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Desarrollo y optimización de métodos de análisis para la determinación de aceites minerales en distintos materiales en contacto con alimentos

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DESARROLLO Y OPTIMIZACIÓN DE MÉTODOS DE ANÁLISIS PARA LA DETERMINACIÓN DE ACEITES MINERALES EN DISTINTOS MATERIALES EN CONTACTO CON ALIMENTOS

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Memoria presentada por

JANIRA E. JAÉN PRADO

Para optar al título de Doctor en Ciencia Analítica en Química

Dirigida por

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La **Dra. Cristina Nerín de la Puerta**, Catedrática de la Universidad de Zaragoza en el Departamento de Química Analítica y la **Dra. Celia Domeño Recalde**, Profesora Titular del Departamento de Química Analítica de la Universidad de Zaragoza.

CERTIFICAN:

Que la presente Memoria, titulada: "***Desarrollo y optimización de métodos de análisis para la determinación de aceites minerales en distintos materiales en contacto con alimentos***" presentada por **Doña Janira E. Jaén Prado** para optar al grado de Doctor en Ciencia Analítica, ha sido realizada bajo nuestra codirección en el grupo GUIA, ubicado en la Escuela de Ingeniería y Arquitectura (EINA) de la Universidad de Zaragoza, de acuerdo con los objetivos presentados en el Proyecto de Tesis aprobado por el Departamento de Química Analítica. Por tanto, autorizamos su presentación para proseguir con los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

En Zaragoza, a 15 de enero de 2022.

Dra. Cristina Nerín de la Puerta

Dra. Celia Domeño Recalde

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Universidad Zaragoza

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A mis padres, Humberto y Juana

A mis hermanos y sobrinos

A mi esposo Amir

ÍNDICE

GLOSARIO DE TÉRMINOS

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ACE: Acenaphthene

ACN: Acetonitrile

APCI: Atmospheric Pressure Chemical Ionization

APGC: Atmospheric Pressure Gas Chromatography

API: Atmospheric Pressure Ionization

ASAP: Atmospheric Pressure Solid Analysis Probe

BfR: German Federal Institute for Risk Assessment

BLL: German Federation for Food Law and Food Science

CAR/PDMS: Carboxen/Polydimethylsiloxane

CE: Collision Energy

CEPI: Confederation of European Paper Industries

DCM: Dichloromethane

EFSA: European Food Safety Authority

EU: European Union

EupIA: European Printing Ink Association

EVA: Ethylene/Vinyl Acetate

FCM: Food Contact Materials

FCM-EURL: European Union Reference Laboratory for Food Contact Materials

FID: Flame Ionization Detector

GC: Gas Chromatography

GCxGC: Comprehensive Two-Dimensional Gas Chromatography

HCA: Hierarchical Cluster Analysis

HPLC: High-Performance Liquid Chromatography

HS: Headspace

HS-SPME: Headspace Solid-Phase Microextraction

IAS: Intentionally Added Substances

JRC: Joint Research Centre

KLZH: Labor Kantonales Zurich

LC: Liquid Chromatography

LOD: Limit of Detection

LOQ: Limit of Quantification

MOAH: Mineral Oil Aromatic Hydrocarbons

MOH: Mineral Oils Hydrocarbons

MOSH: Mineral Oil Saturated Hydrocarbons

MPPO: Modified Polyphenylene Oxide

MS: Mass Spectrometry

NIAS: Non-Intentionally Added Substances

NMR: Nuclear Magnetic Resonance

PAH: Polycyclic Aromatic Hydrocarbons

PAO: Poly Alpha Olefins

PCA: Principal Component Analysis

PDMS/DVB/CAR: Polydimethylsiloxane/Divinylbenzene/Carboxen

PDMS: Polydimethylsiloxane

PET: Polyethylene Terephthalate

PLSR: Partial Least Squares Regression

POSH: Polyolefin Oligomeric Saturated Hydrocarbons

PSA: Pressure Sensitive Adhesives

Q: Quadrupole

R^2: Correlation coefficient

rPET: Recycled Polyethylene Terephthalate

RSD (%): Relative Standard Devation

RSM: Response Surface Methodology

S/N: Signal to Noise

SIM: Selected Ion Monitoring

SPE: Solid-Phase Extraction

SPME: Solid-Phase Microextraction

TOF: Time of Flight

UCM: Unresolved Complex Mixture

PRESENTACIÓN

PRESENTACIÓN

La presente Tesis se enmarca en el campo de investigación de la seguridad alimentaria de materiales en contacto con alimentos, con énfasis en los envases, que es la línea principal de investigación del Grupo Universitario de Investigación Analítica (GUIA) del Departamento de Química Analítica de la Universidad de Zaragoza. El grupo GUIA está liderado por la Dra. Cristina Nerín de la Puerta y está integrado en el Instituto de Investigación en Ingeniería en Aragón (I3A).

Esta tesis se realizó gracias a la Beca otorgada a Janira E. Jaén Prado por la Secretaría Nacional de Ciencias, Tecnología e Innovación (SENACYT) y el Instituto para la Formación y Aprovechamiento de Recursos Humanos (IFARHU) de la República de Panamá para realizar estudios de Doctorado.

Esta tesis doctoral se centra en el desarrollo de métodos analíticos para la extracción e identificación de compuestos que pueden servir como marcadores químicos de los hidrocarburos aromáticos de aceites minerales (MOAH) en envases alimentarios. Para ello, se han examinado distintos tipos de materiales destinados al contacto con alimento, como cartón o papel, plásticos, tintas y adhesivos.

Además, se ha estudiado la migración de MOAH desde el envase a los alimentos y se determinan algunos de los parámetros más relevantes que influyen en la migración. Todo ello ha sido posible gracias a la optimización e implementación de potentes técnicas analíticas empleadas en la detección y cuantificación de estos compuestos.

La memoria se ha estructurado en cinco (5) secciones:

En la sección I se presenta una introducción general del marco en el que se ha desarrollado la tesis doctoral.

En la sección II se plantean los objetivos que se pretenden alcanzar durante el desarrollo del trabajo.

En la sección III se muestra el desarrollo experimental, que se divide en cuatro capítulos. Todos los capítulos son autocontenidos, es decir, cada capítulo contiene un resumen junto a sus objetivos principales en español; seguido de introducción, materiales y métodos, resultados, discusiones, conclusiones y referencias bibliográficas, en inglés.

- **Capítulo 1:** Se realizó un estudio para seleccionar marcadores disponibles MOAH. Para ello, se utilizaron técnicas alternativas como la Sonda de Análisis de Sólidos Atmosféricos (ASAP) y la Cromatografía de Gases a Presión Atmosférica (APGC) acoplada a Espectrometría de masas Cuadrupolo-Tiempo de Vuelo (QTOF-MS) para rastrear los marcadores en distintos tipos de envases alimentarios.

- **Capítulo 2:** Se desarrolla un método rápido de análisis para detectar hidrocarburos aromáticos como marcadores químicos de la contaminación por MOAH proveniente de tintas de impresión offset a base de aceite mineral mediante extracción en fase sólida y cromatografía de gases acoplada a espectrometría de masas (SPE-GC-MS).

- **Capítulo 3**: Se desarrolla un método de micro-extracción en fase sólida acoplado a cromatografía de gases (HS-SPME-GC-MS) para analizar marcadores de MOAH en muestras comerciales de adhesivos termofusibles; y se estudia de la migración de marcadores MOAH y de la fracción MOAH desde algunos laminados fabricados con estos adhesivos.

- **Capítulo 4:** En este capítulo se estudia el comportamiento migratorio de MOAH desde envases primarios de cartón a un simulante alimenticio (Tenax) y dos alimentos secos (cuscús y polenta), en las peores condiciones de tiempo y temperatura. Para ello, se utilizaron dieciséis compuestos modelos que cubrían el rango volátil de MOAH. Se estudia la migración de hidrocarburos aromáticos de

aceite minerales (MOAH) desde envases de cartón a alimentos secos y se hace uso del análisis multivariante como herramienta de predicción.

En la sección IV se recogen las conclusiones más relevantes; y en la V se muestran las publicaciones derivadas de este trabajo.

A continuación, se presenta un esquema con la estructura de la tesis.

Sección I: Introducción General

Sección II: Objetivos

Sección III: Desarrollo Experimental

Capítulo 1: Atmospheric Solids Analysis Probe (ASAP) and Atmospheric Pressure Gas Chromatography (APGC) coupled to Quadrupole Time of Flight Mass Spectrometry (QTOF-MS) as alternative techniques to trace aromatic markers of mineral oils in food packaging.

Capítulo 2: Development of a rapid method for determination of aromatic hydrocarbons in food packaging by solid phase extraction and gas chromatography-mass spectrometry.

Capítulo 3: Migration of mineral oil aromatic hydrocarbon (MOAH) from hot melt adhesives used in food packaging materials

Capítulo 4: Migration of toxic mineral oil aromatic hydrocarbons (MOAH) from cardboard containers to dry food and prediction tool.

Sección V: Publicaciones

Sección IV: Conclusiones Generales

SECCIÓN I: Introducción General

INTRODUCCIÓN

1. Aceites minerales

1.1. Aspectos Generales

Los hidrocarburos de aceites minerales (MOH, por sus siglas en inglés) son una mezcla compleja de compuestos químicos, que provienen del petróleo crudo; pero también puede ser elaborada sintéticamente a partir del carbón, el gas natural o la biomasa (EFSA, 2012).

La complejidad química de MOH se debe a la gran variedad estructural del petróleo crudo; pero también, al hecho de que los derivados del petróleo se producen para cumplir con ciertas especificaciones técnicas y operativas que no guardan relación con una composición química definida (McKee & White, 2014). En la Figura 1 se muestran ejemplos de las diferentes estructuras de los hidrocarburos que componen los MOH.

Se han identificado, tres tipos de estructuras básicas en MOH (EFSA, 2012):

- Parafinas: alcanos de cadenas lineales y ramificados.
- Naftenos: alcanos cíclicos, alquilados y no alquilados
- Aromáticos: hidrocarburos aromáticos y poliaromáticos (PAHs) generalmente alquilados, que pueden incluir cantidades menores de compuestos con heteroátomos de azufre y nitrógeno en su estructura.

Es importante señalar que, en el Dictamen de la EFSA, solo se consideran los hidrocarburos, que contienen entre 10-50 átomos de carbono. Los hidrocarburos con menos de 10 átomos no se consideraron por su alta volatilidad y los mayores de 50 átomos por su baja absorción. Además, el término MOH no incluye: hidrocarburos naturales (n-alcanos impares de C21-C35), hidrocarburos de origen terpénico, polímeros

oligoméricos de hidrocarburos saturados (POSH) ni polialfaolefinas (PAO) (EFSA, 2012).

MOH puede separarse en dos fracciones: hidrocarburos saturados (MOSH), que incluye a las parafinas y naftenos, y otra de hidrocarburos aromáticos (MOAH), que incluye los compuestos con anillos aromáticos, especialmente alquilados (Biedermann & Grob, 2009; EFSA, 2012).

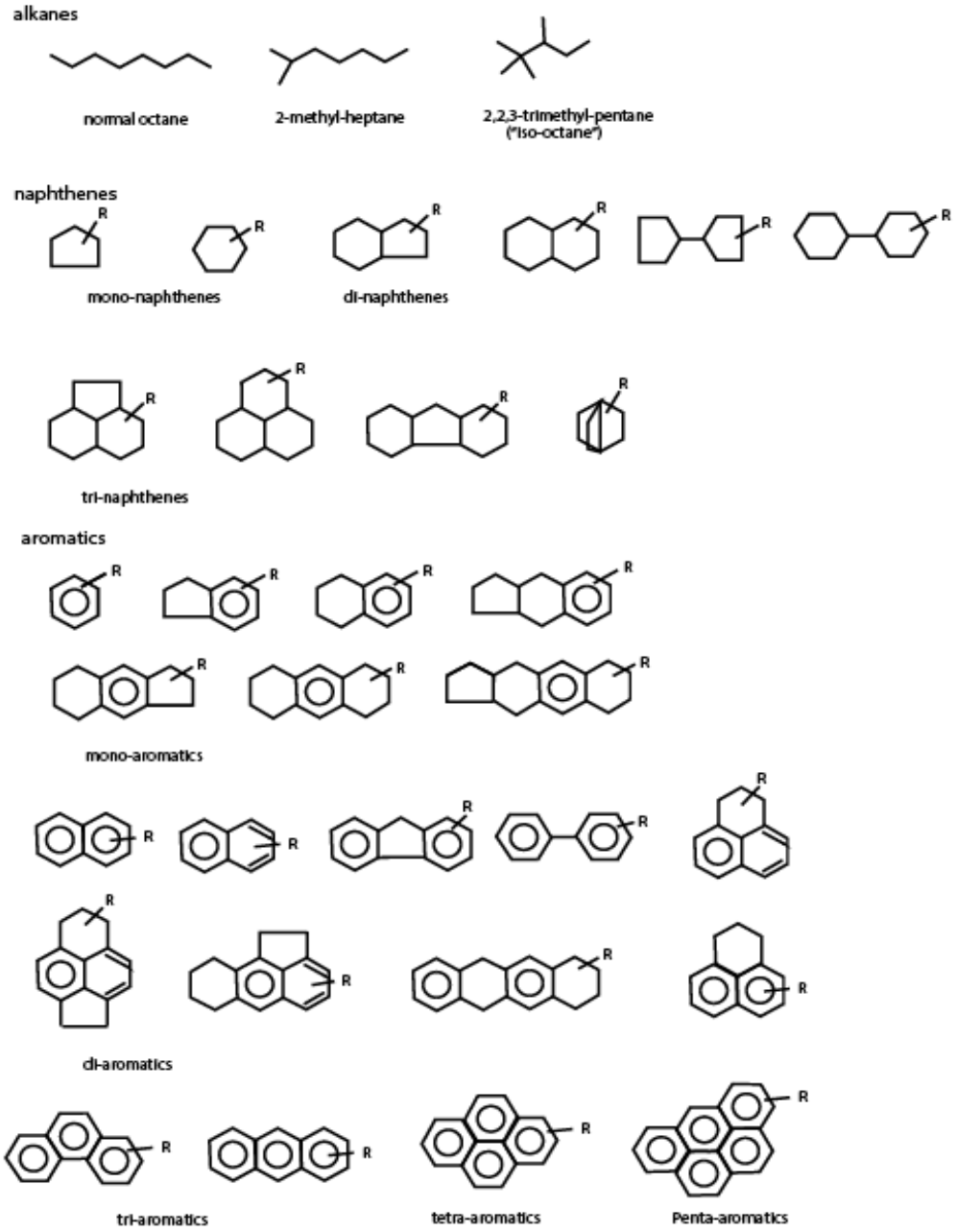

Figura 1. Ejemplos de las distintas estructuras químicas encontradas en MOH (EFSA, 2012).

MOH puede separarse en dos fracciones: hidrocarburos saturados (MOSH), que incluye a las parafinas y naftenos; e hidrocarburos aromáticos (MOAH), que incluye los compuestos con anillos aromáticos, especialmente alquilados (Biedermann & Grob, 2009; EFSA, 2012).

Generalmente, la relación MOSH:MOAH en los aceites minerales fósiles es de 4:1; esta relación se cumple, en el caso de los MOH, de grado técnico, que contienen alrededor de 15-35% de MOAH; no así, en el caso de MOH grado alimentario, que son sometidos a procedimientos adicionales de refino para minimizar la fracción MOAH (EFSA, 2012; Food Federation Germany, 2018). Es decir, que la proporción MOSH:MOAH, en los productos comerciales de aceites minerales, no solo depende del origen del petróleo crudo, sino también, de los procesos de refino (extracciones con solventes, hidrogenación o tratamientos con ácidos) a los que es sometido el aceite (Carrillo et al., 2019).

1.2. Toxicidad

La toxicidad de MOSH y MOAH es diferente; los MOSH se bioacumulan en los seres humanos y aunque su presencia se ha asociado con la aparición de microgranulomas hepáticos en ratas Fischer 344 (EFSA, 2012), no hay evidencia de que MOSH cause efectos similares en los seres humanos. Además, el número de personas que padecen de lipogranulomas hepáticos en la Unión Europea es bajo, por lo tanto, es poco probable que represente un problema de salud pública (Fleming & Carrillo, 2018). La mayor preocupación sobre la toxicidad de los aceites minerales se centra en MOAH y su potencial mutagénico.

Aunque la información toxicocinética de MOAH es bastante escasa, se ha encontrado evidencia de que PAHs alquilados simples, como el 2-metilnaftaleno, 2,6-diisopropilnaftaleno y 2,6-dimetilnaftaleno, administrado por vía oral a cobayas, tienen una alta adsorción gastrointestinal (entre 85-100%) (Barrowman et al., 1989). También, se ha demostrado, que los compuestos aromáticos que contienen azufre se absorben en el tracto gastrointestinal de las ratas (EFSA, 2012).

Cabe esperar que el comportamiento de MOAH absorbido por el cuerpo humano sea parecido a MOSH; sin embargo, en un estudio reciente, se analizaron tejidos humanos con altas concentraciones de MOSH y en ninguna de las muestras se detectó MOAH; por lo que se deduce que MOAH no se acumula en los tejidos humanos (Barp et al., 2014).

Por otro lado, los PAHs no alquilados o poco alquilados, después de la ingestión, se absorben en el tracto gastrointestinal por medio del sistema linfático y la vena porta, para posteriormente metabolizarse y luego excretarse (Pirow et al., 2019). Por lo tanto, es probable que MOAH en lugar de bio-acumularse, se metabolice como los PAHs, y se excrete a través de las heces, igual que MOSH. La metabolización de MOAH explicaría por qué no se detecta en tejidos humanos con altas concentraciones de MOSH (Hochegger et al., 2021).

MOAH con tres o más anillos aromáticos no alquilados o parcialmente alquilados, pueden ser activados por las enzimas P450, en metabolitos carcinógenos genotóxicos. Además, algunos MOAH fuertemente alquilados, pueden originar tumores; mientras que, algunos MOAH sencillos, como el naftaleno pueden ser cancerígenos no genotóxicos (EFSA, 2012).

La actividad carcinogénica de los aceites minerales suele comprobarse, por medio de bioensayos en la piel del ratón, basados en el hecho, de que los ratones y los seres humanos, desarrollan el mismo tipo de tumores de piel. Fueron los resultados obtenidos mediante de este tipo de ensayos los que llevaron a la conclusión que no todos los compuestos aromáticos, son cancerígenos; y que los hidrocarburos aromáticos capaces de causar cáncer en la piel de los ratones poseen poli anillos aromáticos no alquilados o con baja alquilación (Carrillo et al., 2019). Esto se debe a que la oxidación de los hidrocarburos poliaromáticos altamente alquilados, tiende a ocurrir en los grupos alquilos de la cadena lateral, formando metabolitos no cancerígenos, que posteriormente, se conjugan y se excretan por la bilis (Carrillo et al., 2019). Los MOAH también pueden actuar como disruptores endocrinos; y contribuir con la carga estrogénica total en los humanos (Tarnow et al., 2016).

1.3. Presencia en los alimentos

La mayor parte de los datos obtenidos sobre la presencia de MOH en los alimentos, en los últimos años, provienen de Foodwatch, Specialised Nutrition Europe y de la información proporcionada por los Estados miembros de la Comisión Europea.

En 2015, la organización Foodwatch, informó sobre la presencia de MOSH y MOAH en productos alimenticios envasados en cartón y disponibles en Alemania, Francia y Holanda. Tras el análisis de 120 productos alimenticios diferentes y su respectivo envase, se descubrió, que 100 de los productos estaban contaminados con MOSH y 51 tenían MOAH. Entre los productos estudiados se encontraban: arroz, pasta, sémola, cereales lentejas, cuscús, pan rallado, avena, mezcla para hornear, almidón, cacao, copos de chocolate, azúcar en polvo, etc. En algunos casos, se encontró que las concentraciones de MOSH y MOAH estaban correlacionadas con el envase, pero, en otros casos, no hubo tal correlación. La correlación entre las concentraciones de MOSH y MOAH tampoco fueron concluyentes en todos los casos (Buijtenhuijs & Van De Ven, 2019).

En un estudio realizado en el año 2018 en los Países Bajos, se analizaron 217 muestras de alimentos envasados altamente consumidos y con sospechas de contener aceites minerales, en dicho estudio, se detectó MOH en 142 de las muestras analizadas y MOAH en 23 muestras con concentraciones de hasta 2.24 mg kg^{-1} (Van Heyst et al., 2018).

En octubre de 2019 Foodwatch, informó sobre la presencia de MOAH en productos lácteos para bebés. Foodwatch hizo analizar un total de 16 muestras de fórmulas para lactantes y fórmulas de continuación envasadas en latas recolectadas en Francia, Alemania y los Países Bajos; la mitad de los productos lácteos, tenían MOAH en un rango entre 12 a 27% del MOH total (Arcella et al., 2019).

Specialized Nutrition Europe también informó sobre los resultados obtenidos, tras el análisis de 696 muestras de fórmula infantil y fórmula de continuación, en polvo y líquido, recolectadas entre 2016 y 2019. Se detectó MOAH en 28 de 677 muestras de

fórmula infantil en polvo; la muestra más contaminada tenía una concentración MOAH de 1.6 mg kg^{-1}. No se detectó MOAH en las fórmulas líquidas (Arcella et al., 2019).

Es importante señalar que la Oficina Federal de Protección al Consumidor y Seguridad Alimentaria de Alemania, indicó que en el análisis de autocontrol de Nestlé de dos muestras de reservas del mismo lote para el que Foodwatch había informado las concentraciones más altas, no mostraron niveles detectables de MOAH (Arcella et al., 2019).

En los Países Bajos también se analizó la muestra de reserva de Nutrilon perteneciente al mismo lote analizado por Foodwatch y no se detectó MOAH. Las diferencias pueden estar relacionadas con variabilidades en el lote, o problemas del método analítico (Arcella et al., 2019).

Un informe publicado en 2020 sobre MOH en aceites comestibles (manteca de cacao, aceite de palma y aceite de girasol) recolectados en el mercado alemán entre 2013-2017 reportó la presencia de MOSH en todas las muestras y MOAH en algunas de las muestras de manteca de cacao y aceite de palma (Stauff et al., 2020)

En base a las concentraciones de MOH en los alimentos y a los datos de consumo, se estimó la exposición dietética de MOH en el mercado belga para niños de 3 a 9 años. Los valores de exposición para MOAH a nivel medio (percentil 50) y alto (percentil 95) fueron 0.00015 y 0.00038 mg kg^{-1} de peso corporal por día, respectivamente (Hochegger et al., 2021).

También se estimó la exposición dietética de MOAH a partir de las concentraciones encontradas en las fórmulas para lactantes y en las fórmulas de continuación. Los niveles de exposición fueron más altos para los lactantes que para los niños pequeños. Se estima que a nivel medio, la exposición de los lactantes oscila entre 0.8 y 44.6 μg kg^{-1} de peso corporal por día, y a nivel alto, entre 1.7 y 78.8 μg kg^{-1} de peso corporal por día (Arcella et al., 2019).

2. Fuentes de contaminación

Los alimentos pueden contaminarse con aceite mineral en distintos puntos de la cadena de suministro, debido a que en nuestro entorno existe una gran variedad de productos que contienen aceites minerales, como por ejemplo: lubricantes, combustibles, aceites en aerosoles agrícolas, tintas de impresión, aceites para neumáticos, adhesivos, aditivos alimentarios, productos fitosanitarios, cosméticos, piensos, materiales en contacto con los alimentos y coadyuvantes tecnológicos (EFSA, 2012).

El aceite mineral contenido en estos productos puede llegar a los alimentos en distintas etapas de su procesamiento y por diversas rutas, directamente por medio de los materiales en contacto con alimentos, aditivos alimentarios y coadyuvantes tecnológicos, o indirectamente a través del medioambiente o materiales en contacto con alimentos (EFSA, 2012). En la Figura 2 se ilustran las formas en la que los alimentos pueden contaminarse con MOH, en distintas etapas de la cadena de suministro de alimentos.

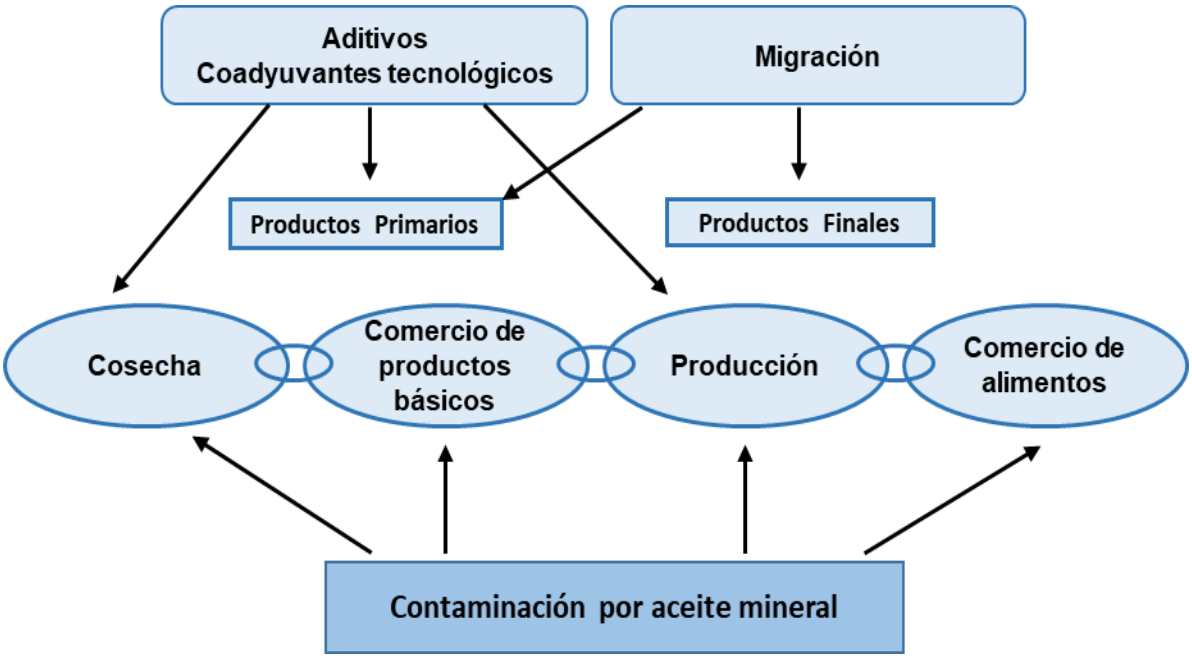

Figura 2. Posibles formas de contaminación de los alimentos con MOH en distintos puntos de la cadena de suministro de alimentos-Figura modificada (Bevan et al., 2020).

2.1. Contaminación ambiental

La contaminación ambiental tiene su origen en los lubricantes y combustibles de las maquinarias empleadas durante la cosecha y procesamiento de alimentos, en los gases de escape de motores de combustión, quemadores de calefacción, residuos de neumáticos o alquitrán de las carreteras. Este tipo de MOH puede contaminar las plantas y los alimentos de origen vegetal por absorción desde la fase gaseosa o por deposición de material particulado presente en la atmósfera (Brühl, 2016; Neukom et al., 2002). Los MOH absorbidos por las plantas son hidrocarburos volátiles, con cadenas de aproximadamente C24 y los que participan en la deposición son hidrocarburos con cadenas mayores a C16 (EFSA, 2012).

2.2. Aditivos alimentarios y coadyuvantes tecnológicos

Los alimentos también pueden contaminarse a partir de los aceites adicionados como aditivos durante la elaboración de plásticos, papel y cartón encerado, lubricantes para latas, sacos de yute o sisal tratados con aceites minerales; o bien, pueden entrar en contacto con los alimentos de forma intencional, como en el caso de los aditivos alimentarios o auxiliares tecnológicos agregados intencionalmente a los alimentos (EFSA, 2012).

