo de extracción (ultrasonidos o agitación magnética) y además de todas esas variables, en la UAE también el tipo de sonicación (pulsado o continuo) y la amplitud. Sin embargo, ambas técnicas de extracción pueden dar extractos con elevada turbidez e interferentes de matriz que pueden provocar un mayor deterioro de las columnas cromatográficas y de la fuente de ionización. Además, no son técnicas de extracción selectivas por lo que, en el caso de alimentos, como ya se ha comentado, es necesario realizar una etapa de purificación y/o preconcentración posterior que mejore la etapa de análisis posterior.

1.2.1.2 Técnicas de extracción y purificación basadas en el uso de materiales adsorbentes

Dentro de todas las técnicas que hay disponibles para la preparación de muestra destaca principalmente el uso de materiales adsorbentes, siendo la extracción en fase sólida (SPE) la técnica más clásica para muestras líquidas y extractos de muestras sólidas [48], [49]. El aislamiento, la concentración y la purificación son los enfoques principales en las prácticas de este método. La SPE es un método de preparación de muestras con una

gran diversidad de aplicación debido a sus numerosas ventajas y su gran versatilidad. De hecho, esta técnica ha sido utilizada en analitos de diversas naturalezas tales como residuos de medicamentos veterinarios, contaminantes del procesado, compuestos fenólicos, entre otros [50]–[52].

La SPE en cartucho es el modo utilizado habitualmente, en el que el material adsorbente o fase sólida se empaqueta dentro de un cartucho generalmente entre dos fritas de polipropileno [53], [54]. Básicamente, la SPE se puede emplear de dos formas, o bien los analitos diana quedan retenidos en el material adsorbente mientras que la mayoría de las interferencias pasan por el cartucho y se eliminan y, posteriormente se eluyen con un disolvente adecuado o también se retienen las interferencias en el material y los analitos pasan sin interactuar por el mismo. Esta segunda estrategia suele elegirse cuando el analito está presente en la muestra en concentraciones elevadas y las interferencias pueden retenerse en gran medida. Sin embargo, la forma más empleada en el análisis de contaminantes es la primera estrategia, ya que además se intenta pasar un mayor volumen de extracto que de disolvente de elución, permitiendo realizar una preconcentración [55]. Por tanto, esta estrategia cuenta con cuatro etapas básicas tal y como se muestra en la Figura 7. La primera de ellas es un acondicionamiento del adsorbente con el objetivo de facilitar la posterior interacción de los analitos. Esta etapa consiste en pasar un disolvente para humedecer el material y activar sus grupos funcionales, en el caso que los tenga. En términos generales, el disolvente que se emplea para acondicionar es el mismo que el del extracto o muestra a purificar. En segundo lugar, está la etapa de carga en la que se pasa el extracto por el cartucho y los compuestos más afines se retienen en el adsorbente (analitos) y los menos afines no (interferencias de la matriz). De esta forma, se consigue separar a los analitos diana del resto de componentes de la matriz, los cuales pueden interferir en el análisis posterior. A continuación, se puede hacer una etapa de lavado para eluir a los interferentes de matriz que se hayan quedado retenidos en el adsorbente y evitar que sean eluídos con los analitos en la etapa posterior. Para ello, se añade un disolvente adecuado para eluir los interferentes sin eluir los analitos. Esta etapa de lavado a veces se sustituye por una etapa de secado. Por último, se realiza la etapa de elución donde se recuperan los analitos retenidos en el adsorbente. Para ello es importante utilizar un

disolvente afín a ellos que sea capaz de romper las interacciones entre el analito y el material adsorbente.

Figura 7. Esquema de las etapas del procedimiento de extracción en fase sólida (SPE).

Debido a que la SPE es la técnica de preparación de muestra más clásica y utilizada durante décadas, existen una gran cantidad de materiales comerciales de diferente naturaleza química. De tal forma que permiten cubrir las diferentes interacciones de retención que pueden existir con diversos analitos diana [53]. Los modos comúnmente más empleados en la SPE son la fase normal y la fase inversa. El primero de ellos utiliza un material adsorbente polar y la retención se debe a la interacción entre los grupos funcionales polares del analito y los grupos polares de la superficie del adsorbente. Por otro lado, la fase inversa implica un material adsorbente apolar y se utiliza generalmente para muestras acuosas cuya principal fuerza de interacción son las fuerzas de Van der Waals. También se encuentra la SPE de intercambio iónico que puede utilizarse para compuestos cargados en disolución. Por último, en la SPE de modo mixto se utilizan dos grupos funcionales diferentes en el mismo adsorbente, como por ejemplo grupos de fase inversa y de intercambio catiónico, con el fin de mejorar el potencial de extracción. Entre los tipos de adsorbentes (por ejemplo, alúmina, silicato de magnesio o grafito), la sílice es el más común debido a que presenta muchas ventajas como su idoneidad para la modificación y estabilidad [53].

También hay que destacar que las cantidades de material adsorbente empleado en este tipo de técnicas son en ocasiones algo elevadas. Sin embargo, las tendencias actuales en

el desarrollo de etapas más respetuosas con el medio ambiente son la reducción de las cantidades de material adsorbente y de los volúmenes de disolventes orgánicos [22]. De esta forma surge la variante µ-SPE, que es la forma miniaturizada. Entre los investigadores, existen diferentes criterios sobre qué cantidad de adsorbente o volumen de elución debe utilizarse para que se considere una SPE miniaturizada. Algunos autores han establecido que por debajo de 100 mg puede considerarse miniaturizado, ya que la mayoría de los cartuchos comerciales envasados para SPE suelen estar disponibles con cantidades elevadas de adsorbentes (100-500 mg) [53].

La SPE o µ-SPE puede presentar algunos inconvenientes como el bloqueo del paso del extracto debido a la obstrucción de los poros del adsorbente sobre todo con extractos más complejos o viscosos. Además, para llevar a cabo cada uno de estos pasos un parámetro crítico es controlar el caudal del extracto que debe ser constante y controlado, dejando caer dos gotas aproximadamente por segundo, para permitir la correcta interacción entre los analitos y el material adsorbente o disolvente de elución correspondientes (también para evitar la canalización). Es por ello que se han desarrollado algunas alternativas más novedosas que permitan solucionar estas limitaciones [56]. Una de las más populares utilizada en la última década es la extracción en fase sólida dispersiva (dSPE) y su variante miniaturizada (µ-dSPE) [57]. A diferencia de en la SPE en la (µ-)dSPE, no se empaqueta el material en un cartucho, sino que se pone en contacto directamente con el extracto que contiene los analitos y se mantiene en agitación durante un tiempo (discontinuo), evitando el paso de acondicionamiento y el tener que controlar y mantener el caudal de forma constante y adecuada, teniendo que controlar el tiempo. Posteriormente, se puede hacer una etapa de lavado para eliminar los interferentes de la matriz y, finalmente, para separar el material se hace un paso de filtración, sedimentación o centrifugación para separar el material adsorbente y recuperar el extracto purificado con los analitos para su posterior análisis [58]. Por este hecho, la (µ-)dSPE es una técnica alternativa con una gran variedad de campos de aplicación debido a que en algunas ocasiones simplifica el procedimiento, reduce el tiempo de extracción/limpieza y las cantidades de material empleadas [55], [59].

El inconveniente de este proceso es que separar el material de los analitos adsorbidos puede ser una tarea tediosa. Por este hecho, una alternativa que ha ganado popularidad en los últimos años es la extracción en fase sólida magnética (MSPE) donde el material adsorbente empleado es de naturaleza magnética y se separa de la muestra mediante el uso de un imán externo [60], permitiendo que esta etapa sea más rápida y sencilla [37]. Además, el alto potencial de los materiales magnéticos ha permitido miniaturizar los procedimientos de extracción, reduciendo las cantidades de adsorbentes y disolventes en los, dando lugar también a la aparición de su versión miniaturizada (µ-MSPE) [56]. Tal y como se muestra en la Figura 8, la (µ-)MSPE consiste en dispersar el material magnético en un extracto durante unos minutos en un baño de ultrasonidos o en un agitador orbital. Una vez que se ha alcanzado el equilibrio de reparto del analito entre el líquido y el material, el material se recupera con la ayuda de un imán externo y se desecha el sobrenadante. Posteriormente, se puede llevar a cabo una etapa de lavado para desorber los interferentes de la matriz. Y, finalmente, los analitos se desorben con un disolvente de polaridad adecuada poniéndolo en contacto con el material durante unos minutos en el agitación (generalmente en baño de ultrasonidos). Una vez que se han desorbido los analitos, se vuelve a utilizar el imán para aislar el material y recuperar el sobrenadante que en este caso contendrá los analitos a analizar [61], [62]. Debido a las ventajas que presenta esta variante hay una tendencia en su empleo en una gran variedad de analitos, desde trazas de contaminantes en muestras de alimentos, como residuos de pesticidas y medicamentos veterinarios, aditivos poliméricos e iones de metales pesados [60].

Figura 8. Esquema de las etapas del procedimiento de extracción en fase sólida magnética (MSPE).

Los adsorbentes magnéticos son nanopartículas (MNPs, que proviene de *magnetic nanoparticles* en inglés) que se basan principalmente en un núcleo magnético generalmente formado por óxidos de hierro, entre los que se encuentra la magnetita (Fe_3O_4), la hematita (α-Fe_2O_3) y la maghemita (γ-Fe_2O_3). Aunque también existen otros materiales con respuesta magnética como los óxidos mixtos de Ni, Co, Mn, Cu y Fe. Sin embargo, los óxidos de hierro, especialmente las MNP de Fe_3O_4 son los materiales más populares debido a que presentan excelentes características como alto poder magnético, síntesis simple y bajo coste de producción, además de pequeño tamaño y alta área superficial que aumenta su capacidad de sorción [61], [63]. Los métodos de síntesis de MNPs incluyen la co-precipitación química, la pirólisis, la síntesis solvotermal y el método de micro-emulsión [64]–[66]. De todos ellos, la co-precipitación química es el método más simple y más comúnmente utilizado para preparar MNPs debido a su corto tiempo de reacción, operación simple, bajo coste y presenta un tamaño controlado de partícula con buenas propiedades magnéticas [67]. Para llevarlo a cabo, se utilizan agentes de precipitación compuestos por una determinada proporción de solución salina ferrosa y férrica para producir una reacción de co-precipitación química en condiciones alcalinas. La reacción de co-precipitación es la siguiente:

$$Fe^{2-} + 2Fe^{3+} + 8OH^- = Fe_3O_4 + 4H_2O$$

Teóricamente, la reacción procede sin problemas en la proporción molar 1:2 de Fe^{2+} y Fe^{3+}. Como el Fe^{2+} es extremadamente fácil de oxidar en el aire, es necesario aumentar el contenido de Fe^{2+} en el proceso para compensar su pérdida por oxidación durante la reacción [61]. Durante el proceso de co-precipitación, factores como la temperatura de reacción, pH y fuerza iónica y la relación $Fe2^+/Fe3^+$ pueden influir en la morfología, el tamaño y la composición de los MNPs preparados por este método. Sin embargo, debido a la precipitación no homogénea de los iones metálicos, los materiales sintéticos se aglomeran fácilmente, y el rango de distribución del tamaño de partícula puede ser amplio.

Además de los avances y tendencias de miniaturización de las técnicas mencionadas, también se tiende hoy día a la búsqueda de su automatización [71]. Es por ello por lo que, se ha desarrollado la técnica de micro-extracción por adsorbentes empaquetados (MEPS).

La técnica MEPS es una miniaturización interesante de la técnica SPE que se caracteriza por tener una pequeña cantidad de sorbente (generalmente de 1 a 4 mg, 50 µm de diámetro) empaquetado en el cilindro de la jeringa, como un tapón, o entre la aguja y el cilindro como un cartucho [69]. Como el empaque está integrado directamente en la jeringa, MEPS puede manejar volúmenes entre 10 y 1000 µL, sin comprometer la eficiencia de extracción. A partir de esta, se ha desarrollado otra variante de micro-extracción automatizada llamada µ-SPEed® (Figura 9). Esta técnica consiste en un novedoso formato introducido en el mercado por la empresa EPREP (Victoria, Australia) y permite extracciones más eficientes que el sistema MEPS [69], [70]. Específicamente, los cartuchos µ-SPEed® contienen sorbentes con partículas aún más pequeñas que los MEPS (≤ 3 µm), que proporcionan un área de superficie mayor y separaciones más eficientes. También contienen una válvula de retención unidireccional accionada por presión que soporta altas presiones de hasta 1200 psi. Esto permite una conexión de bajo volumen muerto y una ruta de flujo de una sola dirección a través del lecho adsorbente en cada paso del protocolo de extracción.

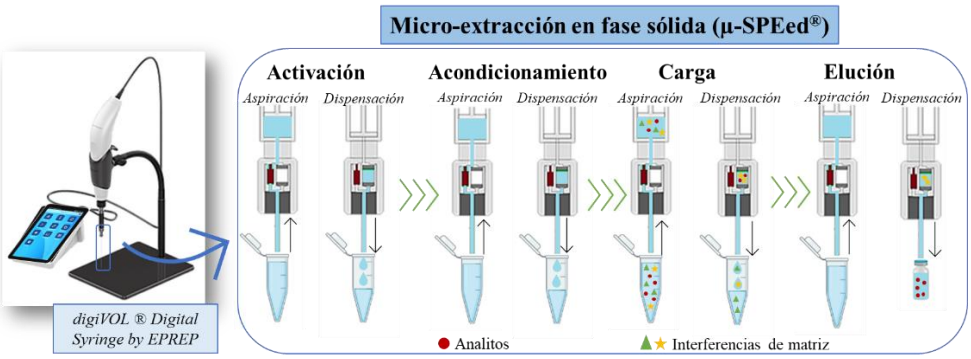

Figura 9. Diagrama de las etapas del procedimiento de micro-extracción en fase sólida (µ-SPEed®).

Actualmente se comercializan diferentes tipos de cartuchos µ-SPEed® con diferentes adsorbentes [69]. Se han desarrollado metodologías para el análisis de polifenoles en diferentes matrices alimentarias utilizando µ-SPEed® que demuestran que esta técnica proporciona métodos más rápidos y económicos que los SPE convencionales [71], [72]. Y además, cada vez se están comenzando más a implementar en la seguridad alimentaria, como por ejemplo para el análisis de alcaloides pirrolizidínicos [73] y alcaloides

tropánicos [74]. Además, estos cartuchos pueden ser reutilizados multitud de veces sin verse afectado la eficacia de la extracción, por lo que supone un ahorro económico importante y una ventaja más de esta técnica miniaturizada.

Preparación de nuevos materiales adsorbentes

En las técnicas de preparación de muestra basadas en el uso de fases sólidas adsorbentes, el uso de materiales que extraigan eficazmente los analitos diana es un factor de vital importancia [75]. Hoy día debido a la expansión que ha sufrido el campo de desarrollo y aplicación de diferentes tipos de materiales avanzados en la etapa de preparación de muestra, uno de los parámetros más críticos para su eficacia es el tipo de material adsorbente empleado, ya que para que el procedimiento sea efectivo se deben de formar las interacciones necesarias para poder retener los analitos diana de forma correcta [76]. Las diferentes interacciones que pueden producirse son las de Van der Waals, enlace de hidrógeno, dipolo-dipolo o electrostática (intercambio eiónico). Por este motivo, la selección del material puede hacerse en función de la matriz alimentaria, los analitos de interés y las interferencias de matriz [53], [56].

Además, para conseguir miniaturizar los procedimientos convencionales, muchos autores han desarrollado nuevos materiales, no sólo para empaquetar sus propios cartuchos, sino también con el objetivo de diseñar adsorbentes potenciales para la preparación de muestras con características específicas [56]. Esto tiene como objetivo hacer los procesos más eficientes y que permitan disminuir la cantidad de material empleado en cada extracción, lo que hace que también se reduzca el uso de disolventes empleados y sea una metodología más respetuosa con el medio ambiente siguiendo los criterios GAC y GSP [56].

Aplicación de sílices mesoestructuradas como materiales adsorbentes en (µ-)SPE

Las sílices mesoestructuradas han sido ampliamente utilizadas como materiales adsorbentes en SPE para extraer y/o purificar extractos de distintas matrices alimentarias con diversos tipos de compuestos debido a sus numerosas ventajas. Es por ello por lo que

nuestro grupo de investigación cuenta con una amplia experiencia en el campo de las sílices mesoestructuradas ya que han sintetizado y aplicado este tipo de materiales en numerosas metodologías para analizar alimentos.

Estos materiales tienen una estructura mesoporosa ordenada que según la I.U.P.A.C. (Unión Internacional de Química Pura y Aplicada), se definen con tamaños de poro comprendidos entre 20 y 50 nm, alto volumen de poro y de área superficial [54], [77]. El descubrimiento de estos materiales fue por parte de investigadores de *Mobil Research and Development Corporation* en 1992 y se denominaron M41S. Los materiales que formaron esta familia fueron MCM (*Mobil Composition of Matter*), concretamente MCM-41 con fase hexagonal, MCM-48 con fase cúbica y MCM-50 en capas.Estos materiales tienen una estructura mesoporosa ordenada que según la I.U.P.A.C. (Unión Internacional de Química Pura y Aplicada), se definen con tamaños de poro comprendidos entre 20 y 50 nm, alto volumen de poro y de área superficial [54], [77]. El descubrimiento de estos materiales fue por parte de investigadores de *Mobil Research and Development Corporation* en 1992 y se denominaron M41S. Los materiales que formaron esta familia fueron MCM (*Mobil Composition of Matter*), concretamente MCM-41 con fase hexagonal, MCM-48 con fase cúbica y MCM-50 en capas.

Para llevar a cabo la síntesis de estos materiales mesoestructurados hay varios métodos diferentes como por ejemplo el método sol-gel, el hidrotérmico, a temperatura ambiente, con microondas, mediante precipitación y no acuoso. Sin embargo, generalmente, se suele utilizar de forma general precursores empleando el proceso hidrolítico sol-gel en el que participa un agente de plantilla o director de estructura, frecuentemente un tensioactivo en disolución acuosa [54], [77]. La síntesis de estos materiales se basa en la utilización de tensioactivos para dirigir la estructura del material, una fuente de silicio, que la más común suele ser tetraetilortosilicato (TEOS), un agente mineralizante y agua. La estructura de la sílice está influenciada por las condiciones de la síntesis, tales como la relación agente director/fuente de silicio, naturaleza del surfactante, temperatura de reacción, pH y tiempo [78].

Por otro lado, hay que destacar que el grupo de Zhao y col. en 1998 sintetizó un grupo de estructuras de sílice mesoporosas altamente ordenadas mediante el uso de tensioactivos

oligoméricos de poli(óxido de etileno) y copolímeros tribloque hidrofílico como plantilla. De esta forma, sintetizó una nueva serie de sílice mesoestructurada denominada SBA-n (*Santa Barbara Amorphous*), siendo n = 1-16 que representa a distintos materiales, según su estructura y características texturales. SBA-15 tiene una estructura hexagonal y es el más estudiado del grupo ya que presenta una alta estabilidad hidrotérmica al tener una pared más gruesa, amorfa y con poros más grandes que los materiales M41S [54], [78]. La síntesis de la SBA-15 es similar a los materiales M41S, usando una fuente de silicio (generalmente TEOS), un copolímero tribloque denominado Pluronic 123 (EO20-PO70-EO20) como plantilla y condiciones ácidas con HCl.

A partir de estas series de materiales se han desarrollado numerosos materiales de sílice mesoporosa como HMS (*Hexagonal Mesoporous Silica*) con una geometría hexagonal desordenada conocida como "agujero de gusano". Para su síntesis se utilizan surfactantes neutros y sílice no ionizada. [54].

Los materiales de sílice mesoestructurada con tamaño de partícula desde micrómetros hasta nanómetros se han utilizado ampliamente en diversos campos como la adsorción, separación, almacenamiento de energía, biofarmacia y para su uso como adsorbente para SPE debido a su estructura mesoporosa ordenada y su gran área superficial (1600 m^2/g). Durante la síntesis de estos materiales, tanto en la superficie como en el interior de los poros hay átomos de silicio que se unen a grupos OH^- dando lugar a la formación de los denominados grupos silanol (Si-OH). Por este motivo, es muy común funcionalizar estas sílices con diversos tipos de ligandos, como grupos orgánicos, compuestos de coordinación, polímeros, entre otros. De esta forma, las sílices mesoestructuradas se han aplicado para extraer diversos analitos, tales como iones metálicos, contaminantes orgánicos o fármacos tanto de muestras medioambientales, alimentarias, como biológicas, mediante interacciones hidrofóbicas, hidrofílicas, π-π, quelación, enlace de hidrógeno, electrostáticas y por reconocimiento molecular [54].

Para llevar a cabo la funcionalización de estos materiales de sílice mesoestructurada existen principalmente dos estrategias: el método post-síntesis y el método de co-condensación. El primero de ellos también se denomina método de anclaje, en el que se modifica la sílice una vez que ya ha sido sintetizada. Y el método de co-condensación

implica una síntesis directa de una sola etapa mediante la condensación simultánea de la sílice con el ligando o precursor orgánico. Con esta última estrategia se pueden obtener mayores grados de funcionalización. Sin embargo, en la funcionalización post-síntesis existe un ordenamiento estructural en todo momento, las estructuras son más definidas y con mayor resistencia. Por otro lado, se requiere el uso de un gran volumen de disolvente para eliminar el surfactante del material en la estrategia por co-condensación, lo que con la estrategia de post-síntesis se evita calcinando la sílice directamente ya que todavía no tiene anclados los ligandos orgánicos y por tanto no se degrada el material.

Aplicación de partículas magnéticas como materiales adsorbentes en (µ-)MSPE

En el caso de la (µ-)MSPE, todavía no se han comenzado a comercializar materiales adsorbentes magnéticos. Esto es debido a que este tipo de técnica se ha desarrollado en mayor medida en los últimos años y todavía se están optimizando las síntesis de este tipo de materiales. Es por ello por lo que el desarrollo y la optimización de la síntesis de materiales magnéticos es un objetivo muy importante en la etapa de preparación de la muestra con (µ-)MSPE [61].

Para que las MNPs sean estables, evitar que se oxiden, proporcionar una dispersión coloidal adecuada y lograr una extracción selectiva de los analitos diana, es necesario modificar su superficie con algunos grupos funcionales [79]. Por esta razón, MNPs suelen recubrirse con un material orgánico o inorgánico adecuado, como por ejemplo con carbono, polímeros, sílice, estructuras metal-orgánicas, entre otros [60], [63]. En base al tipo de funcionalización, el mecanismo de interacción que se puede originar entre el material y el analito objetivo puede ser, tal y como se muestra en la Figura 10, de diversas formas, como, por ejemplo, meso y microporoso, de impresión molecular, interacción hidrofóbica, interacción anticuerpo-antígeno, de conjugación o iónica.

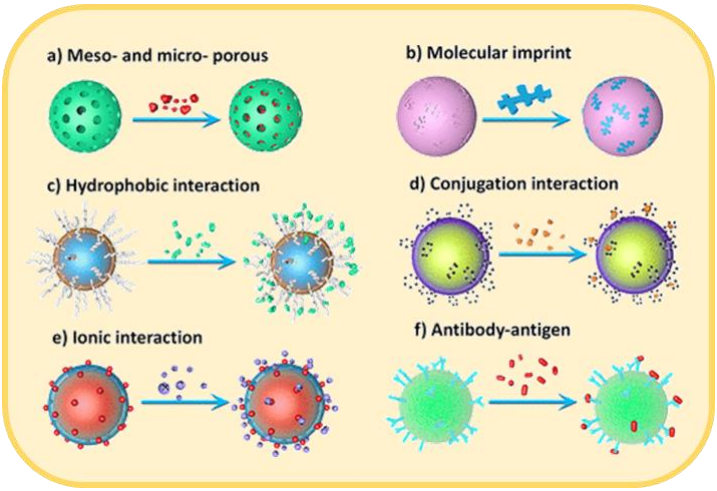

Figura 10. Mecanismo de interacción entre MNP y el analito objetivo utilizando diferentes modificaciones superficiales de MNP: (a) meso y microporoso; (b) impresión molecular; (c) interacción hidrofóbica; (d) interacción anticuerpo-antígeno; (e) interacción de conjugación; (f) interacción iónica (adaptado de Yu y col. 2022 [61]).

Una de las modificaciones superficiales de las MNP más populares es con sílice ya que puede proteger eficazmente a las MNP de la oxidación al tiempo que preserva su magnetismo. Además, también les hace térmicamente estables, de tamaño controlable y estables a un entorno ácido, lo que es favorable cuando se utilizan como adsorbentes. Y una de las características más importantes es que los MNP recubiertos de sílice son muy versátiles para la funcionalización con otros grupos funcionales como -NH_2, grafeno, cadenas alquílicas, así como de otras moléculas orgánicas como por ejemplo son las ciclodextrinas (CD). Cabe destacar que en los últimos años se ha producido un creciente interés de la comunidad científica en el uso de ligandos más respetuosos con el medio ambiente como es el caso de las CD. Estas moléculas son productos naturales obtenidos a partir de degradación enzimática del almidón y por tanto tienen un bajo coste y se consideran verdes y no tóxicas [80], [81].

La mayoría de los métodos para la adición de una capa de sílice utilizan TEOS como reactivo de recubrimiento en un entorno alcalino débil en una solución de etanol o isopropanol/agua. La hidrólisis del TEOS para formar óxido de silicio se produce fácilmente y se puede injertar una capa uniforme de sílice en las MNPs obteniendo partículas esféricas, monodispersas y su tamaño puede ajustarse con un delicado control

de las condiciones de reacción, principalmente la agitación [61]. Esta síntesis produce una modificación superficial de la Fe_3O_4 con sílice amorfa, la cual presenta una estructura desordenada. En los últimos años, ha crecido el interés de añadir una segunda capa de sílice mediante la adicción de un surfactante-plantilla que provoca una mayor ordenación de los poros de la sílice, consiguiendo una estructura mesoestructurada, la cual aporta una mayor ordenación de los poros y una mayor área superficial. El procedimiento de síntesis se basa en depositar bromuro de cetiltrimetilamonio (CTAB) como plantilla con sílice sobre las partículas magnéticas recubiertas de sílice amorfa. Posteriormente se elimina la plantilla mediante calcinación dando lugar a las partículas magnéticas recubiertas de sílice mesoestructurada [82].

Otro de los tipos de modificación superficial más empleados es el de la utilización de compuestos hidrófobos, conjugados e iónicos directamente sobre las MNPs [61]. Concretamente, la modificación superficial conjugada se refiere a la de múltiples grupos aromáticos, dando lugar a interacciones π-π entre los grupos aromáticos en la superficie de las MNPs y el analito diana, dando lugar a una MSPE más selectiva.

Caracterización estructural de los nuevos materiales adsorbentes

Una vez sintetizados los diversos materiales adsorbentes, antes de ser empleados como materiales adsorbentes en la etapa de preparación de muestra, se pueden utilizar distintas técnicas de caracterización para poder conocer sus propiedades estructurales, morfológicas, texturales o químicas. De esta forma, se puede verificar que la síntesis de un material o su funcionalización se ha realizado de forma correcta. En la Tabla 1 se recogen las técnicas de caracterización aplicadas en la presente Tesis Doctoral con la principal información que aporta cada una de ellas.

Tabla 1. Tipos de técnicas de caracterización empleadas en la presente Tesis Doctoral con la información que aporta cada una de ellas.

Técnica de caracterización	Información aportada
Microscopía electrónica de barrido (SEM)	Imágenes para determinar la morfología, si las partículas que componen el material tienen forma esférica o más alargada. Además, permite evaluar el tamaño de las partículas y ver si tienen un tamaño homogéneo.
Microscopía electrónica de transmisión (TEM)	Imágenes para determinar el tamaño de las partículas y al permitir obtener imágenes con más aumentos permite hacer una estimación de la distribución del tamaño de poro.
Difracción de Rayos-X (DRX)	Permite realizar la determinación a bajo y a alto ángulo. La medida a bajo ángulo permite identificar fases cristalinas y ver si el material silíceo sintetizado tiene una estructura mesoestructurada. La medida a alto ángulo permite determinar si el material magnético sintetizado todavía posee las bandas características de la magnetita y en ese caso se demuestra que tras la modificación superficial y las funcionalizaciones no se ha alterado la estructura interna del material.
Rayos X de fluorescencia (XRF)	Permite evaluar la composición química del material, obteniendo, entre otros, el contenido de hierro y sílice (expresado en % de peso).
Espectroscopía infrarroja de transformada de Fourier (FT-IR)	Permite evaluar la composición química del material, obteniendo las bandas de enlaces presentes en el material que son característicos de los mismos. De esta forma, permite hacer una determinación rápida, siendo muy útil para evaluar si la incorporación de grupos funcionales se ha realizado de forma correcta o si se ha llevado a cabo una total eliminación del surfactante. Además, la intensidad de las bandas está directamente relacionada con la concentración con lo que se puede utilizar para cuantificar o para hacer una comparación cualitativa.
Fisisorción de N_2	Permite determinar el volumen de poro, el área superficial o la distribución del tamaño de poro del material. Los resultados dan lugar a una isoterma de adsorción-desorción cuya forma está relacionada con la porosidad del material. El área superficial específica (S_{BET}) se calcula con el método Brunauer-Emmett-Teller (BET). Y el modelo Barret-Joyner-Halenda (BJH) es usado para calcular el volumen de poro mediante las ramas de desorción de las isotermas y el volumen total de poro a partir de la cantidad desorbida a una presión relativa dada.
Análisis Elemental (AE)	Permite determinar el contenido orgánico de un material, concretamente la cantidad de N, H, C y S (%). De esta forma, se evalúa el grado de funcionalización de los materiales y sirve para comparar distintas condiciones de síntesis y confirmar con cuál de ellas se obtiene una mayor funcionalización.

1.2.2 Técnicas de análisis cromatográficas

La etapa de preparación de muestra es crucial para el desarrollo de una metodología de análisis eficaz y con buen rendimiento. Sin embargo, también es muy importante la aplicación de una técnica de análisis que permita identificar y cuantificar una amplia gama de analitos que puedan encontrarse en muestras de alimentos a bajos niveles de concentración y que dicho análisis se produzca de manera rápida, selectiva y sensible. Actualmente las técnicas cromatográficas como la cromatografía de gases (GC) y la cromatografía de líquidos de alta eficacia (HPLC) son las más empleadas para llevar a cabo la determinación de contaminantes en alimentos [83].

La cromatografía es una técnica de análisis separativa que permite separar de forma individual compuestos presentes en una muestra compleja. La separación consiste en la distribución de los analitos entre dos fases, una fase estacionaria (líquida o sólida) y una fase móvil (líquida, gaseosa o de fluido supercrítico). Concretamente, en GC se inyecta de manera general una muestra líquida a una temperatura lo suficientemente alta como para volatilizar los compuestos que pasan impulsados por la fase móvil a la columna cromatográfica que contiene la fase estacionaria y se encuentra en un horno a temperatura programada. Como fase móvil se emplea un gas inerte cuya función es arrastrar los componentes hasta el detector a través de la fase estacionaria con la que interaccionan en función de su grado de afinidad o polaridad. Por tanto, la separación de los componentes o analitos se debe tanto a su polaridad como a su grado de volatilidad, de manera que a polaridad similar será el más volátil el que menor tiempo de retención presente en el análisis. La GC ha sido una técnica de análisis muy empleada para la determinación de compuestos volátiles y térmicamente estables en alimentos. Además, se puede empelar a compuestos no volátiles añadiendo un paso previo de derivatización para hacerlos más volátiles, que por otro lado puede implicar un mayor tiempo de análisis, coste y energía [83].

En los últimos años se ha utilizado cada vez más la técnica de HPLC ya que puede analizar tanto compuestos volátiles como no volátiles con independencia de su estabilidad térmica, ya que en la mayoría de los casos se trabaja a temperatura ambiente. En este caso,

la separación se produce cuando los analitos son empujados por una fase móvil líquida a través de la fase estacionaria y debido a la diferente afinidad que presenten los componentes entre ambas fases. Actualmente, el tipo de cromatografía de líquidos más empleado es la cromatografía de reparto (los analitos se distribuyen entre ambas fases líquidas) y ésta puede ser a su vez en fase normal, cuando la fase estacionaria es polar, generalmente se utiliza sílice recubierta con grupos polares como cianopropilos (CN) o aminopropilos (NH_2) o en fase reversa, cuando la fase estacionaria es apolar, generalmente sílice con octadecilsilano (C_{18}), siendo éste último el tipo de cromatografía más común [84]. La fase móvil cuando se emplea la fase normal es 100% orgánica (disolventes de baja polaridad) y cuando se emplea la fase reversa consiste en un disolvente o mezcla de disolventes, siendo lo más común una mezcla de una disolución acuosa y un disolvente orgánico como el MeOH o el AcN. Tras inyectar la muestra en la columna cada analito avanza a lo largo de la columna cromatográfica con una velocidad diferente que estará influenciada por su afinidad por cada una de las dos fases [83]. En HPLC las partículas empleadas para la fase estacionaria son de sílice con un diámetro de partícula entre 3 y 5 µm. Sin embargo, una variante que ha ganado popularidad en los últimos años es la cromatografía de líquidos de ultra-alta resolución (UHPLC). La principal diferencia es que las columnas cromatográficas empleadas son más cortas y están empaquetadas con partículas de un tamaño inferior a 2 µm. Estas características permiten mejorar la eficacia de la separación mejorando la resolución de los compuestos en tiempos más cortos, lo que produce además una reducción del consumo de disolventes [85].

Los distintos tipos de cromatografía pueden ir acoplados a diversos tipos de detectores, los cuales son los dispositivos que permiten medir una propiedad física del eluyente a la salida de la columna cromatográfica. Para GC los detectores más comunes son el de ionización de llama (FID), de conductividad térmica (TCD), de captura de electrones (ECD), de fotoionización (PID), y de espectrometría de masas (MS), entre otros. Y, para HPLC los detectores que se pueden emplear son el ultravioleta-visible (UV-Vis), diodo array (DAD), fluorescencia, electroquímico, quimioluminiscencia, infrarrojo y MS, entre otros. Tanto para GC como para HPLC, la MS ha sido la técnica más importante en los últimos años, sobre todo para análisis de componentes a muy baja

concentración, debido a que es una técnica universal, específica, altamente sensible y recomendada en la mayoría de las normas internacionales descritas para el análisis de contaminantes en alimentos. Esto se debe a que además de que permite identificar y cuantificar los compuestos, permite confirmar su presencia, debido a que proporciona información sobre la masa y la estructura de los compuestos. Por ello, la mayoría de los estudios que se realizan en la actualidad sobre contaminantes químicos en alimentos emplean este tipo de detector [86].

El principio de la espectrometría de masas es la formación de iones a partir de compuestos neutros en estado gaseoso y la medida de la subsiguiente descomposición de estos iones en fragmentos. Los iones descompuestos (fragmentos que también poseen carga) se mueven rápidamente (sometidos a campos eléctricos) y son "clasificados" de acuerdo con su relación *m/z* (masa/nº de cargas del ion) y contados (se mide la cantidad de cada ion formado), dando lugar a un espectro de masas, que puede considerarse como la "huella digital de la sustancia". Cuando a una molécula en estado gaseoso se le suministra una determinada energía la molécula se descompone siguiendo un patrón concreto en el que se obtienen los mismos fragmentos y en la misma relación de intensidad. Para que todo esto tenga lugar, los espectrómetros de masas están formados por tres componentes fundamentales: la fuente de ionización, el analizador de masas y el detector, y dependiendo de cómo son la fuente de ionización y el analizador se puede obtener un espectro de masas con más o menos fragmentos. Cuando la molécula se ioniza, generalmente lo hace con una carga de +1 o -1. Por tanto, la relación *m/z* corresponde a la masa molecular del analito (M) más 1 (M+1) o menos 1 (M-1). En el caso de perder un electrón o ganar un protón, el analito estará cargado positivamente, mientras que al ganar un electrón o perder un protón el compuesto estará cargado negativamente. Estos iones moleculares pueden fragmentarse y una vez son todos analizados en el analizador, llegan al detector donde se produce una señal eléctrica que se representa en el espectro de masas [86], [87].

Las fuentes de ionización más empleadas dependen del tipo de cromatografía que se esté empleando. En GC las fuentes más usadas son la de impacto de electrones (EI) y la de ionización química (CI), siendo la de EI la más común y la que se empleó en una de

las metodologías de análisis desarrolladas en esta Tesis Doctoral. Particularmente, tal y como se muestra en la Figura 11, el eluyente de la columna de GC llega a la fuente donde colisiona con un haz de electrones a alta energía (70 eV generalmente), producidos por un filamento incandescente de tungsteno. Si la energía transferida supera la energía de ionización de las moléculas se produce la ionización de esta, mediante pérdida de un electrón, por lo que se generan iones positivos. Generalmente debido a la alta energía utilizada se produce también la formación de fragmentos de esta molécula ionizada. Después, los iones son acelerados mediante la aplicación de un voltaje hasta el analizador de masas. La eficacia de la ionización se aumenta colocando la fuente interna entre los polos de un pequeño imán, lo que hacen que los electrones vayan con una trayectoria helicoidal [88].

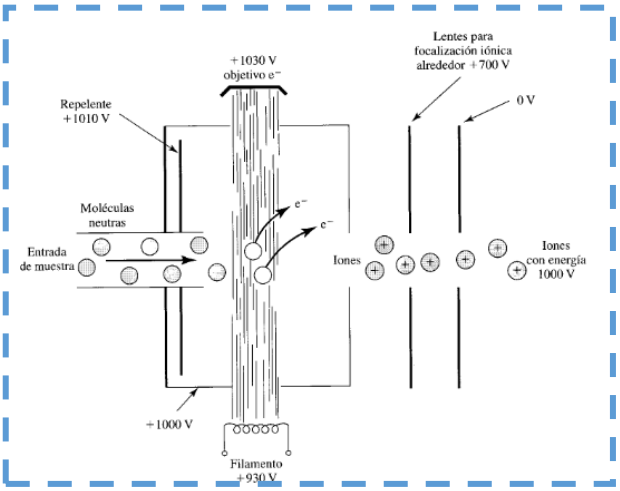

Figura 11. Esquema de la fuente de ionización de tipo impacto de electrones (EI) (extraído de Rubinson y Rubinson, 2001 [86]).

Por otro lado, en cromatografía de líquidos (LC) también hay diversos tipos de fuentes de ionización, las más utilizadas son la ionización por electrospray (ESI), la ionización química a presión atmosférica (APCI) o la desorción/ionización láser asistida por matriz (MALDI). En esta Tesis Doctoral se ha utilizado la ESI, la cual es la fuente de ionización más empleada debido al amplio espectro de moléculas que es capaz de ionizar, ya que permite la ionización de moléculas que puedan cargarse tanto de forma positiva como negativa, así como de moléculas con carga en su estructura interna y moléculas que no poseen carga en su estructura, para las que se adicionan en la fase móvil un ácido o base

volátil que favorezca la formación de la carga. Concretamente, tal y como se muestra en la Figura 12, la fase eluyente de la columna del (U)HPLC llega a la fuente donde pasa a través de un capilar, al cual se le aplica un alto potencial eléctrico. De esta manera, se forma una nube de finas gotas cargadas eléctricamente. El gas nebulizador, generalmente N_2, facilita la evaporación del disolvente hasta que se forma el gas "nebulizado" y los iones pasan de estado líquido a gaseoso que son atraídos hasta un capilar gracias a un campo eléctrico, a través del cual son transportados hasta el analizador de masas [89]. Con este tipo de fuente de ionización pueden obtenerse iones multicargados y generalmente se obtienen iones moleculares, con poca fragmentación.

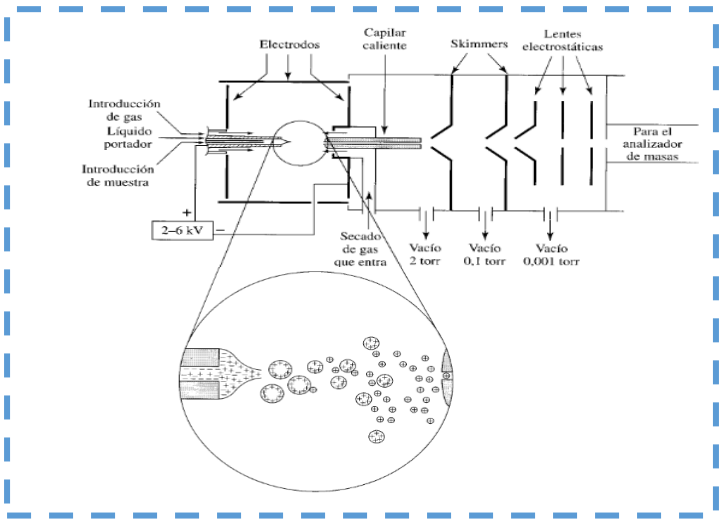

Figura 12. Esquema de la fuente de ionización de ionización por electrospray (ESI) (extraído de Rubinson y Rubinson, 2001 [86]).

En relación con los analizadores de masas destacan el cuadrupolo (Q), la trampa de iones (IT), el tiempo de vuelo (TOF) y la transformada de Fourier (FT). El analizador empleado en esta Tesis Doctoral ha sido el cuadrupolo (Q), en el caso del equipo de GC y el triple cuadrupolo (TQ) en el caso de los equipos de (U)HPLC. Los analizadores de masas cuadrupolares constan de cuatro barras paralelas, cuyos extremos muestran la forma de un diamante, espaciadas a igual distancia de un punto central. Todos los iones son acelerados a través del canal entre las barras, pero sólo aquellos iones con valores *m/z* especificados pueden alcanzar el detector. Los iones fuera del rango *m/z* especificado

colisionan con las barras y se convierten en moléculas neutras. Las corrientes alternas tienen ciclos positivos y negativos, durante los ciclos positivos, los iones cargados positivamente son repelidos y se desplazan hacia el centro del canal entre las barras. Mientras que, durante los ciclos negativos, los iones cargados positivamente son atraídos y desviados hacia las barras.

En el caso del analizador de TQ, se disponen tres cuadrupolos colocados linealmente (Figura 13) y se utilizan para obtener más información sobre el compuesto analizado. En este caso, los iones seleccionados en función de su masa por el primer cuadrupolo (generalmente iones moleculares) colisionan con un gas de colisión en el segundo cuadrupolo y sufren una disociación inducida o fragmentación. Los diferentes iones fragmento generados como resultado de estas colisiones se someten a un análisis de masa (*m/z*) en el tercer cuadrupolo antes de entrar en el detector [90].

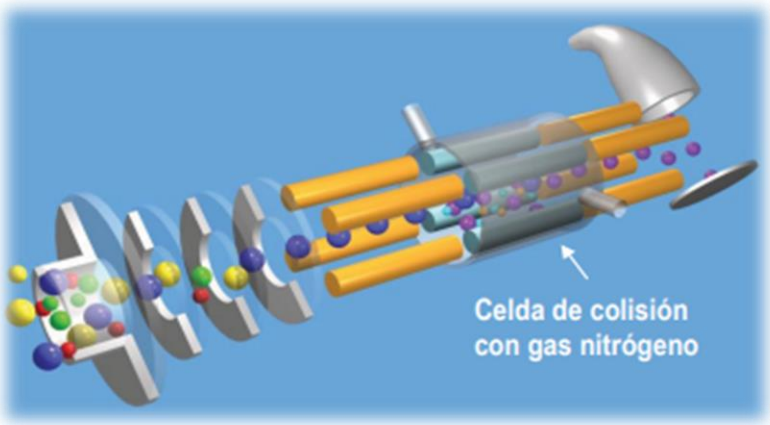

Figura 13. Esquema de un analizador de tipo triple cuadrupolo (extraído de Agilent Technologies [91]).

Dependiendo del tipo de analizador empleado en MS, hay diferentes modos de trabajo. Se puede trabajar en modo full scan, es decir, se hace un barrido de masas entre un determinado intervalo de *m/z* y en modo de monitorización de iones seleccionados (SIM), es decir, fijando las condiciones para que sólo pase un ion con una determinada *m/z*. En esta Tesis Doctoral se empleó el modo SIM una vez optimizada la detección en full scan para llevar a cabo la cuantificación de los analitos de una forma más selectiva y sensible en el caso de GC-MS. Por otro lado, los modos de trabajo más empleados para

registrar multitud de analitos cuando se emplea un analizador de TQ, son el modo de monitorización de reacción múltiple (MRM) y el modo MS/MS de barrido completo. En esta Tesis Doctoral se empleó MRM, después de optimizar la detección de los analitos realizando una infusión directa de los mismos. El modo MRM consiste en que los iones precursores con una *m/z* única son seleccionados en el Q1 y pasan a la celda de colisión donde se generan los fragmentos iónicos (por colisión con moléculas de N_2) y en el Q3 se seleccionan los iones con una *m/z* única de fragmento específico. La principal diferencia entre barrido completo y MRM es la función del barrido, que hace que sea menos sensible que el MRM. En este caso, Q3 se escanea secuencialmente permitiendo el paso por el detector de 1 *m/z* cada vez, generando un espectro de ion del producto [91]. Este permite registrar espectro de MS^2 para cada analito mejorando la selectividad.

OBJETIVOS

2. OBJETIVOS

Como se ha puesto de manifiesto en la Introducción, mejorar la seguridad alimentaria forma parte de los ODS marcados por la ONU y es por tanto un reto muy importante en la investigación actual, ya que aún quedan muchos aspectos por resolver. Uno de estos aspectos está relacionado con la mejora del control de las toxinas naturales en los alimentos. Para ello, es necesario desarrollar nuevas metodologías analíticas que sean eficaces para cuantificar estas toxinas, concretamente OAs, que en los últimos años han originado un elevado número de alertas sanitarias y casos de intoxicaciones alimentarias. Estas metodologías deben ser lo más respetuosas con el medio ambiente posible, reduciendo el uso de disolventes orgánicos, la producción de residuos etc., contribuyendo de esta manera, a mejorar la sostenibilidad de los análisis, como otro de los grandes retos actuales.

Por todo ello, el objetivo general de esta Tesis Doctoral ha sido el desarrollo de métodos analíticos avanzados basados en novedosas etapas de preparación de muestra miniaturizadas mediante la aplicación de nuevos materiales adsorbentes para la mejora de la extracción y/o purificación por un lado y en el uso de técnicas micro-extractivas por otro, seguidos de análisis mediante cromatografía acoplada a espectrometría de masas con la finalidad de mejorar el control de OAs en alimentos de una forma más eficaz y sostenible.

Para llevar a cabo este objetivo general se han planteado los siguientes objetivos específicos:

- **Objetivo 1:** Preparación de nuevos materiales adsorbentes con el fin de evaluar su potencial empleo en la etapa de preparación de muestra para la extracción de OAs.
    - **Objetivo 1.1:** Síntesis y funcionalización de materiales magnéticos adsorbentes con núcleo de Fe_3O_4 y su posterior caracterización.
    - **Objetivo 1.2:** Síntesis y funcionalización de materiales adsorbentes basados en sílices mesoestructuradas, tales como SBA-15 y HMS y su posterior caracterización.

- **Objetivo 2:** Optimización, desarrollo y validación de métodos analíticos para la determinación de OAs en distintas matrices alimentarias.
    - **Objetivo 2.1:** Optimización de la separación y detección de OAs mediante (U)HPLC-MS/MS y GC-MS.
    - **Objetivo 2.2:** Optimización de la etapa de extracción de los OAs en las distintas matrices alimentarias mediante el empleo de SLE o de UAE.
    - **Objetivo 2.3:** Optimización de la etapa de extracción y/o purificación de los OAs aplicando los materiales adsorbentes sintetizados en el procedimiento de MSPE o SPE.
    - **Objetivo 2.4:** Optimización de la etapa de extracción y purificación de OAs aplicando la novedosa técnica micro-extractiva µ-SPEed®.
- **Objetivo 3:** Aportar información sobre los niveles de OAs presentes en distintos alimentos comerciales con el objetivo de determinar la exposición de los consumidores a esta familia de toxinas.
- **Objetivo 4:** Estudiar la influencia de distintos tipos de procesado, como infusionado, fermentación o molienda, en los niveles de OAs presentes en los alimentos.

RESULTADOS ORGANIZADOS POR ARTÍCULOS

3. RESULTADOS ORGANIZADOS POR ARTÍCULOS

Tal y como se ha mencionado anteriormente en la Introducción de esta Tesis Doctoral, los OAs son toxinas naturales que pueden estar presentes en las semillas de la planta *Papaver somniferum* L., comúnmente conocida como adormidera o amapola real que se destinan para consumo humano. Tras el consumo de las semillas contaminadas con altas concentraciones de OAs se han dado numerosos casos de intoxicaciones e incluso falsos positivos en test de drogas en los últimos años. Por este motivo, desde la EFSA se han reclamado más estudios que permitan establecer la exposición real de los consumidores a esta familia de toxinas, ya que el conocimiento de estos tóxicos es muy limitado [11]. En el caso de morfina y codeína están establecidos los límites máximos que se pueden encontrar en semillas y productos de panadería [31]. Sin embargo, hay más alimentos que se pueden consumir con semillas de amapola contaminadas y, por tanto, es importante analizarlos para establecer una legislación acorde con ello [12]. Además, se ha visto que el resto de OAs también pueden estar presentes en concentraciones elevadas en las semillas de amapola y, según las autoridades sanitarias, podrían llegar a ser incluso más tóxicos. Por este motivo, también se reclaman más estudios que tengan en cuenta todos los OAs mayoritarios que se pueden encontrar en los alimentos [11], [18]. Como consecuencia de las altas concentraciones encontradas se están buscando buenas prácticas de procesado para prevenir o eliminar las concentraciones altas de OAs en alimentos. Por este hecho, las autoridades sanitaras reclaman más estudios que determinen el efecto que puede tener distintos tipos de procesados culinarios en la concentración de OAs finales.

Para dar respuesta a todos estos retos en la presente Tesis Doctoral se han desarrollado y aplicado una serie de metodologías de análisis que incluyen los alimentos y los principales procesados culinarios que se aplican en los alimentos que contienen semillas de amapola, dando lugar, tal y como se muestra en la Figura 14, a 8 artículos científicos y a 1 nota de aplicación para la empresa.

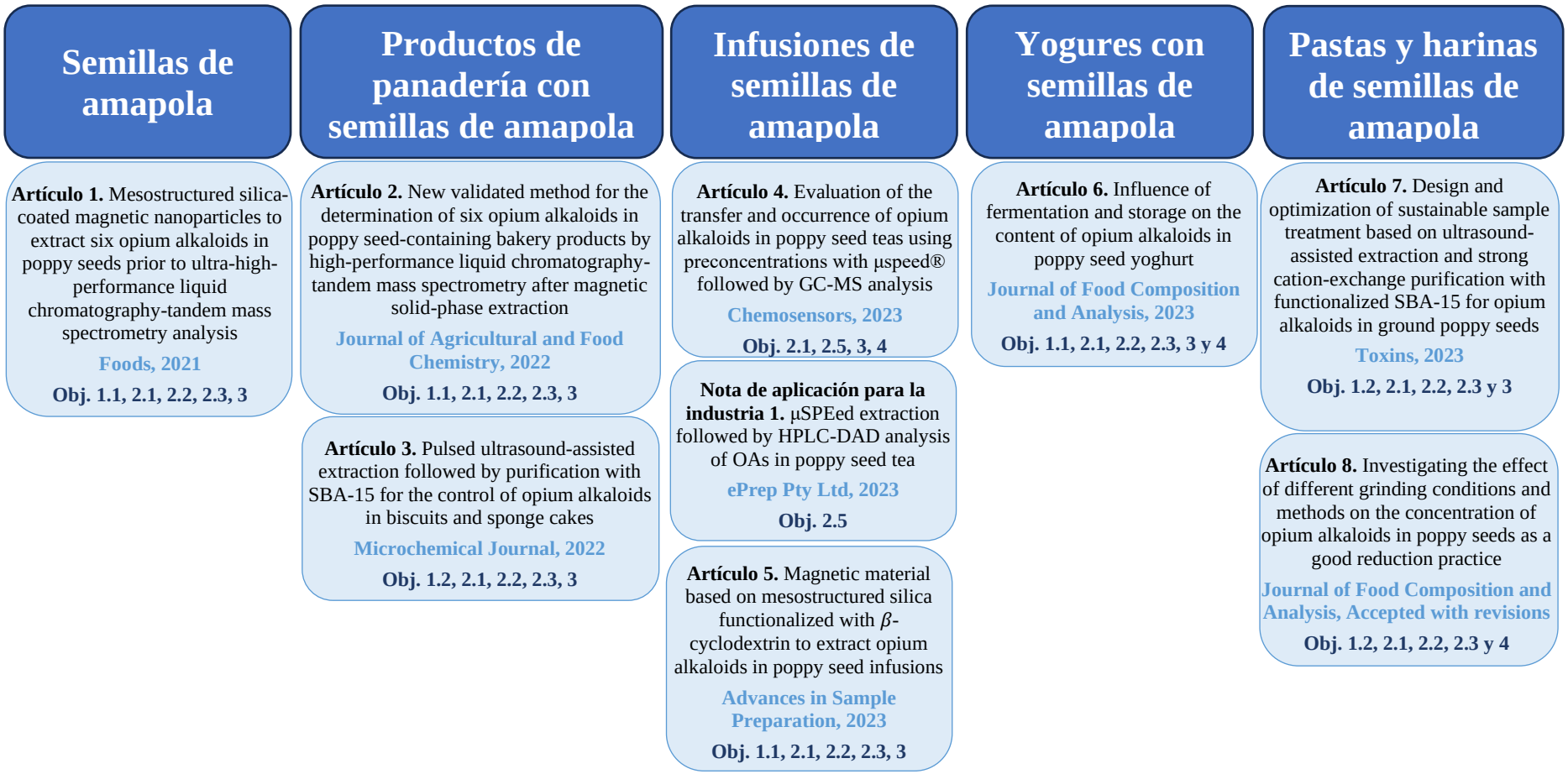

Figura 14. Esquema de los resultados de la presente Tesis Doctoral que relaciona los artículos de investigación con la matriz alimentaria estudiada y los objetivos específicos abordados.

En primer lugar, se desarrollan metodologías para el análisis de OAs en semillas de amapola como la muestra más importante y que da lugar a la problemática de contaminación, ya que es la muestra que se puede contaminar directamente con los OAs presentes en el látex de la propia planta. Posteriormente, se evaluó el contenido de OAs en los productos de panadería con semillas de amapola ya que son los productos más comercializados con semillas de amapola. Por un lado, se desarrollaron metodologías para evaluar el contenido de OAs en el pan de molde y los palitos de pan y, por otro lado, productos dulces como galletas y bizcochos. Después, se evaluaron metodologías para analizar OAs en las infusiones con semillas de amapola, las cuales son consumidas ampliamente a nivel mundial. Por un lado, se estudió la transferencia mediante el infusionado a distintas condiciones (cantidad inicial de semillas, temperatura del agua y tiempo de infusión), y, por otro lado, se evaluaron las cantidades de OAs que se pueden llegar a encontrar en infusiones con distintos tipos de semillas con las condiciones de infusionado que más favorecían la transferencia. También se han estudiado el contenido de OAs en yogures comerciales con este tipo de semillas y se ha estudiado la influencia de distintas condiciones de fermentación y almacenamiento de los yogures sobre el contenido de OAs. Y, por último, se desarrolló una metodología y se aplicó para evaluar el contenido de OAs en pastas y harinas de semillas trituradas de amapola para posteriormente evaluar la influencia de distintos tipos y condiciones de molienda (tiempo o moliendas consecutivas) en el contenido de final de OAs.

Como se ha puesto de manifiesto en el trabajo de revisión, las metodologías desarrolladas hasta la fecha para semillas de amapola se basaban principalmente en una etapa de SLE y un análisis directo mediante cromatografía y para el resto de las matrices alimentarias eran inexistentes [16], [17], [24]. Por este motivo, para todas las metodologías desarrolladas en esta Tesis Doctoral se trató de optimizar una etapa de extracción y/o purificación del extracto eficaz y más sostenible antes del análisis. Esto es un paso crucial en las metodologías analíticas ya que las muestras alimentarias son muy complejas formadas por una alta diversidad de compuestos [41].

Por todo ello, en el primer trabajo de esta Tesis Doctoral (Artículo 1) se desarrolló una metodología analítica para cuantificar los seis OAs mayoritarios en semillas de

amapola mediante una primera SLE, una posterior purificación mediante µ-MSPE, seguido de un análisis por HPLC-MS/MS. Para ello, se optimizaron las variables que influyen en la etapa de SLE, tales como la cantidad de muestra, el tipo de disolvente, el volumen de disolvente, el pH del medio, el tiempo y el número de extracciones consecutivas. Posteriormente, se sintetizaron partículas magnéticas de Fe_3O_4 mediante co-precipitación química. Estas partículas se recubrieron con una capa de sílice amorfa (Fe_3O_4@SiO_2) y una segunda capa de sílice mesoestructurada (Fe_3O_4@SiO_2@mSiO_2). Posteriormente, cada uno de estos tipos de partículas se funcionalizaron con diferentes cadenas alquílicas, unas formadas por octilsilano (C_8) y en otras por C_{18} con el objetivo de seleccionar el material más eficaz para la extracción de los analitos. Para ello, se realizó un estudio sobre la capacidad de adsorción de cada uno de los seis materiales en el que se determinó la adsorción (%) de los OAs con cada uno de ellos a diferentes tiempos (1, 5, 10 y 20 min). Además, se estudiaron las cinéticas y las isotermas de adsorción del material con mayor capacidad de adsorción para evaluar de una forma más completa el material y se optimizó con el más adecuado (Fe_3O_4@SiO_2@mSiO_2) el procedimiento de MSPE de los seis OAs en muestras de semillas de amapola. Para ello, se estudiaron todas las variables que influyen en la eficiencia de la MSPE, tanto de la etapa de adsorción (tiempo, volumen de extracto, pH) como de la etapa de desorción (disolvente, pH, volumen, tiempo y número de desorciones consecutivas) y la cantidad de material adsorbente necesario. Por último, se optimizó el análisis de los OAs mediante UHPLC-MS/MS realizándose mediante modo MRM y con ESI+. Para ello, se optimizó en primer lugar la detección de los analitos mediante infusión directa de cada uno de ellos y, posteriormente, el método cromatográfico para su correcta separación.

Para los productos de panadería, se desarrollaron dos metodologías analíticas, una en pan de molde y palitos de pan (Artículo 2) y otra en galletas y bizcochos (Artículo 3) con semillas de amapola. La metodología analítica desarrollada para analizar las muestras de panadería saladas (Artículo 2) se basó en una SLE, seguida de una purificación con µ-MSPE y un posterior análisis por HPLC-MS/MS. En primer lugar, se optimizó la etapa de SLE y para ello se estudió el tipo de disolvente, el volumen y el tiempo de extracción con el objetivo de emplear la menor cantidad de volumen de disolvente y tiempo posible. Además, para la etapa de µ-MSPE se sintetizó un nuevo material magnético que, en vez

de llevar a cabo el recubrimiento de las partículas de Fe_3O_4 con sílice, se realizó una modificación superficial con ácido tereftálico y cloruro de hierro (III) (Fe_3O_4@TPA-Fe), para evaluar la eficacia de interacción de otros grupos funcionales mediante interacciones con enlaces de hidrógeno, electrostáticas π-π y de ion-dipolo. Una vez sintetizado y caracterizado, se estudiaron las cinéticas y las isotermas de adsorción de este nuevo material y se optimizó el procedimiento µ-MSPE de forma similar al trabajo anterior. Para ello, se evaluó la etapa de adsorción (tiempo, volumen de extracto, pH), la etapa de desorción (disolvente, pH, volumen, tiempo y número de desorciones consecutivas) y la cantidad de material adsorbente necesario. Por último, se optimizó el análisis de los OAs mediante HPLC-MS/MS, de forma similar al trabajando anterior mediante MRM y con ESI+. Para ello, también se optimizó en primer lugar la detección de los analitos mediante infusión directa de cada uno de ellos y, posteriormente, el método cromatográfico para su correcta separación.

Por otro lado, para la metodología analítica desarrollada para analizar las muestras de panadería dulces (Artículo 3) se optimizó la extracción mediante UAE para disminuir el volumen de disolventes orgánicos y de tiempo de extracción y se empleó una posterior µ-SPE seguida del análisis por HPLC-MS/MS. Tal y como se ha mencionado en la Introducción, hay una gran cantidad de parámetros que influyen en el rendimiento de la UAE y que hay que optimizar. Normalmente se ha utilizado la técnica de una variable a la vez, lo que significa modificar una variable con las demás fijas. Sin embargo, la mayor desventaja de esta técnica es que no incluye efectos interactivos entre las variables y, eventualmente, no representa los efectos completos de los parámetros en el proceso. Por este motivo, una forma muy interesante de optimizar este tipo de procedimientos con tantas variables es utilizar un diseño de experimentos. Este método permite además de llevar a cabo la optimización, medir la interacción entre los distintos factores. Para ello, la metodología de superficie respuesta (RSM) es el método de optimización más popular utilizado en los últimos años [92]. RSM es una herramienta que permite evaluar múltiples factores y sus interacciones simultáneamente, siendo un conjunto de técnicas matemáticas y estadísticas útiles para desarrollar, mejorar y optimizar procesos en los que una respuesta de interés está influenciada por varias variables y el objetivo es optimizar esa respuesta [93], [94]. Por este hecho, en el Artículo 3, se optimizó el procedimiento UAE

mediante diseño de experimentos factorial completo de cinco variables a dos niveles para hacer un *screening* completo y ver las variables que más influyen en el procedimiento para posteriormente hacer una re-optimización mediante RSM de las variables más influyentes con un tercer nivel más. Una vez optimizada la extracción se llevó a cabo la purificación mediante µ-SPE aplicando materiales a base de sílice que, tal y como se ha mostrado en la Sección 3.1, presentan numerosas ventajas para su utilización como adsorbentes en la preparación de muestra [54]. En este trabajo se evaluaron dos sílices, concretamente la sílice SBA-15 y HMS previamente sintetizadas y un material comercial de balance hidrofílico-lipofílico (HLB). Para ello, se optimizó el procedimiento µ-SPE con cada uno de los materiales adsorbentes y se compararon los valores de recuperación (%) de cada uno de ellos para seleccionar el más eficaz para la extracción de estos analitos. Una vez seleccionado el material con mayor rendimiento para OAs (SBA-15), se empleó para llevar a cabo la etapa de µ-SPE de los extractos de muestras de panadería dulces y su análisis posterior mediante HPLC-MS/MS optimizado en el trabajo anterior.

Posteriormente, se desarrollaron varias metodologías de análisis para cuantificar OAs en infusiones de semillas de amapola, una mediante la aplicación de la novedosa técnica de micro-extracción µ-SPEed® seguida del análisis mediante GC-MS (Artículo 4) y una nota de aplicación para la industria sobre la µ-SPEed® y el análisis posterior mediante HPLC-DAD (Nota de Aplicación 1). El trabajo de investigación descrito en el Artículo 4 basado en µ-SPEed® se realizó durante una estancia predoctoral de 3 meses en el Centro de Química de Madeira – CQM (Universidad de Madeira, Funchal, Portugal) bajo la supervisión del Doctor José Sousa Câmara y en colaboración con la Doctora Rosa Perestrelo Gouveia. Para su realización, se evaluaron nueve cartuchos comerciales para seleccionar el más eficiente para la extracción de los OAs en infusiones de semillas de amapola, siendo seis cartuchos basados en sílice: C_4 (tetrilsilano), C_8, C_{18}, APS (WAX, intercambio aniónico débil), PFAs (50% WAX, 50% C_{18}) y tres materiales poliméricos: PS/DVB-RP (fase reversa), PS/DVB-SCX (intercambio catiónico fuerte) y PS/DVB-SAX (intercambio aniónico débil). Para ello, se evaluaron los cartuchos con diferentes pH del extracto, ciclos y disolventes de elución. Una vez seleccionado el cartucho PS/DVB-RP, se evaluó su capacidad de preconcentración y se optimizó la etapa de derivatización y la detección y separación mediante GC-MS, la cual se llevó a cabo en

Full Scan y, posteriormente la cuantificación en modo de monitorización de ion simple (SIM). Una vez desarrollada y validada la metodología, se estudió la transferencia de OAs de las semillas a distintas preparaciones de infusionado, con el objetivo de determinar la influencia de distintas variables del infusionado (temperatura del agua, tiempo de infusionado y cantidad de semillas). Además, se determinaron las concentraciones de OAs que pueden encontrarse en las condiciones de infusionado más utilizadas por el consumidor para evaluar el riesgo de realizar esta práctica. La Nota de Aplicación de µ-SPEed® se hizo en colaboración con la empresa australiana EPREP para analizar infusiones elaboradas con semillas de amapola empleando HPLC-DAD para el análisis al ser un equipo más disponible en laboratorios de rutina, demostrando la aplicabilidad del método propuesto en la industria.

Al volver de la estancia pre-doctoral se continuó con el análisis de infusiones con semillas de amapola y para ello se desarrolló una metodología basada en una µ-MSPE (Artículo 5) y un posterior análisis mediante HPLC-MS/MS) para lo que las partículas magnéticas sintetizadas en el Artículo 1 se funcionalizaron con moléculas de β-ciclodextrina (β-CD), con el objetivo de mejorar los valores de recuperación obtenidos en el Artículo 1, aportando interacciones más eficientes mediante enlaces de hidrógeno, interacciones hidrofóbicas y de Van der Waals, que se favorecen cuando la β-CD está en medios acuosos [95], [96]. Se eligió la β-CD como ligando para funcionalizar este material ya que este compuesto se trata de una familia de oligosacáridos cíclicos naturales que provienen del almidón y, por tanto, es un tipo de ligando más respetuoso con el medio ambiente que otros empleados. Para llevarlo a cabo, se optimizó la funcionalización del material con distintos grados de funcionalización con el objetivo de conseguir el mayor grado de funcionalización posible. Posteriormente, se evaluaron las cinéticas y las isotermas de este material para tener un mayor conocimiento del funcionamiento de este material en MSPE y se optimizó la etapa de µ-MSPE, tanto la etapa de adsorción (tiempo, volumen de extracto, pH), como la etapa de desorción (disolvente, pH, volumen, tiempo y número de desorciones consecutivas) y la cantidad de material adsorbente necesario. Una vez optimizada y validada la metodología, se aplicó al análisis de infusiones con distintos tipos de semillas de amapola.

Para cuantificar OAs en yogures con semillas de amapola se desarrolló una metodología basada en una etapa de SLE en la que se empleó agua como disolvente de extracción y posteriormente se llevó a cabo la purificación mediante µ-MSPE con el material Fe_3O_4@SiO_2@mSiO_2@β-CD como adsorbente y un posterior análisis mediante HPLC-MS/MS (Artículo 6). Para ello, se optimizaron en la etapa de SLE, el volumen y tiempo de extracción y en la µ-MSPE los parámetros necesarios para conseguir unos buenos valores de recuperación como en el trabajo anterior, pero con este extracto de yogur. En este trabajo, además de cuantificar los OAs en yogures comerciales, se evaluó si la fermentación ácido-láctica y el almacenamiento en refrigeración podían tener un efecto de degradación de los OAs durante el proceso de elaboración del yogurt. Esto se debe a que este procesado culinario no ha sido previamente estudiado en la influencia de la cantidad de OAs en los yogures finales de la misma forma que si ha sido estudiado previamente en otras familias de alcaloides [97], [98].

La última matriz alimentaria analizada fueron las pastas y harinas de semillas trituradas de amapola. Para ello, en primer lugar, se desarrolló una metodología basada en UAE, una purificación mediante µ-SPE y el análisis por HPLC-MS/MS (Artículo 7). Para ello, también se llevó a cabo la optimización mediante diseño de experimentos factorial completo, pero en este caso se estudiaron tres variables a tres niveles con el objetivo de optimizar con mayor precisión cada una de estas variables influyentes en el procedimiento (tipo de disolvente, ratio sólido-líquido y tiempo de extracción). Y, para realizar la purificación del extracto, se llevó a cabo la funcionalización de la SBA-15 con grupos sulfónicos (SO_3^-). Estos grupos de intercambio catiónico fuerte aportan unas interacciones más específicas y fuertes debido a que además de aportar enlaces de hidrógeno con los grupos OH^-, se dan enlaces catiónicos fuertes con los grupos amino de los analitos utilizando el pH adecuado. Esto podría permitir reducir la cantidad de material, haciendo que la metodología de análisis fuera más respetuosa con el medio ambiente. Una vez optimizada y validada la metodología, se aplicó al análisis de pastas y harinas de semillas trituradas de amapola.

Por último, esta metodología optimizada y validada para cuantificar OAs en pastas y harinas de semillas trituradas de amapola se empleó para evaluar el efecto de la molienda

en el contenido final de OAs en las semillas con el objetivo de evaluar si este tipo de procesado culinario puede considerarse como una buena práctica de reducción de OAs (Artículo 8). Según las autoridades sanitarias y algunos artículos previamente publicados la molienda puede disminuir entre un 25-34% la concentración de morfina debido principalmente a su oxidación [17], [19]. Sin embargo, no están establecidas unas condiciones de molienda específicas para alcanzar esa reducción y se desconoce cómo puede afectar distintos tipos de molienda. Además, solo se tiene indicios de degradación de morfina y se desconocen cómo pueden estar influenciados el resto de OAs presentes en las semillas. Por este motivo, en el Artículo 8 se estudió la influencia de cinco tipos de molienda con distintas condiciones (cantidad de semillas inicial, tiempo o moliendas consecutivas).

A continuación, se presentan cada uno de los artículos de investigación realizados en la presente Tesis Doctoral.

Artículo 1:

Mesostructured silica-coated magnetic nanoparticles to extract six opium alkaloids in poppy seeds prior to ultra-high-performance liquid chromatography tandem mass spectrometry analysis

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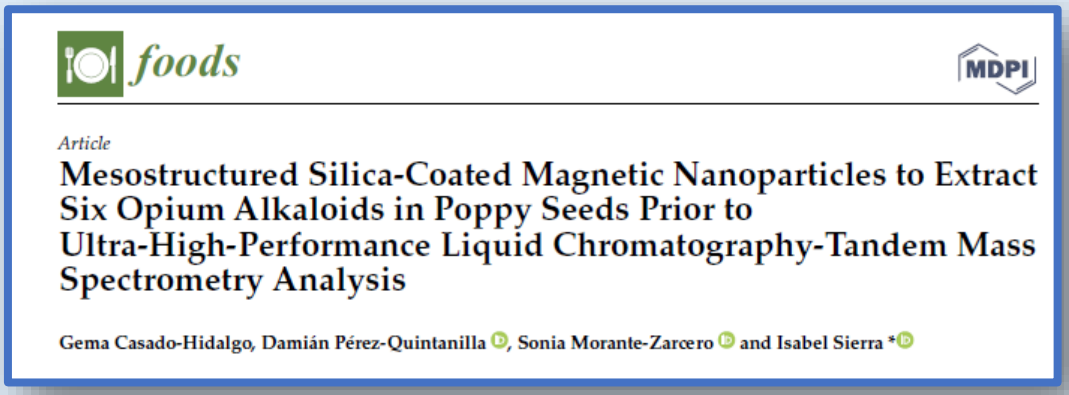


PUBLICATION INFORMATION

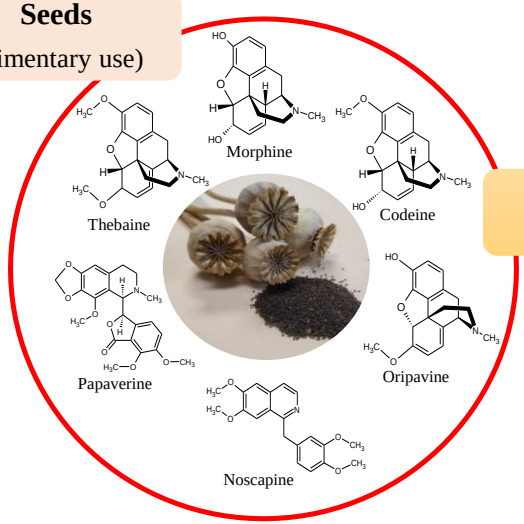
Journal	Foods
Publisher	MDPI
Impact factor	5,561 (JCR, 2021)
Category	Food Science & Technology (35/144, Q1)
DOI	https://doi.org/10.3390/foods10071587

Papaver somniferum L.

Latex
rich in opium alkaloids
(pharmaceutical use)

CONTAMINATION
- Poor harvesting practices
- Insect damage

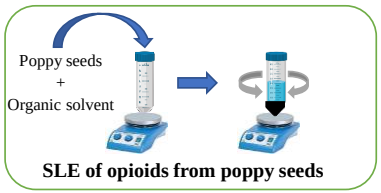
Seeds
(alimentary use)



to ensure food safety

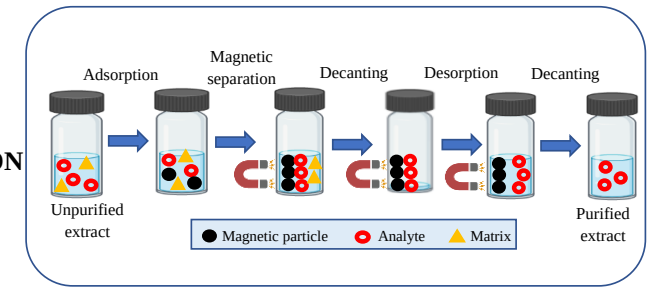
SLE-MSPE-UHPLC-QqQ-MS/MS method

1 EXTRACTION

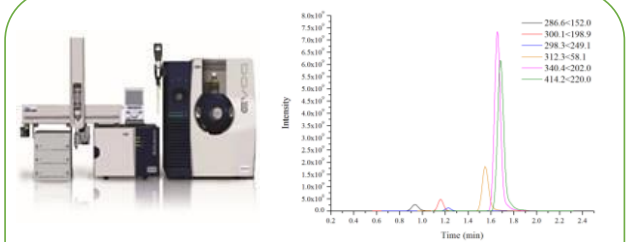


SLE of opioids from poppy seeds

2 PURIFICATION



3 ANALYSIS



UHPLC-QqQ-MS/MS

ABSTRACT

In recent years, health authorities have become increasingly concerned about preventing consumer exposure to opium alkaloids present in *Papaver somniferum* L poppy seeds. In this study, a simple, rapid and efficient method has been optimised to determine all main opioids in poppy seeds (morphine, codeine, thebaine, papaverine, noscapine and oripavine) by UHPLC-QqQ-MS/MS. For this purpose, solid-liquid extraction (SLE) of samples was optimised and six magnetic adsorbent materials with a core of Fe_3O_4 coated with amorphous and mesostructured silica, both functionalised with octadecyl- or octyl-silane were characterized and evaluated for magnetic solid-phase extraction (MSPE). The material with the best results was non-functionalised mesostructured silica and with it, MSPE procedure was optimised. This method was validated and used to quantify six opioids in 14 edible seed samples (eleven poppy seeds and three seed mixes). Considerable amounts were found (1.5-249.0 mg/kg morphine, <0.2 µg/kg-45.8 mg/kg codeine, <2.4 µg/kg-136.2 mg/kg thebaine, <0.2 µg/kg-27.1 mg/kg papaverine, <0.2 µg/kg-108.7 mg/kg noscapine and <240 µg/kg-33.4 mg/kg oripavine), exceeding maximum limits established in some EU countries and the reference level of morphine in the EU. Furthermore, in some commercial samples for human consumption, inadequate labelling was found, because significant amounts of alkaloids were detected even though *Papaver rhoeas* L. seeds were declared on the product label.

KEYWORDS: Opium alkaloids; poppy seeds; *Papaver somniferum;* mesostructured silica; magnetic solid-phase extraction; liquid chromatography-tandem mass spectrometry**.**

1. Introduction

The seeds of the opium poppy, *Papaver somniferum* L., are widely used in the preparation of food products such as bakery, buns, yoghurt, snacks or making tea [1, 2, 3]. Although *Papaver somniferum* L. poppy seeds hardly contain any opium alkaloid, they can be contaminated with latex by poor harvesting practices or insect damage. The main opium alkaloids that may be present are morphine, codeine, papaverine, thebaine, noscapine and oripavine. However, most of the previously published studies have focused mainly on morphine and codeine, without paying attention to the other alkaloids whose concentrations may also be relatively high and of concern due to their possible high levels of toxicity [4]. In addition, another problem observed at the commercial level is that in most cases only poppy seed is indicated on the label and the botanical name is not considered. This is an important aspect because *Papaver rhoeas* L. seeds (corn poppy seeds) does not contain any opium alkaloids unlike *Papaver somniferum* L. [5]. If food products are made from contaminated seeds, there may lead to adverse health effects, especially in babies, infants, the elderly and people with severe health issues [6]. Besides, their consumption can give considerable amounts of opium alkaloids in biological samples such as blood, urine and oral fluid, enough to cause false positive drug abuse testing [7-10].

Nowadays, there is no harmonised European legislation on opium alkaloids in poppy seeds for food purposes, each country is carrying out different actions [4]. For example, in Belgium, only poppy seeds can be used in the production of bakery products [11]. In Austria, a classification of varieties with low morphine content for food use has been established [12]. In Germany, a maximum limit of 4 mg/kg for morphine in poppy seed for use in food has been established and in Hungary of 30 mg/kg for morphine, 20 mg/kg for noscapine, 40 mg/kg for the sum of morphine and noscapine, 20 mg/kg for thebaine and 20 mg/kg for codeine [11]. Due to the absence of harmonised legislation among the EU Member States, a considerably high number of RASFF (Rapid Alert System for Food and Feed) health alerts have been generated in recent years [4]. For this reason, a reference level of 10 mg/kg morphine in poppy seeds for direct human consumption has been established in the EU. This level is not a maximum limit, but an agreement between EU

Member States in November 2016 [13] until the Commission establishes, if necessary, new risk management measures concerning the presence of opium alkaloids in food [5]. In addition, the European Food Safety Authority (EFSA) in 2011 carried out a risk assessment and established an acute reference dose (ARfD) of 10 µg morphine/kg body weight (b.w). In 2014, the European Commission published a set of recommendations for good agricultural and seed processing practices to reduce the morphine content in poppy seeds [14]. In 2018, a new EFSA opinion was published, and its main conclusion was that more studies are needed to determine the presence of the six main opium alkaloids in edible seeds available on the market, to know the real exposure of consumers to all these toxins and to make a harmonised legislation [15].

To carry out these studies, it is necessary to develop methods in food matrices that are effective, simple and rapid. Some of these alkaloids are found at ultra-trace levels in very complex matrices. For this reason, it is required a selective and sensitive analytical technique. The most used is chromatography, such as gas chromatography-coupled mass spectrometry (GC-MS) [10, 16]. However, the costly step of sample derivatising has led to more use of (ultra)high-performance liquid chromatography ((U)HPLC). Multiple detectors can be used, such as the diode array [17] or ultraviolet [18], but the preferred technique for analysis of opium alkaloids is (U)HPLC coupled to triple quadrupole mass spectrometry ((U)HPLC-QqQ-MS/MS) with multiple reaction monitoring (MRM). However, to avoid co-eluted endogenous matrix components and to reduce the matrix effect that can be produced in MS detection, minimizing the possibility of false results, it is very important to do an adequate sample treatment. The commonly is using traditional procedures such as solid-liquid extraction (SLE) with organic solvents [1, 6] or solid-phase extraction (SPE) to do a sample purification [19, 20]. The current trend in sample preparation involves the adoption of more automated, simple, fast and environmentally friendly approaches, mainly by integrating new adsorbent materials in the purification stage by means of microextraction techniques [4, 21]. A powerful new purification alternative is solid-phase magnetic extraction (MSPE) that is faster and simpler. MSPE consists of dispersing the magnetic material in a solution with the sample for a few minutes. Once it is in equilibrium, it is recovered with the help of a magnetic field and, finally, the analytes are desorbed which can avoid tedious filtration, centrifugation, or

sedimentation steps [22]. Adsorbent material type is an important parameter in MSPE procedure because it will determine the ability to purify the samples. Until now, Fe_3O_4 particles covered with amorphous silica are the most used as a magnetic adsorbent, which can be functionalised with different organic groups as natural polymers or graphene [22-25]. However, the use of mesostructured silica is increasingly important because of their larger surface area, which can bind more of the functional groups and their uniform porous structure can facilitate access to the analytes [26, 27]. However, MSPE procedures with this type of adsorbent material have not yet been developed to study all the main opium alkaloids present in poppy seeds.

The aim of this work was to develop and validate a rapid, easy and efficient method to determine the six main opium alkaloids, morphine, codeine, thebaine, papaverine, noscapine and oripavine, in poppy seeds by UHPLC-QqQ-MS/MS. For this purpose, a SLE-MSPE sample preparation protocol was optimized using mesostructured silica-coated magnetic nanoparticles as adsorbent. The method was successfully applied for the quantification of the six opioids in eleven poppy seeds and three seed mixes available in national food supermarkets to know the levels of these alkaloids in seeds destined for food consumption.

2. Materials and Methods

2.1 Reagents and Materials

Standards of morphine, codeine, thebaine and oripavine were received from Alcaliber S.A.U. (Madrid, España). Noscapine, papaverine and morphine-D3 (internal standard) were obtained from Sigma-Aldrich (Sigma-Aldrich, Zwijndrecht, The Netherlands). Individual stocks standard solutions were prepared at 1000 µg/mL in methanol. The intermediate mixed standard solution was prepared at 10 µg/mL in methanol. The working standard solutions were prepared at 1 µg/mL by diluting the intermediate mixed standard in methanol/water 50/50 (v/v). All of them were stored in darkness at -20 °C.

Ferric chloride 6-hydrate ($FeCl_3\,6H_2O$) 99% and ferrous chloride 4-hydrate ($FeCl_2\,4H_2O$) 99% were purchased from Labkem (Barcelona, Spain) and Acros Organics (Geel,

Belgium), respectively. Tetraethylorthosilicate (TEOS) 98%, hexadecyltrimethylammonium bromide (CTAB) 98%, chloro(dimethyl)octylsilane (C_8), chloro(dimethyl)octadecylsilane (C_{18}) were purchased from Sigma-Aldrich. HPLC grade acetonitrile, methanol and formic acid were purchased from Sigma-Aldrich. Ammonia (32%, w/w), isopropanol, toluene, ethanol and diethyl ether were of synthesis grade and acquired from Scharlab (Barcelona, Spain). Ultrapure water (resistance 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). The Nd-Fe-B magnet (5 x 5 x 2 cm) with force 200 kg used in MSPE procedure was obtained from Superimanes S.L. (Sevilla, Spain).

2.2 Reagents and Materials

At the end of 2020, 14 samples of edible seeds were purchased in Spain from some supermarkets and herbalists, of which three are mixtures of different edible seeds (pumpkin, sunflower, sesame, gold flax, brown flax and poppy seeds). In addition, for comparative purposes, two wild samples, one of white opium poppy (*Papaver somniferum* L.) and the other of corn poppy (*Papaver rhoeas* L.) were collected in Madrid and Zaragoza, respectively. Detailed information on each of these samples can be found in Table S1.

2.3 Synthesis of organic functionalized magnetic particles

Magnetic particles (Fe_3O_4) were coated in a first step, with amorphous silica (Fe_3O_4@SiO_2) and, after, with mesostructured silica (Fe_3O_4@SiO_2@mSiO_2) In addition, both materials were functionalized with organic groups (C_8 or C_{18} ligand). The schematic preparation process of different magnetic particles is shown in Figure 1a.

2.3.1 Preparation of Fe_3O_4 particles

First, Fe_3O_4 particles were prepared using chemical co-precipitation according to the work of Zhang and Shi [28]. Briefly, 15 mmol $FeCl_3\,6H_2O$ and 10 mmol $FeCl_2\,4H_2O$ were

dissolved in 80 mL of deoxygenated water stirring at 300 rpm under nitrogen gas. 50 mL of ammonia solution (32%) were dropwise added into the clear yellow solution and it turned black. The reaction was maintained at 80 °C for 30 min. The black precipitates obtained (Fe_3O_4 particles) were collected with the help of a strong magnet and washed repeatedly with deionized water until the pH of the washings became neutral and finally dried under vacuum at 60 °C for 24 h.

2.3.2 Surface modification of Fe_3O_4 particles with amorphous silica ($Fe_3O_4@SiO_2$)

The Fe_3O_4 particles were coated with amorphous silica (SiO_2) according to the reported method of Zeng et al., with minor modifications [29]. Briefly, 1.5 g Fe_3O_4 particles were dispersed in 60 mL of isopropanol/ultra-pure water (5/1, v/v) followed by ultrasonic dispersion for 20 min. Then under continuous stirring, 15 mL of ammonia solution and 8 mL of TEOS were added promptly. After that, the mixture solution was stirred at room temperature for 24 h. Finally, the mixture was separated by an external magnetic field, and the modified magnetic particles were washed with ultra-pure water respectively many times until the pH of the washing fluid was 7. Finally, the modified magnetic particles were dried under vacuum at 60 °C for 24 h.

2.3.3 Surface modification of Fe_3O_4 particles with mesostructurated silica ($Fe_3O_4@SiO_2@mSiO_2$)

With the aim to obtain a material with a higher surface area that allows an increase in the functionalization degree, $Fe_3O_4@SiO_2$ particles were coated with a layer of mesostructured silica ($mSiO_2$) according to the work of Deng et al., but with some modifications, as the amounts of the reagents were optimized [26]. Briefly, 3 g $Fe_3O_4@SiO_2$ were dispersed in a mixed solution containing 160 mL of ultra-pure water, 120 mL ethanol, 1.4 g CTAB and 12 mL ammonia solution. After the mixture was homogenized for 30 min, 6.4 mL of TEOS was dropwise added and stirred for 6 h. The product was collected with the help of a magnet and washed with ethanol and methanol several times. The $Fe_3O_4@SiO_2@CTAB@mSiO_2$ particles were collected with the aid of

a magnet and were calcined to remove the CTAB using a heating program from room temperature to 550 °C at 1 °C/min and holding the temperature for 4 h.

2.3.4 Organic functionalization of Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2@mSiO_2 particles with C_8 or C_{18} groups

Both materials were functionalized with two different organic groups to compare. To carry out this, 0.5 g particles and 0.5 g C_8 or C_{18} were added to 30 mL of anhydrous toluene under a nitrogen atmosphere. The mixture was stirred at 80 °C for 24 h. After the reaction, the particles were separated by an external magnetic field and were washed with toluene, ethanol and diethyl ether. Finally, the organic-functionalized magnetic amorphous and mesostructured particles were dried under vacuum at 60 °C for 24 h.

2.4 Characterization of organic functionalized magnetic particles

Scanning electron microscopy (SEM) were recorded on Nova NanoSEM 230 FEI with an energy-dispersive spectrometry system (EDS). Previously, samples were treated with a sputtering method by dispersing the material in ethanol and coating the sample with a film thickness of 7 nm of gold. The samples have been characterized using XRD to know the structure of the materials. Wide and low angle Powder X-ray diffraction (XRD) patterns of the silicas was done to determine if the material showed the typical spectrum of magnetite, which indicated that the magnetic core had not been disturb by the surface modification and if the material had a long mesoscopic ordered structure, respectively. XRD patterns were obtained on a Philips Diffractometer model PW3040/00 X'Pert MPD/MRD at 45 kV and 40 mA, using Cu Kα radiation (λ = 1.5418 Å). The samples have been treated in power and placed in a sample holder. The sample and detector are rotated and the XRD patterns are collected from 0 to 10° in the low angle and between 20 to 70° in the wide angle. Iron and Silica wt% determination was carried out by X-ray fluorescence (XRF) using an X-ray fluorescence spectrophotometer Phillips MagiX with an X-ray source of 1 kW and a rhodium anode in a helium atmosphere. This quantification method can analyse from 0.0001% to 100% of Fe and Si. Infrared spectra

were carried out on a Thermo Nicolet 380 Fourier-Transform Infrared (FT-IR) spectrophotometer in the region 4000-600 cm^{-1} using the ATR (Attenuated Total Reflection) technique to quantify the functional groups in the magnetic particles and to check the complete removal of the CTAB. For this, a small amount of sample (around 1 mg) previously vacuum-dried was used. The measures were done at room temperature to avoid the signal of the physisorbed water and in the transmittance mode with 64 scans per spectrum at a resolution of 4 cm^{-1}. Nitrogen gas adsorption-desorption isotherms were obtained using a Micromeritics ASAP 2020 analyzer. These isotherms were measured at -196 °C over the interval of relative pressures (P/P_o) from 10^{-4} to 0.994. Before measurements, the samples were degassed in a vacuum at 80 °C for 10 h in the port of degasification of the instrument. These temperatures were chosen to avoid any degradation of the organics groups and to remove adsorbed species, solvents and water. The Brunauer-Emmett-Teller (BET) method was utilized to calculate the specific surface areas (S_{BET}). By using the Barrett-Joyner-Halenda (BJH) model, the pore volumes and pore size distributions were derived from the desorption branches of isotherms, and the total pore volumes (V_t) estimated from the desorbed amount at a relative pressure P/P_o of 0.97. In addition, this characterisation also allowed us to compare the surface areas and to optimize the amounts of TEOS and CTAB to be added at the mesostructured silica coating step. Finally, elemental analysis (% H, % C and % N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. to determine the degree of functionalisation.

2.5 Optimization of UHPLC-QqQ-MS/MS analysis

The determination of opium alkaloids was achieved with a UHPLC system (Advance Elute, Bruker) equipped with a PAL RSI Autosampler (containing a loop of 100 µL) coupled to a triple quadrupole tandem mass spectrometer detector (EVOQTM Elite, Bruker) with an electrospray ion source (ESI). Chromatographic separation was performed on an Intensity Solo 2 C_{18} column (100 mm x 2.1 mm, Bruker) at 30 °C. The injection volume was 10 µL (partial injection) and the flow rate was kept constant of the mobile phase at 0.4 mL/min during the analysis. A gradient elution was used by

combining mobile phase A of water and mobile phase B of acetonitrile, both containing 0.1% formic acid. The linear gradient began in 95% A, in minute 3.5 changed to 30% A, in minute 3.7 returned to 95% B and it was maintained in isocratic until minute 5.

The detection of each analyte was determined by direct infusion of a standard solution in methanol of 1 µL/mL of each opium alkaloid using a syringe pump at a flow rate of 20.0 µL/min. The mass spectrometer was operated in positive electrospray ion source mode (ESI+) with multiple reaction monitoring (MRM) using N_2 as drying gas (350 °C and 40 psi), a cone temperature and gas pressure of 300 °C and 20 psi, Ar as nebulizer (60 psi) and collision gas (2 mTorr) and ion spray voltage of 4200 V, collect delay at 0.6 min and detector voltage at 1.65 V. Compounds were monitored with the transitions shown in Table S2.

2.6 Optimization of sample preparation

For the optimization of the sample preparation, firstly, the SLE parameters and, secondly, the MSPE purification parameters were optimized. The diagram for each of these steps is shown in Figure 1b.

2.6.1 Optimization of SLE of opioids from poppy seeds

First, the conditions for the SLE of opioids from poppy seeds were examined. The parameters that were optimized were: the mass of initial seeds (1, 2.5, 5 and 10 g), the type of solvent (acetonitrile/water/formic acid 80/19/1, v/v/v, methanol, methanol/water 50/50, v/v, and methanol 0.1% acetic acid), the agitation type (ultrasound and magnetic stirring), the volume of solvent (10, 20 and 30 mL), the extraction time (10, 20, 30 min and 1 h) the number of successive extractions (up to 6) and the pH of the solvent (3, 5, 6.8 and 10).

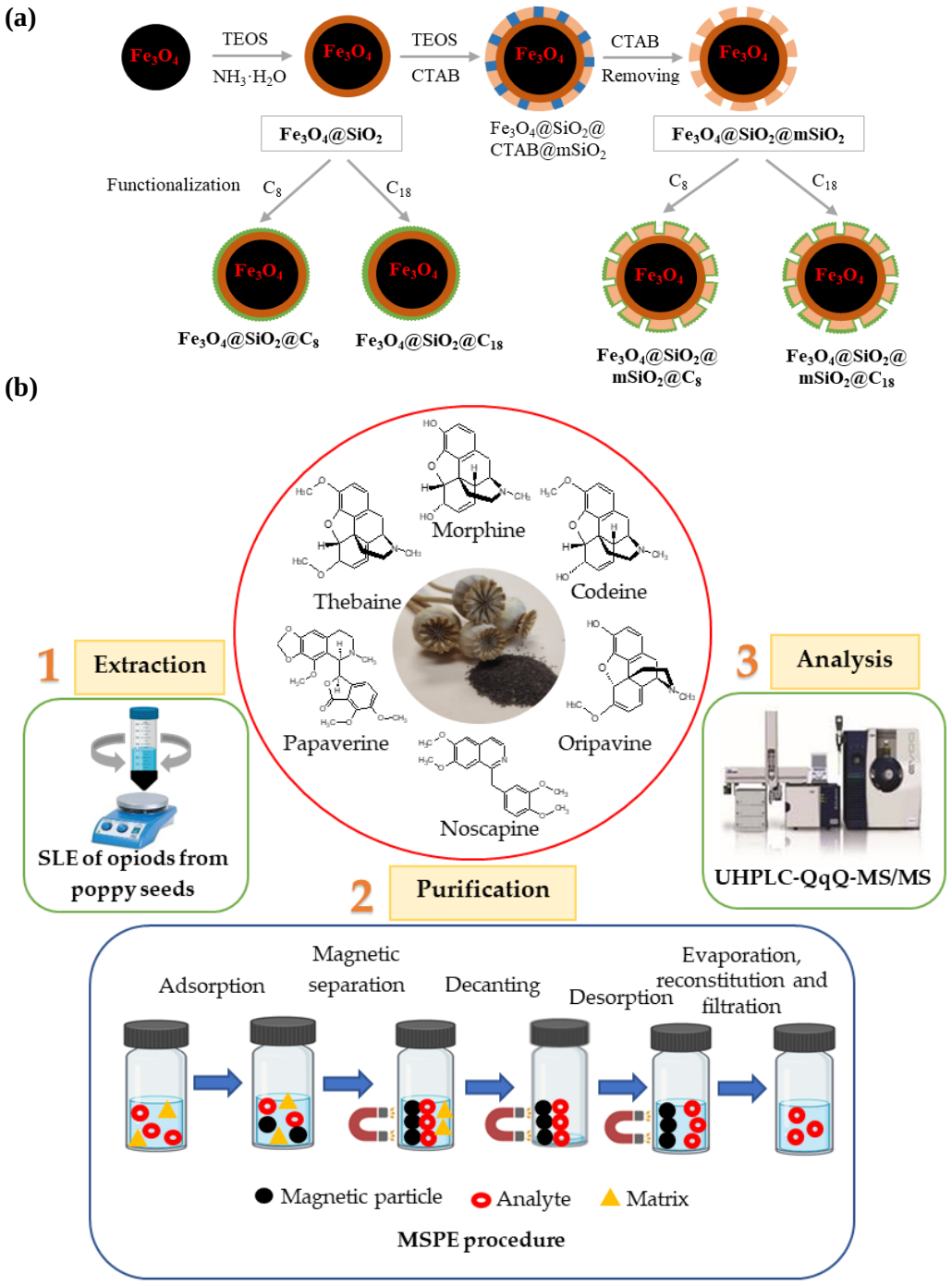


Figure 1. Schematic preparation process of the six types of magnetic particles synthesized (a). Diagram of proposed methodology to quantify opioids from poppy seeds: extraction (SLE), purification (MSPE) and analysis (UHPLC-QqQ-MS/MS) and chemical structures of the six most common opium alkaloids in contaminated poppy seeds (b).

2.6.2 Discontinuous adsorption studies to select the best magnetic material for MSPE procedure

Secondly, discontinuous adsorption studies were performed with all the previously synthesized magnetic materials to evaluate their adsorption capacity and to choose the most suitable one for the subsequent MSPE process. For this purpose, 100 mg of each of the six synthesized materials were added to 2 mL of different types of solvents (methanol/water 50/50, v/v, acetonitrile/water/formic acid, 80/19/1, v/v/v, and methanol 0.1% acetic acid) containing 1 µg/mL of each of the six analytes. Finally, the supernatants of each of the different solvent type were analysed by UHPLC-QqQ-MS/MS after a determined adsorption time (0, 1, 5, 10 and 20 min). All the studies were made in duplicate and the adsorption percentages of each of the materials on the different solvents were calculated and compared to determine which material and solvent were the best.

2.6.3 Adsorption kinetic and isotherm experiments with Fe_3O_4@SiO_2@mSiO_2 material

The adsorption kinetics and isotherms were investigated with the material selected for the MSPE procedure.

To adsorption kinetics, 100 mg of the material was added to a solution containing each of the six analytes (2 mL, 1 µg/mL). The mixtures were shaken for different times (1-20 min) and the supernatants in equilibrium were analysed by UHPLC-QqQ-MS/MS. The adsorption capacity was calculated by equation (1) in Table S3. The adsorption kinetics were determined by Langergren's pseudo-first-order [30], pseudo-second-order [31] and intra-particle diffusion kinetic models [32] (Table S3).

To adsorption isotherm, a series of 2.0 mL solutions of different concentrations of the six analytes (0.1-50 µg/mL) were added 100 mg of the material under optimal time. The isotherms of the six opium alkaloids adsorption on the magnetic particles were analysed using the commonly used Langmuir [33] and Freundlich [34] models (Table S3).

2.6.4 Optimization of MSPE conditions with Fe_3O_4@SiO_2@mSiO_2 material

Conditions of the MSPE procedure were optimized. For this purpose, 2 mL of 1 µg/mL of each of the six analytes were used. The parameters of the MSPE procedure that were optimized were: the amount of adsorbent (between 25 and 100 mg), the adsorption time (from 1 to 20 min), the pH of the initial solution (ranging from 3 to 10), the desorption eluent (diethyl ether, dichloromethane, chloroform, isopropanol, acetonitrile, methanol, water, ethyl acetate and ethanol) with different percentages of acid (formic acid) or base (ammonia), between 0.1 at 20%, or mixtures of some of these solvents at different proportions (50/50, 80/20 or 20/80, v/v), the volume of the desorption eluent (from 2 to 5 mL), and the number of successive desorptions (up to 5). All studies were carried out in triplicate. One factor at a time method was employed to obtain the optimal conditions of each parameter.

2.7 Optimised sample preparation procedure

2.7.1 Optimised SLE of opioids from poppy seeds

In the SLE of opioids from poppy seeds, 2.5 g of seeds were extracted with 30 mL of methanol/water, 50/50 (v/v). The mixture was vortexed for 30 s, and for 30 min it was stirred magnetically. Later the supernatant was recovered, and a second extraction was carried out in the same conditions. Finally, the two supernatants were put together, and 2 mL were taken for purification through the MSPE.

2.7.2 Optimised MSPE procedure

The steps that were carried out in the MSPE procedure are shown in Figure 1b. In the first place, 50 mg of Fe_3O_4@SiO_2@mSiO_2 were added with the extraction supernatant and then the mixture was treated with 1 min ultrasonication until the analytes arrived at an adsorption equilibrium with the adsorbent material. Then, with the help of a magnet, the solution was decanted and the analytes were eluted from the magnetic particles with 2 mL of diethyl ether/methanol 80/20, v/v by ultrasound for 1 min. Later the supernatant was recovered, and a second desorption was carried out in the same conditions. Finally,

2mL of these supernatants with the analytes was vacuum evaporated, 50 µL of a 1 µg/mL solution of morphine-D3 were added and reconstituted in 1 mL, filtered and analysed by HPLC-QqQ-MS/MS.

2.8 Instrumental and method validation

The present methodology was validated in terms of linearity, method and instrumental detection (LOD, MDL) and quantification (LOQ, MQL) limits, matrix effects, accuracy, precision and selectivity. For more details, see section 1.1 supplementary material. Since there is no official regulation for validation methodologies to quantified opium alkaloids in poppy seeds, the validation was done according to the criteria established in SANTE/11813/2017 document for the analytical quality control of pesticide residues in food and feed [35] and EC No 401/2006 [36] establishing the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

For the validation process, the commercial poppy seed sample PS05 (Table S1) was used, as it had low levels of alkaloids. In addition, a double wash with water at 100 °C for 30 min was applied. Signals of the analytes obtained were subtracted when required.