2.3. Materiales en contacto con alimentos

Los materiales en contacto con alimentos incluyen a todos aquellos materiales empleados en la fabricación de materiales u objetos destinados a entrar en contacto con alimentos como pueden ser: envases alimentarios, utensilios de cocina, vajilla, cubertería, equipos de cocina, maquinarias de producción o procesamiento y recipientes para transportar alimentos. En la fabricación de estos, se emplea una gran diversidad de materiales, como plásticos, papel, cartón, vidrio, adhesivos, tintas, resinas, ceras, cerámica, corcho, madera, cauchos, silicona, entre otros (Simoneau et al., 2016).

Los materiales en contacto con alimentos más relevantes son los envases alimentarios, ya que son los encargados de asegurar que los alimentos lleguen al

consumidor en las mejores condiciones posibles. Los envases alimentarios tienen la función de contener, proteger, almacenar, conservar y transportar los alimentos (Robertson, 2013). A nivel mundial, la mayor parte de los envases alimentarios están fabricados a base de plástico (rígido y flexible) y de papel o cartón (All4Pack, 2018).

MOH puede ser transferido a los alimentos desde los materiales en contacto con alimentos, en especial desde los envases alimentarios de papel y cartón; y las principales fuentes de aceite mineral en estos tipos de envases, son las tintas de impresión, tanto las que se aplican directamente sobre el envase, como las que incorporan al envase producto de los procesos de reciclaje (Biedermann & Grob, 2012; Biedermann, Uematsu, & Grob, 2011). Otra fuente importante de contaminación de MOH en el papel, son los adhesivos empleados para ensamblar cajas de papel y cartón destinadas a contener, transportar o almacenar los alimentos (Barp et al., 2015; Lommatzsch et al., 2016).

2.3.1. Plásticos

La popularidad de los plásticos en la producción de envases alimentarios, se debe, a que son materiales económicos, livianos, impermeables al oxígeno y al vapor de agua, fuertes, flexibles, moldeables, resistentes a la corrosión con altas propiedades de aislamiento térmico y eléctrico, (Kumar et al., 2020).

Los plásticos son sustancias orgánicas macromoleculares (polímeros), generalmente de origen fósil. Una amplia gama de plásticos es utilizada en el envasado de alimentos, los más frecuentes son el polipropileno, polietileno de baja densidad, poliestireno expandido, polietileno de alta densidad, poliestireno y el tereftalato de polietileno (Muncke, 2016).

También es habitual el uso de plásticos reciclados en la fabricación de envases alimentarios. El reciclaje de los plásticos puede ocurrir mediante procesos mecánicos y químicos. Los plásticos reciclados en forma mecánica, además de contener productos provenientes de la degradación de los polímeros y aditivos, también, pueden tener contaminantes secundarios derivados del uso previo del material y contaminantes ambientales (Geueke, Groh, & Muncke, 2018).

2.3.2. Cartón y papel

La demanda del papel y cartón, solos o asociados con otros materiales para fabricar envases alimentarios primarios, secundarios o terciarios ha aumentado en los últimos años debido a su versatilidad y a la facilidad para reciclar o formar composta (Ottenio, Escabasse, & Podd, 2004).

El papel y el cartón se producen a partir de fibras de celulosa que pueden ser primarias o recicladas. Las propiedades mecánicas de estos materiales suelen mejorarse mediante el uso de aditivos químicos que se agregan durante su fabricación o se depositan en la superficie del papel o cartón, después de su producción (recubrimiento), por lo general, la cantidad de aditivos utilizadas es menor al 1% del papel. Es usual también, decorar o imprimir el papel para hacerlo atractivo al consumidor (Ottenio, Escabasse, & Podd, 2004).

El papel o cartón reciclado pueden contaminarse con sustancias químicas presentes en el papel de desecho, como: aditivos añadidos durante la producción, tintas de impresión, adhesivos, disolventes, plastificantes, tensioactivos y pigmentos, entre otros; además, de los contaminantes adsorbidos durante el uso o gestión de residuos del cartón o papel (Pivnenko, Eriksson, & Astrup, 2014).

Los aceites minerales también pueden contaminar los envases de papel o cartón ya sea por la aplicación directa de tintas de impresión, o por el uso de fibras recicladas durante su fabricación y migrar hasta los alimentos (Maurus Biedermann & Grob, 2010; Vollmer et al., 2011).

De acuerdo con la EFSA, la exposición de los alimentos a MOSH y MOAH proviene principalmente de la migración de estas sustancias, desde envases de cartón y papel reciclados sin una barrera interna (EFSA, 2012).

2.3.3. Tintas de impresión

Las tintas de impresión son mezclas complejas, cuya formulación depende del sustrato donde se apliquen (offset, flexografía, fotograbado, chorro de tinta) y de la forma en la que seca el disolvente (Poças, 2018). Los componentes principales de las tintas de impresión son: colorantes (pigmentos o tintes), aglutinantes (resinas), disolventes y aditivos (Richter, Gude, & Simat, 2009).

Las tintas de impresión suelen aplicarse sobre el lado externo del envase alimentario con el propósito de decorar o proporcionar información sobre el producto; sin embargo, las sustancias empleadas en la elaboración de las tintas pueden transferirse a los alimentos, a través de las capas del envase o por medio del off-set y de la fase gaseosa (Aznar et al., 2015; Poças, 2018).

Una gran cantidad de las sustancias que contaminan al cartón o papel reciclado provienen de las tintas de impresión (Pivnenko, Eriksson, & Astrup, 2014), incluyendo MOH (EFSA, 2012). Las tintas empleadas en la impresión de periódicos se han considerado como la mayor fuente de MOH para el cartón y papel reciclado (Maurus Biedermann & Grob, 2010).

De acuerdo con el Dictamen de la EFSA en 2012, las tintas de impresión offset convencionales contienen entre 20-30% de aceite mineral, y se producen con altos contenidos de MOAH para mejorar la solubilidad de los aglutinantes y pigmentos (EFSA, 2012).

2.3.4. Adhesivos

Los adhesivos son mezclas complejas formadas por una gran variedad de compuestos químicos con funciones específicas. Los tres componentes principales son: un polímero base, una resina tactificante y un portador. El componente principal de un adhesivo es un polímero, al que se le agregan otras sustancias para dotar al adhesivo de ciertas propiedades, como: catalizadores, endurecedores, aceleradores, inhibidores, retardadores, disolventes, extensores, plastificantes, formadores de película,

antioxidantes, antihidrólisis, antifúngicos, jabones, tensioactivos y agentes humectantes (Mildenberg, Zander, & Collin, 1997; Petrie, 2000).

En base a la composición química del polímero base, los adhesivos se clasifican en: termoendurecibles, curan mediante una reacción química irreversible; termoplásticos, se ablandan o funden cuando se calientan; y elastoméricos con una gran capacidad de alargarse y comprimirse (Petrie, 2000).

Los adhesivos termofusibles pertenecen al grupo de los termoplásticos y son comúnmente empleados en el ensamblaje y etiquetado de envases alimentarios. Este tipo de adhesivo, también, suele encontrase en la estructura del envase en combinación con otros materiales, como plásticos, papel, metal, cartón o vidrio formando estructuras multicapas (Ashley, Cochran, & Allen, 1995).

Es conocido que algunos de los constituyentes de los adhesivos, podrían migrar desde el adhesivo contenido en el envase alimentario y alcanzar los alimentos (Aznar et al., 2011; Vera, Canellas, & Nerín, 2014). Debido a que en la formulación de algunos adhesivos se añaden aceites minerales para mejorar su pegajosidad, los adhesivos son considerados como una posible fuente de migración de MOH (EFSA, 2012).

3. Migración

Los materiales en contacto con alimentos deben garantizar la seguridad química de los alimentos; es decir, deben ser lo suficientemente inertes para no transferir sustancias potencialmente peligrosas a los alimentos en cantidades que puedan ocasionar daño a la salud de los consumidores (European-Parliament, 2004). Sin embargo, es bien conocido que una gran cantidad de las sustancias químicas contenidas en estos materiales migran a los alimentos, comprometiendo en algunas ocasiones la seguridad del consumidor (Geueke, Wagner, & Muncke, 2014).

El término migración se refiere a la transferencia de sustancias químicas desde el envase a los alimentos y depende de factores como: la naturaleza del alimento y del

material en contacto, el tipo de contacto (directo o indirecto), tiempo y temperatura de contacto, las características químicas del migrante y su concentración en el material (Arvanitoyannis & Kotsanopoulos, 2014; Poças et al., 2011).

Generalmente, el mecanismo principal de la migración es la difusión, sobre todo en el caso de los plásticos donde la migración está dominada por el gradiente de concentración del polímero; es decir, las moléculas se mueven desde las zonas de mayor concentración a zonas de bajas concentraciones, hasta alcanzar el equilibrio (Simoneau, 2008). Seguidamente ocurre la solvatación de las sustancias migrantes, hasta alcanzar el equilibrio entre las fases alimento-envase y por último, el migrante se dispersa en el alimento mediante mecanismos de difusión (Catalá & Guevara, 2002).

Sin embargo, la migración no ocurre de la misma forma en todos los materiales; en el caso del papel y el cartón que están formados por estructuras porosas y heterogéneas, la migración comprende una combinación de procesos de adsorción y desorción en la fibra celulósica, acompañados de mecanismos de transferencia del migrante en la fibra y difusión en los poros gaseosos del papel o cartón (Poças et al., 2011).

Las características del migrante tienen un papel importante en el proceso de migración. En el caso de la migración indirecta, por vía gaseosa, el migrante debe ser de naturaleza volátil, es decir, debe tener una alta presión de vapor y se espera que este tipo de sustancias migre con mayor rapidez (Poças et al., 2011).

La migración de MOH varía en base a su rango de distribución, a su polaridad y a la capacidad de sorción del embalaje y del alimento. Los MOH de menos de 28 carbonos migran principalmente en la fase gaseosa, es decir se evaporan y precipitan sobre el alimento (Maurus Biedermann & Grob, 2010; Lorenzini et al., 2010; Vollmer et al., 2011); sin embargo, varias investigaciones han reportado que un porcentaje significativo de MOH también migra a través del contacto directo (Barp et al., 2015; Eicher et al., 2015; Pack et al., 2020).

Tradicionalmente las sustancias que migran del envase al alimento se clasifican en dos tipos: añadidas intencionalmente (IAS) o no añadidas intencionalmente (NIAS). Las IAS son agregadas para mejorar las propiedades del material en contacto con alimentos, como por ejemplo monómeros, lubricantes, tensioactivos y estabilizadores UV. Mientras que las NIAS son todas aquellas que no fueron agregadas intencionalmente durante la elaboración del material y que pueden tener diversos orígenes como: la síntesis de los materiales o degradación de material, reacciones entre el material y los alimentos o impurezas de las IAS (Peters et al., 2019). En el caso particular de MOH, estos pueden ser introducidos de manera intencional y no intencional en los alimentos.

4. Métodos de análisis

4.1. Preparación de la muestra

La preparación de la muestra es un paso fundamental en el análisis de MOH que incluye la extracción de los analitos, la eliminación de las posibles interferencias y la obtención de una disolución adecuada para el análisis cromatográfico.

Existe una gran diversidad de métodos de extracción aplicados al análisis de MOH que dependen del estado físico y composición química de la muestra. La extracción de MOH desde alimentos secos suele realizarse con n-hexano (BfR & KLZH, 2012) pero cuando las muestras son liquidas, se aplica la extracción liquido-liquido con solventes no polares como n-hexano o extracción en fase sólida (SPE) (Hochegger et al., 2021).

En el caso de la leche, que es una matriz más compleja, los MOH se extraen mediante hidrólisis ácida (Concin et al., 2008); en alimentos húmedos, se extraen con etanol y luego n-hexano, seguido de la adición de agua para separar la fase de hexano (Biedermann-Brem & Grob, 2011); y en el caso del cartón se extraen con una mezcla

de hexano/etanol y luego agua para separar el hexano (BfR & KLZH, 2012; Vollmer et al., 2011)

Otros investigadores emplearon técnicas de extracción más avanzadas, como la extracción asistida por microondas (Gharbi et al., 2017), saponificación asistida por microondas (Moret et al., 2016) y extracción de líquido presurizado (Moret et al., 2014) que redujeron el tiempo, el consumo de disolventes y proporcionaron mayor rendimiento en la extracción (Hochegger et al., 2021).

4.2. Técnicas convencionales

Comúnmente, el análisis de las fracciones MOSH y MOAH se efectúa mediante cromatografía de gases (GC) acoplada a un detector de ionización de llamas (FID).

La cromatografía de gases es la técnica empleada en el análisis de MOH porque separa a MOH de los hidrocarburos naturales presentes en los alimentos y permite su caracterización por rango de masa molecular. Sin embargo, no es selectiva, no separa a MOAH de MOSH y produce una amplia joroba de componentes no resueltos para ambas fracciones (Maurus Biedermann, Fiselier, & Grob, 2009).

La complejidad del aceite y la falta de estándares de referencia han hecho del FID el detector analítico apropiado para el análisis de MOSH y MOAH, ya que produce prácticamente, la misma respuesta para todos los hidrocarburos y, por tanto, no requiere estándares de referencia y permite el uso de cualquier hidrocarburo que no esté presente en la joroba, como estándar interno para cuantificar las fracciones de MOH pero tiene dos desventaja, baja sensibilidad y falta de selectividad (Maurus Biedermann & Grob, 2012a).

El análisis de MOSH mediante GC-FID requiere la separación previa de las fracciones MOSH y MOAH. Esta separación suele llevarse a cabo por Cromatografía Líquida de Alta Resolución (HPLC) en una columna de gel de sílice usando un gradiente n-hexano/diclorometano (Maurus Biedermann & Grob, 2012a) o manualmente

mediante una columna de vidrio rellena con gel de sílice/nitrato de plata (Fiselier et al., 2013)

El HPLC además de separar las fracciones de MOH debe aislarlas de la matriz de la muestra, lo que implica eliminar las sustancias químicas que puedan interferir con el análisis como los triglicéridos presentes en los extractos de alimentos y ésteres de ceras que podrían sobrecargar la columna HPLC. El acoplamiento en línea a GC automatiza e integra el proceso de preparación de muestras con el análisis final, eliminando la posibilidad de que la muestra se contamine durante la preparación (Maurus Biedermann & Grob, 2012a).

La secuencia de elución de los compuestos que forman MOH está determinada por el efecto de exclusión del tamaño de las moléculas en los pequeños poros de la sílice. Para verificar la separación de las fracciones MOSH y MOAH se utiliza un grupo de estándares que marcan el inicio y el final de cada fracción. El alfa colestano es utilizado para marcar el final de la fracción MOSH, el tri-tert-butilbenceno (TBB) para el inicio de la fracción MOAH y perileno para el final (Maurus Biedermann & Grob, 2012a). Recientemente se ha propuesto di (2-etilhexil) benceno para marcar el inicio de MOAH en lugar de TBB en cosméticos (Maurus Biedermann, Munoz, & Grob, 2017)

Por otro lado, la separación manual es el método de elección para aquellos laboratorios que no cuentan con un HPLC-GC-FID en línea. Varios métodos manuales que involucran óxido de aluminio activado (Wagner et al., 2001) o gel de sílice (Bennett & Larter, 2000; Fiorini et al., 2010) se han propuesto para separar las fracciones de MOH antes del análisis GC-FID. El método que proporcionó los mejores resultados se obtuvo mezclando gel de sílice altamente activada con una baja cantidad de nitrato de plata (Fiselier et al., 2013; Moret et al., 2011).

La fase de sílice/nitrato de plata, además de asegurar la separación de las fracciones MOSH y MOAH, retiene sustancias interferentes como los ésteres de cera y ha proporcionado resultados similares a los obtenidos por HPLC-GC-FID en línea (Fiselier et al., 2013; Moret et al., 2011).

De acuerdo con Fiselier y col (2013), para el análisis de MOH en aceites comestibles, el método manual tiene las mismas limitaciones que HPLC-GC-FID en línea: requiere grandes cantidades de muestra para mejorar los límites de cuantificación y presenta el riesgo de sobrecargar la columna GC con n-alcanos naturales (generalmente C23-C33) u olefinas (Fiselier et al., 2013).

En la actualidad el método recomendado para el análisis de MOH es HPLC-GC-FID en línea. Una versión estandarizada de este método se convirtió en la norma europea EN 16995:2017 para la determinación de MOSH y MOAH en aceites vegetales y productos alimenticios a base de aceites vegetales (AENOR, 2018).

El método descrito en la norma fue sometido a un ensayo inter-laboratorio, en el que se demostró que el método era adecuado para concentraciones superiores a 10 mg kg^{-1} de ambas fracciones (AENOR, 2018). Este método proporciona alta eficiencia de separación, reducción de los límites de detección del consumo de disolventes y del riesgo de contaminación de las muestras (Bratinova & Hoeskstra, 2019).

MOSH y MOAH se cuantifican usando un estándar interno, añadido durante la preparación de la muestra, junto con los estándares de verificación para controlar el fraccionamiento de MOH.

La cuantificación se realiza siguiendo los siguientes pasos (BfR & KLZH, 2012; Maurus Biedermann & Grob, 2012b):

- Primero, se establece la línea base del cromatograma mediante inyecciones repetidas de una muestra en blanco.
- Segundo, se introducen cortes verticales para dividir el cromatograma en sub-fracciones. Los puntos de corte se definen por los tiempos de retención de una mezcla de n-alcanos inyectada en las mismas condiciones que las muestras
- Tercero, se integra en forma manual el área total de cada sub-fracción o MOSH
- Cuarto, se identifican, integran y restan los estándares internos, al igual que los picos agudos sobre la joroba que no forman parte de MOSH o MOAH.

- Y finalmente, se calcula la concentración de cada fracción por medio del método del estándar interno.

En la Tabla 1 se muestran las sub-fracciones de MOSH y MOAH recomendadas en la Guía para la orientación en el muestreo, análisis y presentación de datos para el seguimiento de MOH del Joint Research Centre (JRC). Es importante señalar, que cuando se reporta MOSH y MOAH total se debe integrar todo el intervalo de la señal cromatográfica (Bratinova & Hoeskstra, 2019).

Tabla 1. Sub-fracciones MOSH y MOAH recomendadas por la JRC.

MOSH	MOAH
Total MOSH	Total MOAH
MOSH ≥ n-C10 a ≤n-C16	MOAH ≥ n-C10 a ≤n-C16
MOSH > n-C16 a ≤n-C20	MOAH > n-C16 a ≤n-C25
MOSH > n-C20 a ≤n-C25	MOAH > n-C25 a ≤n-C35
MOSH > n-C25 a ≤n-C35	MOAH > n-C35 a ≤n-50
MOSH > n-C35 a ≤n-C40	
MOSH > n-C40 a ≤n-C50	

4.3. Técnicas auxiliares

En algunas ocasiones es necesario aplicar técnicas auxiliares para mejorar los límites de cuantificación y/o eliminar sustancias que pueden interferir con el análisis MOH o sobrecargar la columna cromatográfica.

Entre estas sustancias interferentes se encuentran (Weber et al., 2018):

- n-alcanos naturales de números impares (en el intervalo de C23- C35) que forman picos aislados en cantidades que pueden sobrecargar el GC e impedir la detección MOSH.
- Hidrocarburos saturados oligoméricos de poliolefina (POSH) proveniente de materiales plásticos en contacto con alimentos, que consisten principalmente en alcanos ramificados, que eluyen con MOSH.

- Olefinas naturales, como el escualeno y sus productos de isomerización, que debido a su polaridad pueden eluir en MOSH o MOAH.
- Lípidos, como los triglicéridos, que pueden sobrecargar la columna, impidiendo la separación exitosa de MOSH y MOAH.

Los n-alcanos naturales (C21-C35) forman picos agudos sobre MOSH que pueden integrarse y restarse del área total. Cuando estos picos se encuentran en cantidades lo suficientemente grandes como para sobrecargar la columna, deben eliminarse por medio de una técnica adicional de limpieza, como por ejemplo, alúmina activada (350-400 ºC), que retiene fuertemente los n-alcanos de cadena larga (Bratinova & Hoeskstra, 2019; Hochegger et al., 2021). Cabe señalar, que el óxido de aluminio también puede eliminar parafinas (Fiselier, Fiorini, & Grob, 2009)

Las polialfaolefinas (PAO), al igual que POSH, eluyen junto con MOSH y aunque su perfil cromatográfico en algunos casos permite distinguirlos de MOSH es muy difícil de separarlos; por lo que se recomienda que se cuantifiquen junto con MOSH y se informe de su presencia (Bratinova & Hoeskstra, 2019).

Las olefinas naturales (carotenoides, escualeno, esterenos), que se encuentran en los aceites vegetales y sus productos derivados, representan un grave problema para el análisis de MOAH debido a que coeluyen con MOAH e interfieren en su cuantificación. Generalmente, las olefinas, se eliminan por epoxidación; sin embargo, la epoxidación puede ocasionar la perdida de algunos MOAH y la eliminación de las olefinas puede quedar incompleta (Bratinova & Hoeskstra, 2019).

Se estima que aproximadamente entre el 20-35% de MOAH se pierde durante la epoxidación; por lo cual, la epoxidación solo debe llevarse a cabo en casos estrictamente necesarios. Después de la epoxidación durante la integración de las joroba MOAH, el analista debe eliminar las interferencias residuales, y si es necesario, confirmar su presencia por espectrometría de masas (Maurus Biedermann, Munoz, & Grob, 2020).

En la actualidad se utilizan varios procedimientos para epoxidar las olefinas en muestras de alimentos. Biedermann y col (2009) propusieron el uso del ácido 3-

cloroperbenzoico en diclorometano (DCM) como reactivo de epoxidación con el propósito de aumentar la polaridad de las olefinas y así eliminarlas (Maurus Biedermann et al., 2009).

Otros investigadores examinaron la hidroboración, la bromohidrina y la epoxidación como posibles técnicas para eliminar olefinas y aumentar el porcentaje de recuperación de MOAH; sin embargo, la epoxidación por ácido meta-cloroperoxibenzoico proporcionó los mejores resultados (Maurus Biedermann et al., 2020).

También se presentó un método de cromatografía liquida acoplado al cromatógrafo de gases (LC-LC-GC), que utiliza dos columnas, la primera para retener la grasa y separar el aceite mineral en sus dos fracciones; y la segunda columna con iones de plata para retener las olefinas (Zoccali et al., 2016). La SPE empleando gel de sílice recubierta con nitrato de plata representa para algunos aceites comestibles (aceite de oliva y el semilla de uva), una alternativa a la epoxidación (Hochegger et al., 2021).

Por otro lado, la capacidad de la columna de HPLC para retener las grasas es limitada, por lo que en algunos casos, se pueden requerir pasos adicionales para mejorar los límites de cuantificación, como: concentrar el extracto de muestra antes de la inyección, saponificación para eliminación de grasas o SPE con alúmina o gel de sílice activado (Weber et al., 2018).

Para simplificar la elección de técnicas auxiliares en el análisis de las fracciones MOSH y MOAH, la JRC propuso el árbol de decisiones que se muestra en la Figura 3.

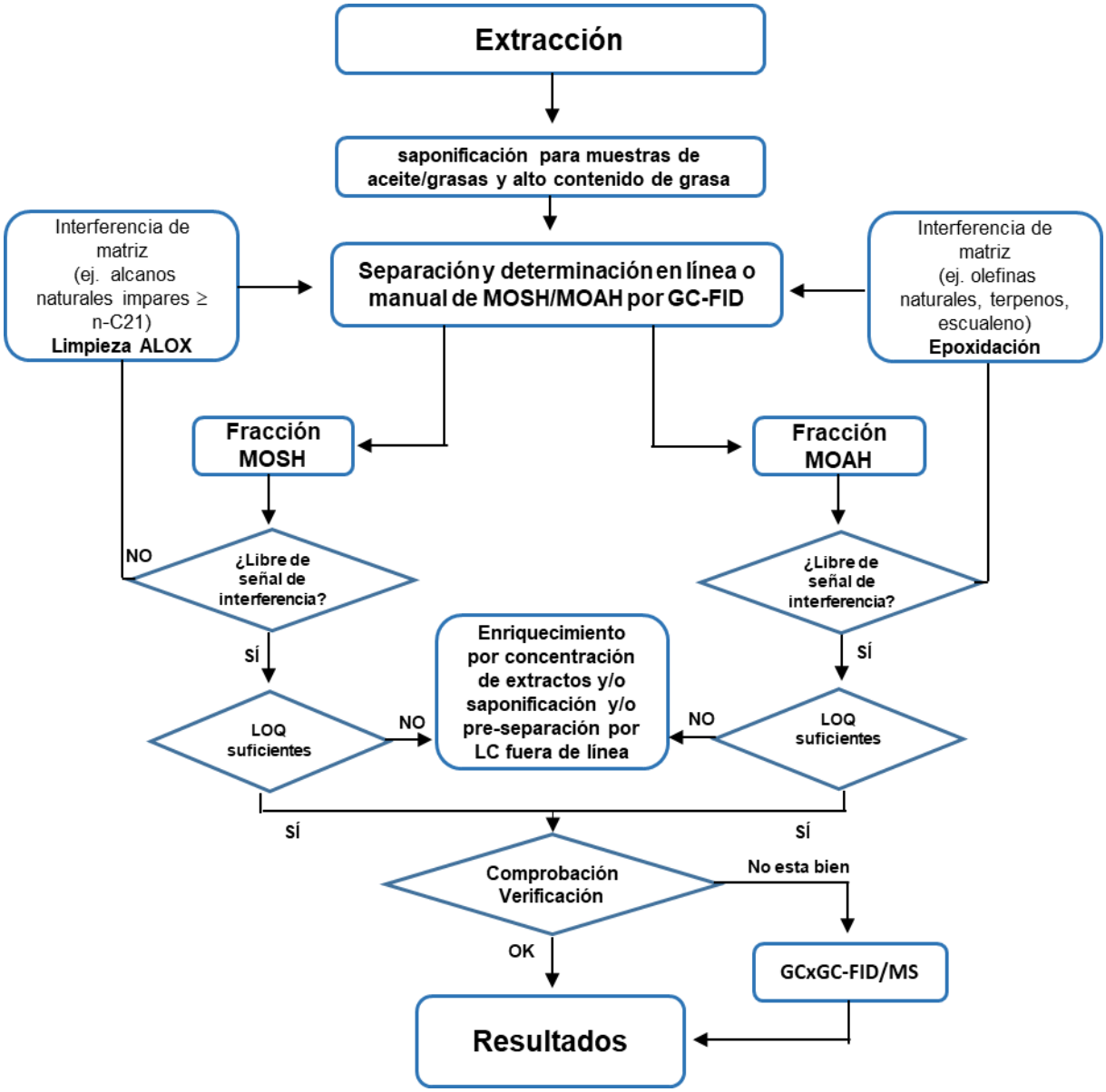

Figura 3. Árbol de decisiones para la selección de técnicas auxiliares-Figura modificada (Bratinova & Hoeskstra, 2019).

4.4. El análisis de MOAH

Algunos científicos consideran de interés particular la fracción MOAH debido al potencial carcinogénico genotóxico de MOAH con tres o más anillos aromáticos, con poca o ninguna alquilación; y la posibilidad de que los alimentos se contaminen con este tipo de compuestos. Además, de las técnicas convencionales de análisis descritas en este documento, se han aplicado otros métodos para caracterizar y estudiar MOAH.

Mediante la aplicación de algunas técnicas instrumentales sofisticadas, como la cromatografía bidimensional de gases integral con espectrometría de masa (GCxGC-MS) y cromatografía líquida en línea con cromatografía de gases bidimensional integral combinada con un sistema de detección dual, espectrometría de masa e ionización de llama (LC-GC×GC-FID/MS) se han logrado grandes avances en la separación y caracterización de los componentes de MOAH (Hochegger et al., 2021).