3. Results

3.1 Characterization of magnetic materials synthetised

3.1.1 SEM

The morphology of the synthesized particles can be seen from the SEM images in Figure S1. The particles have a spherical morphology, and it has experimented a considerable growth in size after the surface modification. The initial Fe_3O_4 particles have a size of 20 nm approximately and the modified particles with amorphous silica (Fe_3O_4@SiO_2) have a size in the range of 0.55-0.91 µm and with mesostructured silica (Fe_3O_4@SiO_2@mSiO_2) of 1.00-1.36 µm. This demonstrated the successful modifications of material, as there is a growth in size with the first coating and another growth with the second coating.

3.1.2 XRD

The XRD patterns of the synthesized magnetic particles are shown in Figure S2. The low-angle XRD pattern reveals the Miller index (100) characteristic of materials with mesoscopic order. In addition, there are several relatively strong diffraction peaks in the 2θ region of 20-70°, which are similar to those of the Fe_3O_4 particles reported by other groups [26, 37, 38], with the six discernible diffraction peaks 220, 311, 400, 422, 511 and 440, that correspond with the Miller index diffraction peaks that appears in the database of magnetite in JCPDS (JCPDS card: 19-629) file. This finding proved that all the silica-coated magnetic particles were composed of Fe_3O_4 core. Besides, the peak positions of the XRD patterns between different materials remain unchanged, which indicates that the magnetic core had not been disturb by the surface modification. This is also seen during the MSPE procedure. Although the separation rate was gradually reduced with the surface modification of Fe_3O_4, the magnetism of the functionalized particles is still strong enough that magnetic decantation with the external magnet can be performed quickly and without sample loss.

3.1.3 XRF

X-ray fluorescence measurement was made to determine the concentration (%) of iron and silica in all the materials synthesized. The percentage of iron decreased from 57.8 in Fe_3O_4 to 31-34% in the functionalized particles covered with amorphous silica (with 15-14% Si) and to 13-20% (with 20-19% Si) in the mesostructured silica shell functionalized particles. Nevertheless, the amount of iron in the materials after functionalization is still big enough to confer high magnetism and ensure a quick and easy separation of the microparticles from the solution in the MSPE procedure with the use of a strong magnet.

3.1.4 FT-IR

An important aspect to consider before the functionalization of the particles with the C_8 or C_{18} ligands is the successful removal of the CTAB. To do this, the amount of CTAB present in the materials was monitored after the removal treatment through FT-IR

spectroscopy before the functionalization process by checking the presence of the bands of the -CH_2 and -CH_3 groups from CTAB at 2855 cm^{-1} and 2925 cm^{-1}, respectively. The first treatment was performed with a Soxhlet acetone extraction, as in most studies [22, 23, 36, 37]. However, the intensity of these bands was remarkable (Figure S3a, spectrum 2), which showed that this treatment was not effective in eliminating the surfactant. For this reason, in this work, we use a calcination treatment as an effective method for the complete elimination of rests of CTAB remaining inside the porous structure of the materials. This was confirmed by the absence of the C-H bands as can be seen in Figure S3a, spectrum 1.

In addition, the materials synthesized in this study were characterized by ATR-FT-IR. The results of each magnetic material are shown in Figure S3b. The absorption peak that can be seen starting around 600 cm^{-1} in all the spectra was attributed to the Fe-O-Fe vibration. This band confirms the existence of a magnetic core of Fe_3O_4 in all the particles. The successful surface modification with silica of the magnetic particles can be confirmed with the appearance of the Si-O-Si band at 1090 cm^{-1} in all the modified materials. After functionalization, the organic groups of C_8 and C_{18} showed two new peaks appearing at 2855 cm^{-1} and 2925 cm^{-1}, due to the C-H stretching bands of -CH_2- and -CH_3 groups. The peak intensity of these bands in the materials functionalized with C_{18} is higher than that functionalized with C_8 because of the bigger number of -CH_2- and -CH_3 groups in the C_{18} ligand (Figure S3b, spectrums 4 and 5, 6 and 7). Moreover, the intensity of this band is higher in the particles modified with mesostructured silica than with amorphous silica (spectrum 6 compared to 4 and 7 compared to 5), which can be attributed to a higher functionalization degree, with is in accordance with the results obtained in the elemental analysis (Table 1).

3.1.5 BET

BET characterization was used to optimize the amount of TEOS and CTAB for coating the magnetic core with a mesostructured silica layer. When coating the Fe_3O_4 particles with the amounts of TEOS and CTAB used in the literature [26], a considerable loss of magnetism of the particles was observed and therefore, in the washing stage of the

material by magnetic decantation a lot of material was lost, resulting in a very low yield of the synthesis. In the washes, it was visually observed that different fractions of particles were formed, some were more coated with mesostructured silica than others and therefore with different levels of magnetism. As shown in Figure S4, the first fraction was darker brown and the second and third (less magnetic) fractions were considerably more whitish due to an excess of the silica layer. To check this, BET surface area measurements were performed on the first three fractions, showing the following surface area results: 380 m^2/g for the first wash, 835 m^2/g for the second wash and 790 m^2/g for the third wash. This showed that TEOS and CTAB were added in excess giving a lower yield and a non-homogeneous coating, therefore, to avoid this issue, the content of these two reagents was reduced. The new amounts were compared with the original amount [26], with half and with a quarter of each of the two reagents and the surface areas were compared. It was found that the surface area did not change significantly (219, 205 and 314 m^2/g respectively) and that decreasing in the amount of reagent resulted in a higher yield since fewer particles were lost by magnetic decantation. Thus, syntheses of $Fe_3O_4@SiO_2@mSiO_2$ were carried out with a quarter of the amounts of TEOS and CTAB used in the literature [26].

In addition, $Fe_3O_4@SiO_2$ and $Fe_3O_4@SiO_2@mSiO_2$ were characterized before and after functionalization. The N_2 adsorption-desorption isotherms of the materials synthesized in this work are shown in Figure S5a and c. For all materials, its adsorption isotherm can be assigned as Type IV, according to the IUPAC classification [39], which is characteristic of mesoporous material. The isotherms show an initial part that is characteristic of monolayer adsorption and a significant increase in the amount adsorbed at intermediate relative pressures characteristic of a multilayer filling mechanism. In the case of $Fe_3O_4@SiO_2$ and their functionalized derivatives presented an H3 hysteresis loop with almost parallel branches confined to relative pressures between 0.8 and 1. This hysteresis loop is characteristic of uniform pores with a slit-like structure. Results obtained a BET specific surface area (S_{BET}) of 147 m^2/g and a pore volume of 0.18 cm^3/g for the non-functionalized and 26-24 m^2/g and 0.09-0.10 cm^3/g for the functionalized with C_8 and C_{18}, respectively, which demonstrates the correct functionalization of the materials (Table 1). As shown in Figure S5b, materials with amorphous silica have a disorganised

pore distribution (width of the peak at half of the height equals to 100 Å) because no template was used. Non-functionalized material presents most of its mesopores centred at 125 Å and a small group of mesopores can also be observed at 35 Å. After the C_8 and C_{18} functionalization, the small pores at 35 Å disappear and only the big pore at 125 Å is shown (Figure S5b and Table 1). The reason for this is that a decrease in the pore volume and pore diameter takes place, which is indicative of the correct anchorage of both ligands into the pores of the materials.

On the other hand, the Fe_3O_4@SiO_2@mSiO_2 and their functionalized derivatives shown also Type IV adsorption isotherm but in this case with an H4 hysteresis loop. Results obtained a S_{BET} of 355 m^2/g and a pore volume of 0.23 cm^3/g for the non-functionalized and 191-14 m^2/g and 0.14-0.04 cm^3/g for the functionalized with C_8 and C_{18}, respectively. This shows the correct functionalization of the material and that the C_8 ligand provides a greater surface area due to its smaller chain. The pore size distribution is close to the micro-pore range, unlike materials with amorphous silica. As can be seen in the pore size distribution (Figure S5d), these materials present most of their pores centered around 40 Å.

Table 1. Textural properties of the six magnetic materials synthesized.

Material	S_{BET} (m^2/g)[a]	Pore volume (cm^3/g)[b]	Pore diameter (Å)[c]	Elemental analysis (%)			
				C	N	H	(mmol ligand/g)[d]
Fe_3O_4	-	-	-	-	-	-	-
Fe_3O_4@SiO_2	147	0.18	125.8	-	-	-	-
Fe_3O_4@SiO_2@C_8	26	0.09	124.4	3.432	0.000	1.070	0.286
Fe_3O_4@SiO_2@C_{18}	24	0.10	124.9	3.227	0.000	0.928	0.134
Fe_3O_4@SiO_2@ mSiO_2	355	0.23	38.9	-	-	-	-
Fe_3O_4@SiO_2@ mSiO_2@C_8	191	0.14	39.0	10.426	0.000	2.174	0.869
Fe_3O_4@SiO_2@ mSiO_2@C_{18}	14	0.04	36.4	11.310	0.000	2.178	0.471

[a] S_{BET}: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method.
[b] Total pore volume was measured at relative pressure (P/P_0) = 0.97.
[c] Pore diameter estimated by using the BJH (Barrett, Joyner and Halenda) model applied on the desorption Branch.
[d] mmol of ligand/g of material calculated with the % obtained by elemental analysis.

3.1.6 Elemental analysis

The percentage of C in each functionalised material, calculated by elemental analysis, showed different levels of functionalization depending on the silica and organic group type, as shown in Table 1. Consequently, the material with the highest level of functionalization was $Fe_3O_4@SiO_2@mSiO_2@C_8$ with 0.869 mmol/g, followed by $Fe_3O_4@SiO_2@mSiO_2@C_{18}$ with 0.471 mmol/g, $Fe_3O_4@SiO_2@C_8$ with 0.286 mmol/g and finally, $Fe_3O_4@SiO_2@C_{18}$ with 0.134 mmol/g, which is coherent seeing the S_{BET}. Looking at these results, it can be confirmed that the use of mesostructured silica allows a higher level of functionalization than amorphous silica. Concerning the ligand, the one that gives the higher levels is C_8. This may be because it is less voluminous due to its shorter chain and has greater accessibility through the pores of the material, allowing for a higher level of functionalization.

3.2 Optimization of UHPLC-QqQ-MS/MS analysis

Triple quadrupole mass spectrometric parameters were optimized for analytes in positive ionization mode by direct infusion of a standard solution in methanol of 1 µg/mL of each opium alkaloid using a syringe pump at a flow rate of 20.0 µL/min. The molecular ion of each compound was detected with a Q_1 resolution of 0.7 at a scan time of 500 ms and to obtain the maximum intensity of the fragment ions of each analyte the collision energy was optimised. For chromatographic separation two mobile phases were tested, using acetonitrile or MeOH as eluent B and in both cases water as eluent A, all of them with 0.1% formic acid. The results were better with acetonitrile than methanol because of their best peak intensity and separation. Successive gradients were tested and finally, the separation of the six analytes was achieved in only 5 min with the following gradient: 5-70% B (0-3.5 min), 70-5% (3.5-3.7 min) and 5% (3.7-5 min). Table S2 lists the parent ion, daughter ion and collision energy (eV) optimal for MRM detection mode and retention time of the opium alkaloids.

3.3 Optimization of sample preparation

3.3.1 Optimization of SLE of opioids from poppy seeds

Many protocols in the literature have been examined for the extraction of opioids from poppy seeds. Some of them have used a large amount of sample and organic solvents, for example, a double extraction with 100 mL acetonitrile/water/acid formic 80/19/1 (v/v/v) to extract 10 g poppy seeds [1]. To minimize the costs and negative impact on the environment, an attempt was made to reduce the amount of sample to be used and with it, the amount of organic solvents. For this reason, the first SLE experiment was carried out in triplicate with 1, 2.5, 5 and 10 g of sample with 10 mL of acetonitrile/water/acid formic 80/19/1 (v/v/v) and for 4 different extraction times, (2.5, 5, 10 and 20 min). As a result, very high RSD values especially with 1 g sample (mean values for all analytes were 82, 66, 53 and 71% for each time, respectively) were observed. However, with 2.5 g of sample, the RSD was lowered (between 16 and 26%) but was not improved with 5 and 10 g of sample. This dispersion in the results could be due to the variability in the opioid content of seeds from the same batch, as the contamination of each seed with plant latex could not be the same as it depends on many different factors (mainly genetic factors and environmental conditions) so that their contamination is finally very heterogeneous. Therefore, it was decided that it was sufficient to use a 2.5 g sample, in order to minimize the use of environmentally harmful organic solvents.

Concerning the extraction solvent in the literature, many authors use methanol to extract opioids from poppy seeds [9, 17, 40-42]. Others use acidified methanol [6, 43] or a mixture of acetonitrile/water/formic acid 80/19/1(v/v/v) [1]. For this reason, in the present study these three types of solvents were evaluated in two agitation modes, ultrasound (US) and magnetic stirring with 2.5 g of poppy seeds and 10 mL of solvent during 10 min. In addition, the solvent methanol/water 50/50 (v/v) was added because it was the medium where the standards were dissolved and conserved in a stable form. As shown in Figure S6, not much difference was shown between acetonitrile/water/formic acid 80/19/1 (v/v/v) and methanol/water 50/50 (v/v) (although acetonitrile was better for morphine and noscapine). So, the solvent finally used for this purpose will depend on the

medium that best favors the subsequent adsorption step in the MSPE procedure to avoid the evaporation process and use less organic solvent.

Successive extractions were then performed to find out how many extractions were needed to complete the extraction. Six successive extractions were carried out with 20 mL of methanol/water 50/50 (v/v) for 10 min under magnetic stirring and complete extraction was not obtained. In the first extraction, most of the opioids present in the seeds were extracted and from the third extraction the values obtained were very low and constant, so it was not considered from the third extraction onwards. Furthermore, the same study was carried out with acetonitrile/water/formic acid, 80/19/1 (v/v/v) and similar results were obtained. Therefore, a double extraction with methanol/water 50/50 (v/v) was proposed.

Afterwards, the solvent volume and the extraction time were optimised. For this purpose, different combinations of double extractions were made with methanol/water 50/50 (v/v) in magnetic stirring where the volumes tested were 10, 20 and 30 mL and times 10, 20, 30 min and 1 hour. As shown in Figure S7, as the solvent volume increased, the amount of opioids extracted increased considerably. In the same way, the more time was used, the more was extracted, except from 30 min to 1 hour that there was no increase in extraction. Furthermore, once the extraction volume and time were optimised, an additional third extraction was performed and only one residual area was observed, therefore it was decided to perform only two extractions of 30mL during 30 min.

Finally, the pH value of the extraction solvent (pH 3, 5, 6.8 and 10) was studied. So, double extraction of 30 mL methanol/water 50/50 (v/v) for 30 min in magnetic stirring with different pH value: 3, 5, 6.8 (non-modified) and 10 was carried out. To obtain the acidified pH, formic acid was used and for the basic pH, ammonia. As can be seen in Figure S8, with the unmodified solvent (pH 6.8) higher extractions were obtained for all the analytes.

3.3.2 Selecting the best material for the MSPE procedure after making discontinuous adsorption study with the synthesized magnetic materials

To select the best magnetic material to do the MSPE procedure a discontinuous adsorption study was carried out with 2 mL of methanol/water 50/50 (v/v) with 1 µg/mL of each of the six analytes and 100 mg of each of the material synthetized. This adsorption medium was selected because it was the medium where the working standard solutions were prepared and were therefore soluble and stable and 100 mg of material because it was the maximum quantity that the material was expected to be used to carry out the MSPE procedure. As can be seen in Figure 2, the result was that the three materials synthesized coated with mesostructured silica showed lower area values of the supernatant as the adsorption time increases and with it, higher percentages of adsorption than amorphous silica materials. This agrees with the characterization results since the mesostructured silica-coated material had a larger surface area than amorphous silica-coated material (355 vs 147 m^2/g). In addition, more adsorption was obtained with non-functionalized materials than functionalized materials with C_8 and C_{18} group.

To confirm the higher adsorption of non-functionalised material, an additional experiment was carried out with the three mesostructured materials with different adsorption media. The solvent selected to carry out the adsorption step with the material were the two solvents that showed similar results in the extraction of the opioids from the seeds, acetonitrile/water/formic acid 80/19/1 (v/v/v) and methanol 0.1% acetic acid (Figure 3). Therefore, the results obtained previously with methanol/water 50/50 (v/v) were compared with the results obtained with acetonitrile/water/formic acid 80/19/1 (v/v/v) and with acidified methanol 0.1% acetic acid. By comparing Figure 2 (orange colour) and Figure 3, with all solvents more adsorption (%) was observed with the non-functionalized material. This can be due to two possible reasons when functionalizing the pore size has been reduced so much that the molecules do not penetrate and only a surface interaction takes place, or the analytes do not interact effectively with the C_8 and C_{18} groups. Therefore, to carry out the MSPE, the material Fe_3O_4@SiO_2@mSiO_2 was finally selected. In addition, the results were significantly better with methanol/water 50/50 (v/v) than with the other two solvents. Therefore, this solvent mixture was selected to carry out the adsorption step of the MSPE as for the extraction of opioids in the poppy seeds to avoid the evaporation step.

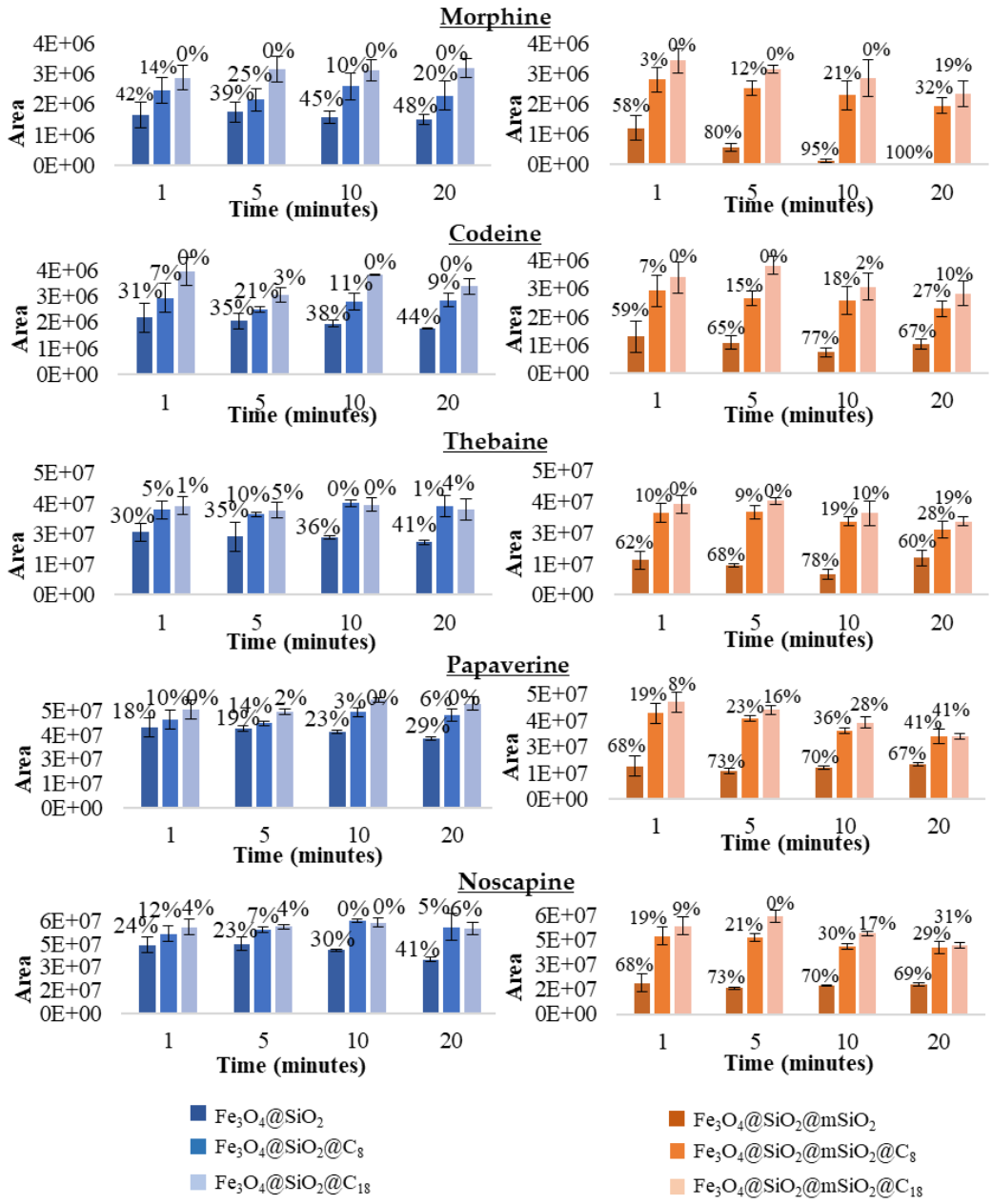


Figure 2. Areas of the supernatant with their respective percentages of adsorption (%) at different times with each of the 3 amorphous silica materials (blue colour) and mesostructured silica magnetic materials (orange colour) with methanol/water 50/50 (v/v) as adsorption solvent.

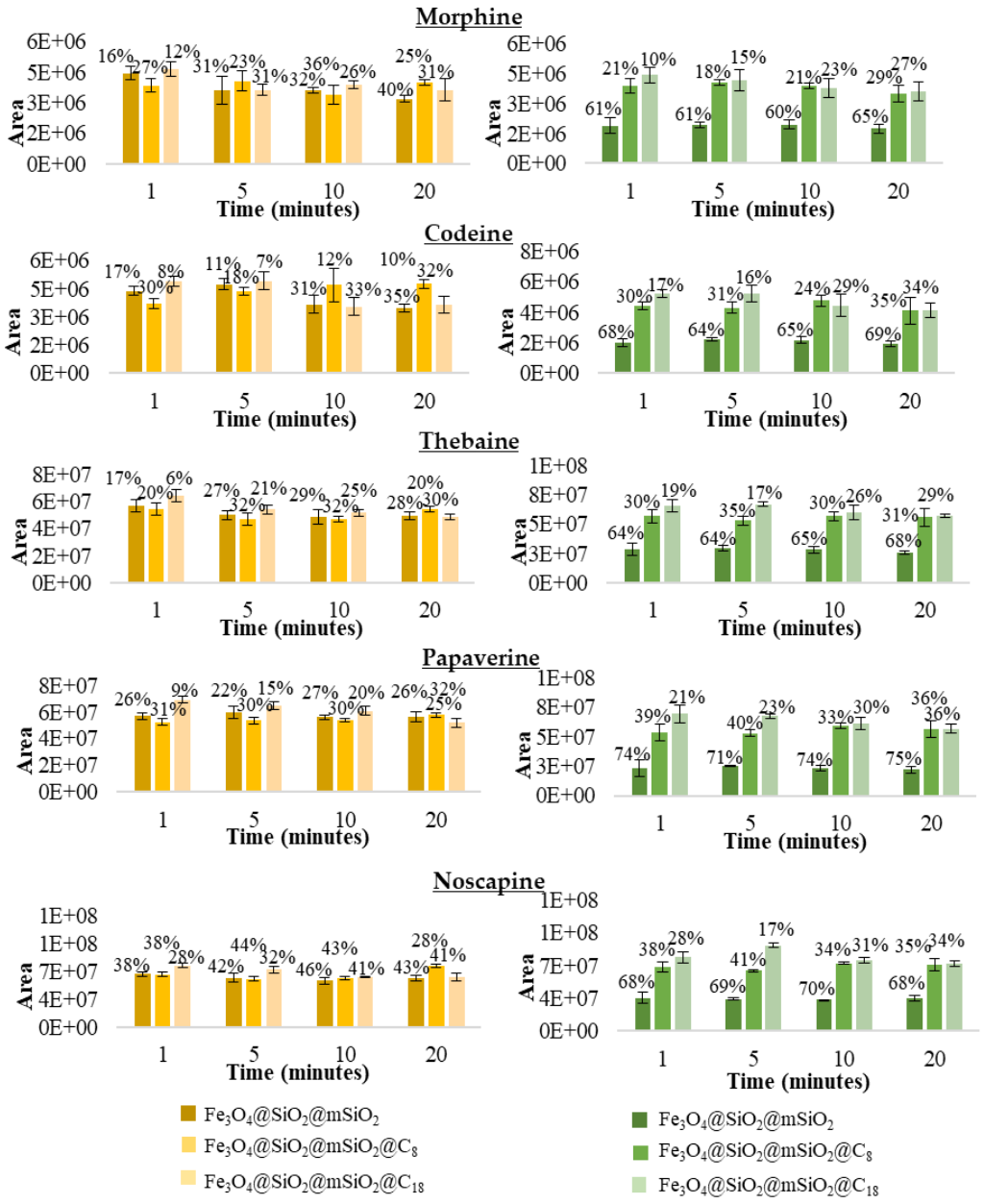


Figure 3. Areas of the supernatant with their respective percentages of adsorption (%) at different times with each of the 3 mesostructured silica magnetic materials with different adsorption solvent: acetonitrile/water/formic acid 90/19/1 (v/v/v) (yellow colour) and methanol acidified with 0.1% acid acetic (green colour).

3.3.2 Adsorption kinetic and isotherm experiments of $Fe_3O_4@SiO_2@mSiO_2$ material

As shown in Figure S9a the adsorption kinetics of the six opioid alkaloids is fast. In the first minute, almost all the adsorption is obtained and in the following minutes, it was similar. However, in morphine and oripavine there is a considerable increase in adsorption at minute 20 with respect to minute 1, so it was decided to take 20 min as the adsorption time for the rest of the experiments for the moment. The adsorption kinetics were determined by Langergren's pseudo-first-order [30], pseudo-second-order [31] and intra-particle diffusion kinetic models [32], according to the equations (2)-(4) in Table S3. The results were shown in Figure S10a and the important data of the three kinetics model were compiled in Table S4. The linear regression coefficients (R^2) were more closed to 1 in the pseudo-second-order model and their $Q_{e,\ cal}$ (calculated result) was more like $Q_{e,\ exp}$ (experiment result). For these reasons, the adsorption of the six opium alkaloids accorded mostly to the pseudo-second-order kinetics, indicating a chemical adsorption mechanism [37]. In addition, morphine and oripavine were the analytes with the highest intraparticular diffusion rate with K_p values of 1.97 and 1.53 mg/g min^2 respectively and showed R^2 close to 1 (Table S4), unlike the rest of the analytes that did not show this trend.

The adsorption isotherms were analysed by Langmuir [33] and Freundlich [34] models, according to the equations (5) and (6) in Table S3. As shown in Figure S9b, by increasing the concentration of opioids in the equilibrium, the adsorption capacity was increased. In morphine, the maximum adsorption capacity was determined, but in the other opioids, it was still possible to adsorb more. As shown in Figure S10b, the R^2 obtained by the Langmuir model was more closed to 1 than by the Freundlich model. So, adsorption occurred monolayer on the uniform surface of $Fe_3O_4@SiO_2@mSiO_2$ [37]. Equations (1), (5), (6) allowed calculated Q_{max} was 73.59, 102.04, 303.03, 303.03, 107.353 and 149.25 mg/g for morphine, codeine, thebaine, papaverine, noscapine and oripavine, respectively.

3.3.4 Optimization of MSPE procedure with Fe_3O_4@SiO_2@mSiO_2 material

3.3.4.1 Adsorption conditions (time and pH)

To determine the optimal adsorption time, different time ranges between 1 and 20 min were investigated with 100 mg Fe_3O_4@SiO_2@mSiO_2 material. It can be seen in the adsorption kinetics of six opium alkaloids in Figure S9a that the amounts of alkaloids adsorbed (Q_t) at 1 min are similar to those adsorbed at 20 min, except for morphine and oripavine which have slower adsorption kinetics and at 20 min their maximum adsorption takes place. After determining that these two compounds have a higher intraparticle diffusion than the rest of the analytes (section 3.3.3), in addition to evaluating the adsorption time in the highest or lowest level of adsorption of the analytes on the material, the influence of the adsorption time on the complete MSPE process was studied. For this purpose, the recoveries obtained at 1 and 20 min were calculated. To calculate them, the areas obtained by performing the complete MSPE procedure on a sample at a known concentration were compared with the areas of a blank sample subjected to the same extraction and purification process and spiked with the expected concentration. Finally, the recoveries of morphine and oripavine obtained after performing the complete MSPE procedure were lower at 20 min adsorption than at 1 min adsorption, approximately 20% versus 50%, respectively. The possible reason for this may be that, with 20 min adsorption, a higher percentage of adsorption is obtained, but it penetrates so much into the pores (following the intra-particle model studied in section 3.3.3) that later, in the desorption step, it cannot be desorbed. For this reason, the adsorption time of 1 min was selected for the MSPE procedure.

The influence of the pH of the sample solution on the efficiency of the MSPE process was investigated. For this purpose, 1 µg/mL of each of six opioids was in contact with 100 mg Fe_3O_4@SiO_2@mSiO_2 material in methanol/water 50/50 (v/v) with different pH values between 3.0 and 10.0 were studied for 1 min adsorption. Such as shown in Figure S11, there were no major differences between the values, especially in morphine and oripavine, which were very similar. In the other analytes, it was seen that the pH 6.8 of the methanol/water 50/50 (v/v) without modification was the one that showed more adsorbed area and with lower RSD, since the 7.6 showed more variation. For codeine pH

6.8 and 10 showed no differences, for thebaine pH 10 facilitated the adsorption but as for papaverine and noscapine it decreased, finally pH 6.8 was selected as the best.

3.3.4.2 Desorption conditions (solvent type, time, pH and number of consecutive desorptions)

The type of the desorption solvent selected is a critical parameter to obtain the highest possible desorption of the analytes and thus a higher recovery value (%). First, the four solvents used in the SLE of poppy seeds at different desorption times (1, 5, 10 and 20 min) were used with 20 min of adsorption time. As shown in Table S5, the recoveries obtained were low for morphine and oripavine. Therefore, it was determined that none of these solvents were effective for desorption. Consequently, eight solvents were studied (diethyl ether, dichloromethane, chloroform, isopropanol, acetonitrile, methanol, water and ethyl acetate) at 20 min desorption. All of them were used at three pH values, acidic with formic acid 1%, without modification and basic with ammonia 1%. With the first two conditions, it was not possible to desorb the analytes from the material obtaining low recoveries, for morphine and oripavine (around 1%) and for the rest of the analytes less than 50%. While the basic medium facilitated the desorption of the six opioids, although the recoveries obtained were still low. Therefore, it was decided to increase the basicity of the medium by adding ammonia 10%. As can be seen in Table S6, by increasing the ammonia content, the recoveries improved moderately. However, the recoveries were still low, so different mixtures were performed with the solvents that gave the best results, diethyl ether with methanol or acetonitrile and dichloromethane with methanol or acetonitrile at different proportions (50/50, 80/20 and 20/80, v/v) and for different times (20, 40 and 60 min). As shown in Table S7, the best combination was diethyl ether/methanol, 80/20 (v/v), as all analytes were around 100% recovery, except for morphine and oripavine, that the maximum recovery was 37%. The reason why desorption of these two analytes was so difficult might be because they showed high intraparticle diffusion (section 3.3.3). Finally, the adsorption and desorption times were reduced, and the study was carried out with 1 min of adsoption and with 1 min for up to five consecutive desorptions with diethyl ether/methanol 80/20 (v/v) with 10% ammonia.

As can be seen in Table S8, higher recoveries were obtained with short times in adsorption and desorption steps, between 76 and 109% for all analytes, except to morphine and oripavine that shown recoveries near to 50%. In addition, it was also studied to increase the desorption solvent volume to double, from 2 mL to 4 mL. However, no better results were obtained. Therefore, the best option was to make triple desorption with 2 mL for 1 min.

3.3.4.3 Amount of Fe_3O_4@SiO_2@mSiO_2 material

To make the MSPE procedure effective and obtain good results, different amounts of magnetic particles between 25 and 100 mg were studied. Figure 4 shows the areas of desorbed opium alkaloids achieved with the different amounts of adsorbent material which their respective recovery values (%). The amount selected to perform the MSPE procedure was 50 mg of magnetic particles because with this amount 51, 64, 98, 101, 76 and 48% recovery for morphine, codeine, thebaine, papaverine, noscapine and oripavine were achieved. Increasing the amount of adsorbent material above this did not significantly improve these results.

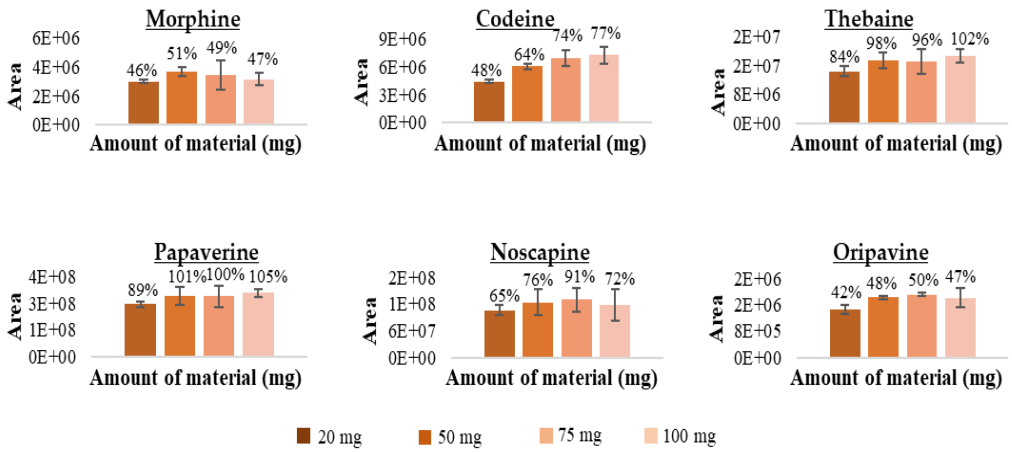


Figure 4. Effect of the different amounts of material in the optimised MSPE procedure with Fe_3O_4@SiO_2@mSiO_2 material.

3.4 Instrumental and method validation

The instrumental validation parameters are showed in Table S9. First, the linear range of each of the six analytes dissolved in pure solvent was evaluated. Instrumental limits of detection and quantification were calculated by analysing dilutions with decreasing concentration until the S/N ratio was approximately 10. The LOQ and LOD were estimated as 10 and 3 times the S/N ratio, respectively, obtaining LOQ and LOD for morphine, codeine and oripavine around 8 x 10^{-2} and 2 x 10^{-2} µg/L, respectively and for thebaine, papaverine and noscapine around 5 x 10^{-3} and 1 x 10^{-3} µg/L, respectively (Table S9). The linear range started at 0.1 µg/L, for morphine, codeine and oripavine or at 0.01 µg/L, for thebaine, papaverine and noscapine, up to 5000 µg/L.

The method validation parameters are showed in Table 2. The MDL and MQL for morphine, codeine, papaverine and noscapine were 0.07 and 0.24 µg/kg, for thebaine were 0.72 and 2.4 µg/kg and for oripavine 72.07 and 240 µg/kg, respectively. The linear range started at 0.01 µg/L for morphine, codeine, papaverine and noscapine, at 0.1 µg/L for thebaine or at 10 µg/L for oripavine up to 5000 µg/L. Calibration lines were obtained with an adequate R^2 (0.999). In addition, the Cm was calculated which was always ≥ 92%, successfully accomplishing the criteria established on the guidelines [35].

To evaluate the ME, the slopes of each of the calibration lines were compared. As shown in Table 2, the ME (%) obtained when comparing the slopes of the purified matrix versus slope of solvent ranged from 80-109% for codeine, thebaine and papaverine, thus these analytes are not affected by any matrix effect that may remain after purification (according to the criteria established in SANTE/11813/2017 [35]). For morphine, noscapine and oripavine values slightly less than 80% were obtained, indicating that there was signal suppression. Therefore, to quantify the target analytes in the real samples, matrix-adjusted calibration curves had to be used to compensate for the errors associated with these matrix effects. In addition, a matrix calibration curve without MSPE purification was also analysed and, in this case, the ME (%) calculated were in almost analytes more than 120%, especially for thebaine and oripavine which were 294 and 258%, respectively. For this reason, a considerable improvement was observed after MSPE purification, which allowed the matrix interferences to be reduced.

Table 2. Validation parameters of the SLE-MSPE-UHPLC-QqQ-MS/MS method for the determination of six opium alkaloids in poppy seeds samples.

Analytes	Linear range (µg/L)	Matrix-matched calibration (R^2)	Cm	ME	MDL (µg/kg)	MQL (µg/kg)	Accuracy		Precision	
							Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-Day (RSD %)	Inter-Day (RSD %)
Morphine	0.01-5000	y = 3269x + 32452 (0.999)	92	64	0.07	0.24	50 ± 1 [a] 42 ± 2 [b]	46 ± 2	4 [a] 2 [b]	11 [a] 5 [b]
Codeine	0.01-5000	y = 2554x + 29642 (0.999)	96	86	0.07	0.24	64 ± 2 [a] 71 ± 6 [b]	68 ± 4	3 [a] 2 [b]	8 [a] 4 [b]
Thebaine	0.1-5000	y = 10093x + 485953 (0.999)	96	109	0.72	2.40	72 ± 3 [a] 76 ± 4 [b]	74 ± 4	5 [a] 0 [b]	7 [a] 4 [b]
Papaverine	0.01-5000	y = 33441x + 2814320 (0.999)	92	80	0.07	0.24	116 ± 5 [a] 101 ± 1 [b]	109 ± 3	5 [a] 2 [b]	9 [a] 7 [b]
Noscapine	0.01-5000	y = 42914x + 1631091 (0.999)	94	65	0.07	0.24	109 ± 1 [a] 97 ± 1 [b]	103 ± 1	4 [a] 3 [b]	10 [a] 8 [b]
Oripavine	10-5000	y = 1080x - 31137 (0.999)	94	31	72.07	240	51 ± 3 [a] 53 ± 3 [b]	52 ± 3	3 [a] 3 [b]	5 [a] 5 [b]

Cm: linearity coefficient calculated by (1 - (SD/average slope)) x 100, where SD is the standard deviation of the calibration slopes obtained on different days; ME: matrix effect calculated by dividing the purified matrix slope by the solvent slope. MDL: method detection limit and MQL: method quantification limit estimated as 3 and 10 times the signal/noise ratio, respectively. Accuracy (mean recovery obtained from six samples, n = 6) and precision were obtained spiking samples at two known concentration level: [a] low spiked level (0.1 mg/kg) and [b] high spiked level (1 mg/kg). Intra-day precision: six consecutive injections (n = 6) on the same day; Inter-day precision: three replicate samples injected in triplicate throughout three diferent days (n = 9).

Accuracy was expressed as the average recovery obtained from six samples (n = 6) spiked with the analytes at a known concentration and subjected to the proposed extraction-purification procedure. Accuracy was evaluated at two concentration levels: low (0.1 mg/kg), and high (1 mg/kg). Recovery values were calculated by comparing the value of the samples with the value of the simulated samples (samples subjected to the same extraction and purification process but spiked at the same concentration level prior to chromatographic analysis). As shown in Table 2, the recovery values obtained were in the range 70-116%, thus favourable according with the guidelines [35] However, for morphine and oripavine the recoveries obtained were around 50%, which may be due to the fact that they were the smallest analytes and showed higher intraparticle diffusion and were, therefore, more difficult to desorb, resulting in lower recoveries. As the molecules were smaller in size, the adsorbent material adsorbed the analytes, but they were not completely desorbed as they remained in the internal and smaller pores of the material due to the fact that they are the compounds with the highest intraparticle coefficient (see section 3.3.3). To check that the recovery value of morphine was associated with the nature of the analyte, a recovery assay with morphine-D3 was also performed and a similar recovery value was obtained (45% ± 1). Satisfactory results of precision were obtained at the two concentration levels evaluated because the RSD values obtained were lower than 6 and 11% for intra-day and inter-day precision, respectively (Table 2). For this reason, morphine and oripavine also could be quantified in real samples using a correction factor. According to SANTE/11813/2017, the recoveries between 30 and 70% and 120 and 140% can be acceptable if RSD is ≤ 20% [35].

A good selectivity of the method was obtained as shown as Figure 5. When comparing the chromatograms and the spectra of each of the analytes, it was found that the variation of the retention time was in all cases ≤ 0.1 min deviation and the ion ratios of the sample extracts were within ±30% (relative) of the average of the calibration standards for each of the analytes, according to with established in the reference guide [35]. All samples showed two peaks to codeine. However, the first peak did not coincide with the retention time of the standard and the ratio of the product ions did not accord. As it did not comply with these two criteria, this first peak was discarded and was considered not to be codeine,

instead, it was a matrix product. In addition, for oripavine, a second peak was also observed in some standards or samples, which was related to thebaine since it appeared in its retention time and since oripavine is its main metabolite and some of its transitions were similar.

Finally, these results indicated that the proposed method showed good analytical performance and could be successfully used for the extraction, purification by MSPE and quantification of poppy seeds samples.

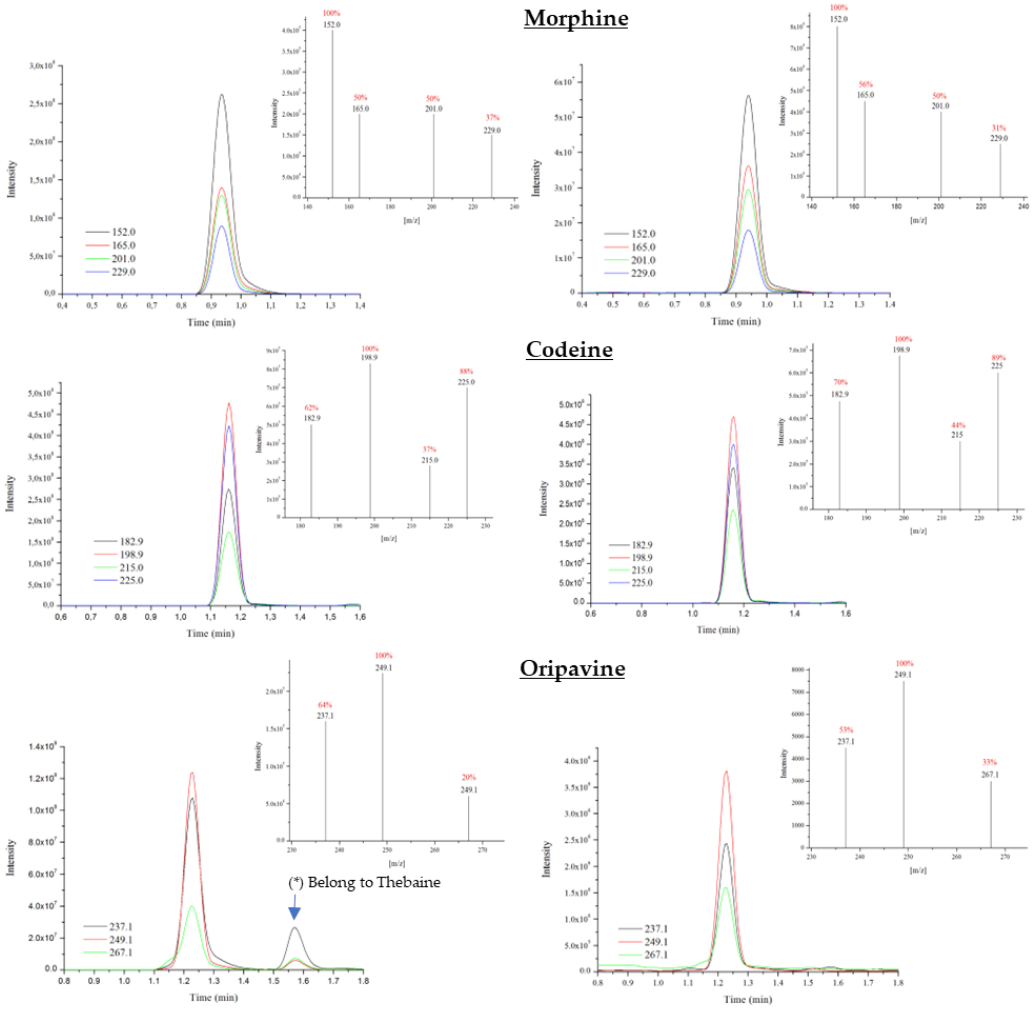


Figure 5. Comparison between the extracted ions chromatograms and intensity of fragment ions mass spectrum (relative abundance (%) in colour red) obtained for each of the compounds in a standard solution mixture 1 mg/L (left) with respect to a poppy seed sample (right).

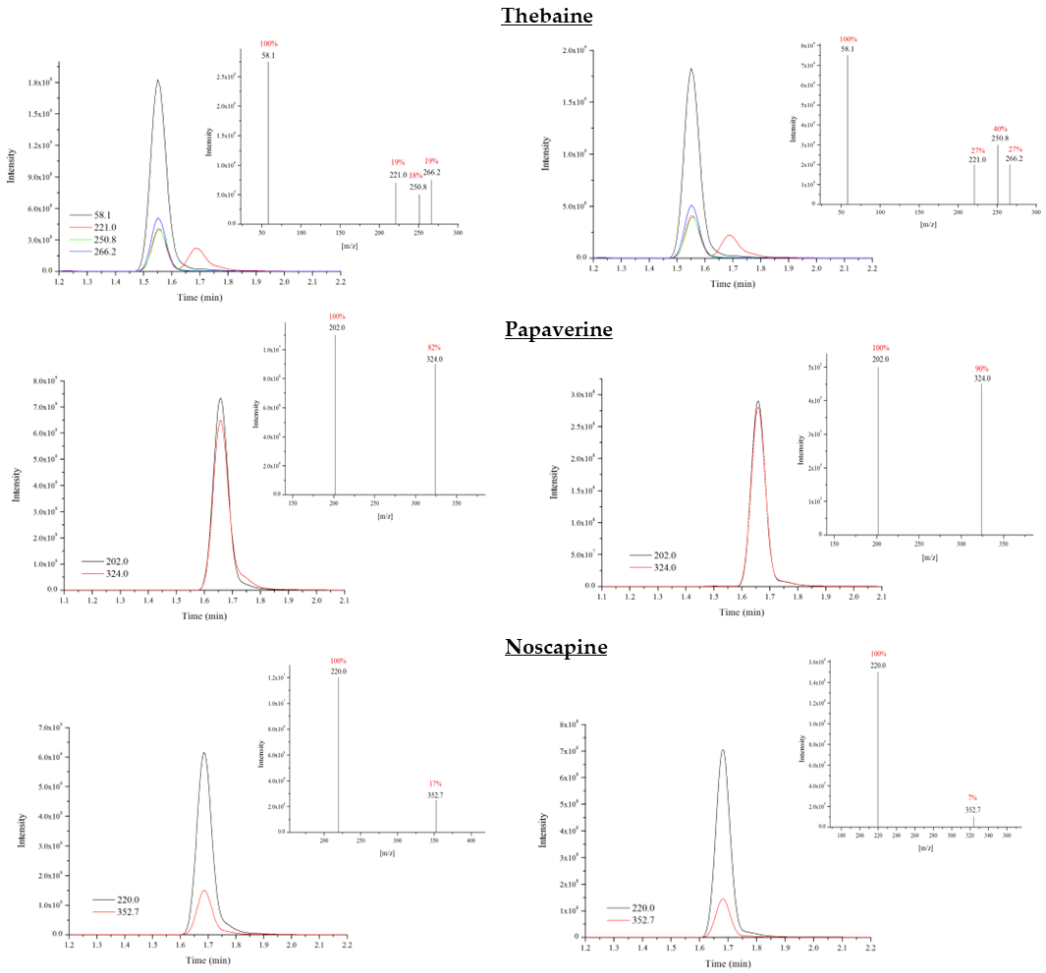


Figure 5. *Cont.*

3.5 Comparison of the proposed methodology with others reported methods

The developed method was compared with others published in recent years for the analysis of opium alkaloids in poppy seeds, straw or capsules, and hot pot, a traditional Chinese food. Table 3 summarizes the extraction, purification and analytical techniques used and the main characteristics of each methodology. As has been seen, the most used separation technique is HPLC, although there are authors who also use UHPLC because it has the advantage of reducing analysis times. Chromatography times for methods developed with HPLC-MS/MS are between 15 and 30 min [6, 20], while methods developed with UHPLC-MS/MS take between 7 and 10 min [1, 22]. A noteworthy aspect of this proposed method is that only 5 min were sufficient to efficiently quantify the six opium alkaloids, considerably less time than the methods already developed to quantify opioids in the literature. This is very important since reducing the analysis time also reduces the cost. In addition, the detector that has been mainly used is the QqQ, which means a much more selective detection with higher sensitivity. Although, there are some studies with other detectors, such as DAD or UV detector [16, 24].

As can be seen in Table 3, the main extraction technique used in the literature is SLE with organic solvents (acetonitrile/water/formic acid, acidified methanol, or pure methanol) [1, 6, 17]. The main disadvantage of these methods is the high consumption of organic solvents, about 200 mL. This is a very important aspect to consider as they are damaging to the environment. In addition, although it gives some good recovery results, it can lead to incorrect results due to matrix interferences, as seen in this work and as some of the authors have commented in their works [1]. Furthermore, it should be considered that, as there are so many impurities that have not been removed, the chromatographic column and the instrument become dirty more easily, shortening its useful life. Therefore, it is very important to perform a purification step that can remove these matrix interferences. The conventional purification technique and which two authors use with these analytes is SPE [19, 20]. However, it is a very complex technique to operate compared to other new purification techniques, such as MSPE, avoiding strict control of the loading and elution flow rate and obstruction problems. There are still few authors who have started to use magnetic adsorbent materials to purify samples to quantify

opioids and none of them uses it for poppy seeds samples. Three have been found in the literature, although one of them only analyses two of the main opioids. In all of them, minimization of matrix interferences and good recoveries have been obtained by selectively adsorbing the analytes. However, in the methodology proposed in this work, lower MDL and MQL have been obtained than in other published works (between 0.07-72.01 µg/kg and 0.24-240 µg/kg, respectively). In addition, the advantage of this method over others is that purification is completed in only 4 min (1 min of adsorption and 3 min of desorption), unlike others that take up to 30 min [23]. Moreover, it is the first methodology developed and validated for the quantification of the six main opium alkaloids in poppy seeds, which is very important to take into account since EFSA claimed in its last opinion of 2018 the absence of studies with all these analytes and, therefore, the need to develop methodologies capable of quantifying all of them [15]. The result is that this method is completely able in a quick, easy, and efficient way to quantify the six target analytes in poppy seed samples.

Table 3. Comparison of the developed SLE-MSPE-UHPLC-QqQ-MS/MS method with other reported methods for the detection of opium alkaloids in poppy (seeds, straw, or capsules) and hot pot.

Sample	Analytes	Sample treatment Extraction	Purification	Analysis technique	MDL (µg/kg)	MQL (µg/ kg)	Recovery (%)	RSD (%)	Ref.
Poppy seeds	MOR, COD, THEB, NOS, PAP	AcN/water/ formic acid, 80/19/1, v/v/v (100 mL, 30 min, x2)	–	UHPLC-QqQ-MS/MS	–	100	77-172	<20.0	[1]
Poppy seeds, cake, buns	MOR, COD, PAP, NOS	MeOH 0.1% acetic acid (30 mL, 60 min)	–	HPLC-QqQ-MS/MS	70-300	200-1000	–	7.4-9.0	[6]
Poppy straw	MOR, COD, THEB, PAP	MeOH (5 mL, 20 min, x2)	–	HPLC-DAD	200-1800	600-5400	97-99	0.2-0.4	[17]
Hot pot	MOR, COD, THEB, PAP, NOS	HCl 0.1 M (20 mL, 10 min) and PE (10 mL)	SPE (Oasis MCX 60 mg)	UHPLC-QqQ-MS/MS	0.003-0.04	0.01-0.1	72-124	7.9-23.7	[19]
Poppy straw	MOR, COD, THEB, PAP	Water 5% acetic acid	SPE (Oasis MCX)	HPLC- Ion trap-MS/MS	400-17500	1100-52200	–	–	[20]
Hot pot	MOR, COD, THEB, PAP, NOS	Water/AcN 50% (20 mg, 5 min)	MSPE (Fe_3O_4@SiO_2@ADME 50 mg)	HPLC-QqQLIT-MS/MS	0.05-0.8	0.25-2.5	80-115	4.3-10.7	[22]
Hot pot	MOR, COD, THEB, PAP, NAR	AcN 0.1% formic acid and n-hexane	MSPE (Fe_3O_4@SiO_2@CS/GO 15 mg)	UHPLC-QqQLIT-MS/MS	0.016-0.092	0.036-0.31	75-104	0.7-9.5	[23]
Poppy capsules	NAR, PAP	SFE	MSPE (Fe_3O_4@Cu@DPTC 50 mg)	HPLC-UV	1-100	–	88-99	5.8-7.7	[25]
Poppy seeds	MOR, COD, THEB, PAP, NOS, ORIP	MeOH/water, 50/50 (v/v)	MSPE (Fe_3O_4@SiO_2@mSiO_2 50 mg)	UHPLC-QqQ-MS/MS	0.07-72.01	0.24-240	46-109	0.4-11	This work

MOR: morphine, COD: codeine, THEB: thebaine, PAP: papaverine, NOS: noscapine, NAR: narceine, ORIP: oripavine, AcN: acetonitrile, MeOH: methanol, HCl: hydrochloride acid, PE: petroleum ether, SFE: supercritical fluid extraction, SPE: solid phase extraction, MCX: mixed-mode, strong cation-exchange, MSPE: magnetic solid phase extraction, DPTC: diphenylthiocarbazone, (U)HPLC: (ultra)-high performance liquid chromatography, QqQ: triple quadrupole, MS/MS: tandem mass spectrometry, DAD: diode-array detector, QqQLIT: quadrupole linear ion trap, UV: ultraviolet detector, MDL: method detection limit, MQL: method quantification limit, RSD: relative standard deviation.

3.6 Application of the method SLE-MSPE-UHPLC-QqQ-MS/MS to real samples of poppy seeds

Finally, the method was applied to analyze 16 seeds samples: 11 commercial poppy seeds, 2 wild poppy seeds and 3 commercial edible seeds mix (Table S1). Once the areas of each of the samples were obtained, the corresponding recovery values (Table 2) were applied and these results were divided by the areas obtained from the internal standard (morphine-D3). Finally, they were interpolated into matrix-matched calibration curves with morphine-D3 to minimize the possible signal errors and give more accurate results.

All samples were found to contain the six opium alkaloids (Table 4), so they were contaminated by latex from the *Papaver somniferum* L. plant. This result was very surprising because three of them were labelled as being from *Papaver rhoeas* L. species, which does not contain opium alkaloids (samples PS03, PS05 and PS06). Moreover, physically they look like the blue poppy seeds of the *Papaver somniferum* L. species. Therefore, these two aspects give enough evidence to say that there is wrong labelling of these products. For comparison, wild corn poppy seeds (PS10), which were black and small, were analysed and none of the analytes was detected, whereas in wild opium poppy seeds (PS9), the six target analytes were found. This confirms the theory that the common poppy (*Papaver rhoeas* L.) does not contain opium alkaloids and the label of the commercial products analysed are incorrect.

In addition, the concentrations of each type of poppy seed were calculated. Due to the surface contamination and the large number of other factors influencing the concentration of these alkaloids in poppy seeds, variable amounts have been found in the same batch of seeds. For this reason, the range of concentration is provided to show the except of the variation and is in keeping with other researchers, who identified a variation in opium alkaloids within a batch and between batches of poppy seeds analyzed [1, 2, 44]. Concentrations of morphine ranged from 1.5 to 249.0 mg/kg, codeine from <0.2 µg/kg to 45.8 mg/kg, thebaine from <2.4 µg/kg to 136.2 mg/kg, papaverine from <0.2 µg/kg to 27.1 mg/kg, noscapine from <0.2 µg/kg to 108.7 mg/kg and oripavine from <240 µg/kg to 33.4 mg/kg.

The opium alkaloid concentrations obtained in poppy seeds agreed with concentrations determined by other researchers. In the case of morphine, wide ranges of concentrations were obtained by López et al., (2018) 0.2-241 mg/kg, Sproll et al., (2006) between <1 and 270 mg/kg, Bjerver et al., (1982) who found 2.6-106.7 mg/kg, Hayes et al. (1987) between 17 and 294 mg/kg [1, 6, 45, 46], lower results were found by Carlin et al., (2020) between 3 and 64 mg/kg [44] and higher levels were found by Powers et al., (2018) from <1 to 2788 mg/kg [3]. Regarding codeine, the results obtained were very similar to the results obtained by Sproll et al., (2006) who found <0.3-56 mg/kg [6] but were lower than the results found by López et al., (2018) who found from <0.1 to 348 mg/kg and Powers et al., (2018) <1 to 247.6 mg/kg [1, 3]. On the other hand, the results obtained for thebaine were similar to those of López et al. (2018), as they found <0.1-106 mg/kg and Powers et al., (2018) <1 to 124 mg/kg [1, 3]. Regarding papaverine and noscapine, the contents found in this study were higher than others, as Sproll et al. (2006) did not detect their presence in any of their samples [6] and López et al. (2018) only determined <0.1-3.8 mg/kg of papaverine and <0.1-5 mg/kg of noscapine [1]. Notably, oripavine had not been previously analysed in poppy seeds by any author.

In addition, many of the samples showed values below the MQL but in all of them considerably high values of morphine were quantified, which meant that all samples except two (87%) exceeded the maximum limit established by Germany (4 mg/kg) and the 73% exceeded the reference level established in the EU (10 mg/kg morphine in poppy seeds for direct human consumption). It, therefore, implies that this agreement is not being fulfilled. In addition, 73% exceeded the Hungarian limit for morphine (30 mg/kg). For the other analytes, the maximum limit established by Hungary was 20 mg/kg, so two samples exceeded the maximum limit for codeine (PS02 and PS03), two samples for thebaine (PS03 and PS11), three for papaverine (PS01, PS02 and PS09) and seven for noscapine (PS01, PS02, PS03, PS07, PS08, PS09 and PS11). If oripavine with the same concentration was considered, two samples would exceed this limit (PS02 and PS03). As it can be seen in Table 4, three seed mixes consisting of sunflower, sesame, brown flax, pumpkin, and poppy seeds were also analysed. In one of them (MIX2) a considerably high amount of morphine was found (54 mg/kg), taking into account that only 5-10% of the seeds in the mix were poppy seeds. Therefore, poppy seeds used in MIX2 also

exceeded the limit maximum established in the EU. All these results confirm the current problems with the marketing of such products in the EU because in the absence of common legislation, a high number of RASFF health alerts are occurring, some 30 notifications since 2005 [4].

Considering the highest concentration obtained in each of the batches as shown in Figure 6, the samples with the highest number of total opiate alkaloids are PS03 with approximately 475 mg/kg (supposedly of the *Papaver rhoeas* L. species) and PS02 with approximately 250 mg/kg (physically blue poppy seed from Turkey). Especially high was the amount of thebaine found in PS11 (physically blue poppy seed from Czech Republic). Oripavine was found in all the commercial samples.

Table 4. Range of occurrence (mg/kg) of each of the six opium alkaloids analysed in three samples (n=3)

Code	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
PS01	20.6-45.4	5.7-19.2	<MQL-7.3	7.3-19.5	9.4-24.8	<MQL-0.6
PS02	23.2-118.7	3.2-26.7	0.9-17.7	2.1-27.1	6.5-42.6	3.8-22.9
PS03	154.9-249.0	21.3-45.8	12.4-31.5	0.8-2.9	0.3-108.7	9.8-33.4
PS04	17.0-19.5	1.0-1.9	3.4-6.5	<MQL	<MQL	<MQL
PS05	1.9-2.2	<MQL	<MQL	<MQL	<MQL	<MQL
PS06	1.5-3.7	<MQL	<MQL	<MQL	<MQL	<MQL
PS07	6.9-59.3	0.4-0.9	<MQL-5.8	<MQL-1.1	0.7-36.3	<MQL-0.8
PS08	28.8-35.1	3.4-4.0	0.8-3.4	10.5-19.9	18.1-22.7	<MQL
PS09	58.6-64.3	2.9-3.6	1.3-3.2	<MQL	19.0-21.7	<MQL
PS10	ND	ND	ND	ND	ND	ND
PS11	33.7-53.0	3.7-8.7	37.4-136.2	<MQL -1.9	2.0-21.1	2.3-13.8
PS12	4.1-22.3	<MQL-0.3	<MQL-1.5	<MQL	<MQL	<MQL-0.5
PS13	49.6-125.4	2.3-16.5	3.9-15.6	<MQL	<MQL	<MQL-0.5
MIX1	6.5-8.7	<MQL-0.04	<MQL	<MQL	<MQL	<MQL
MIX2	44.0-54.0	0.7-3.0	0.68-2.6	<MQL	<MQL	<MQL-0.3
MIX3	7.0-8.6	<MQL	<MQL	<MQL	<MQL	<MQL

MQL: method quantification limit, (for codeine, papaverine and noscapine: 0.24 µg/kg, for thebaine: 2.40 µg/kg and for oripavine: 240 µg/kg). ND: not detected. To quantify seed samples was used matrix-matched calibration with internal standard (morphine: y = 0.006x – 0.182; codeine: y = 0.005x + 0.117; thebaine: y = 0.020x + 1.853; papaverine: y = 0.069x + 6.739; noscapine: y = 0.080x + 6.049; oripavine: y = 0.002x + 0.009).

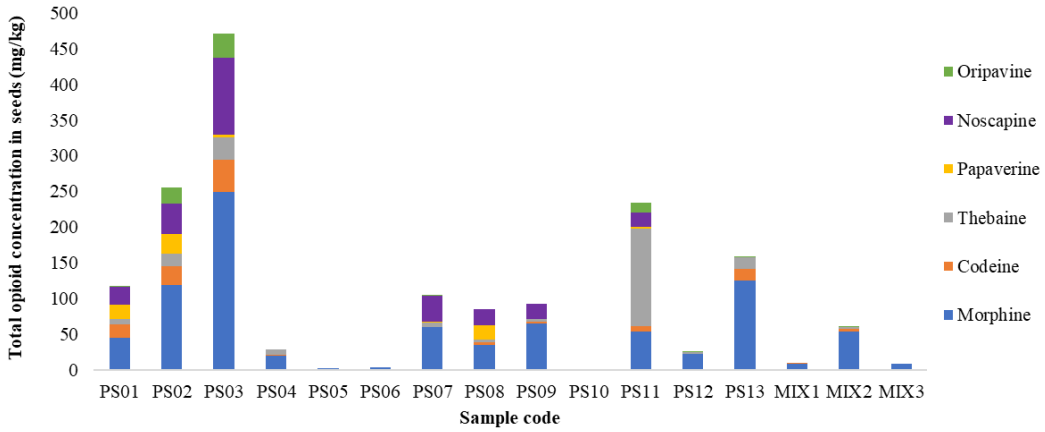


Figure 6. Total content of opium alkaloids found in poppy seeds and seed mixes analyzed by the SLE-MSPE-UHPLC-QqQ-MS/MS method proposed.

4. Conclusions

A rapid, simple and efficient method was developed for the determination of morphine, codeine, thebaine, papaverine, noscapine and oripavine in poppy seeds prior by ultra-high-performance liquid chromatography-tandem mass spectrometry. Mesostructured silica-coated magnetic nanoparticles were used as adsorbent for MSPE and the sample extracts were purified in just 4 min. Furthermore, the chromatographic method was only 5 minutes long, which is very fast, considerably reducing the time and therefore the cost of the analysis. The method was successfully applied to the analysis of commercial poppy seed samples and seed mixes purchased in Spanish supermarkets at the end of 2020. The most surprising result was that wrong labelling is taking place, so the correct naming of the seeds used is necessary. The six opium alkaloids were found in all the commercial samples analysed, and 73% exceeded the reference level for morphine in poppy seeds for direct human consumption established in the EU (<10 mg/kg). All these results confirmed the need to further study the actual exposure to opioid alkaloids of the population, though not only the consumption of poppy seeds as well as other foods containing poppy seeds, in order to establish harmonised legislation in all EU members and thus facilitate the common market by decreasing the number of RASFF alerts.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: SEM images of Fe_3O_4 (a), $Fe_3O_4@SiO_2$ (b) and $Fe_3O_4@SiO_2@mSiO_2$ (c) magnetic particles. Figure S2: XRD patterns of at low angles (a) and wide angles (b): Fe_3O_4 (1), $Fe_3O_4@SiO_2$ (2), $Fe_3O_4@SiO_2@C_8$ (3), $Fe_3O_4@SiO_2@C_{18}$ (4), $Fe_3O_4@SiO_2@mSiO_2$ (5), $Fe_3O_4@SiO_2@mSiO_2@C_8$ (6) and $Fe_3O_4@SiO_2@mSiO_2@C_{18}$ (7). Figure S3: FT-IR spectra of particles with different treatment to remove the surfactant (a): calcination treatment $Fe3O4@SiO_2@mSiO_2$ (1) and particles after treatment with acetone extraction $Fe_3O_4@SiO_2@CTAB/mSiO_2$ (2). FT-IR spectra of 7 synthesized materials (b): Fe_3O_4 (1), $Fe_3O_4@SiO_2$ (2), $Fe_3O_4@SiO_2@mSiO_2$ (3), $Fe_3O_4@SiO_2@C_8$ (4), $Fe_3O_4@SiO_2@C_{18}$ (5), $Fe_3O_4@SiO_2@mSiO_2@C_8$ (6) and $Fe_3O_4@SiO_2@mSiO_2@C_{18}$ (7). Figure S4: Image of the first three washes of the $Fe_3O_4@SiO_2@mSiO_2$ material by magnetic decantation. Figure S5: N_2 adsorption-desorption isotherms (a, c) and pore-size distribution (b, d) of $Fe_3O_4@SiO_2$ and $Fe_3O_4@SiO_2@mSiO_2$, respectively, before and after the modification with C_8 and C_{18} ligands. Figure S6: Extracted areas of target analytes in 2.5 g poppy seeds and 10 mL solvent during 10 min under each of the extraction conditions to be optimised (extraction solvent and agitation type). Figure S7: Extracted areas of the 6 target analytes in 2.5 g of poppy seeds with methanol/water 50/50 (v/v) and magnetic stirring under each of the double extraction conditions (volume and time). Figure S8: Extracted areas of the six target analytes in 2.5 g of poppy seeds with methanol/water 50/50 (v/v), magnetic stirring and double extraction of 30 mL for 30 min under each of the solvent pH values. Figure S9: Adsorption kinetic (a) and isotherm (b) experiments of six opium alkaloids with 100 mg $Fe_3O_4@SiO_2@mSiO_2$ material. Figure S10: Three kinetics models (a) and two isotherm models (b) for the adsorption of six opium alkaloids on the $Fe_3O_4@SiO_2@mSiO_2$. Figure S11: Effect of the solvent pH value in the adsorption step in methanol/water 50/50 (v/v) with 100 mg of $Fe_3O_4@SiO_2@mSiO_2$ material for 1 min. Table S1: Registration of the different poppy seed samples used with their description, species, and origin. Table S2: Optimal parameters of MRM for the analysis of each opium alkaloids by UHPLC-QqQ-MS/MS: precursor and fragment ions, cone voltage (CV), collision energy (CE) and retention time (Rt) of each opium alkaloid. Table S3: Equations of adsorption kinetic and isotherm. Table S4: Kinetic parameters of the adsorption of six

opioid alkaloids with 100 mg Fe_3O_4@SiO_2@mSiO_2 material for different times (1-20 min) based on different kinetic models. Table S5: Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with the solvents used in the SLE of poppy seeds at different desorption times (1, 5, 10 and 20 min). Table S6: Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with eight desorption solvents at different pH values (non-modified, acid with 1% formic acid, basic with 1% ammonia and basic with 10% ammonia). Table S7: Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with the mixture of the solvents that showed the best results in desorption step with different proportions (50/50, 80/20 and 20/80, v/v), all of them at basic pH with solvent/ammonia, 90/10 v/v. Table S8: Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure after a different number of consecutive desorptions with diethyl ether/methanol, 80/20, v/v, with 10% ammonia at 1 min. Table S9: Instrumental validation parameters (solvent linearity, solvent calibration, LOQ and LOD).

Author Contributions: Conceptualization, I.S., S.M.-Z. and D.P.-Q.; methodology, G.C.-H.; validation, G.C.-H. and S.M.-Z.; formal analysis, G.C.H.; investigation, I.S. S.M.-Z. and D.P.-Q.; resources, G.C.-H.; data curation, G.C.-H.; writing—original draft preparation, I.S., S.M.-Z. and G.C.-H.; writing—review and editing, I.S., S.M.-Z., D.P.-Q and G.C.-H.; supervision, I.S., S.M.-Z. and D.P.-Q.; project administration, S.M.-Z.; funding acquisition, I.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by MCIU/AEI/FEDER, UE for project RTI2018-094558-B-I00.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary Information

1.1 Instrumental and method validation

Firstly, the linearity was determined as the six analytes dissolved in the pure solvent, as in the blank extract after extraction and purification by MSPE. Linear regression analysis was applied, and linearity was expressed as the coefficient of determination (R^2) and the linearity coefficient (Cm). Cm was calculated as (1 – (SD/average slope)) x 100, where SD is the standard deviation of the calibration slopes obtained on different days (n = 3). Then, the matrix effect (ME, %) was calculated with the comparison of slope purified matrix versus slope solvent. The ME was considered tolerable from 80 to 120%, less than 80% was considered a signal suppression effect and more than 120% signal enhancement effect. In addition, detection and quantification instrumental and method limits were determined from the analysis of the lowest concentration analysed (0.01 µg/L) and they were estimated as three or ten times the signal/noise ratio (S/N), respectively. Accuracy and precision were evaluated at two different levels of concentration, low (0.1 mg/kg) and high (1 mg/kg). Intra-day precision (repeatability) was evaluated by analysing six times on the same day (n = 6) and inter-day precision (reproducibility) was evaluated on three different days by analysing each day three times (n = 9). In this case, morphine-D3 was also added as an internal standard to compare its results with those of the other analytes. Finally, selectivity was evaluated through the spectra of the different analytes obtained from standards compared with the spectra obtained from samples. Selectivity was considered satisfactory when the variation between spectra was less than 30% and the retention time interval for each analyte was ± 2.5 % [34].

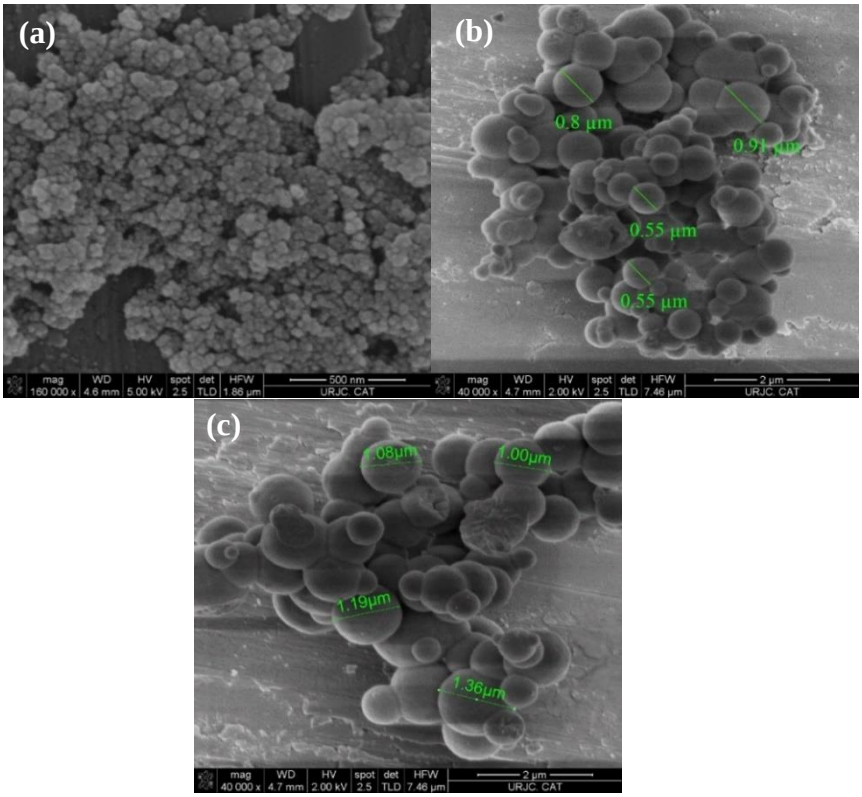


Figure S1. SEM images of Fe3O4 (a), Fe3O4@SiO2 (b) and Fe3O4@SiO2@mSiO2 (c) magnetic particles.

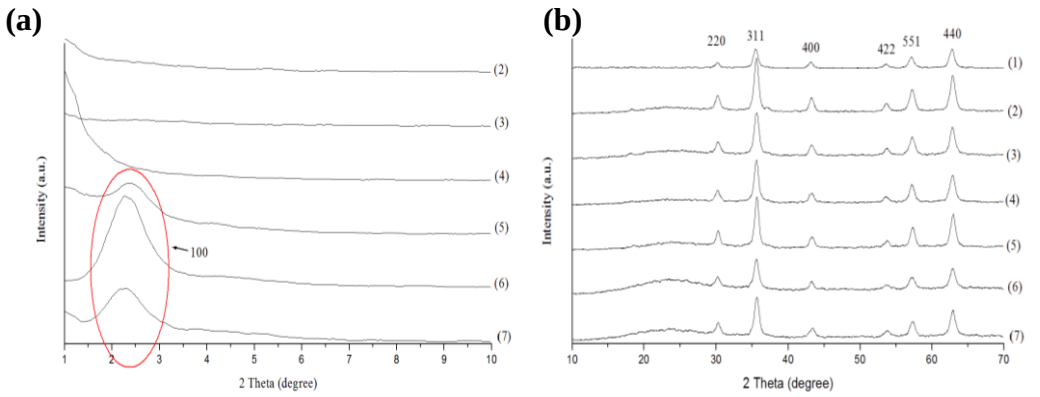


Figure S2. XRD patterns of at low angles (a) and wide angles (b): Fe_3O_4 (1), Fe_3O_4@SiO_2 (2), Fe_3O_4@SiO_2@C_8 (3), Fe_3O_4@SiO_2@C_{18} (4), Fe_3O_4@SiO_2@mSiO_2 (5), Fe_3O_4@SiO_2@mSiO_2@C_8 (6) and Fe_3O_4@SiO_2@mSiO_2@C_{18} (7).

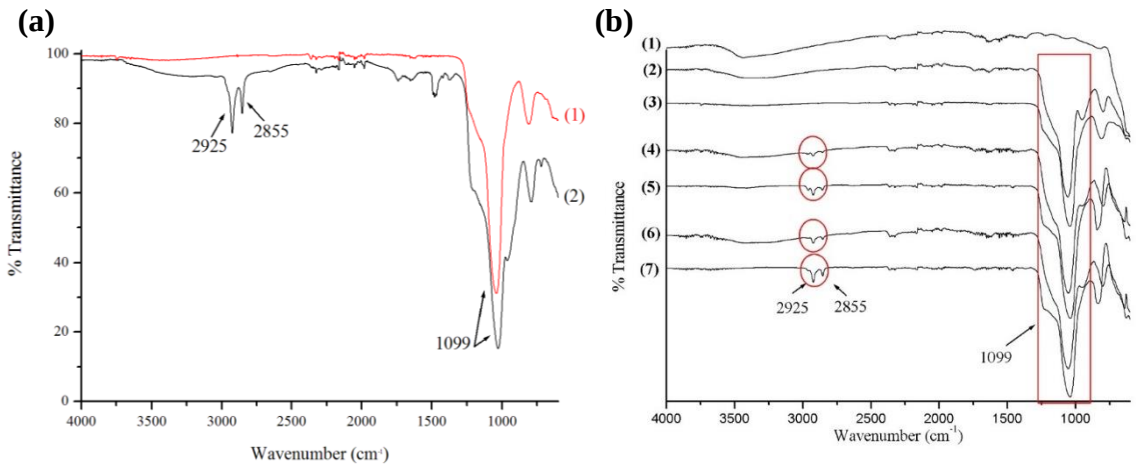


Figure S3. FT-IR spectra of particles with different treatment to remove the surfactant (a): calcination treatment $Fe_3O_4@SiO_2@mSiO_2$ (1) and particles after treatment with acetone extraction $Fe_3O_4@SiO_2@CTAB/mSiO_2$ (2). FT-IR spectra of 7 synthetized materials (b): Fe_3O_4 (1), $Fe_3O_4@SiO_2$ (2), $Fe_3O_4@SiO_2@mSiO_2$ (3), $Fe_3O_4@SiO_2@C_8$ (4), $Fe_3O_4@SiO_2@C_{18}$ (5), $Fe_3O_4@SiO_2@mSiO_2@C_8$ (6) and $Fe_3O_4@SiO_2@mSiO_2@C_{18}$ (7).

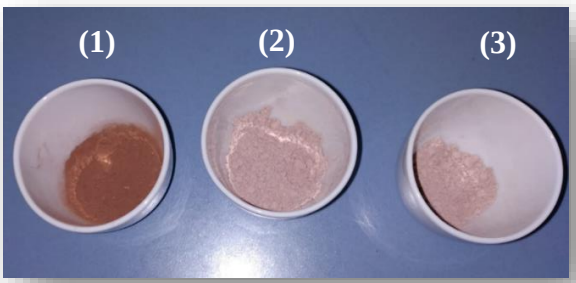


Figure S4. Image of the first three washes of the $Fe_3O_4@SiO_2@mSiO_2$ material by magnetic decantation: first wash (1), second wash (2) and third wash (3).

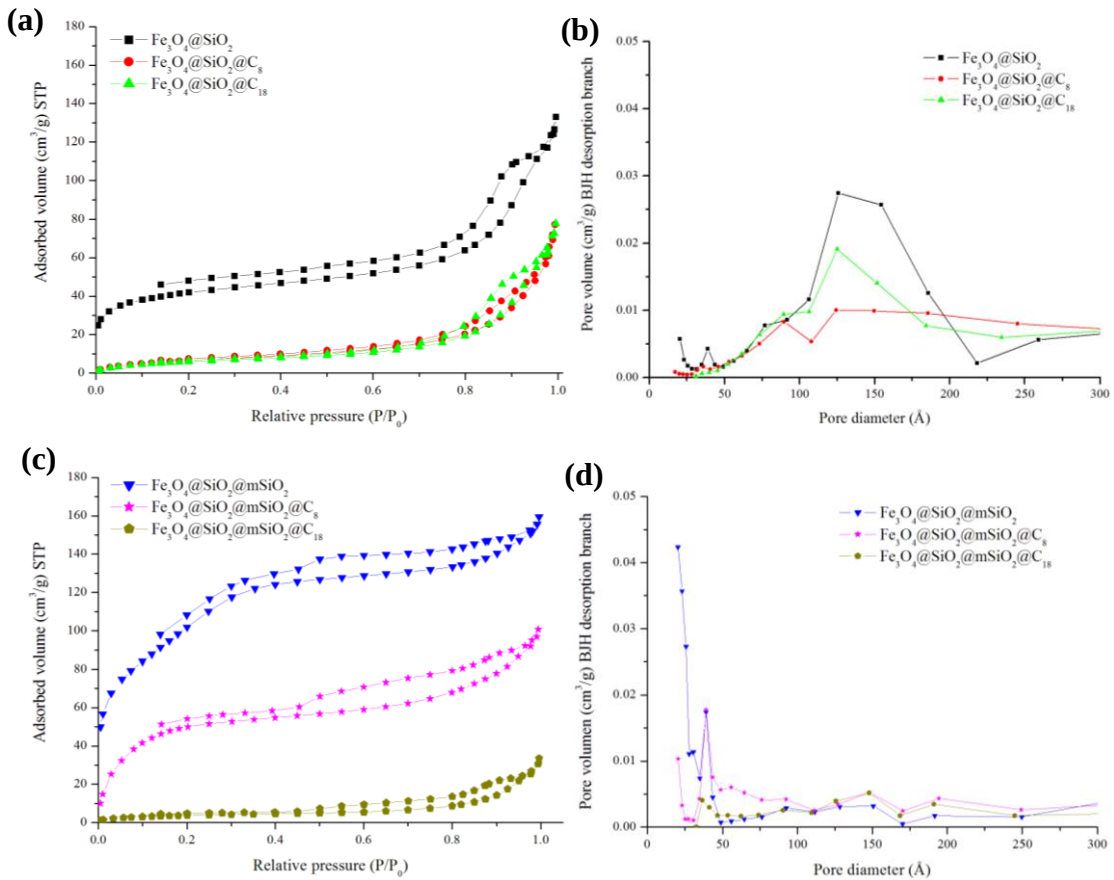


Figure S5. N_2 adsorption-desorption isotherms (a, c) and pore-size distribution (b, d) of $Fe_3O_4@SiO_2$ and $Fe_3O_4@SiO_2@mSiO_2$, respectively, before and after the modification with C_8 and C_{18} ligands

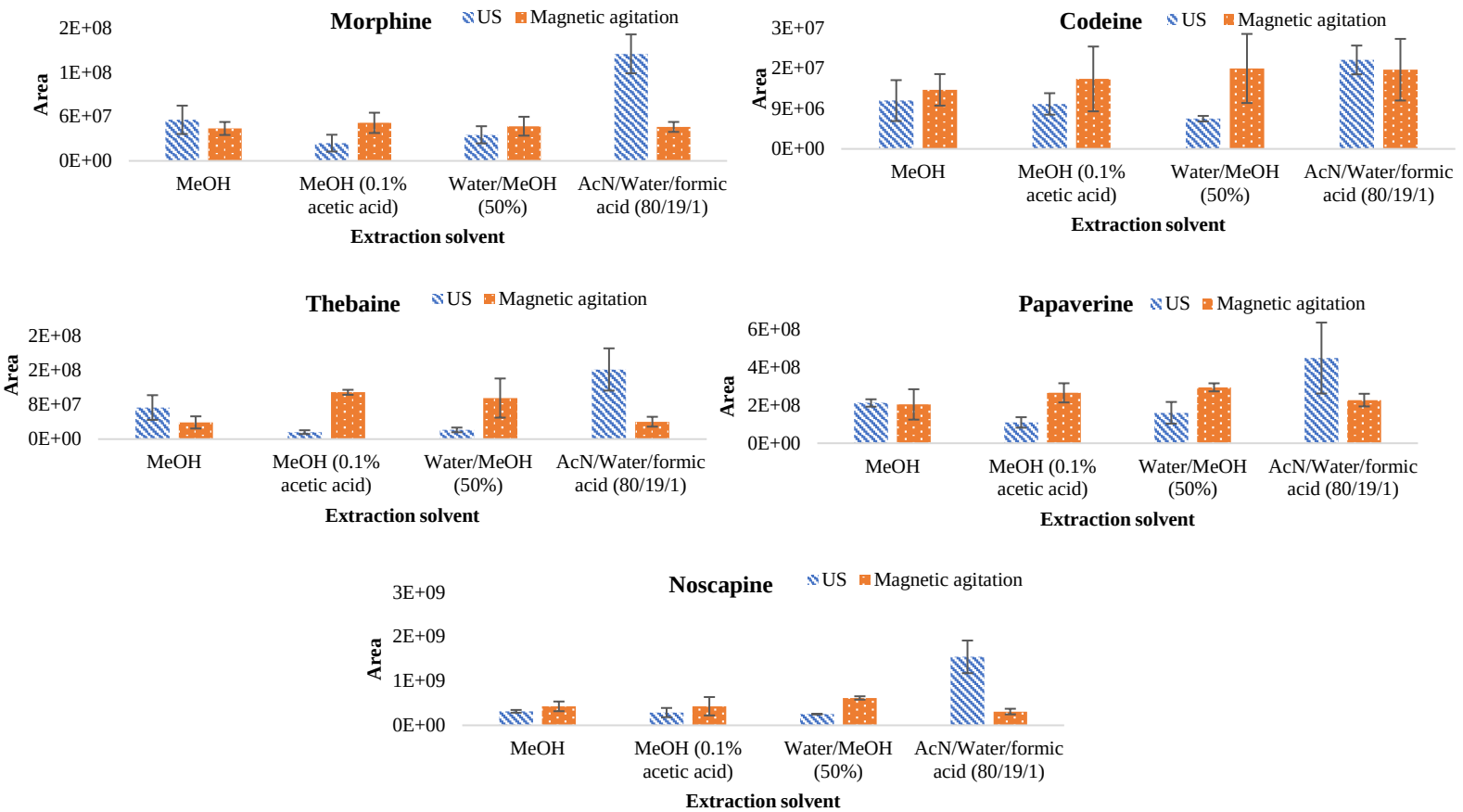


Figure S6. Extracted areas of target analytes in 2.5 g poppy seeds and 10 mL solvent during 10 min under each of the extraction conditions to be optimised (extraction solvent and agitation type).

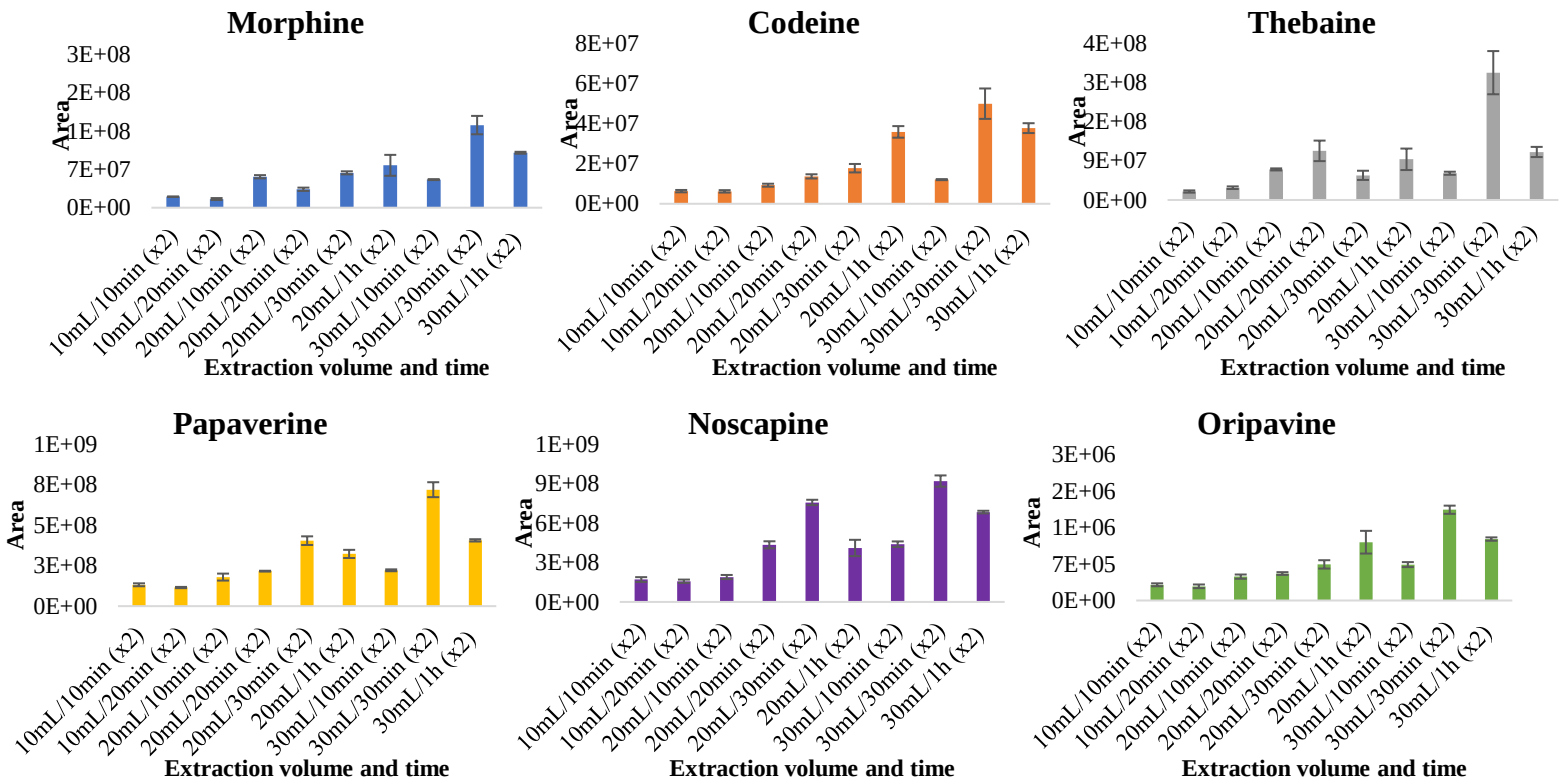


Figure S7. Extracted areas of the 6 target analytes in 2.5 g of poppy seeds with methanol/water 50/50 (v/v) and magnetic stirring under each of the double extraction conditions (volume and time).

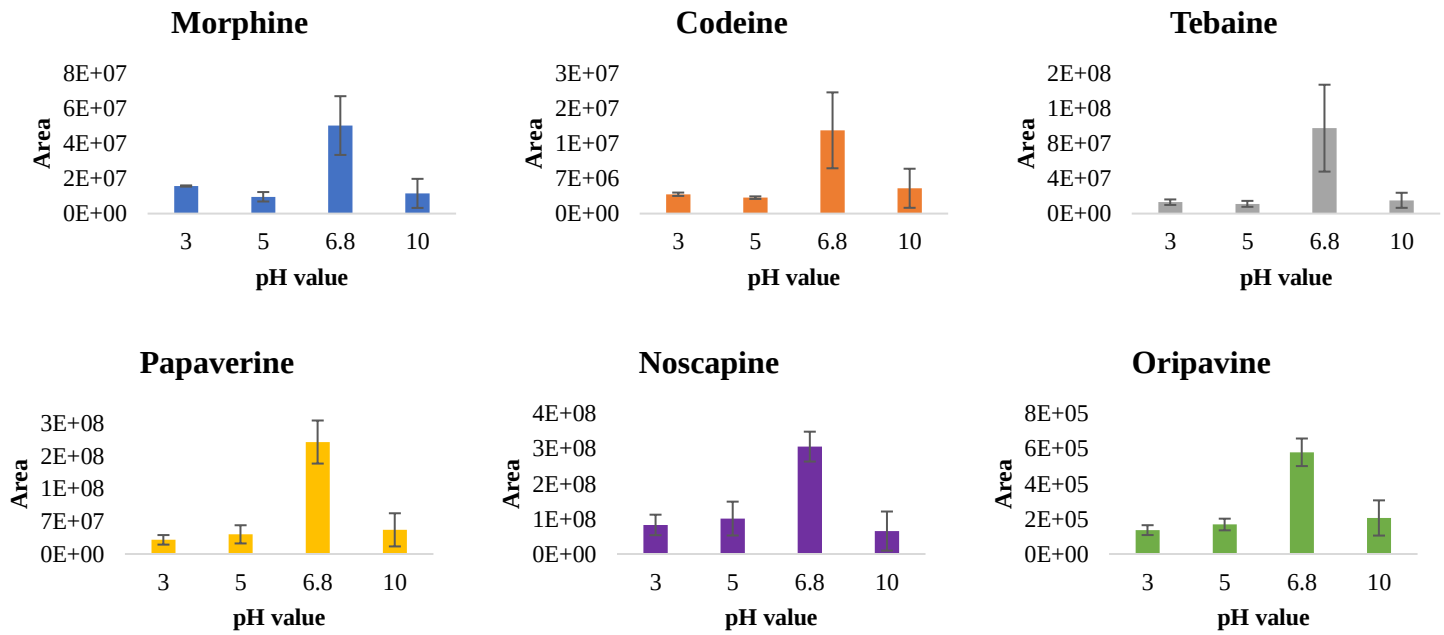


Figure S8. Extracted areas of the 6 target analytes in 2.5 g of poppy seeds with methanol/water 50/50 (v/v), magnetic stirring and double extraction of 30 mL for 30 min under each of the solvent pH values.

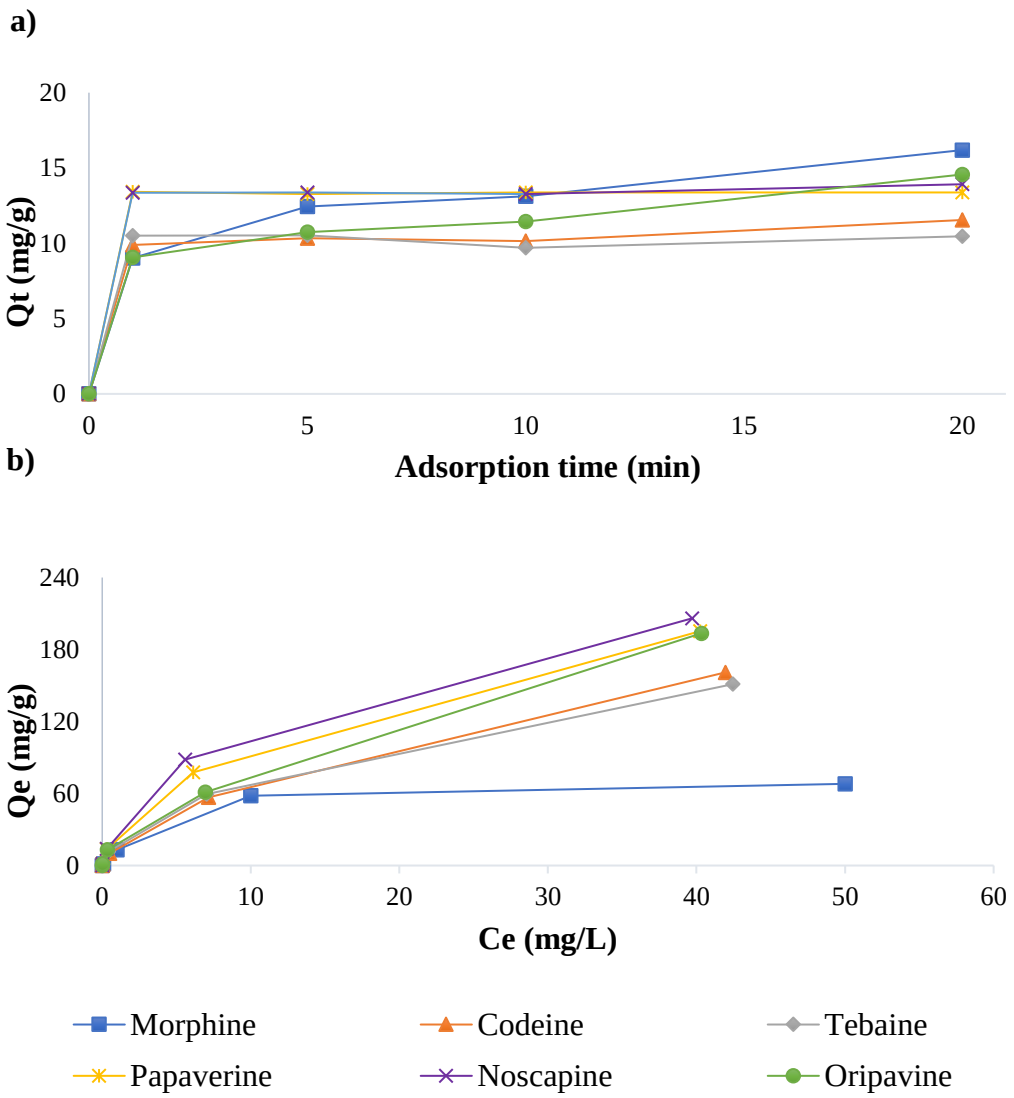


Figure S9. Adsorption kinetic (a) and isotherm (b) experiments of six opium alkaloids with 100 mg Fe_3O_4@SiO_2@mSiO_2 material.

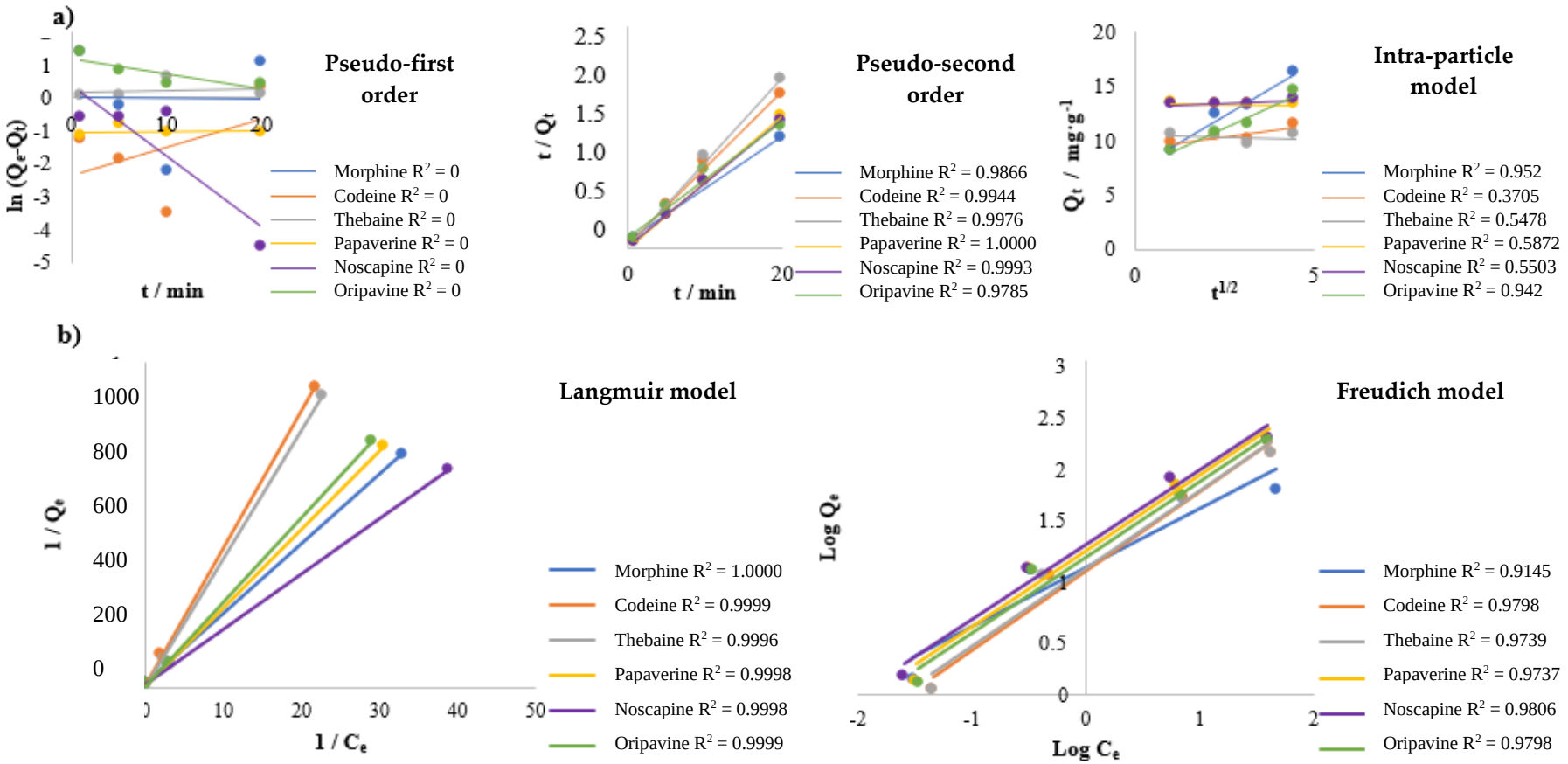


Figure S10. Three kinetics models (a) and two isotherm models (b) for the adsorption of six opium alkaloids on the $Fe_3O_4@SiO_2@mSiO_2$.

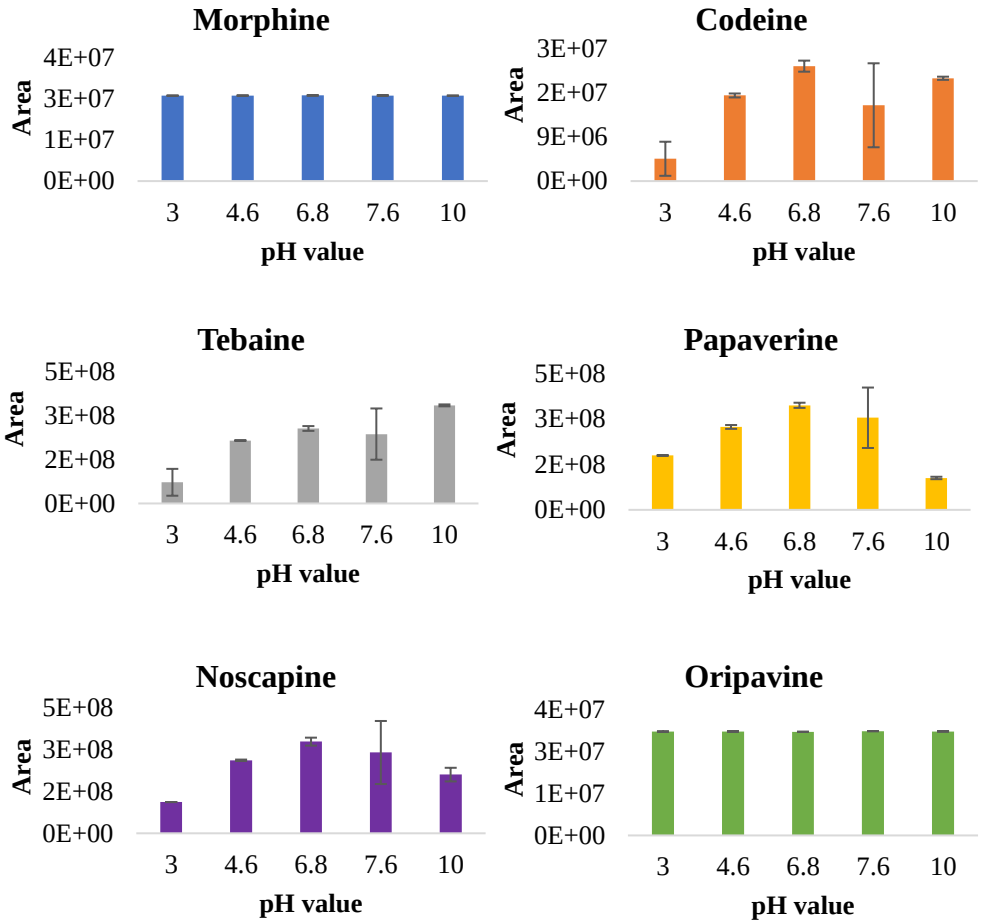


Figure S11. Effect of the solvent pH value in the adsorption step in methanol/water 50/50 (v/v) with 100 mg of Fe_3O_4@SiO_2@mSiO_2 material for 1 min.

Table S1. Registration of the different poppy seed samples used with their description, species, and origin.

Code	Description	Species specified by labelling	E/No-E	Origin
PS01	Blue poppy seeds	*P. somniferum*	E	Turkey
PS02	Not specific on the label (physically: blue poppy seeds)	Unknown	E	Turkey
PS03	Physically: blue poppy seeds	*P. rhoeas*	No-E	Unknown
PS04	Not specific on the label (physically: blue poppy seeds)	Unknown	No-E	Spain
PS05	Physically: blue poppy seeds	*P. rhoeas*	No-E	Unknown
PS06	Physically: blue poppy seeds	*P. rhoeas*	No-E	Unknown
PS07	Blue poppy seeds	*P. somniferum*	E	Turkey
PS08	Not specific on the label (physically: blue poppy seeds)	Unknown	No-E	Turkey
PS09	Wild opium poppy seeds (white)	*P. somniferum*	No-E	Spain
PS10	Wild poppy seeds	*P. rhoeas*	No-E	Spain
PS11	Not specific on the label (physically: blue poppy seeds)	Unknown	No-E	Czech Republic
PS12	Not specific on the label (physically: blue poppy seeds)	Unknown	E	No EU
PS13	Not specific on the label (physically: blue poppy seeds)	Unknown	E	No EU
MIX1	Sunflower, sesame, brown flax, pumpkin, and poppy seeds	Unknown	No-E	Unknown
MIX2	Sunflower, sesame, brown flax, pumpkin, and poppy seeds	Unknown	No-E	Unknown
MIX3	Pumpkin, sunflower, sesame, gold flax, brown flax, and poppy seeds	Unknown	E	Unknown

The code of the samples: PS is because the samples are poppy seeds and MIX is the mixture of the different seeds. E/no-E: ecological/ no ecological cultivation, No EU: not from the European Union

Table S2. Optimal parameters of MRM for the analysis of each opium alkaloid by UHPLC-QqQ-MS/MS: precursor and fragment ions, collision energy (CE) and retention time (Rt) of each opium alkaloid.

Analytes	Precursor ion (Q_1, *m/z*, $[M+H]^+$)	Fragment ion[a] (Q_3, *m/z*)	CE (eV)	Rt (min)
Morphine	286.6	**152.0**	55	1.556
		165.0	35	
		201.0	20	
		229.0	20	
Morphine-D3	289.0	**153.1**	20	1.554
		157.0	35	
		165.0	35	
		201.0	35	
Codeine	300.1	182.9	25	1.935
		198.9	25	
		215.0	20	
		225.0	20	
Oripavine	298.3	237.1	15	2.044
		249.1	15	
		267.1	15	
Thebaine	312.3	**58.1**	15	2.598
		221.0	25	
		250.8	20	
		266.2	20	
Papaverine	340.4	**202.0**	20	2.765
		324.0	25	
Noscapine	414.2	**220.0**	20	2.813
		352.7	20	

[a]The fragment ion used for the quantification are in bold.

Table S3. Equations of adsorption kinetic and isotherm

$Q_e = \frac{(C_o - C_e)V}{W}$	Adsorption capacity	(1)
$\ln(q_e - q_t) = lnqe - k_1 t$	Lagergren's pseudo-first order	(2)
$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left[\frac{1}{q_e}\right] t$	Pseudo-second order	(3)
$q_t = k_p t^{1/2} + C$	Intra-particle diffusion	(4)
$\frac{1}{Q_e} = \frac{1}{Q_{max}} + \frac{1}{K_L Q_{max} C_e}$	Langmuir model	(5)
$Log\ q_e = logK_F + \frac{1}{n} logC_e$	Freundlich model	(6)

C_o and C_e: initial and equilibrium concentrations of the target analytes (µg/L), respectively; V: volume of the solution (L); W: mass of the adsorbent (g); k_1: pseudo-first order rate constant constant (min^{-1}); q_e and q_t: amounts of opium alkaloids adsorbed at equilibrium and time (mg/g), respectively; k_2: pseudo-second order adsorption rate constant (g/mg min); q_t: amount of opium alkaloids adsorbed at time t (mg/g); k_p: intraparticle diffusion rate (mg/L min^2); C: intercept; Q_{max}: maximum monolayer capacity of the adsorbent (mg/); K_L: Langmuir binding constant which is related to the energy of adsorption (L/mg); K_F: is the Freundlich constant (L/mg); n: is the heterogeneity factor (dimensionless).

Table S4. Kinetic parameters of the adsorption of six opioid alkaloids with 100 mg $Fe_3O_4@SiO_2@mSiO_2$ material for different times (1-20 min) based on different kinetic models.

		Pseudo-first order model			**Pseudo-second order model**			**Intra-particle model**		
	$Q_{e,\ exp}$ (mg/g)	**R^2**	**K_1 (min^{-1})**	**$Q_{e,\ cal}$ (mg/g)**	**R^2**	**K_2 (g/mg min)**	**$Q_{e,\ cal}$ (mg/g)**	**R^2**	**K_p (mg/g min^2)**	**C**
Morphine	13.23	0.0004	0.0042	1.05	0.9866	2925.58	17.04	0.9705	1.9732	7.33
Codeine	10.18	0.2047	-0.0864	0.10	0.9944	2472.37	11.68	0.7544	0.4352	9.29
Thebaine	11.61	0.0211	-0.0049	1.21	0.9976	7535.20	10.42	0.0594	-0.0670	10.48
Papaverine	13.74	0.0001	-0.0002	0.37	1.0000	162916.89	13.39	0.0093	-0.0040	13.37
Noscapine	13.91	0.7720	0.2132	0.21	0.9993	12749.19	13.97	0.5173	0.1454	13.08
Oripavine	13.02	0.7028	0.0449	0.04	0.9785	2004.18	15.27	0.9546	1.5342	7.28

$Q_{e,\ exp}$: amounts of opium alkaloids adsorbed at equilibrium, experimental; K_1: pseudo-first order rate constant constant; $Q_{e,\ cal}$: amounts of opium alkaloids adsorbed at equilibrium, calculated; K_2: pseudo-second order adsorption rate constant; K_p: intraparticle diffusion rate; C: intercept.

Table S5. Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with the solvents used in the SLE of poppy seeds at different desorption times (1, 5, 10 and 20 min).

Desorption time (min)	Desorption solvent	Morphine	Codeine	Tebaine	Papaverine	Noscapine	Oripavine
1	AcN/water/formic acid, 80/19/1	0.31 ± 0.22	32 ± 9	28 ± 9	47 ± 16	42 ± 14	0.71 ± 0.12
	MeOH/water 50/50	0.24 ± 0.11	22 ± 4	18 ± 3	22 ± 3	22 ± 3	0.52 ± 0.01
	MeOH 0.1% acetic acid	0.21 ± 0.04	12.81 ± 0.12	16.21 ± 0.31	26 ± 1	25.62 ± 0.82	0.34 ± 0.01
	MeOH	0.14 ± 0.02	10 ± 1	12.12 ± 0.72	23.03 ± 0.34	24 ± 1	0.33 ± 0.11
5	AcN/water/formic acid, 80/19/1	0.51 ± 0.31	45 ± 12	56 ± 4	67 ± 23	58 ± 18	1.21 ± 0.11
	MeOH/water 50/50	0.43 ± 0.32	35 ± 2	30.61 ± 0.21	42 ± 6	40 ± 3	0.71 ± 0.52
	MeOH 0.1% acetic acid	0.43 ± 0.13	18 ± 1	22.43 ± 0.63	38.51 ± 0.02	37.41 ± 0.52	0.71 ± 0.13
	MeOH	0.22 ± 0.03	11 ± 1	13.64 ± 0.72	29.22 ± 0.24	31 ± 1	0.74 ± 0.14
10	AcN/water/formic acid, 80/19/1	0.21 ± 0.01	62 ± 2	67 ± 3	93 ± 1	96.42 ± 0.23	0.72 ± 0.12
	MeOH/water 50/50	0.23 ± 0.12	21 ± 2	19 ± 2	13 ± 1	19 ± 2	0.44 ± 0.01
	MeOH 0.1% acetic acid	0.24 ± 0.12	12 ± 2	12.21 ± 0.61	15 ± 2	20 ± 2	0.52 ± 0.01
	MeOH	0.11 ± 0.03	9 ± 1	9 ± 1	11.03 ± 0.32	17 ± 1	0.33 ± 0.12
20	AcN/water/formic acid, 80/19/1	0.40 ± 0.13	79 ± 2	106 ± 26	121 ± 4	12 ± 3	1.52 ± 0.31
	MeOH/water 50/50	0.32 ± 0.14	39 ± 2	36 ± 2	28 ± 5	38 ± 2	0.93 ± 0.12
	MeOH 0.1% acetic acid	0.40 ± 0.12	16 ± 1	17.14 ± 0.72	22 ± 1	32 ± 1	1.25 ± 0.13
	MeOH	0.11 ± 0.03	11 ± 1	11.34 ± 0.83	14 ± 1	22 ± 2	0.33 ± 0.13

AcN: acetonitrile; MeOH: methanol.

Table S6. Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with eight acidified, non-modified or basified desorption solvents (acid with 1% formic acid, basic with 1% ammonia and basic with 10% ammonia).

pH	Desorption solvent	MOR	COD	THE	PAP	NOS	ORI
Non-modificated pH	Ether	-	10.61 ± 0.21	10.62 ± 0.12	14.01 ± 0.01	33.71 ± 0.51	0.71 ± 0.01
	DCM	-	2 ± 2	12.61 ± 0.41	77.01 ± 0.42	93 ± 1	-
	Chl	-	1.12 ± 0.11	11.21 ± 0.52	44.01 ± 0.53	47.41 ± 0.62	-
	Isopropanol	-	4.23 ± 0.23	4.64 ± 0.44	8.02 ± 0.42	13.92 ± 0.73	0.92 ± 0.02
	AcN	-	16 ± 3	16.21 ± 0.73	28.13 ± 0.71	29.51 ± 0.12	-
	MeOH	-	8.34 ± 0.32	9.40 ± 0.92	15.24 ± 0.92	18.02 ± 0.11	1.10 ± 0.03
	Water	-	7.12 ± 0.91	4.32 ± 0.41	2.91 ± 0.44	3.23 ± 0.42	0.74 ± 0.02
	EtOAc	-	2 ± 1	2.93 ± 0.32	22.30 ± 0.32	36.92 ± 0.84	-
Acid pH with 1% formic acid	Ether	-	0.4 ± 0.21	0.81 ± 0.11	1.50 ± 0.12	7.84 ± 0.12	-
	DCM	-	2.92 ± 0.51	38 ± 2	40.32 ± 0.21	51.83 ± 0.52	-
	Chl	-	1.51 ± 0.11	24 ± 1	26.73 ± 0.23	38.13 ± 0.21	-
	IPA	-	5.92 ± 0.31	5.21 ± 0.12	3.01 ± 0.90	6.14 ± 0.03	-
	AcN	-	29.64 ± 0.32	25.31 ± 0.33	31.71 ± 0.42	36.12 ± 0.04	-
	MeOH	-	25.93 ± 0.14	24.02 ± 0.44	21.01 ± 0.23	26.21 ± 0.12	-
	Water	-	11 ± 1	3.73 ± 0.41	2.01 ± 0.02	2.41 ± 0.32	-
	EtOAc	-	7 ± 1	12.91 ± 0.22	14.14 ± 0.11	29.61 ± 0.34	-
Basic pH with 1% ammonia	Ether	3.31 ± 0.12	48.81 ± 0.22	69.41 ± 0.21	73.31 ± 0.31	82.01 ± 0.04	16.72 ± 0.01
	DCM	4.24 ± 0.31	68.5 ± 1.11	67.42 ± 0.32	82.82 ± 0.43	75.72 ± 0.23	20.32 ± 0.42
	Chl	1.80 ± 0.33	23 ± 1	16.72 ± 0.31	17.72 ± 1.12	16.22 ± 0.03	5.22 ± 0.21
	IPA	1.81 ± 0.12	19.11 ± 0.21	20.71 ± 0.72	30.22 ± 0.83	31.41 ± 0.14	6.31 ± 0.31

	AcN	0.32 ± 0.04	45.41 ± 0.22	49.11 ± 0.43	62.54 ± 0.22	61.62 ± 0.04	2.11 ± 0.11
	MeOH	2.81 ± 0.01	44.43 ± 0.33	42.54 ± 0.04	55.64 ± 1.53	54.51 ± 0.32	9.51 ± 0.22
	Water	0.43 ± 0.11	10.30 ± 0.42	9.72 ± 0.11	24.93 ± 0.24	19.92 ± 0.42	1.91 ± 0.01
	EtOAc	1.41 ± 0.40	28 ± 1	29.60 ± 0.31	40.83 ± 0.51	39.51 ± 0.41	4.51 ± 0.52
Basic pH with 10% ammonia	Ether	3.22 ± 0.21	70 ± 2	98 ± 1	113.7 ± 0.21	115.72 ± 0.01	16.70 ± 0.22
	DCM	15.72 ± 0.32	85 ± 2	81.12 ± 0.21	99.62 ± 0.22	92.63 ± 0.02	22.60 ± 0.42
	Chl	3.81 ± 0.84	54 ± 1	60.12 ± 0.81	73.12 ± 0.04	57.64 ± 0.14	13.31 ± 0.33
	IPA	0.91 ± 0.02	12.64 ± 0.51	14.84 ± 0.33	23.24 ± 0.12	21.03 ± 0.13	1.71 ± 0.84
	AcN	3.81 ± 0.14	44.43 ± 0.32	51.04 ± 0.72	60.73 ± 0.32	57.22 ± 0.22	10.30 ± 0.12
	MeOH	3.21 ± 0.51	55.93 ± 0.81	62.52 ± 0.74	72.63 ± 0.33	68.73 ± 0.74	8.50 ± 0.03
	Water	2.61 ± 0.31	16.74 ± 0.22	20.92 ± 0.12	45.21 ± 0.14	29.44 ± 0.53	5.22 ± 0.33
	EtOAc	1.32 ± 0.01	17.21 ± 0.71	21.23 ± 0.33	22.50 ± 0.14	21.22 ± 0.42	4.32 ± 0.24

MOR: morphine; COD: codeine; THE: thebaine; PAP: papaverine; NOS: noscapine; ORI: oripavine; Ether: diethyl ether; DCM: dichlorometane; Chl: chloroform; IPA: isopropapol; AcN: acetonitrile; MeOH: methanol; EtOAc: ethyl acetate; -: no detected

Table S7. Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with 20 min adsorption with the mixture of the solvents that showed the best results in desorption step with different proportions (50/50, 80/20 and 20/80, v/v), all of them at basic pH with solvent/ammonia, 90/10 v/v.

Time (min)	Desorption solvent	MOR	COD	THE	PAP	NOS	ORI
20	Ether/AcN, 50/50	10.21 ± 0.21	63.91 ± 0.62	72.41 ± 0.64	63.62 ± 0.41	67 ± 3	21.82 ± 0.62
	Ether/AcN, 80/20	12 ± 1	74.61 ± 0.81	85.11 ± 0.24	91.81 ± 0.13	93.12 ± 0.12	29.62 ± 0.43
	Ether/AcN, 20/80	6.71 ± 0.42	51 ± 1	50.81 ± 0.04	66.02 ± 0.24	63.21 ± 0.23	13.51 ± 0.21
	DCM/AcN, 50/50	7.54 ± 0.23	67 ± 1	72.34 ± 0.12	72.54 ± 0.71	66.12 ± 0.64	24.12 ± 0.32
	DCM/AcN, 80/20	5.01 ± 0.22	55.84 ± 0.62	56.33 ± 0.23	59.12 ± 0.52	53.24 ± 0.42	19.93 ± 0.44
	DCM/AcN, 20/80	3.92 ± 0.01	38.31 ± 0.74	42.91 ± 0.32	42.96 ± 0.44	40.11 ± 0.51	13.71 ± 0.42
	Ether/MeOH, 50/50	10.23 ± 0.84	37.08 ± 0.83	38.52 ± 0.32	43 ± 1	49.04 ± 0.54	11.52 ± 0.13
	Ether/MeOH, 80/20	20.74 ± 0.82	74.12 ± 0.72	79.71 ± 0.21	102.71 ± 0.01	106.62 ± 0.42	24.11 ± 0.22
	Ether/MeOH, 20/80	7.52 ± 1.13	33.81 ± 0.52	32.80 ± 0.53	43.14 ± 0.12	44.23 ± 0.21	9.93 ± 0.21
	DCM/MeOH, 50/50	8.34 ± 0.84	41.01 ± 0.54	46.40 ± 0.42	58.64 ± 0.34	56.62 ± 0.14	10.12 ± 0.14
	DCM/MeOH, 80/20	8.52 ± 0.52	82.61 ± 0.33	86.61 ± 0.34	112.92 ± 0.11	109.44 ± 0.22	22.71 ± 0.52
	DCM/MeOH, 20/80	11.22 ± 0.43	40.43 ± 0.12	42.70 ± 0.23	53.20 ± 0.43	53.71 ± 0.21	12.22 ± 0.91
40	Ether/AcN, 50/50	11.31 ± 0.22	82.22 ± 0.34	90.83 ± 0.82	82.41 ± 0.14	84.10 ± 0.14	25 ± 1
	Ether/AcN, 80/20	14.42 ± 0.34	94.51 ± 0.32	102.61 ± 0.91	112.64 ± 0.02	105.81 ± 0.02	38 ± 2
	Ether/AcN, 20/80	7.34 ± 0.22	59.31 ± 0.23	58.64 ± 1.12	75.43 ± 0.21	71.52 ± 0.35	15.24 ± 0.51
	DCM/AcN, 50/50	9.02 ± 0.83	75.22 ± 0.83	80.32 ± 0.33	79.54 ± 0.42	72.30 ± 0.57	27.81 ± 0.62
	DCM/AcN, 80/20	6.71 ± 0.82	59.31 ± 0.79	60.54 ± 0.42	64.01 ± 0.14	58.31 ± 0.54	21.92 ± 0.84
	DCM/AcN, 20/80	4.52 ± 1.14	64.24 ± 0.48	69.22 ± 0.54	68.64 ± 0.83	63.52 ± 0.65	16.90 ± 0.91
	Ether/MeOH, 50/50	11.71 ± 0.82	59.85 ± 0.19	61.11 ± 0.63	74 ± 1	75.73 ± 0.37	14.50 ± 0.54
	Ether/MeOH, 80/20	33.84 ± 0.46	105.83 ± 0.24	109.93 ± 0.52	146 ± 1	142.20 ± 0.24	36 ± 2

	Ether/MeOH, 20/80	11.33 ± 0.19	65.45 ± 0.12	59.70 ± 0.41	75.53 ± 0.74	78.01 ± 0.11	13 ± 1
	DCM/MeOH, 50/50	10.10 ± 0.26	54.91 ± 0.51	61.01 ± 0.82	76.42 ± 0.24	72.72 ± 0.62	12.80 ± 0.12
	DCM/MeOH, 80/20	12.12 ± 0.43	96.64 ± 0.22	98.90 ± 0.43	130.33 ± 0.12	125 ± 4	26.94 ± 0.82
	DCM/MeOH, 20/80	13.42 ± 0.53	60.44 ± 0.11	63.10 ± 0.32	78.44 ± 0.14	78 ± 2	14.7 ± 0.71
60	Ether/AcN, 50/50	11 ± 1	86 ± 1	95.34 ± 0.41	86.22 ± 0.52	87.61 ± 0.32	25.61 ± 0.42
	Ether/AcN, 80/20	14 ± 1	96.65 ± 0.89	103.41 ± 0.82	114.04 ± 0.31	106 ± 1	39.01 ± 0.11
	Ether/AcN, 20/80	7.32 ± 0.36	60 ± 2	59.93 ± 0.44	76.74 ± 0.32	72.62 ± 0.98	15.84 ± 0.22
	DCM/AcN, 50/50	9.01 ± 0.12	76.04 ± 0.34	81.14 ± 0.32	80.72 ± 0.44	73.31 ± 0.24	28.41 ± 0.44
	DCM/AcN, 80/20	6.71 ± 0.67	59.82 ± 0.87	61.11 ± 0.13	64.83 ± 0.83	59.42 ± 0.35	22.44 ± 0.63
	DCM/AcN, 20/80	4.51 ± 0.34	67.52 ± 0.65	72.64 ± 0.01	72.44 ± 0.42	67 ± 4	17.30 ± 0.82
	Ether/MeOH, 50/50	12 ± 1	11.64 ± 0.15	72.21 ± 0.44	90.32 ± 0.21	89.32 ± 0.12	15.21 ± 0.44
	Ether/MeOH, 80/20	35.64 ± 0.12	114.53 ± 0.35	114.22 ± 0.42	153.31 ± 0.14	146.21 ± 0.85	37.24 ± 0.72
	Ether/MeOH, 20/80	13.22 ± 0.34	89 ± 1	76.80 ± 0.41	96.04 ± 0.02	98.12 ± 0.67	14.74 ± 0.81
	DCM/MeOH, 50/50	10.70 ± 0.96	65.64 ± 0.96	70.44 ± 0.34	88 ± 1	83.44 ± 0.54	14.01 ± 0.43
	DCM/MeOH, 80/20	12.71 ± 0.68	121 ± 3	100.10 ± 0.83	132.42 ± 0.14	127 ± 2	27.21 ± 0.63
	DCM/MeOH, 20/80	13.50 ± 0.18	62 ± 2	67.73 ± 0.74	84 ± 2	83.23 ± 0.48	15.01 ± 0.42

MOR: morphine; COD: codeine; THE: thebaine; PAP: papaverine; NOS: noscapine; ORI: oripavine; Ether: diethyl ether; DCM: dichlorometane; AcN: acetonitrile; MeOH: methanol.

Table S8. Recovery values (%) ± standard deviation (SD) obtained in MSPE procedure with 1 min adsorption after different number of consecutive desorptions with diethyl ether/methanol, 80/20, v/v, with 10% ammonia at 1 min.

Number of consecutive desorptions	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
1	34.69 ± 0.39	58.12 ± 0.03	78.51 ± 0.12	78.92 ± 0.52	45.82 ± 0.02	36.21 ± 0.33
2	44.42 ± 0.51	72.81 ± 0.89	97.42 ± 0.13	99.71 ± 0.11	66.41 ± 0.11	45.24 ± 0.22
3	46.81 ± 0.08	77.02 ± 0.48	102.52 ± 0.18	105.42 ± 0.33	72.04 ± 0.12	46.63 ± 0.14
4	47.44 ± 0.22	77.58 ± 0.78	103.18 ± 0.57	106.48 ± 0.22	73.10 ± 0.04	46.92 ± 0.21
5	47.57 ± 0.14	78.37 ± 0.41	104.07 ± 0.12	109.34 ± 0.64	76.01 ± 0.12	47.11 ± 0.19

Table S9. Instrumental validation parameters (solvent linearity, solvent calibration, LOQ and LOD).

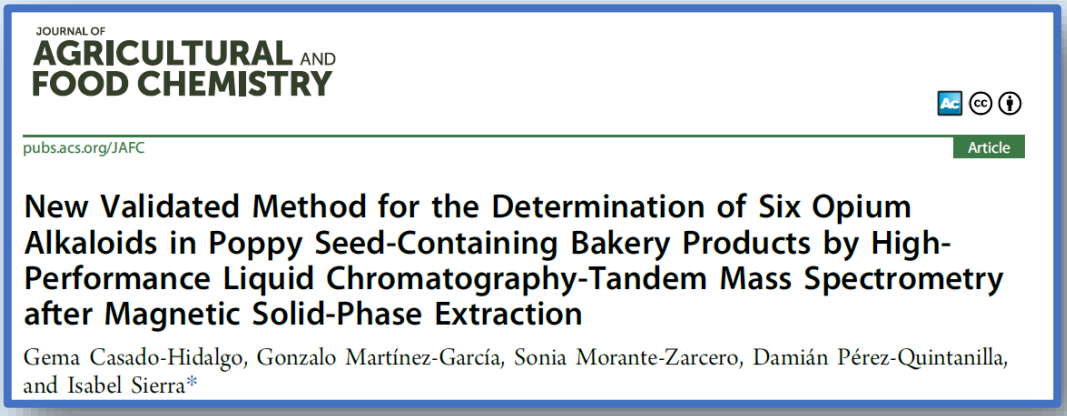
Analytes	Solvent linearity (µg/L)	Solvent calibration (R^2)	LOQ (µg/L)	LOD (µg/L)
Morphine	0.1-5000	y = 5090x + 102761 (0.999)	8×10^{-2}	2×10^{-2}
Codeine	0.1-5000	y = 2986x + 451315 (0.999)	9×10^{-2}	3×10^{-2}
Thebaine	0.01-5000	y = 9235x + 905583 (0.999)	5×10^{-3}	1×10^{-3}
Papaverine	0.01-5000	y = 41638x + 2235050 (0.999)	5×10^{-3}	1×10^{-3}
Noscapine	0.01-5000	y = 66219x + 4092136 (0.999)	9×10^{-3}	3×10^{-3}
Oripavine	0.1-5000	y = 3531x + 204673 (0.999)	6×10^{-2}	2×10^{-2}

LOQ: limit of quantification; LOD: limit of detection.

Artículo 2:

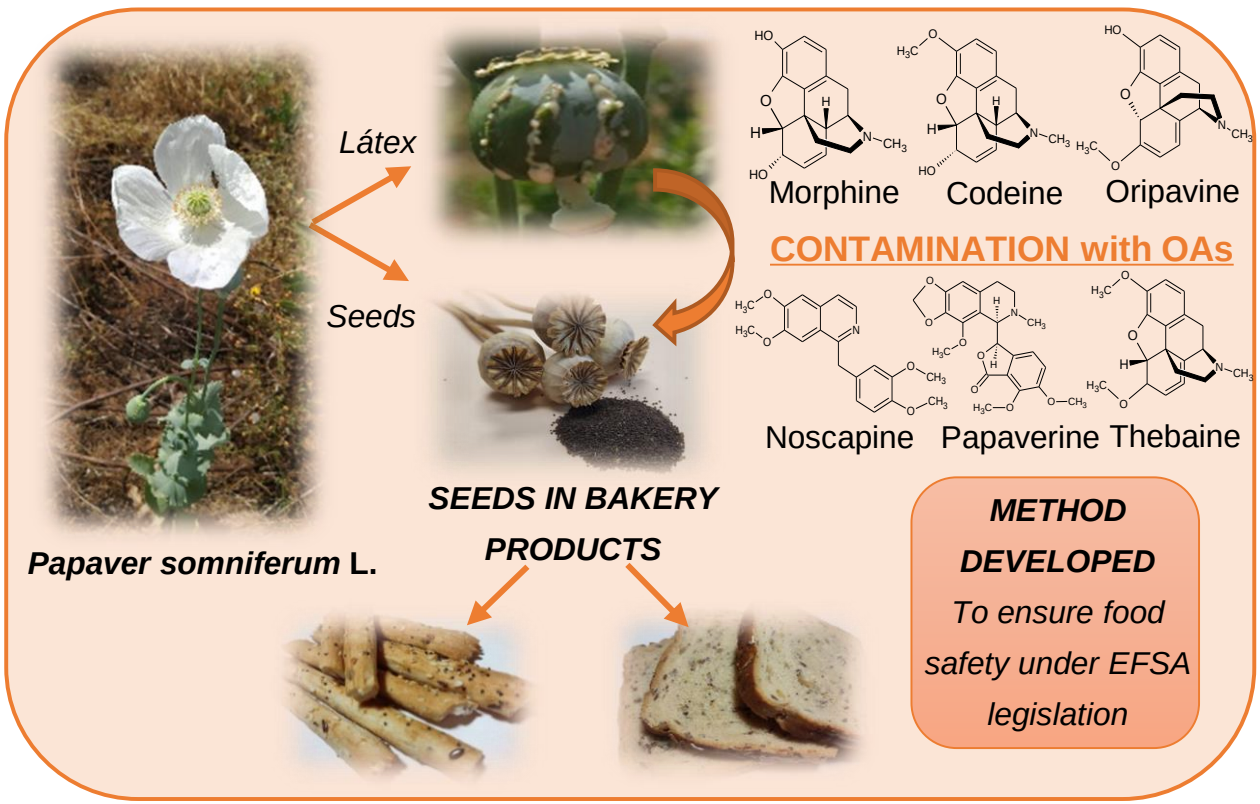
New validated method for the determination of six opium alkaloids in poppy seed containing bakery products by high-performance liquid chromatography tandem mass spectrometry after magnetic solid-phase extraction

Journal of Agricultural and Food Chemistry (2022) 7594-7606



PUBLICATION INFORMATION

Journal	Journal of Agricultural and Food Chemistry
Publisher	ACS Publications
Impact factor	5,50 (JCR, 2021)
Category	Chemistry (13/163, Q1)
DOI	https://doi.org/10.1021/acs.jafc.2c01664


Látex
Seeds
Morphine
Codeine
Oripavine
CONTAMINATION with OAs
Noscapine
Papaverine
Thebaine
Papaver somniferum L.
SEEDS IN BAKERY PRODUCTS
METHOD DEVELOPED
To ensure food safety under EFSA legislation

Highlights:

- Efficient, simple and rapid method developed to quantify opium alkaloids.
- First validated methodology for the analysis of opium alkaloids in bakery products.
- Magnetic purification adsorbent for magnetic solid-phase extraction clean-up.

ABSTRACT

Bakery products containing poppy seeds are increasingly being commercialised. These seeds may be contaminated with latex from the *Papaver somniferum* L. plant, rich in opium alkaloids (OAs). Therefore, health authorities demand the development of analytical methods to control them. In this study, an efficient and simple method was developed and validated for the first time to analyse six OAs in bakery products by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). For this purpose, a solid-liquid extraction was optimised and then a magnetic material (magnetite surface-modified with Fe (III) terephthalate, denoted as Fe_3O_4@TPA-Fe) was used for a fast magnetic solid-phase extraction. The method has been validated with adequate recoveries (70-110%) and relative standard deviations (< 20%) and without matrix effects. Nine bakery samples (five breadsticks and four sliced bread) were analysed and showed low amounts in the breadsticks but two sliced bread showed amounts higher than the new Commission Regulation (EU) 2021/2142 (1.5 mg/kg).

KEYWORDS: Opium alkaloids; bakery products with poppy seeds; magnetic solid-phase extraction; validation; liquid chromatography-tandem mass spectrometry.

1. Introduction

The seeds of the *Papaver somniferum* L. plant, commonly known as opium poppy, are increasingly being used in bakery products (bread, buns and biscuits), as topping for salads or yoghurts, or for the elaboration of tea and oil. The most traded food with poppy seeds is bakery products, mainly breadsticks and sliced bread[1–4]. Poppy seeds hardly contain opium alkaloids (OAs), but they can be contaminated due to harvesting practices or insect damage with the OAs present in the latex of this plant (morphine, codeine, thebaine, papaverine, noscapine and oripavine)[5,6]. Its consumption can lead to false positive drug tests and cause adverse health effects, including cases of intoxication[5-7].

The European Commission has published on 3 December 2021 the Regulation (EU) 2021/2142, which comes into application on 1 July 2022. This regulation sets maximum levels for OAs, expressed in morphine equivalents (morphine + 0.2 codeine) for bakery products (1.5 mg/kg) and for whole, ground or milled poppy seeds (20 mg/kg). Furthermore, it is claimed that those levels should be set considering that food processing may reduce OAs content of raw poppy seeds by 25-100 % in the final product. In this regard, the supplier of poppy seeds should provide the morphine equivalent content of the seeds used as an ingredient to the bakery products manufacturers[9]. Besides, the European Commission published in 2014 recommendations for good agricultural and seed processing practices to reduce the morphine content[10] and several articles published that washing, grinding and baking treatments can decrease the content of opium alkaloids[4,7,11]. Furthermore, in 2018 European Food Safety Authority (EFSA) and German federal institute of risk assessment claimed new effective analytical methods to quantify all main OAs (such as thebaine, papaverine, noscapine and oripavine), not only morphine and codeine as in previous studies, and thus be able to legislate because they can be even more toxic as declared by health authorities and some recent studies[12-14], such as the review by Eisenreich et al. 2020 where the high toxicity of thebaine is reported[8]. Considering that these compounds are found at low concentrations in very complex food matrices, analytical methods based on sensitive and selective analytical techniques are essential. The most used technique is high-performance liquid chromatography (HPLC) with mass spectrometer (MS) detector as recommended by EFSA. It is especially used the triple

quadrupole (TQ) detector with electrospray ionization in positive mode (ESI+) and multiple reaction monitoring (MRM) for multiple analyte detection[3,4,6,15-17]. Regarding sample treatment, until now, most studies simply performed a solid-liquid extraction (SLE) of OAs from poppy seeds[3,4,18,19]. However, it is essential to carry out an adequate sample treatment that includes a preconcentration and/or purification step to eliminating possible matrix effects, thus avoiding erroneous results[3] and extending the useful life of the chromatographic column and MS detector. For this reason, a solid-phase extraction (SPE) step is used in some studies[15,17,20-23]. In addition, the magnetic dispersive SPE version (MSPE) has also been evaluated for this task[6,24-28], as the sorbent material can be quickly separated from the solution by using an external magnetic field, instead of filtration or high-speed centrifugation as required in common dispersive SPE. Then, MSPE is a simpler, faster, easily miniaturised and environmentally friendly preconcentration/purification technique[24]. The most widely used magnetic nanoparticles consist of a magnetite (Fe_3O_4) core and it is popular to add a layer of silica[6,25,26]. However, these materials only offer hydrogen bonding interactions, so alternative functionalisations are being explored to achieve other types of interactions (such as π-π electrostatic and ion-dipole) that improve the interaction with OAs, either by attaching a ligand to the silica or directly to the magnetite.

The aim of this work is to develop an efficient, rapid and very simple method to quantify six OAs in bakery products. For this purpose, a novel magnetic material composed of magnetite surface-modified with Fe (III) terephthalate (Fe_3O_4@TPA-Fe) was synthesized and evaluated as sorbent. Then, a SLE-MSPE sample treatment procedure was optimised and successfully validated to apply for the quantification of six OAs in sliced bread and breadsticks by HPLC-MS/MS.

2. Materials and methods

2.1 *Reagents and materials*

Standards of morphine, codeine, thebaine and oripavine were received from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine and morphine-d_3 (internal standard, IS)

were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1000 µg/mL in methanol and working standard solutions were prepared at 1 µg/mL in water/acetonitrile 90/10 (v/v) with 0.1% formic acid. All of these were stored in darkness at -20 ˚C. Ferric chloride 6-hydrate ($FeCl_3 \cdot 6H_2O$) 99% and ferrous chloride 4-hydrate ($FeCl_2 \cdot 4H_2O$) 99% were purchased from Labkem (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Terephthalic acid (TPA) was obtained from Análisis Vinicos S.L. (Ciudad Real, Spain). Ethanol absolute, formic acid (98%) and ammonia 32%, (w/w) were purchased from Scharlab (Barcelona, Spain). n-Hexane and N,N-dimethylformamide (DMF) were purchased from Merk (Darmstadt, Germany). Acetonitrile and methanol used were HPLC-MS quality and were purchased from Scharlab (Barcelona, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). The Nd-Fe-B magnet (5 × 5 × 2 cm) with force 200 kg used in the MSPE procedure was obtained from Superimanes S.L. (Sevilla, Spain).

2.2 *Bakery samples*

In the middle of 2021, four different brands of sliced bread and five breadsticks samples were purchased from supermarkets in Madrid and Zaragoza (Spain). From each sample, three packets were taken to obtain a more representative sample, as OAs content of poppy seeds can be very variable even from the same batch[6]. The poppy seed content of these bakery products was in the range of 1 to 6% (Table S1). To obtain a representative and homogeneous sample with a small particle size, three packets of each sample were ground with a manual mortar so as not to grind the poppy seeds and reduce OAs levels. To facilitate grinding, sliced bread samples were frozen with liquid nitrogen and all the samples were sieved through a pore size of 1 mm. Later, the three packets were homogenised to obtain a more representative sample. Then, sliced bread was stored at -20 ˚C until further analysis and breadsticks at room temperature for their longer shelf life.

2.3 *Preparation of the Fe_3O_4@TPA-Fe material*

First, Fe_3O_4 nanoparticles were prepared by chemical co-precipitation according to the work of Zhang and Shi[29]. To do this, 15 mmol of $FeCl_3 \cdot 6H_2O$ and 10 mmol of $FeCl_2 \cdot 4H_2O$ were dissolved in 80 mL of degas ultrapure water with stirring at 300 rpm and 80 ˚C under nitrogen atmosphere. Then, 50 mL of ammonia solution (32%, v/v) was added, and the mixture was stirred for 30 min. The black precipitate obtained (Fe_3O_4 nanoparticles) was collected with the help of a strong magnet and washed several times with deionized water until neutral pH. Finally, Fe_3O_4 particles were dried under vacuum by a vacuum line at 60 ˚C for 24 h.

For the Fe_3O_4@TPA-Fe synthesis, 1 g of magnetic particles were mixed with 25 mL of 0.84 M $FeCl_3 \cdot 6H_2O$ solution in DMF through ultrasound (US) (Elmasonic S30, Elma, Singen, Germany) for 10 min. Then, 50 mL of 0.12 M TPA solution in DMF was added and US was applied for 10 min. The mixture was placed in a 530 mL teflon-coated stainless-steel reactor (V 1.0 L, PS 131 bar, Parr Instrument Company, Moline, Illinois, USA) and maintained at 100 ˚C for 10 h. The final product (2 g) was collected using a magnet, washed with hot ethanol, and dried under vacuum at 60 ˚C for 24 h. TPA-Fe material was also synthesized in a similar way.

2.4 *Characterization of the Fe_3O_4@TPA-Fe material*

The synthetized material was characterized by scanning and transmission electron microscopy (SEM, TEM), Attenuated Total Reflection Fourier-Transform Infrared (ATR-FT-IR), Powder X-ray diffraction (XRD), N_2 gas adsorption-desorption isotherms and elemental analysis. Details on the equipment and conditions can be found in Supporting Information S1.

2.5 *Study of adsorption and desorption conditions on the Fe_3O_4@TPA-Fe material with standards*

Firstly, the adsorption was optimised. To do this, the studies were made in duplicate and with a standard solution of 1 µg/mL of each of the six OAs. The parameters evaluated

were solvent type (methanol, acetonitrile, acetone, isopropanol, ethyl acetate, dichloromethane, and hexane), at different times (1, 5, 10 and 20 min) with 50 mg (maximum expected amount) and then, the proportion of added ammonia or formic acid (10%) were evaluated. Subsequently, different quantities of material were studied (1, 2.5, 5, 10, 20 and 50 mg) to decrease it without affecting adsorption. Finally, to optimise desorption, the type of solvent (methanol, acidified methanol, water, acetonitrile and a mixture of water/acetonitrile, 90/10, v/v, containing 0.1% acid formic) and the desorption time (1, 5 and 10 min) were evaluated.

2.6 *Optimized bakery sample preparation procedure by SLE-MSPE*

Firstly, the optimisation of the SLE of OAs from bakery samples was carried out. To do this, two types of extraction solvents (methanol with 0.1% acetic acid and hexane) and two sample amounts (2.5 and 5 g) were studied. For this, a double SLE was performed with 10 mL for 30 min under magnetic stirring, according to the conditions previously used by other authors in the literature[4] and in our previous work[6]. To select the best conditions, recoveries obtained for the different parameters evaluated were compared. The values obtained for samples spiked at two concentration levels were compared with the values obtained for blank samples subjected to the same SLE process but spiked at the end, prior to analysis by HPLC-MS/MS. The spiking of the samples was done considering that the average proportion of poppy seeds in the bakery samples was around 5% (as shown in Table S1). Consequently, two spiked levels were evaluated, estimating that a high amount (5 mg/kg) and a low amount (0.25 mg/kg) of OAs could be found in the sample, based on our previous work in which different poppy seeds were analysed[6].

Once all the conditions were optimised, the method developed after grinding, homogenising, and sieving, consisted of (as shown in Fig.1) a double extraction of 2.5 g of sample with 10 mL of methanol acidified with 0.1% acetic acid for the SLE. The mixture was vortexed for 30 s (Rx^3 Velp Scientifica, Usmate, MB, Italy) and magnetically stirred for 30 min. Later, it was centrifuged at 6000 rpm (3992 rcf) for 10 min to recover the supernatant (ROTOFIX 32A Hettich, Tuttlingen, Germany). Then, the extract was frozen at -24 ˚C and filtered through a 0.45 µm nylon filter to remove fats and 2 mL of

extract solution were evaporated to dryness under vacuum and reconstituted in 1 mL of acidified hexane. Next, 1 mg (weighed in an Excellence Plus XP-6 Mettler with a deviation of 1µg) of Fe_3O_4@TPA-Fe (conditioned with 1 mL of acidified hexane for 1 min in the US) was added into the reconstituted extract followed by US for 1 min. The material was separated by a magnet from the solution, and the analytes were desorbed with 2 mL of water/acetonitrile (90/10, v/v) with 0.1% formic acid for 1 min in the US. Finally, the solution was decanted for 2 min with the magnet and an aliquot of 950 µL was taken and 50 µL of 1 µg/mL morphine-d_3 (IS) were added before the analysis by HPLC-MS/MS (Fig.1).

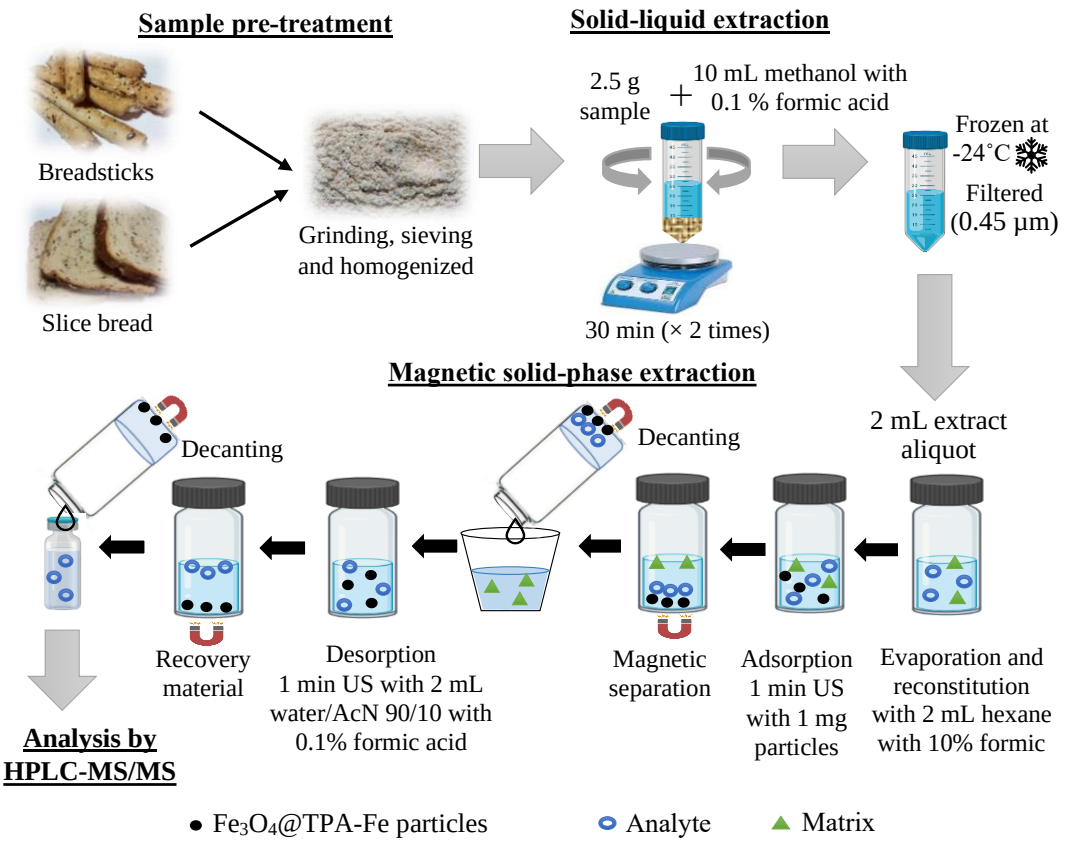


Figure 1. Diagram of the proposed methodology to quantify OAs in breadsticks and slice bread samples.

2.7 *HPLC-MS/MS analysis*

The quantification of OAs in bakery products was performed with a Varian 1200/1200 LC (Varian Ibérica, Madrid, España) equipped with a ProStar 410 autosampler (100 µL loop) coupled to a triple quadrupole tandem mass spectrometer detector (1200 L TQ) with electrospray ionisation (ESI) ion source. The data acquisition system was MS Workstation Varian version 6.8. Chromatographic separation was performed similar to our previous work[6], using a C_{18} Kromaphase 100 column (150 × 2.0 mm, 3.5 µm particle size, Scharlab, Barcelona, Spain) at 30 ˚C. The injection volume was 10 µL (partial injection) and the flow rate was set at 0.25 mL/min. A gradient elution similar to our previous work[6] was used with a mobile phase of water (A) and acetonitrile (B), both with 0.1% of formic acid as follows: 90-30% A (0-6min), 30-90% A (6-9min) and 90% A (9-11min) for column re-equilibration. Mass spectrometry acquisition was with electrospray ionization in positive mode (ESI+) with MRM mode. N_2 was used as drying and nebulizer gas. Drying gas was at 350 ˚C and 22 psi and nebulizer gas were at 58 psi. The capillary voltage was held at 5000 V and shield at 600 V. Collision gas was Argon at 1.90 mTorr and detector voltage 1480 V. The detection of each analyte was performed by direct infusion of a standard solution of 1 µg/mL in methanol using a syringe pump at a flow rate of 20 µL/min. Mass peak width Q_1 2.5, mass speak width Q_3 2.5 and scan width in MRM 2 s.

2.8 *Method validation*

The methodology was validated for analysing breadsticks and for sliced bread because although they are bakery products, they are relatively different samples. This was done by following SANTE/12682/2019 document[35] since there is currently no official regulation on analytical performance requirements for OAs in food or feed. The validation was done in terms of linearity, method detection and quantification limits (MDL, MQL), matrix effect (ME), accuracy, precision and selectivity (more details in Supporting Information S.2).

3. Results and discussion

3.1 *Preparation and characterization of the Fe_3O_4@TPA-Fe material*

SEM images of Fe_3O_4 (Fig. S1a) and Fe_3O_4@TPA-Fe (Fig. S1b) showed small spherical particles, with a tendency to aggregate, which is very common in magnetic materials. TEM images suggested that F_3O_4@TPA-Fe particles (Fig. S1c and d), were assembled on each other in 3D network macroporous structures with an average size of around 300 x 700 nm.

The FTIR spectra of the Fe_3O_4, TPA, Fe-TPA and Fe_3O_4@TPA-Fe are shown in Fig. S2. The band at 520-530 cm^{-1} can be assigned to the Fe-O bond stress, which is observed in Fe_3O_4, TPA-Fe and Fe_3O_4@TPA-Fe. The signals between 3,200-3,500 cm^{-1} are stretching bands of the -OH groups on the surface of the magnetite, due to the functionalization with the TPA-Fe compound decrease a lot, indicating the interaction between TPA-Fe and Fe_3O_4. The FTIR spectrum of TPA shows the characteristic bands of this organic compound at 925, 1,272 and 1,417 cm^{-1}, corresponding with bending bands of the carboxylate group (COO-) and the stress band of the carbonyl group (C=O) which appear around 1,680 cm^{-1}. The carbonyl signal around 1,680 cm^{-1} in TPA, practically disappears in the TPA-Fe compound, because of the interaction with the Fe atoms. Between 3000 and 2,500 cm^{-1} appear the stretching bands corresponding to the carboxylate group (COO-). The FTIR spectrum of the Fe_3O_4@TPA-Fe particles also shows the characteristic bands of the carbonyl group of the TPA at 1,291 and 1,634 cm^{-1} corresponding to the stretching of the C=O and the stretching bands of the carboxylic acid functional group (COO^-) are also observed around 3,040-3,116 cm^{-1} [36]. Therefore, FTIR analysis confirmed the interaction between the TPA-Fe compound and the surface of Fe_3O_4.

XRD pattern (Fig. S3) of Fe_3O_4 and Fe_3O_4@TPA-Fe agreed with the theoretical pattern of Fe_3O_4 described in the bibliography[37]. There are six discernible diffraction peaks in the 2θ region of 20–70˚ (220, 311, 400, 422, 511 and 440) that correspond with the Miller index diffraction peaks (JCPDS card: 19-0629), showing that the magnetite core is still present after modification. Size of particles was calculated using Scherrer Equation (1):

(1) $$d = \frac{k\lambda}{\beta cos\theta}$$

where k is a constant (k=0.9), λ is the wavelength of X-rays (1.5418Å), β is the full width at half maxima of diffraction peak line (in radians) and θ is the half of the diffraction angle. Fe_3O_4 was estimated to have a size of ~9 nm and Fe_3O_4@TPA-Fe of ~13 nm.

In addition, N_2 gas adsorption-desorption isotherms were made. Fe_3O_4 presents a Type IV isotherm according to the IUPAC classification[38] (Fig. S4a). As seen in Table S2, the surface area of 105 m^2/g, pore volume 0.30 cm^3/g and pore distribution at 41.3 Å of Fe_3O_4, according with other studies with chemical co-precipitation method[39]. The pore diameter that appears at 130.1 Å corresponds to the inter-particle space, this phenomenon is also observed in other porous materials that can give rise to particle agglomerates or overlapping layers of the material[40]. This coincides with the type of hysteresis, which is of Type H1, typical of agglomerates as can be observed in SEM (Fig. S1a). Fe_3O_4@TPA-Fe presented an isotherm Type II with a H1 hysteresis (Fig. S4b). In this case, the surface area and pore volume were lower (47 m^2/g and 0.14 cm^3/g, respectively), showing the correct functionalisation of the Fe_3O_4 particles. Moreover, the pore distributions obtained were 21.0 and 93.3 Å, corresponding to the pores in TPA-Fe, and 131.8 Å, 237.8 and 488.3 Å corresponding to the inter-particle spaces between the Fe_3O_4 particles (Table S2), which present irregular distribution as shown in TEM images (Fig. S1c and d).

Finally, the %C calculated by elemental analysis was around 3% and the functionalisation degree estimated was 0.31 mmol TPA/g of material and the %N was 0% N, which confirms the complete elimination of the synthesis solvent (DMF).

3.2 *Study of adsorption and desorption conditions on the Fe_3O_4@TPA-Fe material with standards*

The adsorption solvent was first determined to ensure the highest adsorption of the analytes. For this purpose, 2 mL of a 1 µg/mL solution of each of the six OAs in solvents of different polarity (methanol, acetonitrile, acetone, isopropanol, ethyl acetate, dichloromethane, and hexane) were mixed with 50 mg of the Fe_3O_4@TPA-Fe material through US for 1, 5, 10 and 20 min, and the supernatants were analysed. As shown in Table 1, different behaviours were observed depending on the analytes. For morphine, codeine and oripavine high adsorptions were obtained with all solvents except methanol. However, thebaine, papaverine and noscapine only showed high adsorption with hexane, and so it was the solvent selected for adsorption. Besides, all the analytes were completely adsorbed in 1 min, except noscapine which showed its maximum adsorption percentage (98%) after 20 min. Later, the addition of formic acid or ammonia was evaluated, so adsorption values were calculated after different times (1, 5, 10, 20, 30 and 60 min) in hexane, with 10% formic acid and with 10% ammonia. As can be seen in Fig. S5, with hexane with 10% ammonia thebaine, papaverine and noscapine adsorption were near 0%. However, hexane with 10% formic acid showed the best adsorption for noscapine after 1 min. Once adsorption has been optimised, the influence of the amount of material on the recovery of the analytes was also studied. For this task, different amounts (1, 2.5, 5, 10, 20 and 50 mg) were mixed for 1 min with 2 mL of a 1 µg/mL solution in acidified hexane of each of the six OAs. Desorption was carried out with 2 mL of methanol for 1, 5 and 10 min. As shown in Fig. 2, amounts higher than 1 mg showed recoveries invariant. Therefore, 1 mg of Fe_3O_4@TPA-Fe material was selected as the optimized adsorbent amount for the MSPE procedure. Subsequently, 2 mL of different types of desorption solvents (methanol, methanol with 0.1% acetic acid, acetonitrile, water and a water/acetonitrile, 90/10, v/v, with 0.1% formic acid) were tested. As shown in Fig. 3, the best recovery values were achieved with water/acetonitrile (90/10, v/v) with 0.1% formic acid in 1 min. Therefore, 2 mL of water/acetonitrile (90/10, v/v) with 0.1% formic acid and 1 min were selected as the optimum desorption conditions. Finally, the whole MSPE procedure developed was evaluated under optimized conditions using 1 mg of Fe_3O_4 and, as it was expected, recoveries were near 0%. These results highlight the role of the TPA-

Fe in the adsorption of the target analytes. Fig. S6 and Supporting Information S.3 show a proposal of possible molecular interactions that can occur between the adsorbent material and the target analytes (for example with morphine).

Table 1. Adsorption percentages (%) ± standard deviation (SD) obtained for each of the OAs with seven types of solvents for different times with Fe_3O_4@TPA-Fe material.

Adsorption solvent	Adsorption time (min)	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
AcN	1	98±3	60±3	52±0	49±0	20±1	90±2
	5	98±1	60±1	52±2	46±2	19±2	91±1
	10	98±2	67±4	52±1	44±1	19±0	92±3
	20	98±2	68±2	50±3	42±3	20±3	92±2
MeOH	1	39±4	38±4	33±4	20±2	19±2	35±3
	5	32±2	30±4	24±3	14±4	13±2	28±4
	10	30±10	24±7	18±1	12±1	10±3	18±6
	20	13±7	11±4	6±2	2±1	0±0	14±3
DCM	1	0±1	30±5	2±1	30±5	28±2	60±5
	5	100±0	72±3	46±3	45±4	46±7	70±1
	10	100±0	63±4	43±4	42±2	42±2	72±1
	20	100±0	80±5	45±1	44±1	42±1	85±3
EtOAc	1	94±2	72±9	66±1	16±1	13±2	84±1
	5	95±1	72±3	67±2	18±1	15±1	86±1
	10	95±2	70±2	67±1	19±1	17±2	88±1
	20	95±2	71±3	66±1	20±2	15±1	88±3
IPOH	1	76±3	44±10	53±1	32±1	5±1	76±1
	5	77±1	54±9	60±1	43±1	11±1	88±0
	10	84±2	56±3	62±1	44±1	13±1	89±2
	20	85±3	53±3	59±0	41±0	12±2	92±2
Hx	1	100±0	100±0	99±0	98±1	60±8	100±0
	5	100±0	100±0	100±0	99±1	80±10	100±0
	10	100±0	100±0	100±0	100±0	93±5	100±0
	20	100±0	100±0	100±0	100±0	98±1	100±0
Ace	1	97±1	76±7	68±7	29±16	27±15	91±3
	5	98±1	78±8	69±7	28±15	14±14	93±2
	10	98±2	76±7	67±7	26±17	13±13	95±2
	20	99±1	75±6	67±7	22±17	20±17	95±1

AcN: acetonitrile; MeOH: methanol; DCM: dichlorometane; EtOAc: ethyl acetate; IPOH: isopropanol; Hx: hexane; Ace: acetone.

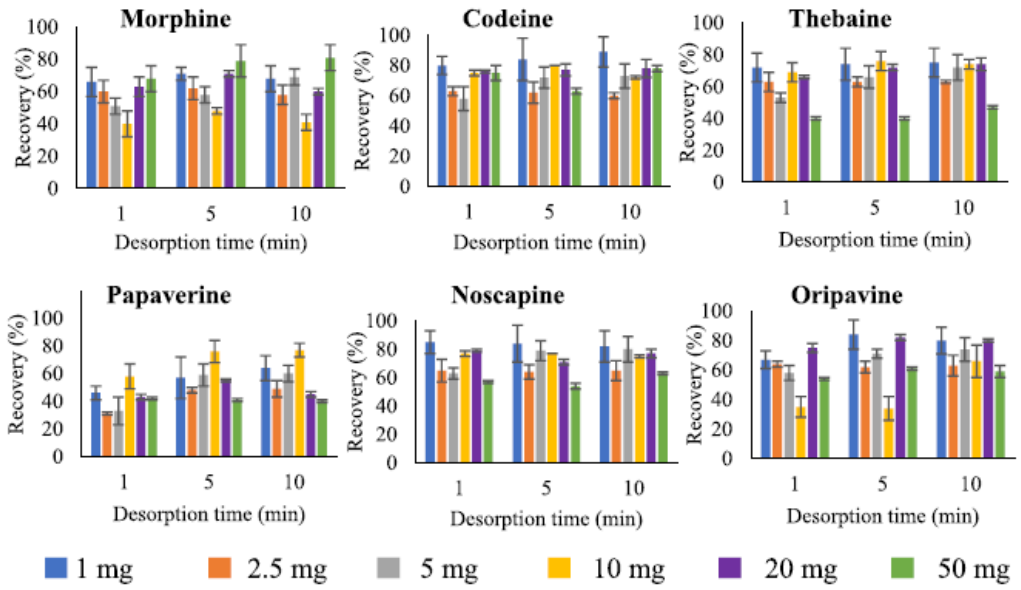


Figure 2. Comparison of the recovery (%) between different amounts of Fe_3O_4@TPA-Fe material (1, 2.5, 5, 10, 20 and 50 mg) in 2 mL of hexane with 10% formic acid with 1 µg/mL of each of the six analytes during 1 min adsorption and different desorption times (1, 5 and 10 min).

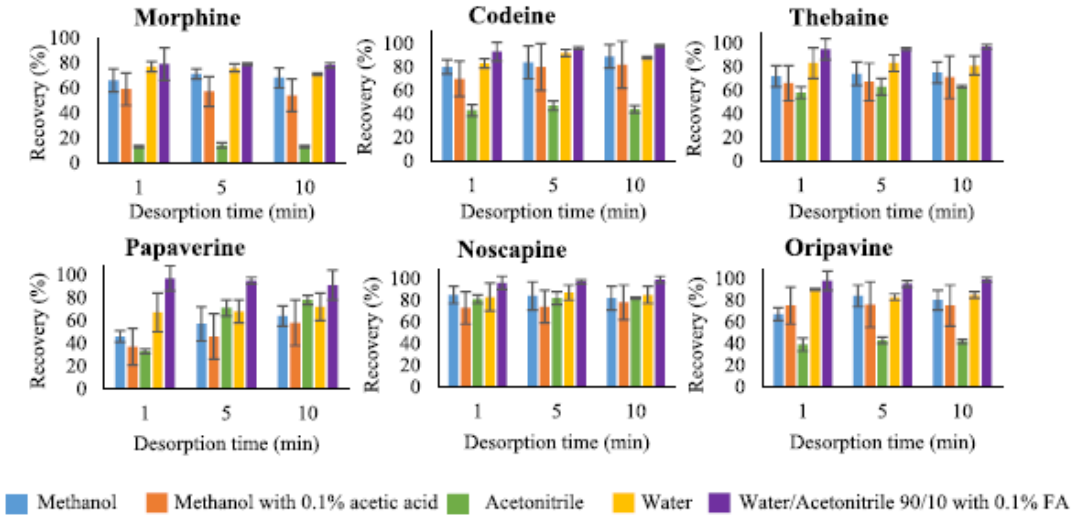


Figure 3. Comparison of the recovery (%) between different desorption solvents with 1 mg of Fe_3O_4@TPA-Fe material in 2 mL of hexane with 10% formic acid with 1 µg/mL of each of the six analytes during 1 min adsorption and different desorption times (1, 5 and 10 min).

3.3 Adsorption kinetics and isotherm experiments with the Fe_3O_4@TPA-Fe material

To study adsorption kinetics, 1 mg of the material was added to a 2 mL hexane with 10% formic acid with each of the six OAs (1 µg/mL) and through US at different times (1, 5, 10 and 20 min). After equilibrium, aliquots of the supernatant were analysed by HPLC-MS/MS. The adsorption capacity (Q_e) was calculated by equation (1) in Table S3 and the adsorption kinetics were determined by Lagergren's pseudo-first order[30], pseudo-second order[31] and intra-particle diffusion kinetic models[32] (Table S3). As shown in Fig. S7a, the adsorption of all analytes is very fast, because in 1 min 100% adsorption was obtained and in the following time remained constant. In addition, the important results of the three kinetic models were compiled in Table S4 and Fig. S8a. The linear regression coefficients (R^2) more close to 1 in the pseudo-second order model and their $Q_{e,cal}$ (calculated result) was more similar to $Q_{e,exp}$ (experiment result), so it is shown a chemical adsorption mechanism. Besides, all compounds did not show intra-particle diffusion tendency, as their R^2 were much lower than 1.

For adsorption isotherms, 2 mL solutions of different concentrations of the six OAs (0.01, 0.1, 1, 10, 20, 30 and 40 µg/mL) were added to 1 mg of the material and 1 min US was applied. After equilibrium, aliquots of the supernatant were analysed by HPLC-MS/MS and determined by Langmuir[33] and Freundlich[34] models (Table S3). As shown in Fig. S7b, by increasing the initial OAs concentration, the adsorption capacity was increased until the last point where the adsorption capacity of all analytes remains constant. In addition, the R^2 obtained by the Freundlich model was closer to 1 than by Langmuir, especially for thebaine, papaverine and noscapine (Fig. S8b).

3.4 Optimization of SLE of OAs from bakery products

To optimise the SLE, two types of extraction solvents (methanol çwith 0.1% acetic acid and hexane) and two sample amounts (2.5 and 5 g) were studied. For this, a double SLE was performed with 10 mL for 30 min under magnetic stirring to ensure a complete extraction. Optimisation studies were performed with sliced bread and breadsticks samples at two concentration levels, high (5 mg/kg) and low (0.25 mg/kg). First, the solvent type was evaluated. To do this, 2.5 g for each sample were extracted with 10 mL of acidified hexane (× 2). This solvent was tested, as it was the best adsorption solvent for

the MSPE procedure. However, the recovery values obtained did not exceed 2% for any of the analytes. Therefore, a different solvent had to be used and consequently, a vacuum evaporation step had to be introduced between the SLE and the MSPE. In this regard, the most widely used solvents to extract OAs from poppy seeds or poppy seed food products are methanol with 0.1% acetic acid[4] and acetonitrile/water/formic acid, (80/19/1, v/v/v)[3,41]. Considering that methanol with 0.1% acetic acid would be evaporated easily, this solvent was tested for the extraction of the alkaloids from the poppy seed-containing bakery samples. Results suggested that this solvent provided a good extraction efficiency due to the polarity and miscibility of the alkaloids in acidified polar solvents. Thus, satisfactory recovery values were obtained for all the analytes at the two concentration levels, being 81-102% and 99-110% (for the high level) and 94-121% and 82-93% (for the low level) in breadsticks and sliced bread samples, respectively. Furthermore, an additional study at the higher concentration level was performed with 5 g of the sample but using the same amount of extraction solvent and extraction time. However, in this study the recovery values obtained were lower, 68-89% for sliced bread sample and 86-95% with breadsticks. For this reason, the sample amount selected for the studies was 2.5 g because it was enough to quantify the analytes at the low spiking level.

3.5 Optimization of HPLC-MS/MS analysis

The parameters were optimised for the OAs with electrospray ionization in positive mode (ESI+). To do this, a 1 µg/mL methanol standard solution of each analyte was directly infused through a syringe pump at 20.0 µL/min. First, the molecular ion was detected with a Q_1 resolution of 0.7 at a scan time of 500 ms and, to obtain the maximum fragment ion intensity, the collision energy was optimised such as shown in Table S5. For chromatographic separation, different mobile phases were evaluated. Water containing 0.1% formic acid was used as eluent A and acidified acetonitrile or methanol (with 0.1% of formic acid) as eluent B. Finally, higher peak intensities and better separation were obtained with acidified acetonitrile. Besides, different gradients were tested, starting with a higher proportion of water at the beginning and increasing the organic phase, depending on the retention time, longer ramps were made. Finally, the selected gradient was: 90% A

(at 0 min), 30% A (at 6 min), 90% A (at 9 min to 11min). The retention time obtained for each analyte is shown in Table S5.

Standard working solutions were analysed in the HPLC-MS/MS to evaluate the instrumental parameters. Results are shown in Table S6. Linearity was evaluated in a 0.001-1 µg/mL range for thebaine, papaverine and noscapine and 0.01-1 µg/mL range for morphine, codeine and oripavine, with $R^2 \geq 0.999$. As can be seen, low LOD and LOQ were obtained, between 0.06 (noscapine) and 1.5 (codeine) µg/L and between 0.1 (papaverine and noscapine) and 6 (oripavine) µg/L, respectively.

3.6 Method validation

The validation results of the proposed SLE-MSPE-HPLC-MS/MS method for the quantification of six OAs in breadsticks and sliced bread samples are shown in Table 2. Calibration lines with R^2 between 0.999 and 1.000 were obtained and the deviation of the back-calculated concentrations of the calibration standards from the true concentrations in the matrix calibration lines were -7 and -19% for breadsticks and -0.7 and -20% for sliced bread. Therefore, these results demonstrated the good linearity of the method, which states good linearity when the deviation of the back-calculated concentrations is ≤ ± 20%[35]. In addition, the deviation of the slopes of the calibration lines for different days (n = 3) was calculated to ensure their reproducibility, obtaining RSDs between 3 and 8% in the case of breadsticks and between 1 and 9% in the case of sliced bread. On the other hand, ME was calculated by comparing the slopes of both matrix-matched and solvent-based calibration curves. ME was not observed in the breadsticks samples (< ±20%) and in the sliced bread samples a slight signal suppression was observed for thebaine, papaverine and oripavine, being ME -36, -22 and -25%, respectively (Table 2). This means that the developed purification procedure was able to eliminate almost all possible matrix effects for the six target analytes in both bakery samples. MDL and MQL values were low for the two sample matrices. For the breadsticks samples, the MDL and MQL obtained were, for noscapine 1.3 and 5 µg/kg, for thebaine 1.6 and 5 µg/kg, for papaverine 3 and 8 µg/kg, morphine 6 and 20 µg/kg, and for codeine and oripavine with 13 and 42 µg/kg, respectively. For sliced bread, MDL and MQL obtained were, for thebaine and

noscapine 0.3 and 1 µg/kg, papaverine 0.5 and 1.5 µg/kg, codeine and morphine 2 and 7 µg/kg and oripavine with 12 and 40 µg/kg, respectively. Accuracy and precision were evaluated at two different levels of concentration, low (0.25 mg/kg) and high (5 mg/kg), showing adequate recovery values in both samples, between 70 and 120% (Table 2). On the other hand, as shown in Table 2, satisfactory results were obtained for intra-day and inter-day precision at two concentrations levels because the RSD values were lower than 20%. Furthermore, as shown in Fig. 4, a good selectivity of the method was obtained. The chromatograms of the extracted ions obtained for each of the OAs in a standard solution were compared with the extracts of each sample. It was obtained that the variation of the t_R was $\leq$ 0.1 min and the ion ratios of the sample extracts were within ± 30% (relative abundance) of the mean of the standards for each analyte.

Table 2. Validation parameters of the SLE-MSPE-HPLC-MS/MS method for the quantification of six OAs in bakery products.

Analytes	Linear range (µg/mL)	Matrix-matched calibration (R^2) [a]	ME [b]	MDL [c] (µg/kg)	MQL [d] (µg/kg)	Accuracy [e]		Precision [e]	
						Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-Day Precision (RSD %)	Inter-Day Precision (RSD %)
Method validation with breadsticks samples									
Morphine	0.01-1	$y = 3.2\times10^6x + 1.2\times10^5$ (0.999)	-11	6	20	71 ± 9 [L] 83 ± 3 [H]	77 ± 6	17 [L] 4 [H]	20 [L] 9 [H]
Codeine	0.01-1	$y = 4.1\times10^6x + 7.0\times10^4$ (1.000)	-7	13	42	75 ± 8 [L] 84 ± 3 [H]	80 ± 6	13 [L] 4 [H]	18 [L] 5 [H]
Thebaine	0.001-1	$y = 2.7\times10^7x + 4.8\times10^5$ (1.000)	-36	1.6	5	88 ± 10 [L] 77 ± 3 [H]	83 ± 7	15 [L] 4 [H]	19 [L] 7 [H]
Papaverine	0.001-1	$y = 4.6\times10^7x + 1.1\times10^6$ (0.999)	-22	3	8	66 ± 11 [L] 73 ± 7 [H]	70 ± 9	19 [L] 10 [H]	19 [L] 12 [H]
Noscapine	0.001-1	$y = 7.2\times10^7x + 1.2\times10^6$ (1.000)	-11	1.3	5	79 ± 9 [L] 83 ± 2 [H]	81 ± 6	14 [L] 2 [H]	17 [L] 5 [H]
Oripavine	0.01-1	$y = 4.1\times10^6x + 4.7\times10^4$ (0.999)	-25	13	42	85 ± 9 [L] 82 ± 3 [H]	84 ± 6	14 [L] 3 [H]	17 [L] 5 [H]
Method validation with sliced bread									
Morphine	0.001-1	$y = 4.7\times10^6x + 9.5\times10^3$ (1.000)	20	2	7	89 ± 9 [L] 90 ± 9 [H]	90 ± 9	10 [L] 9 [H]	18 [L] 11 [H]
Codeine	0.001-1	$y = 5.2\times10^6x + 2.7\times10^5$ (1.000)	17	2	7	66 ± 11 [L] 120 ± 7 [H]	93 ± 9	20 [L] 6 [H]	20 [L] 7 [H]
Thebaine	0.001-1	$y = 3.9\times10^7x + 3.5\times10^7$ (1.000)	**-2**	0.3	1	78 ± 10 [L] 104 ± 9 [H]	91 ± 10	17 [L] 9 [H]	20 [L] 11 [H]
Papaverine	0.001-1	$y = 6.4\times10^7x + 5.3\times10^5$ (1.000)	15	0.5	1.5	95 ± 14 [L] 103 ± 8 [H]	99 ± 11	15 [L] 8 [H]	19 [L] 9 [H]
Noscapine	0.001-1	$y = 8.8\times10^7x + 1.6\times10^6$ (1.000)	12	0.3	1	106 ± 4 [L] 114 ± 9 [H]	110 ± 7	4 [L] 8 [H]	13 [L] 12 [H]
Oripavine	0.01-1	$y = 5.6\times10^6x + 5.3\times10^4$ (1.000)	3	12	40	110 ± 10 [L] 100 ± 8 [H]	105 ± 9	9 [L] 8 [H]	10 [L] 9 [H]

Linear range expressed in µg/kg is 80-8000 in the case of morphine, codeine, oripavine in breadsticks and in oripavine in sliced bread and 8-8000 in all other cases. [a] The calibration line is in the units: µg/mL; [b] ME: matrix effect (dividing the purified matrix slope by the solvent slope). [c] MDL: method detection limit. [d] MQL: method quantification limit. [e] Accuracy and precision were obtained by spiking samples at two known concentration levels: low (L, 0.25 mg/kg) and high (H, 5 mg/kg).

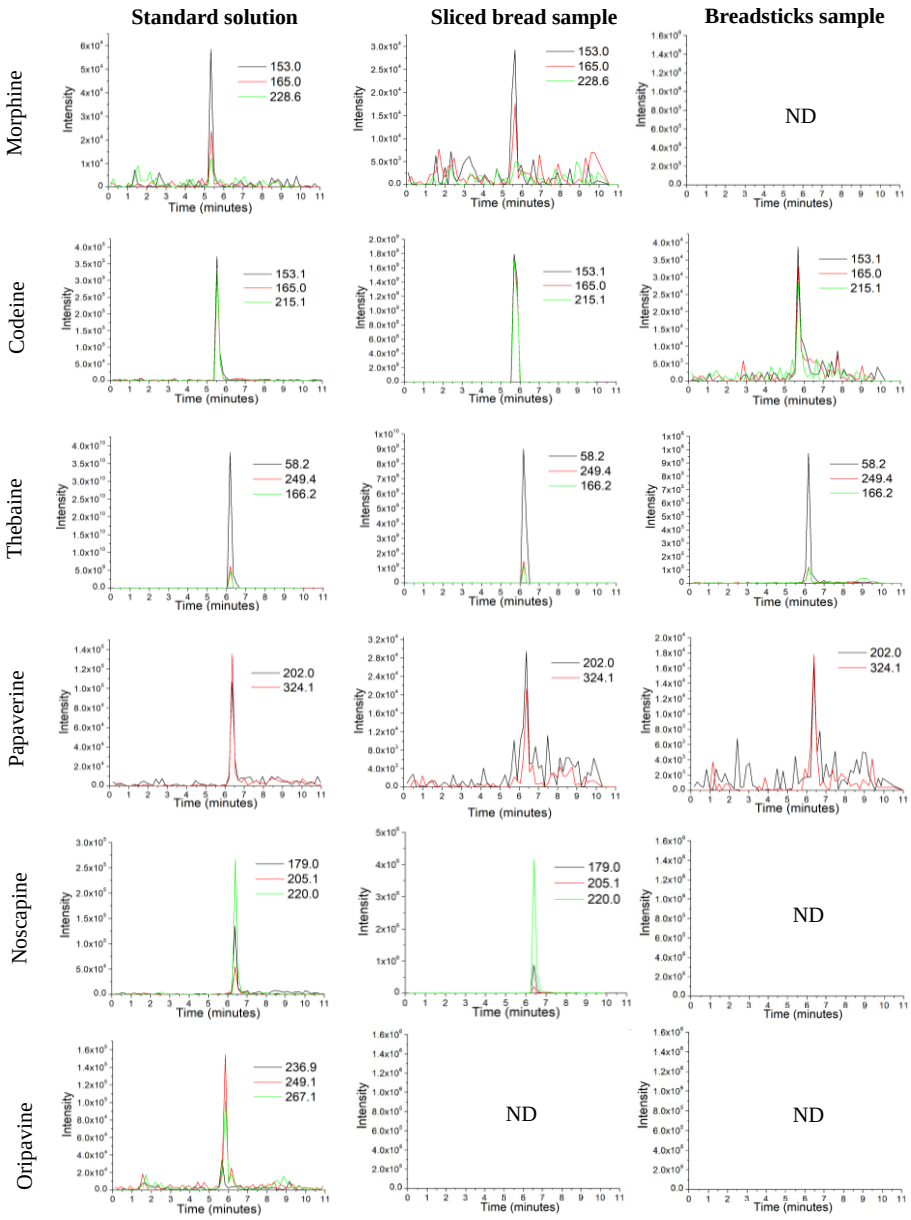


Figure 4. Comparison between the extracted ions chromatograms obtained for each of the OAs in a standard solution mixture of 0.001 µg/mL (thebaine, papaverine and noscapine) and 0.01 µg/mL (morphine, codeine and oripavine) with respect to the extracts of sliced bread and breadsticks. ND: not detected.

3.7 Comparison with others reported methods

The proposed methodology was compared with other methods previously published (Table 3). To the best of our knowledge, this is the first validated method for the simultaneous analysis of six OAs in bakery products with poppy seeds. There are only three articles in bakery products, but their methods used were validated in poppy seeds, which are less complex matrices. In addition, a simple SLE was performed to extract the alkaloids from the bakery products, without a purification step to eliminate/reduce possible matrix effects prior to chromatographic analysis[2,3,4]. This is a very important step in the analytical process as matrix effects can cause false results and increase equipment deterioration. There are three studies analysed hotpot seasoning samples (a Chinese popular food) with SPE and MSPE for clean-up purposes[15,27,28]. For example, Guo et al. used 60 mg of a commercial adsorbent (Oasis ® MCX) for the SPE step[13]. However, novel magnetic materials have also been evaluated for this task, looking for the reduction of sorbent quantities and time. Thus, Xu et al. used 50 mg of amantadine-functionalized magnetic microspheres (Fe_3O_4@SiO_2@ADME)[28] and Tang et al. used 15 mg of a magnetic chitosan composite material (Fe_3O_4@SiO_2@CS/GO)[27] for the MSPE step. In these protocols, the adsorption step required 8 min[28] and 20 min[27] and for desorption 2 min. Finally, in our recent work[6], mesostructured silica-coated magnetic nanoparticles (Fe_3O_4@SiO_2@mSiO_2) were used as adsorbent for MSPE to poppy seeds sample extracts were purified in just 4 min, but 50 mg of the material was needed for this purpose. Thus, regarding these previous studies, emphasis should be put on in the current work since only 1 mg of adsorbent material is need and the purification step takes only 1 min for adsorption and another for desorption. Furthermore, with the Fe_3O_4@TPA-Fe material, matrix effects were avoided to a greater extent for all analytes, and for both sample types. In contrast, with other methods that reflect serious signal suppression[13,27] or enhancement[3,15] for some analytes. Another point to highlight is that the recoveries obtained with the Fe_3O_4@TPA-Fe material were all in the adequate range and with the previous material, two of them were around 50% (morphine and oripavine) because they showed a higher intraparticle effect in the adsorption kinetics and therefore, it was not possible to completely desorb them from the material[6]. In addition, the analytical

characteristics of the method were also compared with those of previously reported methods for the determination of OAs but in other, simpler sample matrices (Table 3). The MDL and MQL achieved with this methodology were sufficiently low for these analytes in these types of samples (0.3-13 µg/kg and 1-42 µg/kg, respectively) and better or comparable accuracy (70-110%) and an adequate precision (≤ 20%) were also obtained. Therefore, the proposed method is a good alternative for an efficient, rapid and simple determination of six OAs in bakery products with poppy seeds.

Table 3. Comparison of the proposed methodology with other methods previously published for the quantification of OAs in food.

Sample analysed	Sample validation	Analyte	Sample Treatment		Analysis Technique	Validation parameters					Ref.
			Extraction	Purification		MDL (µg/kg)	MQL (µg/kg)	ME (%)	Recovery (%)	RSD (%)	
Poppy seeds, poppy seed topped rolls, muffins	Poppy seed (200 mg)	MOR, COD, THEB, NOS, PAP	Chl/IPOH (90/10, v/v) at pH 3.5 (1 mL, 10 min)	–	HPLC-IT-MS/MS	–	–	–	–	≤6	[2]
Poppy seeds, filling for bakery and cakes	Poppy seed (10 g)	MOR, COD, THEB, NOS, PAP, NAR	AcN/water/ formic acid, 80/19/1, v/v/v (100 mL, 30 min ×2)	–	UHPLC-TQ-MS/MS	–	100	100-130	77–172	≤20	[3]
Poppy seeds, cakes, buns	Poppy seed (10 g)	MOR, COD, PAP, NOS	MeOH 0.1% acetic acid (30 mL, 60 min)	–	HPLC-TQ-MS/MS	70–300	200–1000	–	–	≤9	[4]
Poppy seeds	Poppy seeds (2.5 g)	MOR, COD, THEB, PAP, NOS, ORIP	MeOH/water, 50/50 (v/v) (30 mL, 30 min ×2)	Fe_3O_4@ SiO_2@ $mSiO_2$ (50 mg)	UHPLC-TQ-MS/MS	0.07–72.01	0.24–240	31-109	46–109	≤11	[6]
Hot pot	Hot pot (5 g)	MOR, COD, THEB, PAP, NOS	HCl 0.1 M (20 mL, 10 min) and PE (10 mL)	Oasis ® MCX SPE (60 mg)	UHPLC-TQ-MS/MS	0.003–0.04	0.01–0.1	61-201	72–124	≤23.7	[15]

Hot pot	Hot pot (5 g)	MOR, COD, THEB, PAP, NOS	AcN 0.1% formic acid (20 mL, 10 min) and n-hexane (20 mL)	Fe_3O_4@ SiO_2 @CS/GO (15 mg)	UHPLC-TQLIT-MS/MS	0.016–0.092	0.036–0.31	40-92	75–104	≤10	[27]
Hot pot	Hot pot (2 g)	MOR, COD, THEB, PAP, NOS	Water/AcN 50% (20 mL, 5 min)	Fe_3O_4@ SiO_2 @ADME (50 mg)	HPLC-TQLIT-MS/MS	0.05–0.8	0.25–2.5	76-80	80–115	≤10.7	[28]
Breadsticks and sliced bread	Breadsticks and sliced bread (2.5 g)	MOR, COD, THEB, PAP, NOS, ORIP	MeOH 0.1% acetic acid (10 mL, 30 min ×2)	Fe_3O_4@ TPA-Fe (1 mg)	HPLC-TQ-MS/MS	0.3-13	1-42	64-120	70-110	≤20	This work

MOR: morphine, COD: codeine, THEB: thebaine, PAP: papaverine, NOS: noscapine, NAR: narceine, ORIP: oripavine, Chl: chloroform; IPOH; isopropanol; AcN: acetonitrile, MeOH: methanol, HCl: hydrochloric acid, PE: petroleum ether, SPE: solid phase extraction, MSPE: magnetic solid phase extraction, (U)HPLC: (ultra)-high performance liquid chromatography, TQ: triple quadrupole, IT: ion trap, MS/MS: tandem mass spectrometry, TQLIT: triple quadrupole ion trap, MDL: method detection limit, MQL: method quantification limit, ME: matrix effect, RSD: relative standard deviation, Ref.: references.

3.8 Application of the proposed method to real samples of bakery products

The proposed method was applied to the analysis of nine bakery samples, five breadsticks and four sliced breads (Table S1). To give the result of each sample, a range of concentrations obtained in the lowest and highest sample replicate is shown (Table 4). This is because the concentration that can be found in poppy seeds is highly variable, even in seeds from the same commercial batch[2,6], as OAs content depends on several factors such as climate, harvesting method, harvesting time or plant variety[14]. For this reason, each replicate (n = 6) is a proportion different with maybe different contamination. Such as shown in Table 4, the concentrations found in all breadsticks were low, only one of them could be quantified thebaine, showing 0.22 ± 0.01 mg/kg (BS-4). Regarding the analytes identified, codeine was detected in one of them (BS-4), thebaine in three (BS-3, BS-4 and BS-5), papaverine in all except one (BS-5), noscapine in only one (BS-3) and morphine and oripavine were not detected in any of them. On the other hand, higher amounts were found in sliced bread samples (Table 4). Thus, morphine was found in two samples, with a maximum concentration of 0.16 ± 0.02 mg/kg (SB-4). For papaverine, all samples were below the MQL, except for one sample in which was not detected (SB-4), noscapine was found in two (SB-1 and SB-3), where the highest concentration was 0.24 ± 0.01 mg/kg and oripavine was not detected in any sample. In addition, considerably high levels for codeine and thebaine were found, which were identified in all samples, giving concentrations up to 8.3 ± 0.5 and 2.4 ± 0.2 mg/kg, respectively. Therefore, the poppy seeds used in the preparation of these products were highly contaminated. In this regard, considering that the average seed content in the product is 5%, the seeds would have approximately 166 mg/kg of codeine (SB-4) and 48 mg/kg of thebaine (SB-2). In addition, two sliced breads (SB-3 and SB-4) exceeded the EU maximum limit of 1.5 mg/kg morphine equivalents (morphine + 0.2 codeine) in the replicate with the highest amount. Comparing the results obtained with those of other authors on bakery samples, similar results were seen. Sproll et al. analysed 12 samples of bread mix made with baked poppy seeds in which codeine, papaverine and noscapine were not detected, and the morphine content found was between the MQL (<0.3 mg/kg) up to 4 mg/kg[4]. Lopez et al. analysed two ready-to-eat bakery products (cakes) and found up to 0.6 mg/kg of morphine, and <0.1 mg/kg of the rest of the compounds[3]. Carlin et al. in 2020 analysed

untreated poppy seeds and obtained considerable amounts of OAs, and then made muffins and bread coated with poppy seeds and they did not determine any OAs[2].

Therefore, relatively low amounts of OAs were shown in this article as well as in other published articles. However, the OAs levels were much lower than the obtained on poppy seeds in our previous work[6], where concentrations found were of up to 249 mg/kg of morphine, 6 mg/kg codeine, 136 mg/kg thebaine, 27 mg/kg papaverine, 109 mg/kg noscapine and 33 mg/kg oripavine. Estimating that these bakery products have 5% of seeds in their composition, it could be found up to 5% of these values previously found in seeds. For this reason, it could be suggested that the high temperatures used in the elaboration of these products may reduce the OAs content, as reported in previous studies and in the recommendation of European Commision[3,4,7,10,11,41]. Therefore, one might consider a higher decrease in breadsticks samples than in sliced bread samples, which may be attributed to the fact that in sliced bread the poppy seeds are inside the bread whereas in breadsticks the poppy seeds are on the surface and the effect of heating is more pronounced. Hence, in the case of products that are subjected to high temperatures, like bakery products, a treatment should be established to ensure their reduction as much as possible. However, to confirm this factor, more studies should be carried out on how the food processing can interfere in different matrices and, especially, how it affects the other OAs that are also present in high concentrations and are even more potentially toxic than morphine and codeine[8].

Table 4. Range of occurrence (mg/kg) ± SD (standard deviation) of the six OAs in six replicates (*n* = 6) for each of the nine bakery products analysed.

Sample code	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
BS-1	ND	ND	ND	<MQL	ND	ND
BS-2	ND	ND	ND	<MQL	ND	ND
BS-3	ND	ND	<MQL	<MQL	<MQL	ND
BS-4	ND	<MQL	<MQL-0.22 ± 0.01	<MQL	ND	ND
BS-5	ND	ND	<MQL	ND	ND	ND
SB-1	ND	<MQL	<MQL	<MQL	<MQL	ND
SB-2	ND	<MQL-1.03 ± 0.06	<MQL-2.4 ± 0.2	<MQL	ND	ND
SB-3	<MQL-0.09 ± 0.01	1.39 ± 0.08-7.4 ± 0.4	<MQL	<MQL	<MQL-0.24 ± 0.01	ND
SB-4	<MQL-0.16 ± 0.02	<MQL-8.3 ± 0.5	<MQL	ND	ND	ND

BS: breadsticks; SB: sliced bread; ND: not detected; <MQL: lower than method quantification limit but higher than method detection limit (MDL). SD: standard deviation calculated with the corresponding validation level at intraday precision.

4. Conclusions

An efficient, simple and rapid method to quantify six OAs in bakery products with poppy seeds has been developed and validated for the first time. For this purpose, an SLE followed by MSPE purification using 1 mg of Fe_3O_4@TPA-Fe was performed in only 2 min and a posterior analysis by HPLC-MS/MS. The method was successfully validated with recovery values between 70 and 110%, RSD values ≤ 20% and without matrix effects. The method was applied to nine bakery samples, five of them breadsticks and four sliced breads, showing lower amounts than poppy seeds, especially in breadsticks samples. However, two sliced bread samples exceeded the maximum level of new Commission Regulation (EU) 2021/2142. Therefore, in addition to morphine and codeine, further studies are needed on other OAs that may be even more toxic. In addition, it is necessary to study the possible effects of food processing to establish a treatment that guarantees their reduction as much as possible. And the study of more types of samples to be able to legislate according to the levels of contamination.

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

ACKNOWLEDGEMENTS: This research was funded by MCIU/AEI/FEDER, UE for project RTI2018-094558-B-I00.

ABBREVIATIONS: Opium alkaloids (OAs), solid-liquid extraction (SLE), magnetic solid-phase extraction (MSPE), magnetite surface-modified with Fe (III) terephthalate (Fe_3O_4@TPA-Fe), high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

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Supporting Information

Table S1. Different bakery products (breadsticks and sliced bread) analysed in the present work to determine the concentration of six OAs.

Bakery product type	Code	Poppy seeds amount (%)
Breadsticks	BS-01	6
	BS-02	6
	BS-03	Not specified
	BS-04	Not specified
	BS-05	1
Sliced bread	SB-01	2.3
	SB-02	5
	SB-03	6
	SB-04	3

BS: breadsticks; SB: sliced bread. Poppy seed contents were obtained from the ingredients list on the product packages.

Supporting information S1. Characterization of the Fe_3O_4@TPA-Fe material.

Scanning electron microscopy (SEM) images were scanned by a Nova Nano SEM230 (FEG-SEM) (Denton, USA) with an energy-dispersive spectrometry system (EDS). The metalliser used was Leica ACE600 (Wetzlar, Alemania), using a gold target and depositing a layer of less than 5 nm. Transmission electron microscopy (TEM) images were performed on JEOL JEM 1010 (Tokyo, Japan) with an accelerating voltage of 80kV. The samples were dispersed in acetone and deposited on carbon-supported grids. Attenuated Total Reflection Fourier-Transform Infrared (ATR-FT-IR) spectra were recorded with a Spotlight 200i, Perkin Elmer (USA) spectrometer in the region 4000-400 cm^{-1}. Powder X-ray diffraction (XRD) patterns were recorded on a Philips Diffractometer model PW3040/00 X'Pert MDP/MRD (Eindhoven, Netherlands) at 45 kV and 40 mA by Cu Kα radiation (λ = 1.5418 Å). Nitrogen gas adsorption-desorption isotherms were performed with a Micromeritics ASAP 2020 (Norcross, USA) analyser. The isotherms were measured at -196 ˚C with an interval of relative pressures (P/P_0) from 10^{-4} to 0.994. Previously, samples were degassed at 80 ˚C under vacuum for 10 h. The Brunauer-Emmett-Teller (BET) method was utilized to obtain the specific surface area (S_{BET}), the Barrett-Joyner-Halenda (BJH) model was used to calculate the pore volume and pore size distribution by the desorption branches of isotherms and the total pore volume (V_t) was estimated from the desorbed amount at a relative pressure P/P_0 of 0.97. Finally, elemental analysis (% C and % N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. (Hampton, USA).

Supporting information S2. Method validation.

Linearity was assessed with matrix-matched calibration curves prepared in three consecutive days. All these curves were prepared for blank breadsticks (BS-1) and sliced bread (SB-1) samples at six known concentration levels within the linear range evaluated. For this purpose, the sample extracts obtained after the SLE-MSPE procedure were spiked with an aliquot of a standard solution containing the target alkaloids according to the desired concentration level of the calibration curve. In addition, quantification of morphine by means of isotope labelled IS correction was carried out. To do this, 50 µL of 1 µg/mL IS were added to each point of the matrix-matched calibration curves. The criteria for good linearity involve values $\leq \pm$ 20% for the deviation of the back-calculated concentrations of the calibration standards from the true concentrations. Matrix effects were determined by comparing the slopes of the calibration equations obtained from both matrix-matched and solvent-based calibration curves (both expressed in the same units µg/mL), calculating (1 - the ratio slope matrix-matched/slope solvent-based) × 100 for each analyte. The ME is lower when closer to 0% and according with the guideless the ME is negligible when is lower than ±20%. Positive values greater than 20% indicate signal enhancement and negative values indicate signal suppression. However, when the signal suppression or enhancement is greater than this margin of 20%, matrix effects must be considered in calibration. The sensitivity of the method for each sample was determined through the MDLs and MQLs of the OAs from the analysis of the lowest concentration analysed (0.01 or 0.001 µg/mL), which were estimated as the minimum concentration yielding a signal-to-noise ratio (S/N) of 3 or 10, respectively. The recovery assays were assessed by comparing the areas obtained for samples spiked (n = 6) with a known concentration of analytes and subjected to the SLE-MSPE procedure with those areas obtained for simulated samples (samples spiked at the same concentration but at the end of the procedure prior to their chromatographic analysis). The recovery assays were performed spiking the bread samples at a concentration of 5 mg/kg (high value) and 0.25 mg/kg (low value). According to the validation guidelines, the recovery values should be between 70 and 120%. On the other hand, the method precision was evaluated in terms of repeatability and reproducibility, using the same validation levels (low and high) as for the accuracy. For repeatability (expressed as RSD%), a sample spiked with the OAs at

the corresponding validation level was consecutively carried out six times (n = 6) on the same day. The reproducibility (also expressed as RSD %) was calculated by the analysis of three replicates of a sample (spiked with the analytes at the corresponding validation level), which were carried out in triplicate through three different days (n = 9). According to the validation guidelines, the RSD values for these precision parameters should be ≤ 20%. The selectivity of the method was determined by comparing the spectra of the different analytes obtained from standard solutions with the spectra obtained in the samples. It was considered satisfactory when the variation in the spectra was less than ± 30 % and the retention time of the target analytes was within the interval ± 2.5 %.

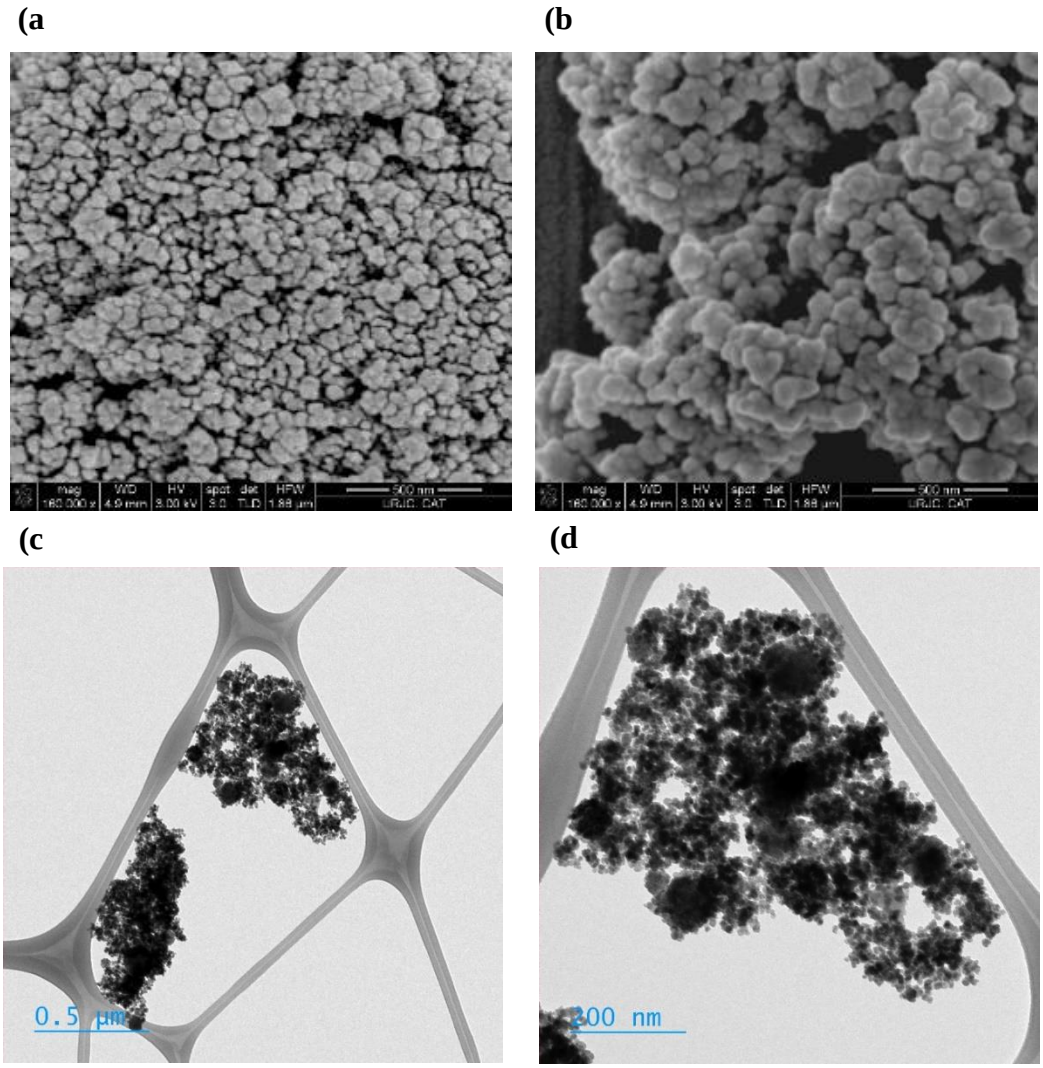


Fig. S1. SEM images of Fe_3O_4 (a) and Fe_3O_4@TPA-Fe (b) and TEM images of Fe_3O_4@TPA-Fe (c and d).

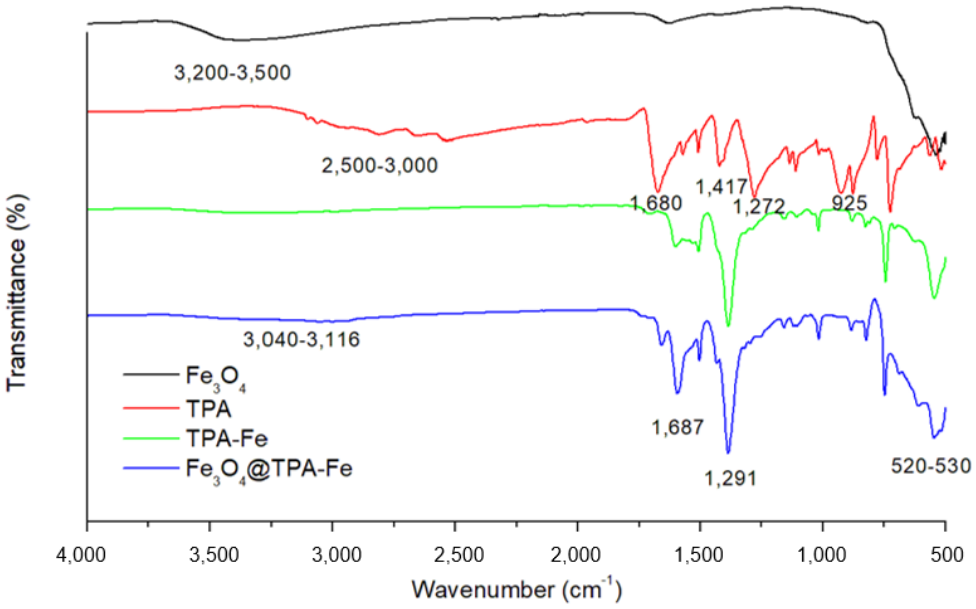


Fig. S2. ATR-FTIR spectrum of magnetite (Fe_3O_4), terephthalic acid (TPA), TPA-Fe and Fe_3O_4@TPA-Fe materials.