Recientemente, se publicó un método LC-GC×GC-FID/MS para separar, caracterizar y cuantificar MOAH en función de sus anillos aromáticos. El método permitió separar los MOAH con uno o dos anillos aromáticos de los MOAH con tres o más anillos aromáticos. Sin embargo, los compuestos con tres o más anillos aromáticos no pudieron separarse de los diaromáticos con heteroátomos, como dibenzotiofenos, debido a la similitud en su ionización y limitaciones en la selectividad de la columna cromatografica (Koch et al., 2020).

La detección ultravioleta también se ha aplicado a la caracterización de MOAH. Garcia-Circourel y col (2019) utilizaron una columna de sílice cargada con plata para separar la muestra en función de su aromaticidad (alifáticos, monoaromáticos y poliaromáticos), seguidamente se aplicó una separación por GC; y la detección se realizó con FID o ultravioleta al vacío (VUV). El VUV proporcionó además, información estructural sobre MOAH (García-Cicourel et al., 2019).

4.5. Dificultad del análisis

Entre los grandes problemas del análisis de MOH se encuentran la variabilidad de los resultados proporcionados por los laboratorios, y la falta de fiabilidad de las metodologías actuales como cuando las concentraciones de MOAH son bajas (Koster et al., 2020)

De acuerdo con algunas publicaciones, las posibles causas de esta variabilidad, son (Hochegger et al., 2021; Koster et al., 2020):

- Falta de muestras validadas para distintos tipos de matrices.

- La diversidad de rangos de carbono reportados por los laboratorios.
- Poco uso de técnicas de confirmación para comprobar los resultados obtenidos por GC-FID.
- Falta de protocolos de análisis apropiados para matrices definidas.
- Poca uniformidad en el tratamiento de las muestras.
- Incertidumbre en la integración de las jorobas e interpretación del cromatograma.
- La necesidad de métodos estandarizados y validados para analizar MOH en distintas clases de matrices.
- La falta de estándares de aceite mineral disponibles comercialmente.

Las interferencias de la matriz y la detección FID representan otro reto. La falta de selectividad del FID y su modesta sensibilidad constituyen serias desventajas ante la gran cantidad de componentes no identificados que forman las jorobas de MOH (EFSA, 2012). Se ha comprobado, que en algunas ocasiones las jorobas detectadas con FID pueden no estar relacionadas con MOH o enmascarar otros compuestos distintos de MOH; es decir, que puede dar resultados falsos positivos, sobre todo cuando no se utilizan técnicas de confirmación como MS (Spack et al., 2017), espectroscopia de resonancia magnética nuclear (NMR) (Lachenmeier et al., 2017) o GCxGC (Maurus Biedermann & Grob, 2015). Además, ante la potencial peligrosidad de MOAH se requiere de un estudio más cuidadoso de esta fracción y verificación mediante marcadores (Spack et al., 2017).

4.6. Técnicas de confirmación

Las técnicas de confirmación son utilizadas para complementar al FID y brindan mayores detalles sobre la composición de MOH. Entre las más comunes se encuentran: MS, GCxGC o NMR (Weber et al., 2018).

La espectrometría de masas es utilizada como técnica auxiliar para confirmar la presencia de MOH en los alimentos o materiales en contacto con alimentos. No cabe duda sobre los beneficios que la MS puede brindar al análisis de MOH. La MS puede ser utilizada para identificar los picos agudos sobre la joroba y detectar marcadores

específicos, como por ejemplo dibenzotiofeno para aceites poco refinados o diisopropilnaftaleno para el cartón reciclado. Aunque, es necesario señalar, que se requieren más investigaciones para identificar de forma concreta compuestos marcadores para diferentes fuentes de contaminación (Hochegger et al., 2021).

Por otro lado, la combinación de la espectrometría de masas con la cromatografía de gases bidimensional integral (GCxGC-MS) contribuye a obtener información más detallada sobre la composición de MOH (Purcaro et al., 2013). La fracción MOAH, por ejemplo, ha logrado separarse de acuerdo con el número de anillos aromáticos de sus componentes en bencenos, naftalenos, fenantrenos y antracenos alquilados (Axel Semrau, 2009).

La separación de MOH, a menudo se realiza utilizando en la primera dimensión, una columna no polar para separar los compuestos en función de sus puntos de ebullición y en la segunda, una columna polar para separarlos en base a su polaridad, aunque en algunos casos, la disposición inversa también puede ser efectiva (M Biedermann & Grob, 2015).

Sin embargo, a pesar de su alto poder de resolución, GCxGC no logra separar MOSH de MOAH, algunos naftenos coeluyen con MOAH altamente alquilados; por lo que se requiere que MOSH y MOAH sean separados antes del análisis GCxGC (M Biedermann & Grob, 2015; Purcaro et al., 2013).

Algunos investigadores, sostienen que la espectroscopia NMR también puede ser utilizada como o como técnica de confirmación, sobre todo en el caso de MOAH porque ofrece la posibilidad de detectar los anillos aromáticos tóxicos (Lachenmeier et al., 2017; Weber et al., 2018).

5. Legislación

El propósito principal de la Legislación, en el ámbito de los materiales en contacto con alimentos, es proteger la salud de los seres humanos. Estos reglamentos

pueden ser de tres tipos, los generales, que se aplican a todos los materiales y artículos, los específicos, que se aplican a un tipo concreto de materiales; y los que regulan sustancias individuales (Arvanitoyannis & Kotsanopoulos, 2014).

5.1. Reglamentos generales

En la Unión Europea, todos los materiales y objetos destinados a entrar en contacto con alimentos deben cumplir los requisitos del Reglamento Marco (CE) 1935/2004. Este Reglamento Marco dicta las bases legales para garantizar que los materiales en contacto con alimentos no constituirán un peligro para la salud humana, ni darán lugar a cambios inaceptable en la composición de los alimentos o en sus características organolépticas (European-Parliament, 2004).

Con miras a alcanzar estos objetivos, el Reglamento (CE) 2023/2006 sobre buenas prácticas de fabricación de materiales y objetos destinados a entrar en contacto con alimentos, establece las pautas para asegurar la calidad y seguridad de los materiales en contacto con alimentos (European Commission, 2006).

5.2. Reglamentos específicos

Los reglamentos específicos se han adoptado para cumplir con el Reglamento Marco, no obstante, solo existen normas específicas para cuatro materiales; y, por consiguiente, los materiales que no cuenten con normas específicas deben cumplir con la regulación general.

Los materiales plásticos se encuentran regulados por el Reglamento (UE) 10/2011, que establece los requisitos específicos para materiales y artículos plásticos destinados a entrar en contacto con alimentos. Este Reglamento proporciona un listado de sustancias autorizadas para la fabricación de envases plásticos junto a una serie de requisitos generales y específicos que deben cumplir dichas sustancias, hace referencias a las NIAS y establece límites de migración para los distintos tipos de sustancias; además proporciona las normas y condiciones para realizar ensayos de migración (European Commission, 2011).

Todavía no se han desarrollado regulaciones específicas para los envases de papel y cartón ni para los adhesivos empleados en su fabricación; por este motivo la migración desde estos materiales se regula por medio del Reglamento Marco (CE) 1935/2004 y el Reglamento (UE) 10/2011.

En España, las sustancias empleadas en la fabricación de adhesivos, son reguladas por el Real Decreto 847/2011 para materiales poliméricos destinados a entrar en contacto con los alimentos (RD 847/2011, 2011).

En cuanto al papel y cartón, la asociación industrial Confederation of European Paper Industries (CEPI) ha elaborado un documento voluntario y no vinculante, que proporciona recomendaciones para garantizar el cumplimiento del Reglamento (CE) 1935/2004 (CEPI, 2012).

A excepción de la Ordenanza Suiza, no existe en Europa un reglamento específico para las tintas de impresión; por lo que la Asociación Europea de Tinta de Impresión (EuPIA) ha elaborado una colección de documentos para orientar a los fabricantes a cumplir con la legislación general y proteger la seguridad del consumidor (Lara-Lledó et al., 2018).

5.3. Reglamentos aplicados a los aceites minerales y recomendaciones

Actualmente, en Europa no existe una normativa específica para los aceites minerales, por lo que la presencia de MOH en alimentos o envases alimentarios, se regula según lo establecido en los reglamentos generales (Food Federation Germany, 2018).

Sin embargo, algunas ceras refinadas y aceites minerales blancos, que cumplen con las especificaciones señaladas en el Reglamento (UE) 10/201 están autorizados paras ser empleados como aditivos de materiales plásticos destinados al contacto con alimentos (European Commission, 2011). De igual forma, los aceites minerales empleados como aditivos alimentarios deben cumplir con los requisitos señalados en los

Reglamentos (EU) 231/2012 y (EC) No 1333/2008 (Commission European, 2012; European Parliament, 2008).

En el 2012, la EFSA publicó un Dictamen Científico sobre MOH en los alimentos, en el cual consideró como preocupante la exposición de los seres humanos a MOAH; pero la escasa información sobre la presencia de MOAH en los alimentos y su toxicidad, impido a la EFSA establecer límites específicos para MOAH (EFSA, 2012).

Ante el peligro potencial que MOAH representa, la Comisión Europea (CE), en 2017, publico la Recomendación (UE) 2017/84 sobre la vigilancia de MOH en alimentos y en materiales en contacto con alimentos. Esta recomendación busca crear una base de datos, que sirva para realizar una evaluación científica sobre la exposición y riesgos de MOH para los seres humanos, en especial MOAH (European Commission, 2017).

Otros países miembros de la Unión Europea, han hecho sus recomendaciones y han tomado medidas internas para prevenir la migración de MOH desde los envases alimentarios a los alimentos.

En el 2017 en Bélgica, el Comité Científico de la Agencia Federal para la Seguridad de la Cadena Alimentaria recomendó límites para MOSH en varios productos alimenticios; y en el 2019, en Alemania, el Consorcio de Protección al Consumidor de los Estados Federales (LAV) y la Federación Alemana de Legislación y Ciencia de los Alimentos (BLL) propusieron niveles de referencia para MOH en varios productos alimenticios (Hochegger et al., 2021).

En Alemania, además se han preparado varios borradores para establecer límites legales a la migración de MOAH. Recientemente la Comisión Europea público una versión actualizada del Proyecto del Ministerio Federal de Alimentación y Agricultura para modificar el vigesimosegundo Decreto relativo a la modificación del Decreto sobre los materiales y objetos destinados al contacto con alimentos.

El proyecto alemán define MOAH como: “hidrocarburos aromáticos alquilados con un número de carbonos en el intervalo de C16 a C35, que contienen uno o varios

anillos, con excepción de los diisopropilnaftalenos", limita la migración de MOAH en alimento a 0.5 mg kg^{-1} y 0.15 mg kg^{-1} en simulante alimentario, y obliga a los comerciantes a utilizar barreras funcionales para evitar la migración de MOAH, en el caso de materiales u objetos destinados al contacto con alimentos de papel o cartón (BMEL, 2020).

Por otro lado, en Suiza, la normativa sobre tintas de impresión, establece un listado de MOH permitidos en las tintas, bajo el término de sustancias no evaluadas, sujetas a un límite de migración menor a 0.01 mg kg^{-1} (Hochegger et al., 2021).

Tampoco existen normas específicas, para regular los ensayos de migración de MOH. Por lo que la mayoría de los investigadores utilizan como simulante alimenticio, el óxido de polifenileno modificado (MPPO) (Buscaroli et al., 2017; Conchione, Picon, Bortolomeazzi, & Moret, 2020; Guazzotti et al., 2015); ya que es el simulante recomendado por el Reglamento UE 10/2011 para alimentos secos (European Commission, 2011); y por la norma UNE-EN 14338 para los ensayos con papel y cartón (AENOR, 2004).

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SECCIÓN II: Objetivos Generales

OBJETIVOS GENERALES

El objetivo principal de esta Tesis Doctoral es el desarrollo y la optimización de métodos analíticos para la identificación y cuantificación de compuestos que puedan servir como marcadores químicos de MOAH (hidrocarburos aromáticos de aceites minerales) en distintos materiales destinados a entrar en contacto con alimentos. Se ha incluido, además, el estudio de la migración de estos compuestos desde los envases alimentarios a un simulante alimenticio y a alimentos secos.

Con el propósito de alcanzar estos objetivos se han planteados los siguientes objetivos específicos:

- Selección de compuestos que puedan utilizarse como marcadores para identificar la presencia de MOAH en muestras de aceites minerales mediante dos técnicas analíticas: ASAP-QTOF-MS y APGC-QTOF-MS.

- Identificación de los compuestos seleccionados como marcadores de MOAH en muestras de PET reciclado, cartón reciclado y envases de cuscús y sémola mediante APGC-QTOF-MS.

- Desarrollo y optimización de un método de extracción en fase sólida (SPE) y posterior análisis por GC-MS para identificar y cuantificar los marcadores químicos de MOAH en envases de cartón impresos.

- Aplicación del método SPE-GC-MS desarrollado para identificar y cuantificar los compuestos seleccionados como marcadores MOAH en envases alimentarios de cartón contaminados con aceites minerales.

- Desarrollo y optimización de un método de microextracción en fase sólida (SPME) y análisis por GC-MS para identificar y cuantificar los marcadores

MOAH en muestras de adhesivos termofusibles utilizados en materiales de envasado a los alimentos.

- Estudio de la migración de MOAH y compuestos marcadores de MOAH desde adhesivos termofusibles utilizados en laminados multicapa a simulantes alimentarios por GC-FID y SPME-GC-MS respectivamente.

- Estudio del comportamiento migratorio de MOAH desde muestras de cartón a alimento secos, utilizando compuestos modelo que cubren el rango volátil de MOAH, en las peores condiciones de tiempo y temperatura.

- Evaluación de las diferencias entre la migración de MOAH en alimentos secos y la migración en Tenax mediante el uso de técnicas estadísticas de análisis multivariante.

SECCIÓN III: Desarrollo Experimental

Capítulo 1: *Atmospheric Solids Analysis Probe (ASAP) and Atmospheric Pressure Gas Chromatography (APGC) coupled to Quadrupole Time of Flight Mass Spectrometry (QTOF-MS) as alternative techniques to trace aromatic markers of mineral oils in food packaging.*

Capítulo 2: *Development of a rapid method for determination of aromatic hydrocarbons in food packaging by solid phase extraction and gas chromatography-mass spectrometry.*

Capítulo 3: *Migration of mineral oil aromatic hydrocarbon (MOAH) from hot melt adhesives used in food packaging materials.*

Capítulo 4: *Migration of toxic mineral oil aromatic hydrocarbons (MOAH) from cardboard containers to dry food and prediction tool.*

Capítulo 1

Atmospheric Solids Analysis Probe (ASAP) and Atmospheric Pressure Gas Chromatography (APGC) coupled to Quadrupole Time of Flight Mass Spectrometry (QTOF-MS) as alternative techniques to trace aromatic markers of mineral oils in food packaging.

1. Resumen

Este trabajo se realizó con el propósito de seleccionar e identificar los mejores marcadores químicos para el estudio de MOAH en envases de alimentos. Para ello, inicialmente se analizó una serie de aceites minerales, y se exploraron compuestos como: PAHs, isómeros alquilados de metilnaftaleno, diisopropilnaftalenos, dibenzotiofenos, metildibenzotiofeno, dimetildibenzotiofenos y benzonaftotiofenos. Su presencia fue confirmada mediante el análisis directo de varios aceites minerales con una Sonda de Análisis de Sólidos Atmosféricos con Espectrometría de Masas Cuadrupolo-Tiempo de Vuelo (ASAP-QTOFMS). Se utilizó, además Cromatografía de Gases a Presión Atmosférica Acoplada a Espectrometría de Masas Cuadrupolo-Tiempo de Vuelo (APGC-QTOFMS) para confirmar los marcadores en diferentes muestras de aceites, en tereftalato de polietileno reciclado (rPET), cartón reciclado y en empaques de cuscús y sémola. Se encontraron 27 marcadores en las muestras de aceite mineral, 22 de ellos en rPET, 8 en cartón reciclado y no se encontró MOAH en los envases de cuscús y sémola.

2. Objetivos

El objetivo principal de este capítulo fue seleccionar marcadores MOAH disponibles para confirmar la contaminación por MOAH en distintos tipos de materiales destinados al contacto con alimentos.

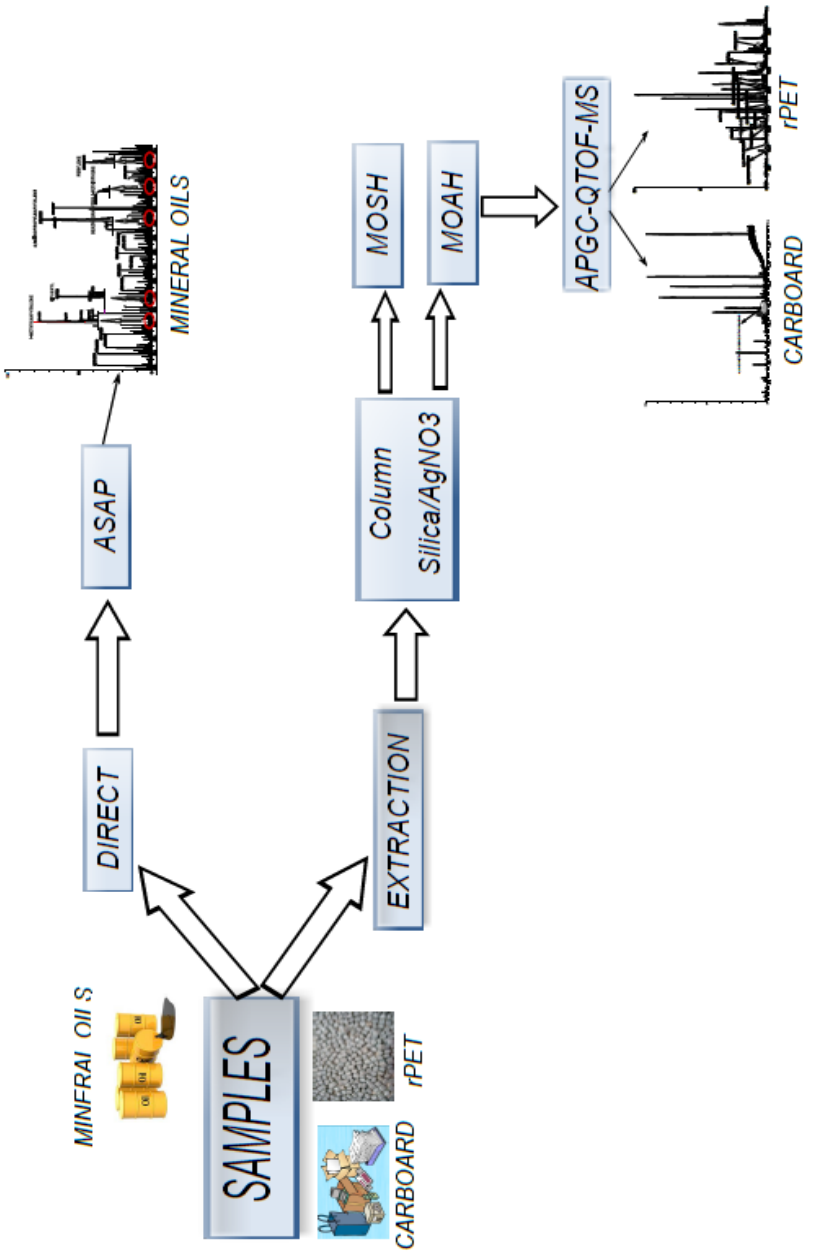
Los objetivos específicos fueron:

- Identificación de hidrocarburos aromáticos que puedan ser utilizados como marcadores de la fracción MOAH y posibles indicadores de las fuentes de contaminación.
- Verificación de la presencia de los marcadores MOAH en muestras de rPET, cartón reciclado y en envases de cuscús y sémola.

Para alcanzar estos objetivos se realizaron las siguientes tareas:

- Optimización de los distintos métodos utilizados para extraer MOH en aceites, rPET y materiales de cartón.
- Optimización de técnicas de análisis como ASAP-QTOFMS y APGC-QTOFMS para detectar los marcadores MOAH en las muestras.
- Identificación de los marcadores MOAH en muestras de aceites analizadas por ASAP-QTOFMS.
- Identificación de marcadores en las muestras de rPET y materiales de cartón por APGC-QTOFMS.
- Separación de la fracción MOAH mediante extracción en fase sólida con sílice activada impregnada de AgNO3.
- Análisis y caracterización de la fracción MOAH por el método convencional en su versión manual (SPE-GC-FID).

3. Esquema de trabajo



Esquema 1. Diseño experimental del Capítulo 1.

4. Introduction

Despite the large amount of testing applied to both food and food packaging materials, consumers are increasingly concerned about food safety. In-depth chemical analysis and toxicity studies often show that some families of chemicals need to be removed from the food area. This is the case of mineral oils. Once their toxicity has been demonstrated (Buist et al., 2020; Grob, 2018; Gude, 2019; IARC, 2012; Pirow et al., 2019), their presence in the food context should be avoided. Mineral oils were found in food packaging and the first series of data came from the study of recycled paper and board (Grob et al., 1997; Grob et al., 1991). Mineral oils hydrocarbons (MOH) are mixtures of different chemical molecules derived from petroleum. Chemical analysis of mineral oils is very complex because they are very heterogeneous structures with hundreds of isomers, which may be linear alkanes, branched alkanes and multiple aromatic rings, which in turn may be alkylated and include sulfur. To facilitate the work, MOH are grouped into two families called MOSH and MOAH. The MOSH open chain hydrocarbons are often branched (paraffins and isoparaffins) and saturated cyclic (commonly naphthenes) hydrocarbons (EFSA, 2012). The individual identification is really difficult. For this reason, several approaches have been suggested by different research groups (Grob, 2018; Moret et al., 2016; Spack et al., 2017; Vollmer et al., 2011) However, in all cases the final quantification is done from the total area of the hump obtained after fractionation of mineral oils into the MOSH and MOAH, and no individual confirmation of the identity is done.

The MOAH fraction depends on the composition of mineral oil and can vary between 15 and 35% and can contain mutagenic and carcinogenic activity. Recently estrogenic activity was also attributed to MOAH and probably behaviour as endocrine disruptors (Tarnow et al., 2016). In 2016 the European Union (EU) published the recommendation EU 2017/84 of monitoring mineral oils in food and food packaging, with special emphasis on MOAH.

Several food products were analysed in different EU countries, pasta (Barp et al., 2015), cereals (Moret et al., 2016), dry food (Moret et al., 2014), oats or cheesecakes showed different concentrations of MOAH. In 2015, many foodstuffs were analysed in 120 countries, and 43% of the samples showed MOAH (Forum, 2017). It is suggested that MOAH could come not only from mineral oils but from atmospheric pollution. However, it has been confirmed that MOAH from mineral oils are mainly those alkylated and when these alkyl derivatives are detected, about 97% are coming from mineral oils (Koster et al., 2020).

The analysis of MOHs and MOAHs has been developed by Kantonales Labor Zurich (KLZH) and the National Reference Laboratory for Food Contact Materials at the Federal Institute for Risk Assessment (BfR) (BfR & KLZH, 2012). The method is quite complex and involves the extraction, fractionation and analysis by HPLC-GC-FID (Barp et al., 2013; Maurus Biedermann & Grob, 2012). Recently, they have improved the method to analyse better the MOAH using LC-GC-GC-MS, in which more information about the chemical structure of aromatic rings can be obtained (M Biedermann & Grob, 2015; Maurus Biedermann & Grob, 2009; Fiselier et al., 2013). Although the method is good for screening, it does not solve the quantitative problem, and there is still great variability in the results reported by different laboratories (Koster et al., 2020). Recently, the Joint Research Center (JRC) prepared a packaging material with a known concentration of mineral oils to help the laboratories in this task. However, the material is not certified yet.

Without a doubt, the most difficult task in this analysis is quantification, since it is based on the humps containing the MOSH and MOAH fraction, without identifying the individual hydrocarbons under the hump. This way, several hydrocarbons different from MOAH can be coeluted, and these facts provide higher quantitative values, which will be assigned to MOAH. To eliminate this risk, some MOAH markers have been suggested, such as anthracene y perylene (Maes et al., 2019), but this is not enough. Another approach for confirming the MOAH values was the use of m/z 91, 105, 113 and 119, but these mass fragments coincide as well with many natural substances such

as terpenes, phytosterols, olefins and carotenoids, what would overestimate the MOAH values (Bratinova & Hoeskstra, 2019; Spack et al., 2017). These studies concluded that more MOAH chemical markers would be necessary to confirm the quantification of MOAH (Maurus Biedermann, Munoz, & Grob, 2017; Spack et al., 2017). This is the main objective of the present work.

Chemical markers have the advantage of being useful tools for verifying the source of contamination and providing detailed and reliable chemical evidence of MOAH contamination, helping to avoid misinterpretations in MOAH analysis (Koster et al., 2020; Spack et al., 2017)

To find the right and useful markers for MOAH is not an easy task, as there are hundreds of aromatic hydrocarbons present in mineral oils (Fang et al., 2017; Li et al., 2012; Machado et al., 2013). Due to the complexity of mineral oils, conventional GC-MS technique is not enough to identify all potential markers and other techniques could be used. Atmospheric pressure gas chromatography coupled to high resolution MS, named APGC- MS-QTOF is a powerful technique that allows the accurate mass and thus, facilitates the identification of chemical compounds (Carrizo et al., 2015; Vera, Canellas, & Nerín, 2014). This technique also provides the use of MSE, where all compounds are driven to the collision cell and alternatively exposed to low and high collision energy, which provides high selectivity and sensitivity.

The present work shows the study carried out to select available markers of MOAHs, which could be used to identify the contamination of MOAHs in any sample. 27 potential markers were explored, including Polycyclic Aromatic Hydrocarbons (PAHs) and their alkyl and branched derivatives as well as DBTs and BNTs isomers. 16 out of the 27 under study were finally selected as representatives of the different families present in mineral oils, in order to identify MOAH in the samples. The hyphenated techniques ASAP-QTOF-MS and APGC-QTOF-MS were used for analysing MOAH in mineral oils, recycled PET, recycled paperboard and packaging of couscous and semolina samples. The results are shown and discussed.

5. Materials and methods

5.1 Reagents

The standards used were: 1,2,3-trimethylbenzene, 1,3-diethylbenzene, 2-methylnaphthalene, 1-methylnaphthalene, 4-tertbutyltoluene, biphenyl, acenaphthene, 2,6-dimethylnaphthalene, cyclohexylbenzene, fluorene, anthracene, phenanthrene, 4-methyldibenzothiophene, pyrene, 3,3',5,5'-tetramethylbiphenyl, 2,6-diisopropylnaphthalene, 4,6-dimethyldibenzothiophene, 1-methylpyrene, benz(a)anthracene, chrysene, benzo(b)naphtho(1,2-d)thiophene, 1,3,5-tri-tert-butylbenzene, benzo(b)fluoranthene, benzo(a)pyrene, and perylene, all supplied by were Sigma-Aldrich (Madrid, Spain). 9,9'-dimethylfluorene from Tokyo Chemical Industry CO., LTD. and 3,6-dimethylphenanthrene from Dr. Ehrenstorfer (Augsburg, Germany). A stock solution containing an accurate concentration of 100 μg g^{-1} of each compound was prepared in toluene. Lower concentrations were prepared by appropriate dilution in n-hexane.

The standards used for MOSH and MOAH analysis by GC-FID were n-undecane, n-tridecane, bicyclohexyl, 5α-cholestane, pentylbenzene, 1-methylnaphthalene, 2-methylnaphthalene, 1,3,5-tri-tert-butylbenzene and perylene, all acquired to Sigma-Aldrich. A solution containing: n-undecane (175 μg g^{-1}), n-tridecane (350 μg g^{-1}), bicyclohexyl (350 μg g^{-1}), 5α-cholestane (350 μg g^{-1}), pentylbenzene (350 μg g^{-1}), 1-methylnaphthalene (350 μg g^{-1}), 2-methylnaphthalene (350 μg g^{-1}), 1,3,5-tri-tert-butylbenzene (350 μg g^{-1}) and perylene (350 μg g^{-1}) in toluene was used as internal standard. All solutions were stored at 4°C. All standards and solutions were under gravimetric control.

The solvents used were: toluene, n-hexane, methanol, dichloromethane (DCM) and acetone, all HPLC grade, supplied by Scharlab SL (Barcelona, Spain). Standard of saturated alkanes (C7-C40) of 1000 μg mL^{-1} each component in n-hexane was purchased from Sigma Aldrich.

Silica gel of high-purity grade with 60 Å (70-230 mesh) pore size for chromatographic columns and silver nitrate on silica gel (~ 10 wt% loading, 230 mesh) were obtained from Sigma-Aldrich (Madrid, Spain). Anhydrous sodium sulfate and cellulose extraction thimbles were purchased from Scharlab SL (Barcelona, Spain). The silanised glass wool was from Supelco (Bellefonte, USA) and ultrapure water type I (reactive grade) was obtained from Ultramatic GR water purification system (Wasserlab, Spain). Liquid nitrogen was supplied by cryogenic liquids service of Zaragoza University.

5.2. Samples

The mineral oil samples were hydraulic oil (oil 01), multigrade lubricant oil (oil 02), oil for rotary vane pumps and roots pumps (oil 03), lubricating oil different uses (oil 04), oil for gasoline engine (oil 05) and industrial oil use (oil 06). These samples were purchased in the local retail market. The oils chosen cover a wide range of uses in industry.

Samples of recycled polyethylene terephthalate (rPET) intended for food packaging from different companies coded as AB, FFT, TP, GE and IN were analysed. The samples of recycled cardboard were provided by a manufacturing company, and two samples of cardboard packaging in direct contact with the food, couscous and semolina, were obtained from the retail market.

5.3. Sample preparation

Sample extraction and treatment was different for each kind of matrix. 50 mg of mineral oil samples were diluted to 1000 mg with n-hexane. Then a fractionation on silica impregnated with silver nitrate was applied and the MOAH fraction was separated, following the analytical method developed by KLZH and BfR (BfR & KLZH, 2012) and recommended by JRC (Bratinova & Hoeskstra, 2019). The mixture of silica gel with silver nitrate (0.3%) was prepared according to the procedure described by Spack et al.

(Spack et al., 2017) and homogenised for 12 h in a laboratory shaker Vibromatic JP Selecta (Spain).

The recycled PET pellets were cooled in liquid nitrogen and milled as powder to increase the surface and facilitate the extraction process of mineral oils. In a cellulose thimble, 35 g of the cryogenically powdered rPET were introduced and extracted with n-hexane in a 250 mL Soxhlet for 24 h. The extract obtained was concentrated in a rotary evaporator at 40º C up to 0.5 g, and the MOAH fraction was separated using the KLZH & BfR protocol.

Recycled cardboard samples were cut into small pieces of 0.5 x 0.5 cm and 2.0 g exactly weighed were placed in a 20 mL glass vial with 10 mL of ethanol/n-hexane (1:1) mixture. The vial was shaken at room temperature for 2 h to extract the MOH, according to the procedure proposed by the KLZH & BfR. The extract was then added to a glass column containing 33 g of silica previously activated at 400 ºC for 24 h in the oven and 1 g of silica coated with silver nitrate 10% (w/w). MOSH and MOAH fractions were obtained using an n-hexane/dichloromethane gradient.

All glassware was cleaned with methanol, acetone and n-hexane and dried in the oven before using. The samples analysed by ASAP-QTOF-MS did not require previous extraction or sample treatment. Blank samples were always prepared and analysed simultaneously with the samples throughout the process.

All samples were analysed in triplicate by APGC-QTOF-MS and GC-FID to demonstrate the presence of contamination according to the Grob method (Grob et al., 1997).

5.4. ASAP-QTOF-MS

ASAP-QTOF-MS XevoG2 QTOF from Waters Corporation (Manchester, UK) was used. The instrument has a mass range up to m/z 100,000 and a solving power of >22.500 full wide at half maximum (FWHM). Constant temperature at 120 ºC was used in the ionisation chamber. The key ion source parameters to be optimised were: corona

current (mA), sample cone voltage (V) and desolvation gas temperature (ºC). Acquisition mode used was full-scan MS data from 45 to 450 amu. The working conditions were: 4 min of acquisition time, 650 L h^{-1} desolvation gas, resolution mode, sampling cone 4.0, 5 mA crown, 4.0 cone extraction, 120 scan. Cone flow was not needed for this technique.

To facilitate the analysis of all markers in a single acquisition, a gradient of temperature from 200 to 450 ºC was applied to the gas desolvation step, so that the compounds would progressively appear according to their size. To set the limit of detection (LOD) solutions of individual standards in n-hexane were analysed at different concentrations (0.1, 0.5, 1, 5, 10, 20 µg mL^{-1}) under the same experimental conditions as the samples. Data were collected and processed using MassLynx (Waters Corporation) software. LODs were calculated as three times the standard deviation of the blank at the same characteristic mass of each compound.

Before the analysis, the single use glass rod was inserted into the source at high temperature for 2 min to remove any contamination from the tip. Then it was cooled and introduced into the sample for 10 s. After that it was inserted into the ionisation chamber of ASAP. Blanks were performed without loading the sample, during the first 120 s of acquisition, applying the same temperature ramp. The samples were analysed in continuous mode for 4 min. From each MS spectrum, the characteristic masses were selected, and this information was used for searching the presence or absence of each compound in the samples. Five replicates of each sample were analysed.

5.5. APGC-QTOF-MS

The analysis was performed using a 7890A GC system (Agilent, Santa Clara, CA, USA) equipped with a CTC Analytics Combipal autosampler. The chromatographic separation was performed in a DB-5 MS capillary column, 30 m, 0.25 mm id, 0.25 µm film thickness. The oven temperature program was: 40 ºC for 1 min, 7 ºC min^{-1} ramp to 100 °C, 9 ºC min^{-1} ramp to 240 ºC and held for 1 min, 5 ºC min^{-1} ramp

to 300 ºC and held for 7 min. 1 µL was injected in splitless mode. Helium was used as carrier gas at a constant flow of 0.07 L h^{-1}.

The APGC source was coupled to a quadrupole-time of flight analyser (Q-TOF) Xevo G2 from Waters (Milford, MA, USA). API positive polarity and sensitivity analyser mode were selected. The ion source (API+ mode) with a corona current of 3 kV was used. Sampling and extraction cone voltages were 30 V for sampling cone and 3 V for extraction cone. Cone and desolvation gas flow were 20 and 175 L h^{-1} "respectively". N_2 was used as a makeup gas at 300 mL min^{-1} and 300°C, while the source temperature was 150 °C. Acquisition was performed in MSE mode, with collision energy of 6 EV in function 1 (low energy), while a collision energy ramp 20-40 V was used in function 2 (high energy). Scan time was 0.5 s, and the mass range was 45-450 acquisition m/z. The lockmass reference used was perfluorotributylamine.

5.6. GC-FID

The GC-FID analysis was performed with a Trace GC Ultra (Thermo Electron Corporation, Milan, Italy) equipped with an AS 300 autosampler and a flame ionisation detector (FID). The analytical column used was HP-5 (60 m x 0.25 mm i.d., 0.25 µm film thickness) from Agilent Technologies. The oven temperature program started at 50 °C held for 2 min; then increased by 30 °C min^{-1} to 310 °C and held for 15 min. The total run was 25.7 min. The flow rate of the carrier gas (helium, 99.999%) was 2 mL min^{-1}. Splitless injection and injector temperature 250 °C were used. The injection volume was 5 µL. The FID detector temperature was 350 °C. Data were acquired and processed with Chrom-Card GC Software (Thermo Electron).

6. Results and discussion

6.1. Direct identification of markers of mineral oils by ASAP-MS-Q-TOF

The most complex and expensive step of any analysis is the sample treatment, and mineral oils are very difficult samples in which this step is tedious and time consuming. The major advantage of ASAP is that it allows the direct analysis without sample pretreatment and can be applied to targeted volatile or semi-volatile compounds. The presence or absence of specific compounds can be quickly determined by comparing their exact mass with that of a pure standard. As another technique, ASAP needs the optimisation of certain key parameters, such as the corona current (A), sample cone voltage (V) and desolvation gas temperature (ºC). These parameters will influence the total number of ions reaching the detector.

The first step was to optimise the experimental conditions for the selected standard compounds. The key parameters above mentioned were optimised using standard solutions of the pure compounds. Cone voltage sampling varied from 20 V to 80 V and the cone voltage extraction was set at 0.1 V. Target samples were analysed in continuous mode (3 min) with a cone voltage ramp (20–80 V) and desolvation gas temperature ramp (200–500 ºC) Atmospheric Pressure Ionisation (API) in positive polarity was selected, and source temperature was 120 ºC. The parameters of the XEVO G2 QTOF were: scan time 1 s and the mass range considered was m/z 45–450 to ensure the presence of all standards. Each sample was analysed in triplicate. A blank sample was also analysed under the same experimental conditions. In addition to the high resolution mass achieved, isotopic ratios (C12/C13, N14/N15, O16/O18) and software tools were used to confirm the target compounds. MassLynx software from Waters was used, which considers the isotopic model and the elemental composition. From the mass spectrum of each standard, the characteristic masses were selected and later used to determine the presence or absence of the compound in the sample.

Table 1 shows the results of all analysed oils with their molecular structure, molecular weight, and CAS number of the standards. As can be seen, twelve compounds

1,2,3-trimethylbenzene, biphenyl, 2,6-dimethylnaphthalene, anthracene, 9,9'-dimethylfluorene, 4-methyldibenzothiophene, pyrene, 3,6-dimethylphenanthrene, 3,3',5,5'-tetramethylbiphenyl, benz(a)anthracene, 1-methylpyrene and perylene, were detected in all oil samples. Thus, these compounds could be used as markers of aromatic mineral oils, as they take part in the composition of different types of oils (Fang et al., 2017, 2016; Li et al., 2012; Stevens, Shi, & Hsu, 2013; Wu, Qian, Walters, & Mennito, 2015).

Figure 1 A shows the spectrum of a mixture of potential markers of 2 μg g^{-1} of MOAH analysed by ASAP-QTOF-MS in positive mode. The exact mass allows to check the presence or absence of these compounds in the obtained spectra of mineral oils, with an error lower than 0.5 Dalton as shows Figure 1 A. The molecular ion $[M]^+$ and protonated ion $[MH]^+$ were the most abundant ions in all compounds. This performance was also observed in previous work (Domeño, Canellas, Alfaro, Rodriguez-Lafuente, & Nerín, 2012). LOD was found to determine the order of magnitude in which the markers could be detected in the samples.

Direct analysis provides the exact masses of all compounds present in the sample but without chromatographic separation, isobaric isomers, such as 2-methylnaphtalene and 1-methylnaphtalene isomers, or biphenyl and acenaphthene among others, which have exactly the same m/z ratio, cannot be unequivocally identified.

Figure 1 B shows the MS spectrum of oil 04, where the masses of 1-methylnaphthalene, biphenyl, 2,6 dimethylnaphthalene, 9,9-dimethylfluorene, 3,6-dimethylphenanthrene, 3,3',5,5'-tetramethylbiphenyl, 2,6-diisopropylnaphthalene, 1-methylpyrene, benzo(b)naphtho(1,2-d)thiophene and perylene, can be seen. Fluorene, dibenzothiophene and dibenzofuran and their alkylated homologs are the most important aromatic compounds in crude oils.

Although ASAP was an excellent tool for fast screening of MOAH in difficult samples, some of the common isomers could not be properly identified and additional in-depth analysis using a chromatographic separation was required.

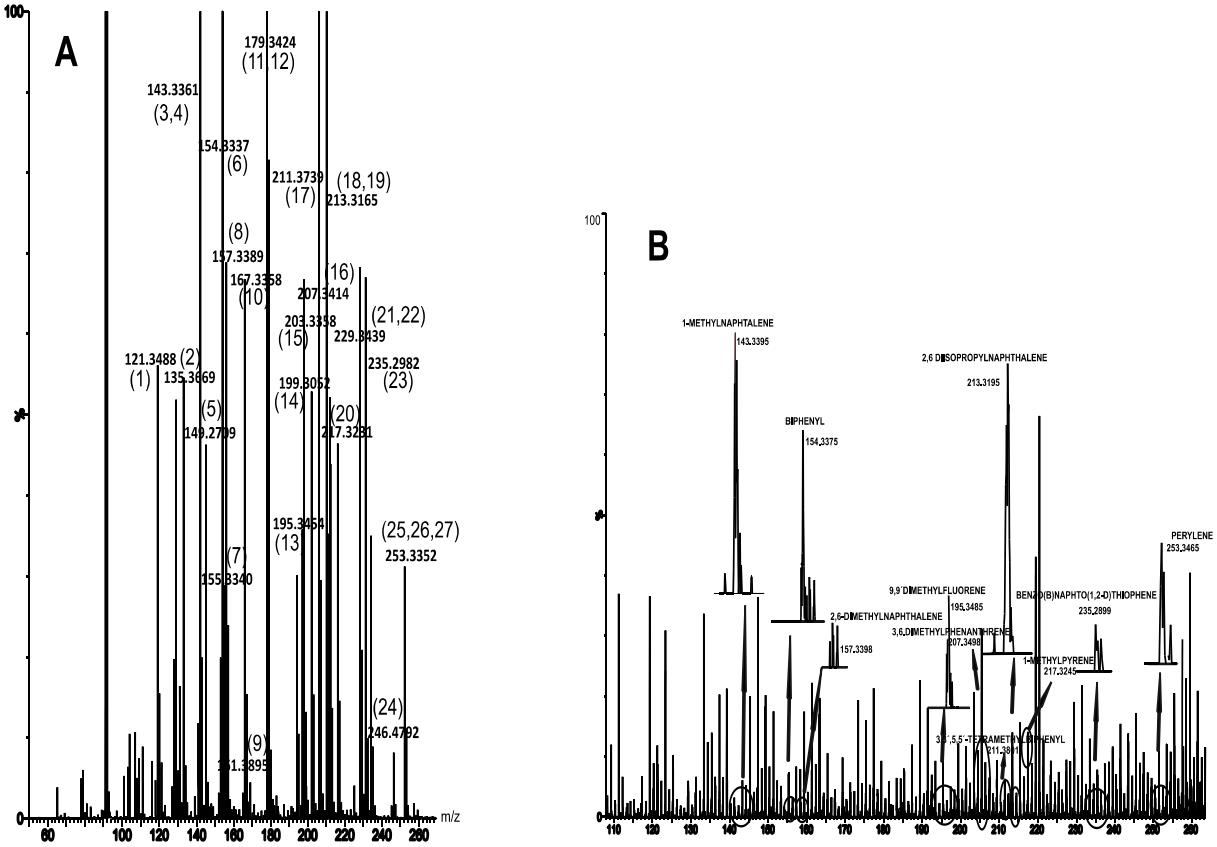
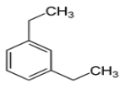
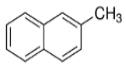
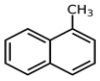
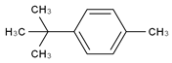
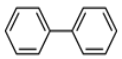
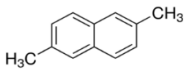
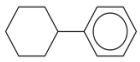
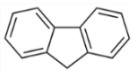
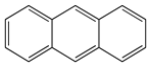
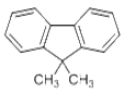
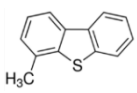
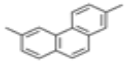
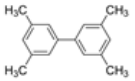
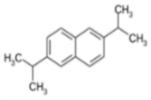
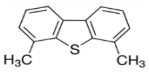
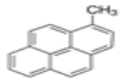
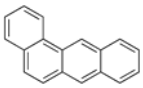
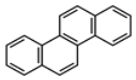
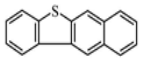
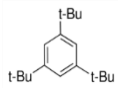
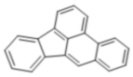
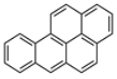


Figure 1. ASAP-Q-TOF spectrum of standard solution of markers of 2 μg/g (A) and of mineral oil 04 (B). Compounds1,2,3-trimethylbenzene: (1); 1,3-diethylbenzene (2); 2-methylnaphthalene; (4) 1- methylnaphthalene (3); 4-tert-butyltoluene (5); biphenyl(6); acenaphthene(7); cyclohexylbenzene (8); fluorine (9); anthracene (10); phenanthrene (11); 4-methyldibenzothiophene (12); pyrene (13); 2,6-diisopropylnaphthalene (14); 4,6-dimethyldibenzothiophene (15); benz(a)anthracene (16); chrysene (17); benzo(b)naphto(1,2-d)thiophene (18); 1,3,5-tri-tert-butylbenzene(19); benzo(b)fluoranthene (20); benzo(a)pyrene (21); perylene(22).

Table 1. Markers of aromatic mineral oils, chemical characteristics and oil samples where they were detected.

Nº	Compounds	CAS	Molecular structure	Molecular weight	Oil samples
1	1,2,3-trimethylbenzene	526-73-8		120.20	1,2,3,4,5,6
2	1,3-diethylbenzene	141-93-5		134.22	2,3,4,5,6
3	2-Methylnaphthalene	91-57-6		142.20	1,3,4,6
4	1- Methylnaphthalene	90-12-0		142.20	1,3,6
5	4-tert-Butyltoluene	98-51-1		148.24	1,2,3,4,5
6	Biphenyl	92-52-4		154.21	1,2,3,4,5,6
7	Acenaphthene	83-32-9		154.21	1,2,4,5,6
8	2,6 Dimehtylnaphthalene	581-42-0		156.22	1,2,3,4,5,6
9	Cyclohexylbenzene	827-52-1		160.26	3,4,5,6
10	Fluorene	86-73-7		166.22	2,3,4,5,6
11	Anthracene	120-12-7		178.23	1,2,3,4,5,6
12	Phenanthrene	85-01-8		178.23	1,2,4,5,6
13	9,9'-Dimethylfluorene	4569-45-3		194.28	1,2,3,4,5,6
14	4-Methyldibenzothiophene	7372-88-5		198.28	1,2,3,4,5,6
15	Pyrene	129-00-0		202.25	1,2,3,4,5,6

Nº	Compounds	CAS	Molecular structure	Molecular weight	Oil samples
16	3,6-Dimethylphenanthrene	1576-67-6		206.28	1,2,3,4,5,6
17	3,3',5,5'-Tetramethylbiphenyl	25570-02-9		210.31	1,2,3,4,5,6
18	2,6-Diisopropylnaphthalene	24157-81-1		212.33	1,2,3,4,5
19	4,6-Dimethyldibenzothiophene	1207-12-1		212.31	1,3,4,5,6
20	1-Methylpyrene	2381-21-7		216.28	217.3231
21	Benz(a)anthracene	56-55-3		228.29	1,2,3,4,5,6
22	Chrysene	218-01-9		228.29	2,3,4,6
23	Benzo(b)naphto [1,2-d]thiophene	205-43-6		234.32	1,2,4,6
24	1,3,5-Tri-tert-butylbenzene	1460-02-2		246.43	3,4,5
25	Benzo(b)fluoranthene	205-99-2		252.31	2,3,4,5,6
26	Benzo(a)pyrene	50-32-8		252.31	2,3,4,5,6
27	Perylene	198-55-0		252.31	1,2,3,4,5,6

6.2. Markers analysis by APGC-Q-TOF-MS

Once a set of 12 markers were initially identified, the objective was to detect them in different food packaging materials. The selected samples were recycled PET, recycled cardboard and two samples of cardboard packaging of couscous and semolina from the retail market.

As was above mentioned, one of the problems in ASAP is the inability to differentiate the isomers without prior separation. This problem is solved with chromatography. However, the usual GC-MS analysis of mineral oils provides a forest of peaks that make almost impossible the identification of individual compounds and additional tools are required for this purpose. Using QTOF analyser and MSE mode, the unequivocal identification of MOAH in the samples was achieved. Data for accurate molecular mass ions were processed using MassLynx, a software program that provides the corresponding elemental composition and the difference between experimental and theoretical mass. It was also necessary to optimise the chromatographic conditions to achieve a good separation of all standards including the isomers. Figure 2 shows the chromatogram obtained of a mixture of standards 3 μg g^{-1} in n-hexane. As can be seen, the isomers: 2-methylnaphthalene and 1-methylnaphthalene (4,5) with molecular mass 142.20; biphenyl and acenaphthene (7,10) with molecular mass 154.21; anthracene and phenanthrene (15,16) with more molecular mass 178.23; 2,6-diisopropylnaphthalene and 4,6-dimethyldibenzothiophene (13, 18) with molecular mass 212.31, benz(a)anthracene and chrysene (23, 24) with molecular mass 228.29 and the three isomers benzo(b)fluoranthene, benzo(a)pyrene and perylene (25, 26, 27) with molecular weight 252.31 were perfectly separated by chromatography.

The spectra of standards showed gently ionized analytes with low or any fragmentation. The most abundant compounds were protonated $[M+H]^+$ and deprotonated $[M-H]^-$, in addition to some methylated $[M-CH3]^+$. The ionisation process with APGC can be driven towards protonation by using modifiers within the source enclosure, typically H_2O or MeOH. The modifier is simply a vial of reagent which is located within either the source door (cool position) or ion block holder (heated position). Individual standard solutions in n-hexane at different concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20 μg g^{-1}) were analysed to determine the analytical features.

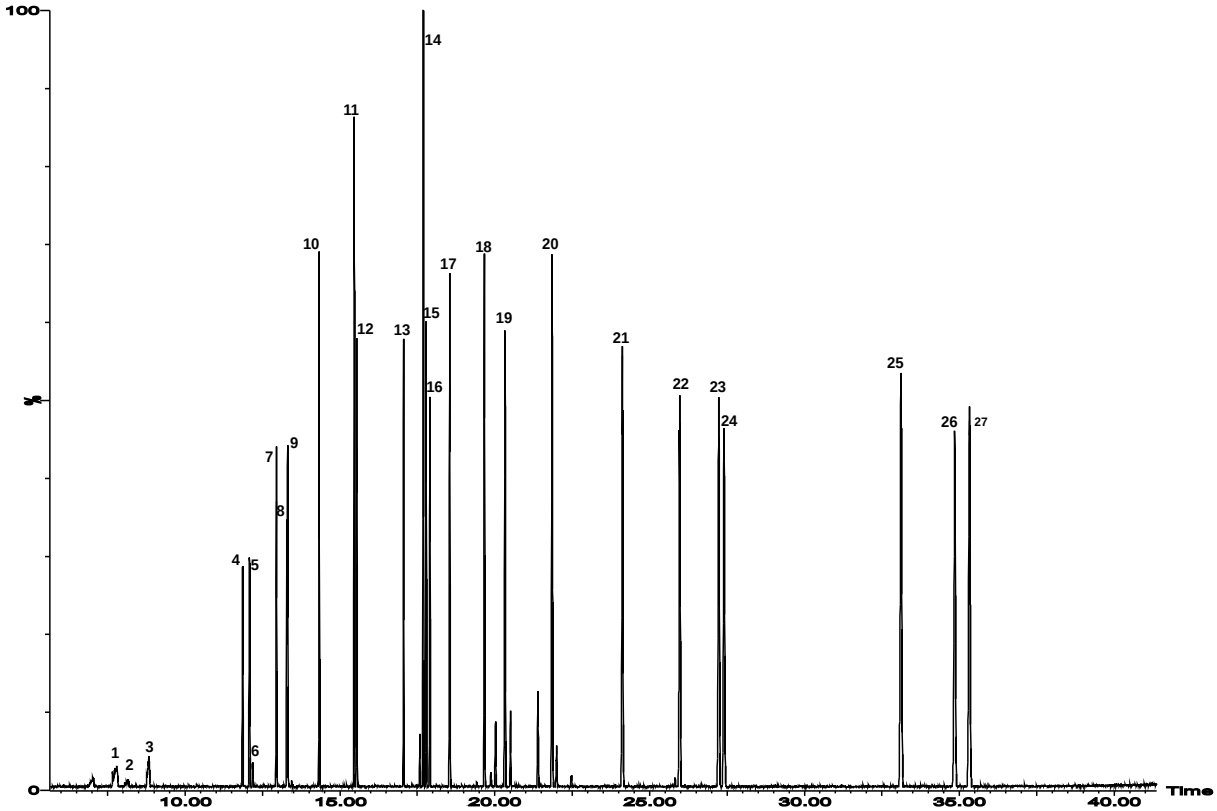


Figure 2. Chromatogram of the standard solution (1 μg g^{-1}) obtained by APGC-Q-TOF. Compounds: 1,2,3-trimethylbenzene (1), 1,3-diethylbenzene (2), 4-tert-butyltoluene (3), 2-methylnaphthalene (4), 1- methylnaphthalene (5), cyclohexylbenzene (6), biphenyl (7), 1,3,5-tri-tert-butylbenzene (8), 2,6-dimethylnaphthalene (9), acenaphthene (10), 9,9′-dimethylfluorene (11), fluorine (12), 2,6-diisopropylnaphthalene (13), 3,3′ ,5,5′ -tetramethylbiphenyl (14), anthracene (15), phenanthrene (16), 4-methyldibenzothiophene (17), 4,6-dimethyldibenzothiophene (18), 3,6-dimethylphenanthrene (19), pyrene (20), 1-methylpyrene (21), benzo(b)naphto [1,2-d]thiophene (22), benz(a)anthracene (23), chrysene(24), benzo(b)fluoranthene (25), benzo(a)pyrene (26) and perylene (27).

Table 2 shows the characteristics of the method with retention times, characteristic mass, LOD of the compounds as well as the reproducibility and accuracy. LOD was calculated as three times the standard deviation “respectively” of the blank at the same characteristic mass of each compound. All LOD values were in the range of 0.01 to 0.06 μg g^{-1}.

Table 2. Analytical features for method APGC-QTOF-MS

Nº	Compounds	Accurate mass (Da)	Retention time (min)	Ion type	LOD (μg g^{-1})	Intraday precision (RSD, %)	Interday precision (RSD, %)
1	1,2,3-Trimethylbenzene	121.3488	5.62	$[M+H]^+$	0.05	3.3	6.1
2	1,3-Diethylbenzene	135.3669	6.25	$[M-CH_3]^+$	0.06	4.1	6.2
3	4-tert-Butyltoluene	149.2709	7.68	$[M-CH_3]^+$	0.05	3.9	5.9
4	2-Methylnaphthalene	143.3293	11.86	$[M+H]^+$	0.04	2.1	4.4
5	1- Methylnaphthalene	143.3293	12.09	$[M+H]^+$	0.03	1.9	3.6
6	Cyclohexylbenzene	161.3895	12.18	$[M+H]^+$	0.06	3.1	4.8
7	Biphenyl	155.3337	12.96	$[M+H]^+$	0.04	2.8	3.6
8	1,3,5-tert-Butylbenzene	246.4792	13.29	$[M+H]^+$	0.04	1.8	2.2
9	2,6-Dimethylnaphthalene	157.3389	13.32	$[M+H]^+$	0.03	2.1	2.3
10	Acenaphthene	155.3340	14.33	$[M+H]^+$	0.03	2.2	3.1
11	9,9'-Dimethylfluorene	195.3454	15.47	$[M+H]^+$	0.02	1.9	2.1
12	Fluorene	167.3358	15.55	$[M+H]^+$	0.03	1.9	2.5
13	2,6-Diisopropylnaphthalene	213.3165	17.06	$[M+H]^+$	0.04	1.6	2.3
14	3,3',5,5'-Tetramethylbiphenyl	211.3739	17.70	$[M+H]^+$	0.2	1.5	1.9
15	Anthracene	179.3424	17.79	$[M+H]^+$	0.03	1.9	3.6
16	Phenanthrene	179.3369	17.92	$[M+H]^+$	0.02	1.8	3.4
17	4-Methyldibenzothiophene	199.3052	18.55	$[M+H]^+$	0.02	1.4	1.9
18	4,6-Dimethyldibenzothiophene	213.3165	19.68	$[M-H]^+$	0.02	1.1	2.2
19	3,6-Dimethylphenanthrene	207.3414	20.34	$[M+H]^+$	0.03	1.5	1.9
20	Pyrene	203.3358	21.86	$[M+H]^+$	0.02	2.6	5.4
21	1-Methylpyrene	217.3231	24.14	$[M+H]^+$	0.03	1.6	1.8
22	Benzo(b)naphto[1,2-d]thiophene	235.2982	25.99	$[M+H]^+$	0.01	1.1	2.1
23	Benz(a)anthracene	229.3439	27.26	$[M+H]^+$	0.01	1.9	4.4
24	Chrysene	229.3439	27.42	$[M+H]^+$	0.01	3.2	5.7
25	Benzo(b)fluranthene	253.3352	33.14	$[M+H]^+$	0.02	3.6	6.2
26	Benzo(a)pyrene	253.3350	34.88	$[M+H]^+$	0.02	4.2	10.1
27	Perylene	253.3452	35.37	$[M+H]^+$	0.05	5.6	11.2

To evaluate the reproducibility, repeatability and accuracy, the samples were analysed five times in the same day (intra-day precision) and eleven times in five different days (interday precision) and the resulting relative standard deviation of five injections were calculated. The accuracy values vary from 1.1 to 5.6 for intra-day and from 1.9 to 11.2 for interday with relative standard deviation between 5.6 and 11.2% (n = 5), being the highest value to perylene. These parameters were used to identify the markers in the samples.