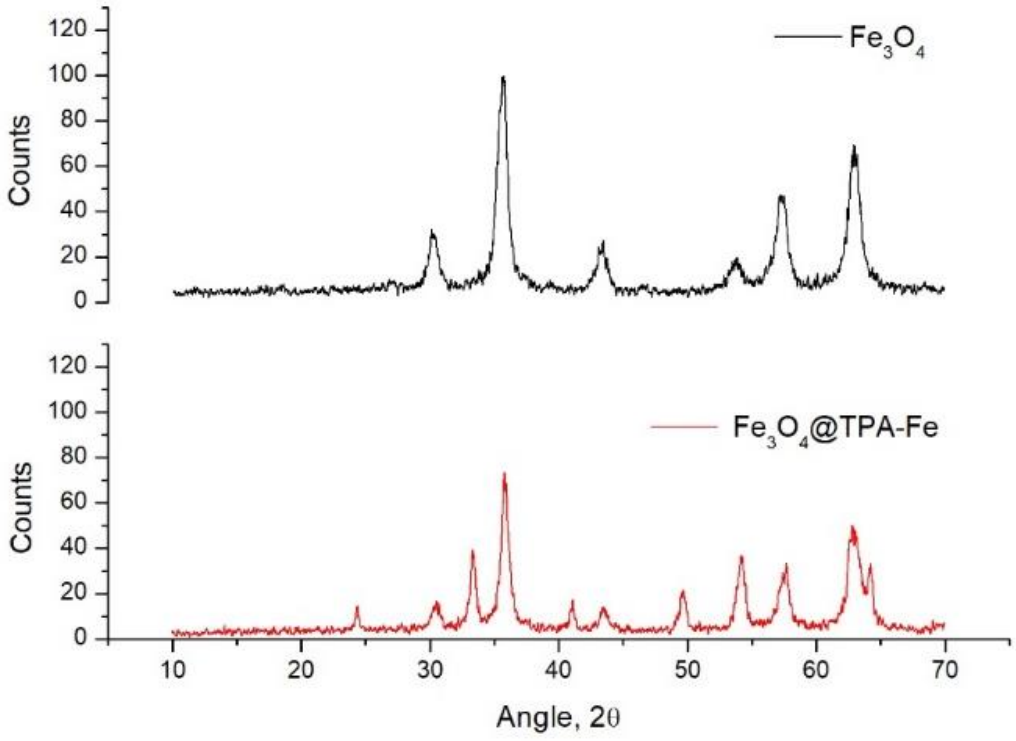
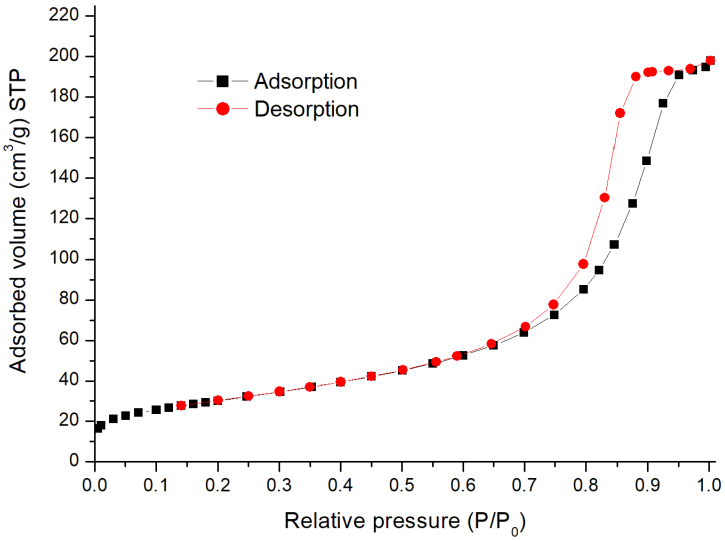


Fig. S3. XRD patterns obtained for Fe_3O_4 and Fe_3O_4@TPA-Fe.

(a)



(b)

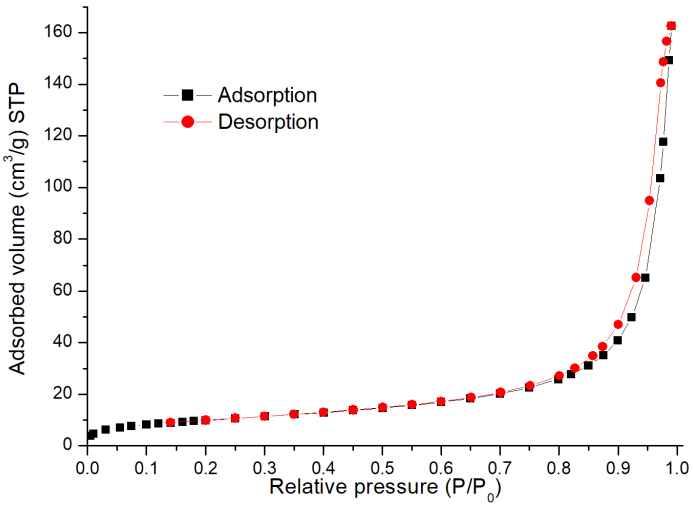


Fig. S4. N_2 adsorption-desorption isotherms of Fe_3O_4 (a) and Fe_3O_4@TPA-Fe (b). STP: standard temperature and pressure.

Table S2. Textural properties of materials synthesized.

Material	S_{BET} (m^2/g)[a]	Pore volume (cm^3/g)[b]	Pore diameter (Å)[c]
Fe_3O_4	105	0.30	41.3 and 130.1
TPA-Fe	718	0.50	20.8, 92.9 and 279.2
Fe_3O_4@TPA-Fe	47	0.14	21.0, 93.3, 131.8, 237.8 and 488.3

[a] S_{BET}: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method.

[b] Total pore volume was measured at relative pressure (P/P_0) = 0.97.

[c] Pore diameter estimated by using the BJH (Barrett, Joyner and Halenda) model applied on the desorption Branch.

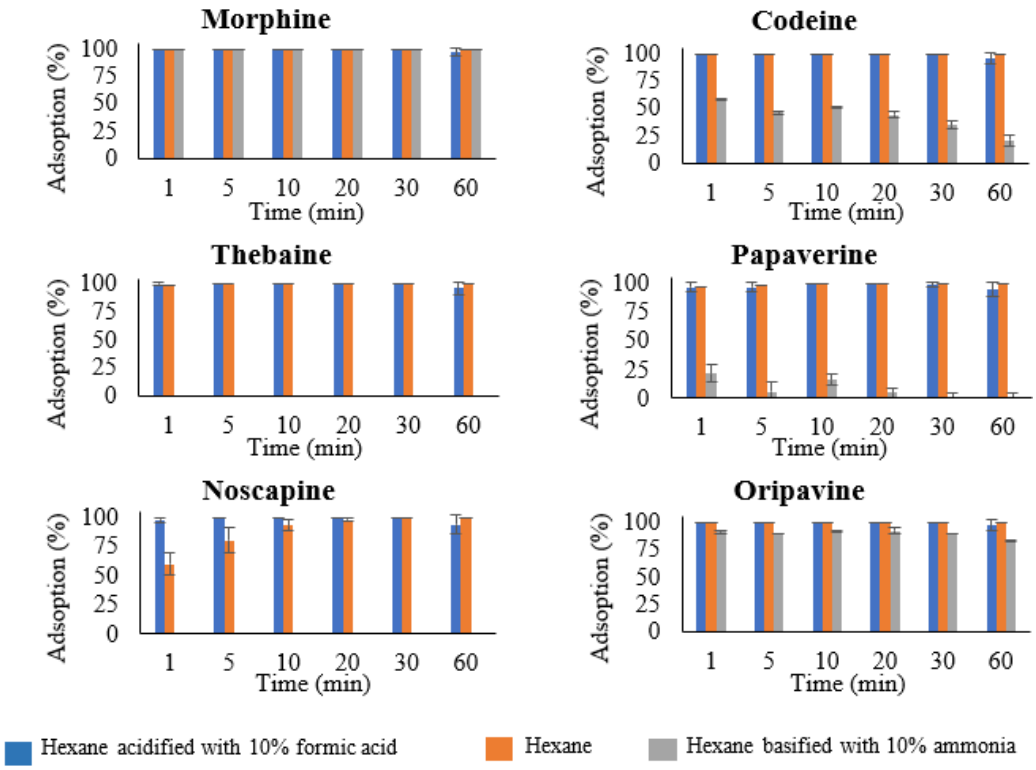


Fig. S5. Effect of pH on adsorption for each of the analytes at different times (1, 5, 10, 20, 30 and 60 min) with 2 mL of hexane with 10% formic acid, hexane and hexane with 10% ammonia with 50 mg Fe_3O_4@TPA-Fe material.

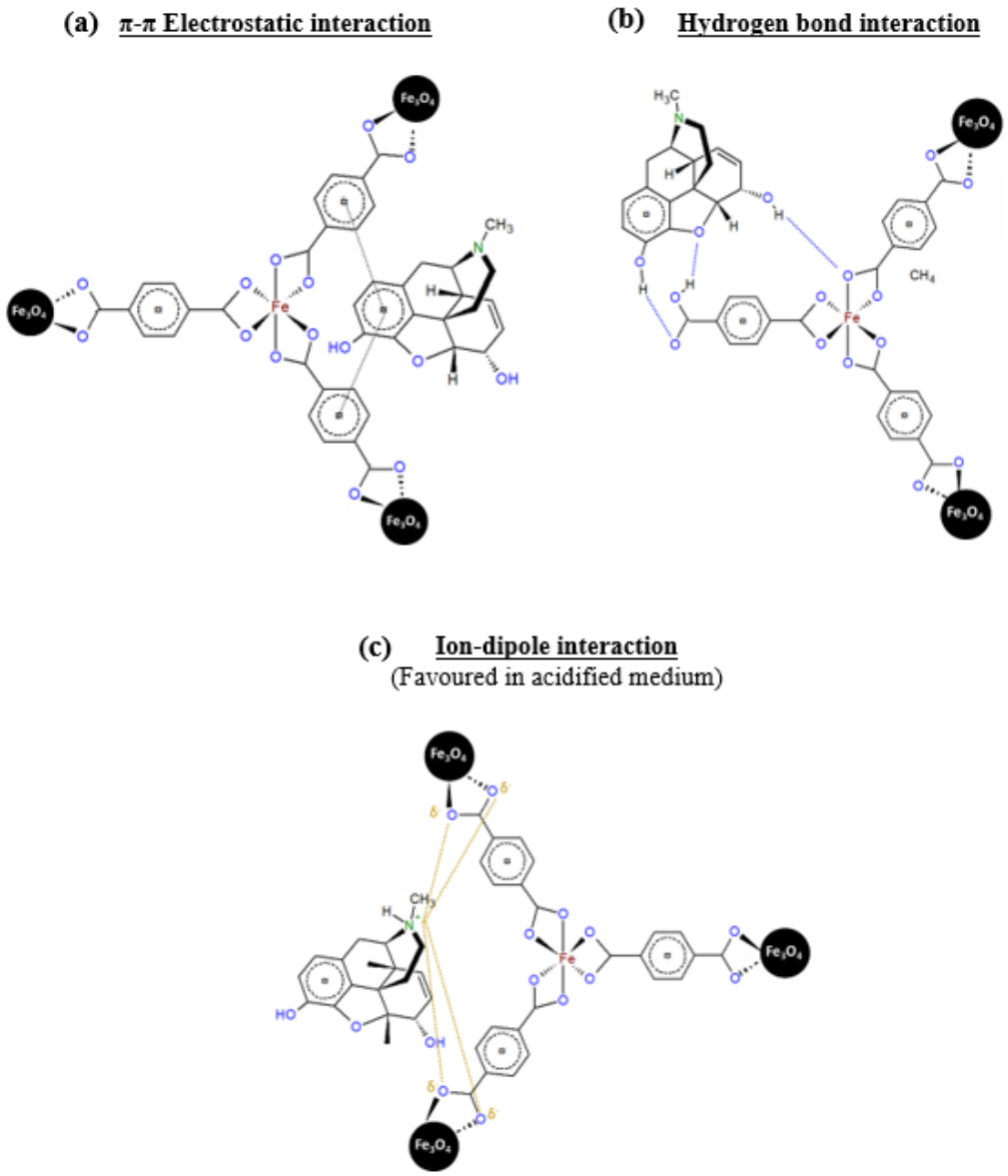


Fig. S6. Diagram of possible molecular interactions π-π (a), hydrogen bond (b) and ion-dipole (c) between the adsorbent material and OAs (in the example, morphine).

Supporting information S.3. Possible molecular interactions between the adsorbent material and OAs.

As OAs have a structure composed of aromatic rings, possible electrostatic interactions were considered, especially π-π, between the π cloud of the opium alkaloid and that of the TPA (Fig. S6a). In addition, hydrogen bonds between the -OH of the analytes and the acids groups of the material were expected (Fig. S6b). Besides, with acidic pH, the nitrogen of the amino groups of the alkaloids can be protonated giving a positive charge that interacts ionically with the polar group of the TPA (Fig. S6c). This last interaction was important for noscapine as it allowed 100% adsorption in the first minute when the adsorption medium was acidified.

Table S3. Equations of adsorption kinetics and isotherms

$Q_e = \frac{(C_o - C_e)V}{W}$	Adsorption capacity	(1)
$\ln(q_e - q_t) = lnqe - k_1 t$	Lagergren's pseudo-first order	(2)
$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left[\frac{1}{q_e}\right] t$	Pseudo-second order	(3)
$q_t = k_p t^{1/2} + C$	Intra-particle diffusion	(4)
$\frac{1}{Q_e} = \frac{1}{Q_{max}} + \frac{1}{K_L Q_{max} C_e}$	Langmuir model	(5)
$Log\ q_e = logK_F + \frac{1}{n} logC_e$	Freundlich model	(6)

C_o and C_e: initial and equilibrium concentrations of the target analytes (µg/L), respectively; V: volume of the solution (L); W: mass of the adsorbent (g); k_1: pseudo-first order rate constant (min^{-1}); q_e and q_t: amounts of OAs adsorbed at equilibrium and time (mg/g), respectively; k_2: pseudo-second order adsorption rate constant (g/mg min); q_t: amount of OAs adsorbed at time t (mg/g); k_p: intraparticle diffusion rate (mg/L min^2); C: intercept; Q_{max}: maximum monolayer capacity of the adsorbent (mg/g); K_L: Langmuir binding constant which is related to the energy of adsorption (L/mg); K_F: is the Freundlich constant (L/mg); n: is the heterogeneity factor (dimensionless).

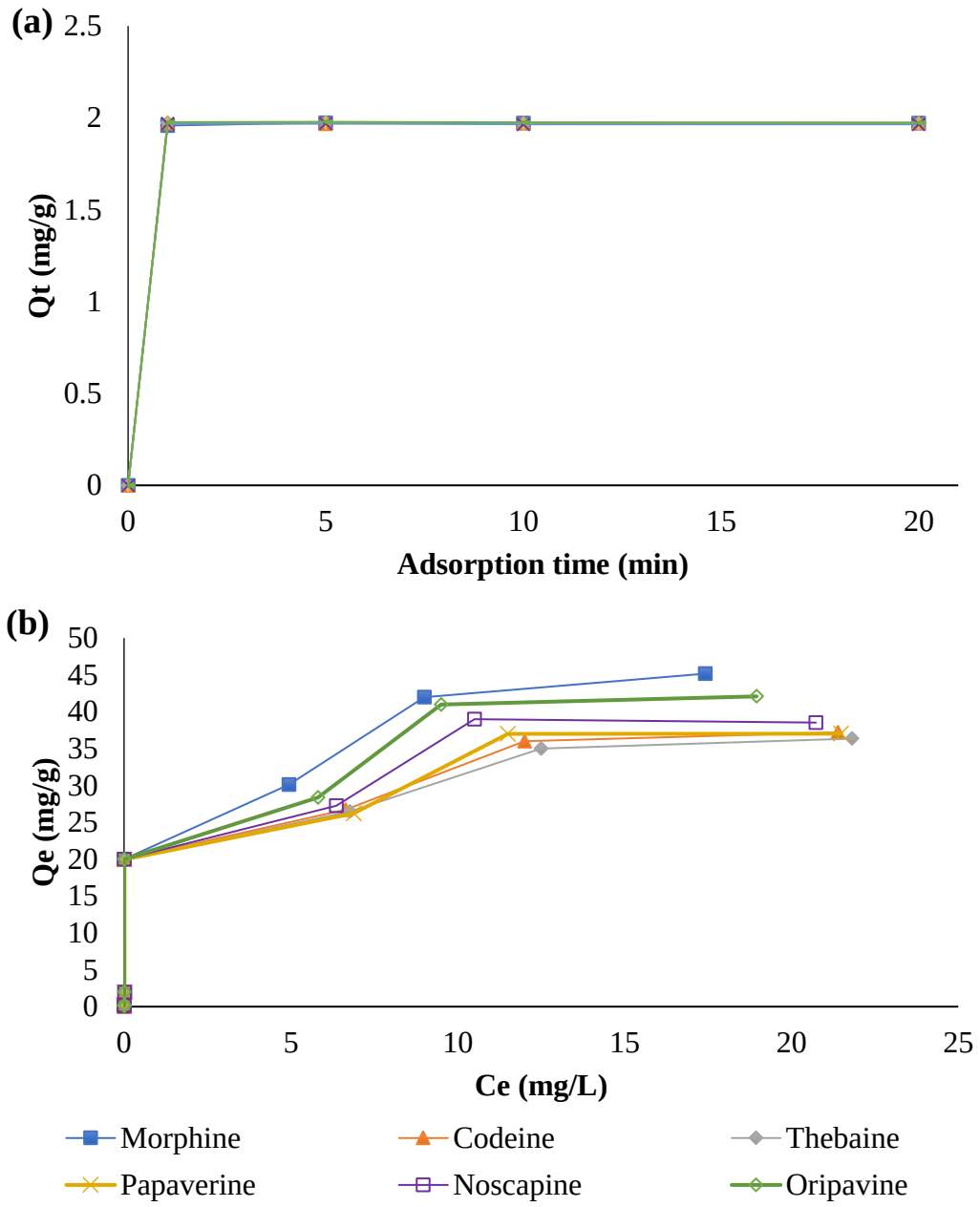


Fig. S7. Adsorption kinetic (a) and isotherm (b) experiments of the six OAs with 1 mg of Fe_3O_4@TPA-Fe material. Qe and Qt: amounts of OAs adsorbed at equilibrium and time (mg/g), respectively; Ce: initial and equilibrium concentrations of the target analytes (µg/L).

Table S4. Kinetic parameters of the adsorption of six opioid alkaloids with 1 mg Fe_3O_4@TPA-Fe material for different times (1-20 min) based on different kinetic models.

		Pseudo-first order model			Pseudo-second order model			Intra-particle model		
	$Q_{e,\ exp}$ (mg/g)	R^2	K_1 (min^{-1})	$Q_{e,\ cal}$ (mg/g)	R^2	K_2 (g/mg min)	$Q_{e,\ cal}$ (mg/g)	R^2	K_p (mg/g min^2)	C
Morphine	1.96	0.018	-0.061	0.028	1	5804	1.703	0.472	0.003	1.961
Codeine	1.97	0.031	0.070	0.018	1	1344	1.972	0.278	0.001	1.969
Thebaine	1.97	0.799	0.378	5.312	1	758	1.968	0.707	-0.002	1.976
Papaverine	1.97	0.799	0.312	3.975	1	1292	1.968	0.256	-0.001	1.972
Noscapine	1.97	0.671	0.343	1.116	1	1015	1.965	0.575	-0.001	1.970
Oripavine	2.00	0.570	-0.008	0.022	1	1053	1.972	0.476	-0.001	1.979

$Q_{e,\ exp}$: amounts of OAs adsorbed at equilibrium, experimental; K_1: pseudo-first order rate constant constant; $Q_{e,\ cal}$: amounts of OAs adsorbed at equilibrium, calculated; K_2: pseudo-second order adsorption rate constant; K_p: intraparticle diffusion rate; C: intercept.

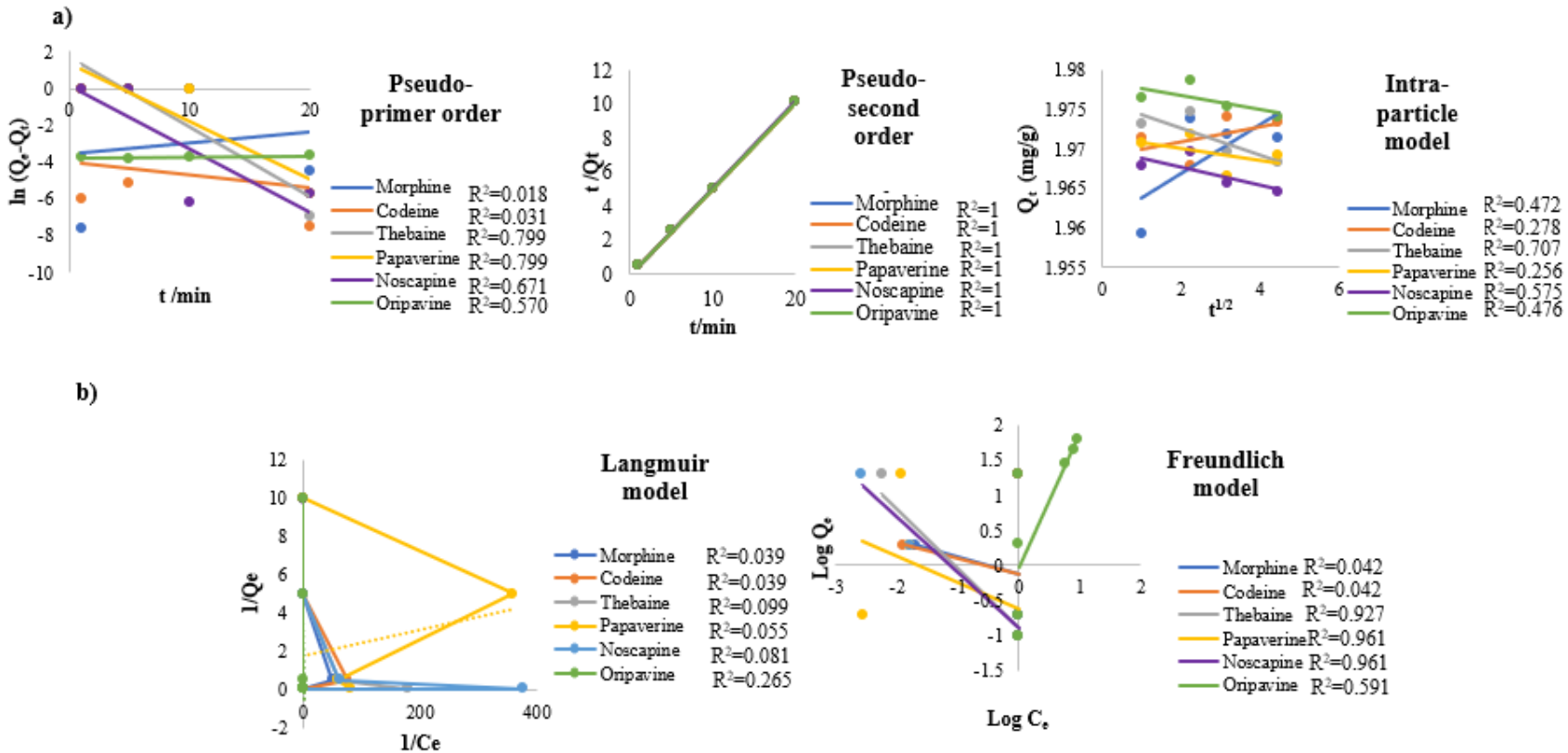


Fig. S8. Three kinetics (a) and two isotherm models (b) for the adsorption of the six OAs with 1 mg of Fe_3O_4@TPA-Fe material. Qe and Qt: amounts of OAs adsorbed at equilibrium and time (mg/g), respectively; Ce: initial and equilibrium concentrations of the target analytes (µg/L).

Table S5. Optimal parameters of MRM for the analysis of six OAs by HPLC-MS/MS.

Analytes	t_R[a] (min)	Ionization mode	Precursor ion (Q_1, m/z, $[M+H]^+$)	Fragment ion[b] (Q_3, m/z)	CV[c] (V)	CE[d] (eV)
Morphine	5.778	ESI (+)	286.1	**153.0**	72	45
				165.0		24
				228.6		22
Morphine-d3	5.819	ESI (+)	288.7	**152.3**	72	45
				164.2		37
				200.6		25
Codeine	5.869	ESI (+)	300.2	153.1	72	45
				165.0		45
				215.1		24
Oripavine	6.020	ESI (+)	298.3	236.9	72	14
				249.1		17
				267.1		12
Thebaine	6.245	ESI (+)	312.3	**58.2**	72	8
				249.4		16
				166.2		16
Papaverine	6.270	ESI (+)	340.2	**202.0**	72	24
				324.1		30
Noscapine	6.303	ESI (+)	414.3	179.0	72	24
				205.1		42
				220.0		20

[a] t_R: retention time; Gradient elution with a mobile phase of acetonitrile (A) and water (B), both with 0.1% of formic acid. The gradient started at 90% B, in min 6 changed to 30% B, in min 9 returned to 90% B and it was maintained until min 11 to equilibrate.
[b] The quantitation ion transitions are in bold.
[c] CV: cone voltage.
[d] CE: collision energy.

Table S6. Instrumental validation parameters of HPLC-MS/MS analysis.

Instrumental validation				
Analytes	**Linear range (µg/mL)**	**Solvent calibration (R^2)**	**LOQ (µg/L)**	**LOD (µg/L)**
Morphine	0.01-1	$y = 3.6\times10^5x + 2.3\times10^4$ (1.000)	3	1
Codeine	0.01-1	$y = 4.2\times10^6x + 1.9\times10^4$ (1.000)	5	1.5
Thebaine	0.001-1	$y = 4.0\times10^7x + 5.4\times10^5$ (1.000)	0.3	0.1
Papaverine	0.001-1	$y = 5.5\times10^7x + 7.6\times10^5$ (1.000)	0.1	0.04
Noscapine	0.001-1	$y = 7.7\times10^7x + 1.0\times10^6$ (0.999)	0.1	0.06
Oripavine	0.01-1	$y = 5.3\times10^6x + 2.4\times10^4$ (1.000)	6	1

LOQ: limit of quantification; LOD: limit of detection.

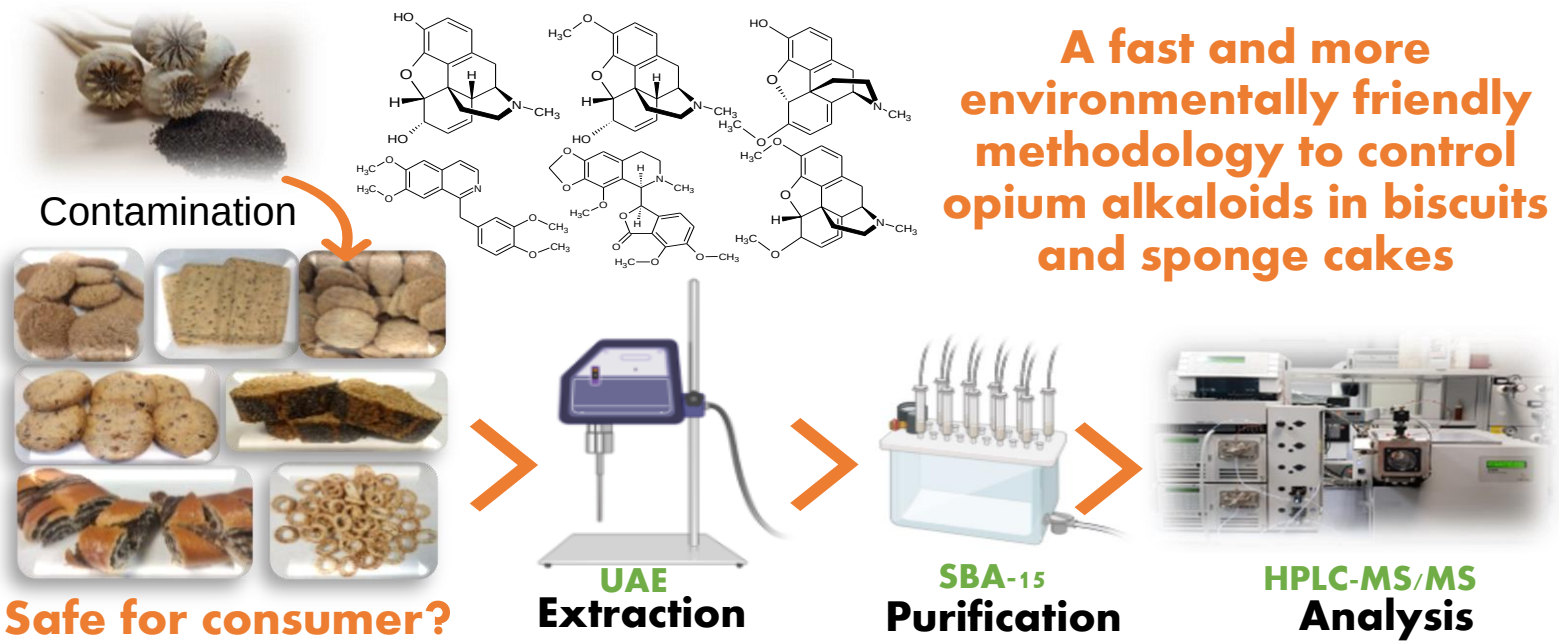
Artículo 3:

Pulsed ultrasound-assisted extraction followed by purification with SBA-15 for the control of opium alkaloids in biscuits and sponge cakes

Microchemical Journal (2022) 108059

PUBLICATION INFORMATION

Journal	Microchemical Journal
Publisher	Elsevier
Impact factor	5,304 (JCR, 2021)
Category	Analytical chemistry (26/130, Q1)
DOI	https://doi.org/10.1016/j.microc.2022.108059


Contamination
A fast and more environmentally friendly methodology to control opium alkaloids in biscuits and sponge cakes
Safe for consumer?
UAE
Extraction
SBA-15
Purification
HPLC-MS/MS
Analysis

Highlights:

- A fast and more eco-friendly methodology to control opium alkaloids in food products.
- First validated methodology for opium alkaloid analysis in biscuits and sponge cakes.
- SBA-15 was used for the solid-phase extraction to avoid matrix interferences.
- Ultrasound-assisted extraction allowed solvent volume and extraction time reduction.
- Morphine was detected in all samples studied below the legislated maximum limits.

ABSTRACT

Food products containing poppy seeds are increasingly consumed. These seeds may be contaminated with opium alkaloids (OAs) from the latex of the plant (*Papaver somniferum* L.), which may present a health hazard to the consumer. Therefore, the aim of this work was to develop an efficient, fast and environmentally friendly methodology to control OAs in biscuits and sponge cake products by analysis with liquid chromatography coupled to a triple quadrupole tandem mass detector (HPLC-MS/MS). For this purpose, the ultrasound-assisted extraction (UAE) step was optimised using a 5-variable full factorial design at two levels, obtaining lower solvent volume and time than with other classical methods. Then, a solid phase extraction (SPE) was used to remove matrix effects. A commercial material (HLB) and two silica synthesised materials (HMS and SBA-15) were evaluated and optimised, selecting SBA-15 (50 mg). Finally, the method was validated and applied to real samples, showing morphine concentrations in 5 of 7 products but below the maximum permitted limit.

KEYWORDS: Opium alkaloids; biscuits; sponge cakes; ultrasound-assisted extraction; solid phase extraction with SBA-15; HPLC-MS/MS.

1. Introduction

Nowadays, the consumption of food with *Papaver somniferum* L. (opium poppy) seeds, such as bakery products (bread, biscuits and sponge cake), salads, yoghurts, teas and oils, is increasing, due in large part to their nutritional value [1]–[5]. However, it is reported that the seeds used may be contaminated with opium alkaloids (OAs; i.e. morphine, codeine, thebaine, papaverine, noscapine and oripavine) from the latex of this plant [3]–[7] and therefore even foods with poppy seeds [8]. Consumption of contaminated food can cause false positive drug tests and intoxication in consumers [9], [10]. As a control measure, Regulation (EU) 2021/2142 was published in December 2021, establishing maximum morphine equivalent limits (morphine + 0.2 x codeine) in seeds and bakery products [11]. However, this legislation only includes morphine and codeine, and health authorities are demanding further studies to regulate the other OAs, which can be even more toxic [10]. Therefore, in order to know consumer exposure and to legislate accordingly, there is a need to develop an efficient, rapid and environmentally friendly analytical methodology to analyse the six main OAs in biscuits and sponge cakes with poppy seeds and to validate for the first time for this type of samples [1].

Sample preparation takes a crucial role in the analysis of organic contaminants in food samples, as these compounds are often present in very low concentrations and their analytical determination is strongly influenced by the matrix components because they are very complex matrices. Extraction methods for opium alkaloids have been described in the literature, such as liquid-liquid extraction, using high solvent volumes and extraction time, and being less environmentally friendly. For example, López et al. used 100 mL of AcN/water/formic acid, 80/19/1, v/v/v/v and 1 h of extraction for each sample, [4] and Sproll et al. [5] used 30 mL of methanol 0.1% acetic acid for 1 h of extraction.

To reduce the extraction time and the volumes of solvent used, ultrasound-assisted extraction (UAE) may be applied, which is one of the most exploited modern extraction techniques in the last years for its many advantages [12]–[14]. However, to our knowledge, none of them has used in OAs extraction yet. Due to the cavitation effect of the ultrasounds, this technique is a simple, cheap and efficient extraction method that allows obtaining high extraction yields with minimum damage to structural and molecular

properties of interest compounds, reduces solvents use, and assures a better penetration of the extracted matrices [15], [16]. The efficiency of UAE is strongly affected by several factors, including solvent volume, acid proportion, extraction time, amplitude and mode of sonication (continuous or pulsed). Optimisation of these parameters is considered essential to maximise extraction yield. To carry out the optimization and to measure the interaction between factors the response surface methodology (RSM) experimental design is applied in this study [17]. RSM is a powerful tool to evaluate multiple factors and their interactions simultaneously, being a set of sophisticated mathematical and statistical techniques, helpful in developing, improving and optimizing processes by establishing empirical models [12]. RSM is based on fitting a polynomial equation to experimental data, which, in turn, must describe the behaviour of the data set and make statistical predictions [18].

In addition to efficient extraction, performing a purification step before analysis is necessary to eliminate possible matrix effects of biscuits and sponge cakes that can lead to erroneous results or further damage to equipment. For this purpose, solid-phase extraction (SPE) with different types of commercial sorbents such as diatomaceous earth [19], Chem Elute column [20], Clean Screen ® DAU [21], Oasis ® MCX and Oasis ® HLB [22], has been used in OAs. The Oasis ® HLB is a universal polymeric adsorbent with hydrophilic-lipophilic balance developed to extract a wide range of acidic, basic and neutral compounds from various matrices using a simple and generic protocol. In addition, more and more efforts are being made to synthesise and produce sorbent materials instead of using commercial ones because of their lower cost and greater control of the desired textural characteristics. Due to the chemical nature of OAs and in consideration of their interactions with the -OH groups of other mesostructured silica based materials [7], the application of unfunctionalised SBA-15 (Santa Barbara Amorphous-15) and HMS (hexagonal mesoporous silica) materials in the purification step can be evaluated, avoiding functionalisation steps with the consequent cost of reagents and time. This materials are increasingly being used as sorbent materials in other compounds due to the advantages in terms of their ordered structure with controlled pore size, large surface area and pore volume [23]–[26].

The aim of this work was to develop and validate an efficient, fast and environmentally friendly methodology for quantification of the six main OAs in samples of biscuits and sponge cake using a UAE-SPE sample preparation protocol previous to the analysis by liquid chromatography coupled to a triple quadrupole tandem mass detector (HPLC-MS/MS).

2. Materials and methods

2.1 Reagents and materials

Supel ™-Select HLB SPE cartridges (30 mg/1 mL) were purchased in Sigma-Aldrich (St. Louis, MO, USA), tetraethylorthosilicate 98% (TEOS, MW= 208.33 g/mol CAS 78-10-4), poly(ethylene glycol)-block-poly (propylene glycol)-block-poly (ethylene glycol) (EO20PO70EO20, Pluronic® 123, P123, MW= 5800 g/mol) and dodecylamine (DDA). 98% (M = 185.36) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid 37% was acquired from Scharlab (Barcelona, Spain). Standards of morphine, codeine, thebaine and oripavine were received from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine, morphine-d_3 and codeine-d_3 (internal standards, IS) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1000 µg/mL in methanol and working standard solutions were prepared at 1 µg/mL in water/acetonitrile 90/10 (v/v) with 0.1% formic acid. All of these were stored in darkness at -20 ˚C. Acetic acid and ammonia 32% (w/w) were purchased from Scharlab (Barcelona, Spain). Acetonitrile and methanol used were HPLC-MS quality and were purchased from Scharlab (Barcelona, Spain). Polyethylene frits (0.20 µm) and nylon filter membranes (0.45 µm) were obtained from Scharlab (Barcelona, Spain). Formic acid 99% Optima™ LC-MS grade was from Fisher Chemical (Madrid, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

SBA-15 and HMS materials were prepared and characterized according to previous works [27], [28] (see Supplementary Information S1, for preparation details). The surface area of SBA-15 and HMS was 702 and 778 m^2/g, the pore volume was 0.74 and 0.99 cm^3/g and the pore distribution was 56.2 and 26 Å, respectively.

2.2 Commercial samples for analysis

Five different brands of biscuits (B-1 to B-5) and two sponge cakes (SC-1 and SC-2) with poppy seeds were purchased from supermarkets in Madrid and Valencia (Spain). The poppy seed content of these products was in the range of 4 to 13%. To obtain a representative and homogeneous sample with small particle size, each packet was ground with a manual mortar so as not to grind the poppy seeds and reduce OAs levels. To facilitate grinding, sponge cake samples were frozen with liquid nitrogen and all the samples were passed through a 18 mesh sieve (1 mm).

2.3 Optimised analysis methodology for quantification of opium alkaloids in biscuits and sponge cakes

The analysis methodology developed was based, as shown in Figure 1, on a first sample pre-treatment step, a subsequent UAE, purification by SPE and analysis by HPLC-MS/MS.

Then, the ground, sieved and homogenised sample was exposed to acoustic waves under controlled UAE conditions (according to the experimental design) by Bandelin Sonopuls 529 (Amplichron ®-System, Bandelin, Berlin, Germany) with MS 73 probe with a diameter of 13 mm. Therefore, for extraction by UAE, 2.5 g of sample were mixed with 10 mL of methanol with 1% formic acid for 30 s in the vortex (Rx^3 Velp Scientifica, Usmate, MB, Italy). Then, the mixture was exposed to 75% amplitude for 5 min in pulse mode in a 50 mL falcon tube immersed in a glass with crushed ice to prevent heating. After the UAE, the mixture was centrifuged at 6000 rpm (3992 rcf) for 5 min to recover the supernatant (ROTOFIX 32A 204 Hettich, Tuttlingen, Germany). Then, the SPE procedure was performed using 50 mg of SBA-15. For this purpose, a conditioning stage was first carried out with 2 mL of methanol/water 0.1% acetic acid, loading with 1 mL of the extract diluted 1/10 with methanol/water 1% acetic acid, drying under vacuum for 3 min and elution with 2 mL of methanol with 10% ammonia. Subsequently evaporated on a vacuum line to dryness, reconstituted with 50 µL of a 0.1 µg/mL dilution of each of the internal standards (morphine-d3 and codeine-d3) and 150 µL of water/AcN (90/10, v/v) with 0.1% formic acid and for subsequent analysis by HPLC-MS/MS.

For the chromatographic analysis of OAs in biscuits and sponge cakes, a Varian 1200/1200 LC (Varian Ibérica, Madrid, España) with a ProStar 410 autosampler (100 µL loop) coupled to a triple quadrupole tandem mass spectrometer detector (1200 L TQ) with electrospray ionisation (ESI) ion source was used (data acquisition system was MS Workstation Varian version 6.8). Chromatographic separation was performed like our previous work [8] using a C_{18} Kromaphase 100 column (150 × 2.0 mm, 3.5 µm particle size, Scharlab, Barcelona, Spain) at 30 ˚C. The injection volume was 10 µL (partial injection) and the flow rate was set at 0.25 mL/min in a gradient elution of water (A) and acetonitrile (B), both with 0.1% of formic acid as follows: 90-30% A (0-6min), 30-90% A (6-9min) and 90% A (9-11min) for column re-equilibration. Mass spectrometry acquisition was with electrospray ionization in positive mode (ESI+) with MRM mode like our previous work [8]. N_2 was used as drying (at 350 ˚C and 22 psi) and nebulizer gas (at 58 psi). The capillary voltage was 5000 V and shield 600 V. Argon at 1.90 mTorr was collision gas and detector voltage 1480 V. Mass peak width Q_1 2.5, mass speak width Q_3 2.5 and scan width in MRM 0.5 s. Compounds were monitored at cone voltage at 72 V. Table S1 shows the optimal mass spectrum parameters.

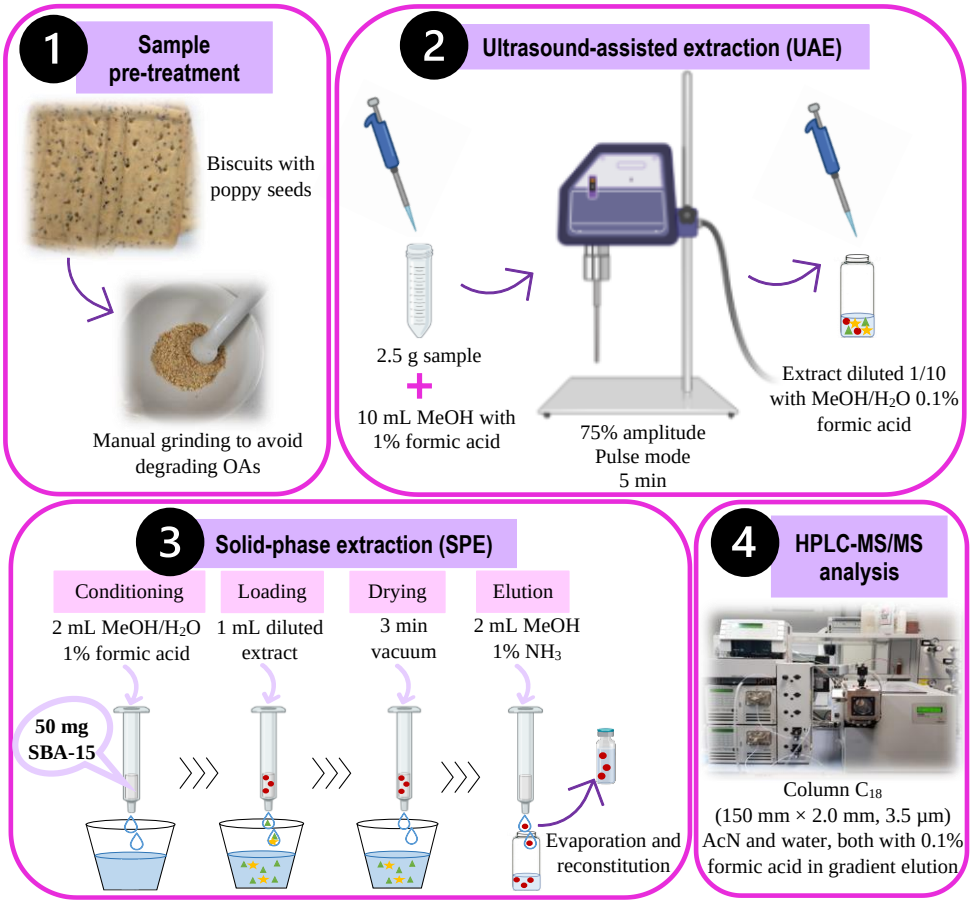


Fig. 1. Diagram of the proposed UAE-SPE methodology to quantify OAs in biscuits and sponge cakes.

2.4 Optimisation of the extraction and purification methodology of opium alkaloids from biscuits and sponge cakes.

For the optimisation of the sample preparation, first, the parameters of the SPE purification step were optimised to determine the recovery values of this step. For this purpose, the two mesostructured silicas were evaluated with 80 µg/L solutions of each of the OAs and the one that showed the highest adsorption of the opioids was selected. To calculate the adsorptions of each material, the solvent remaining after the loading step in the cartridge was analysed by HPLC-MS/MS. Subsequently, the elution step of the analytes was optimised. After optimisation of the SPE with standards, the sample clean-

up step was optimised. For this, a sample extract (sample B-1) was spiked just before the purification process with a known concentration (80 µg/L) to calculate the recovery values of each of the analytes in this step. To spike the extracts, surface contamination of poppy seeds was simulated by adding the corresponding aliquot, homogenizing it with the vortex for 10 s and allowing it to stand at room temperature for 30 min for evaporation of the solvent (MeOH). After a small re-optimisation with the sample extract, the sample extraction was optimised with UAE. For this purpose, sample B-1 was spiked with 3 mg/kg of each of the analytes to calculate the recovery values for the complete proposed UAE-SPE methodology.

2.4.1 Sorbent material selection and preliminary optimisation of the SPE with standards

To select the best sorbent to be used in this work in the purification stage, the procedure was optimised with standards to compare and choose the most efficient one.

First, the adsorption capacity and retention values of the commercial cartridge (HLB) under study were evaluated. For this, the method recommended by the manufacturer was followed, which was based on conditioning with methanol followed by water, loading of the dissolved analytes in an aqueous medium and subsequent elution in AcN/MeOH, 50/50 (v/v). Subsequently, the conditions were optimised with standards. In addition, the necessary conditions for the SBA-15 and HMS silicas were optimised. To this, their adsorption capacity was evaluated in the loading stage with the extraction solvents used in previous work [7], [8]. Therefore, two loading solvents were evaluated (methanol/water (50/50, v/v) and methanol with 0.1% acetic acid). Firstly, the elution solvent was ethyl acetate/methanol (50/50, v/v) with 10% ammonia, similar to previous work with mesoporous silica [7] but with a less volatile organic solvent to avoid excessive evaporation during the SPE process. Once the sorbent material and the loading solvent had been selected, the elution solvent was optimised. To do this, different solvent mixtures were evaluated (ethyl acetate/methanol (50/50, v/v) with 10% ammonia, methanol, acetonitrile, methanol and acetonitrile with 0.1% ammonia). In addition, the ammonia proportion (0.1, 1 and 10%) and volume of eluent (1 or 2 mL) were also studied. All

studies were performed in triplicate with a first conditioning step with 2 mL of the same solvent as the loading, loading with 2 mL of an 80 µg/L standard solution of each of the OAs and a vacuum drying step for 3 min prior to elution. To do this, a Supelco Visiprep SPE vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 7.6 psi. 3 mL polypropylene empty cartridges (length of 65 mm and i.d. 10 mm) were packed with 50 mg of each silica type and plugged with polyethylene frits at both ends. In addition, a pore size nylon filter membrane (0.45 µm) was inserted at the bottom of the material bed to prevent the material lost during sample loading.

2.4.2 Purification of sample extracts with selected cartridge

Once the SPE procedure was optimised with standards, the recovery of the purification step was determined with 2 mL of the sample extract obtained after the UAE and spiked with a high validation level of OA at 3 mg/kg (double the established maximum limit) [11]. However, due to the variation of the pH of the extract with the sample and the need to add a proportion of water to the extract to improve the retention of the analytes in the cartridges, a 1/10 dilution of the extract with 50% methanol/water with 1% acetic acid was performed and the loading volume was modified (1 mL).

2.4.3 Experimental design and statistical analysis of UAE

For both screening and optimisation of the UAE parameters, different experimental design methodologies were employed sequentially. First, a five-factor two-level full factorial design (2^5) was applied to determine the effect of the volume of solvent used (A), the proportion of acid added (B), the extraction time (C), the amplitude of sonication (D) and the mode of sonication (E) on the recovery of opium alkaloids. Each factor was examined at the two most promising levels, as shown in Table S2. The election of the levels of each independent variable was based on preliminary experiments and previous related research. Following the results obtained, the critical factors influencing both pulsed and continuous extraction modes were confirmed. Solvent volume and extraction time were the two most important independent factors selected based on the preliminary experiments, the optimal levels of which were determined for each extraction mode by

RSM. The experimental design, the analysis of the results and the predicted responses were carried out with Statgraphics Centurion software (version 19.3.03).

2.5 Method validation

Since there is currently no official regulation on analytical performance requirements for OAs in food or feed, the method validation was done in terms of linearity, method detection and quantification limits (MDL, MQL), matrix effect (ME), accuracy, precision and selectivity, following the criteria described in the SANTE/12682/2019 document, in regulation EC No 401/2006, and in the Q2(R1) ICH guidelines (Internation Council for Harmonisation, 2005), [29]–[31]. Moreover, the only reference materials to our knowledge are biological samples and only for morphine and codeine. For this reason, the validation was carry out with a spiked sample (S-1). Accordingly, linearity was assessed with matrix-matched calibration curves prepared on three consecutive days. All these curves were prepared for sample B-1 at six known concentration levels within the linear range evaluated. For this purpose, the sample extracts obtained after the UAE-SPE procedure were spiked with an aliquot of a standard solution containing the target alkaloids according to the desired concentration level of the calibration curve. In addition, quantification of morphine and codeine by means of isotope labelled IS correction was carried out. To do this, 50 µL of 0.1 µg/mL of each IS were added to each point of the matrix-matched calibration curves. The criteria for good linearity involve values ≤ ±20% for the deviation of the back-calculated concentrations of the calibration standards from the true concentrations [29], [30]. Matrix effects were determined by comparing the slopes of the calibration equations obtained from both matrix-matched and solvent-based calibration curves (both expressed in the same units µg/mL), calculating (the ratio slope matrix-matched/slope solvent-based -1) × 100 for each analyte. The ME is lower when closer to 0%, and according to the guideless the ME is negligible when is lower than ±20%. Positive values greater than 20% indicate signal enhancement, and negative values indicate signal suppression. However, when the signal suppression or enhancement is higher than this margin of 20%, matrix effects must be considered in calibration. The sensitivity of the method for each sample was determined through the MDLs and MQLs

of the OAs from the analysis of the lowest concentration analysed (0.01 or 0.001 µg/mL), which were estimated as the minimum concentration yielding a signal-to-noise ratio (S/N) of 3 or 10, respectively [31]. The recovery assays were assessed by comparing the areas obtained for samples spiked (n = 6) with a known concentration of analytes and subjected to the UAE-SPE procedure with those areas obtained for simulated samples (samples spiked at the same concentration but at the end of the procedure prior to their chromatographic analysis). The recovery assays were performed by spiking the samples at three concentration levels of 3 mg/kg (high value), 1.5 mg/kg (medium value) and 0.75 mg/kg (low value), according to the Regulation (EU) 2021/2142 was published in December 2021, establishing maximum morphine equivalent limits in bakery products at 1.5 mg/kg [11]. The recovery values should be between 70 and 120%. On the other hand, the method precision was evaluated in terms of repeatability and reproducibility, using the same validation levels (low and high) as for the accuracy. For repeatability (expressed as RSD%), a sample spiked with the OAs at the corresponding validation level was consecutively carried out six times (n = 6) on the same day. The reproducibility (also expressed as RSD %) was calculated by the analysis of three replicates of a sample (spiked with the analytes at the corresponding validation level), which were carried out in triplicate over three different days (n = 9). According to the validation guidelines, the RSD values for these precision parameters should be ≤20%. The selectivity of the method was determined by comparing the spectra of the different analytes obtained from standard solutions with the spectra obtained in the samples. It was considered satisfactory when the variation in the spectra was few than ±30 % and the retention time of the target analytes was within the interval of ±2.5 % [29].

3. Results and discussion

3.1 Optimisation of the extraction and purification methodology

3.1.1 Optimisation of the purification by SPE

3.1.1.1 Sorbent material selection and preliminary optimisation of the SPE with standards

To select the best material in this work to use in the purification step, the adsorption capacity (%) and recovery values (%) of the commercial cartridge (Supel ™-Select HLB SPE) and two synthesised silicas (SBA-15 and HMS) were evaluated.

Firstly, the commercial HLB cartridge was evaluated for the purification step with standard solutions of each of the opium alkaloids at a concentration of 30 µg/mL. For this, the method recommended by the manufacturer was followed. The results obtained were not favourable as the recoveries obtained did not exceed 70% for any of the analytes, so the conditions were optimised. To evaluate both loading and elution, adsorptions (%) and recoveries (%) were calculated by analysing the solvent after passing through the cartridge in each of the stages. In the case of adsorptions (%), variable results were obtained with water, especially in the case of morphine, which showed an RSD of 32%. Therefore, it was tried to add small percentages of organic solvent, methanol and acetonitrile (both at 1 and 10%), but the adsorptions of morphine, codeine and oripavine were lower than 30% in all cases. Subsequently, acidification with 1% formic acid was tested, and although all analytes showed good adsorption, morphine only showed 19%. The next step was to test a basic medium, which, according to the manufacturer's recommended method, for basic polar compounds that are difficult to retain, adjusting the pH two units above the pKa of that analyte would aid retention on the cartridge. Therefore, to improve the adsorption of morphine, which was the most difficult analyte to retain, a 25 mM ammonium formate buffer adjusted with ammonia at pH 12 was tested, since morphine shows a pKa around 9. However, only 50% adsorption was achieved in the case of morphine and oripavine. To calculate the recovery values, the adsorption solvent was maintained with water, and the elution solvent was modified. Different conditions were tested with 100% methanol, acidified at 1% with formic acid and with different percentages of acetonitrile (1, 10, 20 and 30%). And with all these conditions good recoveries were obtained (around 85%). However, the recoveries were not satisfactory in the case of morphine, the highest being 60 ±20% with MeOH with 1% AcN. Therefore, the retention of morphine in the cartridge was not reproducible and in some replicates was very low due to its high polarity, so the use of this cartridge in the SPE stage was discarded.

Secondly, the purification step was optimized with the synthesized SBA-15 and HMS cartridges. To do this, the loading step was with the extraction solvents used in previous work [7], [8]. Therefore, 2 mL of 80 µg/L of each of the opium alkaloids were tested in two different solvents (methanol/water (50/50, v/v) and methanol with 0.1% acetic acid) with 50 mg of each material. The elution solvent for this assay was 2 mL of ethyl acetate/methanol (50/50, v/v) with 10% ammonia, similar to previous work with mesoporous silica material [7] but with a less volatile organic solvent to avoid excessive evaporation during the SPE process. The results obtained from this assay are shown in Figure 2. Regarding the adsorption values (%), those of the SBA-15 were higher than those of the HMS with the two loading solvents, being the highest with methanol 0.1% formic acid with adsorptions between 93 and 100% of all the analytes. Regarding the recovery values obtained, they were low in all cases, especially for morphine, where the maximum was 39 % and oripavine 35 %. Therefore, SBA-15 was selected over HMS because of its higher adsorption capacity for opium alkaloids and methanol with 0.1% acetic acid as a solvent in the loading stage. Once the sorbent material and the loading solvent had been selected, the elution solvent was optimised to improve the low values obtained in the previous assay. To do this, different solvent mixtures were evaluated (methanol, acetonitrile, methanol and acetonitrile with 0.1% ammonia). As shown in Figure S1, the highest recovery values were obtained by eluting with methanol with 0.1% ammonia, ranging from 66 to 89% for all analytes. Once the elution solvent was selected, different ammonia proportions to elution solvent (0.1, 1 and 10%) were tested. As shown in Figure S2, the recovery values were higher with 1% ammonia, showing values between 91 and 95% for all analytes. At 10%, some values decreased, especially for oripavine, which dropped to 69%. In addition, the elution solvent volume was decreased from 2 to 1 mL, but lower recovery values of 30-50% were obtained for all analytes.

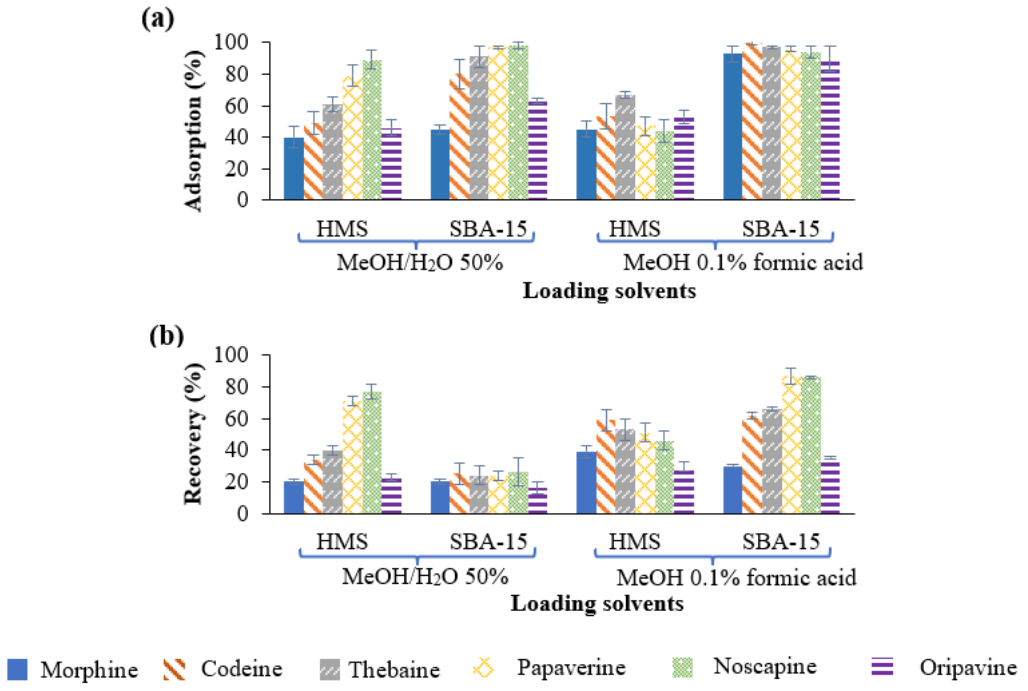


Fig. 2. Comparison between adsorption (a) and recovery (b) values (%) obtained with 50 mg of HMS y SBA-15 materials with 2 mL of 30 µg/L of opium alkaloids in two different loading solvents (MeOH/H_2O at 50% and MeOH with 0.1% formic acid) and using 2 mL of ethyl acetate/methanol at 50% with 10% ammonia for elution.

3.1.1.2 Purification of sample extract with SBA-15 material

Once the SPE procedure was optimised with SBA-15 material with standards, the recovery of the purification step was determined with 2 mL of the sample extract obtained after the UAE and spiked with the high validation level (80 µg/L) of each OA.

The recovery results obtained with the sample extract were significantly lower than those obtained with standards, being around 20 % for papaverine and noscapine, 40 % for morphine and oripavine and 60 % for codeine and thebaine. After these low results, the solvent was analysed after the loading step in the cartridge to identify where the problem was, in the loading or in the elution. After calculating the adsorptions, it was found that the problem was at this stage, as the adsorptions were like the previous recovery values. This could be due to the change in pH of the methanol solution 0.1% acetic acid in the sample, so it was decided to acidify the extract with a higher proportion of acetic acid

(1%). After further acidification, higher recovery values were obtained (for morphine and oripavine close to 90%, for codeine and thebaine close to 80% and papaverine and noscapine around 50%). These results showed that pH adjustment is a key aspect for OAs to interact effectively with the SBA-15 material. Furthermore, to improve the interaction of papaverine and noscapine with the material, a proportion of water was added to the extract as these two compounds show the lowest water solubility (0.013 and 0.18 g/L, respectively), in contrast to morphine and oripavine, which are the most water soluble (10.20 and 0.87 g/L, respectively) [1]. Therefore, the extract was diluted 1/10 with a 50% methanol/water mixture with 1% formic acid, and the recovery values obtained were 74% for morphine, 87% for codeine, 97% for thebaine, 99% for papaverine, 96% for noscapine and 80% for oripavine.

Subsequently, to ensure the correct loading of the analytes in the material in the case of obtaining a more concentrated extract with the UAE (instead of 10 mL, with 5 mL), the same test was performed at a higher concentration (150 µg/L of each OA). The recovery results obtained were lower (around 60% for morphine, codeine and oripavine and around 75% for thebaine, papaverine and noscapine), and it was determined that the adsorptions were like these recovery results, so it was considered that the 50 mg of material used might be saturating. To avoid increasing the amount of material, it was decided to pass 1 mL of the diluted extract through the cartridge instead of 2 mL and the results obtained were again satisfactory (75% for morphine and oripavine, 86% codeine, 94% for thebaine, 105% noscapine and 107% papaverine).

3.1.2 Optimisation of UAE

3.1.2.1 Evaluation of UAE variables influencing extraction efficiency, their main effects and statistical analysis.

A screening of the variables was done to estimate the influence of the different independent variables and to identify the factors that have significant effects on the dependent variables. To do this, the experimental factorial design methodology was used, specifically a screening design. Therefore, five possible independent variables that affect the process of UAE: solvent volume (A), acid proportion (B), extraction time (C),

sonication amplitude (D) and sonication mode (E) were investigated using a five-factor two-level full factorial design (2^5) and the dependent variables were the recovery values of each of the analytes: morphine (Y_1), codeine (Y_2), thebaine (Y_3), papaverine (Y_4), noscapine (Y_5) and oripavine (Y_6). Each factor was examined at the two most promising levels based on preliminary experiments and previous related research (Table S2).

The results obtained from the experimental design are shown in Table S3. It should be noted that for each condition, extraction was performed in triplicate, and the responses presented in Table S3 are the mean values of the three replicates ± standard deviation (SD). Under the set conditions, the experimental values for each of the responses ranged from 23 ± 1 to 75 ± 5% for morphine, 26 ± 1 to 88 ± 1% for codeine, 46 ± 2 to 97 ± 2% for thebaine, 50 ± 1 to 101 ± 8% for papaverine, 56 ± 1 to 104 ± 2% for noscapine and 26 ± 1 to 82 ± 2% for oripavine.

To determine the effects of each variable and the possible interactions between them, the Pareto Chart was plotted, as it shows the absolute values of the standardised effects from the largest effect to the smallest effect and allows to determine whether a factor has a positive or negative effect on the response. In addition, the chart also plots a reference line indicating which effects are statistically significant. Therefore, as shown in Figure 3, it was confirmed that all independent variables studied individually showed a positive effect on all analytes, that is the recovery values (%) of all analytes increased with increasing solvent volume, acid ratio, extraction time, sonication amplitude and, in addition, the pulsed mode also showed a higher performance than the continuous mode.

All variables showed statistically significant differences individually for all analytes, except for sonication mode, which only showed statistically significant differences for morphine and acid proportion for codeine and papaverine. Furthermore, the Pareto Charts (Figure 3) revealed that extraction time (C) was the parameter that most influenced the response of all analytes, showing a much higher standardised effect than the rest of the factors.

The fact that an increase in the solvent has a positive effect on the extraction yield may be due to the fact that a small solvent-solid ratio may lead to an increase in the viscosity of the solvent, decreasing the diffusion of the analytes through the extraction

medium, or that saturation may be reached early, and the extraction process cannot be completed [32]. This factor was the second most influential in the extraction of all OAs except morphine and codeine which was the third and in the case of oripavine showed non-significant differences.

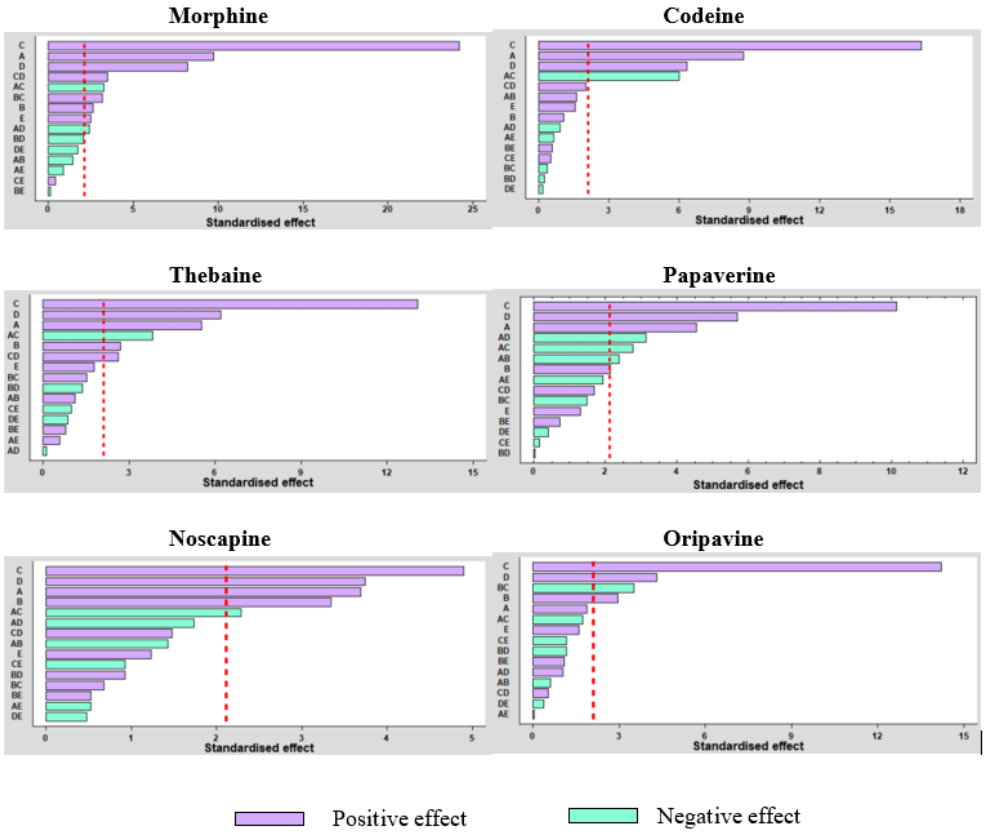


Fig. 3. Response from full factorial design 2^5 by the Pareto Chart of the standardised effect of each of the responses (recovery of each analyte), showing the five factors: (A) solvent volume (mL), (B) acid proportion (%), (C) extraction time (min), (D) sonication amplitude (%), (E) sonication mode to UAE optimisation.

A higher acid proportion having a positive effect on the extraction may be since a more acidic pH of the medium increases the solubility of the analytes and thus increases the extraction yield. A longer extraction time may increase the extraction yield, but in the case of the use of UAE it may exhibit the opposite effect when the extraction time is longer than optimal due to the possibility of degradation due to heat generation, which

may lead to degradation of the compounds [12]. In sonication mode, there were no significant differences except for morphine where pulsed mode showed a higher performance. Other studies claim the advantages of pulsed mode over continuous mode due to lower energy input, better temperature control, and reduced ultrasound probe tip erosion [33].

Regarding the interactions between the variables that showed statistically significant differences, the interactions of solvent volume (A) with acid proportion (AB) in papaverine, with amplitude (AD) in morphine and papaverine and with time (AC) in all the analytes except oripavine were the most significant. Other statistically significant interactions shown by time (C) were with acid proportion (BC) in morphine and oripavine and with amplitude (CD) in morphine and thebaine.

3.1.2.2 Optimization of UAE parameters based on an RSM approach

After performing the statistical analysis of the full factorial design of the 5 variables at 2 levels, a final optimisation of the solvent volume (A) and extraction time (C) was considered necessary. These variables were the most influential in the efficiency of the extraction, and the aim of the present work was to try to reduce these two variables as much as possible to achieve the most environmentally friendly and fastest possible methodology. Therefore, the critical variables (A, C) were studied in detail (A: 5, 7.5 and 10 mL, C: 1, 3 and 5 min) both in continuous and pulsed mode. The acid proportion and amplitude of the sonication were set at their previously determined optimal values, acid proportion 1% and amplitude 75%, as these were the levels that showed a significantly higher extraction performance. The final optimisation procedure was based on an RSM approach, and the experimental design, as well as the results obtained for the continuous and pulsed modes are presented in Tables S4 and S5 of the Supplementary Material, respectively.

The statistical parameters obtained from the statistical analysis (ANOVA) (p-values, R^2, R^2adj., R^2pred.) were compiled in Table S6 and indicated that the resulting quadratic models have very high predictability and could be used in RSM to optimise the extraction procedure by maximising the recovery values of each of the analytes. The statistical

analysis also confirmed the significant terms of the response surface quadratic models obtained ($p < 0.05$). The variables with the highest impact on extraction performance using continuous ultrasound-assisted extraction (CUAE) were the linear terms of solvent volume (A) and extraction time (C), followed by the interaction term between the two critical variables (AC) and the quadratic term of extraction time (C^2). In the case of pulsed ultrasound-assisted extraction (PUAE), the results indicated that the first order linear term of the extraction time (C) was the most significant, followed by its quadratic term (C^2) and the linear term of the extraction volume (A) and the interaction between the two variables (AC). Once again, extraction time proved to be the most critical factor affecting the recovery of all analytes. In addition, these results were introduced into a multiple regression analysis to generate the mathematical models shown in Table S6, which adequately describe the empirical relationship between the two variables examined and the responses of each of the analytes with both extraction modes (CUAE and PUAE). Furthermore, to visualise the relationship between the independent and dependent variables, response surface and contour plots were generated based on the acquired polynomial equations, as shown in Figure S3. In the case of CUAE, the recoveries increased with increasing solvent volume in the same way as they increased with increasing extraction time. Furthermore, it is evident that the mutual interaction between solvent volume and extraction time was very significant due to the elliptical contour plot obtained for most of the analytes. In particular, the different shapes of the contour plots indicate whether the mutual interactions between the studied variables are significant [12]. In the case of PUAE, increasing the solvent volume showed few increases in yield and a more significant increase in extraction time (Figure S3).

Numerical optimisations were performed to determine the optimal level of each independent variable. For some analytes, the optimal value for the extraction volume variable was closer to 5 mL, while for codeine, thebaine and papaverine the optimal level was 10 mL, the same as for the extraction time, where some analytes needed 5 min to obtain the maximum recovery (morphine, thebaine, papaverine and noscapine). Therefore, the optimal conditions selected for optimal recovery of all OAs were 10 mL of solvent for 5 min of extraction.

To verify the reliability of the response surface models for the quantitative predictions, the results experimentally obtained were compared to the results predicted with the mathematical equations with the estimated optimal conditions. As shown in Table S7, the experimental results were very similar to the predicted values. Thus, the effectiveness and validity of the response surface models to reflect the response values and to be able to determine the best extraction conditions are confirmed.

3.2 Method validation

The results of the validation of the proposed UAE-SPE-HPLC-MS/MS method for the quantification of six AOs in biscuit and cake samples are shown in Table 1. The calibration lines were obtained with R^2 between 0.998 and 0.999 for all analytes, and the deviation of the back-calculated concentrations of the calibration standards with respect to the real concentrations in the matrix calibration lines was between -12 and -17 for all analytes, demonstrating the good linearity of the method as they are ≤ ±20% values according to the reference document [29]. In addition, the deviation of the slopes of the calibration lines for different days (n = 3) was calculated to ensure reproducibility, obtaining RSDs between 3 and 16%.

Regarding the MDL and MQL values obtained, they were low for all analytes: 1 and 2 μg/kg for papaverine, 1 and 3 μg/kg for noscapine, 1 and 4 μg/kg for codeine and thebaine, 3 and 10 μg/kg for morphine and 6 and 20 μg/kg for oripavine, respectively.

On the other hand, the ME was calculated for both the complete methodology and the methodology without the purification step, to determine the need to introduce this step in the methodology. For this purpose, the slopes of both matrix and solvent calibration curves were compared. As shown in Table 1, the ME of the complete methodology was negligible, as all values were within + / - 20%. However, the ME of the methodology without the purification step showed increased signal enhancement values for papaverine and noscapine (25 and 43%). On the other hand, for oripavine, a ME of decreased signal (-51%) was shown and its MDL and MQL were also affected, being worse. This means that the developed purification procedure was able to eliminate all possible matrix effects

for all six target analytes. As no matrix effect is observed, solvent calibration lines could be used in the analysis of samples to quantify which would simplify the analysis.

Accuracy and precision were evaluated at three different concentration levels, low (0.75 mg/kg), medium (1.5 mg/kg) and high (3 mg/kg), showing adequate recovery values, between 77 and 103% (Table 1). On the other hand, as shown in Table 1, satisfactory results were obtained for intra-day and inter-day precision at the three concentration levels, since the RSD values were lower than 20%, the lowest value is 11%. Furthermore, as shown in Figure S4, a good selectivity of the method was obtained. The chromatograms of the extracted ions obtained for each of the OAs in a standard solution were compared with the sample extracts. It was found that the variation of the t_R was ≤0.1 min, and the ion ratios of the sample extracts were within ±30% (relative abundance) of the mean of the standards for each analyte.

Table 1. Validation parameters of the UAE-SPE-HPLC-MS/MS method for the quantification of six OAs in biscuits and sponge cakes.

Analytes	Linear range (mg/L)	Matrix-matched calibration (R^2) [a]	MDL (µg/kg) [b]	MQL (µg/kg) [c]	ME [d]	Accuracy [e]		Precision	
						Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-Day Precision (RSD %)	Inter-Day Precision (RSD %)
Morphine	0.005-1	$y = 6.7\times10^{7}x + 3.3\times10^{5}$ (0.999)	3	10	9	77 ± 2^{L} 77 ± 4^{M} 82 ± 5^{H}	79 ± 4	2^{L} 5^{M} 6^{H}	3^{L} 5^{M} 7^{H}
Codeine	0.001-1	$y = 9.9\times10^{7}x + 7.1\times10^{5}$ (0.998)	1	4	15	90 ± 4^{L} 88 ± 9^{M} 93 ± 4^{H}	90 ± 6	4^{L} 10^{M} 4^{H}	5^{L} 11^{M} 5^{H}
Thebaine	0.001-1	$y = 9.3\times10^{8}x + 7.1\times10^{6}$ (0.998)	1	4	5	97 ± 6^{L} 97 ± 5^{M} 100 ± 3^{H}	98 ± 5	6^{L} 5^{M} 3^{H}	8^{L} 7^{M} 4^{H}
Papaverine	0.001-1	$y = 1.4\times10^{9}x + 1.6\times10^{7}$ (0.998)	1	2	16	101 ± 2^{L} 103 ± 3^{M} 100 ± 4^{H}	101 ± 3	2^{L} 2^{M} 4^{H}	3^{L} 3^{M} 5^{H}
Noscapine	0.001-1	$y = 1.5\times10^{9}x + 2.2\times10^{7}$ (0.998)	1	3	16	97 ± 2^{L} 98 ± 3^{M} 99 ± 2^{H}	98 ± 2	2^{L} 3^{M} 2^{H}	4^{L} 5^{M} 3^{H}
Oripavine	0.005-1	$y = 9.6\times10^{7}x + 1.0\times10^{6}$ (0.998)	6	20	4	78 ± 7^{L} 86 ± 4^{M} 95 ± 4^{H}	86 ± 5	9^{L} 5^{M} 4^{H}	9^{L} 6^{M} 7^{H}

The linear range expressed in µg/kg is 20-4000 in the case of morphine and oripavine and 4-4000 in codeine, thebaine, papaverine and noscapine [a] The calibration line is in the units: µg/mL; [b] MDL: method detection limit; [c] MQL: method quantification limit; [d] ME: matrix effect (dividing the purified matrix slope by the solvent slope); [e] Accuracy and precision were obtained by spiking samples at three concentration levels: low (L, 0.75 mg/kg), medium (M, 1.5 mg/kg) and high (H, 3 mg/kg).

3.3 Comparison of the proposed UAE-SPE method with previously methods for OAs

The most significant advantages of the proposed method over previously published methods were the lower solvent volume and extraction time optimised. This allowed the development of a faster, cheaper and, above all, more environmentally friendly methodology [34]. In this method, only 10 mL and 5 min are enough to achieve complete extraction. However, in previously published works, when not using the UAE and opting for simple SLE, up to 100 mL of AcN/water/formic acid, 80/19/1, v/v/v/v and 1 h of extraction are used for each sample, as in the work of López et al. [4]. Another example with lower solvent volume but with the same extraction time is the work of Sproll et al. [5] which used 30 mL of methanol 0.1% acetic acid for 1 h of extraction or our previous work on seeds with 30 mL of MeOH/water, 50/50 (v/v) for 1 h [7] or that on breadsticks and sliced bread with 20 mL of MeOH 0.1% acetic acid for 1 h [8].

In addition to the advantages of efficient extraction, the advantages of the purification step are also important. As has been shown in this work, these samples have a considerable matrix effect that can lead to an increase in the signal of two analytes (papaverine and noscapine) and a decrease in another (oripavine). However, the purification step was able to completely eliminate all matrix effects using a similar or even lower amount of sorbent than in other studies (50 mg) and lower solvent volume in the process (1mL to load and 2 mL to elution) [7], [22], [35]. Most of the previously published studies have not performed this purification step and have therefore obtained really matrix effects such as López et al. [4] with +30% or have not even been evaluated [3], [5]. In addition, there are some works that even with the purification step, considerable matrix effects are obtained, usually signal decrease effects such as that of Tang et al. [36] with -60% in some analytes and in Casado-Hidalgo et al. [7] with -69% in oripavine. Other works show a mixture of signal increase/decrease, such as the work of Guo et al. [22] where some analytes have a ME of -39% and others of +101%.

It is also interesting to note the economic advantages of using a synthesised material rather than commercial materials that are often considerably more expensive [22]. In addition, the synthesis of SBA-15 is very simple and uses reagents that are cheap and give a high synthesis yield in only 3 days. This is very different from other syntheses that are

more complex, longer and more costly after functionalisation steps with specific organic molecules [35], [36]. Therefore, the methodology presented in this work is simpler, faster, cheaper and more environmentally friendly than any of the previously published methodologies to quantify OAs in food samples.

3.4 Application of the proposed method to commercial samples of biscuits and sponge cake

As the validation of all parameters was successful (section 3.2) according to the reference documents, [29]–[31] the proposed methodology was applied to the analysis of seven commercial samples, five of them biscuits and two sponge cakes.

The solvent calibration line could be used for quantification, which would simplify and reduce the cost of routine analysis of this type of sample. This is because the SPE purification step is able to remove all matrix effects and the recovery values are within the desired range. However, to confirm that similar values were obtained, the signals were corrected with the recovery values obtained at the low level of the validation (Table 1). Subsequently, the signal was corrected with the internal standards and interpolated in the calibration line of the matrix of internal standards. Only the two internal standards of morphine-d3 and codeine-d3 were purchased as these are the compounds that are legislated [11]. So, to signal correct morphine-d3 was used for morphine and codeine-d3 was used for codeine and thebaine due to their similar characteristics like pKa and water solubility [1].

The samples analysed showed low levels of opium alkaloid contamination as shown in Table 2. Especially, oripavine could only be detected in sample SC-1, noscapine was detected in all samples except B-4 and SC-2 and papaverine was detected in all samples, but below the MQL in all cases. Codeine and thebaine were detected in SC-1 and were quantified in sample SC-2, which showed 0.004 ± 0.02 mg/kg of codeine and 0.015 ± 0.003 mg/kg of thebaine. On the other hand, morphine concentrations were found in all samples except B-2 and B-4. However, these results were low and in all cases below the legislated maximum limit for bakery products (1.5 mg/kg of morphine equivalents) [11]. In sample B-1 it was 0.013 ± 0.006 mg/kg, in B-3 0.010 ± 0.008 mg/kg, in B-5 0.010 ±

0.002 mg/kg, in SC-1 0.03 ± 0.01 mg/kg and in SC-2 0.153 ± 0.009 mg/kg. Therefore, the most contaminated sample was SC-2, which coincides with a higher proportion of seeds (13%) than the rest of the samples.

These low concentrations could be due to the heat treatment the seeds have undergone in the baking of biscuits and sponge cakes as stated in some previous studies and EU recommendations [3]–[5], [37]–[40]. In addition, as demonstrated in recent work by Vera-Baquero et al. the location of the seeds in the sample could also influence the resulting opium content [40]. For example, in sample SC-1 all seeds were located on the surface of the product, so degradation due to baking temperature could be higher as they are more exposed and can reach higher temperatures. However, in general, all the biscuits studied are small and thin and the dough might not protect the seeds as much, therefore no differences are seen between SC-1 where the seeds are located on the surface and the rest of the biscuits where they are added in the dough. This aspect might be more relevant in the bakery products studied in the previous work [8], where differences were shown between breadsticks where the seeds were on the surface and sliced bread where the seeds were in the dough and higher opium concentrations were found (with two samples exceeding the maximum limit). However, this aspect should be further studied to determine how temperature really influences these compounds and whether it really allows them to degrade and therefore to be safe samples for the consumer.

Table 2. Occurrence of opium alkaloids in the commercial samples analysed (n = 3).

		B-1	B-2	B-3	B-4	B-5	SC-1	SC-2
Sample [a]								
Poppy seeds amount (%) [b]		5	4	5	Not specified	4	Not specified	13
Range of occurrence (mg/kg) ± SD	Morphine	0.013 ± 0.006	ND	0.010 ± 0.008	ND	0.010 ± 0.002	0.03 ± 0.01	0.153 ± 0.009
	Codeine	ND	ND	ND	ND	ND	<MQL	0.004 ± 0.002
	Thebaine	ND	ND	ND	ND	ND	<MQL	0.015 ± 0.003
	Papaverine	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
	Noscapine	<MQL	<MQL	<MQL	ND	<MQL	<MQL	ND
	Oripavine	ND	ND	ND	ND	ND	<MQL	ND
Morphine equivalents (mg/kg) [c]		0.013	ND	0.010	ND	0.010	0.03	0.154

[a] B: biscuits; SC: sponge cakes; [b] Poppy seed content was obtained from the ingredients list on the product packages; [c] maximum limit of morphine equivalents in bakery products of 1.5 mg/kg in Commission Regulation (EU) 2021/2142; ND: not detected; <MQL: lower than method quantification limit but higher than method detection limit.

4. Conclusions.

A fast, efficient and environmentally friendly method for the control of opium alkaloids in biscuit and sponge cake samples has been developed. For this purpose, UAE was used for the first time in OAs, which allowed using lower solvent volumes and extraction time than with other classical methods. This was followed by a purification step using SBA-15 in SPE as it showed more efficiency than HMS silica and the commercial HLB sorbent, eliminating all matrix effects. After successful validation, the method was shown to have low MDL and MQL and favourable recovery values (79-101%). The method was applied to seven commercial samples of biscuits and sponge cakes, obtaining morphine concentrations in five of the samples below the established maximum limit (1.5 mg/kg morphine equivalents). The rest of the analytes were detected below the limit of quantification except in one of the samples where the proportion of seeds was higher (13%) than in the rest of the products (around 5%).

Declaration of competing interest. The authors declare that they have no conflict of interest.

Acknowledgemts. This research was funded by MCIU/AEI/FEDER, UE for project RTI2018-094558-B-I00.

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Supplementary Information

Supplementary Information S1. Preparation of SBA-15 and HMS materials.

SBA-15 material was prepared following the method proposed by Zhao et al. [99]. Firstly, 19.36 g of P123 were dissolved in 576 mL of 2 M HCl and 144 mL of distilled water. The mixture was stirred at 35 °C in a silicone bath until P123 was completely dissolved in the HCl solution. Then, 40.8 g of TEOS were added drop by drop and stirred for 20 h. After 20 h, the stirring was stopped, and the temperature was raised to 80 °C it was left for 24 h at this temperature to carry out an ageing process. The material was collected by filtration, washed with distilled water, air-dried and calcined (1 °C/min ramp until 500 °C and 12 h at 500 °C).

HMS material was prepared according to Pérez-Quintanilla et al. [100]. HMS silica was synthesized at room temperature using TEOS, DDA, ethanol and distilled water at a composition ratio of 1:0.27:6.5:36. The solution was stirred for 18 h, yielding a thick white suspension that was filtered and dried at 80ºC for 1 h. The amine was removed by calcination in an atmosphere of air at 550 °C for 12 h.

Table S1. Optimal parameters of MRM for the analysis of six OAs by HPLC-MS/MS.

Analytes	t_R[a] (min)	Ionization mode	Precursor ion (Q_1, m/z, $[M+H]^+$)	Fragment ion[b] (Q_3, m/z)	CE[c] (eV)
Morphine	5.179	ESI (+)	286.1	**153.0**	45
				165.0	24
				228.6	22
Morphine-d3	5.819	ESI (+)	288.7	**152.3**	45
				164.2	37
				200.6	25
Codeine	5.528	ESI (+)	300.2	153.1	45
				165.0	45
				215.1	23
Codeine-d3	5.533	ESI (+)	303.4	182.2	30
				199.0	30
				215.1	24
Oripavine	5.648	ESI (+)	298.3	236.9	14
				249.1	17
				267.1	12
Thebaine	6.292	ESI (+)	312.3	**58.2**	8
				166.2	16
				249.4	16
Papaverine	6.507	ESI (+)	340.2	**202.0**	24
				324.1	30
Noscapine	6.554	ESI (+)	414.3	205.1	24
				220.0	42
				280.1	20

[a] t_R: retention time; [b]: the fragment ions used for the quantification are in bold; [c] CE: collision energy.

Table S2. Summary of the 5 factors studied with their codes and the levels of independent variables used in two-level full factorial screening.

Factor	Symbol	Factor levels	
		Low (-1)	High (+1)
Solvent volumen (mL)	A	5	10
Acid proportion (%)	B	0.1	1
Extraction time (min)	C	1	5
Sonication amplitude (%)	D	50	75
Sonication mode (pulse duration: pulse interval (sec))	E	Continuous (0:0)	Pulsed (2:1)

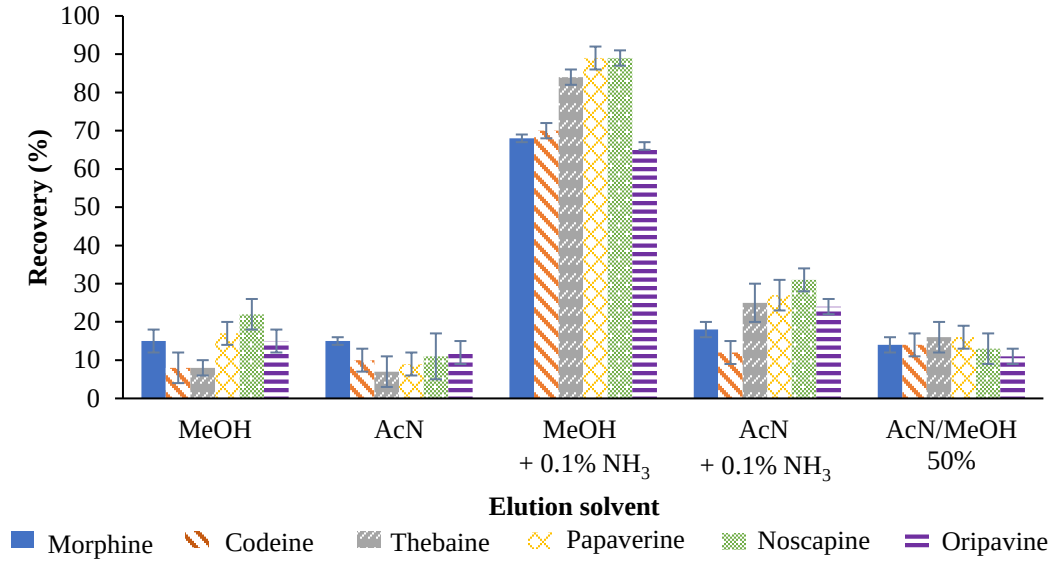

Fig. S1. Comparison between recovery values (%) obtained with 50 mg SBA-15 material with 2 mL of 30 µg/L of opium alkaloids in MeOH with 0.1% formic acid as loading solvent and 2 mL of different elution solvents (methanol, acetonitrile, methanol and acetonitrile with 0.1% ammonia).

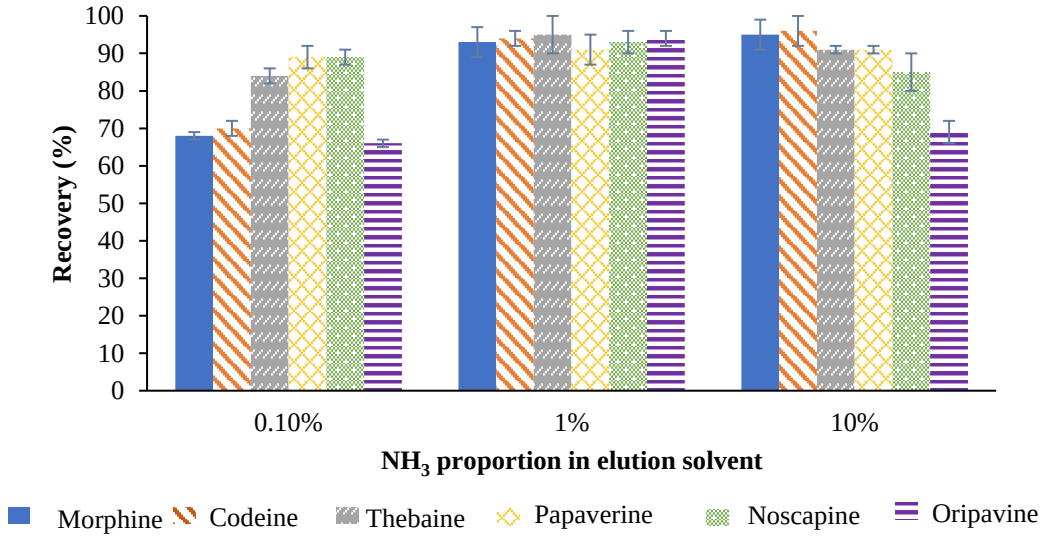

Fig. S2. Comparison between recovery values (%) obtained with 50 mg SBA-15 material with 2 mL of 30 µg/L of opium alkaloids in MeOH with 0.1% formic acid as loading solvent and 2 mL of methanol with different proportions of ammonia to elution (0.1, 1 and 10%).

Table S3. Results obtained from the factorial experimental design to UAE optimization.

RUN	Factor*					Responses (recovery mean (%) ± SD)					
	A	B	C	D	E	Morphine (Y_1)	Codeine (Y_2)	Thebaine (Y_3)	Papaverine (Y_4)	Noscapine (Y_5)	Oripavine (Y_6)
1	-1	1	-1	-1	-1	23 ± 1	26 ± 1	46 ± 2	50 ± 1	56 ± 1	51 ± 1
2	-1	1	-1	-1	1	25 ± 1	27 ± 1	49 ± 1	60 ± 1	72 ± 1	53 ± 3
3	-1	1	-1	1	-1	29 ± 1	29 ± 1	50 ± 2	72 ± 1	81 ± 3	43 ± 1
4	-1	1	-1	1	1	34 ± 1	39 ± 2	56 ± 1	82 ± 1	90 ± 2	60 ± 1
5	-1	1	1	-1	-1	58 ± 2	66 ± 1	84 ± 1	84 ± 1	93 ± 1	69 ± 2
6	-1	1	1	-1	1	62 ± 2	72 ± 1	86 ± 1	88 ± 4	101 ± 2	72 ± 2
7	-1	1	1	1	-1	69 ± 4	79 ± 1	94 ± 3	93 ± 7	103 ± 3	76 ± 4
8	-1	1	1	1	1	74 ± 1	85 ± 3	97 ± 3	101 ± 3	101 ± 2	76 ± 4
9	-1	-1	-1	-1	-1	23 ± 1	28 ± 1	50 ± 1	53 ± 1	66 ± 1	26 ± 1
10	-1	-1	-1	-1	1	26 ± 2	30 ± 1	52 ± 2	51 ± 1	68 ± 1	37 ± 1
11	-1	-1	-1	1	-1	35 ± 2	45 ± 1	60 ± 1	60 ± 2	73 ± 4	47 ± 2
12	-1	-1	-1	1	1	35 ± 2	40 ± 1	59 ± 1	65 ± 1	77 ± 1	46 ± 1
13	-1	-1	1	-1	-1	39 ± 1	58 ± 2	65 ± 2	61 ± 1	68 ± 1	75 ± 14
14	-1	-1	1	-1	1	51 ± 3	67 ± 2	70 ± 1	78 ± 1	71 ± 4	69 ± 11
15	-1	-1	1	1	-1	64 ± 2	80 ± 3	92 ± 2	96 ± 1	92 ± 1	77 ± 5
16	-1	-1	1	1	1	64 ± 6	83 ± 2	90 ± 1	95 ± 1	92 ± 1	73 ± 5
17	1	1	-1	-1	-1	42 ± 1	56 ± 1	63 ± 3	77 ± 1	90 ± 1	54 ± 4
18	1	1	-1	-1	1	44 ± 1	66 ± 2	76 ± 2	77 ± 1	92 ± 3	57 ± 3
19	1	1	-1	1	-1	45 ± 1	70 ± 2	78 ± 1	78 ± 7	88 ± 4	54 ± 3
20	1	1	-1	1	1	45 ± 3	69 ± 4	84 ± 1	83 ± 1	95 ± 4	62 ± 2
21	1	1	1	-1	-1	63 ± 4	73 ± 6	83 ± 2	82 ± 6	87 ± 6	65 ± 2
22	1	1	1	-1	1	67 ± 5	68 ± 2	89 ± 1	84 ± 1	82 ± 5	66 ± 4
23	1	1	1	1	-1	72 ± 2	84 ± 3	95 ± 1	95 ± 3	104 ± 2	78 ± 4
24	1	1	1	1	1	72 ± 3	88 ± 1	95 ± 6	88 ± 11	104 ± 7	82 ± 2
25	1	-1	-1	-1	-1	38 ± 1	55 ± 3	60 ± 1	76 ± 1	82 ± 2	39 ± 2
26	1	-1	-1	-1	1	47 ± 1	52 ± 1	70 ± 1	75 ± 2	89 ± 2	45 ± 2
27	1	-1	-1	1	-1	45 ± 1	55 ± 1	65 ± 2	73 ± 1	80 ± 1	55 ± 3
28	1	-1	-1	1	1	43 ± 1	56 ± 1	68 ± 1	69 ± 5	81 ± 1	48 ± 3
29	1	-1	1	-1	-1	57 ± 1	70 ± 1	74 ± 3	89 ± 3	90 ± 1	65 ± 1
30	1	-1	1	-1	1	60 ± 1	75 ± 1	73 ± 2	86 ± 1	91 ± 1	73 ± 3
31	1	-1	1	1	-1	75 ± 5	85 ± 2	98 ± 10	101 ± 8	95 ± 2	80 ± 1
32	1	-1	1	1	1	74 ± 3	87 ± 1	97 ± 2	99 ± 8	96 ± 3	80 ± 2

SD: standard deviation (n=3). * (A) solvent volume (mL), (B) acid proportion (%), (C) extraction time (min), (D) sonication amplitude (%), (E) sonication mode to UAE optimisation.

Table S4. Results of factorial experimental design data for RSM using continuous mode to UAE optimization.

RUN	Factor*		Responses (recovery mean (%) ± SD)					
	A	C	Morphine (Y_1)	Codeine (Y_2)	Thebaine (Y_3)	Papaverine (Y_4)	Noscapine (Y_5)	Oripavine (Y_6)
1	1	-1	45 ± 1	70 ± 2	78 ± 1	78 ± 7	88 ± 4	54 ± 3
2	1	0	56 ± 5	70 ± 5	70 ± 7	78 ± 1	82 ± 1	70 ± 6
3	1	1	72 ± 2	84 ± 3	95 ± 1	95 ± 3	104 ± 2	78 ± 4
4	0	-1	42 ± 1	51 ± 7	60 ± 1	75 ± 2	85 ± 5	45 ± 2
5	0	0	52 ± 7	67 ± 5	68 ± 5	76 ± 8	82 ± 3	60 ± 1
6	0	1	70 ± 2	82 ± 6	94 ± 2	94 ± 2	104 ± 3	77 ± 6
7	-1	-1	29 ± 1	29 ± 1	50 ± 2	72 ± 1	81 ± 3	43 ± 1
8	-1	0	46 ± 8	61 ± 1	67 ± 2	75 ± 4	81 ± 1	54 ± 4
9	-1	1	69 ± 4	79 ± 1	94 ± 3	93 ± 7	103 ± 3	76 ± 4

SD: standard deviation (n=3). *(A) solvent volume (mL), (C) extraction time (min).

Table S5. Results of factorial experimental design data for RSM using pulse mode to UAE optimization.

RUN	Factor*		Responses (recovery mean (%) ± SD)					
	A	C	Morphine (Y_1)	Codeine (Y_2)	Thebaine (Y_3)	Papaverine (Y_4)	Noscapine (Y_5)	Oripavine (Y_6)
1	1	-1	45 ± 3	69 ± 4	84 ± 1	83 ± 1	95 ± 4	62 ± 2
2	1	0	55 ± 4	69 ± 3	57 ± 6	76 ± 1	82 ± 5	80 ± 4
3	1	1	72 ± 3	88 ± 1	95 ± 6	88 ± 11	104 ± 7	82 ± 2
4	0	-1	39 ± 8	48 ± 4	63 ± 3	82 ± 1	92 ± 4	61 ± 1
5	0	0	52 ± 5	68 ± 3	55 ± 2	75 ± 3	80 ± 1	75 ± 1
6	0	1	70 ± 2	86 ± 2	95 ± 5	97 ± 3	100 ± 4	79 ± 1
7	-1	-1	34 ± 1	39 ± 2	56 ± 1	82 ± 1	90 ± 2	60 ± 1
8	-1	0	43 ± 7	60 ± 4	53 ± 3	74 ± 8	79 ± 2	72 ± 3
9	-1	1	74 ± 1	85 ± 3	97 ± 3	101 ± 3	101 ± 2	76 ± 4

SD: standard deviation (n=3). *(A) solvent volume (mL), (C) extraction time (min).

Table S6. ANOVA report and mathematical equations for the models of CUAE and PUAE.

Variable	Morphine recovery (%)		Codeine recovery (%)		Thebaine recovery (%)		Papaverine recovery (%)		Noscapine recovery (%)		Oripavine recovery (%)	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
CUAE Model												
A	4.8333	0.0084*	9.1667	0.0173*	5.3333	0.0488*	1.8333	0.0014*	1.5	0.0349*	4.8333	0.0393*
C	15.8333	0.0003*	15.8333	0.0037*	15.8333	0.0024*	9.5	0.0000*	9.5	0.002*	14.8333	0.0017*
AA	-1.8333	0.2663	-1.1667	0.7479	1.6667	0.6025	0.1667	0.5836	-0.5	0.5305	1.8333	0.4984
CC	3.1667	0.1000	-0.1667	0.9630	10.1667	0.0384*	8.1667	0.0001*	12.5	0.0004*	0.8333	0.7501
AC	-3.25	0.0420*	-9.0	0.0311*	-6.75	0.0450*	-1.0	0.0138*	-1.5	0.0577	-2.25	0.2748
R^2	0.9937		0.9726		0.9766		0.9994		0.9966		0.9775	
R^2 Adj.	0.9832		0.9269		0.9377		0.9983		0.9909		0.9401	
R^2 Pred.	0.9251		0.6662		0.7181		0.9930		0.9584		0.7263	
Equations	$y = 52.56 + 4.83A + 15.83C - 3.25AC$		$y = 66.78 + 9.17A + 15.83C - 9.0AC$		$y = 67.22 + 15.83C + 10.17C^2 - 6.75AC$		$y = 76.22 + 9.5C + 8.17C^2$		$y = 82.0 + 1.5A + 9.5C + 12.5C^2$		$y = 60.11 + 4.83A + 14.83C + 0.83C^2$	
PUAE Model												
A	3.5	0.0755	7.0	0.0240*	5.0	0.0523	-1.6667	0.2505	1.8333	0.0128*	2.6667	0.0181*
C	16.3333	0.0011*	17.1667	0.0019*	14.0	0.0031*	6.5	0.0116*	4.6667	0.0009*	9.0	0.0005*
AA	0.1667	0.9460	1.0	0.7497	2.6667	0.4070	-0.6667	0.7644	1.1667	0.1439	0.3333	0.7565
CC	5.6667	0.0878	3.5	0.3084	26.3333	0.0024*	13.8333	0.0065*	16.6667	0.0001*	-5.6667	0.0103*
AC	-3.25	0.1358	-6.75	0.0445*	-7.5	0.0314*	-3.5	0.0929	-0.5	0.3189	1.0	0.2452
R^2	0.9830		0.9788		0.9848		0.9659		0.9970		0.9904	
R^2 Adj.	0.9547		0.436		0.9595		0.9092		0.9921		0.9744	
R^2 Pred.	0.8186		0.7836		0.8258		0.5877		0.9668		0.8867	
Equations	$y = 49.89 + 16.33C$		$y = 65.0 + 7.0A + 17.17C - 6.75AC$		$y = 53.22 + 14.0C + 26.67C^2 - 7.5AC$		$y = 75.44 + 6.5C + 13.83C^2$		$y = 79.56 + 1.83A + 4.67C + 16.67C^2$		$y = 75.44 + 2.67A + 9.0 \cdot C + 1.0AC$	

*significant $p < 0.05$.

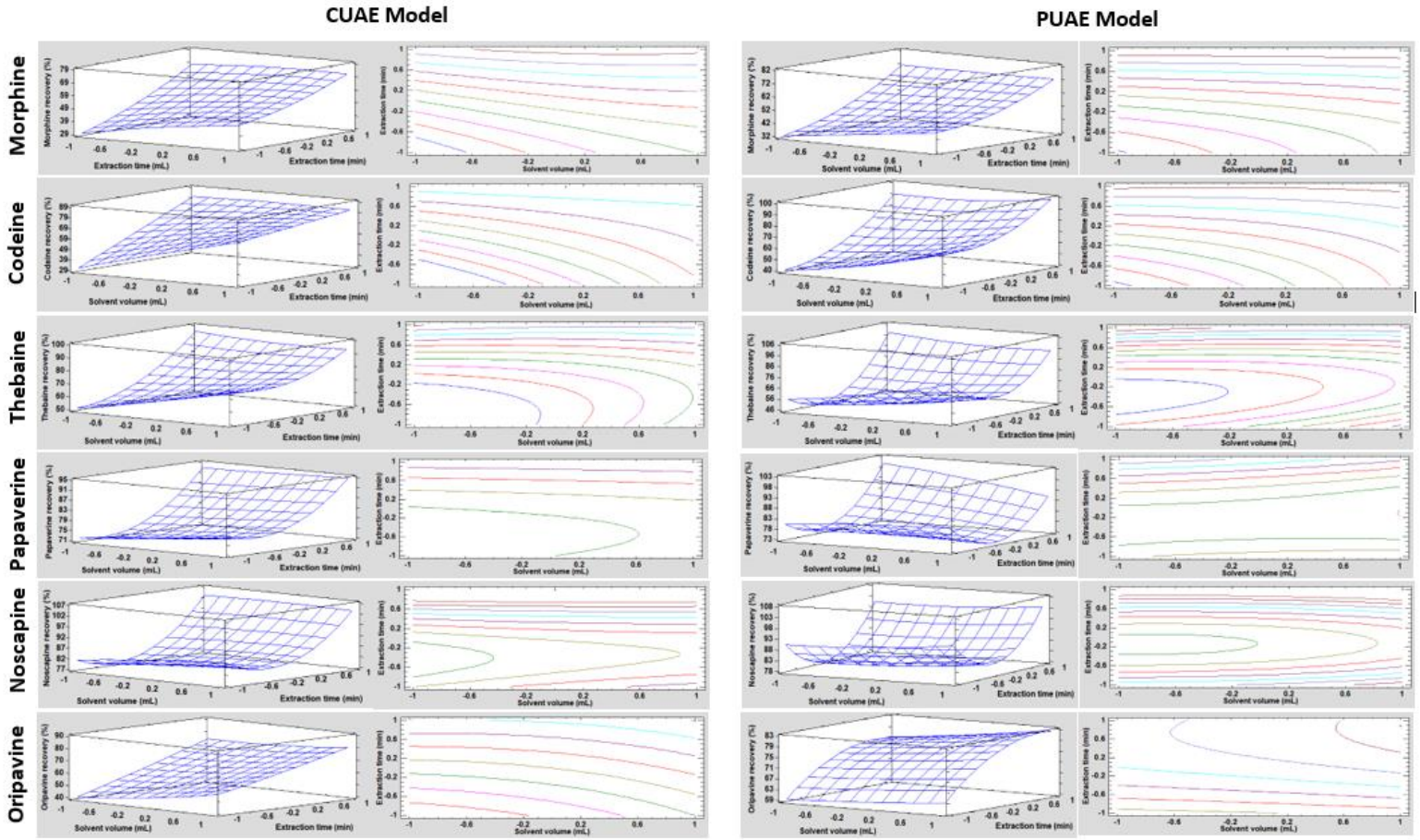

Fig. S3. Response surface and contour plots demonstrate the effects of solvent volume and extraction time on the recovery of each analyte in CUAE and PUAE models to UAE optimisation.

Table S7. Comparison of the experimentally obtained results with the results predicted by the mathematical equations with the estimated optimal conditions in both sonication modes.

Analyte	CUAE		PUAE	
	Experim. results	Pred. values	Experim. results	Pred. values
Morphine	72	70	72	66
Codeine	84	83	88	82
Thebaine	95	87	95	86
Papaverine	95	94	88	103
Noscapine	104	81	104	103
Oripavine	78	81	82	88

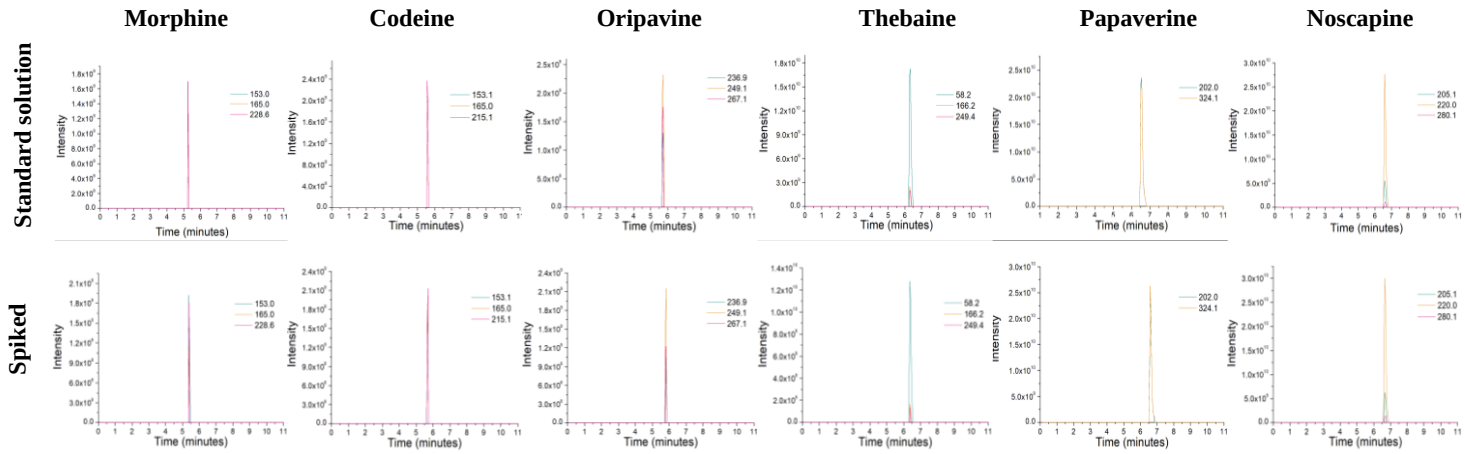

Fig. S4. Comparison between the extracted ion chromatograms obtained for each of the six OAs in a standard solution mixture of 0.1 µg/mL with respect to the extract spiked with the same concentration in the sample.

Artículo 4:

Evaluation of the transfer and occurrence of opium alkaloids in poppy seed tea by a preconcentration with µSPEed® followed by GC-MS analysis

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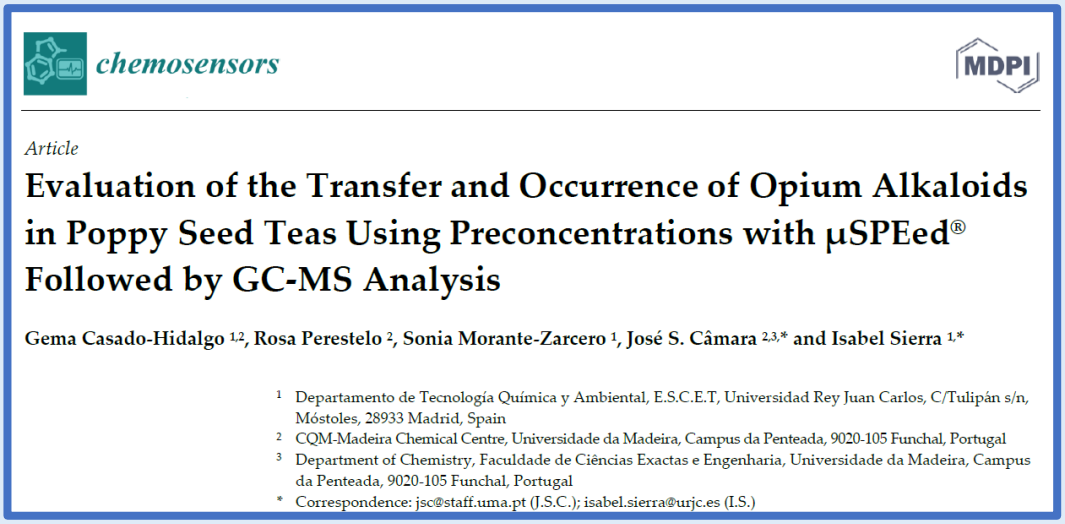

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Evaluation of the Transfer and Occurrence of Opium Alkaloids in Poppy Seed Teas Using Preconcentrations with µSPEed® Followed by GC-MS Analysis

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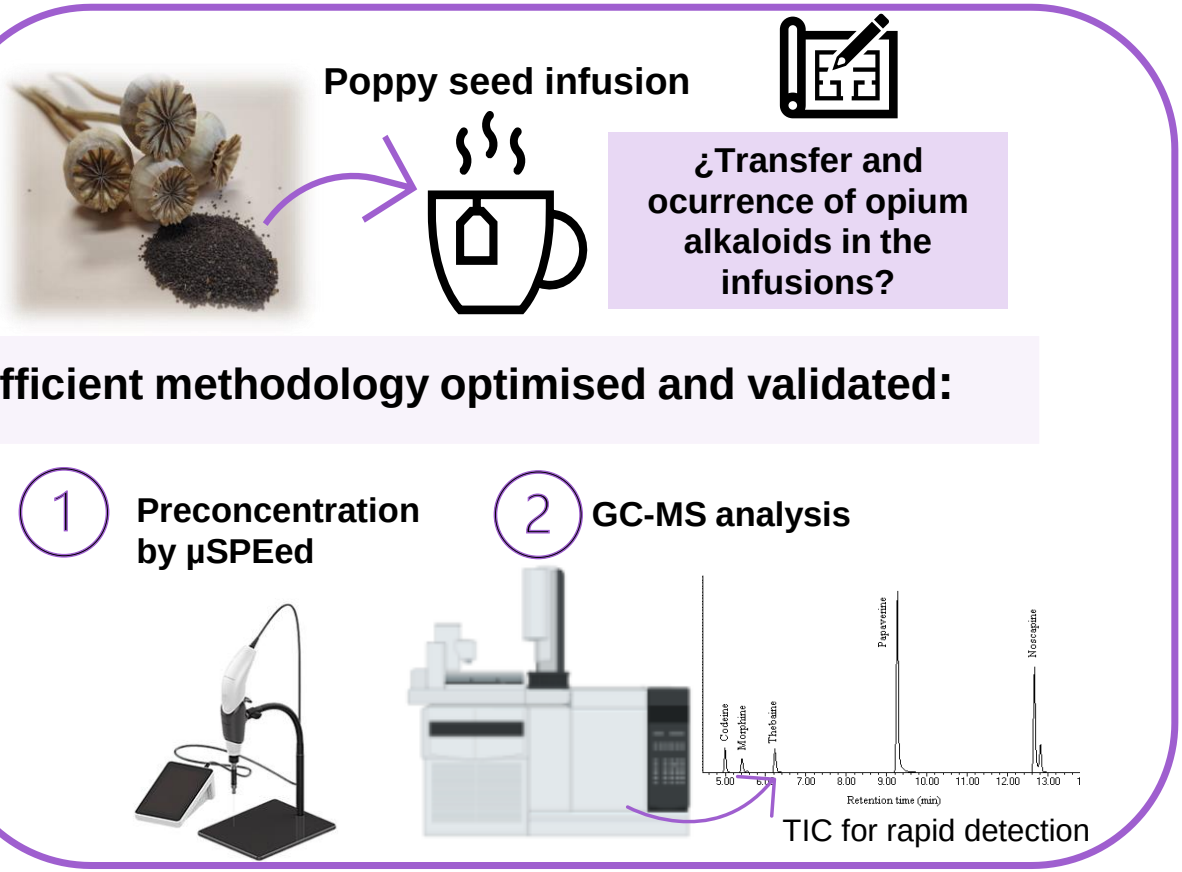
PUBLICATION INFORMATION

Journal	Chemosensors
Publisher	MDPI
Impact factor	4.229 (JCR, 2021)
Category	Instruments & Instrumentation (16/64, Q1)
DOI	https://doi.org/10.3390/chemosensors11020094


Poppy seed infusion
¿Transfer and ocurrence of opium alkaloids in the infusions?
Efficient methodology optimised and validated:
1
Preconcentration by µSPEed
2
GC-MS analysis
Codeine
Morphine
Thebaine
Papaverine
Noscapine
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
Retention time (min)
TIC for rapid detection

Highlights:

- An efficient methodology to control opium alkaloids in poppy seed teas
- µSPEed® allowed pre-concentration of 10 times to achieve lower limits in GC-MS
- Determination of transfer of opium alkaloids from seeds to tea
- All opium alkaloids were found in high concentrations in different poppy seed teas

ABSTRACT

Intoxications due to the consumption of poppy seed teas have been reported due to their contamination with opium alkaloids (OAs). In this work, an efficient methodology based on micro-solid phase extraction (µSPEed®) followed by gas chromatography-mass spectrometry (GC-MS) has been optimised to quantify five OAs in poppy seed tea. Nine cartridges (C4, C8, C18, silica, APS, PFAs, PS/DVB-RP, PS/DVB-SCX and PS/DVB-SAX), pH, cycles and elution solvent were evaluated. The method was validated and applied to study the transfer of OAs by evaluating water temperature, infusion time and seeds amounts. The highest transfer (71% morphine, 96% thebaine and 100% codeine, noscapine and papaverine) was achieved at 90°C, 5 minutes, and 4 g. These conditions were used to quantify the OAs in four teas from different seeds. A high amount of morphine (1563 µg/L) was found in a tea, indicating that the seeds had a concentration twice maximum limit and highlighting the need to warn the population of this dangerous practice.

KEYWORDS: Food safety; opium alkaloids; µSPEed; GC-MS; poppy seed tea; transfer; occurrence.

1. Introduction

Poppy seeds tea have been used for centuries as a home remedy to relieve pain, anxiety and stress [1-4]. Although they do not contain opium alkaloids (OAs) by themselves, they can be contaminated by the latex of the plant itself (*Papaver somniferum* L.), which is rich in OAs (e.g., morphine, codeine, thebaine, papaverine and noscapine). This contamination may be due to poor harvesting practices or insect damage. In recent years, numerous studies on poppy seeds have confirmed the presence of OAs, being in many cases considerably high and very dispersed due to their heterogeneous contamination and the influence of numerous external conditions (e.g., climate, harvesting time, variety, among others) [5-9]. For example, in previous work, widely varying amounts of each of the analytes were determined, between 1.5-249.0 mg/kg morphine, <MQL-45.8 mg/kg codeine, <MQL-136.2 mg/kg thebaine, <MQL-27.1 mg/kg papaverine and <MQL-108.7 mg/kg noscapine [5]. Until now, this topic was quite unknown to the general population and, as there was no control, constituting an important food safety issue. The consumption of poppy seeds tea has caused false positives in drug tests because, after consumption, OAs concentrations have been found in biological samples as studied in some works, such as blood, urine, and serum [10-16]. It may cause adverse health effects, such as nausea and vomiting, drowsiness, respiratory problems, and dependence, especially for the most vulnerable people and including more serious cases of intoxication [2], [17-22]. Such is the seriousness that since 2019 in the United States poppy seeds have been considered Schedule II controlled substances, making their commercialization illegal [1]. However, in Europe it was not until June 2022 that Regulation (EU) 2021/2142 entered vigour, setting the maximum morphine equivalent limits (ME = morphine + 0.2 × codeine) in seeds at 20 mg/kg [23]. To gain toxicologically relevant exposure data, it is important to know the transfer of OAs from seeds to tea, it has not yet been studied. To this end, it is necessary to develop and validate analytical methods able to quantify the OAs present in tea infusions.

For this, it is important to consider the crucial role that sample treatment plays in methods for food analysis, from purifying the sample extract to remove possible matrix interferences, to preconcentrating to achieve lower detection limits. One of the most used

techniques to date is solid phase extraction (SPE) [24]. However, miniaturized techniques are increasingly being selected as faster, cheaper, and more environmentally friendly due to the use of smaller volumes of organic solvents and lower amounts of sorbents required [25], following the principles of green analytical chemistry (GAC). Many microextraction procedures with different formats and configurations have been developed. Microextraction by packed sorbents (MEPS) is an interesting miniaturization of the SPE technique with a small amount of sorbent. However, another configuration that incorporates some improvements over MEPS is the micro-solid phase extraction (μSPE) technique [26]. This technique has been introduced to the market by the company EPREP like μSPEed® (Victoria, Australia) and is based on cartridges containing sorbents with very small particle sizes, smaller than MEPS (≤3 μm versus 50-60 μm), resulting in a larger surface area. In addition, it has a pressure-operated unidirectional valve (up to 1200 psi) to remove sample flow in one direction only, unlike the MEPS technique which has two-directional flow potential (up and down) [26], [27]. In addition, there are a variety of sorbents for μSPEed® cartridges, such as unmodified and functionalized silica and polymeric materials, which allows covering the different retention interactions that may exist with the various target analytes [27]. This technique has been used for the extraction of different families of compounds in food, from bioactive compounds [27], [28] to natural toxins [29], [30]. However, as far as we know, this is the first time that μSPEed® is used for quantifying OAs in foods.

One of the most widely used analytical techniques for the analysis of opium alkaloids is gas chromatography coupled with mass spectrometry (GC-MS) and liquid chromatography tandem mass detector (HPLC-MS/MS) [5-31]. Much of the popularity of GC is based on its very high selectivity and resolution, good accuracy, and precision, as well as wide dynamic concentration range and high sensitivity [32]. GC-MS methods are very useful for the identification of individual compounds using standards or specific libraries and their subsequent quantification. However alternative approach can be used for qualitative and quantitative determination, considering GC as a sensor, with the application of a chemometric tool for data treatment [32].

Hence, the aim of this work was to select the most suitable sorbent and evaluate the efficacy of the µSPEed® technique followed by a GC-MS analysis for the quantification of OAs in poppy seed teas, to propose an efficient and sensitive analytical methodology to determine the transfer of these alkaloids in teas prepared from poppy seeds with different conditions (water temperature, infusion time, and seed amount). In addition, to monitor the occurrence in home-made teas prepared with different commercially available poppy seeds with the most unfavorable conditions and to evaluate the risk of consuming poppy seed tea.

2. Materials and Methods

2.1. Reagents and materials

Standards of morphine, codeine and thebaine were received from Alcaliber S.A.U. (Madrid, Spain) and noscapine and papaverine were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1000 µg/mL in methanol (MeOH). The intermediate mixed standard solution was prepared at 100 µg/mL in MeOH. The working standard solutions were prepared by diluting the intermediate mixed standard in MeOH. All of these were stored in darkness at −20 ˚C.

Methanol (MeOH), chloroform ($CHCl_3$), sodium hydroxide (NaOH) in HPLC grade, were purchased from Fischer Scientific (Loughborough, UK). Hydrochloric acid (HCl, 37% v/v,) was purchased from Panreac (Barcelona, Spain) and trifluoroacetic anhydride (TFA) from Sigma-Aldrich (Zwijndrecht, The Netherlands). Ultra-pure deionized water (18.2 MΩ cm quality) was obtained using a Millipore Milli-Q-System (Billerica, MA, USA) and was used for the preparation of aqueous solutions. Nylon syringe filters (0.45 µm) for the filtration of tea infusions were acquired from Scharlau (Barcelona, Spain). The digiVOL® Digital Syringe and the commercial µSPEed® cartridges tested were acquired to EPREP. Nine types of cartridges of different chemical structure, six silica-based: C_4 (tetrylsilane), 3 µm/300 Å; C_8 (octylsilane), 3 µm/120 Å; C_{18} (octadecylsilane), 3 µm/120 Å; APS (WAX, weak anion exchanger), 3 µm/120 Å; PFAs (50% WAX, 50%

C_{18}), 3 µm/120 Å; and silica, 3 µm/120 Å) and three polymeric-based: PS/DVB-RP (reversed phase), 3 µm/300 Å; PS/DVB-SCX (strong cation exchanger), 3 µm/non-porous; PS/DVB-SAX (strong anion exchanger), 3 µm/non-porous), were tested.

2.2. OAs in poppy seeds and their study of transfer to tea

Four samples of edible seeds (S-1, S-2, S-3 and S-4) were purchased in Spain in the middle of 2021 from supermarkets and herbalists. Specifying in the labelling of some of them (S-2) recommendations for the preparation of calming and sedating infusions. Detailed information on each of these samples can be found in Table S1.

For the study of the transfer of OAs to the tea, seeds S-1 were used previously washed and dried following our previous work [5] and the Recommendation published by the European Union in 2014 to reduce the OAs content in seeds [33]. This was done because the concentrations of OAs that can be found in the same batch of seeds can be widely dispersed due to the heterogeneous contamination that can occur in them. Once the seeds were washed and free of OAs, they were spiked to the intermediate level of concentration of validation (20 mg/kg), which is the maximum permitted limit legislated [23].

The spiked seeds were subjected to the transfer study which consisted of studying three factors of the infusion (e.g., temperature, time, and seeds amount) at two levels each with 100 mL of deionized water. The levels of each factor to be studied were selected according to International Standard ISO 3103 protocol [34] and to the instructions of manufacturers to resemble the real conditions that consumers carry out in their preparations. The temperature was studied at two levels, 90 and 100 ˚C, since in online forums and even in some of the seed labels they recommend infusing at 90 ˚C to avoid degradation of the OAs. The time was studied at 5 and 10 min and the amount was studied at 2 and 4 g. Therefore, a matrix of 8 studies was obtained as shown in Table S2. For it, 2 or 4 g of poppy seeds were weighed in an analytical balance (± 0.1 mg) and infused with 100 mL of deionized water at 90 or 100 ˚C for 5 or 10 min. Then, the infusion was strained and cooled to room temperature. Later, the sample was filtered through a nylon syringe filter (0.45 µm) before purified by µSPEed® and analysed by GC-MS. The areas obtained

from each replicate were interpolated on the matrix-matched calibration to obtain the concentration of OAs in the tea. In addition, transfer rates (%) were calculated by comparing the concentration obtained in the tea and the concentration spiked in the seeds. Finally, with the conditions that gave a higher transfer, infusions of the four poppy seeds were made to evaluate the concentration that can be ingested through the infusion and to assess the risk. Three consecutive infusions were prepared with each poppy seed by taking three different portions from each sample to obtain the mean of the concentrations with the ± standard deviation (SD).

2.3. µSPEed® extraction procedure of OAs in tea infusion

The µSPEed® procedure was performed with digiVOL® Digital Syringe with automatic syringe of 250 µL at a constant flow rate of 2500 µL/min. Nine available sorbents (C_4, C_8, C_{18}, PFAs, APS, silica, PS/DVB-RP, PS/DVB-SCX, and PS/DVB-SAX) were evaluated and compared to select the most efficient for OAs.

First, the µSPEed® parameters were optimized. To do this, it was evaluated whether the derivatization of OAs was more appropriate before or after the µSPEed® procedure. Subsequently, the pH of the sample load (pH 3, 7 and 9) was evaluated with all cartridges. The medium selected for loading was water as it was infusions and the sample medium. Then, the loaded onto the cartridge can be in two modes: draw-effect (sample is discarded in the same vial after each extraction cycle to be sucked in again to ensure complete adsorption) or extract-discard (sample is discarded in a waste vial after each extraction to ensure pre-concentration). First, the draw-effect mode was tested with 3, 5 and 10 cycles to evaluate whether retention increased with increasing cycles. Subsequently, the elution solvent was optimized (MeOH with 10% formic acid, unmodified MeOH, MeOH with 10% NaOH, and $CHCl_3$). At last, the number of cycles in extract-discard was optimized (10, 12 and 15 cycles) to ensure the maximum possible preconcentration once the other parameters had been optimized.

Finally, the µSPEed® procedure was carried out using PS/DVB-RP cartridge previously selected as the most suitable sorbent to extract OAs in the optimization step. Figure 1 shows the µSPEed® procedure under the optimised conditions.

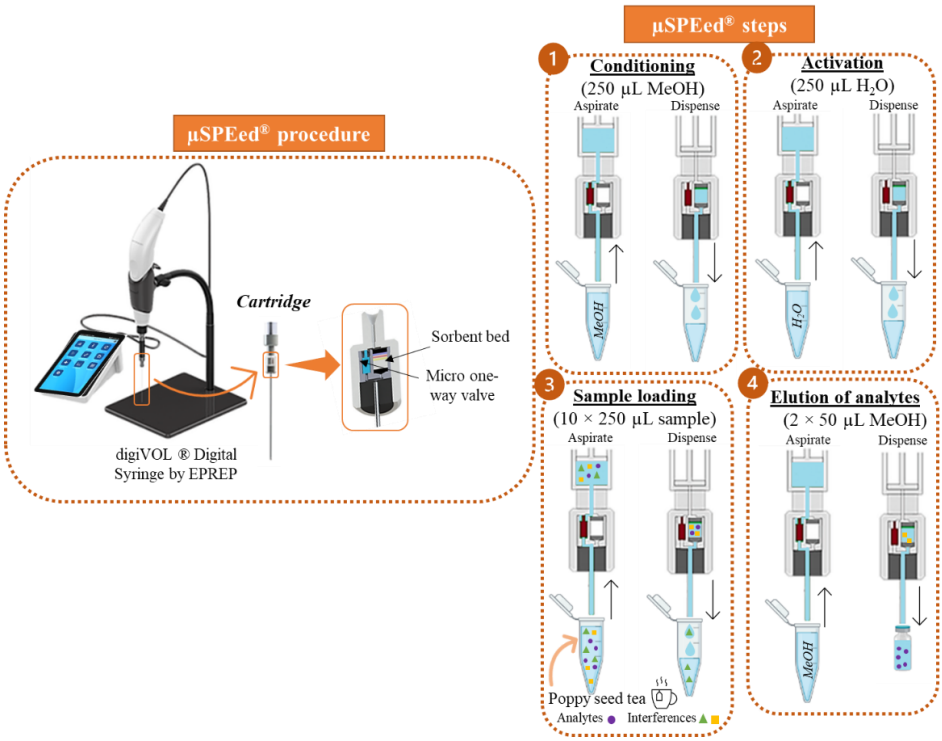


Figure 1. Graphical scheme of the optimised µSPEed® procedure for opium alkaloids extraction using the digiVOL® Digital Syringe (EPREP, Australia).

The first step was the activation of the cartridge with 250 µL of MeOH, later it was conditioned with 250 µL of water. Finally, the extract-discard mode was choosing and ten different 250 µL aliquots of the filtered infusion were used (250 µL, 10 cycles in extract-discard mode). No washing step was carried out. In the end, analytes were eluted into a vial with two different 50 µL aliquots of MeOH (2 × 50 µL). The extract collected in the vial was evaporated and derivatized for subsequent GC-MS analysis. Between each extraction, to ensure proper reuse of the cartridge and to avoid memory effect (carry-over) and to act as a conditioning step before the next extraction, the cartridge was washed with two different aliquots of 250 µL of MeOH (2 × 250 µL). Each cartridge was reused more than 100 times.

2.4. Derivatization of OAs and GC-MS analysis

After µSPEed® procedure, the extract collected in the vial was evaporated and reconstituted with 100 µL of $CHCl_3$ and 100 µL of TFA at 60 ˚C for 20 min for derivatization [11]. Finally, the extract derivatised was evaporated and reconstituted in 20 µL of MeOH to GC-MS analysis.

GC-MS analysis was performed using an Agilent 6890 N (Palo Alto, CA, USA) gas chromatograph system coupled to an Agilent 5975 quadrupole Mass Selective Detector. The injection volume was 5 µL and the mode was in splitless mode. The injector temperature was 280 ˚C and helium was used as a gas carrier at 1.3 mL/min. For the chromatographic separation an HP-5 capillary column ((5%-phenyl)-methylpolysiloxane nonpolar phase, 30 m × 320 µm i.d., 0.25 µm film thickness, Hewlett Packard, Palo Alto, USA)) was used, being thermostated at 220 ˚C for 1 min, then raised up to 300 ˚C at 6 ˚C/min and held for 1 min, resulting in a method of 15.33 min. The ionization was achieved using an electron ionization source (EI) at 70 eV. The transfer line, ion source, and quadrupole analyser temperatures were maintained at 220, 180, and 200 °C, respectively, and a solvent delay of 4 min was selected.

Initially, a full scan (FS) MS acquisition mode with a mass range of fragment ions from 50 to 500 amu, was employed, for qualitative detection, to select the characteristic fragment ions for the target OAs. For this purpose, a standard solution of the analytes at a concentration of 50 µg/mL previously derivatized with the optimized protocol was used. The selected ion monitoring (SIM) mode was used as a quantitative scan whereby the molecular ion of the analyte was monitored in a narrow amu window. For each compound the most abundant fragment ions were used (Table 1). In this MS acquisition mode only the selected fragment ions with m/z values of interest rather than over a wide range of m/z values, are collected, significantly improving the selectivity and consequently the detection limits of the technique.

Table 1. Retention time, and *m/z* of fragment ions selected for the quantification and confirmation of the five opium alkaloids.

Compound name	Quantification 1 (m/z)	Ion 2 (m/z)	Ion 3 (m/z)
Codeine	299	229	162
Morphine	285	215	162
Thebaine	311	296	242
Papaverine	338	324	154
Noscapine	220	215	205

2.5. Analytical method validation

There is no official regulation on analytical performance requirements for quantifying OAs in food or other matrices. Therefore, the validation of the method was performed following the criteria described in SANTE/11312/2021 for pesticides, in EC Regulation No. 401/2006 and in the ICH Q_2(R1) guidelines [35-37]. The validation method was performed for linear dynamic range (LDR), matrix effect (ME), method detection and quantification limits (MDL, MQL), accuracy, precision, and selectivity. To do this, the validation was carried out with a spiked sample previously washed and dried (S-1).

Accordingly, LDR was evaluated at six concentration levels on standard solutions prepared and analyzed using the proposed µSPEed® procedure. Concentration ranges were selected according to the sensitivity of the GC-MS system to each target analyte, as well as the amount expected in the tea infusions. Matrix-matches calibration lines were prepared in the same way but instead of in standard solutions, spiking the tea obtained from the poppy seed (S-1) previously washed and dried. All calibration curves were obtained with the mean peak area of each analyte versus analyte concentration and fitted by least squares linear regression. Matrix effects were determined by comparing the slopes of the calibration equations obtained from matrix-matched and solvent-based calibration curves for each analyte, calculating with the following formula: (slope matrix-matched/slope solvent-based -1) × 100. The ME is negligible when is lower than + / -20% [35], being signal enhancement when ME values are greater than 20% and signal suppression when values are greater than -20%. The sensitivity of the method for each sample was determined through the MDLs and MQLs of the OAs from the analysis of the

lowest concentration analysed (5 µg/L), which yielded a signal-to-noise (S/N) ratio of 3 and 10 (when the quantification ion was monitored), respectively.

The accuracy, expressed as recovery percentage (%), was assessed by comparing the areas obtained for samples spiked (n = 6) with a known concentration of analytes and subjected to the µSPEed® procedure with those areas obtained for simulated samples (samples spiked at the same concentration but at the end of the µSPEed® procedure prior to the derivatization step for GC-MS analysis). The recovery assays were performed by spiking the samples at three concentration levels of 800 µg/L (high level, HL), 400 µg/L (medium level, ML) and 100 µg/L (low level, LL). In the absence of legislation setting a maximum limit for OAs in poppy seed tea infusions, the Regulation (EU) 2021/2142 maximum limit of 20 mg/kg in seeds was followed and the concentration of OAs in the tea was estimated assuming complete transfer. Thus, in the seeds with 20 mg/kg of OAs, the concentration in tea (2 g of seed in 100 mL of water) was 400 µg/L, being the intermediate level of validation. The recovery values should be between 70 and 120%. On the other hand, the method precision was evaluated in terms of repeatability (intra-day) and reproducibility (inter-day), using the same validation levels as for the accuracy assays. For repeatability (expressed as the percentage of relative standard deviation, RSD %), six replicates (n = 6) of the whole procedure were performed on the same day. The reproducibility (also expressed as RSD %) was calculated by the analysis of three replicates of a sample, which were carried out in triplicate over three different days (n = 9). According to the validation guidelines, the RSD values for these precision parameters should be ≤20%. Selectivity was assessed by the absence of interfering chromatographic peaks at the retention time of the target analytes.

2.6. Statistical analysis

Statistical analyses were performed using SPSS 25.0 statistical package (SPSS Inc., Chicago, IL, USA) by one-factor ANOVA analysis. Significant differences were considered significant for p values ≤ 0.05. For the Pareto charts, Statgraphics Centurion software (version 19.3.03) was used to show the significant effects of each of the variables and their respective interactions.

3. Results and Discussions

3.1. OAs derivatization step and GC-MS analysis

First, derivatization was performed following the protocol of Hayes & Krasselt (1987) [11] using 100 µL of $CHCl_3$ and 100 µL of TFA for 20 min at 60 ˚C. One µL of a 50 µg/mL standard solution was injected into the GC-MS equipment and the MS acquisition was carried out in FS mode within a mass-to-charge ratio ranging from 50 to 500 (m/z). to identify each OAs based on its mass spectrum. Considering the low levels that OAs can occur, this means that the full scan MS detection is not the most adjusted approach to this end. The low sensitivity and selectivity of this MS-acquisition mode were overcome by using SIM mode, in which the most abundant OAs fragment ions seen in the full scan were selected for quantitative purposes (Table 1). This MS-acquisition mode presents high selectivity for every single OA in the sample, high sensitivity, and allows the confirmation of analyte identity and accurate quantification of the analytes. This allows achieving lower detection and quantification limits by improving the selectivity and sensitivity of the instrumental analysis. A chromatographic method with a starting temperature of 80 ˚C and rising to 300 ˚C with a ramp of 4 ˚C/min, corresponding to an analysis time of 45 min, was used. Thus, it was possible to separate and identify each of the analytes with their respective ions to create the SIM method that allows increasing specificity and eliminating interferences.

Once the SIM method was created, the analysis time was reduced by modifying the oven temperatures. For this, the first step was to increase the starting temperature from 80 ˚C to 180 ˚C since the retention time of the first analyte was 24.14 min. This decreased the retention time of the first analyte to 12.10 min and the analysis time to 31 min. Then, to shorten it further, the starting temperature was still raised to 200 ˚C, achieving a codeine retention time of 7.30 min and a total time of 21 min. And finally, the starting temperature was raised to 220 ˚C and the ramp was made faster, instead of at 4 ˚C/min to 6 ˚C/min and thus, the retention times for each of the analytes were: for codeine 5.06 min, morphine 5.46 min, thebaine 6.31 min, papaverine 9.44 min and noscapine 12.77 min, respectively, with a total time of 14.33 min, such as shown in Figure 2. In addition, 1 min of hold time was increased at the end of the method to ensure the cleanliness of the column between

injections. Besides, the spectra of each compound can be used for rapid identification of the compounds before quantification, i.e. from the TIC (Figure 2) it can be extracted and quickly checked and identified.

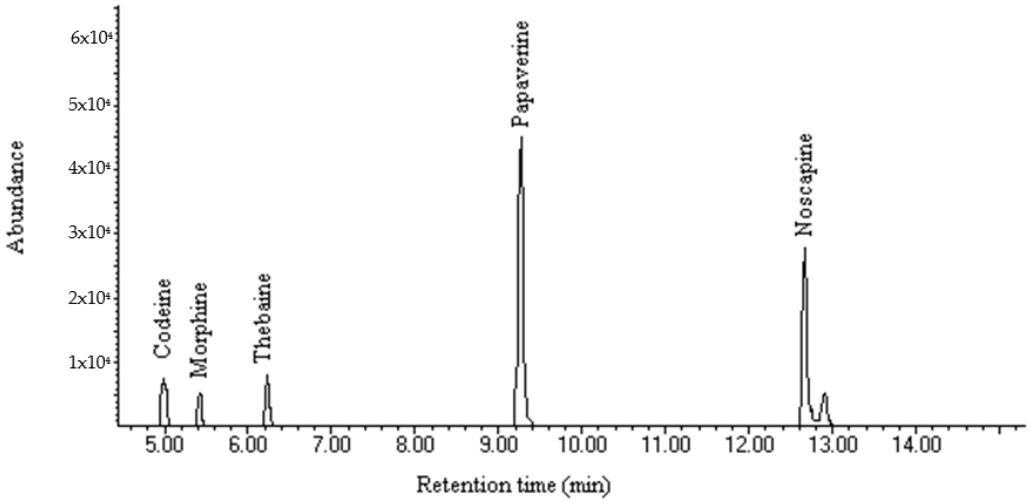


Figure 2. Total Ion Chromatography (TIC) obtained for each opium alkaloid using SIM mode in a poppy seed tea extract spiked at low level of validation (100 µg/L).

Once the chromatographic method was optimized, some tests were carried out to optimize the derivatization step. For this, different derivatization solvents were tested ($CHCl_3$, MeOH, ethyl acetate, and hexane) at different temperatures (40 and 60 ˚C) and times (10, 20, and 40 min). Finally, the optimum conditions were $CHCl_3$ with 60 ˚C for 20 min, thus confirming that the protocol used in the work of Hayes & Krasselt (1987) [11] to derivatize morphine and codeine was also the most suitable for the derivatization of all OAs.

3.2. µSPEed® procedure optimisation

To optimize the µSPEed® procedure, nine cartridges were tested due to the different chemical properties of the OAs, being papaverine and noscapine with the lowest water solubility (0.013 and 0.18 g/L, respectively) and morphine with the highest (10.20 g/L) [31].

First, nine cartridges were evaluated by performing the derivatization step before and after to see when it was more efficient to perform it. For this, the cartridge was activated and conditioned with 250 µL of MeOH and H_2O respectively and the loading step was done with 250 µL of a 5 mg/L standard solution in water in a 3-fold draw-effect cycle. The first studies were performed at a higher concentration of the target analytes to be detected by GC-MS. Then, the analytes were eluted with 50 µL of MeOH twice (50 µL × 2). As shown in Figure 3a, the areas obtained were generally larger when derivatization was performed after the µSPEed® procedure. Thus, it was decided to perform derivatization after the µSPEed® procedure.

Subsequently, as no cartridge was shown to be clearly better than the others for all the analytes due to the different chemical properties of OAs, in addition to testing all the cartridges by performing the loading step at pH 7, it was tested by acidifying with HCl (pH 3) and basifying with NaOH (pH 9) the 5 mg/L standard solution. Comparing the areas obtained at pH 7 (Figure 3b) and those obtained at pH 3 (Figure 3c) and 9 (Figure 3d), the structural and polarity differences between the analytes were even more evident. Since some cartridges showed higher areas with lower pH such as C_4 and others with higher pH such as C_8 or PS/DVB-SAX. However, the areas obtained with pH 3 and 9 for morphine were very low in all cartridges. Therefore, pH 7 was selected and, finally, it was decided to select the silica and PS/DVB-RP cartridges, which showed the best response for morphine and codeine, and for the rest of the analytes, there was little difference with respect to the cartridges that gave the greatest area.

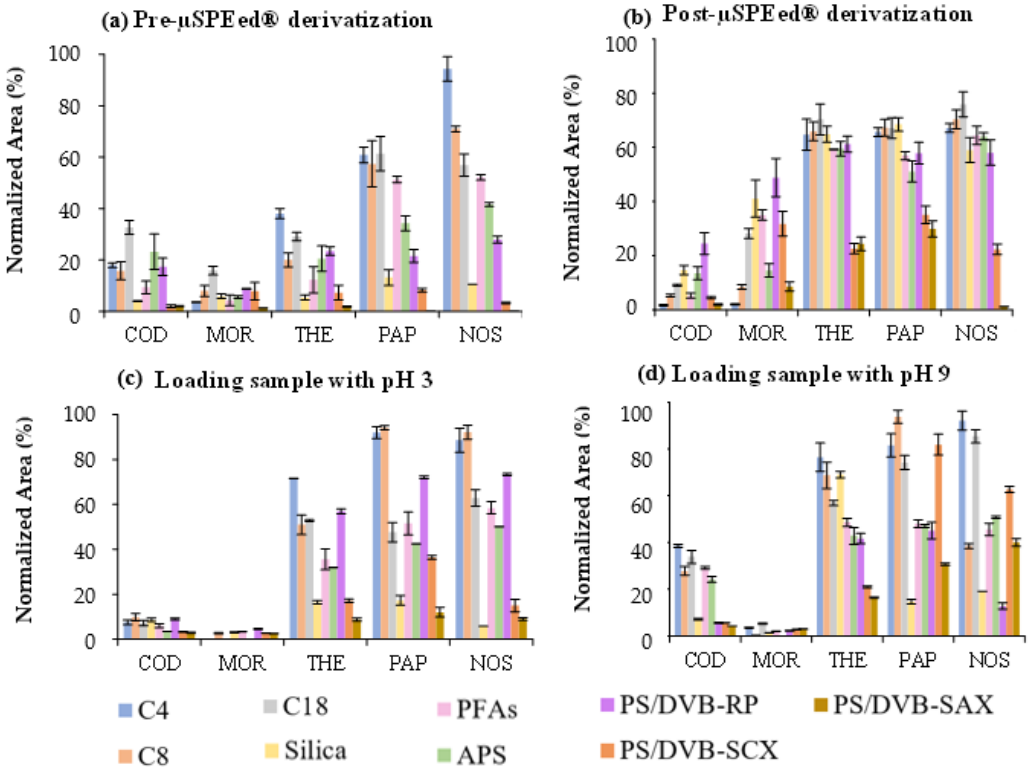


Figure 3. Normalized areas (%) obtained for OAs in the optimization of µSPEed® procedure with nine cartridges: derivatization before (a) and after (b), acidifying to pH 3 (c) or basifying to pH 9 (d) in the loading step. All assays were with 5 mg/L standard solutions in triplicate and error bars indicate the relative standard deviation (RSD, %).

Afterwards, the two selected cartridges were studied by increasing the draw-effect cycles in the loading step to obtain the maximum possible adsorption of 5 mg/L of standard solution and to be able to select the cartridge that could adsorb the most. As shown in Figure 4a, after comparing the areas obtained, it was seen that the PS/DVB-RP cartridge, after 10 cycles, achieved a considerably higher area.

Later, different elution solvents (MeOH unmodified, MeOH with 10% formic acid, MeOH with 10% NaOH and $CHCl_3$) were evaluated with the other optimal conditions with 5 mg/L of standard solution in the loading step. As shown in Figure 4b, MeOH with 10% formic acid was the highest area shown for all analytes.

Finally, as one of the advantages of the µSPEed® technique is the possibility of pre-concentrating the sample extracts to obtain lower instrumental limits, the studies were carried out with the optimal conditions, but with 10, 12 and 15 extract-discard cycles in the loading step. For this purpose, this study was carried out at the low validation level (100 µg/L) to determine whether a pre-concentration could be carried out with the µSPEed® to allow the desired concentration levels to be quantified in the equipment. The recovery values were calculated with their respective simulated values. The values obtained by performing 10 cycles were 91 ± 8% for morphine and around 100% recovery for the rest of OAs; performing 12 cycles, codeine, thebaine and papaverine were around 100% recovery but morphine was 37 ± 12% and noscapine 85 ± 9%; and performing 15 cycles, morphine and noscapine were close to 15% recovery, codeine 40 ± 9%, papaverine 65 ± 9% and thebaine 82 ± 9%. For this reason, the µSPEed® procedure with 10 cycles was selected, which allowed a pre-concentrations factor of 10, obtaining adequate recovery values.

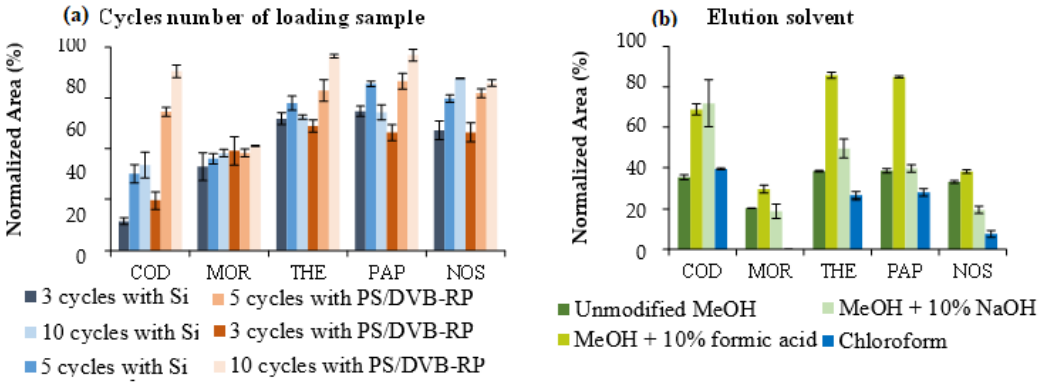


Figure 4. Normalized areas (%) obtained with the silica and PS/DVB-RP cartridge with 3, 5 and 10 draw-effect cycles in the loading step at pH 7 (a) and with PS/DVB-RP cartridge with different elution solvent with pH 7 in the loading step (b). All assays were with 5 mg/L standard solutions in triplicate and error bars indicate the relative standard deviation (RSD, %).

3.3. Validation of proposed method based on µSPEed® followed by GC-MS

The results of the validation of the proposed method based on µSPEed® followed by GC-MS for the quantification of OAs poppy seed teas are shown in Table 2. The regression lines were obtained by least-squares linear regression analysis of the data

provided excellent correlation coefficient (R^2) values between 0.998 and 1.000 for all analytes.

The method showed low MDL and MQL values expressed in µg/L of poppy seed tea (Table 2), obtaining: 0.06 and 0.2 µg/L for papaverine, 0.07 and 0.2 µg/L for noscapine, 0.2 and 1 µg/L for thebaine, 0.3 and 1 µg/L for codeine and 0.5 and 1.6 µg/L for morphine, respectively.

On the other hand, the ME was calculated by comparing the slopes of the matrix and solvent calibration curves. As shown in Table 2, the ME of the proposed methodology was negligible, as all values were within + / - 20%. This means that µSPEed® procedure was able to eliminate all possible matrix effects. For this reason, solvent regression lines could be used to quantify the samples which would simplify the analysis [35].

Accuracy and precision were evaluated at three different concentration levels, 100 µg/L (LL), 400 µg/L (ML) and 800 µg/L (HL). Accuracy was expressed as the average recovery obtained comparing six samples (n = 6) spiked with the corresponding value with their simulated samples. As shown in Table 2, recovery values were adequate according to the guidelines [35] in all validation levels (between 89 and 108%). In addition, satisfactory results were obtained for intra-day and inter-day precision at the three concentration levels, since the RSD values were lower than 20% according to the guidelines [35], the lowest value is 14% (Table 2). Furthermore, as shown in Figure 2, a good selectivity of the method was demonstrated since no interfering peaks were found at the retention time of the target analytes.

The most significant advantages of the proposed method over previously published ones were that this method, in addition to taking into account morphine, codeine and thebaine, also takes into account papaverine and noscapine, which can be even more toxic [17]. In addition, by performing the µSPEed® procedure, it was possible to pre-concentrate the extract 10 times more, thus making the present method have 10-fold lower limits than those of the work of Li et al. (2021) which also uses GC-MS to quantify poppy seed teas, with their MDL and MQL being 2.5 and 10 µg/L, respectively for morphine and codeine and 20 and 50 µg/L for thebaine, respectively [1]. On the other hand, the present method had a large linear response range allowing us to quantify infusions made

with seeds contaminated with double the OAs (40 mg/kg) than the legislated maximum limit (20 mg/kg) as quantified in numerous studies [5-7]. However, the method developed by Powers et al. (2019) that used an HPLC-MS/MS only reached up to 500 µg/mL, i.e. 25 mg/kg in seeds considering a 100% transfer rate [3]. So, different dilutions are needed to quantify the samples when the concentration is outside the calibration range.

Overall, the proposed method based on µSPEed® following by GC-MS analysis proved to be an efficient strategy for the extraction and quantification of five OAs in poppy seed teas, revealing excellent performance in terms of linearity, sensitivity, matrix effects, precision, and accuracy.

Table 2. Validation parameters of the proposed method based on µSPEed® followed by GC-MS for the quantification of opium alkaloids poppy seed teas.

Analytes	Linear range (µg/L)[a]	Matrix-matched calibration (R^2)	MDL (µg/L)[b]	MQL (µg/L)[c]	ME[d]	Accuracy[e]		Precision (%RSD)[e]	
							Recovery (% ± SD)	Intra-day	Inter-day
Codeine	5 - 800	$y = 3.7\times10^{6}x - 4.1\times10^{2}$ (0.999)	0.3	1	8	LL	91 ± 8	9	11
						ML	101 ± 4	4	7
						HL	99 ± 1	1	4
Morphine	5 - 800	$y = 3.0\times10^{6}x - 1.6\times10^{4}$ (0.998)	0.5	1.6	-4	LL	90± 8	8	10
						ML	102 ± 7	7	13
						HL	90 ± 3	3	7
Thebaine	5 - 800	$y = 3.6\times10^{6}x + 9.6\times10^{3}$ (0.999)	0.2	0.7	1	LL	91 ± 4	5	9
						ML	100 ± 10	10	14
						HL	101 ± 3	3	4
Papaverine	5 - 800	$y = 2.9\times10^{7}x - 2.3\times10^{5}$ (1.000)	0.06	0.2	-6	LL	89 ± 4	4	7
						ML	100 ± 7	7	10
						HL	95 ± 6	6	10
Noscapine	5 - 800	$y = 1.6\times10^{7}x - 3.3\times10^{5}$ (0.999)	0.07	0.2	10	LL	93 ± 8	9	13
						ML	102 ± 7	7	10
						HL	108 ± 7	6	11

[a] The linear range is expressed in µg/L of poppy seed tea; [b] MDL: method detection limit is in µg/L of poppy seed tea; [c] MQL: method quantification limit is in µg/L of poppy seed tea; [d] ME (%): matrix effect (dividing the purified matrix slope by the solvent slope – 1) × 100; [e] Accuracy and precision were obtained by spiking samples at three concentration levels: low level (LL, 100 µg/L), medium level (ML, 400 µg/L) and high level (HL, 800 µg/L).

3.4 Transfer study of OAs from poppy seeds to tea infusion

To determine the level of transfer of OAs from poppy seeds to tea infusions, three factors (e.g., water temperature, infusion time, and seed amount) were studied at two levels each (n = 8), studying each condition in triplicate. For this, the seeds (S-1) were spiked to the maximum limit of the legislation (20 mg/kg) [23], previously washed and dried. Each of the studies was performed with 100 mL of deionized water, and after cooling to room temperature (25 ± 1 ºC), they were filtered through a nylon syringe filter and finally, the proposed µSPEed® procedure was applied followed by the GC-MS analysis.

The areas obtained were interpolated on the respective matrix-matched calibration to calculate the concentrations of OAs in the teas. The concentrations obtained were compared as shown in Figure 5a. An ANOVA test was performed to determine the statistically significant differences between each of the studies performed and the transfer ratios (%) were calculated to determine the conditions under which the highest concentration of OAs could be obtained in the tea. By spiking the seeds with the maximum limit established in the legislation and using two quantities of seeds (2 and 4 g), the concentrations that could be obtained in these teas were 0.4 and 0.8 mg/L, respectively if 100% transfer occurs. As can be seen in Figure 5a, with 4 g of seeds at 90 ˚C for 5 min statistically significantly higher concentrations of codeine, morphine and thebaine in tea were obtained.

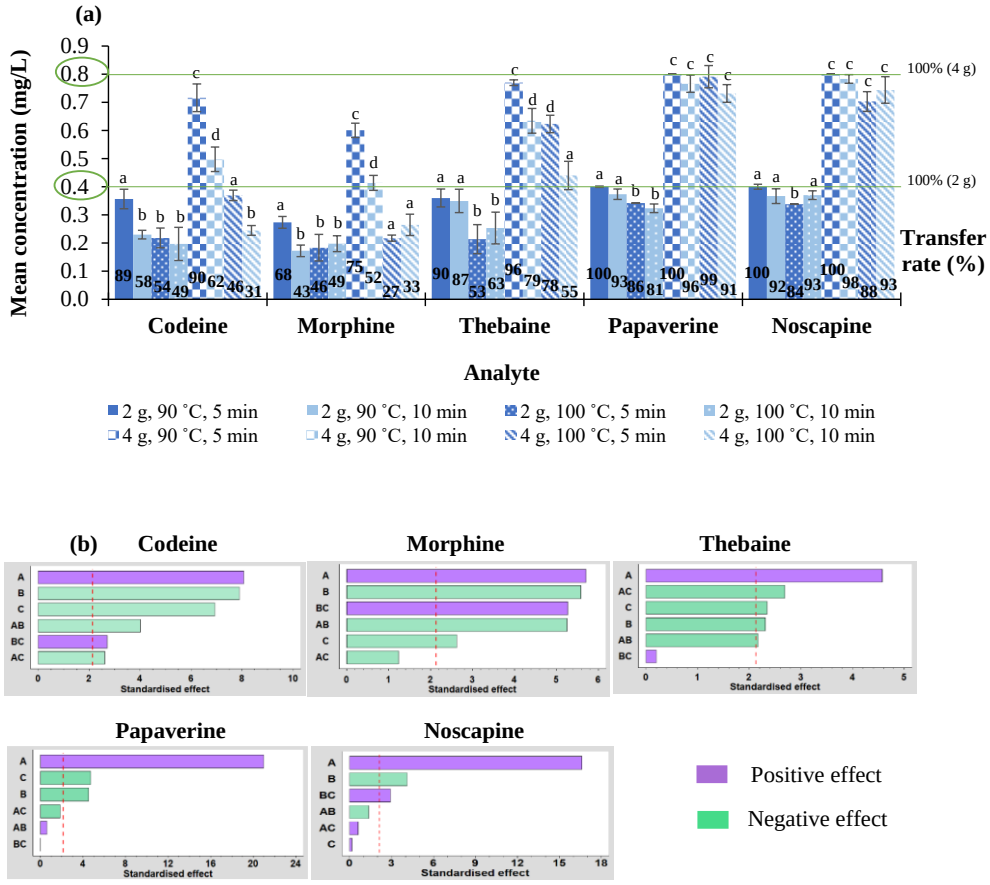


Figure 5. Mean concentrations (n=3) obtained in each transfer study with their transfer rate (%). Equal letters mean that there are no statistically significant differences and different letters mean that there are differences ($p \leq 0.05$) (a). Pareto Chart of the standardized effect of each of the responses (concentration of each analyte), showing the three factors: (A) poppy seed amount (g), (B) infusion time (min), (C) temperature (˚C) (b).

This is because the greater the amount of seeds, the greater concentration of OAs was obtained, i.e., 100 mL of water was still sufficient to be able to extract all the opiates from the seeds. Therefore, transfer ratios (%) lower than 100% should not be due to a lower transfer, but rather to thermal degradation as confirmed by some published works [7], [33], [38], [39]. This degradation is mainly observed in codeine and morphine, which are the most thermally sensitive analytes [38]. Thus, between the studies performed at 90 ˚C and those performed at 100 ˚C statistically significant differences were shown in all analytes, although being much lower in papaverine and noscapine where thermal

degradation was affected less. Regarding infusion time, higher concentrations could be expected with longer times as the contact time between water and seeds increased. However, it was seen that increasing the time could in some cases decrease the concentration of OAs in the tea. This may be due to increased degradation with increasing exposure time of the OAs at high temperatures. All this is confirmed in the Pareto charts in Figure 5b, where effect A (amount of seeds) has a positive effect on all analytes very significantly, effect B (infusion time) has a negative effect on all analytes (being much higher in codeine and morphine) and effect C (temperature) has a negative effect on all analytes except noscapine where there is no statistically significant difference.

Therefore, the conditions selected for their highest rate of transfer or lowest degradation were 4 g at 90 ˚C for 5 min, obtaining 90% transfer in codeine, 75% in morphine, 96% in thebaine, and 100% in papaverine and noscapine.

3.5. Occurrence of OAs in tea infusions from different poppy seeds

To determine the occurrence of OAs in poppy seed tea from different samples of poppy seeds, the conditions with more transfer were used. For this reason, 4 g of each poppy seeds were infused with 100 mL of water at 90 ˚C for 5 min. The studies were done in triplicate, taking a new 4 g in each infusion to determine the variation that the same seed sample can give. After cooling to room temperature, they were filtered through a nylon syringe filter and finally, the µSPEed® procedure was applied followed by the proposed GC-MS analysis. The areas obtained were interpolated on the respective matrix-matched calibration to calculate the concentrations of OAs obtained in each of the teas.

As shown in Table 3, all OAs were quantified in all poppy seed teas analyzed. Furthermore, as can be seen, the calculated standard deviations of the three replicates were in some cases high. This may be due to the heterogeneous contamination that seeds suffer from being an external contamination that depends on multiple factors, such as climate, time of harvest, and variety, among others [5], [6], [8].

Of the four poppy seed teas analyzed, S-1 and S-2 showed the lowest contents of all OAs, being morphine and noscapine the analytes with the highest concentration in both

teas 43 µg/L and 23 µg/L, respectively, and thebaine the lowest, being below the MQL. These samples are labelled as *Papaver rhoeas* L. (Table S1), which does not contain OAs in its latex and therefore its seeds cannot be contaminated. Therefore, the determination of OAs in their infusions confirms the mislabelling of the product as observed in previous studies [5]. S-3 showed a higher content of all OAs, especially morphine 227 µg/L, and S-4 was the sample showing the highest concentration of all OAs, giving up to 1563 µg/L morphine, 254 µg/L codeine, 71 µg/L thebaine, 56 µg/L noscapine and 29 µg/L papaverine. This sample was diluted 1:2 to quantify it within the calibration line and then recalculated to give the final concentration. Considering the transfer rate of 75% for morphine and 90% for codeine, the estimated quantity of poppy seeds S-4 expressed in morphine equivalents is 54 mg/kg which is higher than the legislated limit (20 mg/kg). It should be noted that, the seeds were purchased in 2021 and therefore do not comply with current legislation and the legislation says that if the seeds were marketed before 1 July 2022, they can remain in trade until the best-before date is reached [23]. In addition, the acute dose of morphine equivalents set by EFSA in 2018 is 10 µg per kg body weight. So, for a 20 kg child the acute dose would be 200 µg and for a 60 kg adult it would be 600 µg morphine equivalents. Following the recommendations of some consumer manufacturers of 3 cups of tea per day, if the seeds used for the infusion were S-4, it would be 648 µg morphine equivalent. Therefore, the consumption of tea from these seeds poses a health risk for both adults and children. Furthermore, it should be noted that the legislation only applies to morphine and codeine, and as observed in all teas, considerable amounts of thebaine, papaverine and noscapine are also present, which can be even more toxic as claimed by the health authorities [17]. This highlights the need to control these analytes and the importance of developing analytical methods to analyse them to develop adequate legislation accordingly.

Table 3. Occurrence of opium alkaloids (main concentration (µg/L) of three replicates ± standard deviation) in poppy seed tea with four different samples of poppy seeds (S-1, S-2, S-3, and S-4).

Code sample	Morphine	Codeine	Thebaine	Papaverine	Noscapine	ME[a]
S-1 tea	25 ± 3	4 ± 1	< MQL	8.13 ± 0.02	20.6 ± 0.2	26
S-2 tea	43 ± 25	1.8 ± 0.4	< MQL	8.2 ± 0.2	23 ± 2	43
S-3 tea	227 ± 39	58 ± 42	56 ± 36	11 ± 6	29 ± 6	239
S-4 tea	1563 ± 33	254 ± 35	71 ± 13	29 ± 2	56 ± 1	1614

[a] ME: morphine equivalents = morphine + 0.2 × codeine; <MQL: below the method quantification limit.

Results from other works that quantify OAs in home-brewed poppy see tea also showed considerably high amounts of OAs in the infusions. For example, in the work of Li et al. (2021), the total mean concentration of morphine, codeine, and thebaine estimated for poppy seeds, from the analysis of three repeated infusions of 2 g of seeds with 6 ml of water acidified with 5% lemon juice (heated at 90ºC, 10 min), ranged from 1.1 to 1926, 20.2 to 311, and 9.0 to 100 mg/kg, respectively. These authors indicated that most OAs were extracted in the first brew (around 80% of the total opiate yield), so potential overdose could occur in some tea samples when a large quantity of seeds is used for brewing [1]. In the work of Powers et al. (2019) different teas were prepared (i.e. 85 g of seeds in 150 ml, 6 g of seeds in 30 ml and 35 g of seed powder in 100 ml with 5% lemon juice in water, for 10 min at 23ºC and 94ºC) and the concentrations of morphine, codeine, and thebaine estimated in the seeds from the poppy teas were <1-2788 mg/kg, <1-247.6 mg/kg, and <1-124 mg/kg, respectively [3]. In the work of Montgomery et al. (2019) the average concentrations of morphine (mg/kg of seed) determined from the beverage extractions were found to be between 155-223 mg/kg. In this work, four poppy seed tea samples were prepared utilizing extractants commonly described in online drug forums (i.e. lemonade, lemon flavoured iced tea, 10% concentrated lemon juice and water) and compared with a control solution containing acetic acid (1g of seed in 5 ml). These results showed that all the beverages can extract a significant amount of morphine from the

surface of poppy seeds, despite the extractions being carried out at room temperature for 2 h [4].

In short, the consumption of poppy seed tea can become potentially dangerous, especially since the consumers can add any amount of seeds they want to the tea and do not know the concentration of OAs they may have. In addition, as demonstrated in the present study, despite some thermal degradation, there is a high transfer rate of morphine and codeine as well as the rest of the OAs. Therefore, the study of this type of sample is essential to highlight the danger of this consumption practice and to warn the authorities of the need to control this type of practice that may pose a potential risk to the health of the consumer. Even if the maximum amount is currently set at 20 mg/kg and no new seed samples with higher levels can be marketed from 1 July onwards, the authorities should instruct the manufacturers of these products to establish recommendations as to the method of preparation and the maximum daily amount, with indications that adverse health effects may occur if these recommendations are not followed.

4. Conclusions

A simple and efficient method was developed for the quantification of morphine, codeine, thebaine, papaverine and noscapine in poppy seed teas by µSPEed® following by GC-MS analysis. Of the nine µSPEed® cartridges evaluated, the PS/DVB-RP was the most efficient for OAs and allowed, with optimized conditions, to eliminate possible matrix effects of the extract and to concentrate it 10 times, thus decreasing the MDLs and MQLs. Once the method was successfully validated, it was applied to study the rate of transfer of opiates from poppy seeds to tea. For this, the influence of three factors (e.g., temperature, time, and quantity) was evaluated at two levels and it was determined that 4 g of seeds at 90 ˚C for 5 min were the conditions that produced the highest rate of transfer or less thermal degradation (75% morphine, 90% codeine, 96% thebaine and 100% papaverine and noscapine). With these conditions, four teas made with different poppy seeds were quantified and in all of them all the OAs were determined, and even one of them showed a high morphine content (1563 µg/L) and codeine (254 µg/L), indicating that the seeds used had 53 mg/kg, twice the concentration of the maximum limit legislated

by the EU (20 mg/kg) as morphine equivalents. Therefore, it is necessary to warn the population of the danger of not following the recommended amounts, since it may be thought that the OAs content in the seeds is not very high.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Registration of the different poppy seeds used to make tea infusion with their description, species and origin; Table S2: Summary of the number of transfer studies performed with the three factors under study and the two levels of each.

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Funding: This research was funded by MCIU/AEI/FEDER, UE for project RTI2018-094558-B-I00. This work was also funded by FCT-Fundação para a Ciência e a Tecnologia through the CQM Base Fund - UIDB/00674/2020, and Programmatic Fund - UIDP/00674/ 2020, and by ARDITI-Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação, through the project M1420-01- 0145-FEDER-000005 - Centro de Química da Madeira - CQM+ (Madeira 14-20 Program). The authors also acknowledge the financial support from Fundação para a Ciência e Tecnologia and Madeira 14-2020 program to the Portuguese Mass Spectrometry Network through PROEQUIPRAM program, M14-20 M1420-01-0145-FEDER-000008.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Gema Casado-Hidalgo would like to thank the Rey Juan Carlos University for providing her a mobility grant to carry out a pre-doctoral stay in the CQM – Centro de Química da Madeira.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Information

Table S1. Registration of the different poppy seeds used to make tea infusion with their description, species and origin.

Code	Description	Specified specie by labelling	Cultivation type	Origin	Best-before date	Recommendations for use
S-1	Physically: blue poppy seeds	P. rhoeas	No ecological cultivation	Not specified	01/2025	Not specified
S-2	Physically: blue poppy seeds	P. rhoeas	No ecological cultivation	Not specified	12/07/2022	3 cups per days of 4 g of poppy seeds for 10 min
S-3	Not specific on the label. Physically: blue poppy seeds	Not specified	Ecological cultivation	Turkey	04/2025	Not specified
S-4	Not specific on the label. Physically: blue poppy seeds	Not specified	No ecological cultivation	Not specified	20/11/2022	Not specified

Table S2. Summary of the number of transfer studies performed with the three factors under study and the two levels of each.

RUN	Levels of each factor		
	Temperature (ºC)	Amount (g)	Time (min)
1	90	2	5
2	90	2	10
3	90	4	5
4	90	4	10
5	100	2	5
6	100	2	10
7	100	4	5
8	100	4	10

Nota de aplicación 1:

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ACKNOWLEDGEMENTS

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ABSTRACT

The innovative µSPEed technique has been applied using digiVOL Digital Syringe (Figure 1) for accurate and precise extraction and pre-concentration of the main opium alkaloids (morphine, codeine, thebaine, papaverine, noscapine and oripavine) from poppy seed tea using a PS/DVB-RP cartridge. The infusion sample has been loaded 10 × 250 µL and the elution was with a small amount of methanol with 10% formic acid (2 × 125 µL), pre-concentrating the analytes 10-fold before analysis by HPLC-DAD, demonstrating the high preconcentration capacity of this microextraction technique.

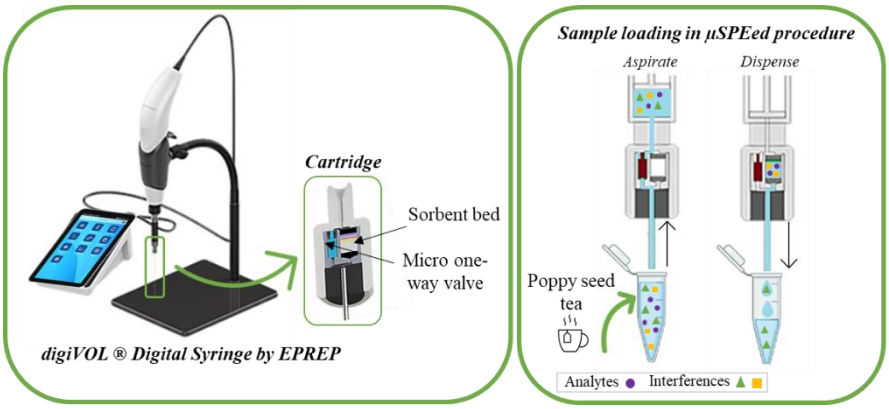

Figure 1. DigiVOL Digital Syringe with cartridge structure and scheme of sample loading in µSPEed procedure.

INTRODUCTION

Opium alkaloids (OAs) are secondary metabolites that are present in milky latex sap of opium poppy plant (*Papaver somniferum* L.). The main OAs present in the latex are morphine, codeine, thebaine, papaverine, noscapine and oripavine (Figure 2). For this reason, this traditional plant is widely used for medicinal purpose due to its pharmacological properties[1,2].

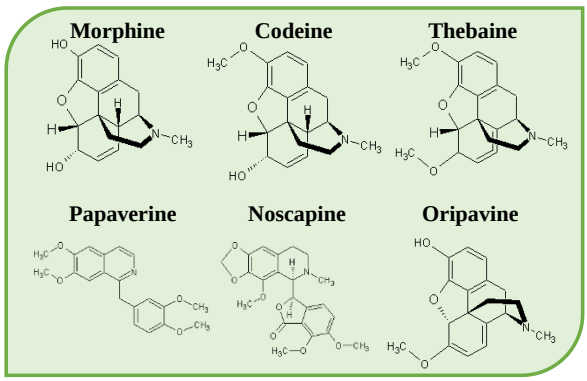

Figure 2. Structure of main opium

OAs can be introduced into the food chain from poppy seed of this plant. These

seeds are increasingly used in some food, such as bakery products, as toppings for salads or yoghurts, or even to produce teas and oil[3,4]. Poppy seeds hardly contain any OAs, but they can be contaminated with the OAs present in the latex of this plant due to harvesting practices or insect damage. Consumption of contaminated food can lead to false positives in drug tests and may cause adverse health effects, such as nausea and vomiting, drowsiness, respiratory problems, and dependence, especially for the most vulnerable people and including more serious cases of intoxication, especially with the consumption of tea made from poppy seeds[5]. For this reason, the European Food Safety Authority (EFSA) has recommended the analysis of OAs in different matrices and has established an acute reference dose (ARfD) of 0.01 µg of morphine/kg body weight[1]. However, due to a lack of control in recent years, numerous food alerts have been reported, 34 since 2005, with high levels of morphine in poppy seeds, up to almost 400 ppm[6]. Therefore, due to the potential health risks described above, the European Commission recently published Regulation (EU) 2021/2142, which has entered vigour on July 1, 2022, that sets maximum levels for OAs, expressed in morphine equivalents (morphine + 0.2 × codeine), in bakery products (1.5 mg/kg) and in poppy seeds (20 mg/kg)[7].

For the analysis of these compounds, liquid chromatography coupled to a tandem mass detector (HPLC-MS/MS) is mainly used. However, many routine food quality control laboratories have diode array detectors (DAD). Therefore, there is a need to develop methodologies with a sample preparation step that allows not only purifying the extract to remove possible matrix interferences, but also pre-concentrating the extract to allow lower instrumental detection limits. To date, there are many works that use solid-phase extraction (SPE), but in addition to requiring more time and solvent consumption, none of them allows to achieve high preconcentration factors of the extract.

The innovative µSPEed technique makes it possible to decrease time and solvent consumption, obtaining methodologies more in line with green chemistry, as well as to obtain high preconcentration factors, contributing to low instrumental limits thanks to high preconcentration capacity. In addition, there is a large variety of commercial cartridges that allow their use with many types of analytes of different natures. The most suitable for opium alkaloids resulted to be the PS/DVB-RP polymer-based cartridge.

Therefore, this application note describes a µSPEed-based analytical methodology for the extraction of opium alkaloids from poppy seed teas. Using a PS/DVB-RP cartridge to load 10 × 250 µL of sample and the target analytes were eluted with 2 × 125 µL of methanol[8].

PROCEDURE

Sample preparation

Teas were elaborated with poppy seeds (Figure 3). They were prepared according to International Standard ISO 3103 protocol[9]. For this purpose, 2 g of poppy seeds was infused with boiling water (100 ˚C) for 5 min. After, the sample was cooled to room temperature and filtered through a nylon syringe filter (0.45 µm) before µSPEed process.

Figure 3. Opium poppy seeds.

µSPEed Extraction Workflow

All extractions were performed on PS/DVB-RP (3 µm/300 Å) µSPEed cartridge by EPREP such as show Figure 4. First, cartridge was activated with an aspiration-dispense cycle of 250 µL of methanol followed by conditioning with 250 µL of water. Then, infusion sample was loaded onto µSPEed cartridge (ten aspiration-dispense cycles of 250 µL) and eluted with two aspiration-dispense cycles of 125 µL, pre-concentrating the analytes 10-fold. All steps were carried out at 15 µL/sec in extract-discard mode (each volume aspirated in all cycles was then discarded, after the dispense step, in a waste vial). Figure 4 shows the µSPEed extraction workflow followed and the aspiration-elution working mode.

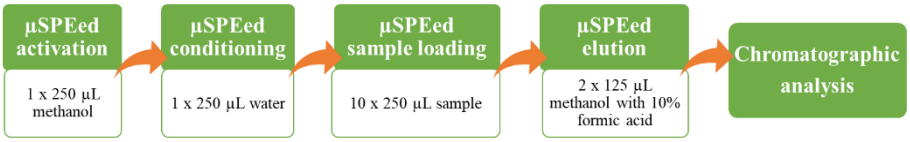

Figure 4. Diagram of µSPEed procedure

Chromatographic analysis

The separation (Figure 5) and determination of each of six opium alkaloids was carried out with an Agilent 1260 Infinity II HPLC system (Agilent Technologies, Madrid, Spain) coupled to diode array detector (G7117C 1260 DAD HS). The column used was an InfinityLab Poroshell 120 EC-C18 column (3.0 mm i.d. × 150 mm, 2.7 µm particle size) equipped with an InfinityLab Poroshell 120 EC-C18 guard column (3.0 mm i.d., 2.7 µm particle size) both from Agilent Technologies (Madrid, Spain) at 30 °C. The mobile phase used was milli-Q water containing 0.1% trifluoroacetic acid and acetonitrile in gradient elution mode with a total analysis time of 9 min. The wavelength used was 212 nm for all analytes.

RESULTS

Analytical parameters

Good performances of the method were demonstrated in terms of precision and accuracy using the proposed methodology with the µSPEed technique. The results obtained were evaluated according to the criteria established for precision and accuracy in the SANTE/11312/2021 document[10].

Accuracy was evaluated in terms of recovery percentage (%) for standard solutions at 0.4 µg/mL prepared in water and three spiked levels were evaluated in poppy seed teas (0.2, 0.4 and 0.8 µg/mL). The 0.4 µg/mL spiked level was selected according to the Regulation (EU) 2021/2142, maximum level concentration established poppy seed[7] and considering 2 g of poppy seeds in 100 mL of water[9]. Standard solutions and samples were both subjected to the µSPEed process prior to HPLC-DAD analysis. Furthermore, an additional water or infusion sample (simulated, post-extraction spiked sample) was subjected to the µSPEed process and spiked immediately after the µSPEed procedure to estimate the percentage recovery (%). After HPLC-DAD analysis, the areas obtained from the sample spiked before the µSPEed process and the samples spiked after the µSPEed process were compared. Table 1 shows the accuracy results of six µSPEed purified standard solutions and samples (n = 6). The results obtained with the proposed

methodology showed good accuracy with recovery percentages between 85 and 104% for the standard solutions and the poppy seed infusions. Since, according to the validation guideline, recovery percentages should be between 70-120%[10].

Furthermore, the precision of the method, expressed as relative standard deviation (RSD %), was evaluated for both standard solutions and poppy seed teas. Precision was evaluated in terms of intra-day precision (repeatability), six replicates in one day (n = 6), and inter-day precision (reproducibility), three replicates in three different days (n = 9). Standard solutions were carried out at 0.4 mg/L and samples were carried out at three concentration levels (0.2, 0.4 and 0.8 mg/L). Table 1 shows the RSD values, which are all below 20% as required by the validation guidelines[10]. Specifically, the intra-day values are ≤9%, and the inter-day values are ≤11% at all concentrations studied.

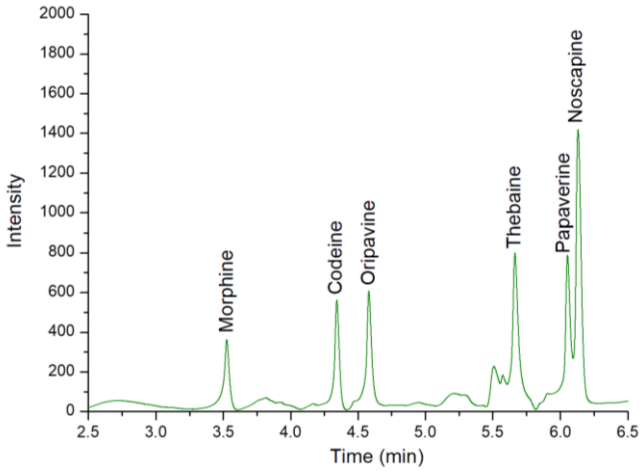

Figure 5. Chromatographic separation of six OAs at 8 µg/mL under optimised conditions with the HPLC-DAD method at 212 nm.

CONCLUSION

The µSPEed technique has proven to be a suitable technique for the extraction and purification of the main opium alkaloids, morphine, codeine, thebaine, papaverine, noscapine and oripavine, in poppy seed tea samples. The method has provided adequate recovery rates between 85-104% and good intra- and inter-day precision (≤ 11%) for all

analytes. Moreover, this technique is simple, fast and environmentally friendly, as it uses significantly lower volumes of organic solvents than other conventional techniques such as solid-phase extraction. Moreover, the solvents used are not very harmful and hazardous, as the sample is loaded in water and eluted with a small aliquot of methanol with formic acid (2 x 125 µL). In this sense, the good performance of µSPEed was demonstrated with its application on poppy seed tea samples for OAs analysis, contributing to the requirements of food safety and green analytical chemistry.

ACKNOWLEDGEMENTS

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Table 1. Accuracy (recoveries ± SD %) and intra-day and inter-day precision (RSD %) obtained using the µSPEed procedure proposed following by HPLC-DAD analysis for the determination of opium alkaloids in standard solutions prepared in water at 0.4 µg/mL and poppy seed tea at 0.2, 0.4 and 0.8 µg/mL concentration.

Analyte	Standard solution			Poppy seed tea samples		
	Accuracy (recoveries ± SD %)	Intra-day precision (RSD %)	Inter-day precision (RSD %)	Accuracy (recoveries ± SD %)	Intra-day precision (RSD %)	Inter-day precision (RSD %)
Morphine	89 ± 3 ML	3 ML	7 ML	95 ± 1 LL 91 ± 5 ML 94 ± 2 HL	1 LL 6 ML 3 HL	2 LL 7 ML 3 HL
Codeine	92 ± 8 ML	9 ML	8 ML	100 ± 1 LL 85 ± 6 ML 97 ± 4 HL	1 LL 7 ML 4 HL	2 LL 8 ML 5 HL
Thebaine	88 ± 6 ML	6 ML	6 ML	96 ± 2 LL 97 ± 2 ML 100 ± 4 HL	3 LL 2 ML 4 HL	3 LL 5 ML 4 HL
Papaverine	91 ± 8 ML	8 ML	11 ML	103 ± 5 LL 94 ± 6 ML 97 ± 5 HL	4 LL 6 ML 5 HL	9 LL 11 ML 11 HL
Noscapine	89 ± 8 ML	9 ML	11 ML	101 ± 2 LL 104 ± 4 ML 97 ± 4 HL	2 LL 4 ML 4 HL	3 LL 8 ML 5 HL
Oripavine	100 ± 1 ML	1 ML	7 ML	92 ± 6 LL 86 ± 3 ML 100 ± 2 HL	7 LL 4 ML 2 HL	5 LL 5 ML 2 HL

Accuracy: n = 6; Intra-day precision: n = 6, 1 day; Inter-day precision: n = 9, 3 days. LL: low level (0.2 µg/mL); ML: medium level (0.4 µg/mL); HL: high level (0.8 µg/mL).

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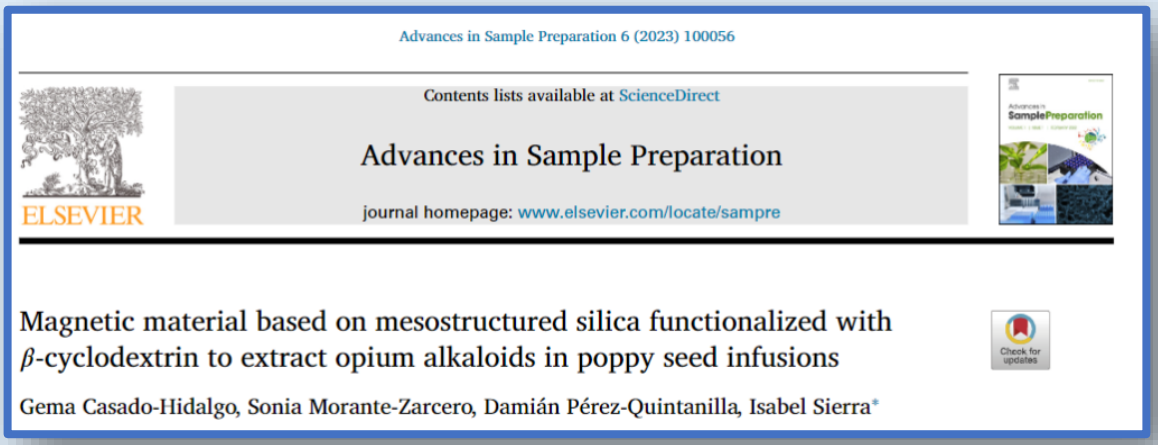
Artículo 5:

Magnetic material based on mesostructured silica functionalized with β-cyclodextrin to extract opium alkaloids in poppy seed infusions

Advances in Sample Preparation 6 (2023) 100056

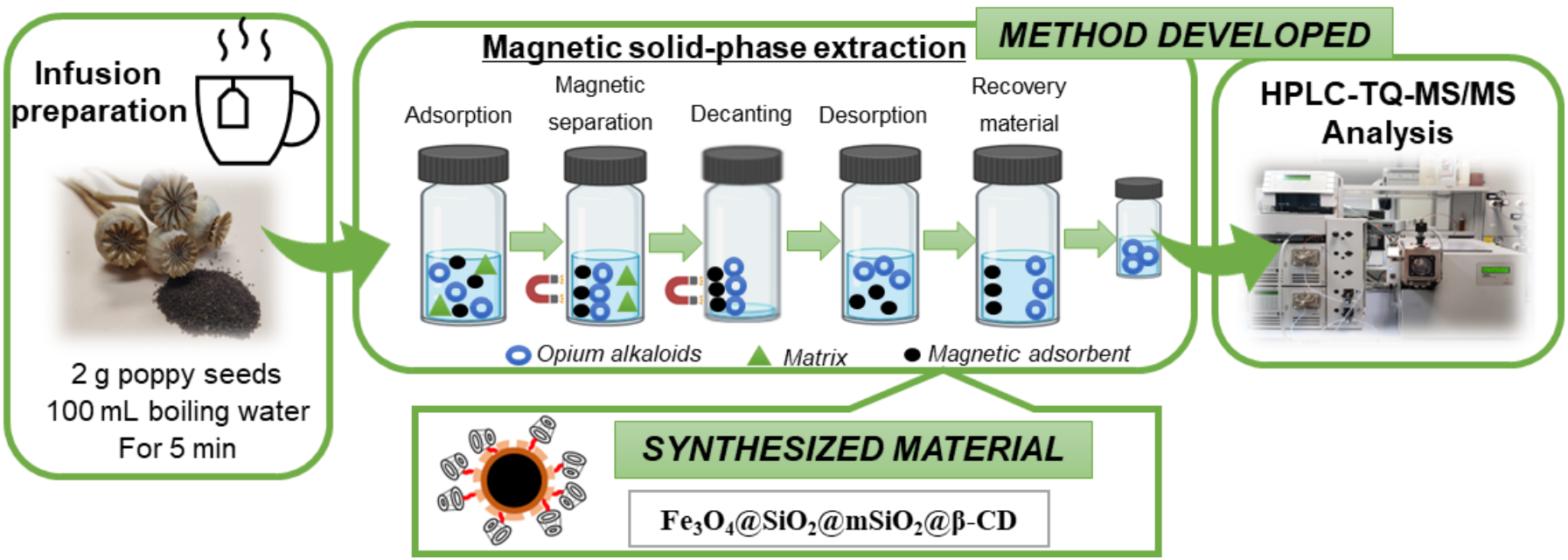


PUBLICATION INFORMATION

Journal Advances in Sample Preparation

Publisher Elsevier

DOI https://doi.org/10.1016/j.sampre.2023.100056


Infusion preparation
2 g poppy seeds
100 mL boiling water
For 5 min
Magnetic solid-phase extraction
METHOD DEVELOPED
Adsorption
Magnetic separation
Decanting
Desorption
Recovery material
Opium alkaloids
Matrix
Magnetic adsorbent
HPLC-TQ-MS/MS Analysis
SYNTHESIZED MATERIAL
$Fe_3O_4@SiO_2@mSiO_2@\beta$-CD

Highlights:

- Efficient, simple and rapid method developed to quantify opium alkaloids
- Optimized synthesis to form magnetic adsorbents functionalized with β-CD
- β-CD is a ligand that favours the OAs interaction significantly for their extraction
- Magnetic mesostructured silica composite with β-CD is efficient for opium alkaloids
- Consumption of poppy seed infusions may be a health risk to both adults and children

ABSTRACT

Sample preparation methods tend to be fast, simple, with the use of new adsorbents and with lower amounts of organic solvents to make them more environmentally friendly. In this work, a magnetic solid-phase extraction (MSPE) protocol for the quantification of six opium alkaloids (OAs) in poppy seeds infusions has been optimised followed by analysis with liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). For this purpose, a mesostructured silica magnetic composite with β-CD (Fe_3O_4@SiO_2@mSiO_2@β-CD) was synthesised and characterized. To obtain the highest level of functionalisation, three proportions of β-CD monomer/material (12.5, 25 and 100%) were determined, demonstrating that 25% showed the highest level. Adsorption studies were carried out in water at different pH (1, 2, 7, 9 and 11) and times (1, 5, 10 and 20 min), confirming the high adsorption capacity at pH 9 for 1 min. Then, the elution conditions of MSPE procedure were optimised (50 mg material, 2 mL of water/EtOH at 50% with 1% formic acid for 1 min). The methodology was successful validated with low detection and quantification limits, negligible matrix effect and good recovery values (89-94%). The methodology was applied to analysis of home-made infusions with four different poppy seeds, observing in one of them a worrying amount of OAs which imply a content in the seeds of at least four times that of the legislation.

KEYWORDS: Opium alkaloids; poppy seed infusions; magnetic solid-phase extraction; β-cyclodextrin; mesostructured silica magnetic composite; liquid chromatography-tandem mass spectrometry.

1. Introduction

Sample preparation prior to instrumental analysis plays an important role in analytical methodologies. In fact, to reduce interferences of complex matrices, efficient sample treatment methods are needed for selective isolation and concentration of target analytes at suitable levels before the analysis. In addition, the purification step can considerably increase the lifetime of the equipment by obtaining cleaner extracts. However, sample preparation is still the most labour-intensive analytical step and can affect both accuracy and precision of the results [1].

In general, sample preparation is based on extraction techniques and one of the most used is solid-phase extraction (SPE). However, SPE have some limitations and drawbacks, including the wide use of organic solvents and waste generated, but also because it requires too much labour, is time-consuming and relatively expensive. For this reason, different extraction techniques have emerged as greener and sustainable alternatives to classical sample preparation procedures, with the aim of improving the selectivity and sensitivity of analytical methods, while simultaneously reducing the deleterious side effects of traditional techniques for both the operator and the environment [2]–[5]. In this regard, some methodologies have been developed in the last years for the analysis of different natural toxins, based on new sample preparation techniques trying to use fewer organic solvents and sorbents, to be more environmentally friendly, and to synthesize new effective sorbent materials, to be able to avoid matrix effects and preconcentrate the extracts to fewer instrumental limits [6]. For example, protocols based on miniaturized SPE (μ-SPE) with functionalized mesostructured silicas have been proposed recently for the analysis of toxic alkaloids [7], [8] and good results have been obtained thanks to the advantages of this type of materials, such as large surface area, porosity, facile functionalization, good mechanical and chemical stability [9].

A powerful SPE alternative is magnetic solid phase extraction (MSPE), based on the use of magnetic sorbents. It has several advantages of high adsorption efficiency, simple operation, low cost, and time saving. MSPE involves dispersing the magnetic material in a solution with the sample/extract for a few minutes. Once it is at equilibrium, it is recovered with the help of a magnetic field and finally the analytes are desorbed, which

can avoid tedious filtration, centrifugation, or sedimentation steps [10]. Magnetic sorbents are mainly based on magnetite (Fe_3O_4) nanoparticles (MNPs), that show excellent features such as high magnetic moment, simple synthesis and low production cost, besides small size and high surface area that increases their sorption capacity [11]. However, to stabilize bare MNPs and to achieve a selective extraction towards the target analytes, it becomes necessary to modify their surface with some functional groups. For this reason, MNPs are usually coated with an adequate organic or inorganic material, and different MNP composites have been prepared with carbon, polymers, silica, metal-organic frameworks, etc. The coating layer prevents oxidation and increases the durability of the MNPs, but also provides an adequate colloidal dispersion [11], [12]. In that respect, core coated MNPs with different mesostructured silicas (SBA-15, MCM-41, KIT-6, etc.) have been successfully applied as sorbents in MSPE for extraction of different analytes [9]. Compared with analogous amorphous silica coated particles (i.e., $Fe_3O_4@SiO_2$), these materials displayed higher binding capacity faster mass transfer and higher extraction efficiencies owing to the mesostructured silica coating [13]. For example, recently MNPs coated with amorphous and mesostructured silica, both functionalized with C_{18} or C_8, were evaluated for MSPE to determine opium alkaloids (morphine, codeine, thebaine, papaverine, noscapine and oripavine) in edible poppy seeds samples. The best results were obtained with the non-functionalised mesostructured silica coated MNPs ($Fe_3O_4@SiO_2@mSiO_2$) and the sample extracts were purified in just 4 min using 50 mg of sorbent. However, for morphine and oripavine the recoveries obtained were around 50%, which may be since they were the smallest analytes and showed higher intra-particle diffusion and were, therefore, more difficult to desorb, resulting in lower recoveries [13].

On the other hand, in recent years, there has been an increasing interest of the scientific community the use of cyclodextrin (CD) with different purposes, which are natural products obtained from the enzymatic degradation of starch and therefore have a low price, negligible environmental impact and non-toxicity [14]–[16]. CDs are a family of natural torus-shaped cyclic oligosaccharides composed of 6 (α-CD), 7 (β-CD), and 8 (γ-CD) D-glucopyranose units linked by α-1,4-glycosidic bonds, possessing hydrophobic inner cavities and hydrophilic outer faces. The unique structures of CD make possible the formation of inclusion complexes with aliphatic and aromatic non-polar compounds of

suitable size by a variety of forces such as hydrogen bonding, hydrophobic and van der Waals interaction, especially when they are in aqueous media [14], [15]. The use of MNPs composites as support is a versatile strategy to enhance the active surface where the CDs must be located. In this regard, some works have been explored these materials for the analysis of PAHs [17], pesticides [18], etc., but to the best of our knowledge, they have not yet been used for opium alkaloids (OAs).

The seeds of the *Papaver somniferum* L. plant, commonly known as opium poppy, are increasingly being used to elaborate infusions [19]–[21]. The problem with this practice is that seeds can be contaminated with OAs [13], [22]. The consumption of infusions with poppy seed could lead to cases of severe intoxications [23]–[27] because the concentration of OAs in poppy seeds can be dangerously high [13], [22]. However, the OAs content in the seeds is not the same as what can appear in the infusion, because in this process some of these compounds can be thermically degraded in different percentages depending on the analyte and heating conditions [28]. Therefore, it is necessary to study the levels of OAs that can be found in poppy seed infusions to estimate the real risk that the ingestion of this product represents for the population.

Accordingly, the potential use of a mesostructured silica magnetic composite functionalized with β-CD (Fe_3O_4@SiO_2@mSiO_2@β-CD) as MSPE sorbent in a new proposal for the extraction and quantification of six OAs in infusions is assessed. After an initial comparison with magnetite and amorphous silica-coated magnetite containing β-CD (Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD) to evaluate its performance, the analytical features of the developed method have been established to obtain a selective and more environmentally friendly protocol and to improve the recoveries obtained with previous methodologies. Finally, home-made infusions prepared with different commercially available poppy seeds have been analysed by using the validated method.

2. Materials and methods

2.1 Reagents and materials

Standards of morphine, codeine, thebaine and oripavine were received from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine, morphine-d3 and codeine-d3 (internal

standard, IS) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1000 mg/L in methanol, and working standard solutions were prepared at 1 mg/L in water/ethanol 75/25 (v/v) with 10% formic acid. All of these were stored in darkness at -20 ˚C.

Ferric chloride 6-hydrate ($FeCl_3 \cdot 6H_2O$) 99% and ferrous chloride 4-hydrate ($FeCl_2 \cdot 4H_2O$) 99% were purchased from Labkem (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Tetraethylorthosilicate (TEOS) 98%, hexadecyltrimethylammonium bromide (CTAB), 3-isocyanatopropyltriethoxysilane 98% and β-cyclodextrin ≥ 97% were purchased from Sigma-Aldrich. Ethanol absolute, formic acid (98%) and ammonia 32% (w/w), isopropanol, toluene, pyridine, and diethyl ether were of synthesis grade and purchased from Scharlab (Barcelona, Spain). N, N-dimethylformamide (DMF) were purchased from Merk (Darmstadt, Germany). Acetonitrile, methanol, and ethanol used were HPLC-MS quality and were purchased from Scharlab (Barcelona, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). The Nd-Fe-B magnet (5 × 5 × 2 cm) with force 200 kg used in the MSPE procedure was obtained from Superimanes S.L. (Sevilla, Spain).

2.2 Infusions with poppy seed samples

Four samples of edible seeds (PS-01, PS-02, PS-03 and PS-04) were purchased in Spain in early to mid-2022 from supermarkets and herbalists. More information detailed in Table S1. To preparate the infusions (PS-I-01, PS-I-02, PS-I-03 and PS-I-04) with these poppy seeds, International Standard ISO 3103 protocol was used [29]. For it, 2.00 ± 0.01 g of poppy seeds were weighted, and 100 mL of boiling water was added. The infusion was covered for 5 min and then, it was removed and cooled to room temperature. Finally, the infusion was filtered through a nylon syringe filter and the MSPE procedure was applied with Fe_3O_4@SiO_2@mSiO_2@β-CD followed by HPLC-MS/MS analysis. The studies were done in triplicate, taking again 2 g of seeds in each infusion to determine the variation that the same seed batch could give, as observed in previous studies [13], [30].

2.3 Preparation of the magnetic materials with β-CD

The schematic preparation process of the magnetic materials is shown in Fig. 1. First, the β-CD monomer was synthetised to functionalise the MNPs. For this purpose, 2.5 g of previously dried β-CD were added to 50 mL of dry pyridine. Once dissolved, 1.56 g of 3-isocyanatopropyltriethoxysilane was added (with a 1:3, β-CD:3-isocyanatopropyltriethoxysilane, stoichiometry). The reaction was maintained at 80 ºC for 24 h under magnetic stirring at 500 rpm in an inert N_2 atmosphere. Then, the solvent was evaporated, and the resulting light-yellow solid was dried at room temperature under vacuum for 24 h. Secondly, such as shown in Fig.1, MNPs were prepared using co-precipitation according to the work of Zhang and Shi [31]. Then, the first layer of amorphous silica (Fe_3O_4@SiO_2) was added to the magnetite, followed by a second layer of mesostructured silica (Fe_3O_4@SiO_2@mSiO_2) (Fig.1), according to the protocol used in our previous work [13]. Thirdly, the final material (Fe_3O_4@SiO_2@mSiO_2) was functionalised with the β-CD (Fig.1). For this purpose, the amount of β-CD monomer (weight percentage related to mass of material) added to the magnetic material were optimised (12.5, 25 and 100%). Finally, 25% by weight of the formed β-CD monomer (0.75 g) was added to the magnetic material (3 g) with approximately 50 mL of DMF. The reaction took place at 70 °C for 24 h under magnetic stirring at 500 rpm and in N_2 atmosphere. After the reaction time, the material was collected with the help of an external magnet, washed with approximately 30 mL of each solvent: DMF, ethanol and diethyl ether and dried under vacuum for 24h.

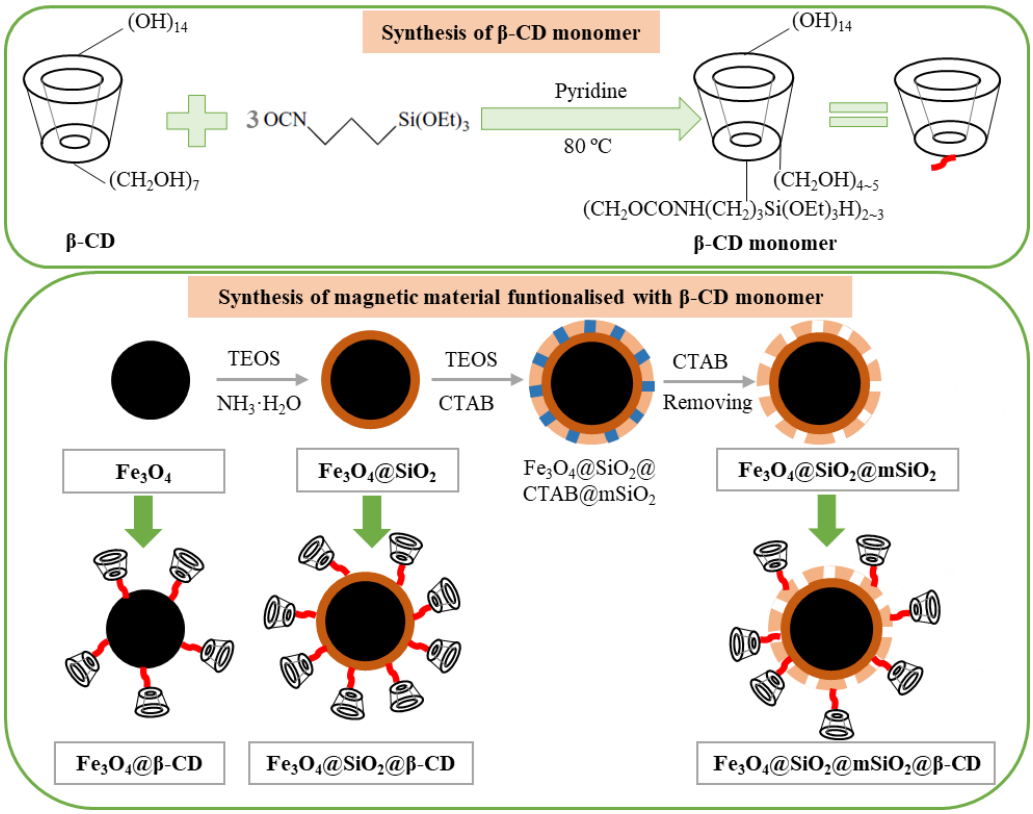


Fig. 1. Scheme of synthesis of β-CD based monomer and synthesis of each of magnetic composite functionalized with β-CD.

2.4 Characterization of the magnetic materials with β-CD

The magnetic materials with β-CD were characterized by Attenuated Total Reflection Fourier-Transform Infrared (ATR-FT-IR), Elemental analysis (% N), Nitrogen gas adsorption–desorption isotherms and X-ray diffraction (XRD). Details of the equipment and conditions can be found in Supporting Information S1.

2.5 Discontinuous adsorption studies of Fe_3O_4@SiO_2@mSiO_2@β-CD as MSPE sorbent and comparison with Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD.

To evidence that the mesostructured silica magnetic composite with β-CD is an efficient magnetic material to do the MSPE procedure a discontinuous adsorption study was performed. In addition, Fe_3O_4@SiO_2@mSiO_2@β-CD was compared with the

Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD materials to confirm their higher adsorption capacity. For this purpose, 50 mg of each of the three synthesised materials was added to 2 mL of water with different pH values (1, 3, 7, 9 y 11) containing 1 mg/L of each of the six analytes. Finally, the supernatants of each of the different solvents were analysed by HPLC-MS/MS after a fixed adsorption time (1, 5, 10 and 20 min). All the studies were done in duplicate, and the adsorption percentages of each of the materials on the different solvents were calculated and compared to determine the optimal adsorption conditions. The adsorption (%) was calculated by the following formula: 100 – (value of the supernatant divided by value of the simulated sample, sample subjected to the same procedure but spiked at the concentration level prior to HPLC-MS/MS analysis).

2.6 Adsorption kinetic and isotherm experiments with Fe_3O_4@SiO_2@mSiO_2@β-CD material.

For adsorption kinetics, 50 mg of the material was added to a water solution at pH 9 containing each of the six analytes under study (2 mL, 1 mg/L). The mixtures were subjected to ultrasound at different times (1 – 20 min), and the supernatants in equilibrium were analysed by HPLC-MS/MS. The adsorption capacity was calculated by Equation (S1) in Table S2. The adsorption kinetics were determined by Lagergren's pseudo-first-order [32], pseudo-second-order [33] and intra-particle diffusion kinetic models [34] (Table S2). For adsorption isotherm, a series of 2.0 mL solutions of different concentrations of the six analytes (0.1 – 40 mg/L) was added to 50 mg of the material under optimum time. The isotherms of the six opium alkaloids adsorption on the magnetic particles were analysed using the commonly used Langmuir [35] and Freundlich [36] models (Table S2).