6.3. Identification of markers in samples by APGC-Q-TOF-MS

The identification was performed using the software ChromaLynx XS (SCN 714) in both targeted and non-targeted mode. For the targeted mode, a database library containing the 27 MOAH species, which were previously identified by GC-MS, was built and used for the purpose.

The accurate theoretical masses of the compounds $[M]^+$, protonated compounds $[M+H]^+$ and deprotonated $[M-H]^-$ were taken into account for building the database.

The chromatograms were processed with ChromaLynx, with a narrow window of mass (± 15 mDa) and the retention time window of ± 0.20 min, to confirm the presence or the absence of the analytes of interest. Based on these data and the comparison with those obtained with standards, the results were confirmed in the samples.

When concentrations were very low, and there were plenty of low intensity ions, the deconvolution of the mass spectrum for each compound under study helped us to confirm the identification. The results of the presence of MOAH found in the samples, after analysing four replicates of each sample, are shown in Table 3.

Five samples of rPET, one recycled cardboard and two samples of food contact packaging, couscous and semolina were analysed. Figure 3 A shows the chromatogram with the identified compounds in the rPET sample coded as TP. Figure 3 B shows the chromatogram of the recycled cardboard with the compounds identified.

22 out of the 27 selected markers were found in rPET AB and FFT samples. In rPET TP sample 17 markers were found, 15 markers were found in rPET GE sample and 14 markers in rPET IN.

PET AB and FFT samples were the most contaminated ones and in which more compounds were identified. Some of the markers, such as the alkylated aromatic 1, 2, 3-trimethylbenzene and 1,3-diethylbenzene, were common to all rPET samples. Among the MNs family, 2-methylnaphthalene was found in all rPET samples and its isomer 1-methylnaphthalene in three samples. As marker of MDBTs family, 4-methyldibenzothiophene was found in all samples except rPET IN and as marker of BNTs family, benzo(b)naphto(1,2-d)thiophene was only found in samples of rPET AB and rPET FFT. Biphenyl was found in all samples of rPET with the only exception of rPET AB sample.

2,6-dimethylnaphthalene and alkylated PAHs appeared in all rPET samples; 9,9'-dimethylfluorene was present in all rPET samples except AB; 3,6-dimethylphenanthrene appeared in all rPET samples except IN and 1-methylpyrene was found in rPET samples except TP.

4,6-dimethyldibenzothiophene considered one of the most abundant compounds in MOAH (Moustafa & Andersson, 2011) and suggested by Spack et al. (Spack et al., 2017) to identify and characterize mineral oils, was identified in all rPET samples. After carbon and hydrogen, sulfur is considered the most important element in crude oils. Thiophene joined to polycyclic aromatic hydrocarbons form sulfur heterocycles and leads to different families, including benzothiophene (BT), dibenzothiophene (DBT), benzonaphthothiophenes (BNT) and their alkylated homologous series (Asif et al., 2009; Li et al., 2012; Yang et al., 2016).

In the recycled cardboard sample only 1,2,3-trimethylbenzene, 1,3-diethylbenzene, 4-tert-butyltoluene, acenaphthene, DIPN, 3,6-dimethylphenanthrene, 1-methylpyrene and 4,6-dimethyldibenzothiophene were found. DIPN is characteristic of recycled board (Spack et al., 2017).

Table 3. Compounds identified in mineral oil and/or paperboard samples

Compounds	PET samples					Cardboard samples		
	AB	FFT	TP	GE	IN	Recycled	Cous-cous	Semolina
1,2,3-Trimethylbenzene	✓	✓	✓	✓		✓	✓	✓
1,3-Diethylbenzene	✓	✓	✓	✓	✓	✓	✓	✓
4-tert-Butyltoluene					✓	✓	✓	✓
2-Methylnaphthalene	✓	✓	✓	✓	✓			
1- Methylnaphthalene	✓	✓	✓					
Cyclohexylbenzene	✓	✓		✓				
Biphenyl		✓	✓	✓	✓			
1,3,5-Tert-butylbenzene	✓							
2,6-Dimethylnaphthalene	✓	✓	✓	✓	✓			
Acenaphthene	✓	✓	✓					
9,9'-Dimethylfluorene		✓	✓	✓	✓	✓		
Fluorene		✓	✓		✓			
2,6-Diisopropylnaphthalene	✓	✓	✓	✓	✓	✓		
3,3',5,5'-Tetramethylbiphenyl		✓		✓	✓			
Anthracene	✓	✓						
Phenanthrene	✓	✓						
4-Methyldibenzothiophene	✓	✓	✓	✓				
4,6-Dimethyldibenzothiophene	✓	✓	✓	✓	✓			
3,6-Dimethylphenanthrene	✓	✓	✓	✓		✓		
Pyrene	✓	✓	✓	✓	✓			
1-Methylpyrene	✓	✓		✓	✓	✓		
Benzo(b)naphto[1,2-d]thiophene	✓	✓						
Benz(a)anthracene	✓							
Chrysene	✓		✓	✓	✓			
Benzo(b)fluoranthene	✓							
Benzo(a)pyrene	✓	✓	✓					
Perylene	✓	✓						

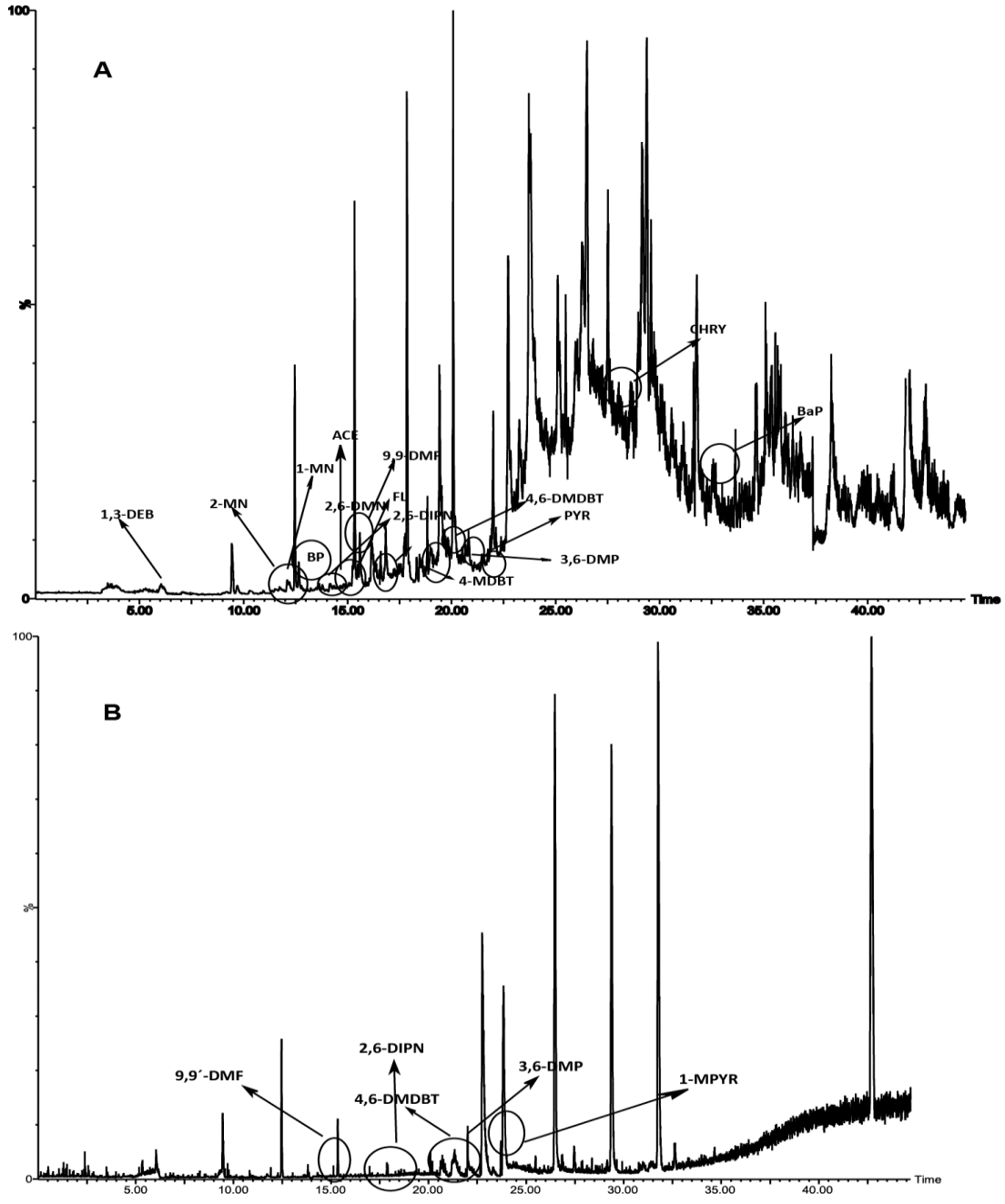


Figure 3. Chromatograms obtained by APGC-Q-TOF for rPET TP (a) and the paperboard (b) samples.

Only 1,2,3-trimethylbenzene, 1,3-diethylbenzene and 4-tert-butyltoluene were detected in food-contact packaging samples (couscous and semolina), but no other MOAH markers of those studied that could demonstrate MOAH contamination in these packaging were found. 1,3,5-tri-tert-butylbenzene was barely present in the samples under study.

The semi-quantitative estimation of the results obtained for the individual markers is in the order of 0.05 μg g^{-1} benz(a)anthracene at 0.15 μg g^{-1} 4,6-dimethyldibenzothiophene for rPET AB, 0.07 μg g^{-1} for benzo(b)naphto[1,2-d]thiophene at 0.12 μg g^{-1} 2,6-diisopropylnaphthalene for rPET FFT, 0.05 μg g^{-1} pyrene at 0.11 μg g^{-1} biphenyl for rPET TP, 0.06 μg g^{-1} chrysene at 0.09 μg g^{-1} 2,6-diisopropylnaphthalene for rPET GE, 0.06 μg g^{-1} 4,6-dimethyldibenzothiophene at 0.09 μg g^{-1} biphenyl for rPET IN and 0.06 μg g^{-1} 2,6-diisopropylnaphthalene at 0.11 μg g^{-1} 3,6-dimethylphenanthrene for recycled paperboard.

From these results it is clear that APGC-Q-TOF-MS identified without a doubt the presence of specific markers of MOAH, which demonstrates the contamination of MOAH in the samples of rPET and cardboard under study.

6.4. Confirmation of mineral oils contamination by GC-FID

All samples were treated according to the BfR & KLZH protocol and analysed by GC-FID to verify the formation of the characteristic humps of the oils and to know the distribution range of their molecular masses.

The characterisation of the molecular mass of the mineral oil was carried out, overlapping the MOSH and MOAH chromatograms obtained by GC-FID and a C7-C40 saturated alkane standard, injected under the same chromatographic conditions as the samples, at concentration 5 μg g^{-1}.

In the case of pure mineral oil samples, both MOSH and MOAH signals ranged from n-C-13 to n-C35 (see Figure 4 A). On the other hand, the rPET samples, analysed during the present investigation, showed the presence of typical chromatographic humps of MOSH and MOAH fractions of mineral oil (see Figure 4 B) with a molecular mass distribution for MOSH and MOAH between n-C14 and n-C25, which corresponds to the hydrocarbon mass range, volatile enough to migrate through the gas phase at room temperature. It should be noted that at higher temperatures, higher molecular weight hydrocarbons can also migrate. The mineral oil chromatograms of the recycled cardboard (see Figure 4 C) ranged from n-C14 to n-C28, thus indicating contamination

with heavier mineral oils. The range found matches that found in mineral oil samples (García-Cicourel, van de Velde, Roskam, & Janssen, 2020). The MOAH content in rPET ranged from 8.62 to 16.33 mg kg^{-1}, and for recycled cardboard, it was 25.12 mg kg^{-1}. The RSD of the samples was less than 10%

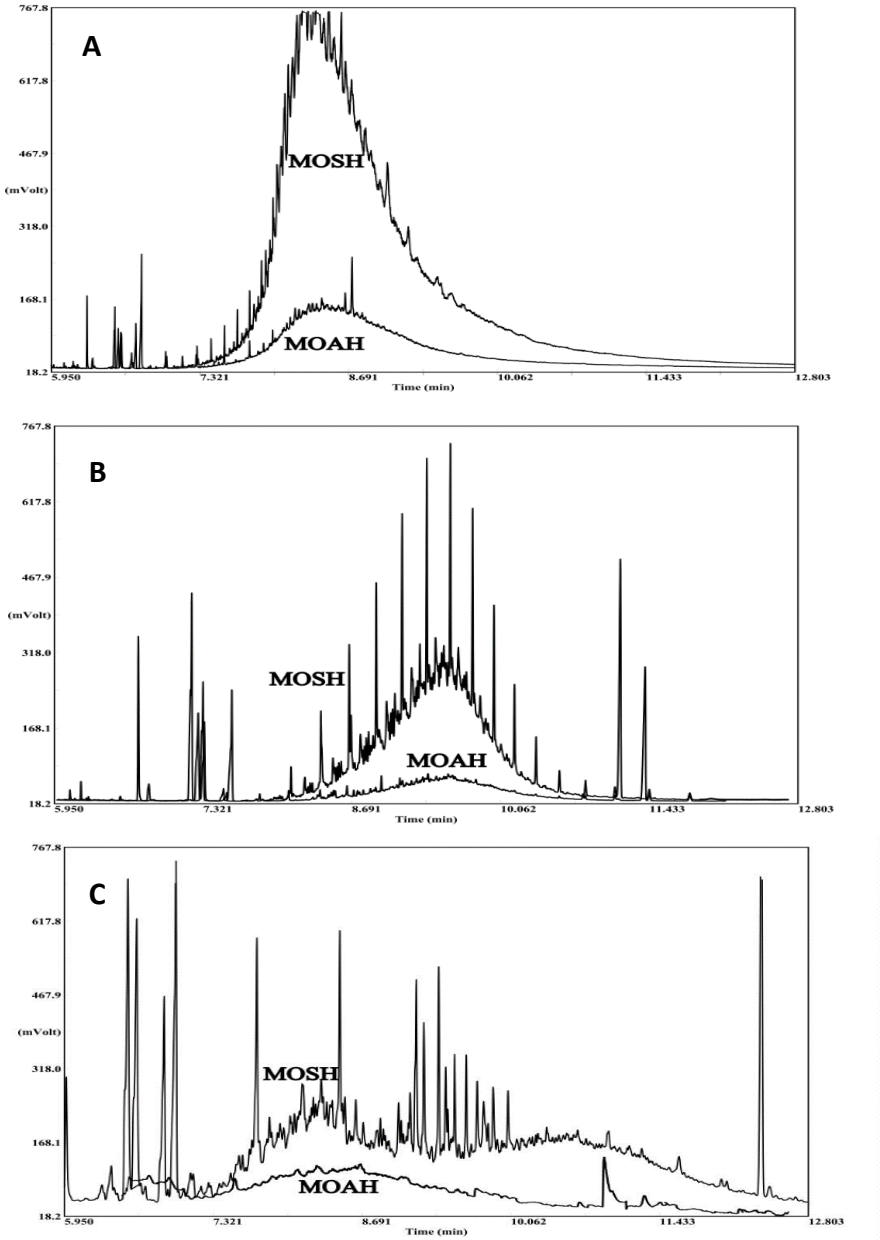


Figure 4. Chromatogram of the mineral oil 2 sample obtained by GC-FID (A), chromatogram of the PET TP sample obtained by GC-FID (B) and chromatogram of the paperboard sample obtained by GC-FID (C).

6.5. Final selection of MOAH markers

To determine without any doubt if MOAH are present in any sample, identification of MOAH markers is a useful way, as it will avoid misidentification due to the lack of knowledge of what is present under the hump.

Markers such as alkylated naphthalene, phenanthrene, dibenzothiophene, fluorene, and chrysene were previously used to determine mineral oils (Wang & Fingas, 2003). Other studies used naphthalene, 1-methylnaphthalene, 1-ethylnaphthalene, acenaphthylene, acenaphthene, 2,3,6-trimethylnaphthalene, fluorene, phenanthrene, 2-methylphenanthrene, 1-methylphenanthrene, 3,6-dimethylphenanthrene, fluoranthene, pyrene, 1-methylpyrene, anthracene, chrysene and perylene (Zrafi et al., 2013) as markers. However, most of them are also common to air pollution and the influence of mineral oils cannot be properly distinguished. Based on the results obtained in this study, a series of 16 MOAH markers can be proposed as representatives of the different families present in mineral oils. These markers are the following ones:

PAHs: biphenyl, acenaphthene, benzo(b)fluoranthene, chrysene and perylene.

Alkylated PAHs: 2,6-dimehtylnaphthalene, 9,9'-dimethylfluorene, 3,3',5,5'-tetramethylbiphenyl and 1-methylpyrene.

Methylnaphthalenes (MNs): 2-methylnaphthalene and 1-methylnaphthalene.

MDBTs: 4-methyldibenzothiophene.

DIPNs: 2,6-diisopropylnaphthalene.

DMDBTs: 4,6-dimethyldibenzothiophene.

BNTs: benzo(b)naphto(1,2-d)thiophene.

They cover different families of mineral oils and represent the most toxic compounds. Consequently, they can be considered the most important ones to detect contamination by MOAH in any sample.

7. Conclusions

This study investigated the application of two techniques, ASAP-QTOF-MS and APGC-QTOF-MS, to select the specific markers of MOAH present in food packaging material. Working with these selective techniques possible confusion with the presence of other substances is avoided and clear identification of MOAH was achieved.

The selection of markers was firstly done by direct targeted analysis of several mineral oils by ASAP-QTOF-MS, in which the exact mass of potential MOAH was used. In the six mineral oils of different origins, the presence of most of these markers was verified. 16 MOAH markers out of 27 under study were selected to be used as tracers of possible mineral oil contamination.

Using APGC-QTOF-MS, a method was developed with the best chromatographic conditions for the analysis of the markers under study. The appropriate sample treatment was optimized to verify the presence of some of them in a series of rPET samples, recycled cardboard and food packaging. This contamination was also confirmed by the GC-FID method proposed by Grob.

Detection of these markers undoubtedly determines the presence of traces of MOAH contamination in food packaging.

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Capítulo 2

Development of a rapid method for determination of aromatic hydrocarbons in food packaging by solid phase extraction and gas chromatography-mass spectrometry.

1. Resumen
2. Objetivos
3. Esquema de trabajo
4. Introduction
5. Materials and methods
6. Results and discussion
7. Conclusions
8. References

1. Resumen

En esta investigación se desarrolló un método rápido de análisis para detectar marcadores químicos de la contaminación MOAH provenientes de tintas de impresión offset a base de aceite mineral. Para ello, se utilizaron 16 hidrocarburos aromáticos como analitos target y se probaron diferentes procedimientos de extracción en fase sólida (SPE) seguido de cromatografía de gases acoplada a espectrometría de masas (GC-MS). El rango de concentración estudiado fue 0.1-7.5 μg g^{-1} con coeficientes de correlación (R^2) superiores a 0.9963, valores de RSD intradía inferiores al 5%, valores de RSD entre días inferiores al 12%, recuperaciones superiores al 80%, LOD y LOQ inferiores a 0.09 μg g^{-1}. Diez de los analitos objetivo de esta investigación se identificaron en tintas de impresión offset en concentraciones entre 2.28-8.59 μg g^{-1}. Nueve de estos compuestos también se identificaron en los envases de alimentos examinados en concentraciones entre 0.10 y 0.33 μg g^{-1}. El aceite mineral en los envases alimentarios también se analizó previamente mediante GC con detección de ionización de llama (FID).

2. Objetivos

El objetivo principal de este apartado fue el desarrollo de un método rápido de análisis para detectar hidrocarburos aromáticos como marcadores químicos de la contaminación por MOAH proveniente de tintas de impresión offset a base de aceite mineral.

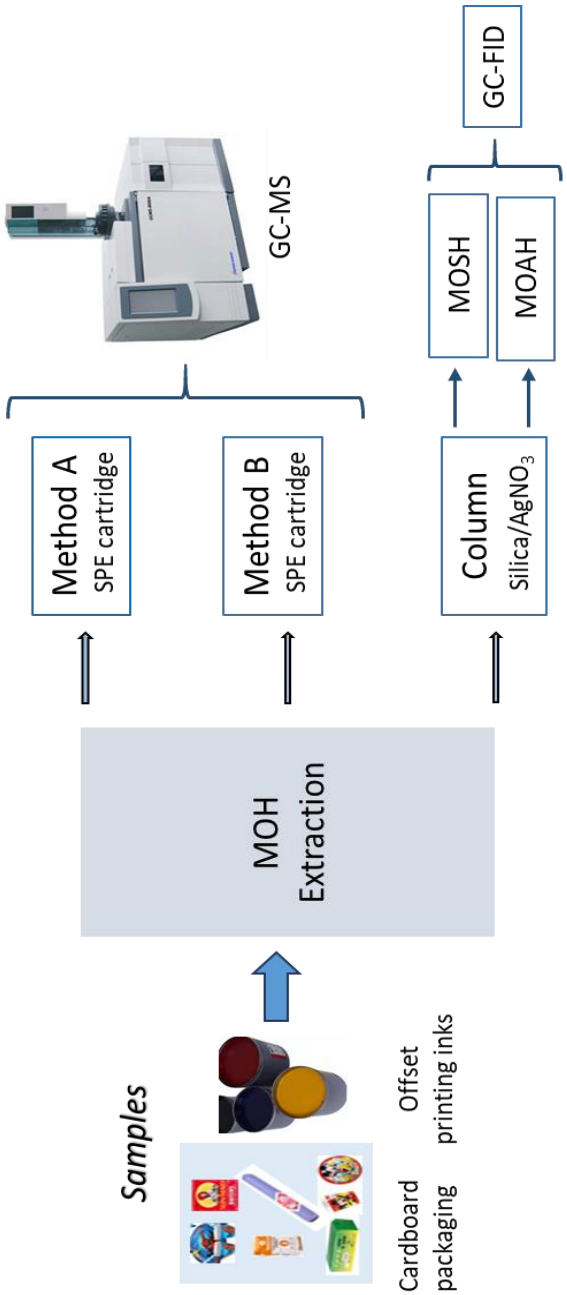
Los objetivos específicos fueron:

- Selección y optimización un método de extracción en fase sólida para el análisis de los marcadores químicos de MOAH.
- Identificación los marcadores químicos presentes en tintas de impresión offset a base de aceite mineral.
- Aplicación del método de extracción seleccionado, en matrices de envases alimentarios de cartón contaminados con MOAH proveniente de tintas de impresión.

Para alcanzar estos objetivos se realizaron las siguientes tareas:

- Aplicación de técnicas de preparación de muestras.
- Se probaron dos métodos SPE y se optimizó el método seleccionado.
- Identificación y cuantificación de los marcadores MOAH en tintas de impresión offset.
- Determinación de los marcadores MOAH en envases alimentarios de cartón.
- Separación de la fracción MOAH mediante extracción en fase sólida con sílice activada impregnada de $AgNO_3$.
- Análisis y caracterización de la fracción MOAH por el método convencional en su versión manual (SPE-GC-FID).

3. Esquema de trabajo



Esquema 2. Diseño experimental del Capítulo 2.

4. Introduction

Recently, contamination by mineral oil aromatic hydrocarbons (MOAH) has become a public interest due to their harmful effects on human health. Scientific studies have shown that MOAHs act as endocrine disruptors (Tarnow et al., 2016) and that MOAHs with three or more rings can be mutagenic and/or genotoxic carcinogens (EFSA, 2012). Given this situation, the European Food Safety Authority (EFSA) suggested that food must be free of MOAH (EFSA, 2012), and the European Commission published a recommendation (EU) 2017/84, indicating the necessity to monitor the presence of mineral oil hydrocarbons (MOHs), in general, in food and food contact materials (European Commission, 2017).

Food contamination from MOHs might come from different sources, such as lubricating oil used in agricultural and industrial food processing machinery, food additives, processing aids, and direct or indirect contact from food packaging (EFSA, 2012). Among food packaging, paper and board tend to be the most contaminated by MOHs, probably because they are often covered both with a layer of printing ink for decorative purposes or made from recycled fibres (Pack et al., 2020; Vollmer et al., 2011)

From the chemical point of view, MOAHs are formed by aromatic and polyaromatic compounds (PAHs), mostly branched. Together with saturated hydrocarbons (MOSH) belongs to the MOHs class of compounds. Therefore, MOAH needs to be separated from MOSH to be analysed (EFSA, 2012).

A commonly used method for analysing MOH in food matrices or food contact materials is on-line liquid chromatography with gas chromatography coupled to a flame ionisation detector (LC-GC-FID) (AENOR, 2018; Bratinova & Hoeskstra, 2019; Weber et al., 2018). However, the off-line separation by solid-phase extraction (SPE) of MOSH and MOAH before the chromatographic injection is an alternative option for laboratories that do not have instrumentation (Fiselier et al., 2013; Moret et al., 2011).

Despite the many achievements made in the study of mineral oils, their chemical analysis continues to be a huge analytical challenge. The chemical complexity of these substances has made the chromatographic separation of their individual components very difficult. Besides, both MOSH and MOAH fractions show humps composed of a broad series of unresolved peaks (Biedermann & Grob, 2015; Gharbi et al., 2017) that could mask compounds of different nature, which could lead to false quantitative analysis, since it is impossible to distinguish between analytes (Bratinova & Hoeskstra, 2019; Koster et al., 2020). In a recent study, Koster et al. indicated that when MOH contamination is low, current methodologies cannot reliably conclude whether or not the sample is contaminated (Koster et al., 2020). Therefore, it is necessary to apply additional confirmatory techniques, such as nuclear magnetic resonance (NMR) spectroscopy, two-dimensional gas chromatography (GCxGC), mass spectrometry, and the identification of chemical markers (Bratinova & Hoeskstra, 2019; Lachenmeier et al., 2017; Spack et al., 2017)

Chemical markers have been proposed as indicators of MOH contamination. The use of diisopropylnaphthalene as an indicator of contamination from recycled paper or cardboard is common (Biedermann & Grob, 2015; Moret et al., 2013). Bratinova et al. recommend the use of mass spectrometry to characterise MOH when interference is suspected, as well as the use of indicative MOSH/MOAH compounds (Bratinova & Hoeskstra, 2019). Spack et al. concluded that identifying a more significant number of marker substances would improve the capacity of mass spectrometry as a confirmatory technique (Spack et al., 2017). Recently, Jaén et al. found 27 substances in different mineral oil samples that could be used as markers of MOAH contamination (Jaén et al., 2021).