2.7 Optimization of MSPE conditions with Fe_3O_4@SiO_2@mSiO_2@β-CD material.

For this purpose, 2 mL of 1 mg/L of each of the six analytes was used. The parameters of the MSPE procedure that were optimised were the following: the amount of adsorbent (between 5 and 50 mg); the adsorption time (from 1 to 20 min); the pH of the initial solution (ranging from 1 to 11); the desorption eluent (water with ethanol, acetonitrile, or

methanol at 50%) with 1% of formic acid. All studies were carried out in triplicate. In addition, different batches of the Fe_3O_4@SiO_2@mSiO_2@β-CD material were evaluated to ensure that the results obtained with all of them were reproducible. For this purpose, RSD (relative standard deviation, %) of the recoveries obtained with 0.4 mg/L (medium validation level) standard solutions at the optimum conditions used for the MSPE procedure was calculated.

2.8 Optimised MSPE methodology and HPLC-MS/MS analysis for quantification of opium alkaloids in poppy seeds infusion

In the optimised analysis methodology, 50 mg of Fe_3O_4@SiO_2@mSiO_2@β-CD (conditioned with 1 mL of water at pH 9 for 1 min in ultrasound were added with 2 mL of the poppy seed infusion (previously filtered and adjusted at pH 9) and then, the mixture was treated with 1 min ultrasound until the analytes arrived at an adsorption equilibrium with the adsorbent material. Later, the material with analytes was separated by an external magnet from the solution, and the analytes were eluted from the magnetic particles with 2 mL water/ethanol at 50% with 1% formic acid for another minute. Subsequently, 950 µL was recovered and 50 µL of a 1 mg/L solution of morphine-d3 and codeine-d3 was added as an internal standard and analysed by HPLC-MS/MS.

Analysis of OAs was carried out with a Varian 1200/1200 LC (Varian Ibérica, Madrid, Spain) equipped with a ProStar 410 autosampler (100 µL loop) coupled with a TQ tandem mass spectrometer detector (1200 L TQ) with an electrospray ionization (ESI) ion source. For data acquisition, the system was MS Workstation Varian with a version 6.8. Chromatographic separation was performed as mentioned in our previous works [37], [38] using a C18 KromaPhase 100 column (150 × 2.0 mm, 3.5 µm particle size, Scharlab, Barcelona, Spain) at 30 °C. The injection volume was 10 µL (partial injection), and the flow rate was set at 0.25 mL/min. The gradient elution was used with a mobile phase of water (A) and acetonitrile (B), both with 0.1% of formic acid as follows: 90−30% A (0−6 min), 30−90% A (6−9 min), and 90% A (9−11 min) for column reequilibration. Positive mode electrospray ionization (ESI +) with MRM mode was used for mass spectrometry acquisition. The drying and nebulizer gas was N_2. The drying gas was set at 350 °C and

22 psi, and the nebulizer gas was set at 58 psi. The collision gas used was argon at 1.90 mTorr and the detector voltage of 1480 V. The capillary voltage was held at 5000 V and shielded at 600 V and the cone voltage 72 V. In Table S3 was showed the optimal mass spectrum parameters.

2.9 Method validation

Currently, there is no official regulation on analytical performance requirements for OAs in food or feed. For this reason, the method validation was performed following the criteria described in the SANTE/11312/2021 document [39], in regulation EC No 401/2006 [40], and in the Q2(R1) ICH guidelines (International Council for Harmonisation, 2005) [41]. The method validation was done in terms of linearity, method detection and quantification limits (MDL, MQL), matrix effect, accuracy, precision, and selectivity (more details in Supporting Information S2). To carry out the validation, washed and dried poppy seeds were used to avoid variation of OAs content in the same batches as confirmed in previous work [13]. After infusion following International Standard ISO 3103 protocol [29] (2.00 ± 0.01 g with 100 mL of boiling water for 5 min) the infusion was cooled to room temperature, filtered through a nylon syringe filter and spiked at three concentration levels: a low (0.2 mg/L), medium (0.4 mg/L) and high (0.8 mg/L). The spiked levels were selected according to the legislation (20 mg/kg in seeds) which calculated at 2 g/100 mL of infusion was 0.4 mg/L.

3. Results and discussion

3.1 Preparation and characterization of β-CD monomer

To attach the β-CD to the mesoporous silica structure, two or three triethoxysilyl groups were covalently attached to the β-CD via carbamate bonds (Fig.1). For this purpose, the β-CD is reacted with 3-isocyanatopropyltriethoxysilane with a 1:3 stoichiometry [42]. However, the reaction medium used in different works to prepare del monomer is different, in DMF [42], in pyridine [43], [44] and in toluene [45]. Therefore, a previous study was carried out to synthetise to monomer with each of the three solvents to determine the most efficient one.

First the ATR-FT-IR spectrum of the β-CD is shown in Fig. S1a where the characteristic bands around 100cm^{-1} can be seen. Subsequently, the spectra of the β-CD monomer were made with the three solvents (Fig. S1b) in which, in addition to the bands characteristic of B-CD, the bands of the 3-isocyanatopropyltriethoxysilane ligand were also seen. In toluene, the characteristic IR bands of the 3-isocyanatopropyltriethoxysilane ligand are less intense. The stretching bands νC-H(-CH_3 groups) (2850 cm^{-1}) and νC-H(-CH_2 groups) (2900 cm^{-1}) of the ethoxy groups (-OCH_2-CH_3) of the 3-isocyanatopropyltriethoxysilane ligand anchored to the β-CD moieties appear much less pronounced than in DMF this also happens with the band at 1083 cm^{-1} due to C-O-C bonds. In addition, a higher yield of monomer was obtained in DMF. These facts suggest a lower yield in the reaction between de CDs groups and the 3-isocyanatopropyltriethoxysilane ligands, using toluene as solvent medium, that can be explained due to a lower solubility of the β-CD in toluene. The β-CD monomer was isolated from the reaction medium through DMF evaporation. Nevertheless, the use of DMF presents some drawbacks, like its high boiling point and hydrogen bonding formation with the hydroxyl groups of the monomer, which means that for total removal of the DMF from the monomer, high vacuum, high temperature and long drying time are required, and even with that extreme conditions some rests of DMF can still be found in some situations, as the presence of carbonyl bands of the DMF in the ATR-FT-IR spectra of the β-CD monomer (1650 cm^{-1}) revealed. To avoid these drawbacks, pyridine was tested, as the reaction solvent, which due to its lower evaporation point and weaker interaction with the monomer, allowed better solvent removal, and produced the highest yield (90%). The ATR-FT-IR spectra of the monomers (Fig. S1b) show all the characteristic adsorption bands of the 3-isocyanatopropyltriethoxysilane ligand anchored to the β-CD moieties. In addition, the main bands due to β-CD groups can be detected in the range of 900-1400 cm^{-1} and at 3000 cm^{-1} that it belongs to the valence vibrations of the C-H bonds in the CH and CH_2 groups of the β-CD. Finally, the band at 3400 cm^{-1} is characteristic of the stretching band of the O-H groups. All these IR bands show the appropriate modification of the β-CD monomer with the 3-isocyanatopropyltriethoxysilane ligand. For all the above-mentioned reasons, pyridine was selected as the solvent to obtain the β-CD monomer.

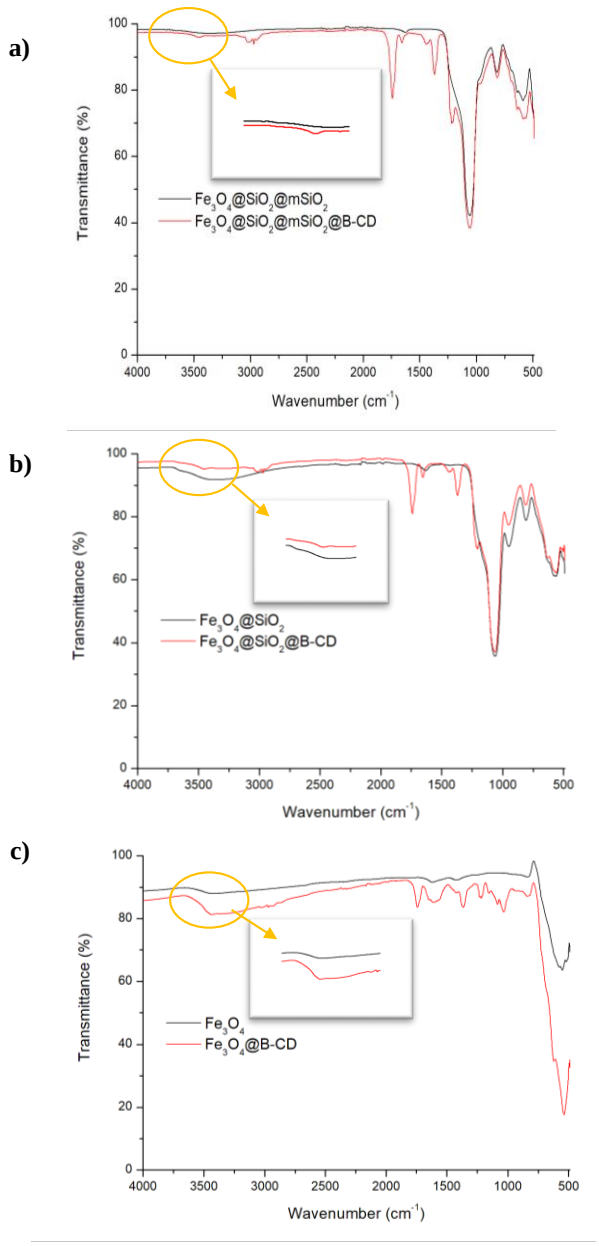


Fig. 2. ATR-FT-IR spectra of mesostructured silica magnetic composite with β-CD (Fe_3O_4@SiO_2@mSiO_2) before and after of functionalization with β-CD (a) and spectra of Fe_3O_4@SiO_2 (b) and Fe_3O_4 (c) before and after of functionalization with β-CD for comparative purposes. Functionalization was carried out with 100% of β-CD.

3.2 Characterization of Fe_3O_4@SiO_2@mSiO2@β-CD material compared with Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD.

The mesostructured silica magnetic composite was characterised before and after functionalisation. In addition, the Fe_3O_4 and Fe_3O_4@SiO_2 materials, before and after functionalisation, were also characterised for comparative purposes.

First, ATR-FT-IR spectra was performed of Fe_3O_4@SiO_2@mSiO_2 material before and after functionalisation with 100% of β-CD monomer to verify the formation of the silica layer and the functionalisation with the β-CD monomer. As shown in Fig. 2a, the infrared bands at 575 cm^{-1} were observed, which were associated with the stretching and twisting vibrational modes of the Fe-O bonds of magnetite at tetrahedral sites. Besides, the band at 3400 cm^{-1} is due to the protonation states by extensive hydrolysis of the interfacial water molecules with Fe_3O_4. Furthermore, the lack of this band in the functionalised materials due to the deprotonation of the hydroxyl groups, suggests a bonding between magnetite and β-CD monomer in the formed complex. In addition, the material both before and after functionalisation showed the characteristic band of Si-O bands at 1000 cm^{-1}. Therefore, it was determined that the material was functionalised with β-CD monomer and that the silica layer has formed around the particles. Besides, ATR-FT-IR spectrum of Fe_3O_4@SiO_2 (Fig. 2b) and Fe_3O_4 (Fig. 2c) before and after of functionalisation are also performed and the signals observed in the spectra also confirm the presence of silica and β-CD.

Then, an elemental analysis was done to optimise the amount of β-CD monomer added to the magnetic material in the synthesis. For this purpose, three different proportions of β-CD monomer (12.5, 25 and 100%) added to Fe_3O_4@SiO_2@mSiO_2 material were evaluated and the functionalisation (mmol β-CD/g material) was estimated with the N content (%). As shown in Fig. 3, the Fe_3O_4@SiO_2@mSiO_2 material synthesised with 100% of β-CD monomer, (i.e., 1g of β-CD monomer + 1g of material), resulted in less functionalisation than with 25%. This fact can be explained because of the pore entrance blocking that can happens when a large amount of ligand is used in the modification process, avoiding the functionalization of free -OH groups inside de pores. Adding 12.5% decreases the degree of functionalisation considerably, with a similar value

that in the case of 100%. Therefore, it was decided to perform the functionalisation with 25% β-CD monomers in all materials, because a similar trend was observed in the other two materials (Fe_3O_4 and Fe_3O_4@SiO_2) such as shown in Fig. 3. However, the one with the highest degree of functionalisation was the one with a single silica layer (Fe_3O_4@SiO_2), with 0.147 mmol ligand/g as a maximum with 25% of β-CD monomer. This could be because, although the mesostructured silica bilayer gives a higher number of -OH groups and a higher surface area (402 vs. 220 m^2/g), its pore size is smaller than the non-ordered silica (34.9 vs. 127.4 Å). Bulky ligand, like the β-CD monomer, could provoke steric impediments in the pores entrance, and the functionalisation taking place mainly the surface, resulting in a lower functionalisation. Nevertheless, with the addition of 25%, a similar functionalization degree was obtained with Fe_3O_4@SiO_2@mSiO_2@β-CD material (0.134 mmol ligand/g). For this reason, 25% was the optimum to allow surface and in-pore functionalisation in Fe_3O_4@SiO_2@mSiO_2@β-CD material.

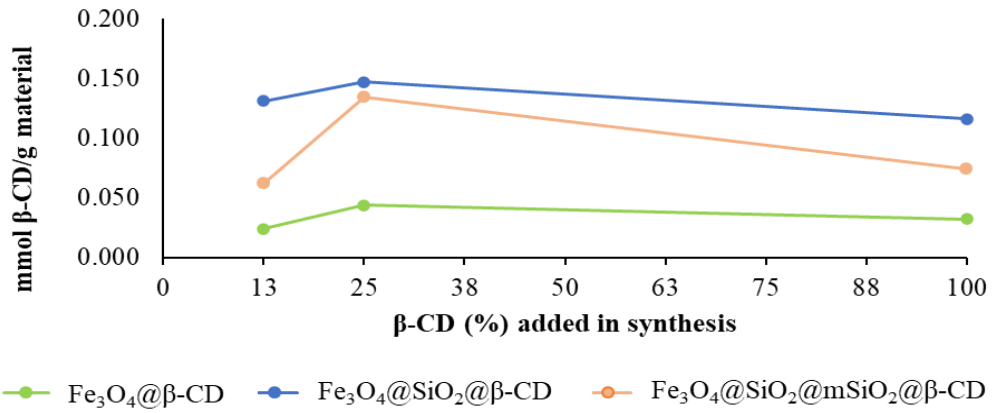


Fig. 3. Functionalization degree of the three synthetized materials (mmol β-CD/g material) at three β-CD monomer proportions (weight percentage related to mass of material) added to the magnetic material (12.5, 50 and 100%) obtained by elemental analysis of N.

In addition, the N_2 adsorption-desorption isotherms of mesostructured silica magnetic composite Fe_3O_4@SiO_2@mSiO_2@β-CD before and after of functionalization were performed (Fig. 4a). The adsorption isotherms can be assigned as Type IV, according to the IUPAC classification [46], which is characteristic of mesoporous materials. The isotherms show an initial part characteristic of monolayer adsorption and a significant

increase of the adsorbed amount at intermediate relative pressures, characteristic of a multilayer filling mechanism. An H4 hysteresis loop is shown in material both before and after of functionalization (Fig. 4a). The textural properties of the Fe_3O_4@SiO_2@mSiO_2 material are shown in Table 1. As it can be seen, the material experiments a decrease in specific surface area BET (S_{BET}) and pore volume after functionalization due to anchoring of the β-CD monomers to the surface and inside the pores. In addition, the Fe_3O_4@SiO_2 material was also characterised before and after functionalisation (Fig. 4b) and as it was expected, lower surface area and pore volume was found in these materials (Table 1).

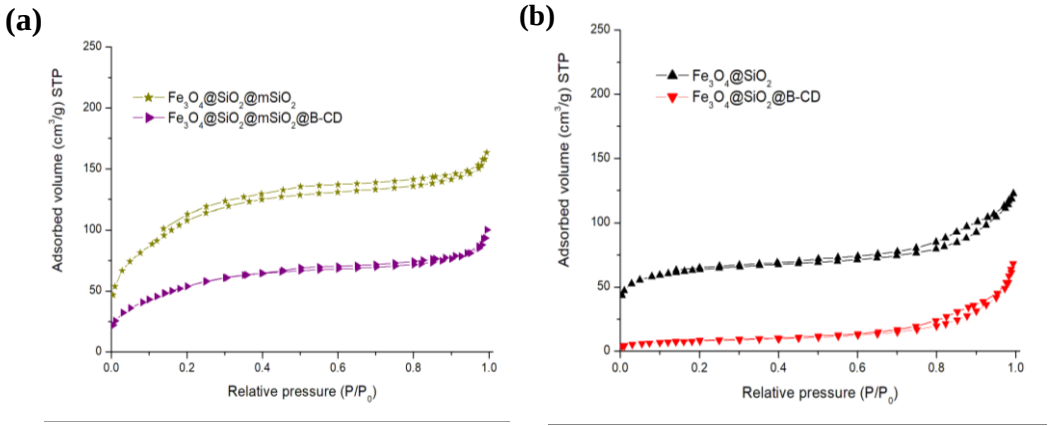


Fig. 4. N_2 adsorption-desorption isotherms of mesostructured silica magnetic composite before and after of functionalization (a) and for comparative purposes those of Fe_3O_4@SiO_2 (b).

Table 1. Textural properties of the magnetic materials synthesized.

Material	S_{BET} (m^2/g)[a]	Pore volume (cm^3/g)[b]	Pore diameter (Å)[c]
Fe_3O_4@SiO_2	220	0.17	38.9, 91.9, 127.4
Fe_3O_4@SiO_2@β-CD	28	0.08	38.6, 91.4, 128.9
Fe_3O_4@SiO_2@mSiO_2	402	0.23	20.4, 34.9
Fe_3O_4@SiO_2@mSiO_2@β-CD	203	0.13	20.5, 39.0

[a] S_{BET}: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method.

[b] Total pore volume was measured at relative pressure (P/P_0) = 0.97.

[c] Pore diameter estimated by using the BJH (Barrett, Joyner and Halenda) model applied on the desorption Branch.

Finally, XRD was done to mesostructured silica magnetic composite before and after of functionalisation with β-CD to know its long-range order. The low-angle XRD pattern (Fig. S2a) reveals the Miller index (100) characteristic of materials with mesoscopic order, and in the wide-angle XRD patterns (Fig. S2b) the material before and after functionalisation showed the theoretical pattern of Fe_3O_4 with six discernible diffraction peaks in the 2θ region from 20º to 70º (220, 311, 400, 422, 511 and 440) that correspond with the Miller index diffraction peaks of the magnetic core (JCPDS card: 19-0629), showing that it is still present after modification.

3.3 Discontinuous adsorption studies with Fe_3O_4@SiO_2@mSiO_2@β-CD as MSPE sorbent compared with Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD

To evidence that the mesostructured silica magnetic composite with β-CD is an efficient magnetic material to do the MSPE procedure a discontinuous adsorption study was performed. To do this, 2 mL of water at different pH values (1, 2, 7, 9 and 11) with 1 mg/L of each of the six analytes and 50 mg of material. For comparative purposes the same studies were carried out with the Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD materials. The addition of the silica layers improved the adsorption capacity of the materials since, as shown in Fig. 5, the lowest adsorption (%) was in general with Fe_3O_4@β-CD material, then with Fe_3O_4@SiO_2@β-CD and the highest adsorption was obtained with Fe_3O_4@SiO_2@mSiO_2@β-CD. On the other hand, the optimum adsorption time was 1 min since increasing the adsorption time does not increase the adsorption. Moreover, the more acidified the medium, the lower the adsorption and at pH 11 it is like that obtained at pH 7, being pH 9 the pH at which the best adsorption results were obtained. Consequently, the highest adsorptions obtained were with Fe_3O_4@SiO_2@mSiO_2@β-CD with water at pH 9 and for 1 min, obtaining adsorptions of 95% for morphine, 94% for codeine, 97% for thebaine, 99% for papaverine, 95% for noscapine and 93% for oripavine. This confirms that the use of a β-CD functionalised mesostructured silica magnetic composite, Fe_3O_4@SiO_2@mSiO_2@β-CD, is more efficient than the material with magnetite alone or amorphous silica, due to its higher adsorption capacity.

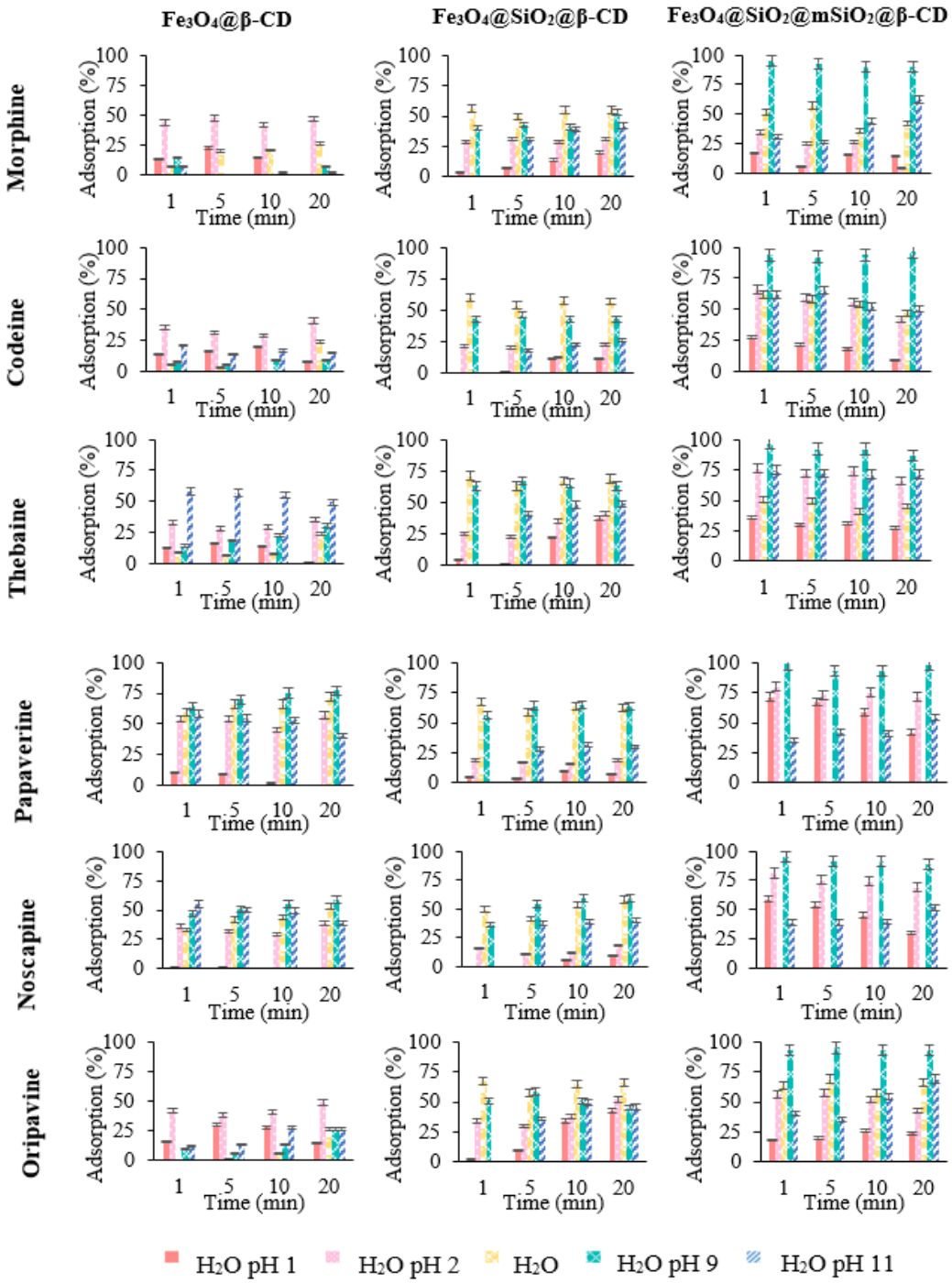


Fig. 5. Adsorption of morphine, codeine, thebaine, papaverine, noscapine and oripavine obtained at different times with the three magnetic materials. The adsorption (%) was calculated: 100 – (value of the supernatant divided by value of the simulated sample).

An additional test was performed with the non-functionalised material (Fe_3O_4@SiO_2@mSiO_2) which was used in a previous work [13] to quantify OAs in poppy seeds. In that work, adequate recovery values were obtained but those of morphine and oripavine only achieved up to 50% at all validation levels. Therefore, to improve this material, it was decided to add a ligand that interacts with these analytes to achieve better recovery values. To verify that the good adsorption values, obtained in the previous assay, are not only due to the interactions between the free -OH groups of the silica, but also to the formation of inclusion complexes with the β-CD, the optimal conditions selected for the material with β-CD and the material without β-CD were compared. For this purpose, the adsorption was determined with a 1 mg/L standard solution in water at pH 9 at different times. The adsorptions obtained with Fe_3O_4@SiO_2@mSiO_2 were significantly lower than with Fe_3O_4@SiO_2@mSiO_2@β-CD, around 70% for thebaine, papaverine and noscapine, 60% for codeine and only 40% for morphine and oripavine. These results show an improvement of this type of material with mesostructured silica and, in addition, the high efficiency of β-CD in the extraction of OAs.

3.4 Adsorption kinetic and isotherm experiments with mesostructured silica magnetic composite Fe_3O_4@SiO_2@mSiO_2@β-CD

In Fig. S3a is shown the adsorption isotherms of the six OAs. This shows how the amount of OAs adsorbed increases over the equilibrium time until it reaches a maximum and remains constant. In addition, Langmuir [35] and Freundlich [36] models were applied (equations 5 and 6 in Table S2). As shown in Fig. S4b, the R^2 obtained by Langmuir model was more closed to 1 than by Freundlich model. So, adsorption occurred monolayer on the uniform surface of material [47].

As shown in Fig. S3b the adsorption kinetics is fast for all OAs. It is due because in the first minute all adsorption is obtained and kept it constant. The adsorption kinetics were determined by Lagergren's pseudo-first-order [32], pseudo-second-order [33] and intraparticle diffusion kinetic models (equations 2-4 in Table S3). The results were compiled in Fig. S4a and the important data of the three-kinetics model were shown in Table S4. The kinetics does not comply with pseudo-first order. The linear regression

coefficients (R^2) were closed to 1 in the pseudo-second-order kinetics, indicating a chemical adsorption mechanism [47]. In addition, all the analytes showed a low intraparticular diffusion rate with Kp values of -0.008 and -0.011 mg/g min^2.

3.5 Optimization of MSPE conditions with mesostructured silica magnetic composite Fe_3O_4@SiO_2@mSiO_2@β-CD

Once the good adsorption capacity of the material has been verified, with the adsorption solvent (water at pH 9) and the equilibrium time (1 min), the desorption and the amount of material required to obtain good recovery values were optimized. For this purpose, 2 mL of 1 mg/L of each of the six analytes in water with pH 9 was used. The desorption eluent was evaluated with 1 min of elution time by ultrasound. The desorption eluent evaluated were water with ethanol, acetonitrile, or methanol at 50% all of them with 1% of formic acid. As shown in Fig. S5, the mixture of 50% water/ethanol with 1% formic acid showed the highest recovery percentages. It was 94% for morphine, 98% codeine, 97% thebaine, 85% papaverine, 90% noscapine and 91% oripavine.

In addition, different amounts of magnetic composite (5, 10, 25 and 50 mg) were evaluated to determine if lower amounts of adsorbent material could be used. As shown in Fig. S6, as the amount of material decreased the recovery values decreased, so the optimum amount of material to obtain good recovery values was 50 mg.

3.6 Method validation

The analytical methodology proposed for the quantification of six OAs in poppy seed infusions was carried out in terms of linearity, limits of detection and quantification of the method, matrix effect, accuracy, precision inter and intra-day and selectivity. The validation results are shown in Table 2. Calibration lines between 0.005 or 0.001 and 1 mg/L with adequate R^2 between 0.996 and 0.999 were obtained and the deviation of the back-calculated concentrations of the calibration standards from the true concentrations in the matrix calibration lines were between -0.3 and 10.6%. Therefore, these results demonstrated the good linearity of the method, which claims good linearity when the

deviation of back calculated concentrations is ≤ ± 20% [39]. In addition, the matrix effect was calculated by comparing the slopes of the matrix calibration curves after the proposed method with the solvent calibration curves. No matrix effect was observed for poppy seed infusions after performing the proposed method with a purification step by MSPE with Fe_3O_4@SiO_2@$mSiO_2$, as the matrix effect values were between -2.1 and 5.8% (Table 2), so for all analytes the matrix effect was < +/- 20%. This means according to the validation guideline [39] that the developed purification procedure was able to eliminate all possible matrix effects for the six target analytes in the poppy seed infusion. The MDL and MQL values were sufficiently low to be able to detect and quantify the concentration of OAs in infusions originating from seeds with the quantity established in the legislation on poppy seeds (20 mg/kg) prepared with the ISO protocol [29], being 1.5 and 5 µg/L for oripavine, 0.3 and 1.3 µg/L for morphine and codeine, 0.1 and 0.3 µg/L for thebaine, papaverine and noscapine, respectively (Table 2). The limits obtained were lower than those achieved by other researchers such as Li et al. which were between 10 and 50 µg/L using gas chromatography coupled to mass spectrometry (GC-MS) [21].

Accuracy and precision were evaluated at three different concentration levels, low (0.2 mg/L), medium (0.4 mg/L) and high (0.8 mg/L). Good recovery values were shown at all validation levels for all analytes, showing values between 83 and 99 % (Table 2), which complies with the appropriate values established in the validation guidelines of between 70 and 120% (Table 2). In addition, satisfactory results were obtained for intra-day and inter-day precision at the three concentration levels, since the RSD values were lower than 10 and 11% respectively (Table 2), which complies with what is established in the validation guides, since they are lower than 20%. In addition, a good selectivity of the method was obtained, since when comparing the chromatograms of the extracted ions obtained for each of the OAs in a standard solution with those obtained in the sample, it was obtained that the variation of the t_R was ≤ 0.1 min and the ion ratios of the sample extracts were within ± 30% (relative abundance) of the mean of the standards for each analyte, as established by the validation guidelines [39]. Regarding the results of the validation parameters for poppy seed infusion samples, in the present work, besides having successful recovery values and inter- and intra-day precision values, good quantification limits were achieved (between 0.3 and 5 µg/L),

Table 2. Validation parameters of the proposed methodology for the quantification of six OAs in poppy seed infusions.

Analytes	Linear range (mg/L)	Matrix-matched calibration (R^2) [a]	MDL (µg/L) [b]	MQL (µg/L) [c]	ME [d]	Accuracy [e]		Precision	
						Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-day Precision (RSD %)	Inter-day Precision (RSD %)
Morphine	0.005-1	$y = 7.3\times10^{7}x + 1.2\times10^{6}$ (0.999)	0.3	1.3	5.8	91 ± 4^{L} 95 ± 3^{M} 90 ± 3^{H}	92 ± 3	4^{L} 3^{M} 3^{H}	8^{L} 4^{M} 9^{H}
Codeine	0.005-1	$y = 1.4\times10^{7}x + 1.1\times10^{6}$ (0.999)	0.3	1.3	-2.1	96 ± 9^{L} 97 ± 2^{M} 88 ± 4^{H}	94 ± 5	10^{L} 2^{M} 4^{H}	11^{L} 5^{M} 11^{H}
Thebaine	0.001-1	$y = 1.3\times10^{9}x + 1.1\times10^{7}$ (0.996)	0.1	0.3	2.5	93 ± 7^{L} 99 ± 6^{M} 90 ± 4^{H}	94 ± 6	7^{L} 6^{M} 4^{H}	8^{L} 7^{M} 10^{H}
Papaverine	0.001-1	$y = 2.7\times10^{9}x - 2.6\times10^{6}$ (0.996)	0.1	0.3	1.9	86 ± 3^{L} 88 ± 2^{M} 94 ± 5^{H}	89 ± 3	3^{L} 2^{M} 5^{H}	10^{L} 9^{M} 6^{H}
Noscapine	0.001-1	$y = 2.9\times10^{9}x + 4.9\times10^{7}$ (0.997)	0.1	0.3	4.7	83 ± 4^{L} 91 ± 2^{M} 93 ± 1^{H}	89 ± 2	4^{L} 2^{M} 1^{H}	11^{L} 8^{M} 9^{H}
Oripavine	0.005-1	$y = 1.1\times10^{8}x + 1.8\times10^{5}$ (0.999)	1.5	5	1.3	83 ± 6^{L} 90 ± 3^{M} 93 ± 2^{H}	89 ± 4	6^{L} 3^{M} 2^{H}	9^{L} 8^{M} 7^{H}

[a] The calibration line is in the units: mg/L; [b] MDL: method detection limit; [c] MQL: method quantification limit; [d] ME: matrix effect (purified matrix slope / solvent slope -1) × 100; [e] Accuracy and precision were obtained by spiking infusion with poppy seeds at three concentration levels: low (L, 0.2 mg/L), medium (M, 0.4 mg/L) and high (H, 0.8 mg/L); Accuracy: n = 6; Intra-day precision: n = 6, 1 day; Inter-day precision: n = 9, 3 days.

3.7 Comparison of the proposed methodology with previously methods for OAs

One of the most important advantages of the methodology proposed in this work is that the adsorbent material used is a magnetic material, which allows the developed methodology to be faster and simpler than the classical solid phase extraction (SPE) used in other works to quantify OAs [38]. Even the developed methodology is faster and simpler compared to other previously published MSPE methodologies. For example, the work of Li et al. (2021) although they only used 5 mg of material, required 20 min of adsorption, a centrifugation to accelerate the subsequent separation with the magnet and 10 min of desorption, after which they had to centrifuge again to remove the material with the magnet [21]. In contrast, the methodology proposed in the present work required only 1 min adsorption and 1 min desorption without any centrifugation step, which meant that it was a much faster and simpler methodology.

In addition, the most remarkable aspect of the method proposed in this work is the use of β-CD as a ligand, which is natural and more environmentally friendly than others [48]. In the validation results, it has been possible to confirm its high effectiveness, showing very satisfactory recovery values. This is a great advantage with respect to the previous work in which the same material was used without this ligand [13]. This improvement is observed especially in the case of morphine and oripavine, rising from 46 and 52% to 92 and 89%, respectively.

All in all, the present methodology presented several advantages that make it very effective for the analysis of this family of natural toxins. However, a future challenge is the reuse of the material for several cycles [49]. In this way, the methodology could be more environmentally friendly by reducing the amount of waste and extending the lifetime of the material.

3.8 Reproducibility of mesostructured silica magnetic composite $Fe_3O_4@SiO_2@mSiO_2@\beta$-CD

An important parameter for adsorbent evaluation is the reproducibility of the material synthesis. That is, to ensure reproducible results during the MSPE procedure. For this

purpose, the recoveries obtained with different batches of mesostructured silica magnetic composite Fe_3O_4@SiO_2@mSiO_2@β-CD were calculated. For this purpose, five batches were synthesized and evaluated at the medium validation level (0.4 mg/L). Table S5 shows that the RSDs were less than 9%, which indicated that the mesostructured silica magnetic composite with β-CD have acceptable reproducibility.

3.9 Application of the developed method to real samples of infusions with poppy seeds

The presence of OAs in poppy seed infusion from different poppy seed samples was determined. The areas obtained could have been interpolated on the solvent calibration line directly since the matrix effect was negligible [39]. However, to confirm the reliability of the proposed methodology, internal standards were used to correct the signals of matrix-matched calibration. Therefore, the obtained areas were corrected with the signals obtained from the internal standards, morphine-d3 was used to correct the signals of all except codeine which was corrected by codeine-d3. The corrected areas were interpolated to the respective calibration lines of the internal standard matrix-matched to calculate the OAs concentrations obtained in each of the infusions.

As shown in Table 3, all OAs were quantified in some of the poppy seed infusion samples analyzed. Moreover, as can be seen, the calculated standard deviations of the three replicates were in some cases high. This is due to the external heterogeneous contamination suffered by the seeds that depends on multiple factors, such as plant variety, climate, harvesting conditions, among others [13], [30], [50]. Of the four poppy seed infusions analyzed, two of them (PS-I-03 and PS-I-04) showed lower contents of all OAs, with morphine being the analyte with the highest concentration in both infusions 0.1 µg/L and 0.01 µg/L, respectively (Table 3). It should be noted that in sample PS-I-02 all analytes were quantified, except for oripavine which was below the MDL. In this PS-I-02 sample the OAs concentration was in general low, but for thebaine a significant amount (0.4 mg/L) was found (Table 3). This opium alkaloid is not legislated and therefore this poppy seed sample complies with current legislation. However, the importance of studying all opium alkaloids and not only morphine and codeine should be emphasized, since, as health authorities state, they can become even more toxic [22]. This

has been observed in recent cases of intoxication due to consumption of poppy seed infusions in Australia, where seeds consumed were contaminated with high concentrations of thebaine [27]. On the other hand, sample PS-I-01 showed a higher OAs content, especially morphine (1.7 mg/L), codeine (0.6 mg/L) and thebaine (1.1 mg/L) (Table 3). This sample was diluted 1:3 to quantify it within the calibration line and then recalculated to obtain the final concentration. Therefore, the estimated amount of OAs in PS-01 poppy seeds will be 91 mg/kg, as morphine equivalents (morphine + 0.2 codeine), assuming a transfer rate of 100% of both analytes. It is well above the legislated limit established by the European Commission on December 2021 (Regulation (EU) 2021/2142) for poppy seeds of 20 mg/kg morphine equivalents [51]. It should be noted that the legislation states that if the seeds were commercialised before 1 July 2022, they can remain on the market until the best-before date is reached, so even if the maximum content is exceeded, they can still be marketed according is best-before date (see Table S1) [51].

Table 3. Occurrence of OAs in poppy seed infusions analysed (n = 3).

Code sample[a]	OAs in poppy seed infusions (mean, mg/L ± standard deviation)					
	Morphine	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Oripavine**
PS-I-01	1.7±0.5	0.6±0.2	1.1±0.1	<MDL	<MDL	1.4±0.2
PS-I-02	0.11±0.04	0.003±0.02	0.4±0.1	0.01±0.01	0.06±0.02	<MDL
PS-I-03	0.10±0.05	0.008±0.02	<MDL	<MQL	0.03±0.05	<MDL
PS-I-04	0.01±0.01	<MQL	<MQL	<MQL	<MQL	<MDL

[a]PS-I: poppy seed infusion; <MDL: lower than method detection limit; <MQL: lower than method quantification limit but higher than method detection limit. PS-I-01 was diluted 1:3 to quantify it within the calibration line and then recalculated to obtain the final concentration.

In addition, the acute dose of morphine equivalents set by EFSA in 2018 is 10 µg/kg body weight. So, for a 20 kg child the acute dose would be 200 µg and for a 60 kg adult it would be 600 µg morphine equivalents. In the infusion with the highest amounts of OAs (PS-I-01), 182 µg of morphine equivalent were found, so for a child this would be close to the acute dose and an adult could drink three cups of this infusion. It is also a main

problem because many packages do not give recommendations for use, and it is up to the consumer to take the amount of seeds they want in each infusion. In addition, it can be found in numerous internet forums that the preparation of infusions with very high quantities of seeds is recommended, as in the reported case of a man who consumes poppy seed tea with dependence, consuming up to 2 L of poppy seed tea per day, which requires about 4 kg of poppy seeds per day [21], [52]. Therefore, the consumption of infusion from these seeds poses a health risk for both adults and children. This highlights the need to continue controlling this public health problem and the importance of developing analytical methods to analyse them to establish a legislation accordingly. In addition, an important aspect to note is that all four seed samples are labelled as poppy (*Papaver rhoeas* L.), which does not contain OAs in its latex and therefore its seeds cannot be contaminated. In this regard, the determination of OAs in their infusions confirms the mislabelling of the product as observed in previous studies [13]. Therefore, the need to correctly label the product and specify that they are opium poppy seeds (*P. somniferum* L.) and, therefore, must comply with the legislated maximum limit is claimed [51].

Conclusions

A fast, efficient, and environmentally friendly method for the control of OAs poppy seed infusions has been developed. For this purpose, the synthesis of a mesostructured silica magnetic composite with β-CD (Fe_3O_4@SiO_2@mSiO_2@β-CD) and the magnetic solid-phase extraction (MSPE) procedure have been optimised for the quantification of six OAs in infusions with poppy seeds followed by liquid chromatography coupled to tandem mass detector (HPLC-MS/MS). This material achieved a fast (1 min adsorption and 1 min desorption) extraction of the analytes in a very efficient way (with successful recovery values between 89 and 94%). It was confirmed that the use of mesostructured silica layer confers higher adsorption capacities when compared by discontinuous adsorption studies with Fe_3O_4@β-CD and Fe_3O_4@SiO_2@β-CD. Furthermore, the efficacy of β-CD as an OAs-interacting ligand was confirmed when compared to the unfunctionalized material, being a more environmentally friendly alternative to other ligands used in the literature. Finally, the method was applied to the analysis of infusions

with four different poppy seeds, showing in one of the seed samples using to make infusion a worrying concentration of OAs (91 mg/kg morphine equivalents) which imply a content in the seeds of at least four times that established in the current legislation (20 mg/kg).

Declaration of competing interest. The authors declare that they have no conflict of interest.

Acknowledgements. This research was funded by MCIU/AEI/FEDER, UE for project RTI2018-094558-B-I00.

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Supplementary Information

Table S1. Commercial samples of poppy seeds used for the preparation of infusions.

Code	Description	Botanical name declared in the labell	Origin	Best-before date	Recommendations for use
PS-01	Physically: blue poppy seeds	*P. rhoeas*	Not specified	07/2024	Not specified
PS-02	Physically: blue poppy seeds	*P. rhoeas*	Not specified	07/2022	Poppy seeds can be used in juices, soups, smoothies, and yoghurts. They can also be eaten ground.
PS-03	Not specific on the label. Physically: blue poppy seeds	*P. rhoeas*	Turkey	02/2022	Not specified
PS-04	Not specific on the label. Physically: blue poppy seeds	*P. rhoeas*	Spain	12/2023	Not specified

Supporting Information S1. Characterization of the magnetic materials with β-CD

Attenuated Total Reflection Fourier-Transform Infrared (ATR-FT-IR) spectra were recorded with a Spotlight 200i, Perkin Elmer (USA) spectrometer in the region 4000-400 cm-1 to identify the presence of the main functional groups after functionalization process, and to select the best reaction conditions for monomer formation. The measurements were done with around 1 mg of sample, previously vacuum-dried at room temperature in the transmittance mode with 64 scans per spectrum at a resolution of 4 cm-1. Elemental analysis (% N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. (Hampton, NH, USA) to determine the degree of functionalisation obtained in each material after the functionalization with each amount of β-CD added. Nitrogen gas adsorption–desorption isotherms were obtained using a Micromeritics ASAP 2020 analyser. These isotherms were measured at -196 °C over the interval of relative pressures (P/Po) from 10-4 to 0.994. Before measurements, the samples were degassed in a vacuum at 80 °C for 10 h in the degasification unit of the instrument. These temperatures were chosen to avoid any degradation of the organics groups and to remove adsorbed species, solvents, and water. The Brunauer–Emmett–Teller (BET) method was employed to calculate the specific surface areas (SBET). By using the Barrett–Joyner–Halenda (BJH) model, the pore volumes and pore size distributions were derived from the desorption branches of isotherms, and the total pore volumes (Vt) estimated from the desorbed amount at a relative pressure P/Po of 0.97. In addition, this characterisation also allowed us to compare the surface areas before and after functionalization. The synthesized materials were characterized by X-ray diffraction (XRD) to evaluate the structure of the materials. Wide and low angle powder XRD patterns of the silicas were performed to determine if the material showed the typical spectrum of magnetite, which means that the magnetic core had not been disturbed by the surface modification and if the material had a long mesoscopic ordered structure, respectively. XRD patterns were obtained on a Philips Diffractometer model PW3040/00 X'Pert MPD/MRD at 45 kV and 40 mA, using Cu Kα radiation (λ = 1.5418 Å). The samples were treated in power and placed in a sample holder. The sample and detector were rotated and the XRD patterns were collected from 0 to 10 ° at the low angle and between 20 to 70 ° at the wide angle.

Table S2. Equations of adsorption kinetic and isotherm.

$Q_e = \frac{(C_o - C_e)V}{W}$	Adsorption capacity	(1)
$\ln(q_e - q_t) = lnqe - k_1 t$	Lagergren's pseudo-first order	(2)
$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left[\frac{1}{q_e}\right] t$	Pseudo-second order	(3)
$q_t = k_p t^{1/2} + C$	Intra-particle diffusion	(4)
$\frac{1}{Q_e} = \frac{1}{Q_{max}} + \frac{1}{K_L Q_{max} C_e}$	Langmuir model	(5)
$Log\ q_e = logK_F + \frac{1}{n} logC_e$	Freundlich model	(6)

C_o and C_e: initial and equilibrium concentrations of the target analytes (µg/L), respectively; V: volume of the solution (L); W: mass of the adsorbent (g); k_1: pseudo-first order rate constant constant (min^{-1}); q_e and q_t: amounts of opium alkaloids adsorbed at equilibrium and time (mg/g), respectively; k_2: pseudo-second order adsorption rate constant (g/mg min); q_t: amount of opium alkaloids adsorbed at time t (mg/g); k_p: intraparticle diffusion rate (mg/L min^2); C: intercept; Q_{max}: maximum monolayer capacity of the adsorbent (mg/); K_L: Langmuir binding constant which is related to the energy of adsorption (L/mg); K_F: is the Freundlich constant (L/mg); n: is the heterogeneity factor (dimensionless).

Table S3. Optimal parameters of MRM for the analysis of each opium alkaloid by HPLC-MS/MS.

Analytes	Precursor ion (Q_1, m/z, $[M+H]^+$)	Fragment ion[a] (Q_3, m/z)	CE[b] (eV)	Rt[c] (min)
Morphine	286.1	**153.0**	45	5.179
		165.0	24	
		228.6	22	
Morphine-D3	288.7	**152.3**	45	5.819
		164.2	37	
		200.6	25	
Codeine	300.2	153.1	45	5.528
		165.0	45	
		215.1	23	
Oripavine	298.3	236.9	14	5.648
		249.1	17	
		267.1	12	
Thebaine	312.3	**58.2**	8	6.292
		166.2	16	
		249.4	16	
Papaverine	340.2	**202.0**	24	6.507
		324.1	30	
Noscapine	414.3	205.1	24	6.554
		220.0	42	
		280.1	20	

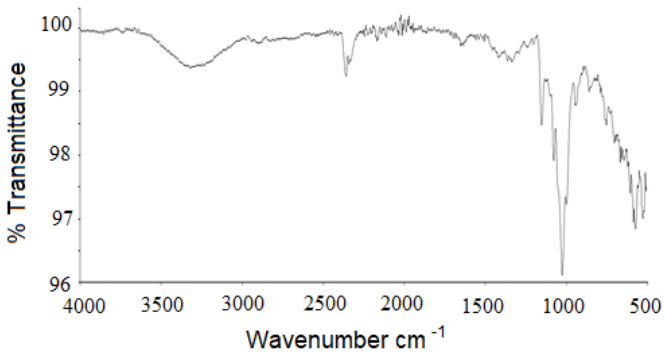
[a]The fragment ion used for the quantification are in bold; [b]CE: collision energy; [c]Rt: retention time.

Supporting Information S2. Method validation

First, linearity was evaluated with calibration curves adjusted to the matrix. For this purpose, the infusion made from washed and dried poppy seeds was used. After performing the MSPE procedure on the extract, it was spiked with an aliquot of a standard solution (six points) containing the target alkaloids according to the concentration level of the calibration curve. In addition, for the quantification of OAs in the samples, morphine-d3 and codeine-d3 were used to correct for possible signal variation. For this purpose, 50 µL of a 1 mg/L dilution of morphine-d3 and codeine-d3 were added to each point of the matrix-adjusted calibration curves. According to the validation guidelines, the criteria for good linearity imply values ≤ ± 20% for the deviation of the back-calculated concentrations of the calibration standards from the actual concentrations [39], [41]. In addition, matrix effects were determined by comparing the slopes of the calibration equations obtained from the matrix-based and solvent-based calibration curves, calculating (matrix-based slope/solvent-based slope -1) × 100 for each analyte. Following validation guidelines, the matrix effect is lower when close to 0%, and is negligible when less than +/- 20%. Positive values greater than 20% indicate signal enhancement, and negative values indicate signal suppression. Therefore, when signal suppression or signal enhancement is greater than this 20% range, matrix effects must be taken into account in the calibration [39]. The sensitivity of the method for each sample was determined through the MDLs and MQLs of the OAs from the analysis of the lowest concentration analysed (1 or 5 µg/L), which were estimated as the minimum concentration yielding a signal-to-noise ratio (S/N) of 3 or 10, respectively. The recovery assays were assessed by comparing the areas obtained for samples spiked (n = 6) with a known concentration of analytes and subjected to the MSPE procedure with those areas obtained for simulated samples (samples spiked at the same concentration but at the end of the procedure prior to their chromatographic analysis). The recovery assays were performed by spiking the samples at the three concentration levels (0.2, 0.4 and 0.8 mg/L). According with the guideless [39], the recovery values should be between 70 and 120%. On the other hand, the method precision was evaluated in terms of repeatability and reproducibility, using the same validation levels as for the accuracy. For repeatability (expressed as RSD %), a sample spiked with the OAs at the corresponding validation level was consecutively

carried out six times (n = 6) on the same day. The reproducibility (also expressed as RSD %) was calculated by the analysis of three replicates of a sample (spiked with the analytes at the corresponding validation level), which were carried out in triplicate over three different days (n = 9). According to the validation guidelines, the RSD values for these precision parameters should be ≤ 20%. The selectivity of the method was determined by comparing the spectra of the different analytes obtained from standard solutions with the spectra obtained in the samples. It was considered satisfactory when the variation in the spectra was few than ± 30 % and the retention time of the target analytes was within the interval of ± 2.5 % [39].

(a)



(b)

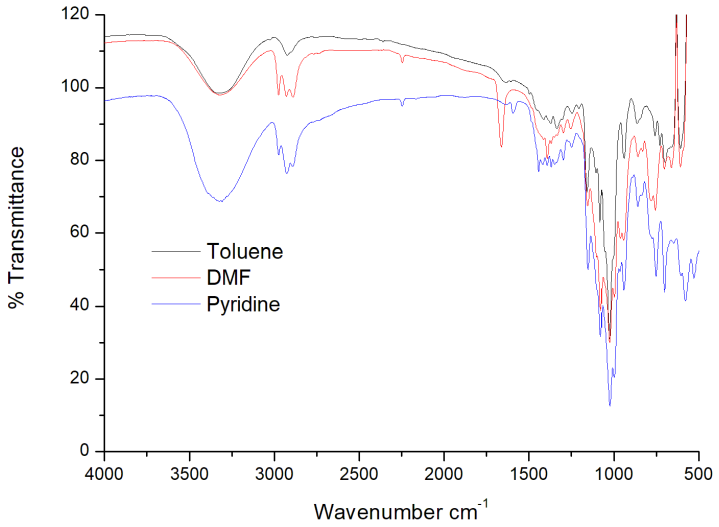


Fig. S1. FT-IR spectra of β-CD (a) and the monomer synthetized in three different mediums: toluene, DMF and pyridine (b).

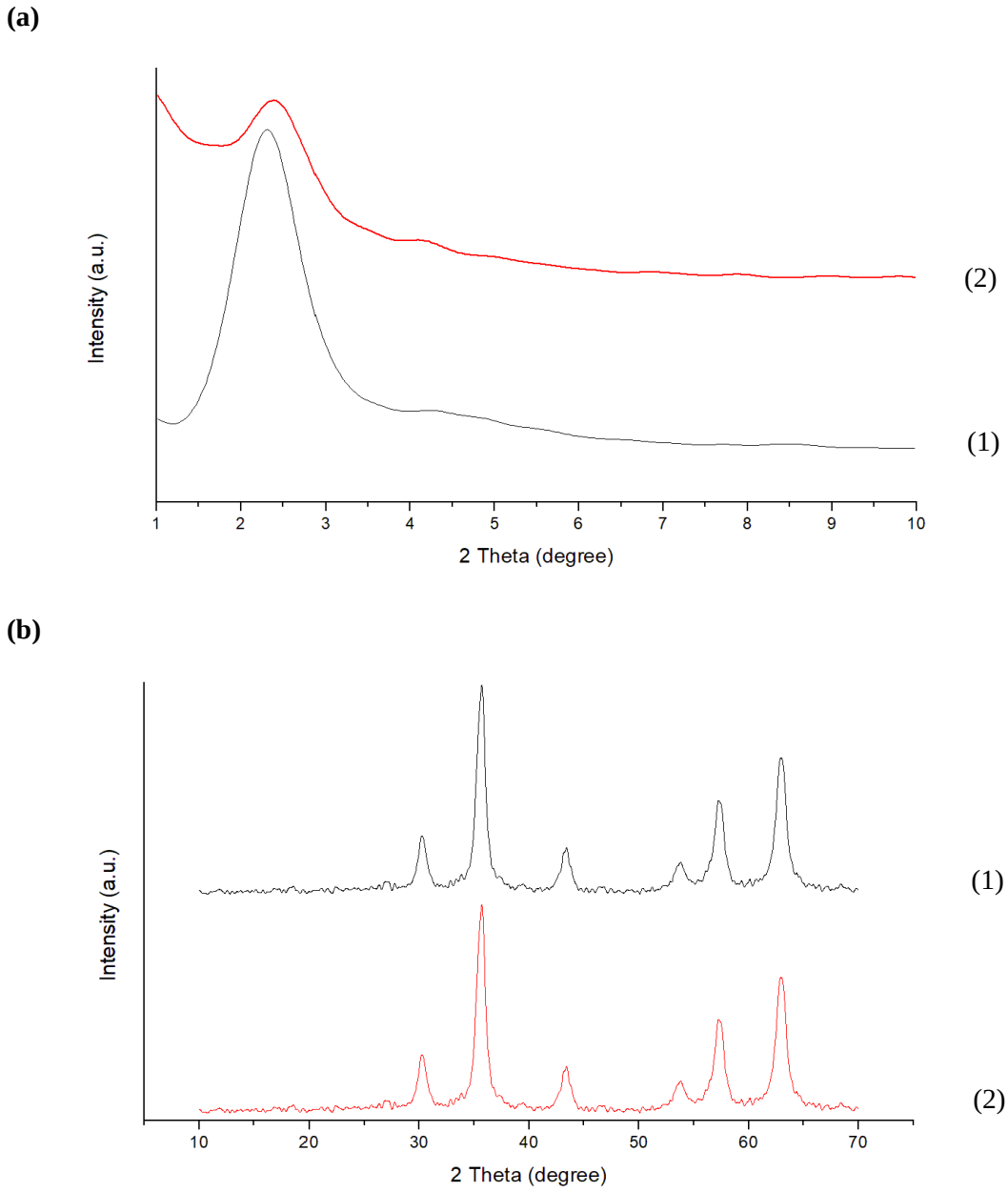


Fig. S2. XRD patterns of at low angles (a) and wide angles (b) of Fe_3O_4@SiO_2@mSiO_2 material before (1) and after (2) of functionalization with β-CD.

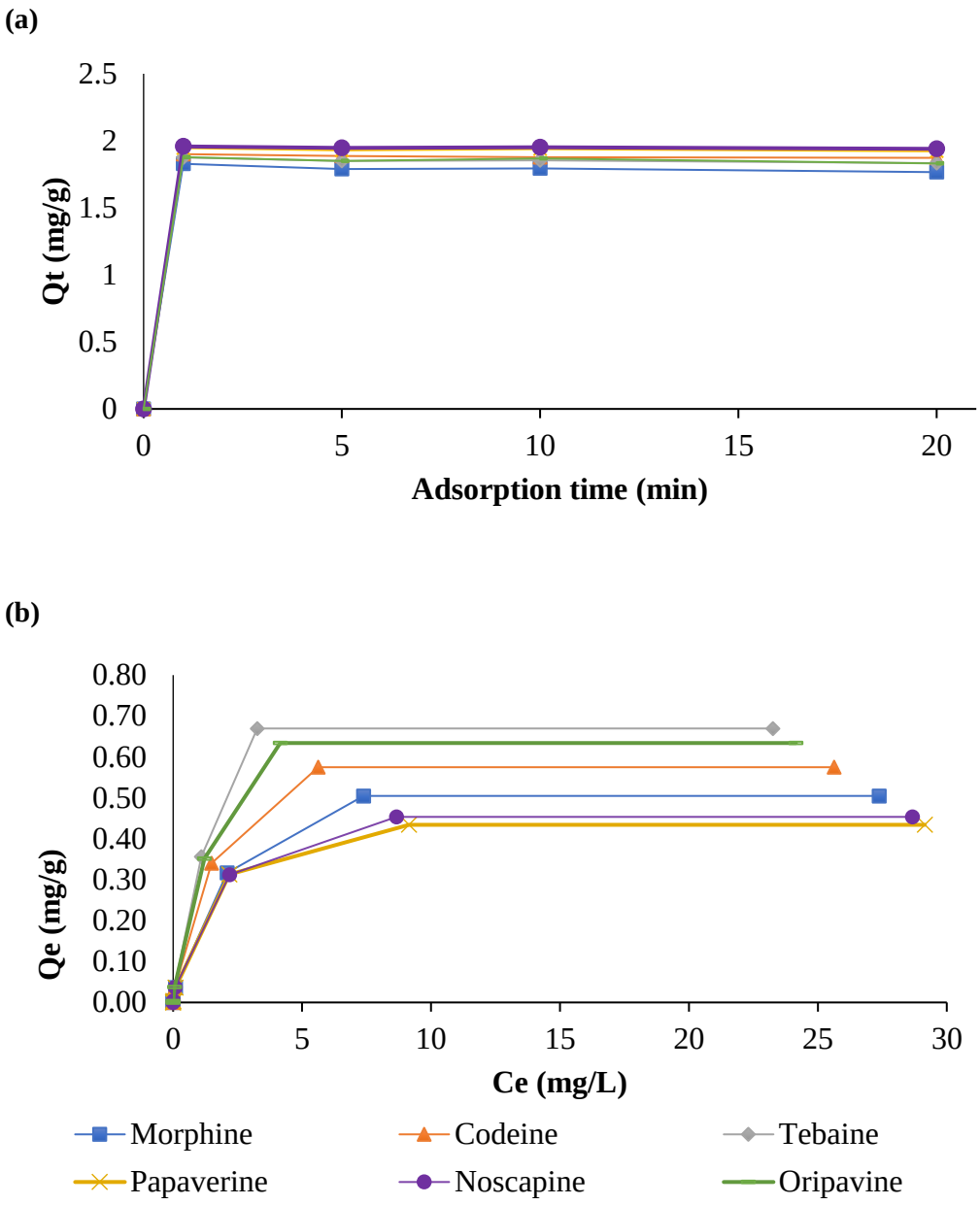


Fig. S3. Adsorption kinetic (a) and isotherm (b) experiments of six opium alkaloids with 50 mg of Fe_3O_4@SiO_2@mSiO_2@β-CD.

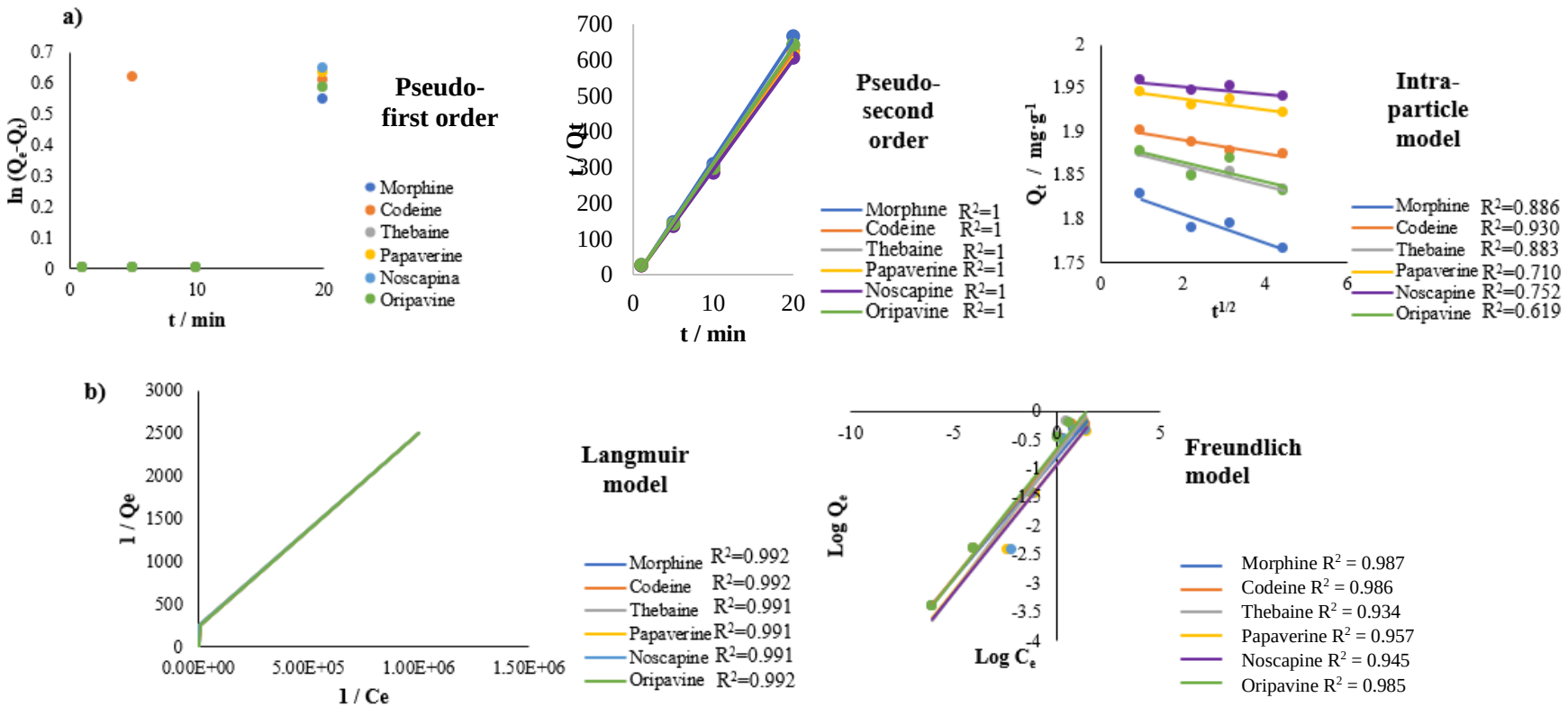


Fig. S4. Kinetics (a) and isotherms models (b) for the adsorption of the six OAs with 50 mg of Fe_3O_4@SiO_2@mSiO_2@β-CD material. Qe and Qt: amounts of OAs adsorbed at equilibrium and time (mg/g), respectively; Ce: initial and equilibrium concentrations of the target analytes (µg/L).

Table S4. Kinetic parameters of the adsorption of six opium alkaloids with 50 mg Fe_3O_4@SiO_2@mSiO_2@β-CD material for different times (1-20 min) based on different kinetic models.

Analyte	**$Q_{e, exp}$ (mg/g)**	**Pseudo-first order model**			**Pseudo-second order model**			**Intra-particle model**		
		R^2	**K_1 (min^{-1})**	**$Q_{e, cal}$ (mg/g)**	**R^2**	**K_2 (g/mg min)**	**$Q_{e, cal}$ (mg/g)**	**R^2**	**K_p (mg/g min^2)**	**C**
Morphine	0.036	-	-	-	1	65.81	1.761	0.886	-0.017	1.840
Codeine	0.037	-	-	-	1	240.20	1.873	0.930	-0.008	1.906
Thebaine	0.038	-	-	-	1	106.83	1.832	0.883	-0.011	1.884
Papaverine	0.036	-	-	-	1	206.97	1.920	0.710	-0.006	1.950
Noscapine	0.037	-	-	-	1	307.85	1.938	0.752	-0.004	1.961
Oripavine	0.038	-	-	-	1	79.76	1.828	0.619	-0.011	1.887

$Q_{e, exp}$: amounts of opium alkaloids adsorbed at equilibrium, experimental; K_1: pseudo-first order rate constant constant; $Q_{e, cal}$: amounts of opium alkaloids adsorbed at equilibrium, calculated; K_2: pseudo-second order adsorption rate constant; K_p: intraparticle diffusion rate; C: intercept.

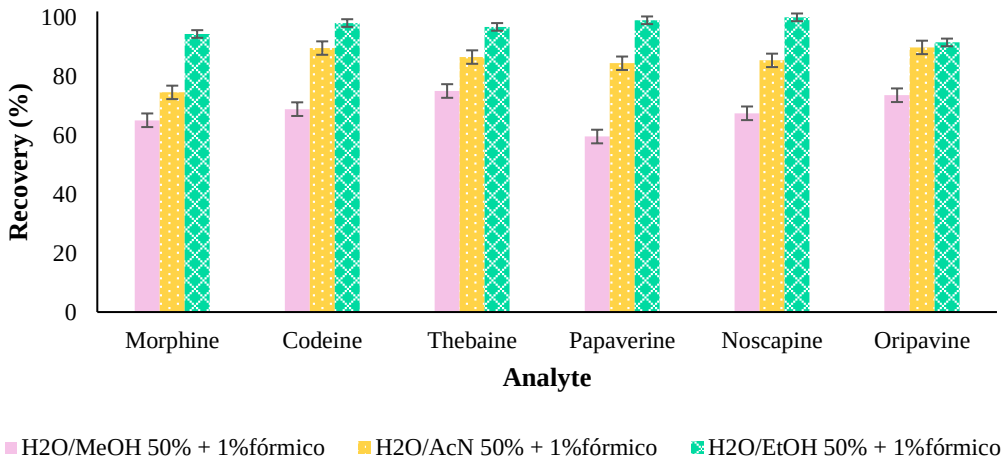


Fig. S5. Recovery obtained with different elution solvent mixtures in the MSPE procedure.

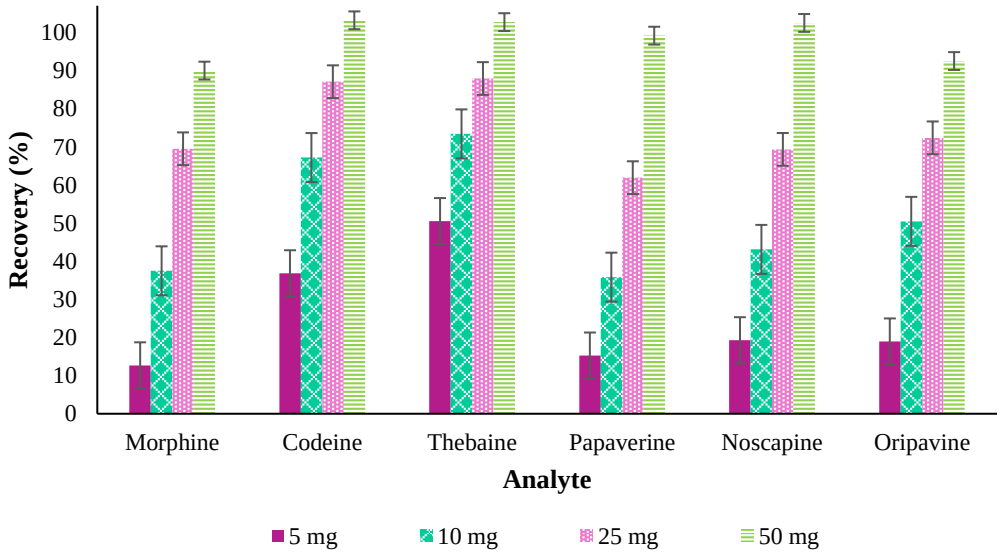


Fig. S6. Recovery obtained with different amounts of material (5, 10, 25 and 50 mg) in the MSPE procedure with optimised conditions.

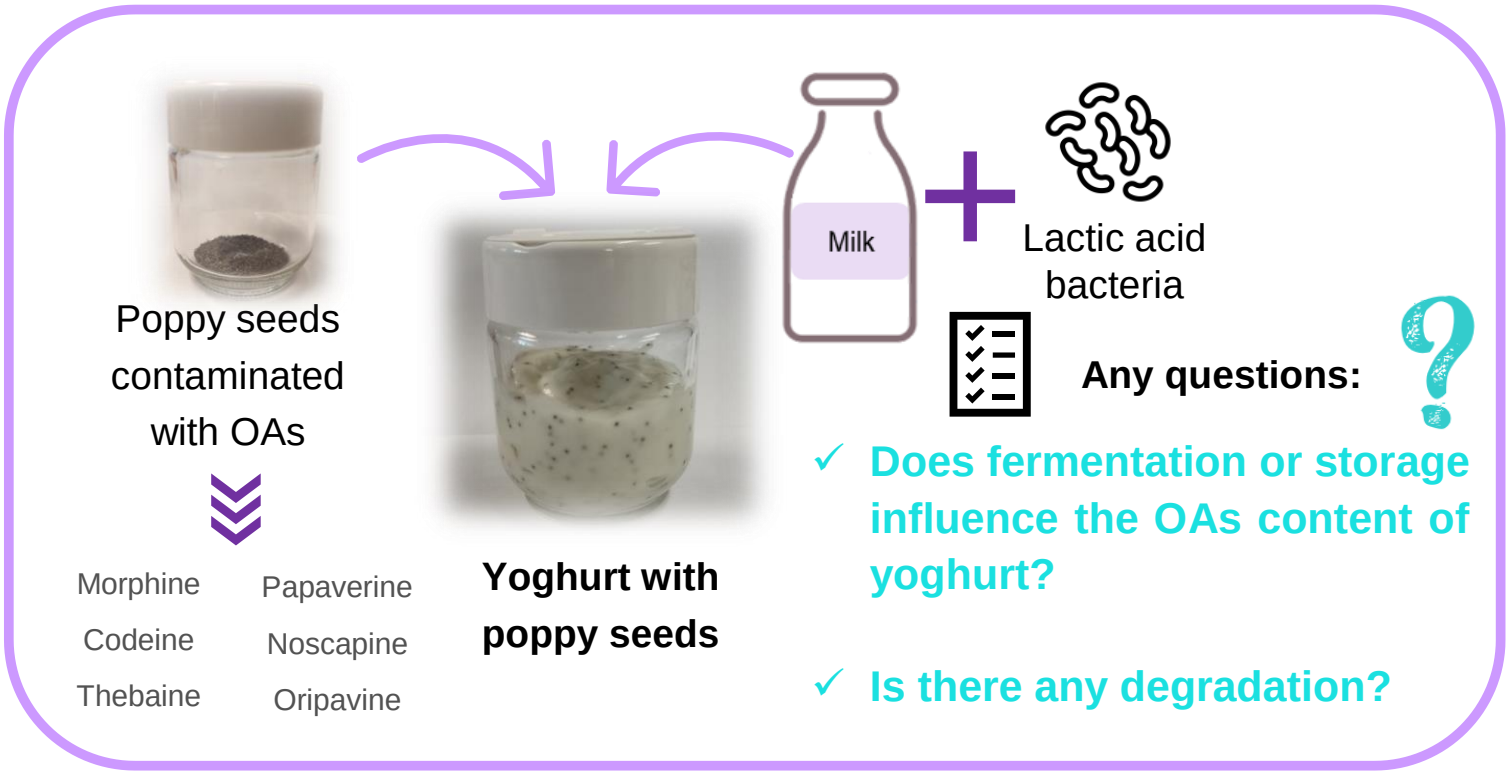
Artículo 6:

Influence of fermentation and storage on the content of opium alkaloids in poppy seed yoghurt

Journal of Food Composition and Analysis 121 (2023) 105412

PUBLICATION INFORMATION

Journal	Journal of Food Composition and Analysis
Publisher	Elsevier
Impact factor	4,3 (JCR, 2022)
Category	Food Science & Technology (43/142, Q2)
DOI	https://doi.org/10.1016/j.jfca.2023.105412


Poppy seeds contaminated with OAs
Morphine
Codeine
Thebaine
Papaverine
Noscapine
Oripavine
Yoghurt with poppy seeds
Milk
Lactic acid bacteria
Any questions:
✓ Does fermentation or storage influence the OAs content of yoghurt?
✓ Is there any degradation?

Highlights:

- A simple and fast method for quantifying OAs in yoghurt by MSPE and HPLC-MS/MS
- Mesostructured silica magnetic composite with β-CD is effective for OAs extraction
- Fermentation significantly decreases the content of all OAs in yoghurt
- Longer fermentation time or refrigerated storage does not lead to a higher effect

ABSTRACT

There is a current trend to make yoghurts with seeds such as poppy seeds to enhance their nutritional benefits. These seeds may be contaminated with opium alkaloids (OAs) present in the own latex of the plant (*Papaver somniferum* L.). Food processing such as heat treatment, grinding or washing may reduce them, but lactic fermentation has not been studied. In this work, an analytical methodology was optimised based on a solid-liquid extraction (SLE) with water, a purification by magnetic solid-phase extraction (MSPE) with a mesostructured silica magnetic composite with β-cyclodextrin (Fe_3O_4@SiO_2@mSiO_2@β-CD) and subsequent analysis by liquid chromatography coupled to a tandem mass detector (HPLC-MS/MS). The methodology was successfully validated in terms of linearity, limits of detection and quantification, matrix effect, precision, accuracy, and selectivity. Therefore, it was applied to determine OAs in commercial yoghurts, detecting morphine, papaverine and noscapine in all of them but below the limit of quantification. Besides, the effect of fermentation was studied (6 and 18 h) and storage (1 week/ 4ºC), showing a considerable degradation effect (33-80%) in the first hours.

KEYWORDS: Opium alkaloids; poppy seeds; yoghurt; fermentation; storage; magnetic solid-phase extraction; mesostructured silica magnetic composite; β-cyclodextrin; liquid chromatography-tandem mass spectrometry.

1. Introduction

The seeds of the *Papaver somniferum* L. plant, commonly known as opium poppy, are increasingly being added to different foods, such as bakery products, yoghurts, salads and for making tea and oil (AESAN (Spanish Food Safety and Nutrition Agency), 2020; Carlin et al., 2020; Casado-Hidalgo, 2022a; Casado-Hidalgo, et al., 2022b; López et al., 2018; Sproll et al., 2006). This increase in popularity is due to their good nutritional properties, as poppy seeds are a great source of essential fatty acids such as linoleic acid and antioxidant compounds such as vitamin E (Ghafoor et al., 2019; Musa Özcan & Atalay, 2006). The problem with this practice is that although the seeds do not naturally contain opium alkaloids (OAs), they can be contaminated with OAs (morphine, codeine, thebaine, papaverine, noscapine and oripavine) present in the latex of the plant itself due to poor harvesting practices or insect damage (Casado-Hidalgo, et al., 2021a; EFSA (European Food Safety Authority, 2018). This could lead to cases of intoxication and even false positives in drug tests (Lachenmeier et al., 2010).

For this reason, the European Commission published on December 3, 2021, the Regulation (EU) 2021/2142, which came into application on July 1, 2022. This regulation sets maximum levels for morphine equivalents (morphine + 0.2 codeine) for bakery products (1.5 mg/kg) and poppy seeds (20 mg/kg) (Commission Regulation, 2021). It only considers morphine and codeine and does not consider the other OAs as is the case in most of the articles published on OAs (Casado-Hidalgo, et al., 2021b). However, it has been seen that seeds can also be contaminated with other OAs (Casado-Hidalgo, et al., 2021a). Therefore, in 2018 EFSA and the German Federal Institute of Risk Assessment (BfR) claimed new effective analytical methods to quantify all main OAs, because they can be even more toxic (BfR, 2006; EFSA, 2018). In addition, this legislation only includes bakery products, and therefore, health authorities are demanding the study of other food matrices to know the real exposure of consumers. One of the food products with poppy seeds that are being consumed more frequently are yoghurts, which in addition to being commercialized, homemade yoghurts are a widespread practice (AESAN, 2020; EFSA, 2018). To date, to our knowledge, a method for determining OAs in this sample type has not yet been developed and validated.

Furthermore, in previously published articles, the degradation of OAs due to high temperatures during baking has been studied (Vera-Baquero et al., 2022). In fact, 25-100% degradation has been considered to establish legislation for bakery products (Commission Regulation, 2021). Furthermore, in 2014 the European Commission published recommendations for good agricultural and seed processing practices to reduce the morphine content (European Commission, 2014), and several articles published that washing, grinding, and baking treatments can decrease the content of OAs (Carlin et al., 2020; Shetge et al., 2020; Sproll et al., 2006; Vera-Baquero et al., 2022). However, to our knowledge, the influence of fermentation on OAs has not yet been studied. It is important to consider how this process can affect the content of these toxins, as depending on the fermentation conditions and the type of microorganisms used as a starter culture, the content of alkaloids can be affected (Casado et al., 2023). In other non-opium alkaloids, lactic and alcoholic fermentation studies have been carried out, and degradation of the compounds has been observed (De Nijs et al., 2017; Marín-Sáez et al., 2019). It would therefore be interesting to study whether lactic fermentation influences OAs in the yoghurt production process.

Considering that OAs can occur in complex matrices such as yoghurt in low concentrations, sample preparation prior to instrumental analysis plays a significant role in the whole analytical process. For this reason, an adequate sample purification or clean-up treatment is necessary to avoid possible matrix effects of yoghurt thus avoiding erroneous results and extending the useful life of the equipment (Casado et al., 2020). The most popular technique to purify OAs is solid-phase extraction (SPE) (Casado-Hidalgo, et al., 2022b; Guo et al., 2013; Meos et al., 2017; Stranska et al., 2013). However, the use of the magnetic dispersive SPE version (MSPE) is becoming increasingly popular because is a faster, simpler, and more environmentally friendly miniaturized technique (Casado-Hidalgo, et al., 2022a; Casado-Hidalgo, et al., 2021a; Jiang et al., 2019; Tang et al., 2020; Xu et al., 2019). Until now, Fe_3O_4 particles are the most used as magnetic adsorbents and are usually coated with silica or graphene that can be functionalized with different organic groups or natural polymers (Casado-Hidalgo, et al., 2022a; Casado-Hidalgo, et al., 2021b; Tang et al., 2020; Xu et al., 2019). However, β-cyclodextrin (β-CD) are growing in popularity because they have a low price, negligible environmental impact and non-

toxicity (Gentili, 2020). Structures of CD make possible the formation of inclusion complexes with aliphatic and aromatic non-polar compounds of suitable size by a variety of forces such as hydrogen bonding, hydrophobic and van der Waals interaction (Gentili, 2020; Majd et al., 2021). Thus, it is a potentially effective ligand for interacting with OAs, as demonstrated in our previous work (Casado-Hidalgo, et al., 2023). Where a mesostructured silica magnetic composite with β-cyclodextrin (Fe_3O_4@SiO_2@mSiO_2@β-CD) showed successful results with low detection and quantification limits and recovery values between 89 and 94% for all opium alkaloids.

The aim of this work is to study the fermentation and storage effect on opium alkaloids in yoghurts with poppy seeds. To do this, it was developed and validated an efficient, simple, and more respectful of the environment method to quantify six OAs in yoghurts with poppy seeds. The method was based on a solid-liquid extraction (SLE) with water, a purification by MSPE with Fe_3O_4@SiO_2@mSiO_2@β-CD followed by HPLC-MS/MS analysis. The proposed methodology was applied to a commercial yoghurt with poppy seeds and to homemade yoghurts with poppy seeds prepared and conserved under different conditions to provide data on the content of OAs in the final product to assess intake and to make legislation accordingly.

2. Materials and methods

2.1. Reagents and materials

Standards of morphine, codeine, thebaine and oripavine were obtained from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine, morphine-d3 and codeine-d3 (internal standards, IS) were received from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock standard solutions were prepared at 1 µg/mL in methanol, and working standard solutions were prepared at 1 µg/L in water/ethanol 75/25 (v/v) with 10% formic acid. They were stored in darkness at -20 ˚C.

The reagents for the synthesis of the material were: ferric chloride 6-hydrate ($FeCl_3 \cdot 6H_2O$) 99% and ferrous chloride 4-hydrate ($FeCl_2 \cdot 4H_2O$) 99%, which were purchased from Labkem (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Tetraethylorthosilicate (TEOS) 98%, hexadecyltrimethylammonium

bromide (CTAB), 3-isocyanatopropyltriethoxysilane 98% and β-cyclodextrin ≥ 97% were purchased from Sigma-Aldrich. Ethanol absolute, formic acid (98%) and ammonia 32%, (w/w), isopropanol, toluene, pyridine, and diethyl ether were of synthesis grade and purchased from Scharlab (Barcelona, Spain). N, N-dimethylformamide (DMF) were purchased from Merk (Darmstadt, Germany). Acetonitrile, methanol, and ethanol used were HPLC-MS quality and were purchased from Scharlab (Barcelona, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). The Nd-Fe-B magnet (5 × 5 × 2 cm) with force 200 kg used in the MSPE procedure was obtained from Superimanes S.L. (Sevilla, Spain).

2.2. Yoghurt samples

To carry out the optimisation of the methodology proposed in this work and, subsequently, to validate it, a natural commercial yoghurt without poppy seeds was used with 2.9 g sugar and 3.8 g fat per 100 g to make controlled contamination. Subsequently, 1% poppy seeds, previously washed and dried and spiked at the concentration set by legislation as the maximum limit (20 mg/kg) were added (Commission Regulation, 2021). To do this, 25 mg of poppy seeds were weighed and spiked and left for 2 h so that the methanol from the standard added had been completely evaporated. Afterwards, 2.475 g of natural yoghurt was added and mixed by vortexing for 10 s and stored in the fridge for 24 h to simulate a commercial yoghurt with poppy seeds.

In addition, a commercial yoghurt sample with poppy seeds was analysed for the presence of OAs to demonstrate the validity of the method developed. Furthermore, homemade yoghurts were made to see the effect of fermentation and storage on the OAs content.

2.2.1. Commercial yoghurt for applied the proposed method

A commercial yoghurt sample with poppy seeds was purchased online from a supermarket in Sevilla (Spain). To obtain a represented sample, four yoghurts of the same batch and brand name were purchased. The ingredients of this commercial yoghurt were milk, cane sugar, poppy seed and lactic ferment. As indicated on the label, the yoghurt contains a total sugar content of 28.1 g of sugar and 5.6 g of fat per 100 g of yoghurt.

2.2.2. Elaboration of homemade yoghurts for study of the fermentation and storage effect

Furthermore, homemade yoghurts with poppy seeds were made to see the effect of the fermentation and storage on the OAs content. To make the homemade yoghurts, as shown in Fig. 1, 1 L of pasteurised whole milk with 3.5% of fat was used, heated to 45 ºC and a packet of a bacterial starter culture (1g Acidophilus yogurt starter culture, Natural Probiotic Selection, Bulgarian) of with 2.0×10^{10} UFC, according to the specifications of the manufacturer *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus acidophilus* were added. Once dissolved, 100 mL of the yoghurt mix was distributed into each of the yoghurt containers, and placed in a yoghurt maker at 42 °C. Before adding the yoghurt, 1 g of poppy seeds, previously washed and dried and spiked at 20 mg/kg, was added to these containers. The ratio of 1 g of seeds per 100 g of yoghurt was considered, as commercial yoghurts containing 1% poppy seeds. In addition, as the variable of fermentation time was studied, some yoghurts were in the yoghurt maker at 42°C for 6 h, and others were for 18 h. After this, the yoghurts were cooled, the pH was measured, and they were frozen until further analysis. An additional test was carried out by storing the yoghurts with a 6-hour fermentation in the refrigerator for 1 week at 4ºC.

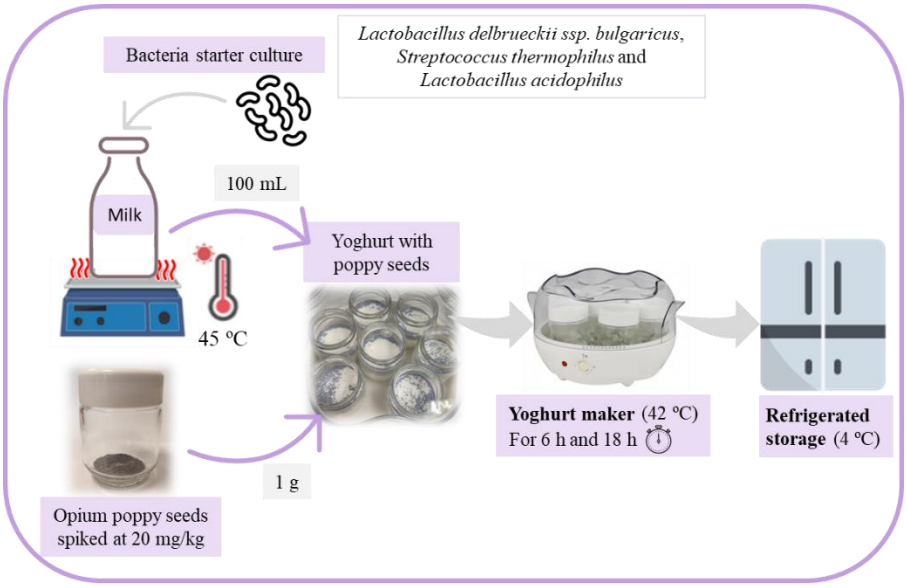


Fig. 1. The process of making home-made poppy seed yoghurts.

2.3. Preparation of the Fe_3O_4@SiO_2@mSiO_2@β-CD material

The adsorbent material used in the present work was a mesostructured silica magnetic composite with β-cyclodextrin denoted as Fe_3O_4@SiO_2@mSiO_2@β-CD. For the synthesis and preparation of this material, the protocol previously optimised in our previous work was followed (Casado-Hidalgo, et al., 2023). The material was characterised by performing nitrogen gas adsorption–desorption isotherms with the Brunauer–Emmett–Teller (BET) method to calculate the specific surface areas (S_{BET}) and using the Barrett–Joyner–Halenda (BJH) model to calculate the pore volumes and pore size distributions. In addition, elemental analysis (% N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. (Hampton, NH, USA) to determine the degree of functionalisation of β-CD. The results obtained agreed with those obtained in the previous work, showing a S_{BET} of 203 m^2/g, a pore volume of 0.13 cm^3/g, a pore diameter distribution of 20.5 and 39.0 Å and a functionalisation of 0.134 mmol β-CD/g material (Casado-Hidalgo, et al., 2023).

2.4. Optimization of the extraction by SLE and MSPE of OAs from yoghurt with poppy seeds

The analysis methodology developed to quantify OAs in yoghurt with poppy seeds was based on a first SLE, purification by MSPE with Fe_3O_4@SiO_2@mSiO_2@β-CD material and analysis by HPLC-MS/MS, as shown in Fig. 2. As a starting point, the conditions developed in the previous work were used with the necessary modifications and optimisations for the sample of yoghurt with poppy seeds (Casado-Hidalgo, et al., 2023).

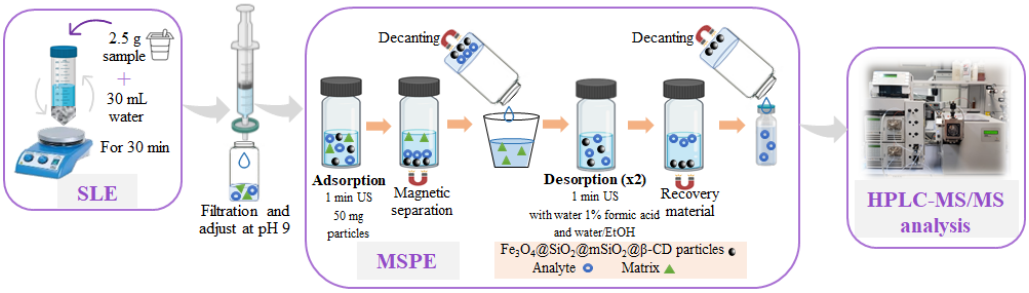


Fig. 2. The proposed methodology to quantify OAs in yoghurts with poppy seeds: extraction (SLE), purification (MSPE with Fe_3O_4@SiO_2@mSiO_2@β-CD particles) and analysis (HPLC-MS/MS).

2.4.1. Optimization of SLE

The first step of the analytical methodology was SLE to extract OAs from yoghurt. To achieve an efficient SLE step, the recovery values obtained after the procedure were calculated and compared with those obtained on a simulated sample (a blank yoghurt sample subjected to the same process but spiked just before the HPLC-MS/MS analysis). When choosing the solvent, it was considered that the magnetic adsorbent material functionalised with β-CD has a higher adsorption capacity with analytes dissolved in water (Casado-Hidalgo, et al., 2023). Therefore, the extraction with water at different pH (2, 7 and 9) was compared.

2.4.2. Optimization of MSPE conditions with Fe_3O_4@SiO_2@mSiO_2@β-CD

First, the MSPE conditions optimised with this adsorbent material for OAs in the previous work were used (Casado-Hidalgo, et al., 2023). However, with the yoghurt sample with poppy seeds, the recovery values obtained were low, especially for morphine, codeine and oripavine. Therefore, both adsorption and recovery values were checked to identify this step that was not effective with this sample and re-optimise it.

Therefore, to evaluate the load, 2 mL of yoghurt extract with poppy seeds (previously washed and dried to ensure that the seeds did not contain OAs) spiked at 20 mg/kg (maximum legislated limit) was used (Commission Regulation, 2021). This extract was adjusted to pH 9, and 50 mg of Fe_3O_4@SiO_2@mSiO_2@β-CD particles (previously conditioned with 1 mL of water at pH 9 for 1 min of ultrasound (US)) were added and

subjected to 1 min of US for adsorption of the OAs on the material. The supernatant was analysed and compared with a simulated sample (a blank sample subjected to the same process and spiked just before the HPLC-MS/MS analysis).

For the evaluation of the desorption conditions, the process was continued. After adsorption, the supernatant was discarded, and the desorption solvent was added and subjected to US for a given time. The desorption conditions evaluated were the number of consecutive desorption (1 and 2), the time (1, 5 and 10 min), the ratio of ethanol/water with 1% formic acid (30/70 and 70/30) and the percentage of formic acid added (1 and 10%).

2.5. Optimised analysis methodology for quantification of OAs in yoghurt with poppy seeds

First, to perform the SLE step, 2.50 g of yoghurt with poppy seeds were weighed, and 30 mL of water were added for the extraction. It was vortexed for 10 s (Rx^3 Velp Scientifica, Usmate, MB, Italy) and stirred magnetically for 30 min. Afterwards, it was centrifuged (ROTOFIX 32A Hettich, Tuttlingen, Germany) at 6000 rpm (3992 rcf) for 10 min to recover the supernatant. Then, the extract was filtered through a 0.45 µm nylon filter and adjusted to pH 9 with ammonia to do MSPE step.

For MSPE, 50 mg of Fe_3O_4@SiO_2@mSiO_2@β-CD particles (previously conditioned with 1 mL of water at pH 9 for 1 min of US) were added and subjected to 1 min of US for adsorption of the OAs on the material. Subsequently, the material with analytes was separated from the solution by an external magnet, and the analytes were desorbed from the magnetic particles in two steps, the first with 1 mL of water with 1% formic acid for 1 min in US and the second, with 1 mL water/ethanol at 50% with 1% formic acid for another minute. Afterwards, 2 mL of these supernatants with the analytes was recovered, and 950 µL with 50 µL of a 1 µg/mL solution of morphine-d3 and codeine-d3 was added and analysed by HPLC-MS/MS.

The analysis of OAs was performed with a Varian 1200/1200 LC (Varian Ibérica, Madrid, España) equipped with a ProStar 410 autosampler (consisting of a 100 µL loop)

and two ProStar 210/215 solvent delivery modules and coupled with a 1200L TQ triple quadrupole mass spectrometer detector with an electrospray ionization (ESI) ion source (data acquisition system was MS Workstation Varian version 6.8). A C18 KromaPhase 100 column (150 × 2.0 mm, 3.5 µm particle size, Scharlab, Barcelona, Spain) with a C18 Kromaphase guard column (10 × 4.0 mm I.D., 5 µm particle size) at 30 °C were used for the chromatographic separation. The injection volume was 10 µL (partial injection), and the flow rate was set at 0.25 mL/min. The mobile phases were water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient elution was performed as follows: 90−30% A (0−6 min), 30−90% A (6−9 min), and 90% A (9−11 min) for column re-equilibration (Casado-Hidalgo, et al., 2022a; Casado-Hidalgo, et al., 2022b). Mass spectrometry acquisition was performed with electrospray ionization in the positive mode (ESI+) with the MRM mode (Multiple Reaction Mode) for all analytes (mass peak width Q1 2.5; mass peak width Q3 2.5 and scan width in MRM 0.70 s). N_2 was used as both drying and nebulizer gas. The drying gas was set at 350 °C and 22 psi, and the nebulizer gas was set at 58 psi. The capillary voltage was held at 5000 V and shielded at 600 V. Argon was used as the collision gas set at 1.90 mTorr and detector voltage at 1480 V. Compounds were monitored at cone voltage of 70 V and Table S1 shows the optimal mass spectrum parameters (ion precursor, product ions, ions used for quantification, collision energy, and retention time).

2.6. Method validation

The analysis methodology proposed in the present work was validated to quantify OAs in yoghurt with poppy seeds. To carry out the validation, poppy seeds were added to yoghurt and previously washed and dried. Then, 25 mg of poppy seeds were weighed and spiked at three known concentrations (100, 200 and 400 µg/kg, representing low, intermediate, and high validation value, respectively) and left for 2 h so that the methanol from the standard added had been completely evaporated. Afterwards, 2.475 g of natural yoghurt was added and mixed by vortexing for 10 s and stored in the fridge for 24 h to simulate a commercial yoghurt with poppy seeds. The validation was performed in terms of linearity, method detection and quantification limits (MDL, MQL), matrix effect (ME),

accuracy, precision, and selectivity, following the recommendation of the method validation guide for pesticide residues in food and feed shown in the SANTE/11312/2021 document, in regulation EC No 401/2006, and in the Q2(R1) ICH guidelines (Internation Council for Harmonisation, 2005). Moreover, the only reference materials to our knowledge are biological samples and only for morphine and codeine. For this reason, the validation was carried out with a spiked sample. For this reason, the validation was carried out with a spiked sample (more details in Supporting Information S1).

2.7. Statistical analysis

Statistical analyses were performed using SPSS 25.0 statistical package (SPSS INC., Chicago, IL, USA) by one-factor ANOVA analysis. Significant differences were considered significant for p values ≤ 0.05.

3. Results and discussion

3.1. Optimization of SLE-MSPE procedure

3.1.1. Conditions of extraction step by SLE

The extraction solvent was optimised at this step of the analysis methodology. In the previous work, the solvent with which the Fe_3O_4@SiO_2@mSiO_2@β-CD material had the highest adsorption capacity was determined to be water (Casado-Hidalgo, et al., 2023). Therefore, the first assay to extract the OAs from yoghurt was carried out with water to avoid an evaporation step between the SLE and the MSPE, thus shortening the analysis time considerably. To do this, the recovery values obtained with water at different pHs (2, 7 and 9) were evaluated. For this purpose, 30 mL of water (with each pH) was added to 2.5 g of yoghurt and mixed by vortexing for 10 s and stirred magnetically for 30 min. Afterwards, it was centrifuged at 6000 rpm (3992 rcf) for 10 min and filtered for subsequent analysis by HPLC-MS/MS. However, both with water at pH 2 and pH 9, the extract was very turbid and even with several centrifugation cycles, very poor decantation of the solid particles was achieved. Therefore, only the extract made with water at pH 7 could be analysed and adequate recovery values were obtained for all analytes (close to 100%).

3.1.2. Conditions of purification step by MSPE

To determine the efficiency of the load step, 2 mL of extract of yoghurt with poppy seeds (previously washed and dried) spiked at 20 mg/kg (the maximum legislated limit) was used (Commission Regulation, 2021). This extract was adjusted to pH 9 and 50 mg of Fe_3O_4@SiO_2@mSiO_2@β-CD particles (previously conditioned with 1 mL of water at pH 9 for 1 min of US) were added and subjected to 1 min of US for adsorption of the OAs on the material. The supernatant was analysed, and the adsorption capacity values of each analyte were calculated, obtaining good results for all analytes (close to 100%).

Subsequently, the desorption conditions were evaluated by calculating the recovery values for each of the analytes. First, desorption was performed with 2 mL of 50% water/ethanol with 1% formic acid for 1 min in US which were the optimised conditions with standard solutions in the previous work (Casado-Hidalgo, et al., 2023). As shown in Fig. 3a, the recovery values with the initial conditions were considerably low below 60% for all analytes. Therefore, different assays were performed by modifying one of the variables of the initial conditions. Firstly, it was evaluated whether a longer desorption time in US (5 and 10 min) resulted in the extraction of a greater amount of analytes. As shown in Fig. 3a, no differences were found between 1, 5 and 10 min, which led to the conclusion that the low recovery values were not due to a lack of time. Subsequently, a test of two consecutive 1 min US desorptions was performed to check whether the second desorption was able to desorb what had not been desorbed in the first step. However, as shown in Fig. 3a, a second desorption was not able to desorb all the adsorbed analyte content from the material. Therefore, it was subsequently tested to change the ratio of ethanol and water in the mixture, instead of 50%, a test was carried out with 70/30 and another with 30/70. With a higher proportion of ethanol, adequate recovery values were obtained for the analytes with a more apolar nature (thebaine, papaverine, and noscapine). However, analytes with a more polar nature obtained low recovery values (morphine, codeine, and oripavine). On the other hand, with a lower proportion of ethanol in the mixture, the recovery values increased for the more polar analytes from approximately 30 to 60% (Fig. 3a). In addition, a test was performed to increase the proportion of formic

acid (from 1 to 10% to the 50% ethanol/water mixture). However, as shown in Fig. 3a, recovery values were not increased compared to those obtained with 1% formic acid.

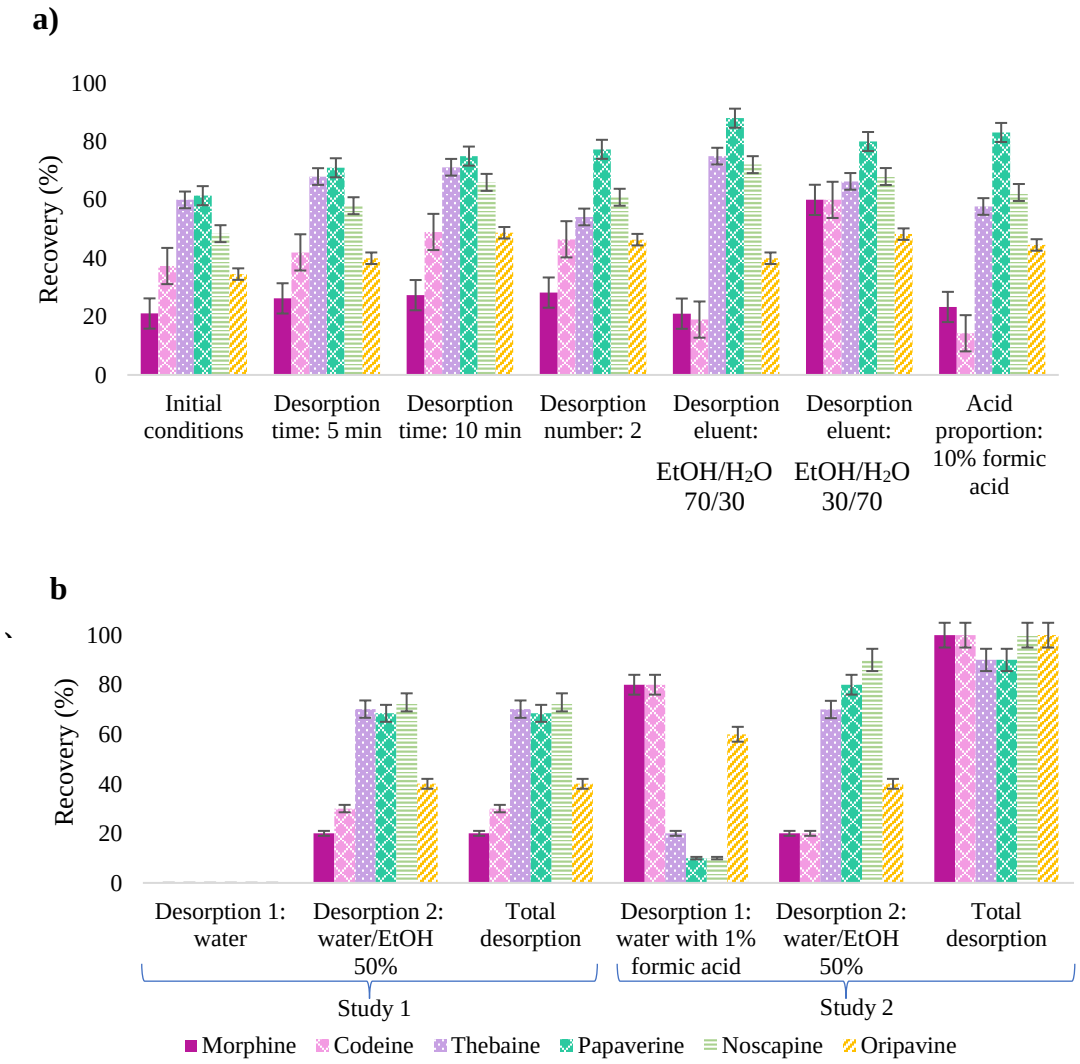


Fig. 3. Optimisation of the desorption conditions of OAs from magnetic particles in the MSPE procedure. (a): recovery values (%) obtain with the initial conditions: desorption with 2 mL of 50% EtOH/H_2O with 1% formic acid for 1 min in US. For the rest of the conditions, one parameter was varied, and the rest were maintained; (b): recovery values (%) obtained with two studies with two consecutive desorption and the sum of each one.

Therefore, observing the different chemical natures of the OAs in terms of polarity, a two-step desorption was carried out, one aqueous to desorb the more polar analytes and

one with a 50% ethanol/water mixture with 1% formic acid. For this, as shown in Fig. 3b, two tests were performed, one with the aqueous desorption step using water with 1% formic acid and the other without acid. Each desorption step was analysed separately to determine the influence of each on the recovery. In the study with unmodified water, hardly any of the analytes were desorbed. However, in the study using the first desorption step with acidified water, 80% was obtained for morphine and codeine, 20% for thebaine, 10% for papaverine and noscapine and 60% for oripavine. In the second step with 50% ethanol/water, approximately 20% was obtained for morphine and codeine, 70% for thebaine, 80% for papaverine, 90% for noscapine and 40% for oripavine. Therefore, adding up the recovery values for each of the desorption stages, approximately 100% was obtained for all the analytes (Fig. 3b). Therefore, once this was obtained, the same study was performed again, but analysing the two desorptions together to confirm the recovery values of the whole process. The results obtained confirmed that performing the desorption in two steps with these solvents was very effective and therefore, it was decided to perform the desorption in this way.

3.2. Method validation

The validation results of the proposed analytical methodology for the quantification of six OAs in yoghurt with poppy seeds samples are shown in Table 1. The calibration lines were obtained with R^2 between 0.999 and 1.000 for all analytes, with a linear range of 0.01-12 mg/kg for thebaine, papaverine and noscapine and of 0.06-12 mg/kg for morphine, codeine and oripavine. The deviation of the back-calculated concentrations of the calibration standards from the true concentrations in the matrix calibration lines were -17% for oripavine, -3% for papaverine, 1% for thebaine, 2% for noscapine, 4% for codeine and 13% for morphine. Therefore, these results demonstrated the good linearity of the method, which states good linearity when the deviation of the back-calculated concentrations is $\leq \pm$ 20% (SANTE/11312/2021). Besides, the deviation of the slopes of the calibration lines for three different days (n = 3) was calculated to ensure their reproducibility, obtaining RSDs between 3 and 9% for all analytes.

Table 1. Validation parameters of the proposed SLE-MSPE and HPLC-MS/MS methodology for the quantification of six OAs in yoghurt with poppy seeds.

Analytes	Linear range (mg/L) [a]	Matrix-matched calibration [b]	R^2	MDL (µg/kg) [c]	MQL (µg/kg) [d]	ME ± SD [e]	Accuracy [f]		Precision	
							Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-day Precision (RSD %)	Inter-day Precision (RSD %)
Morphine	0.005-0.5	$y = 1\times10^{8}x - 2\times10^{5}$	0.999	4.8	16	-9 ± 6	101 ± 5^{L} 100 ± 3^{M} 92 ± 8^{H}	98 ± 5	5^{L} 3^{M} 9^{H}	12^{L} 6^{M} 10^{H}
Codeine	0.005-0.5	$y = 1\times10^{8}x - 5\times10^{5}$	0.999	7	23	-11 ± 5	103 ± 7^{L} 104 ± 3^{M} 95 ± 7^{H}	101 ± 6	7^{L} 2^{M} 7^{H}	9^{L} 6^{M} 8^{H}
Thebaine	0.001-0.5	$y = 1\times10^{9}x - 4\times10^{6}$	0.999	2.9	9.7	-17 ± 1	87 ± 4^{L} 102 ± 3^{M} 93 ± 3^{H}	94 ± 3	4^{L} 3^{M} 3^{H}	12^{L} 4^{M} 5^{H}
Papaverine	0.001-0.5	$y = 3\times10^{9}x - 3\times10^{6}$	1.000	1.6	5	-13 ± 4	87 ± 8^{L} 95 ± 3^{M} 94 ± 3^{H}	92 ± 5	9^{L} 3^{M} 3^{H}	10^{L} 4^{M} 5^{H}
Noscapine	0.001-0.5	$y = 3\times10^{9}x - 9\times10^{5}$	1.000	0.9	2.9	-7 ± 6	81 ± 9^{L} 102 ± 2^{M} 93 ± 3^{H}	92 ± 5	9^{L} 2^{M} 3^{H}	12^{L} 4^{M} 5^{H}
Oripavine	0.005-0.5	$y = 1\times10^{8}x - 3\times10^{5}$	0.999	17	58	-16 ± 2	86 ± 10^{L} 101 ± 2^{M} 99 ± 6^{H}	95 ± 6	11^{L} 2^{M} 6^{H}	15^{L} 3^{M} 10^{H}

[a] The linear range expressed in µg/kg (considering the sample treatment) is 60-12000 in the case of morphine, codeine and oripavine and 10-12000 in thebaine, papaverine and noscapine [b] The calibration line is in the units: mg/L; [c] MDL: method detection limit; [d] MQL: method quantification limit; (both considering the sample treatment); [e] ME: matrix effect (purified matrix slope / solvent slope -1) × 100 ± SD (of three replicates); [f] Accuracy and precision were obtained by spiking yoghurt with poppy seeds at three concentration levels: low (L, 100 µg/kg), medium (M, 200 µg/kg) and high (H, 400 µg/kg); Accuracy: n = 6; Intra-day precision: n=6, 1 day; Inter-day precision: n = 9, 3 days.

Regarding the MDL and MQL values were low for all analytes, noscapine 0.9 and 2.9 µg/kg, papaverine 1.6 and 5 µg/kg, thebaine 2.9 and 9.7 µg/kg, codeine 7 and 23 µg/kg, morphine 4.8 and 16 µg/kg and oripavine was 17 and 58 µg/kg, respectively.

On the other hand, ME was calculated by comparing the slopes of both matrix-matched and solvent-based calibration curves. As shown in Table 1, the ME was negligible because for all analytes the ME values were < +/- 20%. This means that the developed purification procedure was able to eliminate all matrix effects for the six target analytes. Therefore, the quantification could perform with solvent-based calibration curves, which would simplify the analysis.

Accuracy and precision were evaluated at three different levels of concentration, low (100 µg/kg), medium (200 µg/kg), and high (400 mg/kg). As shown in Table 1, all recovery values were adequate between 70 and 120% (Reynolds, n.d.) between 81 and 104% for all levels and analytes. In addition, satisfactory results were obtained for intra-day and inter-day precision at three concentration levels because the RSD values were lower than 20% (SANTE/11312/2021), specifically lower than 11 and 15%, respectively.

Furthermore, the variation of the retention time was ≤ 0.1 min between the chromatograms of the extracted ions obtained for each of the OAs in a standard solution with the extracts of the sample. In addition, the ion ratios of the sample extracts were within ± 30% (relative abundance) of the mean of the standards for each analyte. Therefore, in compliance with the guidelines, the method developed showed good selectivity. In addition, an important point to note is that the proposed methodology is the first method to date developed and validated to analyses OAs in poppy seed yoghurt.

3.3. Application of the proposed methodology to commercial sample of yoghurt with poppy seeds

At the time when the study was carried out, only one yoghurt with poppy seeds could be found on the market, even though many had been available on the market months before. This can be attributed to the recent recommendation that seed suppliers must guarantee a maximum content of morphine equivalents, which may not have been

considered by seed manufacturers so far. It was confirmed that this commercial yoghurt showed good recovery values, and despite showing some matrix effect, it could be corrected for correct quantification with the matrix calibration line made with this same type of yoghurt (Table 2). Therefore, four yoghurts were analysed, and two replicates of each yoghurt were made. Morphine, papaverine, and noscapine were detected in all of them, although below the quantification limit of the method. In conclusion, the consumption of this commercial yoghurt does not pose a health risk to the consumer due to its low OAs content.

3.4. Study of fermentation and storage effect

Finally, once the proposed methodology was validated, its application was confirmed in commercial yoghurts and homemade yoghurts as shown in Table 2. In the case of home-made yoghurts, a calibration line was performed for each condition, i.e., with a fermentation time of 6h, with 18 h and with one week of refrigerated storage. This was done to check that this developed methodology could also be used for the analysis of these types of yoghurts, even if the starting ingredients of the yoghurt are somewhat different. For this purpose, as shown in Table 2, matrix calibration lines were performed, confirming that the linearity in each of the samples did not vary with respect to that already determined in the previous section (Table 1). In addition, the matrix effect that each of them could have been calculated. In the case of homemade yoghurt, the matrix effect was negligible in any of the conditions studied, as it was lower than +/- 20%. However, commercial yoghurt showed some matrix effect in the case of thebaine and papaverine (-52 and -45%, respectively). This could be since this commercial yoghurt had a higher sugar content compared to the others. On the other hand, recovery values were also calculated on three different samples to confirm the good accuracy of the developed method on these types of yoghurts. In all of them, including commercial yoghurts, adequate validation values between 73 ± 3% and 97 ± 6% were shown for all analytes. These last results confirm that the methodology developed in the present work is efficient for the quantification of OAs in different types of yoghurts.

Table 2. Equations of the matrix-matched calibration with yoghurts analysed and the matrix effects calculated.

Analytes	Linear range (mg/L)	Commercial yoghurts		Homemade yoghurts at 6 h of fermentation		Homemade yoghurts at 18 h of fermentation		Homemade yoghurts at 1 week of storage	
		Matrix-matched calibration (R^2) [a]	ME [b]	Matrix-matched calibration (R^2) [a]	ME [b]	Matrix-matched calibration (R^2) [a]	ME [b]	Matrix-matched calibration (R^2) [a]	ME [b]
Morphine	0.005-0.5	$y = 9\times10^7x - 7\times10^5$ (0.999)	-23	$y = 1\times10^8x + 7\times10^4$ (1.000)	23	$y = 1\times10^8x + 7\times10^4$ (1.000)	25	$y = 1\times10^8x + 5\times10^5$ (1.000)	-19
Codeine	0.005-0.5	$y = 1\times10^8x - 4\times10^5$ (1.000)	-22	$y = 2\times10^8x + 6\times10^3$ (1.000)	-7	$y = 2\times10^8x - 9\times10^4$ (1.000)	-4	$y = 2\times10^8x + 3\times10^4$ (1.000)	6
Thebaine	0.001-0.5	$y = 8\times10^9x - 2\times10^6$ (1.000)	-52	$y = 1\times10^9x - 7\times10^5$ (1.000)	2	$y = 2\times10^9x - 3\times10^5$ (1.000)	1	$y = 2\times10^9x + 6\times10^5$ (1.000)	14
Papaverine	0.001-0.5	$y = 2\times10^9x - 1\times10^7$ (1.000)	-45	$y = 3\times10^9x - 5\times10^5$ (1.000)	-1	$y = 3\times10^9x + 2\times10^6$ (1.000)	1	$y = 4\times10^9x + 2\times10^6$ (1.000)	12
Noscapine	0.001-0.5	$y = 3\times10^9x - 9\times10^7$ (0.999)	15	$y = 3\times10^9x + 3\times10^7$ (1.000)	5	$y = 2\times10^9x + 3\times10^7$ (1.000)	-17	$y = 3\times10^9x + 5\times10^7$ (1.000)	-8
Oripavine	0.005-0.5	$y = 1\times10^8x - 1\times10^5$ (1.000)	-19	$y = 1\times10^8x - 2\times10^5$ (1.000)	12	$y = 1\times10^8x - 1\times10^4$ (1.000)	14	$y = 1\times10^8x - 4\times10^4$ (1.000)	14

[a] The calibration line is in the units: mg/L; [b] ME: matrix effect (purified matrix slope / solvent slope -1) × 100.

To carry out the study of fermentation and storage effect, before adding the yoghurt, 1 g of poppy seeds, previously washed and dried, and spiked to the concentration set by legislation as the maximum limit (20 mg/kg) (Commission Regulation, 2021) was added to these containers. The aim of this study was to know the starting concentration of the seeds since there is so wide dispersion within the same batch of seeds.

The study was carried out in duplicate, and two yoghurts were prepared in each of them, with each of the fermentation times studied, 6 and 18 h (Fig. 1). After its elaboration, the yoghurts were cooled, their pH was measured, and they were frozen until further analysis by HPLC-MS/MS. The pH of all the yoghurts was measured to check if there were differences between the values obtained at 6 h of fermentation and 18 h and if, in addition, after one week of refrigeration it was also altered. In all cases, the pH values measured were between 3.92 and 4.39, showing no statistically significant differences between the conditions studied. Subsequently, each of the yoghurts was analysed in duplicate, and the mean OAs concentrations obtained in each of the yoghurts (n=8) were calculated. An additional test was carried out by storing the yoghurts with a 6-hour fermentation in the refrigerator for 1 week at 4ºC to determine whether stored in the refrigerator can reduce the OAs content since although lactic acid bacteria have a slower metabolism at refrigeration temperatures, they are still active and therefore some fermentation is still taking place.

For the analysis of these homemade yoghurts, the analysis methodology proposed in this work was used. It was confirmed that all home-made yoghurts showed good recovery values and negligible matrix effects (Table 2). Despite this, the areas obtained were corrected with the signal of the internal standards to give even more validity to the method. For morphine, thebaine, papaverine, noscapine and oripavine, the morphine-d3 standard was used and for codeine the codeine-d3 standard. Subsequently, the areas were interpolated on the matrix calibration line elaborated with the yoghurt at a specific fermentation time (for the yoghurts with 6 h of fermentation, a matrix calibration line was made with yoghurt containing unspiked poppy seeds with fermentation of 6 h and for the yoghurts with 18 h of fermentation with the yoghurt with 18 h of fermentation). Once the concentrations were obtained, the corresponding calculations were applied to calculate

the mg of each of the OAs per 100 g of yoghurt (the mg of OAs present in each of the yoghurts produced). In addition, considering the concentration at which the seeds were initially spiked, the degradation ratio of each of the yoghurts was calculated using the following formula: 100-(the concentration obtained in each yoghurt/ concentration spiked at the start) x 100. For both the concentrations (mg/100g) and the degradation ratios (%), the means (n=8 for yoghurts with 6 and 18 h of fermentation and n=4 for yoghurts stored for one week under refrigeration) and standard deviations (SD) were calculated as shown in Fig. 4. For all analytes, considerable degradations were obtained with lactic fermentation of yoghurt bacteria, showing values up to 80 ± 7% for papaverine, 74 ± 12% for noscapine, 65 ± 13% for thebaine, and somewhat lower for morphine, codeine, and oripavine, being 56 ± 12, 43 ± 10, and 33 ± 6%, respectively. There were no statistically significant differences at 6 and 18 h, so it can be affirmed that the degradation of OAs that originates with the lactic fermentation of yoghurt occurs in the first hours of fermentation, and even if the fermentation lasts more hours, there is no significantly greater degradation. On the other hand, yoghurts made with a 6 h fermentation were stored under refrigeration for one week and analysed. The degradation values obtained did not show statistically significant differences, so the continued fermentation by the lactic acid bacteria under refrigeration does not continue to degrade the amount of OAs in the poppy seed yoghurt. In the case of tropane alkaloids (TAs), the effect of alcoholic fermentation in bread has been evaluated showing between 19-65% degradation and an increase of the lower molecular weight TAs (Marín-Sáez et al., 2019). In the case of pyrrolizidine alkaloids (PAs), the effect of lactic fermentation has been evaluated in yoghurt and cheese, and in both cases, a reduction of the initial levels of PAs in the starting milk was observed. In yoghurt, a 27% reduction in total PAs content was determined after 6 h of fermentation at 42 °C. For cheese, a 14% reduction was observed during the cheese-making process (De Nijs et al., 2017).

Regarding the highest quantified mean concentrations (n=8) of each of the OAs were 0.01 mg/100 g for morphine, 0.012 mg/100 g for codeine, 0.007 mg/100 g for thebaine, 0.008 mg/100 g for papaverine, 0.006 mg/100 g for noscapine and 0.013 mg/100 g for oripavine. The acute dose of morphine equivalents (morphine + 0.2 x codeine) set by EFSA in 2018 is 10 µg per kg body weight (EFSA, 2018). Thus, for a 20 kg child, the

acute dose would be 200 µg, and for a 60 kg adult, it would be 600 µg of morphine equivalents. Therefore, the morphine equivalent amounts quantified in homemade yoghurt are approximately 12.4 µg. Therefore, in order to exceed the recommended acute intake, many yoghurts should be consumed per day (5 in the case of children), as the amount of seeds in the final product is very small (1%). However, since lactic fermentation decreases the amount of OAs in yoghurt, it would be advisable to add poppy seeds before the yoghurt is formed rather than after, especially if the amount added by the consumer is higher.

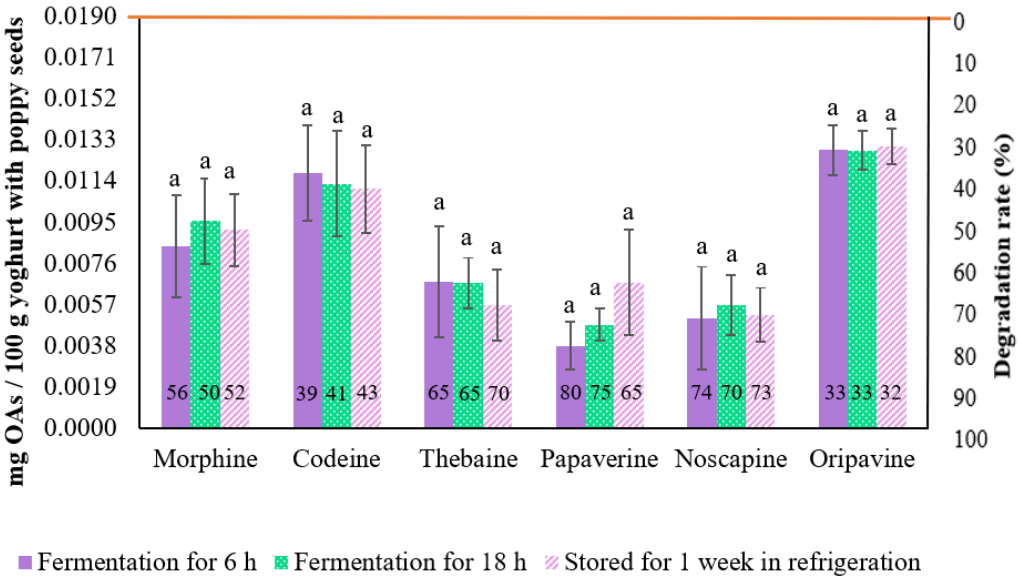


Fig. 4. Mean concentrations (n=8 for 6 and 18 h and n=4 for stored for 1 week) of OAs obtained in each yoghurt with their degradation rate (%) for fermentation effect. All of them spiked at 0.019 mg OAs/100 mg yoghurt with poppy seeds. Equal letters mean that there are no statistically significant differences ($p \leq 0.05$).

4. Conclusions

A rapid, simple, and effective analytical methodology was developed for the quantification of 6 opium alkaloids (OAs) in yoghurt with poppy seeds. This methodology was validated in terms of linearity, limits of detection and quantification in the order of ppb, and the purification step with the magnetic material based on mesostructured silica and functionalised with β-CD allowed the matrix effect to be negligible. Accuracy and

precision were adequate at the three levels of validation evaluated, with average recovery values between 92 and 101% for all analytes and with intra-day and inter-day precision values of less than 11 and 15%, respectively. Therefore, the proposed methodology was applied to quantify OAs in a commercial yoghurt sample, detecting morphine, papaverine, and noscapine in all of them but below the limit of quantification. In addition, it was used to evaluate whether the fermentation process has any effect on the OAs content in yoghurt. Two fermentation times (6 and 18 h) were evaluated, and it was left for one week under refrigeration to see if storage would have any effect. The results showed that considerable degradations of all OAs are achieved with lactic fermentation of yoghurt bacteria, showing values up to 80 ± 7% for papaverine, 74 ± 12% for noscapine, 65 ± 13% for thebaine, and somewhat lower for morphine, codeine, and oripavine, being 56 ±1 2, 43 ± 10, and 33 ± 6%, respectively. Furthermore, this effect is produced in the first hours of fermentation, as no statistically significant differences were shown between 6 and 18 h. Moreover, storage also did not increase the degradation, but was maintained over time. These degradations were only determined with one type of bacterial starter culture strain. An interesting future work could be to see if there are significant differences in degradation after fermentation with different strains. In short, fermentation is a process that allows the reduction of OAs in yoghurt, so it is recommended to add the seeds before fermentation and not to add them directly to the prepared yoghurt to prevent the consumption of this type of food toxins.

Data availability. All data supporting this study are included in the article and supplemental data.

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Supplementary Information

Table S1. Optimal parameters of MRM for the analysis of each opium alkaloid by HPLC-MS/MS.

Analytes	Precursor ion (Q_1, m/z, $[M+H]^+$)	Fragment ion[a] (Q_3, m/z)	CE[b] (eV)	Rt[c] (min)
Morphine	286.1	**153.0** 165.0 228.6	45 24 22	5.179
Morphine-d3	288.7	**152.3** 164.2 200.6	45 37 25	5.819
Codeine	300.2	153.1 165.0 **215.1**	45 45 23	5.528
Codeine-d3	303.4	182.2 199.0 **215.2**	30 30 24	5.533
Oripavine	298.3	236.9 **249.1** 267.1	14 17 12	5.648
Thebaine	312.3	**58.2** 166.2 249.4	8 16 16	6.292
Papaverine	340.2	**202.0** 324.1	24 30	6.507
Noscapine	414.3	205.1 **220.0** 280.1	24 42 20	6.554

[a]The fragment ion used for the quantification are in bold; [b]CE: collision energy; [c]Rt: retention time. The gradient elution was performed with water (A) and acetonitrile (B) as follows: 90−30% A (0−6 min), 30−90% A (6−9 min), and 90% A (9−11 min) for column reequilibration.

Supporting Information S1. Method validation.

The first validation parameter evaluated was linearity. which was assessed with matrix-matched calibration curves prepared on three consecutive days. All these curves were prepared for yoghurt with poppy seeds spiked at seven known concentration levels within the linear range evaluated. For this purpose, the sample extracts obtained after the SLE and MSPE procedures were spiked with an aliquot of a standard solution containing the target alkaloids according to the concentration level of the calibration curve. In addition, quantification of the OAs was performed by isotope-labelled IS correction, for morphine and the rest of the OAs morphine-d3 was used and for codeine, codeine-d3. To do this, 50 µL of 1 µg/mL of morphine-d3 and codeine-d3 were added to each point of the matrix-matched calibration curves. The criteria for good linearity involve values ≤ ±20% for the deviation of the back-calculated concentrations of the calibration standards from the true concentrations (SANTE/11312/2021, EC No 401/2006). The sensitivity of the method was determined through the MDLs and MQLs of the OAs from the analysis of the lowest concentration analysed (0.001 or 0.005 µg/mL), which were estimated as the minimum concentration yielding a signal-to-noise ratio (S/N) of 3 or 10, respectively. Matrix effects were determined by comparing the slopes of the calibration equations obtained from both matrix-matched and solvent-based calibration curves, calculating (slope matrix-matched/slope solvent-based -1) × 100 for each analyte. The ME is lower when closer to 0%, and according to the guideless, the ME is negligible when is lower than +/- 20%. Positive values greater than 20% indicate signal enhancement, and negative values indicate signal suppression. However, when the signal suppression or enhancement is higher than this margin of 20%, matrix effects must be considered in calibration, and therefore, matrix-matched calibration curves are necessary for quantification (SANTE/11312/2021). The recovery assays were assessed by comparing the areas obtained for samples (n = 6) spiked with a known concentration of analytes (low, medium, and high level) and subjected to the SLE-MSPE procedure with those areas obtained for simulated samples (samples spiked at the same concentration but prior to their chromatographic analysis). The recovery values should be between 70 and 120%. On the other hand, the method precision was evaluated in terms of repeatability and reproducibility. For repeatability (expressed as RSD %), a sample spiked with the OAs at

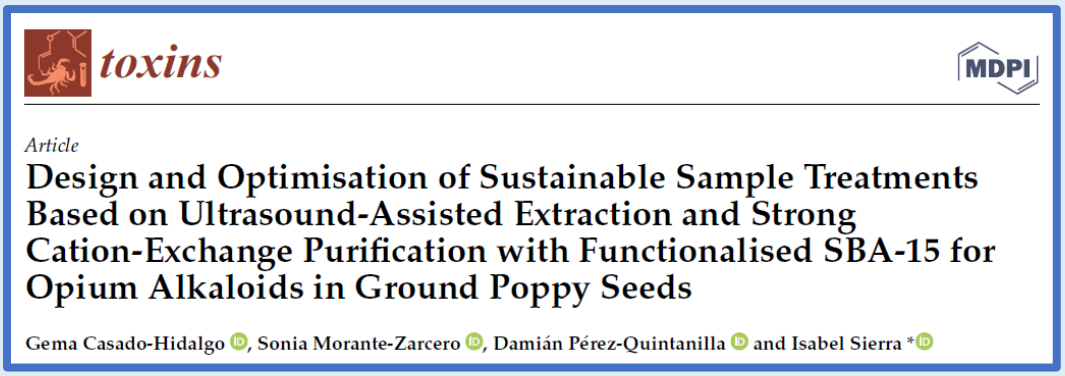
the corresponding validation level was consecutively carried out six times (n = 6) on the same day. The reproducibility (also expressed as RSD %) was calculated by the analysis of three replicates of a sample (spiked with the analytes at the corresponding validation level), which were carried out in triplicate over three different days (n = 9). According to the validation guidelines, the RSD values for these precision parameters should be ≤20% (SANTE/11312/2021). Finally, the selectivity of the method was determined by comparing the spectra of the different analytes obtained from standard solutions with the spectra obtained in the samples. It was considered satisfactory when the variation in the spectra was <±30 % and the retention time of the target analytes was within the interval of ±2.5 % (SANTE/11312/2021).

Finally, once the proposed methodology was validated, matrix-matched calibration curve was performed in commercial yoghurts and in yoghurts elaborated in a homemade way with the aim of confirming the correct application of the developed methodology to these other types of yoghurts. The possible matrix effect was calculated in each of them and, in addition, the recovery values at the low validation level were calculated in triplicate to confirm the good accuracy of the method in these types of yoghurts.

Artículo 7:

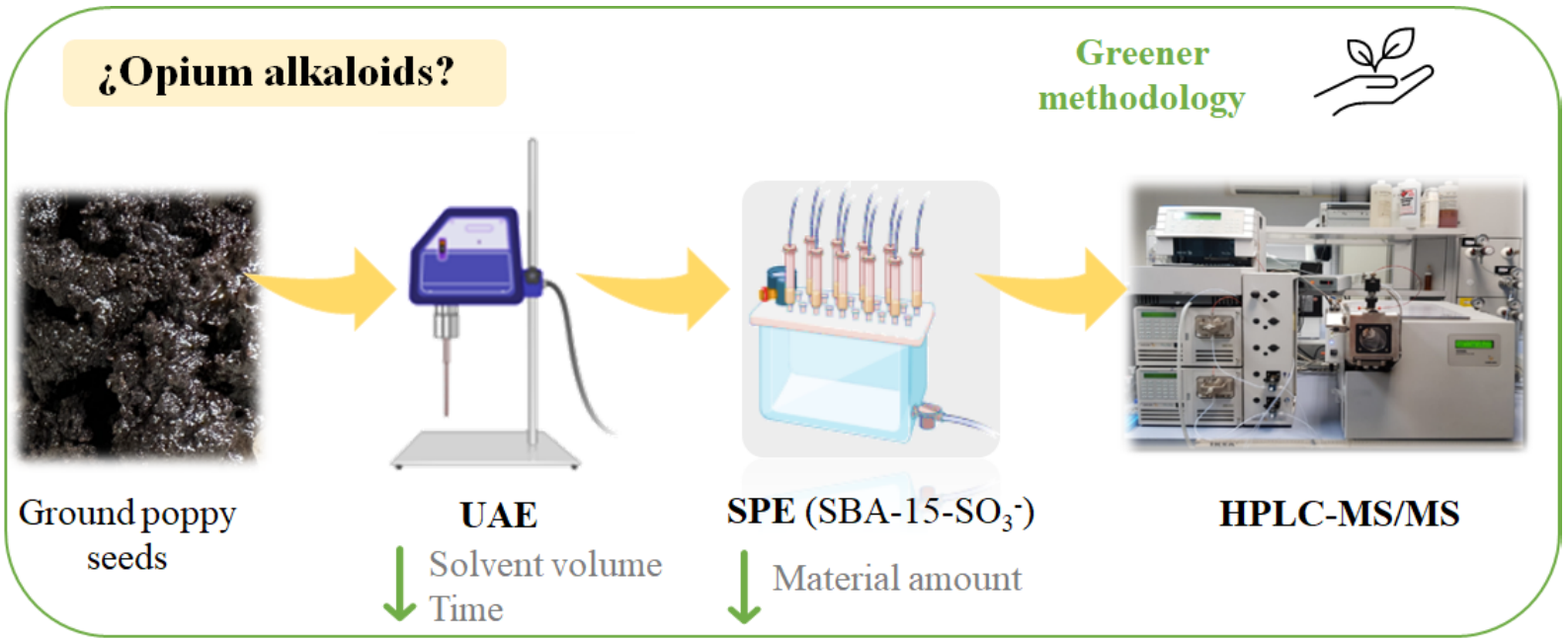
Design and optimization of sustainable sample treatment based on ultrasound-assisted extraction and strong cation-exchange purification with functionalized SBA-15 for opium alkaloids in ground poppy seeds

Toxins (2023) 15, 672.



PUBLICATION INFORMATION

Journal	Toxins
Publisher	MDPI
Impact factor	4.2 (JCR, 2022)
Category	Toxicology (23/94, Q1)
DOI	https://doi.org/10.3390/toxins15120672


¿Opium alkaloids?
Greener methodology
Ground poppy seeds
UAE
Solvent volume
Time
SPE (SBA-15-SO3-)
Material amount
HPLC-MS/MS

ABSTRACT

An analysis methodology was optimised and validated for the quantification of opium alkaloids (OAs) in ground poppy seeds. This involved ultrasound-assisted extraction (UAE) and solid-phase extraction (SPE) purification before analysis using a high-performance liquid chromatography mass spectrometry detector (HPLC-MS/MS). UAE was optimised through the design of experiments with three factors and a three-level full factorial design. For SPE optimisation, a commercial material was compared with a previously synthesised material of SBA-15 silica functionalised with sulfonic groups (SBA-15-SO_3^-). The synthesised material demonstrated superior efficiency with only 25 mg and proved to be reusable for up to four cycles. The methodology was properly validated in terms of linearity, limits of detection and quantification, and selectivity. Matrix effects were negligible; adequate recovery values (85–100%) and inter-day and intra-day precision (≤15%) were obtained. The greenness of the method was evaluated with the AGREEprep metric scale, being more environmentally friendly compared to OA analysis methods. Finally, the method was applied to different samples of ground poppy seeds and revealed a concentration of 140 mg/kg of morphine equivalents in one of the samples, surpassing the legislatively established limits by sevenfold. This highlights the need to analyse these types of samples to mitigate potential public health issues.

Keywords: Opium alkaloids; Ground poppy seeds; Ultrasound-assisted extraction; Solid phase extraction; SBA-15 functionalised with sulfonic groups; HPLC-MS/MS; Food safety; Natural toxins.

Key Contribution: A simple and sustainable method was developed and validated for the analysis of OAs in ground poppy seeds via HPLC-MS/MS, using less solvent and shorter extraction times through ultrasound-assisted extraction and less material for purification via solid-phase extraction.

1. Introduction

There is a growing trend to incorporate seeds into food for both sensory enjoyment and nutritional benefits, such as poppy seeds (from *Papaver somniferum* L.) [1]. These seeds are widely consumed in Central Europe, and their use is increasingly prevalent across various food products. Notably, poppy seeds are used in bakery products (including different types of bread or biscuits), as well as in yoghurt and even for preparing infusions [2]. Additionally, poppy seeds are commonly ground and employed as fillings in traditional sweet dishes and pastries, such as cakes, strudels, fritters, pastries, or poppy dumplings [2,3]. Nevertheless, it is crucial to be aware that poppy seeds have the potential to be contaminated with opium alkaloids (OAs) when they come into contact with the latex of the plant, which is rich in OAs. Significantly elevated concentrations, possibly attributed to automatic harvesting methods or insect damage, have been observed [4]. The consumption of contaminated poppy seeds poses risks, including intoxication and positive results in drug tests for athletes or workers [5]. Consequently, a maximum limit of morphine equivalents (morphine + 0.2 × codeine) in poppy seeds has been established [6]. Nevertheless, this legislation only involves two OAs (morphine and codeine). Given findings from previous studies indicating the presence of other OAs (such as thebaine, papaverine, noscapine, and oripavine) in substantial concentrations, health authorities emphasise the need for further research to control these additional OAs, which may possess higher toxicity than morphine or codeine [5,7]. Presently, there is a lack of a properly validated method specifically designed for the analysis of ground seeds; existing methodologies are tailored only for whole seeds. This is a critical consideration, as ground seeds are widely used and are more complex samples. In addition, it is important to highlight that grinding has been shown to decrease the morphine content in seeds by approximately 25–34%, as is shown in an EFSA recommendation and in other studies [8,9]. However, further studies are required to determine the grinding conditions leading to degradation and to assess the influence of other OAs [10]. Therefore, to study all this, there is a pressing need to develop a rapid, efficient, and environmentally friendly analytical method for quantifying all six OAs in ground poppy seeds.

For this purpose, sample preparation is a key step in the analytical method when dealing with analytes present at low concentrations in highly complex matrices [11]. Historically, the primary methods for extracting OAs have relied on liquid-liquid extraction (LLE). However, this approach typically involves the use of high volumes of solvent, long extraction times, and sometimes successive extractions to achieve full recovery. Unfortunately, these aspects make the methodology less environmentally friendly [4]. For these reasons, there is a trend towards the development of more environmentally sustainable methodologies, characterised by shorter extraction times and smaller solvent volumes [12,13]. An emerging and popular technique in this regard is ultrasound-assisted extraction (UAE), known for its increased sustainability. UAE enables high extraction yields with less solvent consumption, attributed to the cavitation effect induced by ultrasound [14,15]. The efficiency of UAE is influenced by numerous variables, including solvent type, solid/liquid ratio, and extraction time, emphasising the importance of optimisation to maximise extraction efficiency. Response surface methodology (RSM) proves highly valuable in this context, allowing for the systematic optimisation and evaluation of multiple factors at different levels and their possible interactions. RSM is based on fitting a polynomial model equation to various experiments, describing the characteristics of the dataset, and additionally providing statistical prediction equations [16,17]. This extraction technique has previously demonstrated success in extracting OAs from bakery products [18].

On the flip side, achieving an efficient extraction of OAs from the matrix poses a challenge, given that ground poppy seeds are complex samples with many components characterized by diverse physico-chemical properties. Consequently, a crucial step in the process involves purification to eliminate potential interferents from the extract, ensuring a cleaner extract and preventing contamination that may harm the equipment's column or the detector. The widely adopted method for this purpose is solid-phase extraction (SPE), chosen for its notable advantages [19,20]. In previous studies, a variety of commercial sorbent materials have been employed for clean-up purposes. Examples include the use of the Chem Elute column to purify morphine and codeine in serum and urine after ingestion of poppy seeds [21], Clean Screen ® DAU to purify morphine in human urine [22], and Oasis ® MCX and Oasis ® HLB to purify five alkaloids of *Pericarpium*

Papaveris in a hot pot [23]. However, a current trend in analytical chemistry involves the synthesis and development of new absorbent materials with more controlled and improved textural characteristics, allowing stronger and more specific interactions with target analytes [20,23,24]. Mesostructured silicas, such as SBA-15 (Santa Barbara Amorphous-15), have garnered increasing interest in sample preparation research [18]. This is attributed to its fast, cost-effective, and straightforward synthesis, yielding a sorbent with many advantages. These include a highly ordered and size-controlled structure, a high surface area, a large pore volume, good chemical stability, and the ability to be functionalised with different functional groups [25]. Notably, these functional groups contribute to active sites that are more selective than pristine SBA-15. For instance, strong ion-exchangers as sulfonic acids, in addition to providing hydrogen bonds through the free silanol groups of the silica, induce retention towards cationic compounds due to the presence of anionic functionalities (SO_3^-) [26]. The described interactions provide greater strength and selectivity, facilitating a reduction in the required quantity of material. This not only enhances efficiency but also aligns with environmentally friendly practices.

Therefore, the aim of this study was to develop and validate an efficient and environmentally friendly analytical method for quantifying six OAs in ground poppy seed samples. This involved using a UAE-SPE sample preparation protocol with a strong cation-exchange purification step using a functionalised SBA-15 silica with sulfonic groups (SBA-15-SO_3^-) as the solid phase, followed by analysis using liquid chromatography coupled to a triple quadrupole tandem mass detector (HPLC-MS/MS). To obtain the highest recovery values for a faster, simpler, and more environmentally friendly methodology, the extraction conditions in the UAE were optimised using response surface methodology (RSM).

2. Results and Discussion

2.1. Characterisation of the Synthesised Material

To verify the successful synthesis, elemental analysis (EA) was performed to estimate the degree of functionalisation of the material. Nitrogen gas adsorption–desorption isotherms were also employed to evaluate the pore volume, surface area, and pore distribution (for more details, see Supplementary Information S1). First, the EA was carried out for both the final material with the SO_3^- groups and the non-oxidised material with the (SH^-) groups. For the non-oxidised material, the C and S ratios were 4.378 and 1.473%, respectively. In contract, for the final oxidised material, these ratios decreased to 4.042 and 1.344%, respectively. The decrease in both percentages after oxidation validates the correct oxidation and functionalisation of the material. In addition, the quantity of ligand in the final material was estimated at 1.19 mmol S/g of material, aligning with values obtained by our research group [26]. The specific surface area (S_{BET}) of the material was 563 m^2/g, with an average pore volume of 0.67 cm^3/g and an average pore diameter of 49.2 Å. These results coincide with expectations, according to previous results obtained in our research group [26].

2.2 Optimisation of the Sample Preparation

2.2.1 Optimisation of the Purification via SPE

First, a comparison was conducted between the synthesised material (SBA-15-SO_3^-) and a commercial material (MFE-PAK® SCX) using two different amounts of material (25 and 50 mg). Both materials were subject to the same conditions of conditioning (2 mL of water with 1% HCl), loading (2 mL of 0.5 mg/L OAs in water with 1% HCl), and elution (2 mL of methanol with 5% ammonia). The results, as shown in Figure 1a, reveal favourable recovery values (%) with both materials with 50 mg of material were favourable for all analytes: 82–100% in the case of SBA-15-SO_3^- and 72–96% with MFE-PAK® SCX. Notably, with only 25 mg of material, the synthesised material achieved significantly higher recovery values compared to the commercial material (92–103% vs. 52–83%). Therefore, the synthesised material showed enhanced efficiency, as recovery

values did not decrease with lower material amounts. Therefore, 25 mg of SBA-15-SO_3^- was selected for the present work. These results can be attributed to the higher S_{BET} of the SBA-15-SO_3^- material compared to the MFE-PAK® SCX sorbent (563 and 398 m^2/g, respectively) and a higher functionalisation degree (1.19 and 0.8 mmol SO_3^-/g, respectively). Therefore, the SBA-15-SO_3^- material has larger active sites for interaction, resulting in a higher efficiency than the commercial sorbent.

After confirming the efficacy of the synthesised material and optimising the required amount, a re-optimisation of the procedure was carried out. This consisted of determining whether 1% ammonia in methanol (instead of 5%) was sufficient to obtain adequate recovery values. The objective was to determine if this adjustment could enable the injection of purified extracts directly into the equipment without the need for evaporation, thus considerably shortening the analysis time. The results indicated that there were no significant differences in the recovery values obtained when using 1% and 5% ammonia. Consequently, the 1% ammonia solution was selected for subsequent steps.

Following the optimisation of the SPE procedure with standards, the next step involved optimising the procedure using the extract obtained from the sample. First, the recovery values corresponding only to the SPE procedure were evaluated after passing 2 mL of the sample undiluted, as well as extracts diluted 1/10 with water containing 1% HCl and diluted 1/20. To carry out this determination, a sample extract was taken and spiked just before the purification process at a known concentration (1 mg/L). The ground poppy seed sample extract was prepared using the initial UAE conditions (0.5 g sample with 10 mL of 1% HCl water for 10 min of ultrasound). The recovery values are shown in Figure 1b, whereas acceptable results were achieved with dilutions. Consequently, it was concluded that a 1/10 dilution of the extract at the loading stage of the SPE procedure was necessary.

Subsequently, as the solvents used for the optimisation of the UAE will be water, methanol, and ethanol, the SPE procedure was evaluated as showing adequate recovery values with each solvent. Therefore, three extracts were prepared with the initial conditions of the UAE and each of the three types of solvents. Subsequently, each of the extracts was spiked to a known concentration (1 mg/L), and a 1/10 dilution with water

with 1% HCl was carried out. As shown in Figure 1c, no significant differences were shown between the three types of solvents used. Adequate recovery values were obtained in all cases. Therefore, it was concluded that the SPE step was successfully optimised, and an evaluation of the UAE step was carried out with the certainty that lower recovery values than those obtained at this point were due to insufficient extraction in the UAE step.

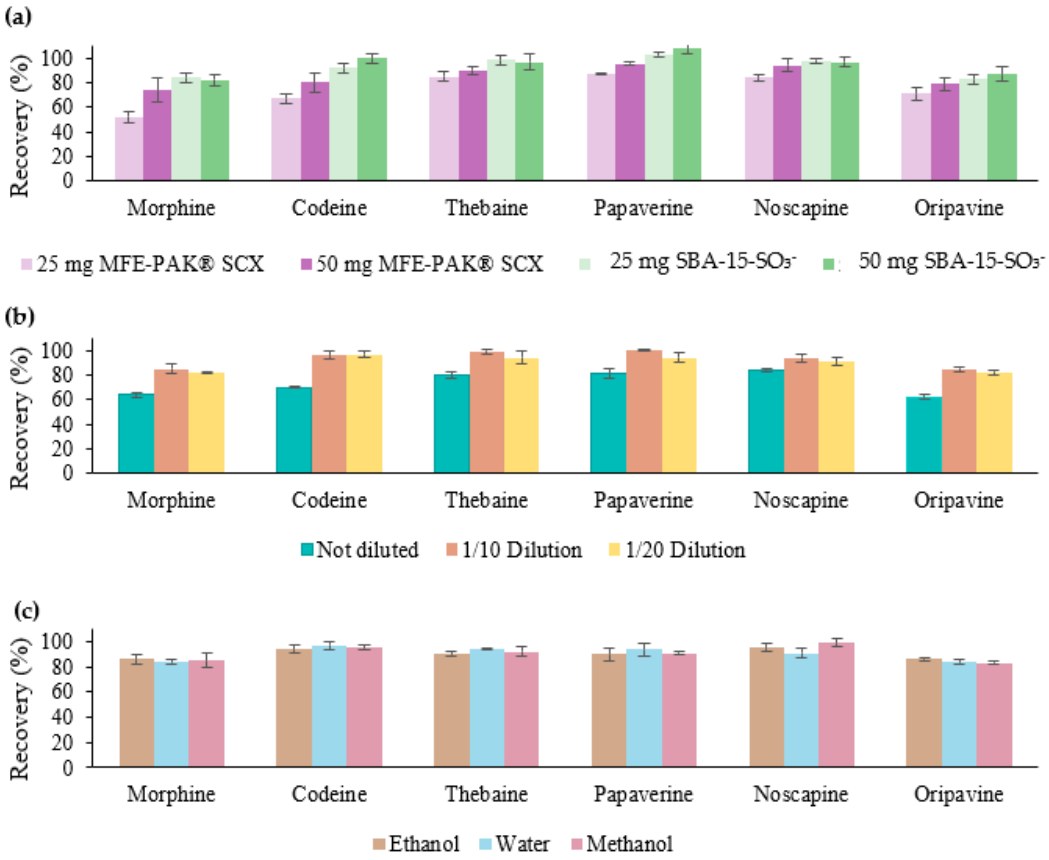


Figure. 1. Recovery values (%) obtained with 25 and 50 mg of MFE-PAK® SCX and SBA-15-SO_3^- material at the same SPE conditions with standards **(a)**; Recovery values (%) obtained with the sample extract not diluted, 1/10 dilution and 1/20 dilution with 25 mg of SBA-15-SO_3^- **(b)**; Recovery values (%) obtained with the sample extract diluted in ethanol, water, methanol 1/10 in water with 1% HCl with SBA-15-SO_3^- **(c)**.

2.2.2. Optimization of UAE

2.2.2.1. Effects on the extraction of the main UAE variables and statistical analysis

The experimental factorial design methodology incorporated a categorical factor, representing the type of solvent (A), and two numerical factors, which are the solid/liquid ratio (B) and the extraction time (C). This approach aimed to investigate how these three independent variables affect the UAE process. Each factor was evaluated at three levels (3^3) to determine the optimal conditions that yield the maximum recovery values (dependent variable) with the lowest extraction time and solvent volume, thereby enhancing the environmental friendliness of the methodology. Thus, a full factorial design of 27 experiments was established, representing a three-factor full factorial design at the three most promising levels, as determined through preliminary experiments and previous research (Table S1). In addition, based on our previous work [18], experiments were carried out with the UAE at a set amplitude (75%) and pulsed sonication mode (2:1). The recovery values for each of the six analytes served as the dependent variables.

The results from the design of the experiment are summarised in Table 1, showing the mean of the values obtained for each response with three replicates of each study ± standard deviation (SD).

Table 1. Results from the factorial experimental design to ultrasound assisted extraction (UAE) optimisation.

RUN	Factor*			Responses (Recovery mean (%) ± SD)					
	A	B	C	Morphine (Y_1)	Codeine (Y_2)	Thebaine (Y_3)	Papaverine (Y_4)	Noscapine (Y_5)	Oripavine (Y_6)
1	1	-1	-1	10 ± 2	17 ± 2	24 ± 3	28 ± 2	18 ± 1	51 ± 1
2	1	-1	0	17 ± 1	34 ± 1	32 ± 2	33 ± 3	29 ± 2	53 ± 2
3	1	-1	1	29 ± 3	42 ± 3	42 ± 3	40 ± 2	38 ± 4	43 ± 4
4	1	0	-1	28 ± 2	48 ± 4	52 ± 2	58 ± 1	49 ± 2	60 ± 1
5	1	0	0	28 ± 1	49 ± 2	53 ± 1	59 ± 2	53 ± 1	69 ± 2
6	1	0	1	37 ± 1	60 ± 3	60 ± 3	71 ± 2	62 ± 2	72 ± 3
7	1	1	-1	27 ± 3	40 ± 2	43 ± 2	47 ± 1	39 ± 3	76 ± 4
8	1	1	0	39 ± 2	69 ± 1	72 ± 4	81 ± 2	65 ± 2	76 ± 5
9	1	1	1	45 ± 1	85 ± 2	88 ± 2	91 ± 1	67 ± 2	26 ± 1
10	2	-1	-1	42 ± 1	40 ± 3	28 ± 1	5 ± 3	40 ± 1	37 ± 2
11	2	-1	0	46 ± 3	50 ± 2	30 ± 2	7 ± 2	46 ± 3	47 ± 2
12	2	-1	1	50 ± 1	55 ± 4	36 ± 3	7 ± 1	52 ± 1	46 ± 1
13	2	0	-1	52 ± 2	57 ± 1	38 ± 2	8 ± 2	52 ± 2	75 ± 6
14	2	0	0	62 ± 3	59 ± 3	38 ± 2	8 ± 3	55 ± 3	69 ± 5
15	2	0	1	64 ± 1	61 ± 2	39 ± 1	11 ± 2	56 ± 1	77 ± 4
16	2	1	-1	67 ± 5	72 ± 1	43 ± 4	12 ± 1	62 ± 2	73 ± 3
17	2	1	0	69 ± 2	73 ± 3	44 ± 2	13 ± 2	63 ± 1	54 ± 2
18	2	1	1	72 ± 3	73 ± 2	45 ± 4	13 ± 1	63 ± 2	57 ± 1
19	3	-1	-1	45 ± 2	48 ± 3	40 ± 2	58 ± 4	48 ± 4	54 ± 3
20	3	-1	0	49 ± 2	52 ± 2	47 ± 3	63 ± 2	55 ± 2	62 ± 2
21	3	-1	1	53 ± 3	57 ± 1	48 ± 2	65 ± 3	60 ± 4	65 ± 3
22	3	0	-1	54 ± 4	86 ± 3	72 ± 3	74 ± 4	75 ± 1	66 ± 2
23	3	0	0	81 ± 1	100 ± 2	94 ± 2	92 ± 5	97 ± 2	78 ± 3
24	3	0	1	81 ± 2	100 ± 4	97 ± 4	93 ± 3	100 ± 5	82 ± 4
25	3	1	-1	68 ± 3	82 ± 2	73 ± 2	80 ± 5	72 ± 2	39 ± 3
26	3	1	0	80 ± 2	100 ± 2	96 ± 3	97 ± 2	98 ± 3	45 ± 2
27	3	1	1	82 ± 1	100 ± 4	99 ± 1	100 ± 4	100 ± 1	55 ± 1

SD: standard deviation (n = 3). * (A) solvent type, (B) solid/liquid ratio, (C) extraction time (min) to UAE optimization.

The experimental values for each response varied within the following ranges: 10 ± 2 and 82 ± 1% for morphine, 17 ± 2 and 100 ± 4% for codeine, 24 ± 3 and 99 ± 1% for thebaine, 5 ± 3 and 100 ± 4% for papaverine, 18 ± 1 and 100 ± 5% for noscapine, and 26 ± 1 and 82 ± 4% for oripavine.

In addition, to evaluate the different types of effects of the variables, graphs were plotted, illustrating each of the main effects of each variable. This graphical representation aids in determining the positive or negative effect of each variable, as shown in Figure 2.

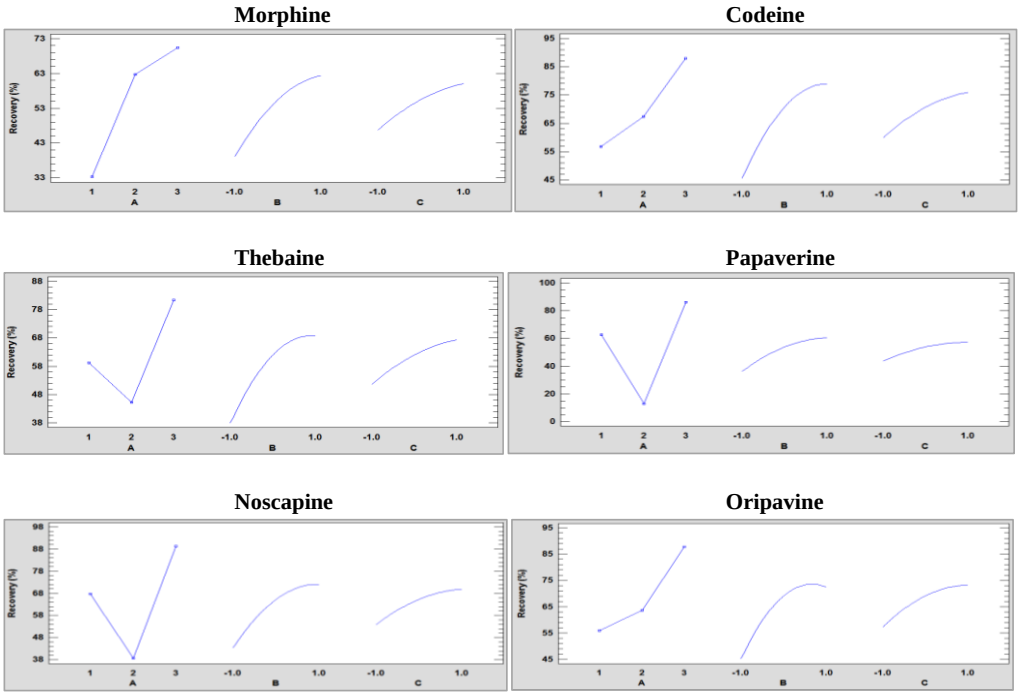


Figure 2. Main effects plots of the full factorial design 3^3 of each of the responses showing the three factors: **(A)** solvent type (1: ethanol, 2: water, and 3: methanol), (**B**) solid/liquid ratio (−1: 0.5 g/3 mL, 0: 0.5 g/5 mL, and 1: 0.5 g/10 mL), and (**C**) extraction time (−1: 3, 0: 5, and 1: 10 min) to UAE optimisation.

The solvent with the highest recovery values was methanol for all analytes. Distinct behaviours were observed between ethanol and water, depending on the analytes. Morphine, codeine, and oripavine exhibited higher recovery values with water, while thebaine, papaverine, and noscapine showed higher recovery values with ethanol. This variation can be attributed to the nature of each compound, as the more polar ones were better extracted with water and the more non-polar ones with ethanol. However, the recovery values were only favourable for all analytes with methanol, which has an intermediate polarity. On the other hand, the solid/liquid ratio displayed a positive effect on recovery values. However, as shown in Figure 2, there is no linearly increasing trend,

and the maximum recovery value did not correspond to the highest solid/liquid ratio studied. A plateau is observed in the final part, which may indicate that there was an intermediate optimum value at these levels. The same occurs when the extraction time increases, and the longer the extraction time, the higher the recovery values.

A statistical analysis (ANOVA) was carried out, and the obtained statistical parameters (R^2, Adj.; R^2, Pred.; and R^2 and *p*-values) were obtained as shown in Table S2. All statistical parameters indicated that the quadratic models exhibited very high predictability, as most coefficients were close to 1. In addition, ANOVA confirmed the variables that showed statistically significant differences in the quadratic models, where values of p were lower or equal to 0.05. For the recovery of codeine, papaverine, and noscapine, the statistically significant individual variables were solvent type (A) and solid/liquid ratio (B). In the case of the recovery of morphine and oripavine, besides those two, it was also the extraction time (C). Thebaine recovery only showed differences with the solid/liquid ratio. In terms of the combinations among the variables, the ones that presented statistically significant differences were the quadratic value of the solid/liquid ratio for all analytes. For the recovery of papaverine and noscapine, it was also the combination of solvent type and solid/liquid ratio (AB). Finally, for the recovery of morphine and oripavine, the quadratic value of the extraction time (CC) also showed statistically significant differences.

2.2.2.2. Optimisation of the Most Influential Variables of the UAE through RSM Approach

After establishing that all the independent variables and certain combinations showed statistically significant differences in responses, a multiple regression analysis was conducted to obtain mathematical models, as represented in Table S3. Table S3 effectively elucidates the empirical relationship among the three studied variables and various responses. In addition, Figure 3 displays the relationship between dependent and independent variables through response surface plots generated from the acquired polynomial equations. Notably, for the three types of solvents, the recoveries increased with increasing solid/liquid ratios in the same way as they increased with increasing extraction time. However, in the case of water, a point was reached where both the

solid/liquid ratio and extraction time no longer contributed to higher recovery values, indicating a plateau in the graph. In contrast, for methanol, the graph shows a much more pronounced increase. This is because the highest recovery values for all analytes were obtained with this solvent.

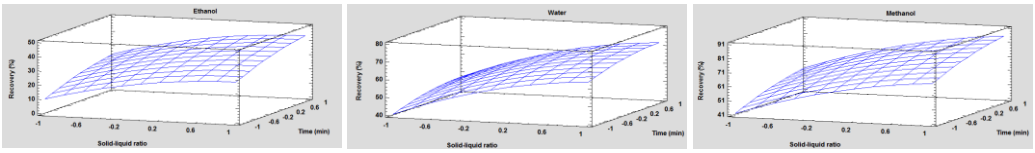
To determine the optimum level of each of the independent variables, numerical optimisations were carried out. For all analytes, a similar value was obtained, between 7.55 and 8.5 mL of solvent volume and 4.12 and 5.5 min of extraction time. Consequently, the selected optimal conditions were 8.5 mL of solvent volume for 5.5 min of extraction time for the optimal recovery of each analyte.

The experimentally determined results were compared with the results predicted using the mathematical equations derived from the RSM models. The aim was to assess the reliability of the RSM for quantitative prediction. The obtained values were very similar, confirming the effectiveness and validity of the response surface models.

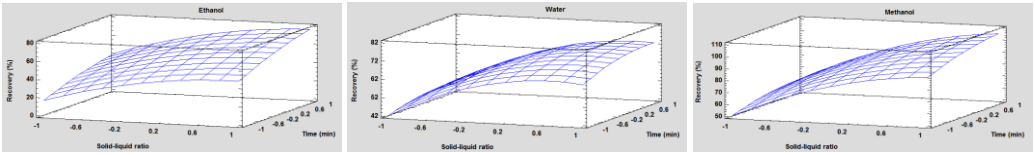
2.3. Method validation

The validation results of the optimised analytical method for the quantification of six OAs in ground poppy seeds are compiled in Table 2. The linear range for most analytes started at 0.001, except for oripavine, which began at 0.005 mg/L. The upper limit for all analytes was 1 mg/L. Additionally, calibration lines showed adequate R^2 values, ranging between 0.994 and 0.996. In addition, slope deviations were calculated for three different days (n = 3) to ensure reproducibility. Low RSDs between 0.3 and 10.9% were obtained, demonstrating good linearity. In addition, the deviations of the back-calculated concentrations of the calibration standards from the real concentrations in the matrix calibration lines were also calculated, yielding adequate values (≤±20%), specifically falling between −1.2 and 17.3% [27].

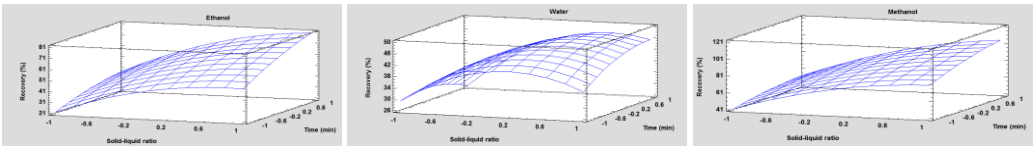
Morphine



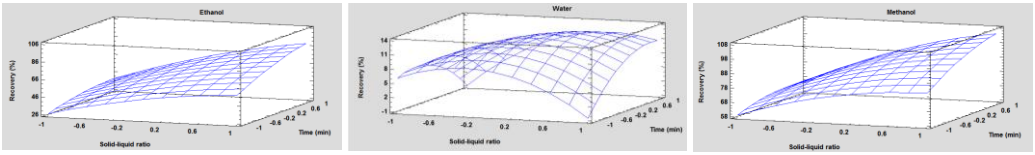
Codeine



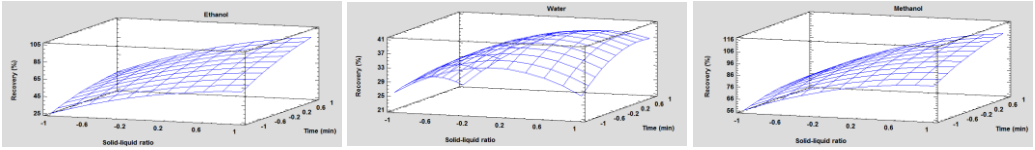
Thebaine



Papaverine



Noscapine



Oripavine

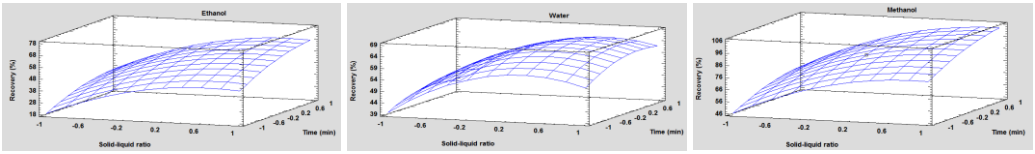


Figure 3. Plots of response surface demonstrate the effects of solid/liquid ratio and extraction time with each of the three solvent types on the recovery of each analyte to UAE optimisation.

To evaluate the possible matrix effect that may remain after doing the purification step, the slope of matrix-matched calibration curves was divided by solvent-based calibration curves. The results, presented in Table 2, ranged between −11 and 9%. In this way, as indicated in the validation guide, the matrix effect is less when it is closer to 0%, and the matrix effect is considered negligible when it is less than ±20%. If the result is more than 20%, this indicates enhancement of the signal, and results below 20% indicate suppression of the signal. In addition, it should be noted that if the signal suppression or enhancement is greater than this 20% margin, matrix effects must be taken into account in the calibration [27]. Therefore, this may mean that the procedure of purification developed was able to eliminate almost all possible matrix effects for all OAs.

To calculate method detection limit (MDL) and method quantification limit (MQL) values used, the lowest concentration was analysed and estimated as the lowest concentration, giving a signal-to-noise ratio (S/N) of 3 or 10, respectively. The obtained results were low enough to quantify the samples, specifically 0.007 and 0.03 mg/kg for noscapine, 0.01 and 0.05 mg/kg for thebaine and papaverine, 0.03 and 0.1 mg/kg for codeine, 0.06 and 0.20 mg/kg for morphine, and 0.2 and 0.5 mg/kg for oripavine, respectively.

Concerning accuracy and precision, both were evaluated at three different concentration levels, specifically a high level of 40 mg/kg, a medium level of 20 mg/kg, and a low level of 3.4 mg/kg. The results indicate satisfactory recovery values between 85 and 100% (Table 2). Additionally, intra-day and inter-day precision were evaluated at these three concentration levels. The RSD values were all below 15%, demonstrating adequate precision according to established guidelines [27].

Furthermore, a good selectivity of the analytical methodology was obtained because the variation of the retention time was ≤0.1 min and the ion ratios were within ± 30% (relative abundance) between the sample extracts and the mean of the standards for each analyte [27].

2.4. Evaluation of material reuse

The potential for material reuse is a crucial aspect to consider in the methodology. Reusing the material not only enhances the economic feasibility of the method but also contributes to its environmental friendliness.

To evaluate this, a study was carried out with three different cartridges (n = 3) with a blank sample extract and spiking it before the SPE procedure with 0.5 mg/L of OAs. Six cycles were run to evaluate at which point the recovery values started to decrease. Between the cycles, the cartridge was washed to remove any retained matrix interference. For this, 2 mL of the elution solvent and 2 mL of the conditioning solvent were passed through, drying for 1 min between the two steps. As shown in Figure 4, the recovery values started to decrease considerably in cycle 5. Therefore, it was concluded that the cartridge could be reused four times without losing efficiency.

Table 2. Validation parameters of the UAE-SPE-HPLC-MS/MS method for the quantification of six opium alkaloids in ground poppy seeds.

Analytes	Linear range (mg/L)	Matrix-matched calibration (R^2)	MDL (mg/kg)	MQL (mg/kg)	ME	Accuracy		Precision	
						Recovery (% ± SD)	Mean recovery (% ± SD)	Intra-Day Precision (RSD %)	Inter-Day Precision (RSD %)
Morphine	0.001-1	$y = 7.02\times10^{7}x + 1.68\times10^{6}$ (0.995)	0.06	0.2	9	99 ± 8^{LL} 82 ± 6^{ML} 73 ± 4^{HL}	85 ± 6	6^{LL} 3^{ML} 3^{HL}	8^{LL} 7^{ML} 6^{HL}
Codeine	0.001-1	$y = 7.65\times10^{7}x + 9.46\times10^{5}$ (0.996)	0.03	0.1	-1	95 ± 9^{LL} 106 ± 15^{ML} 101 ± 10^{HL}	100 ± 11	3^{LL} 4^{ML} 4^{HL}	10^{LL} 14^{ML} 10^{HL}
Thebaine	0.001-1	$y = 6.85\times10^{8}x + 1.49\times10^{7}$ (0.994)	0.01	0.05	-11	89 ± 11^{LL} 102 ± 12^{ML} 98 ± 10^{HL}	96 ± 11	3^{LL} 11^{ML} 7^{HL}	12^{LL} 11^{ML} 11^{HL}
Papaverine	0.001-1	$y = 1.77\times10^{9}x + 1.51\times10^{7}$ (0.995)	0.01	0.05	5	97 ± 15^{LL} 99 ± 7^{ML} 99 ± 6^{HL}	99 ± 12	12^{LL} 6^{ML} 3^{HL}	15^{LL} 15^{ML} 7^{HL}
Noscapine	0.001-1	$y = 2.76\times10^{9}x + 4.87\times10^{7}$ (0.995)	0.007	0.03	5	98 ± 9^{LL} 92 ± 13^{ML} 92 ± 12^{HL}	94 ± 11	6^{LL} 5^{ML} 6^{HL}	9^{LL} 13^{ML} 13^{HL}
Oripavine	0.005-1	$y = 7.60\times10^{7}x + 1.55\times10^{6}$ (0.996)	0.2	0.5	-4	95 ± 11^{LL} 95 ± 10^{ML} 91 ± 10^{HL}	94 ± 10	6^{LL} 3^{ML} 7^{HL}	12^{LL} 10^{ML} 11^{HL}

The linear range expressed in mg/kg is 0.17-170 to all analytes except for oripavine that is and 0.85-170; [a] MDL: method detection limit; [b] MQL: method quantification limit; [c] ME: matrix effect (purified matrix slope / the solvent slope – 1) × 100; [d] Accuracy and precision were obtained by spiking samples at three concentration levels: low (LL, 3.4 mg/kg), medium (ML, 20 mg/kg) and high (HL, 40 mg/kg).

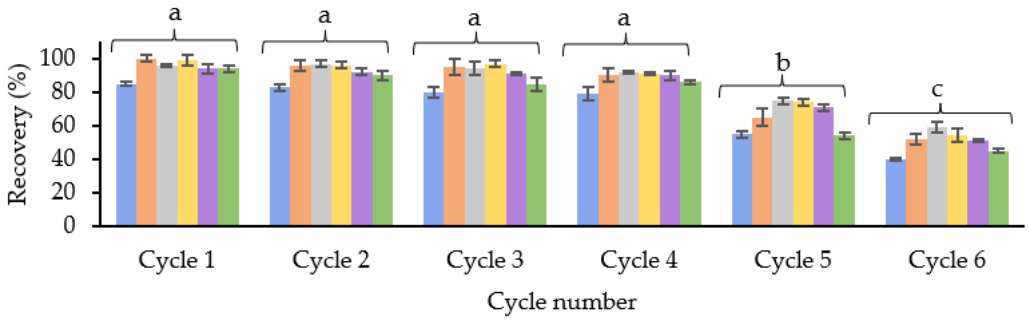


Figure 4. Average recovery values (%) (n = 3) obtained in each of the reuse cycles of the SPE cartridge with 25 mg of SBA-15-SO_3^- material. Different letters mean significant differences if $p \leq 0.05$.

2.4. Comparison to other methods and greenness evaluation

It is noteworthy that, as of now, there is no optimised and validated methodology specifically for ground poppy seeds, which present a more complex matrix compared to whole seeds. Therefore, the analytical methodology of the proposed method was compared to previous methods for the determination of OAs in unground poppy seeds, such as shown in Table S4.

It should also be noted that the previously published works in this field often do not incorporate a purification or cleaning step in their methodologies. The absence of such a step can lead to extracts from complex matrices containing large amounts of matrix interferents. This may result in extracts that are more turbid and dirty, potentially causing damage to the chromatographic column and the ionisation source in the analytical instrument [28]. On the other hand, in other works such as Sproll et al. (2006) [29] and Carlin et al. (2020) [30], neither the matrix effect nor the recovery values of the method have been studied, which shows an incomplete validation of these methodologies. On the other hand, our research group developed a methodology for the analysis of OAs in seeds [4] and obtained a slight matrix suppression effect in some analytes and an increase in others. In the same way, recovery values slightly below 70% and above 100% were obtained. Therefore, it can be stated that the methodology proposed in the present work is the most suitable for the analysis of OAs in ground poppy seeds of those published to date.

On the other hand, when comparing methods, it is also important to consider the environmental impact and safety aspects, often referred to as "greenness". To evaluate these factors, an assessment of greenness was performed based on the recently introduced metric tool AGREEprep [31]. The method proposed in the present work obtained the highest overall score of 0.47 (Figure 5a). Regarding the highest scoring items, the #2 hazardous solvents and reagents were the lowest scoring items due to the use of MeOH for both extraction and elution of the SPE (Table S5). However, the item #Target—sustainable, reusable, and renewable materials—was more favourable and was considered to have the highest score, since in any methodology where a purification step is performed with materials, it is essential that they can be reused and to minimise all costs and residue that can be generated from the performance of a synthesis. In addition, the maximum score was also given to the #minimize sample amount and the #maximize sample throughput that could be performed in one hour, as well as the amount of energy consumed per sample. The greenness assessment result obtained for the proposed methodology was compared with previous analytical methodologies. For instance, a methodology previously optimised to analyse whole seeds relied on SLE and a subsequent purification via magnetic solid-phase extraction (MSPE) [4]. In this case, the score obtained was 0.16 (Figure 5b). This was because the volume of toxic solvent was higher (34 mL), the material used for MSPE could not be reused, the number of seeds used was higher (2.5 g), and the extraction time was longer, namely 1 h of magnetic stirring (Table S5). In addition, the energy required for each sample was high (147.74 Wh) due to the different steps and a solvent evaporation step. Another example in which the number of steps is reduced and no purification is performed despite the matrix effect is the work of Sproll et al. [29]. In this case, the work obtained the highest overall score of 0.18 (Figure 5c). Although it was a methodology that had only one step in sample preparation and did not use much energy, the volume of toxic materials used (30.06 mL) and waste produced was high (50 Wh). In addition, the amount of sample used was higher, and the extraction time was 1 h (Table S5). Another example similar to the previous one is the EURL-MP-method_007 [32], which is a method developed by the EU Reference Laboratory for Mycotoxins & Plant Toxins in Food and Feed (EURL-MP) to determine morphine and codeine in poppy seeds. A highlight of this methodology is that it is the only one

previously published in which poppy seeds are ground to determine the concentration of OAs, which according to the EU recommendation in 2014 would not be very convenient, as grinding is a culinary processing that could decrease the morphine content of the seeds by 25–34% [8]. On the other hand, when subjecting the conditions of the methodology to the AGREEprep metric scale, it obtains the lowest score of the evaluated methodologies (0.12 points). This is mainly due to the large volume of solvent used to perform the SLE (100 mL), the high sample size (10 g), and the high extraction times (30 min stirring, 15 min waiting, and 10 min centrifugation) [32].

In conclusion, the optimisation of the sample preparation step is crucial to achieving a greener analytical methodology. It is not always the case that a purification step worsens the greening of the method. As demonstrated in these examples, it has been clearly demonstrated that this is not the case and that it is also very important to remove matrix interferents to obtain cleaner extracts. The most important factors influencing the environmental impact of these types of methods are solvent volume, solvent type, extraction time, and sample amount. Therefore, it is important to optimise SLE as much as possible to use less sample, solvent, and time, and replacing SLE with UAE can help to further decrease these factors, making the analytical methodology more environmentally friendly.

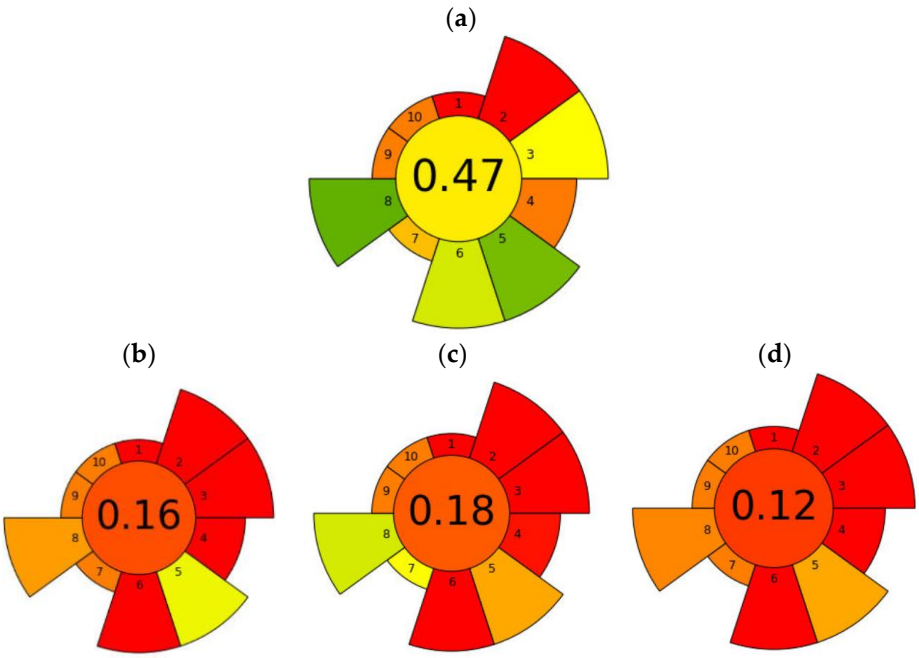


Figure 5. Evaluation of the greener profile of the proposed methodology in this work based on UAE-SPE-LC-MS/MS (**a**), a method based on SLE-MSPE-LC-MS/MS [4] (**b**), a method based on SLE-LC-MS/MS without the purification step [29] (**c**), and another method based on SLE-LC-MS/MS [32] (**d**) using the AGREEprep metric proposed by Pena-Pereira, Tobiszewski, Wojnowski, and Psillakis [31].

2.6. Application of the proposed methodology to OAs analysis in ground poppy seeds

The proposed method was successfully applied to the analysis of three different types of ground poppy seeds. The obtained areas for each analyte could have been interpolated in the solvent calibration line directly because the ME in all cases was negligible. In fact, quantifications were checked with both lines after confirming the absence of a matrix effect, and the concentrations obtained were the same. This indicates that the use of an internal standard may not be necessary, potentially reducing additional costs. However, to further validate the reliability of the developed method, internal standards were used to correct the signals of the matrix-adjusted calibration. Morphine-d3 was used to correct the signals of all analytes except codeine, which was corrected with codeine-d3. Table 3

shows the mean concentrations (mg/kg) of each analyte obtained from six replicates (*n* = 6) for each of the three different samples.

Table 3. Occurrence (mg/kg) ± SD (Standard Deviation) of each of the six opium alkaloids analysed in six replicates (n = 6) for three different samples of ground poppy seeds.

Sample code	Morphine	Codeine	Thebaine	Papaverine	Noscapine	Oripavine
GPS-01	0.8 ± 0.2	0.5 ± 0.1	0.06 ± 0.01	<MQL	0.04 ± 0.01	<MQL
GPS-02	4.2 ± 0.6	1.4 ± 0.4	0.7 ± 0.2	1.2 ± 0.4	3.7 ± 1.1	ND
GPS-03	134.6 ± 1.6	28.2 ± 0.1	75 ± 1	ND	0.9 ± 0.1	82.1 ± 6.3

GPS. Ground Poppy Seeds; ND: not detected; <MQL: lower than method quantification limit but higher than the method detection limit (MDL). These seeds were purchased whole (Table S6) and then they ground in an automatic mortar (Retsch, RM 200, Haan, Germany) for 2 min.

In sample GPS-01, the six analytes were detected, but all were at very low concentrations, the highest being morphine and codeine with 0.8 ± 0.2 mg/kg and 0.5 ± 0.1 mg/kg, respectively. In sample GPS-02, concentrations were higher for morphine and noscapine, with 4.2 ± 0.6 mg/kg and 3.7 ± 1.1 mg/kg, respectively. However, these concentrations were below the maximum limit set by legislation of 20 mg/kg morphine equivalent. However, sample GPS-03 showed high levels of OAs, with 134 ± 1.6 mg/kg morphine, 82.1 ± 6.3 oripavine, 75 ± 1 thebaine, 28.2 ± 0.1 codeine, and 0.9 ± 0.1 mg/kg noscapine. This sample showed a morphine equivalent value exceeding the legal limit (140.24 mg/kg) by seven times, posing a significant risk to consumers' health. This demonstrates the importance of studying these samples to ensure compliance with legislation. In addition, non-legislated analytes also showed significantly high concentrations. Therefore, these analytes should be considered in future studies.

The concentrations obtained in the ground seeds in this study align with those obtained in previous research analysing whole poppy seeds [4,29,30,33]. In all these studies, as in this one, notably high concentrations of OAs were found, which pose a health risk to the consumer. However, in other studies and in accordance with the EFSA

recommendation, it is suggested that grinding could reduce the OA content in seeds by 25–34% [8,9]. This reduction could be attributed to their degradation, primarily through oxygen, leading to the formation of other compounds such as n-oxide morphine and pseudomorphine. Given the variability in the concentration of OAs in poppy seeds, further research challenges would be to carry out additional studies to determine the real influence of this type of processing. Furthermore, understanding the possible degradation compounds formed is crucial to ascertaining whether they are even more toxic [10].

3. Conclusions

The first analytical methodology for quantifying six OAs in ground poppy seeds has been successfully optimised and validated. Moreover, this methodology stands out for its simplicity, speed, and environmental friendliness compared to similar approaches. This is due to the performance of the UAE, which requires shorter extraction times and lower solvent volumes. In addition, the SBA-15-SO_3^- material showed a high efficiency because only 25 mg of material was needed, and it was possible to reuse it up to four times without losing efficiency. Furthermore, the methodology showed negligible matrix effects, adequate recovery, and precision values, as well as the rest of the validation parameters. Finally, the methodology was used to analyse three ground seeds, and it was shown that one of them contained quantities much higher than those established by the legislation and of the rest of the OAs that are not regulated. This emphasises the imperative to rigorously control the concentrations of OAs in such samples, reinforcing the importance of this developed methodology in ensuring the safety and compliance of products derived from ground poppy seeds.

4. Materials and Methods

4.1. Reagents and materials

Standards of thebaine, morphine, oripavine and codeine were acquired from Alcaliber S.A.U. (Madrid, Spain). Papaverine, Noscapine and morphine-d_3 and codeine-d_3, such as internal standards (IS) were purchased from Sigma-Aldrich (Zwijndrecht, The

Netherlands). Stock of each of standard solutions were prepared at 1000 µg/mL in methanol, and working standard solutions were prepared mixing each of the target analytes at 1 µg/mL in methanol with 0.1% formic acid. These solutions were stored at -20 ˚C in darkness.

Hydrogen peroxide (H_2O_2, 30%) and hydrochloric acid (HCl, 37%) were acquired from Scharlab (Barcelona, Spain). 3-mercaptopropyl triethoxysilane (MPTES, 94%) was obtained from Alfa Aesar (Karlsruhe, Germany). Tetraethylorthosilicate (TEOS, 98% CAS 78-10-4, MW= 208.33 g/mol), ethylene glycol (poly(ethylene glycol)-block-poly) (EO20PO70EO20, Pluronic® 123, P123, MW= 5800 g/mol) were acquired from Sigma-Aldrich (St. Louis, MO, USA). MFE-PAK® SCX SPE commercial cartridges with amorphous silica functionalized with sulfonic acid as sorbent were obtained from Análisis Vínicos (Tomelloso, Spain).

Ammonia 32% (w/w) and hydrochloric acid were received from Scharlab (Barcelona, Spain). Formic acid 99% (Optima™ LC-MS grade) was purchased from Fisher Chemical (Madrid, Spain). Methanol and acetonitrile (HPLC-MS quality) were acquired from Scharlab (Barcelona, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was acquired from a Milli-Q water purification system (Millipore, Billerica, MA, USA). To prepare cartridges to do SPE, nylon filter membranes (0.45 µm) and polyethylene frits (0.20 µm) were obtained from Scharlab (Barcelona, Spain).

4.2. Samples

Three different brands of poppy seeds (PS-01, PS-02 and PS-03) were acquired from supermarkets in Madrid (Spain) (Table S6). The seeds indicated on the label that they could be added to cakes and breads, salads, creams, etc., either whole or previously ground. Then, each of these seeds was ground in an automatic mortar (Retsch, RM 200, Germany) for 2 min, until the seeds were completely ground. Therefore, the codes designating the ground seeds are GPS-01, GPS-02 and GPS-03.

4.3. Synthesis of SBA-15-SO_3^- material

SBA-15 material was synthesized according with the method optimised by Zhao et al. [33]. First, 19.36 g of P123 were added to 576 mL of 2 M HCl and in 144 mL of water. This mix was magnetically stirred at 35 °C in a silicon bath until complete dissolution. Later, 40.8 g of TEOS was dropwise added and for 20 h, it was magnetically stirred. Stirring was then stopped, the temperature was increased to 80 °C and it was left for 24 h at that temperature for an ageing period. Afterwards, the material was filtered off and washed with water. Finally, the material was dried in air and at the end, calcined by ramping for 8.5 h to 500 °C and maintanining at 500 °C for 12 h.

SBA-15 silica functionalized with sulfonic acid was prepared according to González-Gómez et al. [26]. For this purpose, a simple synthesis route with two steps was used, which involves an initial functionalisation with thiols groups (-SH) and an oxidation afterwards to sulfonic acid groups (SO_3^-). First, 2.5 g of SBA-15 and 1.06 g of MPTES were added in 250 mL of 0.1 M HCl. After magnetically stirred during 7 h at 180 rpm at room temperature, the mixture was moved to a reactor of Teflon-coated stainless-steel (V 1.0 L, PS 131 bar, Parr Instrument Company, Moline, Illinois, USA) at 100 °C for 24 h. The solid was then filtered and washed with ethanol and Milli-Q water and finally, dried at 50 °C overnight. Afterwards, oxidation was carried out. For this purpose, the solid was suspended in 325 mL of 2 M HCl, and then 11.4 g of H_2O_2 (30%) were added. After magnetically stirring at room temperature for 5 min, the mixture was transferred to the reactor at 100 °C for 6 h. Finally, the solid was filtrated and washed with Milli-Q water and ethanol.

To confirm the successful synthesis of the material, a characterization was performed (for more information, see Supplementary Material S.1).

4.4. Optimization of the sample preparation

The optimisation process for sample preparation involved a sequential optimisation of the SPE purification step, followed by the optimisation of UAE conditions with the design of experiments. This sequential approach was adopted to ensure the purification

of all extracts during the optimisation of the UAE. By injecting cleaner extracts, the risk of equipment deterioration was minimised, enhancing the overall efficiency of the analytical process.

4.4.1. Optimization of the purification via SPE

To carry this out, an SPE Supelco Visiprep 12-port model vacuum manifold (Sigma-Aldrich, St. Louis, MO, USA) coupled to a vacuum pump at 7.6 psi was used. Empty 3 mL polypropylene cartridges (length of 65 mm and i.d. of 10 mm) were filled with the material and plugged at both ends with polyethylene frits. Additionally, a nylon filter membrane with a 0.45 µm pore size was inserted at the underside of the material bed to avoid material loss when loading the sample.

First, a comparison was made between the synthesised material (SBA-15-SO_3^-) and a commercial material based on amorphous silica functionalised with sulfonic groups (MFE-PAK® SCX) with different amounts of material to evaluate the efficiency of the synthesised material. For this purpose, similar conditions were used based on previous work using materials with strong acid exchange groups to analyse OAs in hot pots [23]. Therefore, 2 mL of water with 1% HCl was used to conditionate the cartridge, 2 mL of 0.5 mg/L of OAs in water with 1% HCl was used for sample loading, and 2 mL of methanol with 5% ammonia was used for elution. After elution, the purified extracts obtained were evaporated and reconstituted in water/acetonitrile 90/10 with 0.1% formic acid for subsequent analysis via HPLC-MS/MS. These assays were performed in triplicate. Subsequently, a re-optimisation of the SPE procedure was performed with the material SBA-15-SO_3^-. First, the recovery values obtained with the methanol elution with only 1% ammonia were evaluated. The objective was to determine if this elution medium could be employed to inject it directly without evaporation, thereby significantly reducing the overall analysis time.

Following the optimisation of SPE using standards, the cleaning step with the extract was optimised. For this purpose, the ground poppy seed sample was spiked immediately before the purification process with a known concentration (5 mg/L) to determine the

recovery values for each of the analytes in that step. Subsequently, it was evaluated whether and at what ratio (undiluted, 1/10 dilution, and 1/20 dilution) the extract needed dilution to achieve acceptable recovery values. Dilutions were made in water with 1% HCl. Then, as the solvents used for the optimisation of the UAE will be water, methanol, and ethanol, it was evaluated to ensure that the SPE procedure showed adequate recovery values with each of them.

4.4.2. Optimization of UAE using design experiments

A three-factor, three-level full factorial design (3^3) was used to evaluate the solvent type (A), the solid/liquid ratio (B), and the extraction time (C) more efficiently in the recovery of OAs. A selection of the three most encouraging levels was made, as presented in Table S1. The selection of levels for each independent variable was based on previous preliminary experiments and related research [18]. Subsequently, the optimal levels for the extraction time and the solid/liquid ratio were determined for each solvent type via RSM.

4.5. *Optimized analysis methodology for quantify opium alkaloids in ground poppy seeds*

The developed and optimised method was based on a UAE, following SPE with SBA-15-SO_3^- material and subsequent analysis via HPLC-MS/MS, as presented in Figure 6.

First, the extraction of OAs from the ground seeds was carried out with a UAE. For this purpose, 0.5 g of sample was mixed with 8.5 mL of MeOH with 1% HCl for 10 s using a vortex (Rx^3 Velp Scientifica, Usmate, MB, Italy). Subsequently, the mixture was subjected to acoustic waves in controlled conditions in the UAE as obtained in the experimental design using Bandelin Sonopuls 529 (Amplichron ®-System, Bandelin, Berlin, Germany) with an MS 73 probe with a diameter of 13 mm. The mixture, placed in a 50 mL falcon tube, was exposed to pulsed mode at 75% amplitude for 5 min and 48 s. Afterwards, it was centrifuged for 5 min at 9000 rpm (Digicen 21 R from Ortoalresa, Madrid, Spain).

Then, the extracts were purified via SPE under optimised conditions. For this purpose, 25 mg of silica SBA-15 functionalised with sulfonic groups (SBA-15-SO_3^-) was employed. The process included a conditioning step with 2 mL of water with 1% HCl, a loading with 2 mL of the extract (diluted 1/10 with water with 1% HCl and adjusting the pH to 1), and an elution with 2 mL of methanol with 1% ammonia. Subsequently, an aliquot of 950 µL was taken, and 50 µL of a 1 µg/mL dilution of morphine-d_3 and codeine-d_3 (IS) was added before analysis via HPLC-MS/MS.

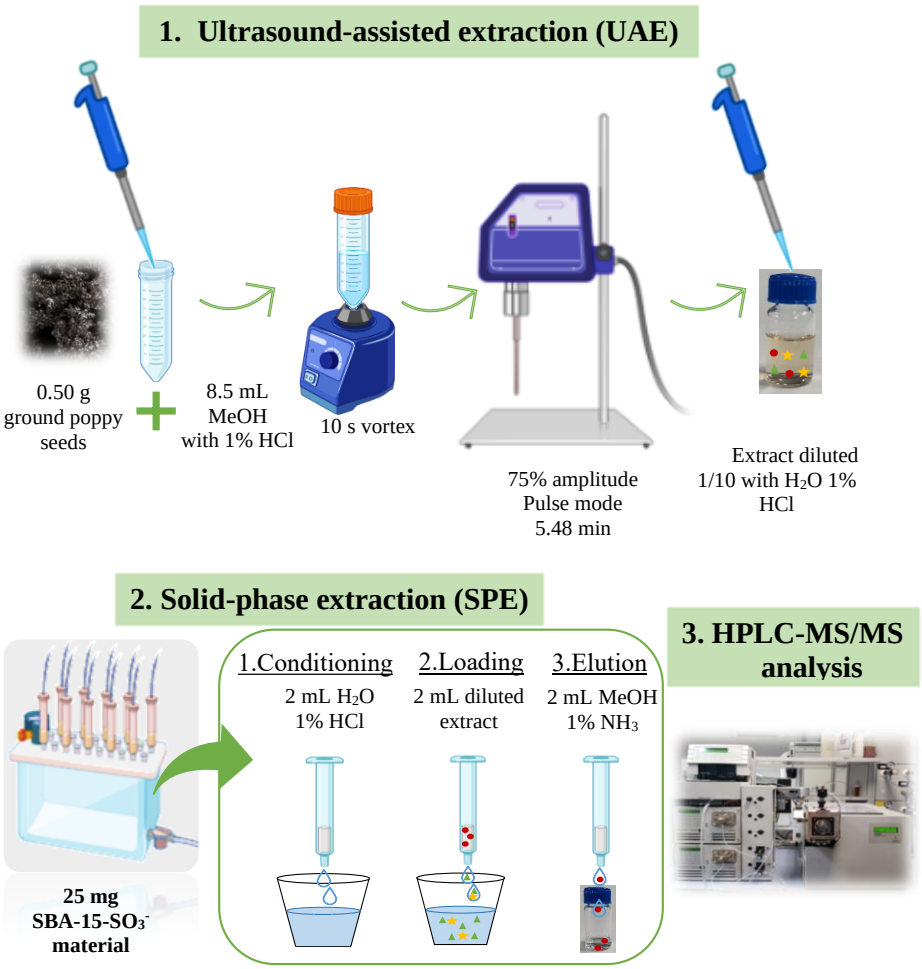


Figure 5. Proposed methodology diagram for the quantification of OAs in ground poppy seeds based on a UAE, SPE to purification and analysis via HPLC-MS/MS.

For the analysis of OAs in ground poppy seeds, a Varian 1200/1200 LC (Varian Ibérica, Madrid, Spain) composed of a ProStar 410 autosampler (100 µL loop) was used.

It was coupled to a tandem mass spectrometer detector with a triple-quadrupole-type analyser (1200 L TQ). The ion source used was electrospray ionisation (ESI), using the MS Workstation Varian data acquisition system (version 6.8). The chromatographic conditions were conducted following the methodology outlined in our prior research [4]. The injection volume was 10 µL via partial injection. The column used was a C18 Kromaphase 100 at 30 °C with dimensions of 150 × 2.0 mm and a particle size of 3.5 µm (Scharlab, Barcelona, Spain). The flow rate was 0.25 mL/min in a gradient elution formed with water (A) and acetonitrile (B), both with 0.1% formic acid. The method started with 90% of A and, in minute 6, changed to 30% A to change again in minute 9 to the initial conditions and maintained 2 min more to re-equilibrate the column. The mass spectrometric acquisition was conducted via electrospray ionisation in positive mode (ESI+) with multiple reaction mode (MRM), as in our previous work [4]. The drying gas was N_2 at 22 psi and 350 °C, and the nebuliser was also N_2 at 58 psi. The capillary voltage was 5000 V and shielding 600 V. The collision gas used was argon at 2.00 mTorr, and the detector voltage was 1553 V. The mass peak width was Q1 2.5, the mass peak width was Q3 2.5, and the MRM scan width was 0.5 s. The cone voltage for the monitored compounds was 72 V. The optimal parameters of MRM for the analysis of six opium alkaloids are shown in Table S7.

4.6. *Method validation*

The validation of the proposed methodology to quantify opium alkaloids in ground poppy seeds was performed in terms of linearity, matrix effect (ME), method detection and quantification limits (MDL and MQL), accuracy, precision, and selectivity. Nowadays, there is no official regulation for validating analytical methods for OAs in food or feed. Therefore, the method validation in this study was carried out in the present work following the SANTE/11312/2021 document [28], regulation EC No. 401/2006 [35], and Q2(R1) ICH guidelines (International council for Harmonisation, 2005) [36]. For the validation, the commercial poppy seed PS01 (Table S6) was used, chosen for its low levels of each OA. Additionally, a double wash with water at 100 °C for 30 min was

applied, following the procedure established in our prior research on whole poppy seeds [4].

First, the linear regression analysis was evaluated with matrix-matched calibration curves made in three different days. These calibration curves were made for sample GPS-01, and six known concentration levels were evaluated between 0.001 and 1 ppm. To complete this, the GPS-01 sample was subjected to the UAE procedure and subsequent purification via SPE, and just before being analysed via HPLC-MS/MS, it was spiked with an aliquot of a standard solution containing the target alkaloids according to the desired concentration level. Furthermore, an isotope-labelled IS correction was performed for quantification. For this purpose, 50 µL of 0.1 µg/mL from each IS was spiked at each point. Additionally, according to the validation guidelines, good linearity criteria dictate that the deviation from back-calculated concentrations of calibrating standards should be within ≤ ±20% of actual concentrations [27, 35].

Regarding the matrix effect, it was established by comparing the slopes of equations of calibration curves from matrix and solvent calibration curves. That is, calculating with the following formula: (slope matrix-matched/slope solvent-based − 1) × 100 for each analyte.

The MDLs and MQLs were calculated to assess the sensitivity of the method with respect to the OAs. To calculate each of these, the lowest concentration analysed was estimated as the concentration giving a signal-to-noise ratio (S/N) of 3 or 10, respectively [27].

To determine the accuracy of the proposed method, recovery tests were performed at three different concentration levels: 40 mg/kg (high validation level), 20 mg/kg (medium validation level), and 3.4 mg/kg (low validation level) by Regulation (EU) 2023/915, establishing maximum morphine equivalent limits in poppy seeds intended for direct human consumption at 20 mg/kg [6]. The blank sample (washed PS01) was used to determine the recovery values. To calculate this, the areas obtained for samples (n = 6) spiked at the corresponding concentration level and subjected to the developed sample preparation were compared with the areas obtained for simulated samples (samples spiked at the respective concentration but before analysis via HPLC-MS/MS). According to the

validation guidelines, the recovery values should be between 70 and 120% [27]. In addition, the precision of the method was determined by its repeatability and reproducibility, using the same validation levels. Intra-day precision (repeatability, RSD %) was calculated six times on the same day (n = 6) on the same day and inter-day precision (reproducibility, RSD %) was evaluated with three replicates on three different days (n = 9). Following the validation guidelines, the RSD (%) values for the parameters should be ≤20% [27].

Finally, the selectivity of the method was evaluated using the spectra obtained for each of the analytes from standards compared with the spectra obtained with samples. It was considered adequate if the variation of the spectra was less than ± 30% and the retention time of the target analytes was within the range of ± 2.5% for each analyte [27].

4.7. *Greenness evaluation of the proposed analytical methodology*

The eco-friendly properties of the proposed analytical methodology for determining OAs in ground poppy seed samples were evaluated for greenness using the Analytical Greenness Metric for Sample Preparation (AGREEprep) [31]. This metric is based on 10 consecutive steps of assessment corresponding to the 10 principles of Green Sample Preparation [37]. Furthermore, it provides insights into the strengths and weaknesses of the procedure. Evaluation categories encompass factors such as the consumption of hazardous reagents, waste generation, sample quantity, and energy consumption, among others. For each assessed item, researchers can assign a score based on its perceived importance to the overall procedure outcome. Finally, a final pictogram is generated to summarise the ecological character of the method.

4.8. *Statistical analysis*

Statistical analysis and design of experiments were performed using Statgraphics Centurion software (version 19.3.03). Differences were considered significant for p values ≤ 0.05.

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/xxx/s1, Table S1: summary of the three factors studied with their codes and the levels of independent variables used in three-level full factorial screening; Table S2: analysis of variance (ANOVA) report for the model; Table S3: adjusted model equation of each type of variable by response surface model; Table S4: comparison of the proposed analytical methodology for determination of six opium alkaloids from ground poppy seeds; Table S5: input used to assign AGREEprep scores of the three methods compared; S.1: characterization of SBA-15-SO_3^- material; Table S6: commercial information on the different poppy seed samples analysed; and Table S7: optimal parameters of multiple reaction modes for the analysis of six opium alkaloids via HPLC-MS/MS.

Author Contributions: G.C.-H.: Formal analysis, Investigation, Validation, Writing original draft. S.M.-Z.: Methodology, Validation, Resources, Supervision, Writing—review and editing. D.P.-Q.: Software, Visualization, Supervision. I.S.: Conceptualization, Supervision, Writing—review and editing, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by MCIU/AEI/FEDER, UE, grant number RTI2018-094558-B-I00 and MCIN/AEI /10.13039/501100011033 / FEDER, UE, grant number PID2022-137278OB-I00.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary Information

Table S1. Summary of the 3 factors studied with their codes and the levels of independent variables used in three-level full factorial screening

Factor type	**Symbol**	**Factor levels**		
- **Factor categorical**		**Type 1**	**Type 2**	**Type 3**
Solvent type	A	Ethanol	Water	Methanol
- **Factor numerical**		**Low (1)**	**Medium (0)**	**High (+1)**
Solid-liquid ratio	B	0.5g/3mL	0.5g/5mL	0.5g/10mL
Extraction time (min)	C	3	5	10

Table S2. Analysis of variance (ANOVA) report for the model.

Variable	Morphine recovery (%)	Codeine recovery (%)	Thebaine recovery (%)	Papaverine recovery (%)	Noscapine recovery (%)	Oripavine recovery (%)
R^2	0.9647	0.9060	0.9110	0.9742	0.9360	0.9222
$R^2_{Adj.}$	0.9388	0.8370	0.8458	0.9553	0.8891	0.8652
$R^2_{Pred.}$	0.9012	0.6842	0.6878	0.9124	0.7679	0.7415
			p-value			
A	0.0006*	0.0146*	0.2048	0.0017*	0.0307*	0.0429*
B	0.0001*	0.0003*	0.0006*	0.0026*	0.0016*	0.001*
C	0.0120*	0.0863	0.1333	0.0713	0.0889	0.0218*
AB	0.4416	0.6007	0.0668	0.0038*	0.0325*	0.3387
AC	0.4006	0.1761	0.2144	0.0876	0.0828	0.2416
BB	0.0004*	0.0009*	0.0012*	0.0035*	0.0028*	0.0002*
BC	0.8551	0.5693	0.2544	0.0640	0.2510	0.7642
CC	0.0361*	0.1236	0.1470	0.0558	0.0974	0.0395*

*significant $p < 0.05$.

Table S3. Adjusted model equation of each type of variable by response surface model.

Variable	Adjusted model equation
Y_1	$Y_1 = 55.4 + 7.2\times A_{[1]} + 14.9\times A_{[2]} + 11.6\times B + 6.7\times C + 0.1\times A_{[1]}B + 2.3\times A_{[2]}B - 2.5\times A_{[1]}C + 1.5\times A_{[2]}C - 4.7\times B^2 + 0.1\times BC - 2.0\times C^2$
Y_2	$Y_2 = 70.7 - 3.3\times A_{[1]} + 17.3\times A_{[2]} + 16.6\times B + 7.9\times C - 4.4\times A_{[1]}B + 4.2\times A_{[2]}B - 4.6\times A_{[1]}C - 1.1\times A_{[2]}C - 8.4\times B^2 + 1.3\times BC - 2.7\times C^2$
Y_3	$Y_3 = 62.0 - 16.7\times A_{[1]} + 19.4\times A_{[2]} + 15.3\times B + 7.8\times C - 9.0\times A_{[1]}B + 6.8\times A_{[2]}B - 6.0\times A_{[1]}C + 2.0\times A_{[2]}C - 8.7\times B^2 + 3.3\times BC - 2.5\times C^2$
Y_4	$Y_4 = 53.\ 9 - 41.0\times A_{[1]} + 32.3\times A_{[2]} + 12.1\times B + 6.7\times C - 10.9\times A_{[1]}B + 3.1\times A_{[2]}B - 5.6\times A_{[1]}C + 0.9\times A_{[2]}C - 5.5\times B^2 + 4.5\times BC - 3.5\times C^2$
Y_5	$Y_5 = 65.2 - 26.6\times A_{[1]} + 24.0\times A_{[2]} + 14.3\times B + 8.0\times C - 9.9\times A_{[1]}B + 3.2\times A_{[2]}B - 6.8\times A_{[1]}C + 0.3\times A_{[2]}C - 7.5\times B^2 + 3.6\times BC - 3.3\times C^2$
Y_6	$Y_6 = 69.1 - 5.4\times A_{[1]} + 18.6\times A_{[2]} + 13.5\times B + 7.9\times C - 5.2\times A_{[1]}B + 4.3\times A_{[2]}B - 5.1\times A_{[1]}C + 2.9\times A_{[2]}C - 10.2\times B^2 + 1.1\times BC - 3.8\times C^2$

Y_1: morphine recovery (%); Y_2: codeine recovery (%); Y_3: thebaine recovery (%); Y_4: papaverine recovery (%); Y_5: noscapine recovery (%); Y_6: oripavine recovery (%); A: solvent type; B: solid-liquid ratio; C: extraction time.