Therefore, the objective of this study is to develop a rapid SPE-GC-MS method to determine aromatic hydrocarbons that could be used as MOAH markers in printing inks. For this, the following steps were carried out: i) To optimize and validate different SPE procedures in combination with GC-MS to analyse the compounds proposed as chemical markers of MOAH; ii) To identify and quantify these markers in mineral oil-

based offset printing inks. iii) To apply the method to food packaging samples contaminated with MOAH.

5. Materials and methods

5.1. Reagents, materials and working solutions

Paraffin oil., Ph. Eur., BP, viscous liquid, silica gel high-purity grade (pore size 60 Å, 70-230 mesh) for chromatographic columns, silver nitrate on silica gel (~10 wt.% loading, 230 mesh), SPE cartridges Supelclean sulfoxide, Supelclean EZ-POP NP, 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2-MN), biphenyl (BP), 2,6-dimethylnaphthalene (2,6-DMN), acenaphthene (ACE), 2,6-diisopropylnaphthalene (2,6-DIPN), 3,3',5,5'-tetramethylbiphenyl (3,3',5,5'-TMBP), 4-methyldibenzothiophene (4-MDBT), 4,6-dimethyldibenzothiophene (4,6-DMDBT), 1-methylpyrene (1-MPYR), benzo(b)naphtha(1,2-d)thiophene (BNT), chrysene (CHRY), benzo(b)fluoranthene (BbF), perylene (PER), undecane (n-C11), n-tridecane (n-C13), bicyclohexyl (Cycy), 5α-cholestane (Cho), pentylbenzene (5B), 1,3,5-tri-tert-butylbenzene (TBB) were purchased from Sigma-Aldrich (Madrid, Spain). While, 3,6-dimethylphenanthrene (3,6-DMP) was from Dr. Ehrenstorfer (Augsburg, Germany) and 9,9'-dimethylfluorene (9,9'-DMF) was from Tokyo Chemical Industry CO., LTD.

Silanized glass wool was acquired from Supelco (Bellefonte, USA). Anhydrous sodium sulphate extra pure Ph. Eur. and solvents of HPLC grade such as toluene, n-hexane, methanol, ethanol absolute, isopropanol, methylene chloride (DCM), acetone and acetonitrile (ACN) were from Scharlab SL (Barcelona, Spain).

MOSH/MOAH kit provided by European Union Reference Laboratory for Food Contact Materials (EURL-FCM) to help in the development and validation of the analytical methods for the determination of MOH in food and FCM (Food contact materials). The test items used were: a C10-C50 mixture of n-alkanes in n-hexane (QC05), Engine oil + Gravex in n-hexane (QC06) prepared at JRC and recycled

cardboard (QC08). Each test item was accompanied by MOSH/MOAH chromatograms and their respective indicative values.

The vacuum manifold system was purchased from Waters Corporation (Massachusetts, USA). The "Vibromatic" mechanical laboratory shaker was obtained from J.P Selecta (Spain), and the Ultramatic GR system for the purification of ultrapure water type I (reactive grade) was obtained from Wasserlab (Spain).

Individual standard solutions of all compounds were prepared at 6000 μg g^{-1} in toluene, except PER and 2,6-DIPN, PER was prepared at 1200 μg g^{-1} in toluene, and 2,6-DIPN was dissolved in n-hexane. Two mixed stock solutions were prepared through appropriate dilutions of the individual standards. Solution A was prepared by dilution in n-hexane of the following aromatic hydrocarbons: 2-MN, 1-MN, BP, 2,6-DMN, 2,6-DIPN, ACE, 3.3',5.5'-TMBP, 4-MDBT, 4,6-DMDBT, 1-MPYR, 3,6-DMP, 9,9-DMF, BNT, CHRY, BbF and PER, up to 100 μg g^{-1}. The second standard mixture (solution B) contained aromatic and saturated compounds with the following concentrations: n-C13, Cycy, Cho, 5B, TBB, 1-MN, 2-MN at 350 μg g^{-1}, n-C11 at 175 μg g^{-1} and PER at 650 μg g^{-1}; this solution was prepared by dilution in toluene and used to verify the separation of MOSH and MOAH. All solutions were stored at -4 ° C, and working solutions were prepared daily.

5.2. Selection of MOAH markers

The substances used as target analytes in this study have been proposed in a previous study as MOAH markers (Jaén et al.,2021). Furthermore, these substances have been identified by different authors in mineral and crude oil samples. A total of sixteen aromatic hydrocarbons were selected: three heterocyclic aromatic sulfur compounds (4-MDBT, 4, 6-DMBT, and BNT), commonly used to track crude oil contamination (Fang et al., 2016, 2017; Yang et al., 2016) and which have been also detected in mineral oils by two-dimensional gas chromatography GCxGC-MS (Biedermann & Grob, 2009, 2015); a diisopropylnaphthalene (2,6-DIPN) to confirm contamination from recycled cardboard (Biedermann & Grob, 2015; Zhang, Noonan &

Begley, 2008) a biphenyl (BP) and an alkylbiphenyl (3.3',5.5'-TMBP) identified in petroleum products (Bundt et al., 1991; Paschke et al., 1992) and used as crude oil indicators (Trolio et al., 1999; Ogbesejana, Oluwasesan & Ali, 2017); four unbranched PAHs (ACE, CHRY, BbF and PER) and six branched PAHs (1-MN, 2-MN, 2,6-DMN, 3,6-DMP, 1-MPYR and 9,9'-DMF), belonging to the homologous series of naphthalene, phenanthrene, anthracene, fluorene, chrysene and pyrene which were previously identified in mineral oils (Biedermann & Grob, 2015; Bartsch et al., 2017; Koch et al., 2020). Another important criterion in the selection of these compounds was the commercial availability of pure reference standards.

5.3. Samples

This study used two different types of mineral oil-based offset printing inks and nineteen virgin cardboard food packaging. The evaluated cardboard containers were: three decorated cardboard plates, two white cardboard plates, four decorated cardboard cups, three boxes for pasta, two boxes for raisins, two paper packaging for wheat flour, and three boxes of tea. All samples were purchased from the local market and analysed in triplicate.

The glassware was sequentially rinsed with methanol, acetone, and n-hexane, then dried in an oven at 100 °C and covered with aluminium foil to avoid contamination of the samples. The cardboard samples were also protected with aluminium foil, and blank samples were analysed throughout the process.

5.4. Instrumental analysis

GC-MS analysis

An Agilent 6890N gas chromatograph equipped with a Combi PAL automatic sampler (CTC Analytics, Zwingen, Switzerland), coupled to a quadrupole mass spectrometry detector (5975, Agilent) was used for the analysis. The temperature of the injector was 250 °C. The injection volume was 1 μL, and the splitless mode was used. The separation of the analytes was performed with a DB-5 column (30 m x 0.25 mm ID,

0.25 μm internal thickness) supplied by Agilent Technologies. The oven temperature was initially set at 50 °C for 1 min, then raised at the rate of 10 °C min^{-1} to 300 °C, and maintained at this temperature for 10 min. The total analysis time was 36 min. The solvent delay was 8 min. The carrier gas was helium (99.999%) at a constant flow rate of 1 mL min^{-1}. Ionisation was performed by electronic impact (EI) at 70 eV. The transfer line and ion source temperatures were 280 ºC and 250 ºC, respectively.

The acquisition was performed in full scan mode (m/z range 45-400) and the selective ion monitoring (SIM) mode for quantitative analysis. SCAN mode was used to select the monitored ions and confirm the retention times of the standards. The monitored ions (Table 1) were selected based on their relative abundance in the SCAN mass spectrum. The data were acquired and processed using the MSD ChemStation data analysis software (version F.01.00.1903, Agilent Technologies).

GC-FID analysis

GC-FID analysis was performed in a Trace GC Ultra chromatograph (Thermo Electron Corporation, Milan, Italy) with an HP-5 analytical column (60 m x 0.25 mm ID, 0.25 μm film thickness) from Agilent Technologies and a flame ionisation detector (FID). A volume of 5 μL of the sample were injected using splitless mode with an AS 300 autosampler, and the injector temperature was 250 °C. The oven temperature was programmed as follows: start at 50 °C for 2 min, then increased at 30 °C min^{-1} to 310 °C and held for 15 min. The total run was 26 min. The flow rate of the carrier gas (helium, 99.999%) was 2 mL min^{-1}. The FID detector temperature was 350 °C. The Chrom-Card GC (Thermo Electron) software was used to analyse the data.

5.5. Sample preparation

The extraction of mineral oil and target analytes from offset printing inks was carried out with n-hexane. In a glass vial 0.050 g of offset ink were weighed and 5 mL of n-hexane was added; then, the sample was placed in an ultrasonic bath with constant

stirring for 1h. The supernatant was then decanted into a clean vial and concentrated under a gentle stream of nitrogen gas to 1 mL.

In the case of cardboard containers intended for food contact, mineral oil extraction was carried out following the conventional methodology reported in the literature by several authors (Barp et al., 203; BfR & KLZH, 2012; Lorenzini et al., 2010; Vollmer et al., 2011) with slight changes. The cardboard was cut into small pieces, approximately 0.5 cm on each side, excluding the parts containing adhesives. Next, 2 g of the sample were placed in a 20 mL glass vial, and 15 mL of an n-hexane/ethanol (1:1) mixture were added. The vial was shaken for two hours by sonication at room temperature. Subsequently, the solvent mixture was decanted, and 5 mL of water were added. The supernatant layer of n-hexane was separated and transferred to another vial where it was concentrated to 1 mL under a gentle stream of nitrogen at 40 °C. It is important to note that 10 µL of solution B were added to the cardboard replicas intended for MOAH analysis by GC-FID.

5.6. MOAH fraction analysis

Manual separation of MOSH and MOAH was performed using a mixture of silver nitrate-coated silica gel, conventionally used to separate aromatic and aliphatic hydrocarbons from crude oils and derivatives (Moret et al., 2011; BfR & KLZH, 2012; Bennett & Larter, 2000). This mixture was prepared by mixing 33 g of high purity silica gel (previously activated in a muffle furnace at 400 °C for 24 h) with 1 g of silver nitrate mixed in silica gel (~ 10% by weight), and it was homogenised for 12 h on a mechanical laboratory shaker. 3 g of this mixture were placed in a glass column (160 mm x 0.8 mm ID) which contained a piece of glass wool at the bottom and then 1 g of sulphate of anhydrous sodium on top of the silica layer. The column was covered with aluminium foil to prevent oxidation of the stationary phase.

The MOSH and MOAH fractions were separated following the workflow recommended by Federal Institute for Risk Assessment (BfR) and Kantonales Labor Zurich (KLZH) (BfR & KLZH, 2012). After separation, both fractions were

concentrated to 0.4 mL under a gentle stream of nitrogen at 40 °C. The analysis was carried out by GC-FID.

Quantitative analysis was performed using the internal standard method. Cycy was used to quantify MOSH fraction, and TBB was used to quantify the MOAH fraction. The area of each fraction was determined by integrating the entire hump and subtracting the sharp peaks found in the hump MOAH as described in the Joint Research Center (JRC) publication (Bratinova & Hoeskstra, 2019) and repeated injections of solvent blanks obtained the baseline. The limit of quantification of the method was calculated using the signal to noise ratio (S/N) generated by direct injection of a sample of pure mineral oil at low concentrations and taking into account the amount of sample analysed and the enrichment steps during sample treatment. The limit was lower than 2.5 mg kg^{-1}.

The efficiency of the analytical methodology for analysing the MOSH and MOAH fraction was verified with the MOSH/MOAH kit provided by FCM-EURL. The test items of the kit were analysed and compared with the indicative values of the kit.

5.7. Analysis of MOAH markers

Two different SPE adsorbents were evaluated: A) SPE with two layers of adsorbents (florisil and Z-Sep/C18 mixture) and B) SPE with silica attached to a sulfoxide group.

In method A, double-layer SPE cartridges containing florisil (upper layer) and a mixture of Z-sep/C18 (lower layer) were used, and the method described by the manufacturer was optimized to work with samples of mineral oil. The composition and volume of the eluent were the parameters evaluated to optimize the method. The solvent combinations tested were: C1) 15 mL of ACN alone; C2) 14 mL of ACN followed by 1 mL of DCM; C3) 14 mL of ACN followed by 1 mL of isopropanol; C4) 13 mL of ACN and 2 mL of DCM; C5) 13 mL of ACN and 2 mL of isopropanol. Actual samples of mineral oils spiked with a standard 5 µg g^{-1} solution of the target analytes were used in all optimization experiments.

The final protocol consisted of conditioning the cartridge with 10 mL of acetone, then vacuum-dried at -15 mmHg for 10 minutes at room temperature. Subsequently, 0.4 mL of sample was added to the cartridge, and penetration through the solid phase was allowed to complete. The target compounds were eluted with 13 mL of ACN, followed by 2 mL of isopropanol. The eluate was collected and concentrated under a gentle stream of nitrogen at 40 °C until 0.5 mL was obtained. These concentrated extracts were directly analysed by GC-MS. The extraction was carried out in a vacuum collecting system from Waters Corporation (Massachusetts, United States). Figure 1 shows an outline of the optimized protocol.

Once the above factors were optimized, the behaviour of the aromatic compounds proposed in this research as MOAH markers was studied. For this purpose, a mineral oil sample free of MOAH (paraffin oil) was enriched with 50 μg g^{-1} of solution A and loaded into the cartridge. The eluate was sequentially collected in 1 mL fractions and analysed by GC-MS.

In method B, an SPE cartridge that contained a stationary phase of silica modified with a sulfoxide group was used. During the experiments, the protocol consisted of conditioning the cartridge with 10 mL of acetone and subsequently equilibrating with 20 mL of n-hexane. Then 1 mL of the mineral oil sample was loaded onto the surface of the modified silica; afterwards, 5 mL of n-hexane were added to collect the first fraction, and the second fraction was collected with 13 mL of n-hexane. Both fractions were concentrated to 0.4 mL under a gentle stream of nitrogen at 40 °C and analysed by GC-MS. The efficiency of the stationary phase was studied by loading onto the cartridge a sample of mineral oil spiked with 5 μg g^{-1} of the target analytes. In addition, different combinations of n-hexane with other solvents such as DCM and isopropanol were tested. Figure 1 shows a summary of the described procedure.

The possibility of evaporation losses of the target analytes during the concentration step of the samples under nitrogen current was also studied, analysing standard solutions of 5 μg g^{-1} of the aromatic compound mixture before and after concentration.

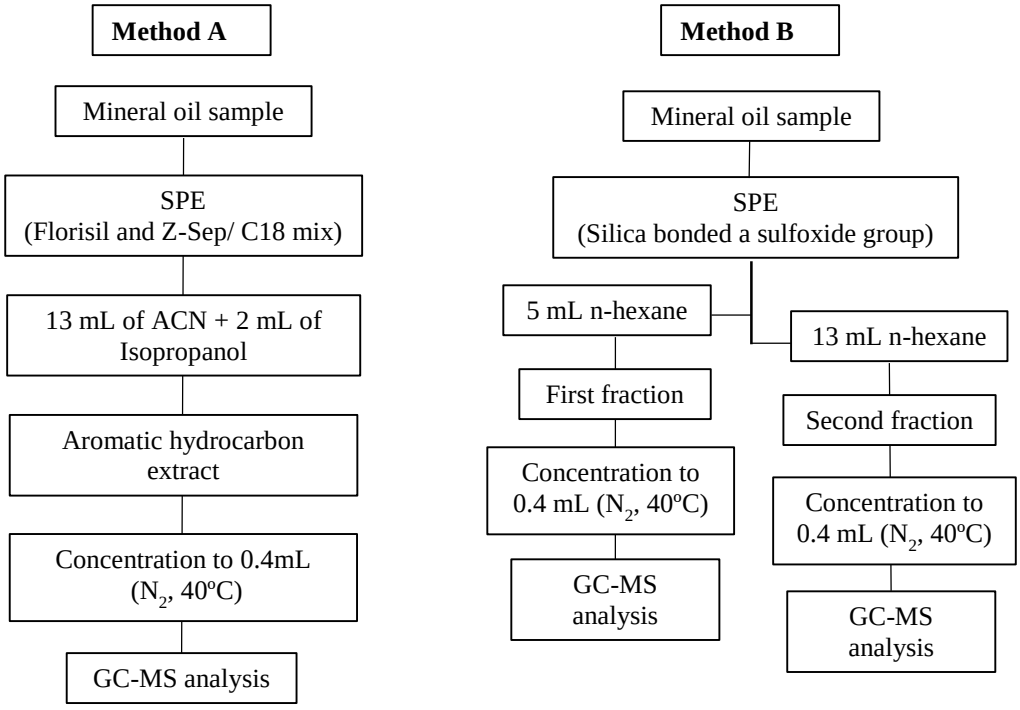


Figure 1. Workflow diagram of the SPE methods

5.7.1. Analytical parameters

The validation of the analytical method for qualitative and quantitative analysis by GC-MS of the target analytes was performed by determining the linearity, linear range, recovery, precision, robustness, detection limits (LOD), and quantification limits (LOQ). Linearity and linear range were determined through external standard calibration using seven solutions prepared in a concentration range from 0.1 to 7.5 μg g^{-1}. Recovery experiments were carried out by fortifying blank samples (paraffin oil) with two different concentrations (1 and 5 μg g^{-1}) of solution A. The spiked samples were analysed following the procedures described in section 5.7. Intra-day precision was evaluated by analysing spiked samples during the same day and under the same working conditions. Inter-day precision was done by analysing fortified samples on three different days. Precision was expressed as a relative standard deviation (RSD). The S/N (signal to noise) was used to determine LOD and LOQ. The S/N value was calculated

at very low and known concentrations of the analytes, close to the noise. LOD was established as the minimum analyte concentration detected at three times S/N and LOQ at ten times S/N. Also, the method quantification limit was estimated considering the amount of sample and the pre-concentration and dilution factors applied in each case.

All samples were done in triplicate. The results are expressed as the mean ± standard deviation of three independent measurements (n = 3) for all experiments.

Table 1. List of aromatic hydrocarbons selected as target analytes with their monitored ions and retention times.

Compound	CAS	Molecular formula	MM (Da)	RT	Monitored ions (m/z)
2-methylnaphthalene	91–57-6	$C_{11}H_{10}$	142.197	10.81	142.1, 115.1
1-methylnaphthalene	90–12-0	$C_{11}H_{10}$	142.197	11.05	142.1, 115.1
Biphenyl	92-52-4	$C_{12}H_{10}$	154.208	11.97	154.1, 76.1
2,6-dimethylnaphtalene	581-42-0	$C_{12}H_{12}$	156.224	12.32	156.1, 141.1
Acenaphtene	83-32-9	$C_{12}H_{10}$	154.208	13.37	153.1, 76.0
9,9'-dimethylfluorene	4569-45-3	$C_{15}H_{14}$	194.272	14.55	179.1, 194.1
2,6-diisopropylnaphtalene	24157-81-1	$C_{16}H_{20}$	212.330	16.16	197.1, 212.2
3,3',5,5'-tetramethylbiphenyl	25570-02-9	$C_{16}H_{18}$	210.314	16.80	210.2, 195.1
4-methyldibenzotiophene	7372-88-5	$C_{13}H_{10}S$	198.283	17.59	198.1, 165.1
4,6-dimethyldibenzotiophene	1207-12-1	$C_{14}H_{12}S$	212.310	18.60	212.1, 105.1
3,6-dimethylphenanthrene	1576-67-6	$C_{16}H_{14}$	206.282	19.10	206.1, 191.1
1-methylpyrene	2381-21-7	$C_{17}H_{12}$	216.277	21.54	216.1, 94.5
Benzo(b)naphtho(2,1-d)tiophene	205-43-6	$C_{16}H_{10}S$	234.316	22.49	234.1, 117.0
Chrysene	218-01-9	$C_{18}H_{12}$	228.288	23.16	228.1, 114.0
Benzo(b)fluoranthene	205-99-2	$C_{20}H_{12}$	252.309	25.49	252.1, 126.0
Perylene	198-55-0	$C_{20}H_{12}$	252.309	26.30	252.1, 126.0

6. Results and discussion

6.1. MOAH fraction analysis

The efficiency of the methodology used for the analysis of MOAH was verified by the analysis of two test elements of the MOSH/MOAH kit. Figure 2 shows the MOSH and MOAH chromatograms of test item QC06, confirming the presence of internal standards in the appropriate MOH fraction. Table 2 shows the MOSH/MOAH values obtained after analysing the QC06 and QC08 test elements, the indicative value of the kit, and the error percentage. The relative error was below 10% for all cases.

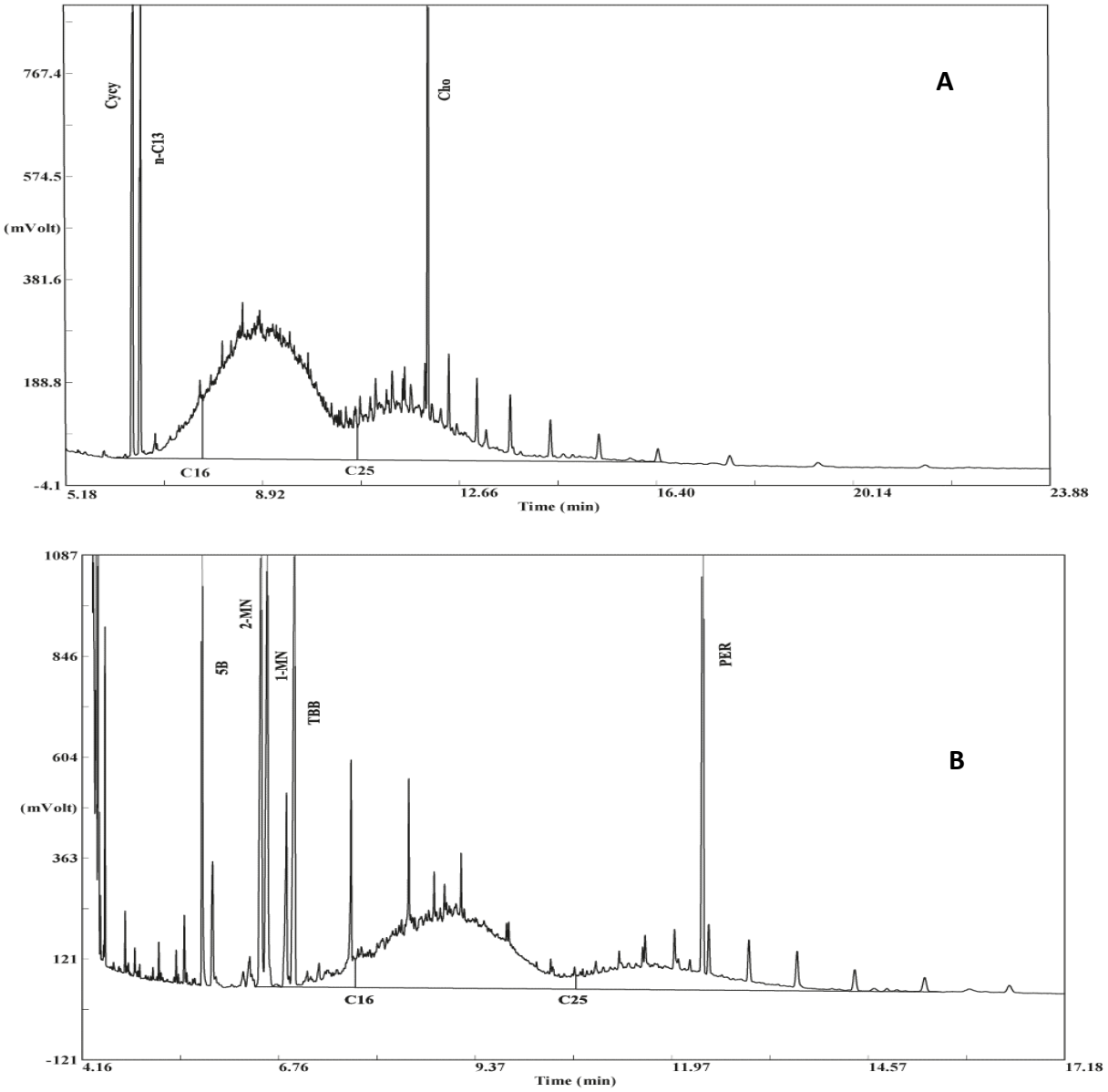


Figure 2. MOSH (A) and MOAH (B) chromatograms of test item QC06.

Figure 3 shows the MOSH/MOAH chromatograms of samples CFPS02 and CFPS04. The MOSH chromatograms were characterised by a large narrow hump of unresolved peaks corresponding to a complex mixture of hydrocarbons; that probably in this case, in addition to MOSH, contained polyolefinic oligomeric saturated hydrocarbons (POSH) derived from the polyolefin film that covered the decorated surface of the contaminated samples (Pack et al., 2020; Dima, Verzera & Grob, 2011), and that elute together with MOSH, forming humps in the same chromatographic region (Biedermann-Brem et al., 2012). In comparison, the MOAH fraction generated a smaller hump.

The GC-FID analysis of the cardboard revealed mineral oils in nine samples, but only in five of them was MOAH detected. The samples contaminated with MOAH were: two decorated cardboard plates (CFPS01 and CFPS02), two decorated cardboard cups (CFPS03 and CFPS04) and a tea box (CFPS05) with coloured prints on the outside.

Table 2. MOSH/MOAH values obtained after analysing the QC06 and QC08 test elements of the MOSH/MOAH Kit for the development of methods for mineral oil provided by EURL-FCM. Indicative value and percentage of error are also provided.

Sample	Mass fraction	Indicative value (mg kg^{-1})	Experimental value (mg kg^{-1} ± SD)	Relative error (%)
QC06	MOSH sum	1156	1110 ± 10	-4.01%
	MOAH sum	360	383 ± 14	6.32%
QC08	MOSH (16-25)	296	322.9 ± 5.6	9.10
	MOSH (25-35)	223	216.4 ± 8.8	-2.96
	MOAH (16-25)	78	81.2 ± 3.8	4.07
	MOAH (25-35)	46.5	41.1 ± 2.87	-9.50

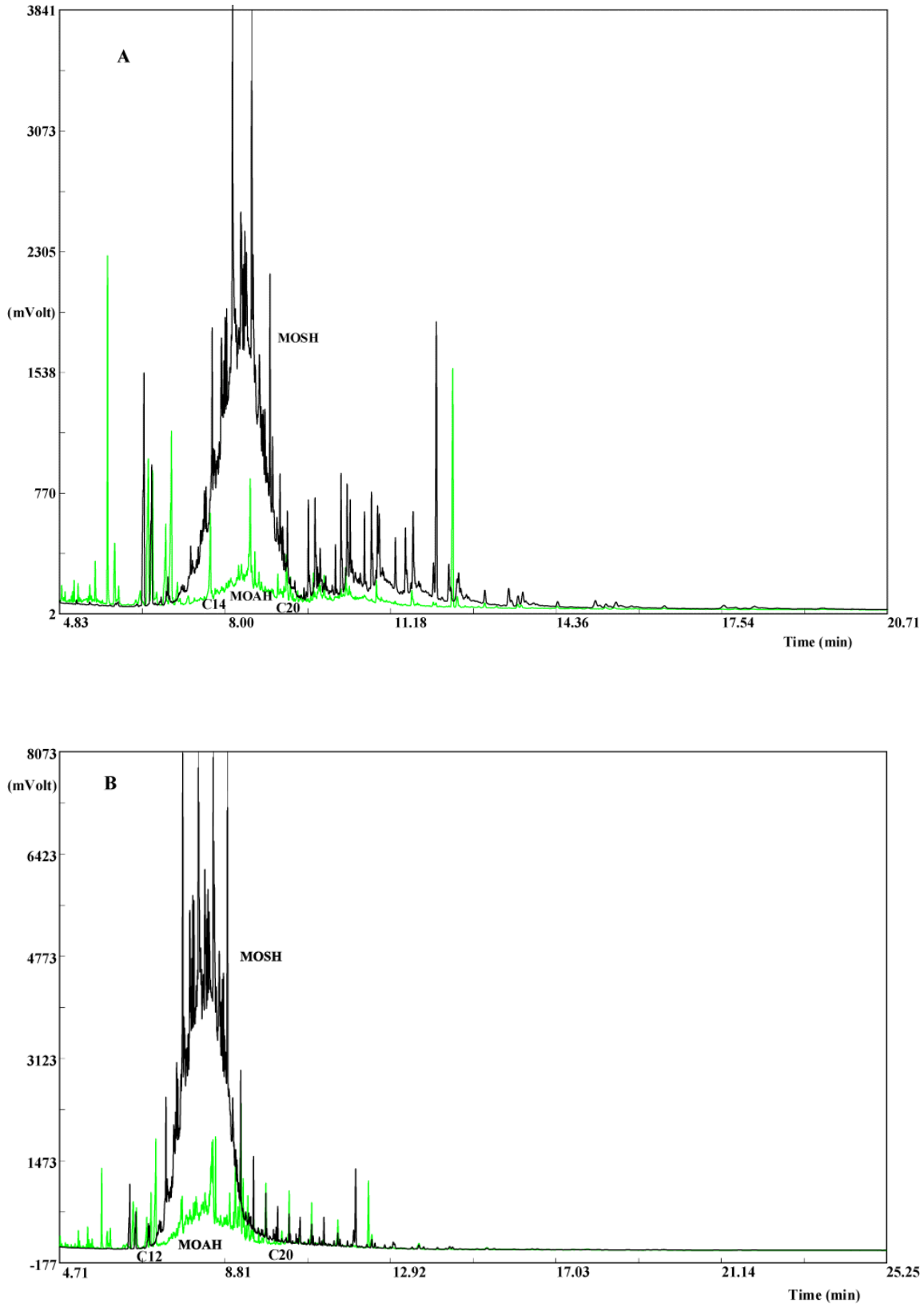


Figure 3. MOSH/MOAH chromatograms of CFPS04 (A) and CFPS02 (B) cardboard food packaging samples.