Table S4. Comparison of the proposed analytical methodology for determination of six opium alkaloids from ground poppy seeds.

Sample (amount)	Sample treatment		Analysis technique	Matrix effect	Recovery (%)	RSD (%)	Refs.
	Extraction	Purification					
Unground poppy seeds (10 g)	MeOH 0.1% acetic acid (30 mL, 60 min)	-	HPLC-TQ-MS/MS	-	-	≤9	[30]
Unground poppy seeds (10 g)	AcN/water/formic acid, 80/19/1, v/v/v (100 mL, 30 min x 2)	-	UHPLC-TQ-MS/MS	20-50	77-172	≤20	[29]
Unground poppy seeds (0.2 g)	Chl/IPOH (90/10, v/v) at pH 3.5 (1 mL, 10 min)	-	HPLC-IT-MS/MS	-	-	≤6	[31]
Unground poppy seeds (2.5 g)	MeOH/water, 50/50, v/v (30 mL, 30 min x 2)	MSPE: Fe_3O_4@SiO_2@$mSiO_2$ (50 mg)	UHPLC-TQ-MS/MS	-39-29	46-109	≤11	[4]
Ground poppy seeds (0.5 g)	MeOH 1% HCl (8.5 mL, 5.5 min)	SPE: SBA-15-SO_3^- (25 mg)	HPLC-TQ-MS/MS	-11-9	85-100	≤15	This work

MeOH: methanol; AcN: acetonitrile; Chl: chloroform; IPOH: isopropanol; HCl: hydrochloride acid; (M)SPE: (magnetic) solid phase extraction; (U)HPLC: (ultra)-high-performance liquid chromatography; IT: ion trap; TQ: triple quadrupole; MS/MS: tandem mass spectrometry; RSD: relative standard deviation; Refs.: references

Table S5. Input used to assign AGREEprep scores of the three methods compared.

Item	Weight	The proposed methodology in this work (UAE-SPE-HPLC-MS/MS)		Example 1 (SLE-MSPE-HPLC-MS/MS) [4]		Example 2 (SLE-HPLC-MS/MS) [30]		Example 3 (SLE-HPLC-MS/MS) [32]	
		Input	Justification for input	Input	Justification for input	Input	Justification for input	Input	Justification for input
1	1	Ex situ	Sample preparation performed in the lab	Ex situ	Sample preparation performed in the lab	Ex situ	Sample preparation performed in the lab	Ex situ	Sample preparation performed in the lab
2	5	10.635	8.5 mL MeOH to UAE + 2 mL MeOH to elution in SPE + 0.115 mL HCl + 0.02 mL NH_3	34	30 mL MeOH to SLE + 4 mL diethyl ether/MeOH	30.06	30 mL MeOH to SLE + 0.06 mL acetic acid	100	39.8 mL MeOH to SLE + 0.4 mL formic acid + 59.8 mL water
3	5	Not sustainable but are used several times	This is because SPE cartridges can be reused 4 times	< 25%	Nothing can be reused, and they are not sustainable	< 25%	Nothing can be reused, and they are not sustainable	< 25%	Nothing can be reused, and they are not sustainable
4	3	11.135	Sum of sample and added reagents	67.5	60 mL MeOH/H_2O to SLE + 2.5 g sample + 4 mL diethyl ether/MeOH + 1 mL to reconstitute	40.06	Sum of sample and added reagents	110	Sum of sample and added reagents
5	5	0.5	Ground poppy seed amount	2.5	Poppy seed amount	10	Poppy seed amount	10	Poppy seed amount
6	5	12	Extraction time are 5 min, so 12 samples can be prepared in one hour	1	Extraction time are 60 min	1	Extraction time are 60 min	1	Extraction time are 55 min
7	1	3 steps	The first step is UAE, the second is centrifugation and the last is SPE	4 steps	The first step is SLE, the second is centrifugation, the third is MSPE and the last, evaporation	1 step	SLE single step	1 step	SLE single step
8	5	20.89 Wh	135 W vortex (10 s) + 40 W ultrasound probe (5 min) + 900 W centrifuge (5 min) + 100 W vacuum bomb (5 min)	147.74 Wh	135 W vortex (10 s) + 50 W stirring (60 min) + 40 W US (3 min) + 900 W centrifuge (5 min) + 100 W vacuum bomb (10 min)	50 Wh	50 W magnetic stirring (60 min)	175 Wh	50 W magnetic stirring (30 min) + 900 W centrifuge (10 min)
9	1	HPLC-MS/MS	Equipment used: HPLC-MS/MS	HPLC-MS/MS	Equipment used: HPLC-MS/MS	HPLC-MS/MS	Equipment used: HPLC-MS/MS	HPLC-MS/MS	Equipment used: HPLC-MS/MS
10	1	3 hazards	3 pictograms: toxic, flammable, carcinogen	3 hazards	3 pictograms: toxic, flammable, carcinogen	3 hazards	3 pictograms: toxic, flammable, carcinogen	3 hazards	3 pictograms: toxic, flammable, carcinogen

Supplementary Information S.1 Characterization of SBA-15-SO_3^- material

Elemental analysis (% N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. (Hampton, NH, USA) to determine the degree of functionalisation obtained and to verify that the SH- groups had been oxidised to SO_3^-. Nitrogen gas adsorption–desorption isotherms were obtained using a Micromeritics ASAP 2020 analyser. These isotherms were measured at -196 °C over the interval of relative pressures (P/Po) from 10^{-4} to 0.994. Before measurements, the samples were degassed in a vacuum at 80 °C for 10 h in the degasification unit of the instrument. These temperatures were chosen to avoid any degradation of the organics groups and to remove adsorbed species, solvents, and water. The Brunauer–Emmett–Teller (BET) method was employed to calculate the specific surface areas (S_{BET}). By using the Barrett–Joyner–Halenda (BJH) model, the pore volumes and pore size distributions were derived from the desorption branches of isotherms, and the total pore volumes (Vt) estimated from the desorbed amount at a relative pressure P/Po of 0.97.

Table S6. Commercial information on the different poppy seed samples analysed.

Code	Description	Best-before date	Cultivation type	Origin	Recommendations for use
PS01	Blue poppy seeds	04/2022	Ecological cultivation	No European Union	For the preparation of infusions
PS02	Blue poppy seeds	02/2024	Ecological cultivation	Turkey	They can be used in juices, soups, smoothies and yoghurts. Also ground
PS03	Blue poppy seeds	07/2024	No ecological cultivation	Unknown	Not specified

PS: poppy seeds.

Table S7. Optimal parameters of multiple reaction mode for the analysis of six opium alkaloids by HPLC-MS/MS.

Analytes	t_R[a] (min)	Ionization mode	Precursor ion (Q_1, m/z, $[M+H]^+$)	Fragment ion[b] (Q_3, m/z)	CE[c] (eV)
Morphine	5.179	ESI (+)	286.1	**153.0**	45
				165.0	24
				228.6	22
Morphine-d3	5.819	ESI (+)	288.7	**152.3**	45
				164.2	37
				200.6	25
Codeine	5.528	ESI (+)	300.2	153.1	45
				165.0	45
				215.1	23
Codeine-d3	5.533	ESI (+)	303.4	182.2	30
				199.0	30
				215.1	24
Oripavine	5.648	ESI (+)	298.3	236.9	14
				249.1	17
				267.1	12
Thebaine	6.292	ESI (+)	312.3	**58.2**	8
				166.2	16
				249.4	16
Papaverine	6.507	ESI (+)	340.2	**202.0**	24
				324.1	30
Noscapine	6.554	ESI (+)	414.3	205.1	24
				220.0	42
				280.1	20

[a] t_R: retention time; [b]: the fragment ions used for the quantification are in bold. [c] CE: collision energy.

Artículo 8:

Investigating the effect of different grinding conditions and methods on the concentration of opium alkaloids in poppy seeds as a good reduction practice

Journal of Food Composition and Analysis (2023). Accepted with revisions

Investigating the effect of different grinding conditions and methods on the concentration of opium alkaloids in poppy seeds as a good reduction practice

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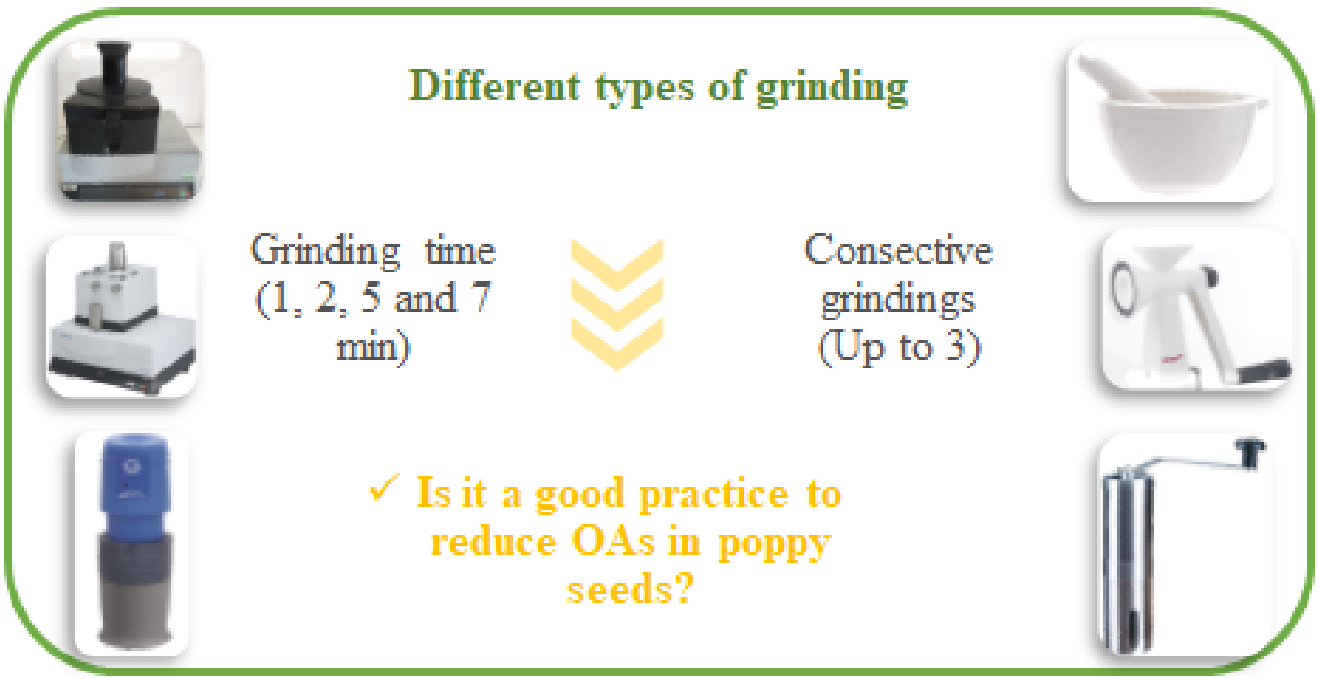
PUBLICATION INFORMATION

Journal	Journal of Food Composition and Analysis
Publisher	Elsevier
Impact factor	4,3 (JCR, 2022)
Category	Food Science & Technology (43/142, Q2)


Different types of grinding
Grinding time (1, 2, 5 and 7 min)
Consective grindings (Up to 3)
✓ Is it a good practice to reduce OAs in poppy seeds?

Highlights:

- Grinding should not be a good practice for OAs reduction in poppy seeds
- Grinding types that extract oil do not exhibit degradation of OAs in poppy seeds
- Morphine is the only alkaloid that exhibits a small reduction with grinding
- Longer grinding time did not result in a degradation trend of OAs

ABSTRACT

In recent years, following intoxications and false positive drug tests for poppy seeds, dangerously high concentrations of opium alkaloids (OAs) have been found. For this reason, health authorities are demanding studies to establish effective practices to reduce OAs in these seeds. Grinding, a common culinary process for various recipes, has previously been associated with potential morphine degradation. However, these studies have not established effective grinding conditions to decrease the concentration of all main OAs. Furthermore, due to the large variation in OAs concentration that can occur even in the same bag, this can be a difficult task. In view of the above, this study aimed to evaluate for the first time the impact of different grinding types, varying grinding time or repetitions, to determine any degradation trends of the main OAs present in poppy seeds. As a result, it was obtained a notable 20% degradation of morphine in the second grinding with grinders where the oil content of the seeds is not released, hinting at oil's protective role against morphine oxidation. Therefore, the choice of grinding method is crucial. However, while acknowledging this, relying solely on this method as a good practice for OAs reduction may be insufficient, as it seems to have minimal impact on other OAs with any of the tested methods and conditions.

KEYWORDS: Natural toxins; Ground poppy seeds; Grinding time; Grinding method; Degradation; Food processing; Food safety.

1. Introduction

There is a trend to incorporate various types of seeds into food due to their beneficial nutritional properties, such as poppy seeds derived from the *Papaver somniferum* L. plant (Ghafoor et al., 2019; Musa Özcan & Atalay, 2006). These seeds have a traditional history of use in many Central European countries and are now gaining popularity in other regions (AESAN (Spanish Food Safety and Nutrition Agency), 2020; EFSA (European Food Safety Authority), 2018). Poppy seeds can serve as toppings for salads, yoghurts, and in various bakery products such as bread, biscuits, and buns. Furthermore, they can be used to create soothing infusions (Powers et al., 2018; Li et al., 2021). However, it is quite common to use ground poppy seeds, incorporating them into salads or as fillings in traditional sweet dishes and cakes.

The concern with using these seeds is their potential contamination with opium alkaloids (OAs) (Meos et al., 2017; Montgomery et al., 2020; Özbunar et al., 2019; Yamaguchi et al., 2011). This phenomenon could be linked to automated harvesting methods that result in the seeds coated with the plant's latex, containing elevated levels of OAs (Casado-Hidalgo et al., 2021a). The consumption of food contaminated with high concentrations of OAs has led to severe cases of intoxication and, in some instances, even false positive drug tests results (Meadway et al., 1998; Newmeyer et al., 2015; Rohrig & Moore, 2003; Van Thuyne et al., 2003). The main five OAs can be found in these seeds are morphine, codeine, thebaine, papaverine and noscapine (EFSA, 2018). However, currently, only morphine and codeine have been considered. Present legislation addresses these two toxins exclusively, setting a limit for seeds at 20 mg/kg of morphine equivalent (calculated as morphine + 0.2 × codeine) (Commission Regulation (EU) 2023/915). Nevertheless, previous studies have shown that seeds may contain notably high concentrations of other OAs, which health authorities suggest could potentially be even more toxic (BfR, (German Federal Institute for Risk Assessment), 2006; EFSA, 2018; Eisenreich et al., 2020). Hence, it is vital not just to test for morphine and codeine in these samples to ensure compliance with regulations, but also to analyse the other OAs present. This step is crucial for accurately assessing consumer exposure and subsequently establishing appropriate legislation.

To ensure that poppy seeds comply with legislation and are safe for consumers, health authorities are calling for good practices to prevent and reduce the presence of OAs in food (European Commission, 2014; Kaltner, 2022). Therefore, studies have been undertaken to assess the impact of thermal processing on bakery products (Carlin et al., 2020; Shetge et al., 2020; Shetge & Redan, 2022; Vera-Baquero et al., 2022) and lactic acid fermentation in yoghurt (Casado-Hidalgo et al., 2023b), demonstrating that high temperatures and fermentation can degrade OAs in poppy seeds. However, although there are some studies that ensure morphine reduction with grinding (Avula et al., 2023; European Commission, 2014; Sproll et al., 2006, 2007), none of them have established specific grinding conditions, and it is unknown whether the type of grinding or other variables, such as grinding time, can significantly influence morphine concentration in seeds. In addition, the impact of grinding on all the main OAs that might be present in seeds has not been studied (Casado et al., 2023). Therefore, it is very important to evaluate which conditions of this widespread culinary practice with poppy seeds can ensure a decrease of OAs and thus ensure food safety.

Hence, the aim of the present work is to evaluate the potential impact of grinding on the concentration of OAs in poppy seeds. To achieve this, an evaluation of five different grinding methods, some of them with a more industrial application and others more for domestic use, was carried out to assess all possible approaches and modes of grinding poppy seeds and to determine whether there are differences between different types of grinders with different grinding times or consecutive grindings. To conduct this research, an analytical methodology previously optimised and validated by our research group was utilized to quantify OAs in whole poppy seed and another validated method developed to analyse OAs concentration in each grinding study of ground poppy seeds.

2. Materials and Methods

2.1. Reagents

HPLC-MS grade acetonitrile (CAS 75-05-8) and methanol (CAS 67-56-1) were obtained from Scharlab (Barcelona, Spain). LC-MS grade formic acid 99% Optima™

(CAS 64-18-6) was purchased from Fisher Chemical (Madrid, Spain). Ammonia 32% (w/w) (CAS 1336-21-6) and hydrochloric acid 36% (w/w) (CAS 7647-01-0) were acquired from Scharlab (Barcelona, Spain). Ultrapure water (resistivity 18.2 MΩ cm) was acquired from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

For the reagents and materials required to carry out the synthesis of each of the adsorbent materials used in the purification step, see Supplementary Information 1.

2.2. Standards and Solution Preparation

Standards of morphine, codeine and thebaine were purchased from Alcaliber S.A.U. (Madrid, Spain). Noscapine, papaverine, morphine-d_3 and codeine-d_3 (internal standards, IS) were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Individual stock solutions were prepared in methanol with concentrations of 1000 µg/mL of each of the OAs. The working standard solutions were prepared by serial dilution of stock solutions with methanol to 1 µg/mL. The internal standard was spiked from a standard of 1 µg/mL of each of them (morphine-d_3 and codeine-d_3). All previous solutions were stored in light-protected at -20 ˚C.

2.3. Samples

A sample of poppy seeds was acquired from a supermarket in Madrid, Spain, specifically for this study. The packaging indicated their origin as Turkey and included usage recommendations, suggesting incorporation into juices, soups, smoothies, and yoghurts, as well as mentioning the feasibility of grinding them.

2.4. Analysis methodology for quantification of opium alkaloids in whole and ground poppy seeds

To compare the concentrations obtained from both whole and ground poppy seeds, optimised and properly validated methods from our previous studies for each sample were employed. To analyse OAs in the sample of whole poppy seeds, the method of Casado-

Hidalgo et al., 2021a was used. This method was based on a solid liquid extraction (SLE), a purification by magnetic solid phase extraction (MSPE) followed by HPLC-MS/MS analysis. First, to do the SLE, 2.5 g of poppy seeds were extracted with a double extraction with 30 mL of MeOH/water, 50/50 (v/v). This mixture was vortexed for 30 s (Rx^3 Velp Scientifica, Usmate, MB, Italy) and stirred magnetically for 30 min. The two supernatants were mixed, and 2 mL were purified by MSPE. For this purpose, 50 mg of Fe_3O_4@SiO_2@$mSiO_2$ material was added to the extract and subjected to 1 min of ultrasound. Then, with an external magnet, the solution was decanted, and the analytes were eluted with a double desorption with 2 mL of diethyl ether/MeOH 80/20, v/v by 1 min of ultrasound. Finally, 2 mL of this supernatant was evaporated under vacuum and reconstituted in 950 µL of water/AcN 90/10, v/v with 1% formic acid and 50 µL of a 1 µg/mL dilution of morphine-d_3 and codeine-d_3 (IS) to analysed by HPLC-MS/MS.

On the other hand, to analyse ground poppy seeds, the protocol by Casado-Hidalgo et al., 2023a was used. In this case, the extraction was carried out by ultrasound assisted extraction (UAE), a purification by solid phase extraction (SPE) followed by HPLC-MS/MS analysis. First, to do the UAE, 0.5 g of ground poppy seeds were extracted with 8.5 mL of MeOH with 1% HCl. The mixture was vortexed for 10 s and sonicated for 5 min and 48 s (Sonopuls HD 3100, Bandelin, Berlin, Germany) with an MS 73 probe with a diameter of 13 mm at 75% amplitude in pulse mode. Subsequently, the mixture was centrifuged at 9000 rpm for 5 min to recover the supernatant (Digicen 21 R from Ortoalresa, Madrid, Spain). Then, the extract was purified by SPE with a silica SBA-15 functionalised with sulfonic groups (SBA-15-SO_3^-) material. In addition, to make the cartridges, polyethylene frits (0.20 µm) and nylon filter membranes (0.45 µm) from Scharlab (Barcelona, Spain) were used with 25 mg of SBA-15-SO_3^- material. First, a conditioning step was performed with 2 mL of water with 1% HCl, a loading with 2 mL of the extract diluted 1/10 with water with 1% HCl and adjusting the pH to 1 and an elution with 2 mL of MeOH with 1% ammonia. Finally, an aliquot of 950 µL was taken, and 50 µL of a 1 µg/mL dilution of morphine-d_3 and codeine-d_3 (IS) was added prior to HPLC-MS/MS analysis.

The analysis of OAs was carried out following our previous works (Casado-Hidalgo et al., 2021a; Casado-Hidalgo et al., 2023a). For this purpose, a Varian 1200/1200 LC (Varian Ibérica, Madrid, Spain) coupled to a triple quadrupole tandem mass spectrometer detector (1200 L TQ) with electrospray ionisation (ESI) ion source was used. The data acquisition system used was MS Workstation Varian (version 6.8). The autosampler used was a ProStar 410 with a 100 µL loop. The column used was a C18 Kromaphase 100 (150 × 2.0 mm, particle size 3.5 µm, Scharlab, Barcelona, Spain) at 30 ˚C. The injection volume was 10 µL (partial injection) with a gradient elution of water (A) and acetonitrile (B), both with 0.1% formic acid, as follows: 90-30% A (0-6 min), 30-90% A (6-9 min) and 90% A (9-11 min) to re-equilibrate the column. The flow rate was set at 0.25 mL/min. On the other hand, mass spectrometric acquisition was by electrospray ionisation in positive mode (ESI+) with multiple reaction mode (MRM). The drying gas was N_2 at 350 ˚C and 22 psi, and the nebuliser N_2 at 58 psi. The capillary voltage was 5000 V, and the shielding was 600 V. The collision gas was argon at 1.90 mTorr, and the detector voltage was 1480 V. The mass peak width of Q1 2.5, mass peak width of Q3 2.5 and the MRM scan width was 0.5 s. The cone voltage was 72 V. Table S1 shows the optimal parameters for the analysis with their retention time, precursor ion, transitions, ionization mode and collision energy of each compound.

2.5. *Different types and conditions of grinding evaluated*

To assess the potential degradation impact of OAs after grinding of poppy seeds, an evaluation was undertaken to determine the presence of any degradation patterns concerning either the grinding time or successive grindings. Five different types of grinding were evaluated to see if there are differences between the different types of grinding on the effect of OAs concentration in poppy seeds. Additionally, varying handling conditions were assessed for each, considering their respective operating capacities. This involved evaluating different parameters such as grinding times (1, 2, 5 and 7 min) or, alternatively, the number of consecutive grindings (up to 3 times) whenever applicable. To achieve this, the sample was introduced initially, and subsequently, at

various time intervals or consecutive grindings, three sample replicates were collected for analysis.

2.5.1 *Grinding experiment type 1*

In this experiment a Mortar Grinder (RM200, Retsch, Haan, Germany), that could be used on an industrial scale, was used. The mortar has a pestle that can crush and mill the material in a mortar containing the sample to be milling while a scraper mixes the milled material and scrapes off material adhering to the mortar. In this case, grinding was evaluated with different grinding times studied with each of these: 1, 2, 5, 7, 10, 30 and 60 min.

2.5.2 *Grinding experiment type 2*

An Ultra Centrifugal (Mill ZM 100, Retsch, Haan, Germany), that could be used on an industrial scale, was used. Grinding takes place in the ultra-centrifugal mill by the impact and shearing action between the rotor and the fixed ring sieve. The sample passes through the funnel onto the rotor. With the centrifugal acceleration, it is hurled outwards with great energy and is pre-crushed on the wedge-shaped rotor teeth before being finely ground between the rotor and the screen. In this case, the grinding times evaluated were 1, 2, 5 and 7 min.

2.5.3 *Grinding experiment type 3*

A Mincer (A11 Basic analytical mill, IKA, Staufen, Germany) with impact milling, which could be employed for home use, was used. This grinder has a beater rotor, and thus, the grinding material is broken up. The granularity of the final product is determined by the duration of the grinding. In this case, the grinding times also were 1, 2, 5 and 7 min.

2.5.5 *Grinding experiment type 4*

A Poppy Seed Mill (Poppy Seed Mill, Westmark, Elspe, Germany), that could be employed for home use, was used. This mill has a hand crank for manual turning, which causes a ceramic worm to rotate and pass the sample through a pinion disc, where it is crushed by friction. In this case, 10 g of sample were taken, and as the grinding time could not be controlled in this device, the alternative that was carried out to see a possible increase in degradation was to grind consecutively the initial 10 g of sample. Therefore, it was evaluated with 1, 2 and 3 grindings.

2.5.6 *Grinding experiment type 5*

A Grinding Coffee (Grinding Coffee, Day Day Fun, Hong Kong, China), that could be employed for home use, was used. To do the assays, 10 g of sample were evaluated by grinding once and twice.

2.6. *Statistical analysis*

Statistical analysis was performed using SPSS 25.0 statistical package (SPSS INC., Chicago, Il, USA) by analysis of variance (ANOVA) using Duncan's multiple range test. Significant differences were considered significant for p values ≤ 0.05. In the case of the test where only two levels of a single factor were assessed, a student's t-test with two tails and a 95% confidence interval was performed. In both cases, significant differences were considered significant for p values ≤ 0.05 and different letters were used to indicate this.

3. Results and Discussion

3. 1. Evaluation of the effect of grinding method on the concentration of opium alkaloids in poppy seeds

Assessing the effect of a culinary processing type on opium alkaloids can be a difficult task. This has been demonstrated by the contradictory results obtained for other types of processing such as heat treatment, with some authors claiming a considerable degradation effect (Vera-Baquero et al., 2022) and others that there is no significant effect (Shetge et al., 2020). For this reason, the consensus emphasizes the need for meticulous study design, considering and accounting for all variables involved (Fleischman et al., 2021; Kuntz et al., 2021). So, in this study, two key issues were considered, specifically the large variability of opium alkaloids in the seeds, and the analytical methodology used and validated for the type of samples involved.

The large variability of opium alkaloids presents in the seeds, even among seeds obtained from the same bag has been confirmed in numerous studies (Carlin et al., 2020; Casado-Hidalgo et al., 2021a; López et al., 2018). This variability may be result from external contamination and is influenced by factors such as plant variety, climate, harvest timing and, and notably, the harvesting method. Presently, automated harvesting methods contribute to seed impregnation with latex, exacerbating this variability (EFSA, 2018; Meos et al., 2017; Stranska et al., 2013). Consequently, obtaining an initial value of OAs in the seeds for studying the influence of processing, especially grinding, becomes challenging due to this variability. This problem is not as acute when the seeds are ground, as the concentration of OAs in that portion of seeds is homogenised. For this reason, it is important to carry out numerous replicates of whole seeds to obtain a mean value with as low a standard deviation as possible. In consequence, twelve replicates of the sample were analysed with the validated method by Casado-Hidalgo et al. 2021a, and the concentration ranges obtained were for morphine from 2.5 to 8.8 mg/kg, for codeine from 0.5 to 2.2 mg/kg, for thebaine from 0.1 to 1.5 mg/kg, for papaverine from 0.5 to 2.8 mg/kg and noscapine from 0.2 to 4.5 mg/kg. These results were according to previous works that large variations of OAs were obtained even in the same bag, and for this reason, they gave the concentration of OAs in ranges (Carlin et al., 2020; Casado-Hidalgo et al., 2021a;

López et al., 2018). For all of this, comparing OAs concentrations in whole and ground poppy seeds can be a complicated task, and it is easy to make wrong conclusions. Therefore, in the present work, it also has been decided to evaluate whether there is a trend of degradation of OAs in the seeds with increasing grinding time or with consecutive grindings (Section 3.1.1 and 3.1.2). In this way, it can be determined whether this type of culinary processing is effective in eliminating or preventing the presence of OAs in seeds, as stated by EFSA in their recommendation of 2014 (European Commission, 2014).

Furthermore, it is important to note that when grinding the seeds, the matrix becomes different and more complex compared to whole poppy seeds. This complexity could stem from the release of fatty acids on the seed's surface, forming a fatty paste. It is crucial to consider this aspect, as inadequate analyte recovery or a significant matrix effect could lead to inaccurate results and consequently wrong conclusions. For this reason, in the present work, another previously developed analytical methodology was used to effectively analyse ground seeds (Casado-Hidalgo et al., 2023a). The analysis method was previously validated in terms of linearity, limits of detection and quantification of the method, inter- and intra-day precision and accuracy at three concentration levels, and the matrix effect and selectivity were also evaluated (Casado-Hidalgo et al., 2023a). As all the results were successful and no matrix effect was present, this methodology can be applied for the quantification of OAs in poppy seeds. However, to ensure the correct quantification of all ground seed studies and all replicates of each of them, matrix-matched calibration curves corrected with IS were used as shown in Table 1 and to ensure their reproducibility, the relative standard deviation (RSD %) was calculated between the slopes of the calibration lines used, obtaining in all cases a result of less than 6% (Table 1). These matrix-matched calibration curves were prepared with a blank poppy seed matrix free of OAs. For this purpose, sample extracts obtained after the same analytical procedure were spiked with an aliquot of a standard solution containing the OAs according to the required concentration level of the calibration curve. In addition, an isotope-labelled IS correction was performed by adding 50 µL of 0.1 µL/mL of each IS to each point of the matrix-adjusted calibration curves (Table 1).

Table 1. Matrix-matched calibration curves corrected with isotope-labelled IS employed to quantification of OAs in ground poppy seeds.

Analytes	Matrix-matched calibration curves	RSD (%)
Morphine	8.79x + 0.64	1
Codeine	21.60x – 0.01	4
Thebaine	54.95x + 3.02	6
Papaverine	170.44x + 10.12	2
Noscapine	239.66x + 41.98	1

RSD (%): relative standard deviation between five replicates of matrix-matched calibration curves adjusted with IS.

Therefore, as shown in Fig. 1, five different types of grinding were studied, and three of the grinding types used in the present work had the possibility to select the grinding time (Section 3.1.1). On the other hand, two other grinder types consisted of a hand crank that rotates a ceramic worm and passes the sample through a pinion disc where it is crushed by friction. Thus, in this case, instead of evaluating different types of grinding or consecutive grindings were evaluated (Section 3.1.2). Subsequently, these seeds were subjected to each of the types of grinding and the specific conditions evaluated, and once ground, three replicates were taken and subjected to the proposed analysis methodology. Once the concentrations of the ground seeds were obtained with each of the conditions, a comparison was made with the concentration obtained in the whole seeds (is the value determined as time zero or control) to determine if there were significant differences and thus to check if there was a tendency towards degradation with increasing grinding time or with successive grindings.

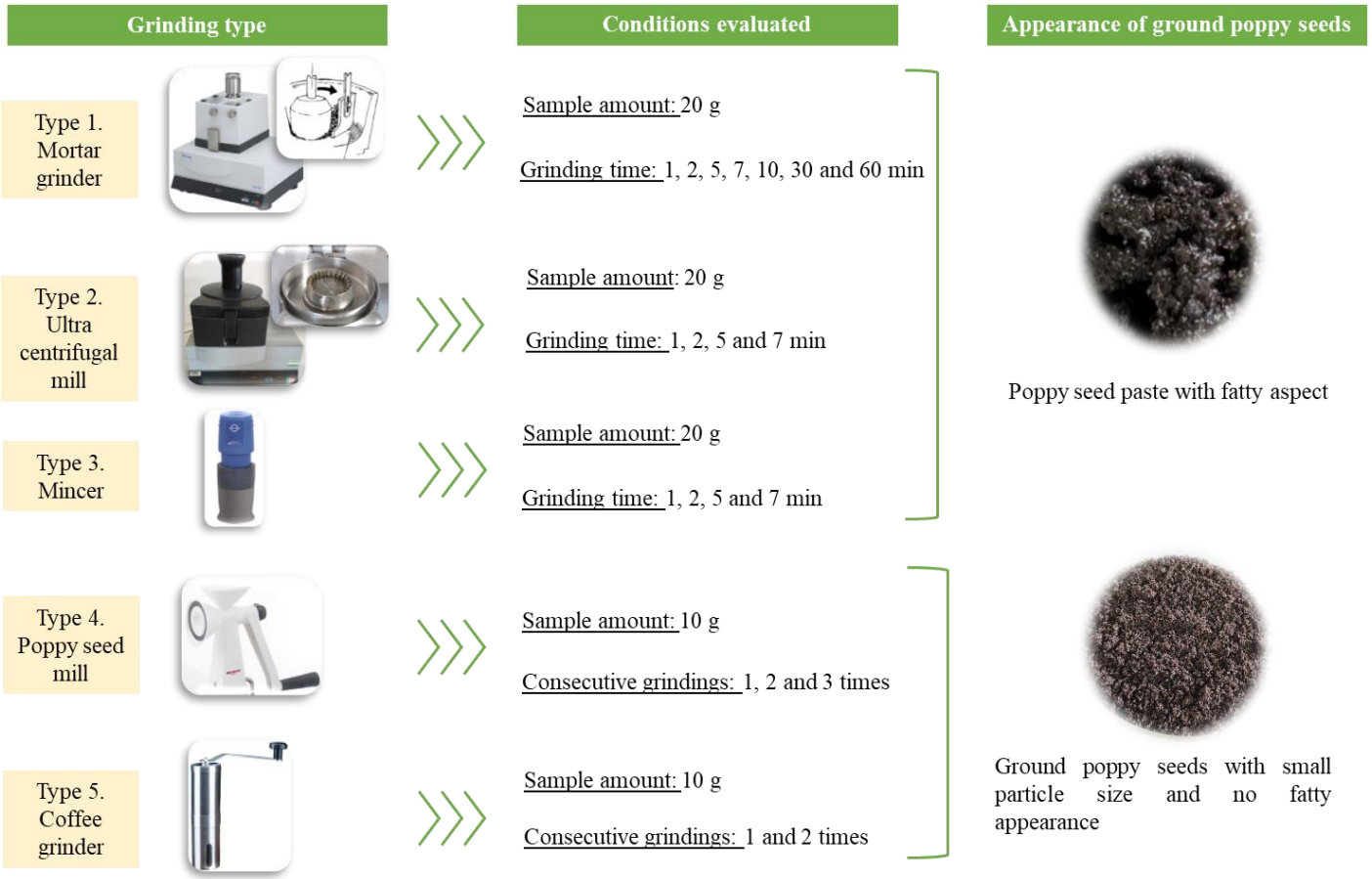


Fig. 1. Types of grindings evaluated with the grinding conditions studied in each of the types (amount of sample and times or consecutive grindings) and the appearance of the ground poppy seed sample.

3.1.1. Effect of grinding time

Different grinding times were used with grinders where the grinding time could be controlled as explained above (mortar grinder, centrifugal mill and mincer), specifically 1, 2, 5 and 7 min for all of them and also 10, 30 and 60 min in the case of the mortar grinder, which higher time can be controlled.

As shown in Fig. 2, none of the studies performed showed significant differences compared to the control (time 0). Besides, Table 2 shows whether the different grinding times showed statistically significant differences ($p < 0.05$) between them and none of the cases showed statistically significant differences in the concentration of OAs in the different conditions. Thus, it was concluded that there was no trend towards degradation of OAs with increasing grinding time with any of the different types of grinders. This was because no significant differences were shown with the control (time 0) as shown in Fig. 2, neither between the different times evaluated as shown in Table 2. The explanation for this result could be because this type of grinding extracts the oil from the seeds to form a paste. The fact that this type of grinding does not result in a significant opium degradation effect could be due to the protective effect of the oil against oxidation of the OAs.

It should be noted that possible differences were shown between the different studies (experiments 1, 2 and 3). But this could be due to the high dispersion of OAs in poppy seeds, even in the same bag, as seen in previously published papers (Carlin et al., 2020; Casado-Hidalgo et al., 2021a; López et al., 2018). On the other hand, in the case of the mortar grinder, longer times were studied as this type of grinder allowed it, but no statistically significant differences were observed with the control (time 0), as shown in Fig. 2, neither between the other times as shown in Table 2.

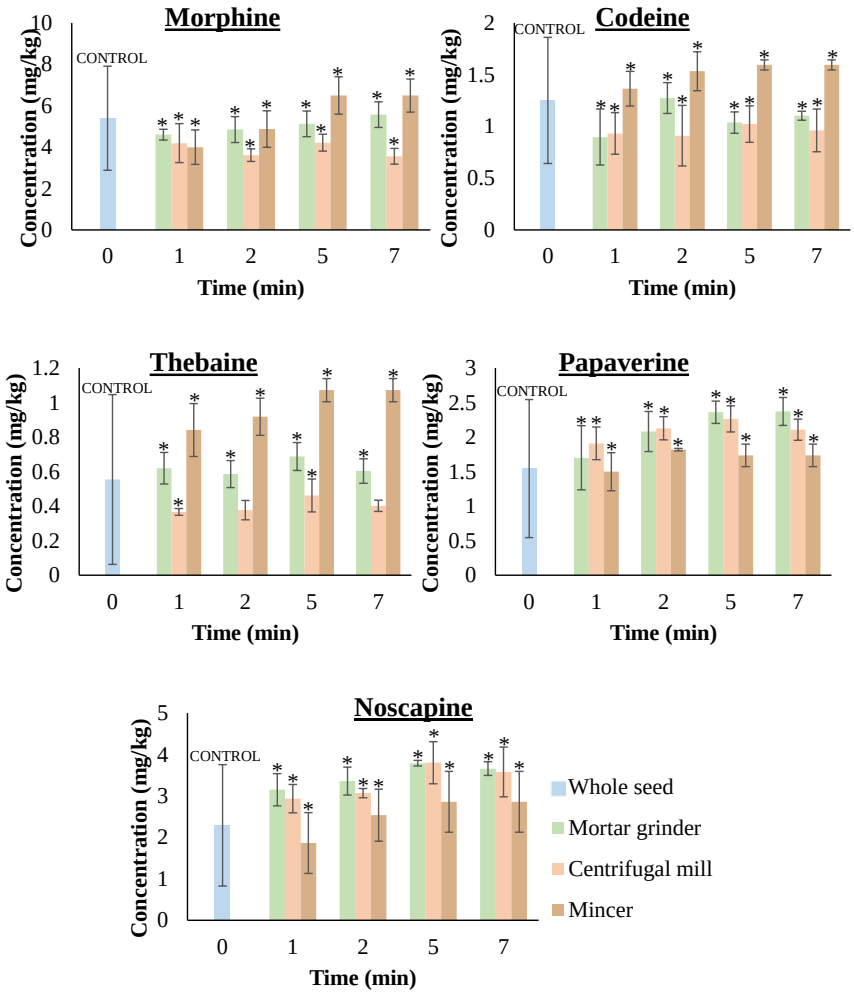


Fig. 2. Concentrations of each of the OAs determined in poppy seeds ground with different types of grinding (mortar grinder, centrifugal mill, and mincer) for different grinding times (1, 2, 5 and 7 min). * Mean that there are no statistically significant differences and ** there are statistically significant differences ($p \leq 0.05$) between the different times with control.

Table 2. Compilation of all the results obtained in each of the grinding studies which is possible to evaluate the grinding time.

Grinding type	Time (min)	Morphine	Codeine	Thebaine	Papaverine	Noscapine
Mortar grinder	1	$4.6 \pm 0.3^{a,b}$	0.9 ± 0.3^{a}	0.6 ± 0.1^{a}	$1.7 \pm 0.5^{a,b}$	3.2 ± 0.4^{a}
	2	$4.9 \pm 0.6^{a,b}$	$1.3 \pm 0.1^{a,b}$	0.6 ± 0.1^{a}	$2.1 \pm 0.3^{a,b}$	3.4 ± 0.4^{a}
	5	5.1 ± 0.6^{b}	$1.1 \pm 0.1^{a,b}$	0.7 ± 0.1^{a}	2.4 ± 0.2^{b}	3.8 ± 0.1^{a}
	7	5.6 ± 0.6^{b}	$1.1 \pm 0.1^{a,b}$	0.6 ± 0.1^{a}	2.4 ± 0.2^{b}	3.7 ± 0.2^{a}
	10	$4.5 \pm 0.6^{a,b}$	1.6 ± 0.4^{b}	0.8 ± 0.1^{a}	1.6 ± 0.4^{a}	3.8 ± 0.6^{a}
	30	$4.5 \pm 0.6^{a,b}$	1.6 ± 0.4^{b}	0.7 ± 0.2^{a}	1.6 ± 0.4^{a}	3.8 ± 1.1^{a}
	60	3.8 ± 0.8^{a}	1.6 ± 0.4^{b}	0.7 ± 0.3^{a}	$1.7 \pm 0.6^{a,b}$	3.6 ± 1.2^{a}
Centrifugal mill	1	4.2 ± 0.9^{a}	0.9 ± 0.2^{a}	0.4 ± 0.1^{a}	1.9 ± 0.2^{a}	2.9 ± 0.3^{a}
	2	3.6 ± 0.3^{a}	0.9 ± 0.3^{a}	0.4 ± 0.1^{a}	2.1 ± 0.2^{a}	$3.1 \pm 0.1^{a,b}$
	5	4.2 ± 0.4^{a}	1.1 ± 0.2^{a}	0.5 ± 0.1^{a}	2.3 ± 0.2^{a}	3.8 ± 0.5^{b}
	7	3.6 ± 0.4^{a}	0.9 ± 0.2^{a}	0.4 ± 0.1^{a}	2.1 ± 0.2^{a}	$3.6 \pm 0.6^{a,b}$
Mincer	1	4.0 ± 0.8^{a}	1.4 ± 0.2^{a}	0.8 ± 0.2^{a}	1.5 ± 0.3^{a}	1.9 ± 0.7^{a}
	2	$4.9 \pm 0.9^{a,b}$	1.5 ± 0.2^{a}	$0.9 \pm 0.1^{a,b}$	1.8 ± 0.1^{a}	2.5 ± 0.6^{a}
	5	6.5 ± 0.9^{b}	1.5 ± 0.1^{a}	1.1 ± 0.1^{b}	1.7 ± 0.2^{a}	2.9 ± 0.7^{a}
	7	6.5 ± 0.8^{b}	1.6 ± 0.1^{a}	1.1 ± 0.1^{b}	1.7 ± 0.2^{a}	2.9 ± 0.7^{a}

Different letters (a and b) mean that there is a statistically significant difference ($p \leq 0.05$) with Duncan's multiple range test.

3.1.2. Effect of consecutive grindings

The effect of grinding was evaluated with crank mills where the grinding time could not be controlled. However, consecutive grinds of 10 g of sample (maintaining the same portion of sample in each consecutive grind) were studied. These experiments (numbered 4 and 5) involved one specifically for poppy seeds and another designed for regular coffee grinding purposes. In the case of the poppy seed mill, it was possible to do 3 consecutive grindings, but in the case of the coffee mill, only 2 were possible. As shown in Fig. 3, no significant differences were shown between the consecutive grindings with the control (time 0), so no degradation trend was shown in any of the analytes.

However, if successive grindings are compared as shown in Table 3, statistically significant effects for morphine can be seen between the first and second grindings with

both the poppy seed mill and the coffee grinder, with approximately 20% degradation of morphine. However, this effect was not observed for any of the other OAs. This could be because the oxidation of OAs primarily involves the formation of N-oxides, where oxidation occurs through the amino groups of these compounds, resulting a bond formation between the nitrogen (N) and oxygen (O) atoms. Nitrogen possesses unshared pairs of electrons that it can offer to oxygen. The accessibility of these electron pair on the nitrogen influences the ease of their donation, thereby determining the compound's susceptibility to oxidation. As shown in Figure S1, the pair of electrons within the N atom of codeine and thebaine exhibits increased delocalization, primarily attributed to the aromatic rings' double bonds in these compounds and their polar ether groups. While these groups are comparatively less polar than those of morphine, the structure of thebaine contains a higher number of conjugated double bonds, making it more difficult to oxidise. Conversely, papaverine and noscapine are more resistant to oxidation due to the nitrogen electron pairs are more delocalized. Papaverine, with its aromatic ring, is more difficult to oxidize than noscapine. Noscapine shares similarities with codeine and thebaine but poses greater difficulty in oxidation due to the presence of multiple polar groups such as ether, carbonyl, and aromatic rings, which collectively reduce the availability of the nitrogen electron pairs and thus increase resistance to oxidation (Figure S1).

The fact that some degradation is observed with this type of mill and not with the others could be due to the protection effect of the oil content of the seeds. In the first 3 experiments, the mechanical cracking releases some of the seed oil, producing a pasty product (as shown in Figure 1). This oil is oxidised by oxygen, having a protective effect on the morphine, and preventing it from oxidising. But in experiments 4 and 5 this effect does not occur; a finely divided flour product is obtained because the oil is not extracted from the seeds and thus oxygen interacts with the morphine and oxidises it.

Besides, as in the previous experiments, possible differences were observed between the different studies (experiments 4 and 5). But this could be due to the high dispersion of OAs in the same bag (Carlin et al., 2020; Casado-Hidalgo et al., 2021a; López et al., 2018).

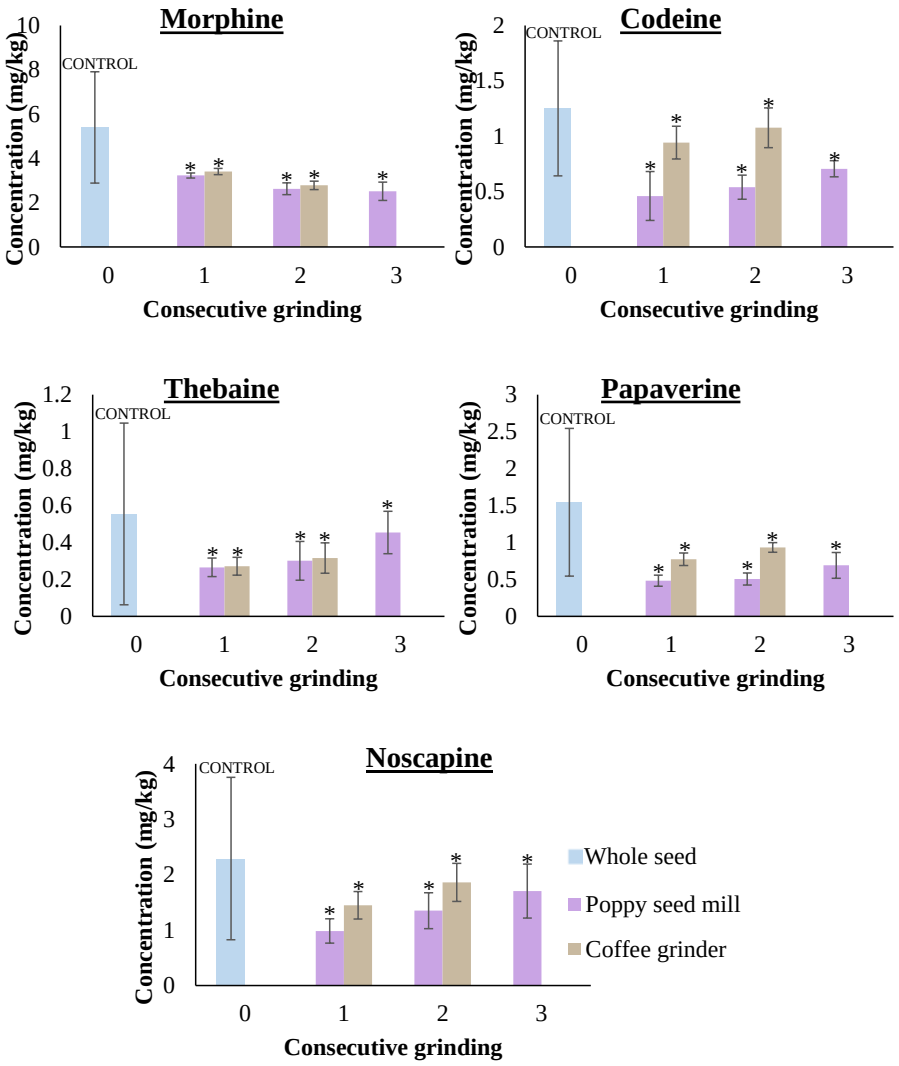


Fig. 3. Concentrations of each of the OAs determined in poppy seeds ground with different types of grinding (poppy seed mill and coffee grinder) with consecutive grindings (1, 2 and 3 times). * Mean that there are no statistically significant differences and ** there are statistically significant differences ($p \leq 0.05$) between the different times with control.

Table 3. Compilation of all the results obtained in each of the grinding studies which is possible to evaluate the consecutive grindings.

Grinding type	Consecutive grinding number	Morphine	Codeine	Thebaine	Papaverine	Noscapine
Poppy seed mill	1	3.2 ± 0.1^{a}	0.5 ± 0.2^{a}	0.3 ± 0.1^{a}	0.5 ± 0.1^{a}	0.9 ± 0.2^{a}
	2	2.6 ± 0.3^{b}	0.5 ± 0.1^{a}	0.3 ± 0.1^{a}	0.5 ± 0.1^{a}	1.4 ± 0.3^{a}
	3	2.5 ± 0.4^{b}	0.7 ± 0.1^{a}	0.5 ± 0.1^{a}	0.7 ± 0.2^{a}	1.7 ± 0.5^{a}
Coffee grinder	1	3.4 ± 0.1^{a}	0.9 ± 0.1^{a}	0.3 ± 0.1^{a}	0.8 ± 0.1^{a}	1.4 ± 0.3^{a}
	2	2.8 ± 0.2^{b}	1.1 ± 0.2^{a}	0.3 ± 0.1^{a}	0.9 ± 0.1^{a}	1.9 ± 0.3^{a}

Different letters (a and b) mean that there is a statistically significant difference ($p \leq 0.05$) in the case of poppy seed mill with Duncan test and in coffee grinder with Student t due to two levels.

3.2. Comparison of the results obtained in the present work with those published previously.

The results obtained from the various ground seeds showed no significant differences compared to the control (time 0). This suggests that there is no degradation effect after grinding. However, it is important to note that the dispersion in OAs concentration in poppy seeds can be quite high, even within the same bag, as demonstrated in previous studies (Carlin et al., 2020; Casado-Hidalgo et al., 2021a; López et al., 2018). This fact may lead to an absence of a significant degradation effect on the OAs concentration after grinding.

On the other hand, the results from each grinding condition were compared to observe potential degradation trends by subjecting the same sample portion to extended grinding times or consecutive grindings. Consequently, it was observed that mills like the poppy seed (experiment 4) or coffee grinder (experiment 5) can exhibit a statistically significant degradation effect reducing morphine levels by approximately 20% between the first and second consecutive grindings. However, no degradation trend was observed for the rest of the OAs. The influence of the milling type appears to be associated with oil extraction from the seeds. In milling methods where a larger portion of the oil was extracted,

resulting in a paste, no degradation effect was observed. This lack of degradation can be attributed to the oil oxidation, preventing morphine oxidation when exposed to oxygen. This aligns with the rationale provided by EFSA in 2014 in their good practice guidance for reducing morphine concentration. According to EFSA, an increased presence of oxygen during seed grinding could lead to a 25-34% oxidation of morphine, forming degradation compounds such as pseudomorphine (European Comission, 2014).

Furthermore, a study conducted by Sproll in 2007, utilizing a poppy seed mill like the one used in this current work (experiment 4), discovered that morphine degradation could reach up to 34 ± 5% (Sproll et al., 2007). Likewise, Avula et al. 2023 similarly determined a potential 10% decrease in certain OAs like morphine, codeine, and thebaine. However, they did not specify the type of grinding method or its conditions (Avula et al., 2023).

3.3. Grinding type and conditions recommended according to results.

According with the results obtained, the type of grinding employed can indeed impact the degradation of morphine, manifesting varying effects among them. Nevertheless, these degradation effects tend to exhibit a notably low percentage and do not seem to affect other OAs, which might exist in considerably high concentrations and possess potentially higher toxicity. It is imperative to recognize that grinding, as a culinary processing method, does not significantly induce OAs degradation. As such, relying solely on grinding to prevent or eliminate OAs in poppy seeds may not be effective. This process proves challenging to evaluate due to substantial variability in seed proportions within the same bag.

Based on the results obtained in the present study and those obtained previously by other authors, the type of grinding that should be performed on poppy seeds to minimize the content of OAs and thus ensure food safety is with crank mills, whether specific for poppy seeds or for coffee. The key to grinding to degrade certain morphine content is that there is no extraction of the oil that would protect the alkaloids from oxidation. In addition, the more consecutive grinds that are performed, the greater the degradation, so it should be recommended to perform at least two grinds to see this effect. However, it should be noted that the effect on the rest of the OAs is negligible and on morphine is

minimal, so the ideal would be to combine this type of processing with others that have shown a significant degradation of the OAs, such as heat treatment (Vera-Baquero et al., 2022) or lactic fermentation (Casado-Hidalgo et al., 2023b), as demonstrated in prior studies. Additionally, alternate methods like washing, as mentioned by EFSA (European Commission, 2014; Sproll et al., 2007), can complement these approaches.

4. Conclusions

In the present work, the influence of grinding on OAs concentrations in poppy seeds was evaluated, since it is one of the most used culinary processes for poppy seeds. For this purpose, five types of grinders were evaluated for the first time, some with potential industrial use and others more commonly used in the household. All methods were evaluated for any significant degradation trends concerning increased grinding time or successive grinding, depending on the grinding type. Significantly, degradation effects on morphine were only observed with grinding methods that produced a finely divided flour product, without loss of oil from the seeds. This phenomenon might be attributed to the protective role of oil against oxygen-induced oxidation. Nonetheless, the results indicate that this processing approach should not be considered an effective practice for OAs reduction in seeds as the observed degradation effect is limited and only shown in morphine. Therefore, other types of culinary processing should be used to reduce the OAs content of seeds or research on combining grinding with other types should be carried out.

Data availability

All data supporting this study are included in the article and supplemental data.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

Funding

This research was funded by MCIU/AEI/FEDER, UE, grant number RTI2018-094558-B-I00 and MCIN/AEI /10.13039/501100011033 / FEDER, UE, grant number PID2022-137278OB-I00.

CRediT authorship contribution statement

Gema Casado-Hidalgo: Formal analysis, Methodology, Investigation, Data curation, Writing – original draft, Visualization. **Sonia Morante-Zarcero:** Conceptualization, Visualization, Methodology, Supervision, Writing – review & editing. **Damián Pérez-Quintanilla:** Conceptualization, Writing – review & editing, Supervision**. Isabel Sierra:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. All authors revised and approved the final manuscript.

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Supplementary Information

Supplementary Information 1. Materials and reagents necessary to carry out the synthesis of each of the adsorbent materials used in the purification step.

To carry out the synthesis of the Fe_3O_4@SiO_2@mSiO_2 material for the purification step by MSPE the following reagents were used: Ferric chloride 6-hydrate ($FeCl_3$ $6H_2O$) 99% (CAS 7705-08-0) and ferrous chloride 4-hydrate ($FeCl_2$ $4H_2O$) 99% (CAS 7758-94-3) were purchased from Labkem (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Tetraethylorthosilicate (TEOS) 98% (CAS 78-10-4) and hexadecyltrimethylammonium bromide (CTAB) 98% (CAS 57-09-0) were purchased from Sigma-Aldrich.

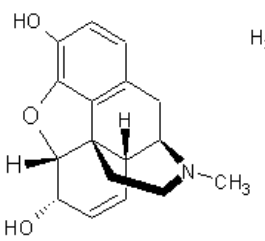
To carry out the synthesis of the SBA-15-SO_3- material for the purification step by SPE the following reagents were used: poly(ethylene glycol)-block (EO20PO70EO20, Pluronic® 123, P123, MW= 5800 g/mol CAS 9003-11-6) and tetraethylorthosilicate 98% (TEOS, MW= 208.33 g/mol), were acquired from Sigma-Aldrich (St. Louis, MO, USA). (3-mercaptopropyl) triethoxysilane (MPTES) 94% (CAS 4420-74-0) was purchased from Alfa Aesar (Karlsruhe, Germany). Hydrochloric acid (HCl) 37% and hydrogen peroxide (H_2O_2) 30% (CAS 7722-84-1) were obtained from Scharlab (Barcelona, Spain).

Table S1. Optimal parameters of multiple reaction mode for the analysis of six opium alkaloids and two internal standards by HPLC-MS/MS.

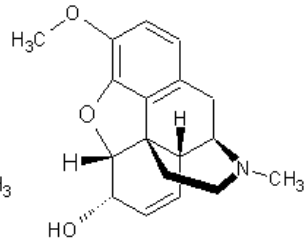
Analytes	Retention time (min)	Precursor ion (Q_1, m/z, $[M+H]^+$)	Fragment ion[a] (Q_3, m/z)	Ionization mode	Collision Energy (eV)
Morphine	5.179	286.1	**153.0** 165.0 228.6	ESI (+)	45 24 22
Morphine-d3	5.819	288.7	**152.3** 164.2 200.6	ESI (+)	45 37 25
Codeine	5.528	300.2	153.1 165.0 **215.1**	ESI (+)	45 45 23
Codeine-d3	5.533	303.4	182.2 199.0 215.1	ESI (+)	30 30 24
Oripavine	5.648	298.3	236.9 **249.1** 267.1	ESI (+)	14 17 12
Thebaine	6.292	312.3	**58.2** 166.2 249.4	ESI (+)	8 16 16
Papaverine	6.507	340.2	**202.0** 324.1	ESI (+)	24 30
Noscapine	6.554	414.3	205.1 **220.0** 280.1	ESI (+)	24 42 20

[a]: The fragment ions used for the quantification are in bold.

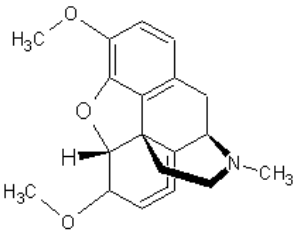
Figure S1. Chemical structures of the five most common OAs in contaminated poppy seeds.



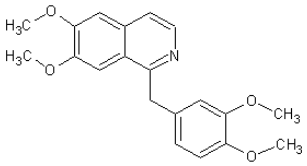
Morphine



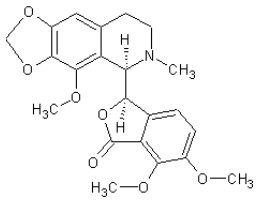
Codeine



Thebaine



Papaverine



Noscapine

DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

La presente Tesis Doctoral se ha centrado en desarrollar metodologías analíticas para cuantificar OAs en semillas de amapola y alimentos elaborados con las mismas para ampliar las insuficientes metodologías que había hasta la fecha y poder con ellas determinar los niveles a los que se encuentran estas toxinas naturales en distintos alimentos comerciales y mejorar la evaluación de la exposición real de los consumidores a esta familia de toxinas. Con las metodologías desarrolladas se ha evaluado también la influencia de distintos tipos de procesado culinario en el contenido de OAs con el objetivo de establecer unas buenas prácticas que permitan prevenir o reducir las altas concentraciones de OAs en las semillas destinadas a consumo humano. Con todo ello, se pretende mejorar el control de los alimentos y mejorar la seguridad alimentaria como parte de los ODS marcados por la ONU.

Al tratarse de analitos que se encuentran en matrices complejas como son los alimentos, es necesario realizar una etapa de purificación previa al análisis para obtener extractos menos turbios y eliminar los interferentes de la matriz. Esto es importante por dos motivos fundamentales. El primero es alargar la vida útil de los equipos como las columnas cromatográficas o el detector. El segundo es reducir el conocido como efecto matriz que se produce cuando se aplica la espectrometría de masas debido a la supresión o el aumento de la ionización de las moléculas que se puede producir y que podría causar resultados erróneos. Por todo ello, las metodologías analíticas desarrolladas se han basado en una primera etapa de extracción de los analitos, una segunda etapa de purificación o clean-up y por último una etapa de análisis por cromatografía de líquidos o de gases acoplada a espectrometría de masas. En todas ellas se ha intentado desarrollar metodologías analíticas más respetuosas con el medio ambiente cumpliendo con los principios de GAC y GSP. Por este motivo, se han optimizado procedimientos de SLE más sostenibles que los existentes y se ha empleado en algunos trabajos la técnica de UAE, la cual ha permitido hacer los procedimientos más respetuosos medioambientalmente, reduciendo los volúmenes de disolventes orgánicos empleados y el tiempo de extracción necesario. Además, para reducir el impacto negativo al medio ambiente en la etapa de purificación, se ha tratado de minimizar la cantidad de material

adsorbente empleado y en consecuencia la generación de residuos. Para ello, se han sintetizado y caracterizado diferentes tipos de nuevos materiales adsorbentes tales como sílices para su empleo en µ-SPE y distintos materiales magnéticos para µ-MSPE. Estos materiales permitieron optimizar procedimientos más efectivos para la extracción de OAs en los que se emplearon entre 1 y 50 mg de material, lo que es considerablemente menor a otros procedimientos convencionales que emplean entre 100 y 500 mg.

Una vez optimizadas todas las metodologías se validaron en términos de linealidad, límites de detección (MDL) y de cuantificación (MQL), efecto matriz, exactitud, precisión y selectividad. Actualmente, no hay una regulación oficial para validar metodologías analíticas para determinar OAs en alimentos, por este motivo, la validación de todas las metodologías desarrolladas se llevó a cabo siguiendo el documento SANTE/11312/2021 que regula la validación de metodologías para analizar pesticidas en alimentos o piensos [99], la regulación CE No. 401/2006 [100] y la guía Q2(R1) ICH (International Council for Harmonisation, 2005) [101]. Posteriormente, se aplicaron tanto para cuantificar OAs en matrices alimentarias comerciales como para evaluar el efecto de algunos tipos de procesado culinarios.

La metodología analítica que se desarrolló para cuantificar OAs en semillas de amapola (Artículo 1) consistió en además de una etapa de SLE, una posterior µ-MSPE para llevar a cabo la purificación del extracto antes del análisis mediante UHPLC-MS/MS. Para llevar a cabo la SLE, se realizó una doble extracción de 2.5 g de semillas con 30 mL de metanol/agua, 50/50 (v/v) durante 30 min de agitación magnética, lo que supuso una disminución de la cantidad de muestra, el volumen de disolvente orgánico y el tiempo de extracción respecto a las metodologías que habían sido previamente publicadas para cuantificar OAs en semillas de amapola. Posteriormente, para la µ-MSPE se sintetizaron unas nuevas partículas magnéticas con el objetivo de llevar a cabo de forma más rápida y sencilla el procedimiento de purificación. Para ello, se sintetizaron partículas de Fe_3O_4 mediante co-precipitación química y se modificaron de forma superficial con una capa de sílice amorfa (Fe_3O_4@SiO_2) y una segunda capa de sílice mesoestructurada (Fe_3O_4@SiO_2@mSiO_2). Posteriormente, cada uno de estos tipos de partículas se funcionalizaron con diferentes cadenas alquílicas, unas formadas por C_8 y en otras por

C_{18}. Después se evaluó la capacidad de adsorción de los seis materiales sintetizados mediante un estudio en el que se determinó la capacidad de adsorción de cada uno de ellos a diferentes tiempos (1, 5, 10 y 20 min), obteniendo como resultado que el mejor material para interaccionar con los OAs fue el Fe_3O_4@SiO_2@mSiO_2 sin funcionalizar. Esto pudo deberse a que las interacciones entre los OH^- libres de la sílice fueron mayores que las interacciones hidrofóbicas que aportaron los grupos C_8 y C_{18} y, además, la doble capa de sílice aportó un mayor área superficial y volumen de poro, dando lugar a una mayor capacidad de adsorción de los analitos. Por este motivo, el material Fe_3O_4@SiO_2@mSiO_2 se empleó en la etapa de µ-MSPE dando lugar a un procedimiento muy rápido y eficaz de purificación, ya que se consiguió con 50 mg de material la máxima adsorción en 1 min (en baño de US) y la desorción en una doble etapa de 1 min cada una. Esta metodología fue exitosamente validada en un (U)PLC-MS/MS y se empleó para la cuantificación de OAs en distintas muestras de semillas de amapola adquiridas en supermercados y herbolarios nacionales. Como resultado se obtuvo que todas las semillas analizadas mostraron concentraciones elevadas de todos los OAs, incluso las que estaban etiquetadas como semillas de amapola de *Papaver rhoeas*, L., la cual no contiene OAs en su látex por lo que sus semillas no podrían estar contaminadas. Estos resultados demostraron la necesidad de exigir un correcto etiquetado del producto y pusieron de manifiesto la necesidad de mejorar el control de los seis OAs mayoritarios para que se pueda establecer una legislación acorde con los niveles de exposición reales y mejorar la seguridad alimentaria.

A continuación, se desarrollaron dos metodologías analíticas para analizar OAs en muestras de panadería con semillas de amapola, una de ellas se aplicó a muestras de panadería saladas, concretamente palitos de pan y pan de molde (Artículo 2) y la otra para muestras dulces, tales como galletas y bizcochos (Artículo 3). La metodología desarrollada para las muestras de panadería saladas (Artículo 2) también se desarrolló con una primera etapa de SLE, una segunda de purificación mediante µ-MSPE y el análisis por HPLC-MS/MS. En este caso, la etapa de SLE se llevó a cabo mediante una doble extracción de 2.5 g de muestra con 10 mL de metanol con 0.1% de ácido acético mediante 30 min de agitación magnética. Y, para la etapa de purificación se sintetizó y caracterizó un novedoso material magnético con un núcleo de Fe_3O_4 y una modificación superficial

con ácido tereftálico y cloruro de hierro (III) (Fe_3O_4@TPA-Fe), aportando interacciones además de enlaces de hidrógeno, electrostáticas π-π y de ion-dipolo. Este material fue muy eficaz para la extracción de OAs ya que, con tan solo 1 mg de material, 1 min de adsorción y otro de desorción, se consiguieron valores de recuperación óptimos. Esta metodología de análisis se empleó para la cuantificación de OAs en muestras de panadería, tales como pan de molde y palitos de pan, mostrando que las concentraciones encontradas en estas muestras eran inferiores a las encontradas en las semillas de amapola (debido al contenido de semillas de este tipo de panes). Sin embargo, alguna de las muestras de pan de molde utilizadas superó el límite máximo establecido por la legislación (1,5 mg/kg de morfina equivalentes), lo que puso de manifiesto la necesidad de su control para asegurar la seguridad alimentaria.

La metodología de análisis para las muestras de panadería dulces (Artículo 3) se basó en una etapa de UAE con el objetivo de disminuir todavía más el volumen de disolventes orgánicos y el tiempo de extracción que se había utilizado en la SLE de trabajos previos. Los resultados confirmaron las potenciales ventajas que presenta la técnica de UAE frente a la SLE, ya que la extracción de 2.5 g de muestra con 10 mL de metanol con 1% ácido fórmico fue completa con tan solo 5 min, lo que supuso la disminución del tiempo de extracción de forma muy considerable respecto a todas las metodologías desarrolladas previamente para el análisis de esta familia de toxinas. Además, también se demostró las ventajas de emplear el diseño de experimentos para llevar a cabo la optimización de esta etapa de preparación de muestra, permitiendo, optimizar de forma más precisa las numerosas variables que influyen en la etapa de UAE, determinar las que son significativas en los resultados y evaluar la interacción entre las mismas. Por otro lado, se demostró la aplicación de las sílices mesoestructuradas, con las que nuestro grupo de investigación cuenta con una amplia experiencia, en la extracción eficaz de OAs mediante SPE. Para ello, se evaluaron dos tipos de sílices, concretamente la sílice SBA-15 y HMS y además un material comercial polimérico de fase reversa tipo HLB, obteniéndose que los dos materiales silíceos sintetizados eran más eficientes que el material comercial HLB. Esto podría deberse a las propiedades texturales de este tipo de sílices, que presentan una mayor área superficial y tamaño de poro más controlado, además de que las interacciones de enlaces de hidrógeno aportadas por los OH^- libres de las sílices podrían ser más

eficientes que las interacciones que aporta el material HLB. Por otro lado, entre la SBA-15 y la HMS, la SBA-15 mostró una mayor capacidad de adsorción, dando lugar a mayores valores de porcentajes de recuperación. Esto podría deberse a que, a pesar de que la HMS tiene una mayor área superficial, la SBA-15 tiene un tamaño de poro más grande. que podría favorecer una difusión a través de los poros superior a la HMS que podría presentar impedimentos estéricos. Una vez seleccionado el material, se optimizó la etapa de SPE dando lugar a unos valores de recuperación óptimos con tan solo 50 mg de material. Una vez validada la metodología desarrollada se aplicó para analizar OAs en galletas y bizcochos, obteniéndose en todos los casos bajas concentraciones de OAs inferiores a las establecidas por la legislación. En definitiva, los resultados de OAs obtenidos mostraron concentraciones bajas, lo que pone de manifiesto el efecto de degradación que pueden sufrir los OAs con las altas temperaturas que requieren los procesos de horneado y elaboración de los productos de panadería. Sin embargo, es necesario seguir controlando estas muestras, ya que en dos panes de molde mostraron concentraciones por encima del límite legislado.

Posteriormente, se desarrollaron varias metodologías de análisis para cuantificar OAs en infusiones con semillas de amapola. La diferencia entre ellas fue el tipo de técnica de análisis empleada y en función de ésta, se seleccionó el tratamiento de muestra más acorde con la técnica y la muestra a tratar. En el trabajo de investigación realizado en la estancia pre-doctoral en el Centro de Química de Madeira, Portugal (Artículo 4) se utilizó como técnica de análisis la GC-MS y la técnica de preparación de muestra fue la µ-SPEed®. Para su optimización, se evaluaron nueve cartuchos comerciales de diferente naturaleza, concretamente seis basados en sílice: C_4, C_8, C_{18}, APS, PFAs y tres materiales poliméricos: PS/DVB-RP, PS/DVB-SCX y PS/DVB-SAX. Como resultado se obtuvo que el cartucho más adecuado fue el PS/DVB-RP, dando lugar a buenos valores de recuperación para todos los analitos. Con este trabajo se demostró la alta eficacia de la técnica miniaturizada µ-SPEed®, ya que permitió una alta capacidad de reutilización de los cartuchos (hasta 100 veces) con una pequeña cantidad de material adsorbente (4 mg) y de volúmenes de disolventes orgánicos empleados (500 µL de metanol). Además, la principal ventaja que mostró esta técnica fue que permitió preconcentrar hasta 10 veces la muestra sin perder eficiencia. De esta manera, se demostró que es una técnica de

preparación de muestra muy útil para utilizarla cuando se emplean otras técnicas de análisis que no son tan sensibles como HPLC-MS/MS, que es la recomendada para OAs. Este es un aspecto para destacar ya que en muchos laboratorios de rutina no tienen equipos tan sensibles y no pueden llegar a detectar o cuantificar niveles de concentración bajos. La metodología desarrollada y validada permitió analizar el contenido de OAs en infusiones de semillas de amapola, bastante consumidas en algunos países europeos. Además, la metodología se aplicó al estudio de la transferencia de los OAs de las semillas de amapola con distintos modos de infusionado. Para ello se estudiaron la influencia de tres factores (temperatura del agua, tiempo de infusionado y cantidad de semillas empleadas). Finalmente se obtuvo que 4 g de semillas a 90 ºC durante 5 min fueron las condiciones que produjeron una transferencia mayor o una menor degradación térmica (entre un 75 y 100% para todos los analitos). Con estas condiciones se analizaron cuatro infusiones de semillas diferentes y en todas ellas se determinaron OAs, obteniéndose en una de ellas, cantidades peligrosamente altas de morfina y codeína. Estos resultados pusieron de manifiesto la necesidad de advertir a la población del peligro de realizar esta práctica.

Por otro lado, a partir del procedimiento µ-SPEed® optimizado en el Artículo 4 para extraer OAs de infusiones con semillas de amapola, se desarrolló una Nota de Aplicación para la empresa, en la que se acopló este procedimiento miniaturizado y más sostenible al análisis mediante HPLC-DAD, de menos sensibilidad que la MS y muy usada en los laboratorios de rutina. Los resultados obtenidos mostraron la buena reproducibilidad y repetitividad de esta técnica además de una alta preconcentración.

La siguiente metodología de análisis de OAs en infusiones se basó en una µ-MSPE seguida de un análisis por HPLC-MS/MS (Artículo 5). En este caso se funcionalizaron las partículas de Fe_3O_4@SiO_2@mSiO_2 con moléculas de β-CD, ligando natural y por ello, respetuoso con el medio ambiente, que presenta mayor eficacia en muestras acuosas. Para obtener el mayor nivel de funcionalización, se determinaron tres proporciones de monómero de β-CD /material (12,5, 25 y 100), demostrando que la relación 25 presentaba el mayor nivel. Posteriormente, se realizaron estudios para evaluar la capacidad de adsorción en agua a diferentes pH (1, 2, 7, 9 y 11) y tiempos (1, 5, 10 y 20 min),

confirmándose la elevada capacidad de adsorción a pH 9 en tan solo 1 min de US. A continuación, se optimizaron las condiciones de elución del procedimiento µ-MSPE con 50 mg de material y 2 mL de agua/EtOH al 50% con 1% de ácido fórmico durante 1 min. Finalmente, se aplicó para llevar a cabo el procedimiento de µ-MSPE y los valores de porcentajes de recuperación fueron muy favorables, superiores a los que se obtuvieron con el material sin funcionalizar del Artículo 1. Esto podría deberse a que las interacciones fueron más fuertes, concretamente enlaces de hidrógeno, interacciones hidrofóbicas y de van der Waals. Además, cabe destacar que se utilizaron disolventes más respetuosos con el medio ambiente para llevar a cabo el procedimiento µ-MSPE (agua y etanol), cumpliendo de esta manera con los objetivos GAC y GSP. Una vez validada la metodología, se aplicó para analizar infusiones de diferentes semillas de amapola comerciales y como resultado, se encontraron concentraciones peligrosamente elevadas, lo que estuvo acorde con las concentraciones obtenidas en el Artículo 4, y confirma la falta de control de los OAs en las semillas de amapola y la peligrosidad de consumir infusiones con las mismas.

Para cuantificar OAs en yogures con semillas de amapola (Artículo 6) se desarrolló una metodología analítica basada en una etapa de SLE y una µ-MSPE con el material Fe_3O_4@SiO_2@mSiO_2@β-CD sintetizado previamente seguido de análisis por HPLC-MS/MS. En este caso se consiguió una extracción completa mediante una etapa de SLE con 2.5 g de yogur y 30 mL de agua durante 30 min de agitación magnética. Posteriormente, se optimizó la etapa de µ-MSPE, realizando una doble desorción, una primera con agua con 1% de ácido fórmico durante 1 min y otra segunda con agua/etanol al 50% con 1% de ácido acético durante 1 min. Una vez que la metodología fue exitosamente validada, se aplicó para cuantificar OAs en yogures comerciales, que presentaron concentraciones bajas de OAs, muy cercanas al límite de cuantificación del método. Por otro lado, se evaluó la influencia de la fermentación ácido-láctica en las concentraciones de OAs con el objetivo de determinar si podía considerarse una buena práctica para reducir su contenido, obteniéndose como resultado que ésta puede degradar algunos de los OAs de forma considerable durante las primeras horas de fermentación (entre el 33 y 80%). Por otro lado, también se evaluó el efecto de almacenamiento del yogurt a temperaturas de refrigeración y no se observaron diferencias significativas, por

lo que se concluyó que debido a la ralentización de la fermentación a temperaturas de refrigeración no se observó degradación durante el almacenamiento.

La metodología de análisis que se desarrolló para cuantificar OAs en pastas y harinas de semillas trituradas de amapola (Artículo 7), se basó en una primera etapa de UAE, seguida de una µ-SPE antes del análisis mediante HPLC-MS/MS. En este caso, se hizo una re-optimización de la UAE con los parámetros que mostraron un efecto más significativo en el rendimiento de la extracción de los OAs en el Artículo 3. De esta forma, se consiguió disminuir el volumen de disolvente orgánico a 8,5 mL y el tiempo de extracción empleado a 5 min, lo que mostró una gran ventaja, ya que para este tipo de muestras con altas concentraciones de OAs se habían utilizado previamente procedimientos considerablemente más largos y con mayor volumen de disolventes orgánicos. Por otro lado, para realizar la etapa de purificación en este caso se funcionalizó la SBA-15 con grupos sulfónicos (SO_3^-), lo que permitió disminuir la cantidad de material empleado a 25 mg, la mitad de lo que se empleó en el Artículo 3 con la sílice SBA-15 sin funcionalizar. Esto puede deberse a que estos grupos de intercambio catiónico fuerte permiten unas interacciones más específicas (cuando se utilizan los pH adecuados) que los enlaces de hidrógeno que aporta la sílice sin funcionalizar. Además, también se estudió la posibilidad de reutilizar los cartuchos con el material y se determinó que se podían utilizar hasta 4 ciclos seguidos sin perder eficacia. Todo esto hizo que esta metodología de análisis resultara ser más respetuosa con el medio ambiente. Una vez optimizada y validada se aplicó para cuantificar OAs en muestras de pastas y harinas de semillas trituradas de amapola, obteniéndose en una de ellas una concentración muy superior al límite máximo establecido por la legislación. De esta forma, se puso de manifiesto la necesidad de controlar este tipo de productos para evitar que supongan un peligro para el consumidor. Además, tras encontrar concentraciones altas de OAs después de la molienda de las semillas, se quiso evaluar la influencia de la molienda ya que en la actualidad esta práctica se contempla como una buena práctica para prevenir o reducir la presencia de OAs en las semillas de amapola destinadas a consumo humano. Según las autoridades sanitarias podría reducir entre un 25-34% de morfina presente en las semillas. Sin embargo, no se habían establecido unas condiciones específicas de molienda para reducir todo lo posible la contaminación en las semillas y se desconocía si el tipo de molienda

podía dar diferencias significativas en el resultado. Por este motivo, en el siguiente trabajo se estudiaron diferentes tipos de molienda con cinco tipos de molinillos en los que se evaluó si podía haber una tendencia a la degradación con el aumento del tiempo de molienda o el número de moliendas consecutivas (Artículo 8). Para ello, la metodología analítica previamente desarrollada se utilizó para analizar las pastas y harinas de semillas trituradas de amapola en cada uno de los estudios realizados. Los resultados mostraron una notable degradación del 20% para morfina, concretamente en los molinos en los que no se libera el contenido de aceite de las semillas, lo que apunta al papel protector de la grasa frente a la oxidación de la morfina. Por lo tanto, la elección del método de molturación resulta crucial. Sin embargo, no se obtuvieron efectos de degradación significativos en el resto de OAs y aun reconociendo que algunos tipos de molienda pueden disminuir la concentración de morfina en el producto final, basarse únicamente en este método como buena práctica para la reducción de los OAs puede ser insuficiente. Por lo tanto, es aconsejable combinar estos procesos con otros métodos para lograr una reducción eficaz de los OAs, tales como el lavado o el tratamiento térmico.

En definitiva, las metodologías analíticas desarrolladas y optimizadas en la presente Tesis Doctoral han supuesto un avance analítico hacia estrategias más sostenibles, debido a que son metodologías más rápidas y sencillas que algunas convencionales y, más respetuosas con el medioambiente. Además, estas metodologías han permitido mejorar la seguridad alimentaria, dando una mayor información sobre los niveles de OAs presentes en los alimentos comerciales. Como resultado, las altas concentraciones encontradas en las semillas de amapola confirmaron la necesidad de seguir mejorando su control para cumplir con la legislación, así como la necesidad de considerar al resto de OAs ya que también pueden encontrarse en altas concentraciones. En lo que concierne a la influencia de algunos tipos de procesado, la fermentación ácido-láctica es uno de los que puede disminuir el contenido de OAs en los yogures. Sin embargo, con el infusionado o la molienda de las semillas, no se disminuyen las concentraciones de OAs de las semillas de forma considerable por lo que no es aconsejable considerarlos como buenas prácticas de reducción de OAs en los productos alimentarios.

CONCLUSIONES GENERALES

5. CONCLUSIONES GENERALES

A partir de los resultados obtenidos durante la realización de la presente Tesis Doctoral, las conclusiones que se pueden obtener son las siguientes:

- Se han optimizado y validado distintas metodologías analíticas avanzadas basadas en novedosas etapas de preparación de muestra y análisis por cromatografía acoplada a espectrometría de masas para determinar las concentraciones de OAs presentes en distintas matrices alimentarias y evaluar distintos tipos de procesado culinario. En todas ellas, la etapa de preparación de muestra fue desarrollada intentando ser lo más respetuosas con el medio ambiente posible, disminuyendo el volumen de disolventes orgánicos, tiempo de extracción y cantidad de material adsorbente empleado, lo que conlleva una menor generación de residuos y consumo energético y por tanto un avance en el cumplimiento de los principios de GAC y GSP y con los ODS.

- Se han sintetizado y caracterizado de forma adecuada distintos materiales adsorbentes magnéticos. Todos ellos estuvieron formados por un núcleo de Fe_3O_4 y fueron funcionalizadas con diferentes rutas y ligandos. Por un lado, las partículas de Fe_3O_4 se recubrieron con sílice amorfa (Fe_3O_4@SiO_2) y sílice mesoestructurada (Fe_3O_4@SiO_2@mSiO_2) y ambas se funcionalizaron con grupos C_8, C_{18} o β-CD. Por otro lado, las partículas de Fe_3O_4 fueron modificadas de forma superficial directamente con ácido tereftálico y cloruro de hierro (III) (Fe_3O_4@TPA-Fe). En todos los casos, la modificación de las partículas de Fe_3O_4 dio lugar a materiales adsorbentes que podían establecer diferentes interacciones con los OAs, presentando además una mayor área superficial, volumen de poro y estrecha distribución de diámetro de poro. Además, en todos ellos se mantuvo la estructura de Rayos-X propia de la Fe_3O_4, lo que supuso que tras su funcionalización no se modificaron las propiedades magnéticas.

- Se han sintetizado y caracterizado de forma adecuada distintos tipos de sílices mesoestructuradas como la SBA-15 y la HMS, ambas con una elevada área superficial y con un tamaño y distribución de poro controlados. Además, la SBA-15 fue funcionalizada con grupos sulfónicos (1.19 mmol S/g material) que posibilitó la extracción de los OAs mediante un intercambio catiónico fuerte dando lugar a un material más específico y con un mayor número de interacciones.

- Se han desarrollado métodos cromatográfico mediante UHPLC-MS/MS y HPLC-MS/MS ambos con detector de triple cuadrupolo utilizando ESI como fuente de ionización en positivo, una columna de C_{18} como fase estacionaria y un gradiente de AcN y agua (ambos con 0.1% de ácido fórmico) como fase móvil que han permitido la separación cromatográfica de seis OAs (morfina, codeína, tebaína, papaverina, noscapina y oripavina) de forma sensible y selectiva en un tiempo inferior a 5 min en el caso de UHPLC-MS/MS y de 11 min en el de HPLC-MS/MS.

- Se ha desarrollado un método cromatográfico mediante GC-MS con detector de cuadrupolo utilizando una fuente de ionización de IE, una columna capilar HP-5 como fase estacionaria y un gradiente de temperaturas desde 220ºC hasta 300ºC con una rampa de 6ºC/min, lo que ha permitido la separación cromatográfica de cinco OAs (morfina, codeína, tebaína, papaverina y noscapina) de forma sensible y selectiva en un tiempo inferior a 15 min.

- Se han evaluado los materiales magnéticos previamente sintetizados y caracterizados $Fe_3O_4@SiO_2$ y $Fe_3O_4@SiO_2@mSiO_2$ y también ambos funcionalizados con C_8 y C_{18}, obteniéndose una mayor capacidad de adsorción de los OAs con el material $Fe_3O_4@SiO_2@mSiO_2$. Por este motivo, se aplicó con éxito este material en el procedimiento de µ-MSPE para purificar un extracto de semillas de amapola, consiguiendo una adsorción completa con 50 mg de material en tan solo 1 min de US y una desorción completa con una doble desorción de 1 min cada una. Este material permitió disminuir el efecto matriz y los valores de recuperación obtenidos fueron aceptables.

- Se ha evaluado el material magnético funcionalizado con ácido tereftálico y cloruro de hierro (III) previamente sintetizado y caracterizado Fe_3O_4@TPA-Fe, obteniéndose una buena capacidad de adsorción de los OAs. Por este motivo, se aplicó con éxito este material en el procedimiento µ-MSPE para purificar el extracto de productos de panadería con semillas de amapola, concretamente palitos de pan y pan de molde, consiguiendo una adsorción completa con tan solo 1 mg de material en tan solo 1 min de US y una desorción completa en otro min. Este material permitió eliminar de forma completa el efecto matriz y los valores de recuperación fueron muy satisfactorios.

- Se han evaluado los materiales magnéticos funcionalizados con β-CD previamente sintetizados y caracterizados Fe_3O_4@β-CD, Fe_3O_4@SiO_2@β-CD y Fe_3O_4@SiO_2@mSiO_2@β-CD, obteniéndose una mayor capacidad de adsorción de los OAs con el material Fe_3O_4@SiO_2@mSiO_2@β-CD. Además, se hizo la comparativa con el material sin funcionalizar, obteniendo una mayor capacidad de adsorción tras la funcionalización. Por este motivo, se aplicó con éxito este material en el procedimiento µ-MSPE para purificar tanto una infusión como un extracto de yogur con semillas de amapola, consiguiendo en ambas metodologías una adsorción completa con 50 mg de material en tan solo un min de US y una desorción completa en otro min. Este material permitió eliminar de forma completa el efecto matriz en ambas metodologías y los valores de recuperación fueron muy satisfactorios.

- Se han evaluado las sílices mesoestructuradas previamente sintetizadas y caracterizadas SBA-15 y HMS y además se han comparado con un material comercial Oasis® HLB, obteniéndose mejores resultados con la sílice SBA-15. Por este motivo, se aplicó con éxito este material en el procedimiento µ-SPE para purificar el extracto de galletas y bizcochos con semillas de amapola. 50 mg de este material fueron suficientes para eliminar de forma completa el efecto matriz y obtener valores de recuperación satisfactorios.

- Se ha evaluado la sílice mesoestructurada funcionalizada con grupos sulfónicos previamente sintetizada y caracterizada SBA-15-SO_3^- y además se han comparado con un material comercial MFE-PAK® SCX, obteniéndose mejores resultados con SBA-15-SO_3^-. Por este motivo, se aplicó con éxito este material en el procedimiento de µ-SPE para purificar el extracto de pastas y harinas de semillas trituradas de amapola. 25 mg de este material fueron suficientes para eliminar de forma completa el efecto matriz y obtener valores de recuperación muy satisfactorios. Además, se consiguió una reutilización del material de hasta 4 veces sin perder eficiencia en el proceso.

- Se ha optimizado mediante diseño de experimentos la UAE para extraer de forma completa y más respetuosa con el medioambiente los OAs tanto de galletas y bizcochos como de semillas de amapola molidas. Para ello, se llevó a cabo un diseño factorial completo de las variables que influyen en el proceso, obteniendo los resultados óptimos de cada una de ellas y, además, permitió conocer los efectos significativos de las distintas variables, cómo afectaban a la respuesta y las relaciones entre las mismas. Tras esta optimización de UAE se obtuvieron menores volúmenes de disolventes orgánicos y tiempo de extracción, lo que supuso que la metodología analítica fuera más respetuosa con el medio ambiente y, por tanto, una mejora en relación con los principios GAC y GSP.

- La novedosa técnica micro-extractiva µ-SPEed® mostró unas potenciales ventajas en su aplicación en la preparación de muestra para la determinación de OAs. Esto se debió a que, además de permitir una extracción y purificación eficiente, con unos buenos valores de recuperación y sin efecto matriz, permitió pre-concentrar el extracto hasta 10 veces, lo que permitió cuantificar OAs a concentraciones por debajo de los límites instrumentales de equipos menos sensibles que el HPLC-MS/MS, como es el caso de GC-MS y HPLC-DAD.

- Las concentraciones de OAs encontradas en las semillas de amapola fueron altamente preocupantes por encima del límite legislado. Además, se cuantificaron

altas concentraciones de los seis OAs, lo que pone de manifiesto la necesidad de incluir todos los OAs mayoritarios en la legislación con el objetivo de mejorar su control. A su vez, se demostró el incorrecto etiquetado de las semillas de *Papaver rhoeas* L., llegando a encontrar altas concentraciones de OAs, lo que indica la importancia de exigir un correcto etiquetado de los productos.

- Las concentraciones de OAs encontradas en los productos de panadería (palitos de pan, pan de molde, galletas y bizcochos) fueron más bajas que en las semillas. Esto pudo deberse al efecto de degradación de las altas temperaturas de horneado que vieron otros autores. Sin embargo, en algunas muestras de pan de molde se obtuvieron valores por encima del límite legislado, lo que pone de manifiesto la importancia de seguir controlándolo.

- Las concentraciones de OAs en infusiones con semillas de amapola fueron significativamente elevadas. Esto concuerda con que la transferencia de los OAs mediante el infusionado de las semillas de amapola resultó prácticamente completa. Por este hecho, sería importante advertir a la población de la peligrosidad de realizar esta práctica y considerar una legislación que obligue a etiquetar unas buenas prácticas con el fin de reducir su peligrosidad.

- Las concentraciones de OAs encontradas en los yogures comerciales analizados fueron muy bajas. Esto concuerda con los resultados obtenidos acerca de que la fermentación ácido-láctica puede degradar de forma considerable a los OAs durante las primeras horas de la fermentación. Por lo que se podría considerar como una buena práctica de reducción de OAs.

- Las concentraciones de OAs encontradas en las pastas y harinas de semillas trituradas de amapola fueron significativamente elevadas, superando el límite establecido por la legislación. Esto concuerda con los resultados obtenidos acerca de que la molienda solo mostró una disminución significativa de morfina del 20% con algunos de los

tipos de molienda empleados. Por este hecho, este procesado no debería considerarse como una buena práctica de reducción de OAs en semillas de amapola.

Based on the results obtained during the present Doctoral Thesis, the conclusions that can be drawn are the following:

- Different advanced analytical methodologies based on novel sample preparation steps and analysis by chromatography coupled to mass spectrometry have been optimized and validated to determine the concentrations of OAs present in different food matrices and to evaluate different culinary processing types. In all of them, the sample preparation step was developed trying to be as environmentally friendly as possible, reducing organic solvent volume, extraction time and adsorbent material amount used, which leads to less waste generation and energy consumption and therefore an advance in compliance with the principles of GAC and GSP and with the SDGs.

- Different magnetic adsorbent materials have been synthesized and adequately characterized. All of them were formed by a Fe_3O_4 core and were functionalized with different routes and ligands. On the one hand, Fe_3O_4 particles were coated with amorphous silica (Fe_3O_4@SiO_2) and mesostructured silica (Fe_3O_4@SiO_2@mSiO_2) and both were functionalized with C_8, C_{18} or β-CD groups. On the other hand, Fe3O4 particles were surface modified directly with terephthalic acid and iron (III) chloride (Fe_3O_4@TPA-Fe). In all cases, the modification of Fe_3O_4 particles resulted in adsorbent materials that could establish different interactions with OAs, also presenting higher surface area, pore volume and narrow pore diameter distribution. Moreover, in all of them the X-ray structure of Fe_3O_4 was maintained, which meant that after their functionalization the magnetic properties were not modified.

- Different mesostructured silica types such as SBA-15 and HMS, both with high surface area and controlled pore size and pore distribution, have been synthesized and properly characterized. In addition, SBA-15 was functionalized with sulfonic groups (1.19 mmol S/g material) which enabled the extraction of OAs by strong cation exchange resulting in a more specific material with a higher number of interactions.

- Chromatographic methods have been developed by UHPLC-MS/MS and HPLC-MS/MS both with triple quadrupole detector using ESI as positive ionization source, a C_{18} column as stationary phase and a gradient of AcN and water (both with 0. 1% formic acid) as mobile phase, which allowed the chromatographic separation of six OAs (morphine, codeine, thebaine, papaverine, noscapine and oripavine) in a sensitive and selective way in less than 5 min in the case of UHPLC-MS/MS and 11 min in the case of HPLC-MS/MS.

- A chromatographic method by GC-MS with quadrupole detector has been developed using an IE ionization source, an HP-5 capillary column as stationary phase and a temperature gradient from 220ºC to 300ºC with a ramp of 6ºC/min, which has allowed the chromatographic separation of five OAs (morphine, codeine, thebaine, papaverine and noscapine) in a sensitive and selective manner in a time of less than 15 min.

- Previously synthesized and characterized magnetic materials $Fe_3O_4@SiO_2$ and $Fe_3O_4@SiO_2@mSiO_2$ and both functionalized with C_8 and C_{18} have been evaluated, obtaining a higher adsorption capacity of OAs with the material $Fe_3O_4@SiO_2@mSiO_2$. For this reason, this material was successfully applied in the µ-MSPE procedure to purify a poppy seed extract, achieving complete adsorption with 50 mg of material in only 1 min of US and complete desorption with a double desorption of 1 min each. This material allowed to decrease the matrix effect and the recovery values obtained were satisfactory.

- The magnetic material functionalized with terephthalic acid and iron (III) chloride previously synthesized and characterized Fe_3O_4@TPA-Fe has been evaluated, obtaining a good adsorption capacity of OAs. For this reason, this material was successfully applied in the µ-MSPE procedure to purify the extract of poppy seed bakery products, specifically breadsticks and sliced bread, achieving a complete adsorption with only 1 mg of material in only 1 min of US and a complete desorption

in another min. This material allowed complete elimination of the matrix effect and the recovery values were very satisfactory.

- Magnetic materials functionalized with β-CD previously synthesized and characterized Fe_3O_4@β-CD, Fe_3O_4@SiO_2@β-CD and Fe_3O_4@SiO_2@mSiO_2@β-CD have been evaluated, obtaining a higher adsorption capacity of OAs with the material Fe_3O_4@SiO_2@mSiO_2@β-CD. In addition, the comparison was made with the unfunctionalized material, obtaining a higher adsorption capacity after functionalization. For this reason, this material was successfully applied in the µ-MSPE procedure to purify both an infusion and a yogurt extract with poppy seeds, achieving in both methodologies a complete adsorption with 50 mg of material in only one min of US and a complete desorption in another min. This material allowed complete elimination of the matrix effect in both methodologies and the recovery values were very satisfactory.

- The previously synthesized and characterized mesostructured silicas SBA-15 and HMS have been evaluated and compared with a commercial material Oasis® HLB, obtaining better results with silica SBA-15. For this reason, this material was successfully applied in the µ-SPE procedure to purify the extract of biscuits and sponge cakes with poppy seeds. 50 mg of this material was sufficient to eliminate the matrix effect and to obtain satisfactory recovery values.

- The previously synthesized and characterized sulfonic group functionalized mesostructured silica SBA-15-SO_3^- has been evaluated and compared with a commercial material MFE-PAK® SCX, obtaining better results with SBA-15-SO_3^-. For this reason, this material was successfully applied in the µ-SPE procedure to purify the extract from ground poppy seed pastes and flours. 25 mg of this material was sufficient to eliminate the matrix effect and to obtain very satisfactory recovery values. In addition, a reuse of the material up to 4 times was achieved without losing process efficiency.

- The UAE has been optimized by experimental design to extract in a complete and more environmentally friendly way the OAs from biscuits and sponge cakes as well as from ground poppy seeds. For this purpose, a complete factorial design of the variables that influence the process was carried out, obtaining the optimum results for each one of them and, in addition, it allowed to know the significant effects of the different variables, how they affected the response and the relationships between them. After this UAE optimization, lower volumes of organic solvents and extraction time were obtained, which resulted in a more environmentally friendly analytical methodology and, therefore, an improvement in relation to the GAC and GSP principles.

- The new micro-extractive technique µ-SPEed® showed potential advantages in its application in sample preparation for the determination of OAs. This was because, in addition to allowing efficient extraction and purification with good recovery values and no matrix effect, it allowed pre-concentration of the extract up to 10-fold, which made it possible to quantify OAs at concentrations below the instrumental limits of less sensitive equipment than HPLC-MS/MS, such as GC-MS and HPLC-DAD.

- The concentrations of OAs found in poppy seeds were of high concentration concern above the legislated limit. In addition, high concentrations of the six OAs were quantified, highlighting the need to include all the main OAs in the legislation to improve their control. At the same time, the incorrect labelling of *Papaver rhoeas* L. seeds was demonstrated, with high concentrations of OAs being found, which indicates the importance of requiring correct labelling of the products.

- The OAs concentrations found in bakery products (breadsticks, sliced bread, biscuits and sponge cakes) were lower than in seeds. This could be due to the degradation effect of high baking temperatures seen by other authors. However, values above the legislated limit were obtained in some sliced bread samples, highlighting the importance of further monitoring.

- The concentrations of OAs in poppy seed infusions were significantly elevated. This is consistent with the fact that the transfer of OAs by infusion of poppy seeds was practically complete. Therefore, it would be important to warn the population of the dangers of this practice and to consider legislation that would make it mandatory to label good practices to reduce its danger.

- The concentrations of OAs found in the commercial yogurts analyzed were very low. This agrees with the results obtained that lactic acid fermentation can considerably degrade OAs during the first hours of fermentation. Therefore, it could be considered as a good OAs reduction practice.

- The OAs concentrations found in the ground poppy seed pasta and flour were significantly elevated, exceeding the limit established by legislation. This agrees with the results obtained that grinding only showed a significant decrease of morphine by 20% with some of the types of grinding used. Therefore, this processing should not be considered as a good practice for reducing OAs in poppy seeds.

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CONTRIBUCIONES A LA TESIS DOCTORAL

7. CONTRIBUCIONES A LA TESIS DOCTORAL

7.1 Artículos científicos

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7.2 Nota de aplicación para la industria

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- Casado-Hidalgo, G., Morante-Zarcero, S., Pérez-Quintanilla D., Sierra, I. Evaluation of fermentation and storage on the concentration of opium alkaloids in poppy seed yoghurt by extraction with a magnetic mesostructured silica composite with beta-cyclodextrin. *XXXIX Reunión Bienal de la Sociedad Española de Química* (Congreso Internacional), Zaragoza (España), junio 2023.
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- Casado-Hidalgo, G., Perestelo, R., Morante-Zarcero, S., Câmara, J. S., Sierra, I. An emerging extraction technique based on micro-solid-phase extraction followed by GC-MS analysis for quantification of opium alkaloids in poppy seed infusion. *The 25th International Symposium on Advances in Extraction*

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7.6 Premios otorgados

- Primer premio a la mejor comunicación póster presentada en el congreso *I Jornadas de jóvenes investigadores de la Sociedad Española de Espectrometría de Masas (SEEM)* por la comunicación titulada "Cuantificación de alcaloides opiáceos en alimentos mediante extracción en fase sólida magnética y análisis por cromatografía de líquidos acoplada a espectrometría de masas de triple cuadrupolo" Marzo 2022.

- Primer premio a la mejor Tesis en 3 min presentada en el concurso *II Edición del concurso online Tu tesis en 3 min* organizado desde el proyecto Avansecal II con el título de "Desarrollo de métodos analíticos avanzados para la determinación de alcaloides opiáceos en alimentos" Marzo 2023.

- Primer premio al mejor vídeo en la categoría de química general y ramas afinas en el congreso de divulgación *II Divulga NextGen* por la comunicación titulada "La cara oculta de los alimentos de origen vegetal: tóxicos naturales" Noviembre 2023.