The MOAH content found in food cardboard packaging ranged from 8.4 to 32.9 mg kg^{-1} (Table 3). The decorated cardboard cup (CFPS03) had the highest concentration, and the lowest concentration was found in the tea box (CFPS05). MOAH concentrations represent between 5 and 9% of the total mineral oil concentration in the samples, while the MOSH content was higher than 90% in all samples with concentrations between 85.9 and 332.6 mg kg^{-1}.

The hydrocarbon distribution in the samples was in the typical range of mineral oils for printing inks (Dima, Verzera & Grob, 2011; Lorenzini et al., 2010; Vollmer et al., 2011). MOAH was detected in the decorated plates from n-C12 to n-C20 centred on n-C17, in the case of decorated cups between n-C11 to n-C20 centred on n-C18 and for the tea box from n-C14 at n-C21 also centred on n-C18. As expected, the protrusions detected were found in the range of printing inks; and typical recycled cardboard chromatograms, with extensive humps, were not observed as a result of contamination of the cardboard by various types of mineral oils (Dima, Verzera & Grob, 2011; Lorenzini et al., 2010). It confirms the fact that mineral oil comes from the printing inks used to decorate cardboard food packaging.

Table 3. MOSH/MOAH concentrations (mg kg^{-1}) in cardboard food packages. The analysis of MOSH and MOAH was carried out by GC-FID.

Samples	Concentration (mean ± SD)	
	MOSH (mg kg^{-1})	MOAH (mg kg^{-1})
CFPS01	173.8± 1.3	9.7 ± 0.6
CFPS02	231.0 ± 1.8	14.2 ± 1.8
CFPS03	332.6 ± 1.2	32.9 ± 0.9
CFPS04	301.8 ± 2.1	23.3 ± 0.63
CFPS05	85.9 ± 2.0	8.4 ± 0.2

6.2. Analysis of MOAH markers

6.2.1. SPE selection

Method A demonstrated high efficiency in terms of the extraction of target analytes. In addition to the good recoveries (Table 4), the cartridge had a high adsorption capacity, which allowed retaining matrix interferences (saturated hydrocarbons). The duality of the stationary phase makes it possible that multiple chemical interactions occur with the different components of the sample. While the florisil layer retains polar interferences that may be present in the matrix, the Z-sep/C18 layer is capable of retaining saturated hydrocarbons from a sample of mineral oil through non-polar interactions with the C18 particles.

In contrast, method B was unsuitable for the treatment of mineral oil samples. The cartridge protocol provided by the manufacturer claimed that interferences eluted with the first 5 mL of n-hexane and that the compounds containing benzene rings can be eluted subsequently with another 13 mL of n-hexane. It was assumed that target analytes would be retained in the stationary phase due to the interactions between the electrophilic sulphur atom and the π-electrons of the aromatic compounds, eluting in the second fraction. Unfortunately, in the first fraction, aromatic compounds with few π-electrons eluted with interference from the matrix. On the other hand, in the second fraction, in addition to aromatic compounds with a greater number of π-electrons, matrix interferences were still detected, while heavier compounds, such as BbF and PER, were strongly retained in the column and were never eluted. Other solvents such as DCM and isopropanol were tested, but recoveries remained low, and the compounds of interest were not collected in a single fraction, and matrix interferences were not separated from the analytes.

Based on the results obtained, method A was considered the most suitable for treating mineral oil samples and extracting the target analytes.

Table 4. Analytical parameters of method A.

Compound	R^2	Slope	LOD ($\mu g\ g^{-1}$)	LOQ ($\mu g\ g^{-1}$)	Spiked concentration (1.0 $\mu g\ g^{-1}$)			Spiked concentration (5.0 $\mu g\ g^{-1}$)		
					Recovery (%)	RSD (%) Intraday (n = 5)	RSD (%) Interday (n = 3)	Recovery (%)	RSD (%) Intraday (n = 5)	RSD (%) Interday (n = 3)
2-MN	0.9964	1.35E+05	0.008	0.056	82	1.4	9.4	86	1.2	7.0
1-MN	0.9989	1.26E+05	0.016	0.056	81	1.4	5.9	85	1.6	6.1
BP	0.9980	1.73E+05	0.016	0.064	98	2.0	7.4	99	3.0	7.0
2,6-DMN	0.9975	1.50E+05	0.016	0.056	104	2.9	11.7	98	2.1	7.5
ACE	0.9991	1.53E+05	0.008	0.032	94	1.8	7.9	92	1.6	6.5
9,9'-DMF	0.9996	2.38E+05	0.016	0.040	94	3.0	9.5	93	2.8	6.4
2,6-DIPN	0.9989	1.79E+05	0.008	0.016	95	3.2	8.3	89	1.3	4.5
3,3',5,5'-TMBP	0.9993	2.02E+05	0.008	0.040	91	3.2	7.5	89	2.0	5.9
4-MDBT	0.9989	1.69E+05	0.008	0.016	94	1.0	8.7	95	2.4	6.5
4,6-DMDB	0.9995	1.76E+05	0.008	0.024	88	2.8	6.7	92	2.0	6.2
3,6-DMP	0.9993	1.67E+05	0.016	0.048	90	3.3	7.4	95	4.4	5.7
1-MPYR	0.9988	2.00E+05	0.008	0.016	84	4.3	6.0	90	3.9	3.3
BNT	0.9979	2.06E+05	0.008	0.024	90	3.8	6.7	95	3.4	7.0
CHRY	0.9974	1.87E+05	0.024	0.072	90	2.4	7.7	97	5.2	6.6
BbF	0.9975	1.74E+05	0.008	0.034	88	3.7	9.8	93	3.6	2.1
PER	0.9965	1.63E+05	0.024	0.080	81	4.4	4.8	89	3.0	2.6

6.2.2. Optimization of the method A

Figure 4 shows the recovery results of the target analytes related to the volume and eluent composition. The first option evaluated was the use of ACN as the only eluent because ACN is a solvent of intermediate polarity with little affinity for the oily matrix, which allows the elution of aromatic compounds without dragging the interferences coming from the matrix. However, as shown in Figure 4, ACN alone (C1) obtained low analytes recoveries, while a moderate decrease in eluent polarity significantly improved the recovery percentage (C2-C5 combinations). The affinity of aromatic compounds for less polar solvents such as DCM and isopropanol advantaged their extraction; nevertheless, an excess of DCM (combination C4) caused the extraction of the matrix from the sample, with the formation of tiny drops of oil that prevented obtaining a clean enough extract to be directly analysed by gas chromatography. For this reason, a small aliquot of the supernatant liquid was taken for chromatographic analysis, thus reducing the percentage of recovery of the analytes. Instead, the C5 combination, which consisted of adding 13 mL of ACN followed by 2 mL of isopropanol, showed the best results, with recoveries greater than 85%, and was considered the optimal option in this study.

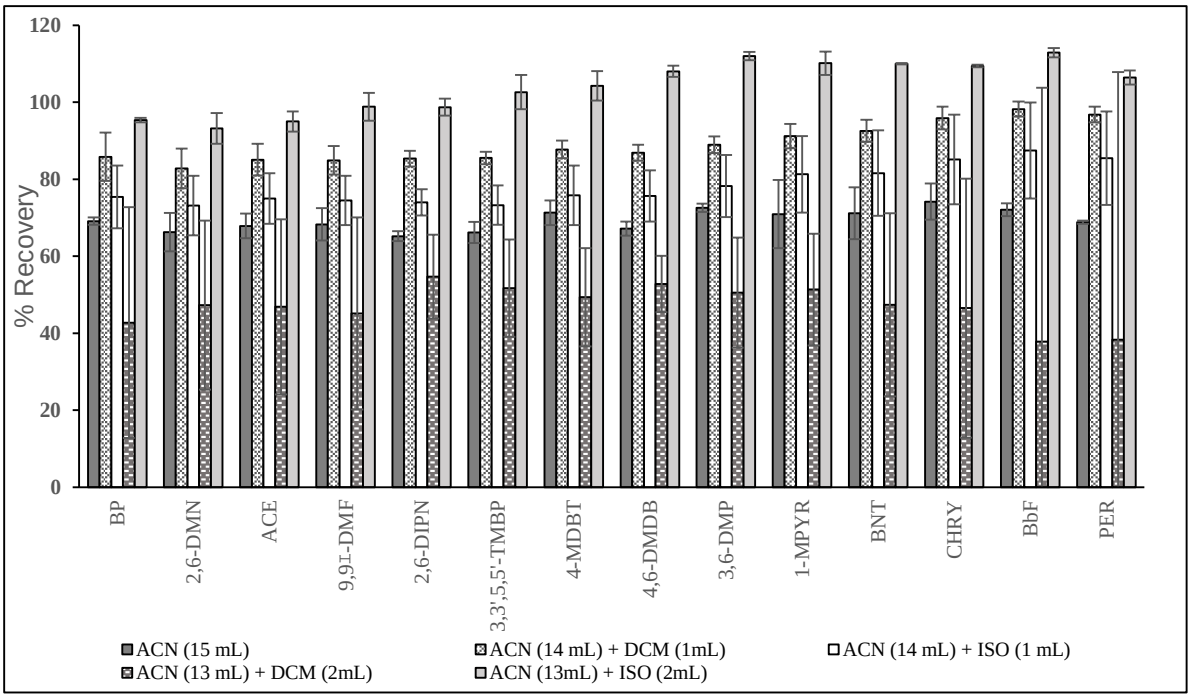


Figure 4. Comparison of recovery of target analytes when method A is applied and different solvent combinations.

The elution curve shown in Figure 5 was constructed to study the behaviour of the target analytes during the solid phase extraction with method A. As can be seen, a high content of the analytes eluted with the first millilitres of the solvent, and most of the compounds showed a similar behaviour during solid phase extraction. However, aromatic hydrocarbons with higher molecular weight and lower polarity eluted slower; this behaviour is likely due to their lower affinity for the eluent. Regarding the evaporation losses during the concentration step under mild nitrogen current, the results revealed no significant differences in the concentration of the target analytes in the solutions analysed; therefore, the evaporation losses are negligible in this case.

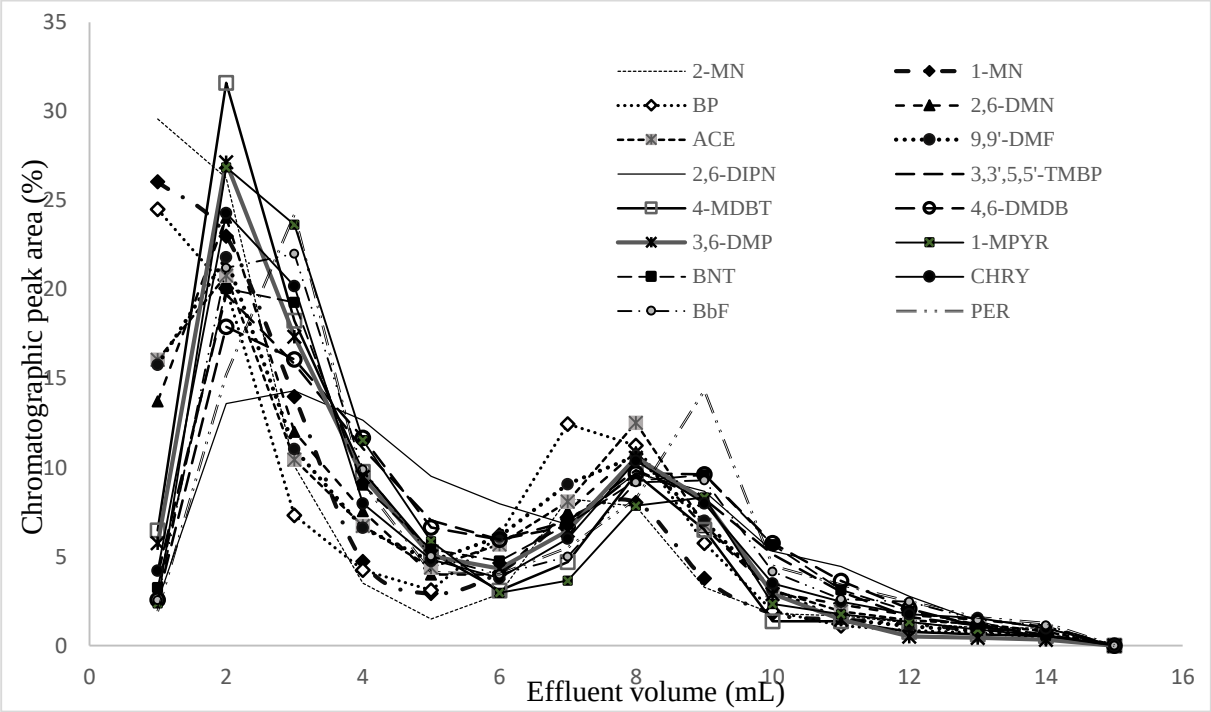


Figure 5. Elution curve of target analytes treated with method A.

6.2.3. Analytical parameters

As shown in Table 4, method A presented good precision and linearity in the range of concentrations tested (0.1- 7.5 μg g^{-1}) with correlation coefficients (R^2) between 0.9964 and 0.9996 and intra-day RSD values below 6% and inter-day RSD values less than 8% for both spiked samples. The recovery assays of the spiked sample with

1 μg g^{-1} showed recovery values in a range from 81 to 104%, while the sample fortified with 5 μg g^{-1} showed recovery values between 85 and 99%. The LODs varied from 0.008 to 0.024 μg g^{-1} and the LOQs from 0.016 to 0.08 μg g^{-1}.

6.2.4. Application in real samples

Optimized procedure A was used to determine the target analytes in the study samples. First, the mineral oil extracted from the offset printing inks was analysed to verify the method's applicability in a natural matrix and identify the target analytes present in the mineral oils used in the oil-based offset printing inks.

The results revealed that ten of the sixteen compounds proposed as MOAH markers by Jaén et al. (Jaén et al., 2020) were found in mineral oil-based offset printing ink samples. The compounds were identified and confirmed by comparing their mass spectra and retention times with standards injected under the same conditions as the samples. The identified compounds were: 2-MN, 1-MN, BP, 2,6-DMN, 9,9'-DMF, 3,3',5,5'-TMBP, 1-MPYR, 4,6-DMDBT, 3,6-DMP and BNT. The presence of ACE, DIPN, or 4-MBT was not detected. Neither was PAHs with more than three aromatic rings such as CHRY, BbF and PER detected. Figure 6 shows the peaks of seven of the target analytes identified in one of the printing ink samples (OPIS01).

Except for 9,9'-DMF, all compounds identified in the printing inks were also identified and quantified in the MOAH-contaminated cardboard samples. Table 5 shows the target analytes identified in the samples and their respective concentrations. As can be seen, sample CFPS01 (decorated cardboard plate) presented the highest amount of analytes, followed by sample CFPS05 (decorated tea box); and CFPS04 (cardboard cup) was the sample with the lowest number of target analytes. The compounds most frequently found in the food packaging under study were the lowest molecular weight target analytes, such as methylated naphthalenes, detected in almost all samples. Among the aromatic hydrocarbons with three or more aromatic rings, which pose a greater risk to consumer health, only 3,6-DMP and 1-MPYR were found.

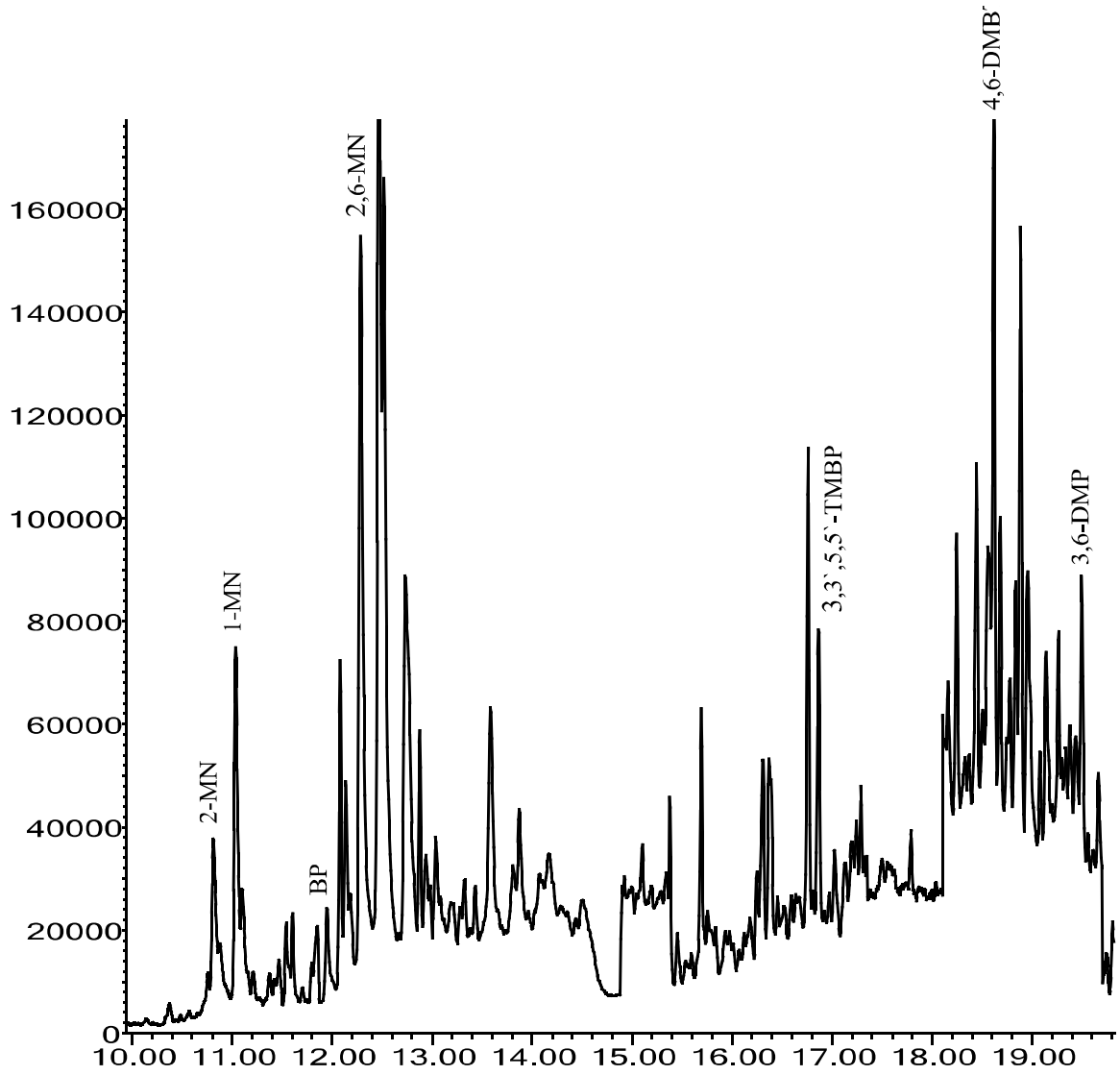


Figure 6. Chromatogram of the target analytes identified in the OPIS01 sample of mineral oil-based offset printing inks by GC-MS.

The concentration of the analytes in the printing inks ranged between 2.28 and 8.59 µg g^{-1}. The compounds with the highest concentration were methyl naphthalenes (2-MN, 1-MN, 2,6-DMN) and BNT, followed by 1-MPYR, 3,3',5,5'-TMBP; and the compound 4,6-DMDBT had the lowest concentrations. The concentrations of the target analytes in the cardboard food package ranged between 0.10 and 0.33 µg g^{-1}. The concentrations were evidently lower than those found in mineral oil-based offset printing inks. However, the concentrations were high enough to be detected and related to MOAH contamination.

Table 5. Concentration of individual aromatic compounds (mean ± SD) in cardboard food packaging samples contaminated with MOAH and offset printing inks. The determination was made by method B.

Compounds	Cardboard food packaging sample (μg g^{-1} ± SD)					Offset printing inks sample (μg g^{-1} ± SD)	
	CFPS01	CFPS02	CFPS03	CFPS04	CFPS05	OPIS01	OPIS02
2-MN	0.17 ± 0.02	0.20 ± 0.01	0.19 ± 0.01	0.16 ± 0.02	0.30 ± 0.01	7.61 ± 0.52	8.59 ± 0.60
1-MN	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.19± 0.01	6.59 ± 0.10	6.74 ± 0.42
BP	0.12 ± 0.02	0.13 ± 0.01	0.11± 0.01	< LOQ	0.22 ± 0.01	3.48 ± 0.33	4.44 ± 0.33
2,6-DMN	0.13 ± 0.01	0.14 ± 0.01	0.12± 0.01	<LOQ	0.24± 0.01	7.37 ± 0.58	5.30 ± 0.19
9,9'-DMF	< LOQ	< LOQ	< LOQ	< LOQ	<LOQ	<LOQ	2.49 ± 0.05
3,3',5,5'-TMBP	0.09 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	<LOQ	0.33 ± 0.01	2.28 ± 0.16	2.77 ± 0.26
4,6-DMDBT	0.10 ± 0.02	nd	<LOQ	nd	0.17 ± 0.01	2.50 ± 0.16	2.83 ± 0.21
3,6-DMP	0.14± 0.03	0.12 ± 0.01	nd	nd	0.20 ± 0.01	3.85 ± 0.13	4.36 ± 0.04
1-MPYR	0.14 ± 0.02	nd	0.12 ± 0.01	nd	0.24 ± 0.01	5.37 ± 1.13	4.65 ± 0.22
BNT	0.11 ± 0.02	nd	nd	nd	nd	7.29 ± 1.83	8.09 ± 0.44

nd: not detected

7. Conclusions

In this study, an offline SPE-GC-MS method has been optimized to determine MOAH contamination markers in both mineral oil-based offset printing inks and cardboard food packaging samples. The results have shown that the developed method is fast, has good sensitivity, precision and linearity within the range of the studied concentrations and presents recovery values of the target analytes above 80%.

Ten of the compounds proposed as MOAH markers were determined in the offset printing inks. These compounds were: 2-MN, 1-MN, BP, 2,6-DMN, 9,9'-DMF, 3,3',5,5'-TMBP, 1-MPYR, 4,6-DMDBT, 3,6-DMP and BNT. Except for 9,9'-DMF, which was detected below the LOQ, all other compounds were also determined in MOAH contaminated cardboard food packaging samples.

In our opinion, these compounds can be used to confirm contamination of cardboard food packaging samples with MOAH from mineral oil-based offset printing inks.

We also consider chemical markers and mass spectrometry valuable tools, complementing the FID analysis of the MOAH fraction, especially in laboratories where other analytical instruments are not available.

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Capítulo 3

Migration of mineral oil aromatic hydrocarbon (MOAH) from hot melt adhesives used in food packaging materials

1. Resumen
2. Objetivos
3. Esquema de trabajo
4. Introduction
5. Materials and methods
6. Results and discussion
7. Conclusions
8. References

1. Resumen

En la presente investigación se estudia la migración de MOAH desde adhesivos termofusibles utilizados en laminados multicapa a simulantes alimentarios. En primer lugar, se determinó la concentración inicial de un grupo de compuestos seleccionados como marcadores MOAH en varios adhesivos mediante Micro-extracción en Fase Sólida de Espacio de Cabeza acoplada a Espectrometría de Masas por Cromatografía de Gases (HS-SPME-GC-MS), haciendo uso del método previamente optimizado. Luego, se estudió la migración de la fracción MOAH y los marcadores MOAH de los laminados. La fracción MOAH se analizó mediante Cromatografía de Gases con Detección de Ionización de Llama (GC-FID) y los marcadores MOAH se analizaron mediante HS-SPME-GC-MS. Se detectaron doce marcadores MOAH, y sus concentraciones iniciales estuvieron entre 0.46-33.8 μg g^{-1}. Solo se identificaron ocho después de la migración, con un rango entre 0.62 y 21.33 μg dm^{-2}, con un porcentaje de migración del 12 al 75%. La fracción de MOAH que migró eluyó principalmente en el rango C16-C25 y alcanzó concentraciones de 19.65 μg dm^{-2} del laminado.

2. Objetivos

Los objetivos más relevantes de esta sección fueron: el desarrollo un método de micro-extracción en fase sólida acoplado a cromatografía de gases (HS-SPME-GC-MS) para analizar marcadores de MOAH en muestras comerciales de adhesivos termofusibles; y el estudio de la migración de marcadores MOAH y de la fracción MOAH desde algunos laminados fabricados con estos adhesivos.

Los objetivos específicos fueron:

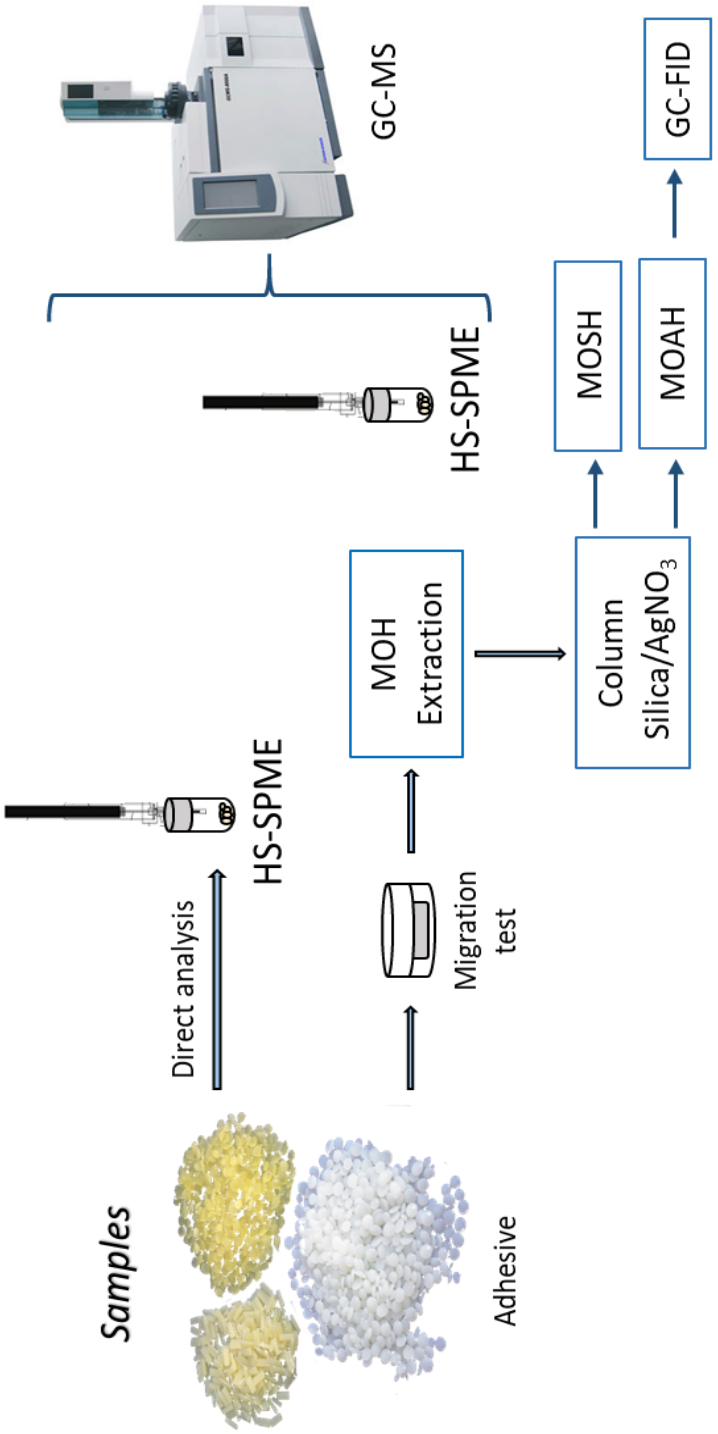
- Optimización de un nuevo método HS-SPME-GC-MS para analizar marcadores de MOAH.
- Determinación de la concentración inicial de los marcadores MOAH en muestras comerciales de adhesivos termofusibles.
- Estudio de la migración de marcadores MOAH y de la fracción MOAH de algunos laminados fabricados con estos adhesivos, al óxido de polifenileno modificado (Tenax®) como simulante de alimentos.

Las tareas realizadas para alcanzar los objetivos propuestos fueron:

- Optimización de los parámetros más importantes en HS-SPME-GC-MS
- Análisis directo de los adhesivos termofusibles curados mediante HS-SPME-GC-MS con el propósito de identificar de los marcadores MOAH presentes en las muestras.
- Cuantificación de la concentración inicial de los marcadores MOAH en los adhesivos termofusibles.

- Aplicación de métodos de extracción y análisis de MOAH en el cartón utilizado como sustrato en los laminados para verificar que se encontraba libre de MOAH.
- Fabricación de los laminados.
- Realización de ensayos de migración con el simulante Tenax, en base a los protocolos establecidos en la Legislación.
- Optimización de la metodología empleada para extraer MOAH desde el Tenax.
- Análisis y cuantificación de los marcadores MOAH que migraron mediante HS-SPME-GC-MS.
- Separación de la fracción MOAH migrante mediante extracción en fase sólida con sílice activada impregnada de $AgNO_3$.
- Análisis y caracterización de la fracción MOAH por el método convencional en su versión manual (SPE-GC-FID).

3. Esquema de trabajo



Esquema 3. Diseño experimental del Capítulo 3.

4. Introduction

Hot melt adhesives are widely used to assemble materials in multilayer laminates commonly found in food packaging. Adhesives are formulated using a combination of chemicals with specific functions. The main component is the base polymer, which gives its name to the adhesive type. Besides, additives as paraffin wax or tacky resins are added to reduce the viscosity and improve the wetting. Also, pigments, fillers and other additives are used to optimise the physical, chemical or mechanical properties of the adhesive (Mildenberg, Zander, & Collin, 1997; Petrie, 2000).

Even though in most cases, the adhesives are not in direct contact with food, scientific studies have shown that many compounds migrate from the adhesives to the food; both additives used in its manufacture and NIAS (non-intentionally added substances) compounds coming from the degradation of components or impurities (Aznar et al., 2011; Nerín et al., 2012; Vera, Canellas, & Nerín, 2014; Yan et al., 2020). However, there is no specific regulation about the use of adhesives as food contact materials and their possible migrations. Only a general Framework Regulation about objects and materials intended to come into contact with food (European Commission, 2004); and the guidelines of the Commission Regulation (EC) 2023/2006 to regulate manufacturing practices (European Commission, 2006) that adhesives producers have to be followed.

Mineral oil hydrocarbons (MOH) are complex substances that come from the distillation of crude oil and contain mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH), which can be separated by liquid chromatography. The presence of MOAH in food is of concern due to the genotoxic carcinogenic potential of MOAH with three or more aromatic rings. MOAH can reach food from recycled paper and cardboard, and printing inks applied to paper and cardboard, as MOH are used in the manufacture of plastic materials, waxes, lubricants and adhesives, among others (EFSA, 2012).

To the best of our knowledge, there are a few publications that relate the presence of this type of compounds in the adhesives. Biedermann et al. showed typical MOSH and MOAH chromatograms for hot melt adhesives (Biedermann & Grob, 2012a). Barp et al. investigated the migration of MOHs from cardboard to food and found that a significant percentage of total MOHs contamination came from adhesives (Barp et al., 2015a, 2015b). Lommatzsch et al. separately analysed the main components of hot melt adhesives and concluded that the primary source of hydrocarbons in adhesives is tacky resin (Lommatzsch et al., 2016).

The recommended technique for MOAH analysis is online liquid chromatography with gas chromatography combined with flame ionisation detection (HPLC-GC-FID) (AENOR, 2018; Bratinova & Hoeskstra, 2019). The chromatographic analysis of MOAH is characterised by the formation of broad chromatographic humps (Biedermann & Grob, 2012b), which could encompass other substances (Koster et al., 2020). Some researchers argue that GC-FID should be supplemented with confirmatory techniques such as mass spectrometry, two-dimensional gas chromatography (GCXGC) or nuclear magnetic resonance (NMR) spectroscopy (Lachenmeier et al., 2017; Spack et al., 2017; Weber et al., 2018).

Given the complexity of mineral oils and the expensive instrumentation required for their analysis, the use of chemical markers is an alternative to certainty identify the presence of MOAH and thus ensure the quality of the results. It is also recommended to identify chemical markers to determine the origin of contamination (Spack et al., 2017). Recently, Jaén et al. identified 16 compounds that can be used as MOAH markers (Jaén et al., 2021).

The main objectives of this study were: (i) to develop and optimise a novel solid phase microextraction method coupled to gas chromatography (HS-SPME-GC-MS) to analyse MOAH markers (ii) to identify and quantify the initial concentration of MOAH chemical markers in commercial samples of hot melt adhesives that had MOH among their components (iii) to study the migration of MOAH markers and MOAH fraction

from some laminates manufactured with these adhesives to modified polyphenylene oxide (Tenax®) as food simulant.

5. Materials and methods

5.1. Reagents

Acetone, n-hexane, toluene, dichloromethane and ethanol absolute, all HPLC grade were from Panreac (Barcelona, Spain). 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2–MN), biphenyl (BP), 2,6-dimethylnaphthalene (2,6-DMN), acenaphthene (ACE), 2,6-diisopropylnaphthalene (2,6-DIPN), 3,3',5,5'-tetramethylbiphenyl (3,3',5,5'-TMBP), 4-methyldibenzothiophene (4-MDBT), 4,6-dimethyldibenzothiophene (4,6-DMDBT), 1-methylpyrene (1-MPYR), benzo(b)naphtha(1,2-d)thiophene (BNT), chrysene (CHRY), benzo(b)fluoranthene (BbF), perylene (PER), undecane (n-C11), n-tridecane (n-C13), bicyclohexyl (Cycy), 5α-cholestane (Cho), pentylbenzene (5B), 1,3,5-tri-tert-butylbenzene (TBB); and standard mixture of saturated alkanes (C7-C40) of 1000 μg mL^{-1} were purchased from Sigma-Aldrich (Madrid, Spain). 3,6-dimethylphenanthrene (3,6-DMP) was obtained from Dr. Ehrenstorfer (Augsburg, Germany) and 9,9'-dimethylfluorene (9,9-DMF) was supplied by Tokyo Chemical Industry CO., LTD.

SPME fibres, Tenax TA 60/100 mesh and salinised glass wool were supplied by Supelco (Bellefonte, USA). Anhydrous sodium sulphate extra pure Ph. Eur was from Scharlab SL (Barcelona, Spain). The "Vibromatic" mechanical laboratory shaker was obtained from J. P. Selecta (Spain). Silica gel high-purity grade (pore size 60 Å, 70-230 mesh), silver nitrate on silica gel (~ 10 wt.% loading, 230 mesh) were purchased from Sigma-Aldrich (Madrid, Spain).

Two standard solutions were prepared. Solution A contained 16 standards (1-MN, 2-MN, BP, 2,6-DMN, ACE, 2,6-DIPN, 3,3',5,5'-TMBP, 4-MDBT, 4,6-DMDBT, 1-MPYR, BNT, CHRY, BbF, PER, 3,6-DMP and 9,9-DMF) at 100 μg g^{-1} in

n-hexane. Solution B contains the standards used to verify the separation of MOSH and MOAH in the following concentrations: n-C11 (175 μg g^{-1}), n-C13 (350 μg g^{-1}), Cycy (366 μg g^{-1}), Cho (350 μg g^{-1}), 5B (355 μg g^{-1}), 1-MN (342 μg g^{-1}), 2-MN (355 μg g^{-1}), TBB (357 μg g^{-1}), and PER (699 μg g^{-1}) in toluene. All solutions were stored at -4 ° C.

5.2. MOAH markers

The compounds used as chemical markers in this study were identified and selected in a previous investigation, as MOAH markers for food packaging (Jaén et al., 2021). In addition, these compounds include branched and unbranched polycyclic aromatic hydrocarbons (PAHs) and benzothiophenes, which other authors have identified in mineral oils (Biedermann & Grob, 2009, 2015; Li et al., 2012; Yang et al., 2016).

The compounds evaluated as MOAH markers in hot melt adhesives were: 1-MN, 2-MN, BP, 2,6-DMN, ACE, 9,9'-DMF, 2,6-DIPN, 3,3',5,5'-TMBP, 4-MDBT, 4,6-DMDBT, 3,6-DMP, 1-MPYR, BNT, CHRY, BbF and PER. The molecular structure of these compounds and their molecular masses are shown in Table 1.

5.3. Samples

5.3.1. Hot melt Adhesives

Eight hot melt adhesives used commercially in food contact materials were studied. Six of them were based on EVA (ethylene/vinyl acetate), and two were pressure sensitive adhesives (PSA). The EVA base adhesives were AD1, AD2, AD3, AD5, AD6 and AD8, and the PSA were AD4 and AD7. Besides, a mineral oil free base hot melt adhesive was used for the construction of the calibration curve.

To cure the adhesives, 1 g of each adhesive was placed in a sealed glass vial; the vial was then heated for approximately 10 min at 160-180 °C. Three replicates of each one were prepared.

5.3.2. Laminates

Laminates with a sandwich structure (substrate-adhesive-substrate) were prepared for migration tests. The cardboard used as substrate had a grammage of 412 g m^{-1} and was obtained from the local trade

The first step in the laminate manufacturing was to cut the cardboard into rectangular sheets with 4 x 2 cm. The adhesive was then melted, and a homogeneous film of the melted adhesive was placed on the cardboard sheet. Finally, the structure was completed, placing a second sheet of cardboard over the melted adhesive.

The amount of adhesive used was calculated as the difference between the laminate mass and the cardboards used as substrates. The average mass of the adhesives in the laminate was 0.10 ± 0.01 g in 8 cm^2.

5.4. Instrumental analysis

GC-MS

GC-MS analysis was performed on an HP 6890 chromatograph coupled to an HP 5975 mass selective detector. It was equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). The column was a DB-5 (30 m x 0.25 mm x 0.25 mm) from Agilent Technologies (Madrid, Spain). The temperature program applied to the oven was: initially 50 ºC, then raised to 310 ºC at 10 ºC min^{-1} and was kept at 310 ºC for 5 min, the splitless mode was selected, and the acquisition was carried out in SIM mode. The quantifier ions and their corresponding retention times are shown in Table 1. Data were processed using MSD ChemStation software (version F.01.00.1903, Agilent Technologies).

The final optimised conditions for the extraction of HS-SPME were as follows: incubation time of 2 min, incubation temperature of 88 ºC, extraction time 17.5 min and desorption time of 2 min.

For the initial concentration of MOAH markers in the adhesives: 1 g of the cured adhesive was placed in a 20 mL vial. And for the migration test, 10 µL of n-hexane after extraction of Tenax were placed into 20 mL vials, both tests were analysed by HS-SPME-GC-MS previously optimised.

GC-FID

GC-FID analysis was performed on a Trace GC Ultra chromatograph equipped with a flame ionisation detector (FID) and an AS 300 autosampler (Thermo Electron Corporation, Milan, Italy). The temperature of the FID was 350 °C. The analytical column was an HP-5 (60 m x 0.25 mm ID, 0.25 µm film thickness) from Agilent Technologies. The oven temperature program was as follows: at 50 °C for 2 min, then increased to 30 °C min^{-1} up to 310 °C and held for 15 min. The total run was 26 min. The carrier gas flow rate (helium, 99.999%) was 2 mL min^{-1}, and the inlet pressure was 70 kPa. The injector temperature was 250ºC, and 5 µL of the MOAH fraction was injected in splitless mode. The data were acquired and processed using the Chrom-Card GC software (Thermo Electron).

Repeated solvent injections determined the position of the baseline. The area of the MOAH sub-fractions was defined by the retention times of n-alkanes injected under the same conditions as the samples. The sharp peaks at the top of the MOAH hump were subtracted from the area of the corresponding sub-fraction, and the quantification was carried out through the internal standard method (Bratinova & Hoeskstra, 2019).

5.5. Optimisation of the HS-SPME method

Optimisation tests were performed by directly injecting 10 µL of a standard solution containing all analytes (10 µg g^{-1}) in n-hexane. The optimised parameters were: type of fibre, extraction temperature (30-80 ºC) and extraction time (5-30 min).

The first step was the selection of the best fibre, that is, the one providing the highest response of the different analytes. For this purpose, the fibres tested were: polydimethylsiloxane/divinylbenzene/carboxen (PDMS/DVB/CAR, 50/30 µm film

thickness), Carboxen/polydimethylsiloxane (CAR/PDMS, 85 μm film thickness) and Polydimethylsiloxane (PDMS, 100 μm film thickness). The conditions of extraction were 15 min and 50ºC.

And the second step was the performance of Face Centred Central Composite Design to select the optimum extraction temperature and time. The statistical design was carried out with the Modde 6.0 software from Umetrics (Umea, Sweden). The experiments to optimise these conditions were carried out with the selected fibre and in random order.

5.6. Initial concentration of MOAH markers in adhesives

The initial concentration of MOAH markers in the adhesives were determined using 1 g of different cured adhesives. To quantify their concentrations, 1 g of the mineral oil free hot melt adhesive was heated at 180 ºC (to be cured) and, once melted, 10 μL of solution A containing different increasing concentrations of the MOAHs were added. Three replicates of each sample were prepared and analysed by HS-SPME-GC-MS method that was previously optimised.

5.7. Analysis of the free MOAHs cardboard used in laminates

Before manufacturing the laminates for the migration test, a previous assay was developed to check the lack of MOAH or marker compounds in the cardboard used to make these laminates. For this purpose, the cardboard was subjected to the mineral oil extraction process described by (Vollmer et al., 2011) with slight modifications. In a 20 mL glass vial, 2 g of the cardboard cut into pieces of 0.5 cm on each side were placed. Then 15 mL of n-hexane: ethanol (1:1) mixture was added, and it was left in the ultrasound for two hours. Next, the extract was decanted into another vial, and 5 mL of water was added. The supernatant n-hexane layer was separated and carefully concentrated under nitrogen gas to 1 mL, and later, it was analysed by HS-SPME-GC-MS.

5.8. Migration tests

The migration tests were carried out with the food simulant Tenax. In accordance with the provisions of the UNE-EN 14338 standard, Tenax was previously purified with acetone in a Soxhlet for 6 h and dried in an oven at 160 ºC (AENOR, 2004).

5.8.1. Optimisation of extraction of MOAHs from Tenax

Previous to migration tests, the methodology for the extraction of these markers from Tenax was optimised. For this, 100 μL of solution A were added to three samples containing 0.32 g of Tenax. The concentration of the analytes was 23 μg of compound per g Tenax. The analytes were extracted four consecutive times with n-hexane. Each extraction was carried out with 4 mL of n-hexane under constant stirring by ultrasound for 1h at room temperature. The extracts were concentrated in a nitrogen gas stream to 0.4 mL at 40 °C. After that, 10 μL of each concentrated extract was analysed by HS-SPME-GC-MS. After establishing the optimal number of extractions, recovery experiments were performed in triplicate to evaluate the extraction efficiency.

The final protocol applied to extract the mineral oil and the marker compounds from Tenax was to add 4 mL of n-hexane to the Tenax sample and place it in an ultrasonic bath at room temperature for one hour. After this time, the extract was collected, and the sample was subjected to two more extractions, following the same procedure. Finally, the extracts were combined and concentrated under a gentle stream of nitrogen gas at 40 °C to 0.4 mL.

5.8.2. Migration test analyses and quantification

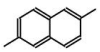
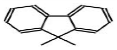
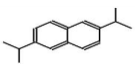
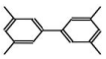
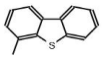
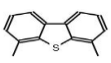
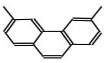
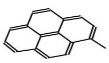
For the migration tests, the laminates were placed in direct contact with 0.32 g of the food simulant according to the 4 g dm^{-2} ratio established by the UNE-EN-14338 standard. Next, the laminates were wrapped in aluminium foil and placed in a glass Petri dish with a diameter of 6 cm. The whole was kept in an oven for 10 days at 40 ºC. The migration conditions were selected following Regulation EU 10/2011 (European Commission, 2011). Three replicates and a blank were prepared for each assay.

After migration, the analytes were extracted from Tenax with n-hexane and concentrated following the optimised protocol described in section 5.8.1. For the study of MOAH markers, 10 μL of the concentrated extract were taken and analysed directly by HS-SPME-GC-MS. For their quantification, 10 μL of increased concentrations of all compounds were analysed by the same methodology.

Besides, to corroborate the migration of MOAH, the MOSH and MOAH fractions were first separated following the protocol developed by (BfR & KLZH, 2012); and subsequently, the MOAH fraction was concentrated under a gentle stream of nitrogen gas to 0.4 mL and analysed by GC-FID. MOAH was quantified using the internal standard method described in (Bratinova & Hoeskstra, 2019).

The concentrations of the migrating compounds were calculated as absolute μg of the compound that migrated to Tenax, and these values were divided by dm^2 of laminate in contact with it.

Table 1. MOAH markers with their monitored ions and retention times.

Compounds	CAS	Molecular formula	Molecular structure	RT	Monitored ions (m/z)
2-methylnaphthalene	91–57-6	$C_{11}H_{10}$		10.04	142.1, 115.1
1-methylnaphthalene	90–12-0	$C_{11}H_{10}$		10.25	142.1, 115.1
Biphenyl	92-52-4	$C_{12}H_{10}$		11.29	154.1, 76.1
2,6-dimethylnaphtalene	581-42-0	$C_{12}H_{12}$		11.61	156.1, 141.1
Acenaphthene	83-32-9	$C_{12}H_{10}$		12.61	153.1, 76.0
9,9'-dimethylfluorene	4569-45-3	$C_{15}H_{14}$		13.63	179.1, 194.1
2,6-diisopropylnaphtalene	24157-81-1	$C_{16}H_{20}$		15.32	197.1, 212.2
3,3',5,5'-tetramethylbiphenyl	25570-02-9	$C_{16}H_{18}$		16.01	210.2, 195.1
4-methyldibenzotiophene	7372-88-5	$C_{13}H_{10}S$		16.90	198.1, 165.1
4,6-dimethyldibenzotiophene	1207-12-1	$C_{14}H_{12}S$		17.94	212.1, 105.1
3,6-dimethylphenanthrene	1576-67-6	$C_{16}H_{14}$		18.31	206.1, 191.1
1-methylpyrene	2381-21-7	$C_{17}H_{12}$		20.70	216.1, 94.5

6. RESULTS AND DISCUSSION

6.1. Optimisation of the HS-SPME method

The success of the HS-SPME extraction depends on the interaction of the analytes with the fibre coating. Therefore, the first step in this research was to select the most efficient fibre for extraction. Three different fibres were tested (PDMS, PDMS/ DVB/ CAR, CAR/ PDMS), and the most appropriate one was selected, considering the number of analytes that could be detected and the intensity of the signal.

As shown in Figure 1, the CAR / PDMS fibre was the one that showed the worst results in the extraction of the analytes. The PDMS/ DVB/ CAR fibre showed good efficiency extracting the most volatile analytes, but it provided lower extraction values with the highest molecular mass compounds compared to PDMS fibre. On the other hand, the PDMS fibre showed good efficiency in extracting most of the analytes, except BbF and PER. In summary, the extraction efficiency of the tested fibres decreased with increasing molecular mass, and the PDMS fibre was the one that showed the best results.

Once the fibre had been selected, the temperature and the extraction time were optimised. Optimal conditions for these parameters were determined using an experimental design. For this statistical analysis, the total chromatographic areas obtained from the random variation of the temperature and the extraction time were used. A good fit of the model was shown, with a fraction of the variation response explained by the model (R2) and a fraction of the response variation that can be predicted by the model (Q2) closed to 1. Figure 2 shows the response surface graph experimental design of the summary area of all compounds. According to the results obtained, the optimal conditions for the HS-SPME extraction were: 100 μm PDMS fibre, extraction time 17.5 min and extraction temperature 60 °C.

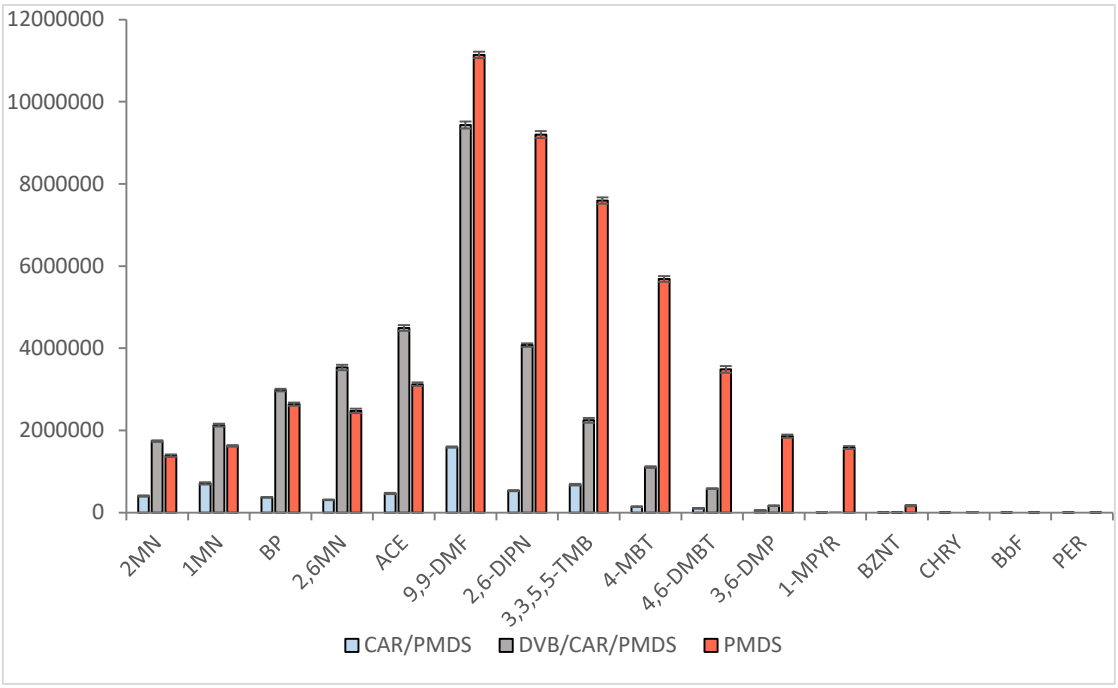


Figure 1. Graph of the areas of the analytes with the three fibres tested (CAR/PDMS, DVB/CAR/ PDMS and PMDS).

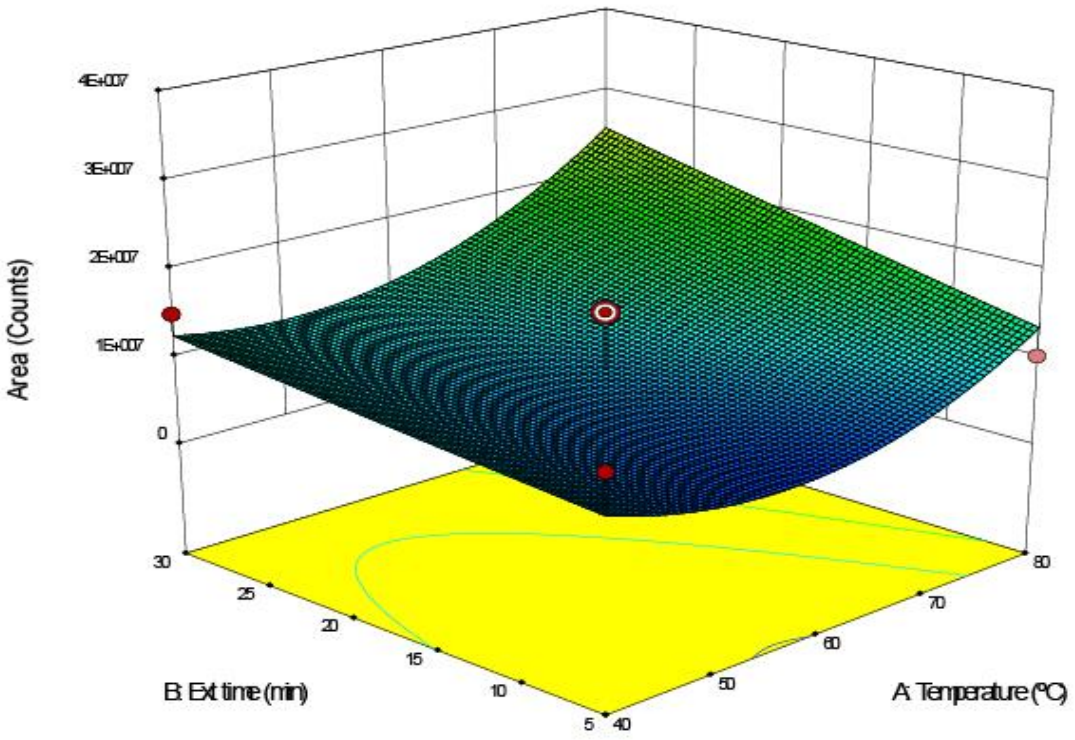


Figure 2. Response surface plot for the optimal HS-SPME extraction conditions.

6.2. Initial concentration of MOAH markers in Adhesives

The first step in determining MOAH markers in the adhesives under study consisted of subjecting the cured adhesives to rapid detection by HS-SPME-GC-MS. The analysis revealed that only five out of the eight adhesives studied had an unresolved chromatographic hump (UCM), which could be MOH. The adhesives that showed this UCM were: AD1, AD5, AD6, AD7 and AD8. As a next step, MOAH markers were identified in the samples by comparing the analytes mass spectra and retention time with pure standards. Figure 3 shows the chromatogram of the MOAH markers in the AD7 sample after the curing process.

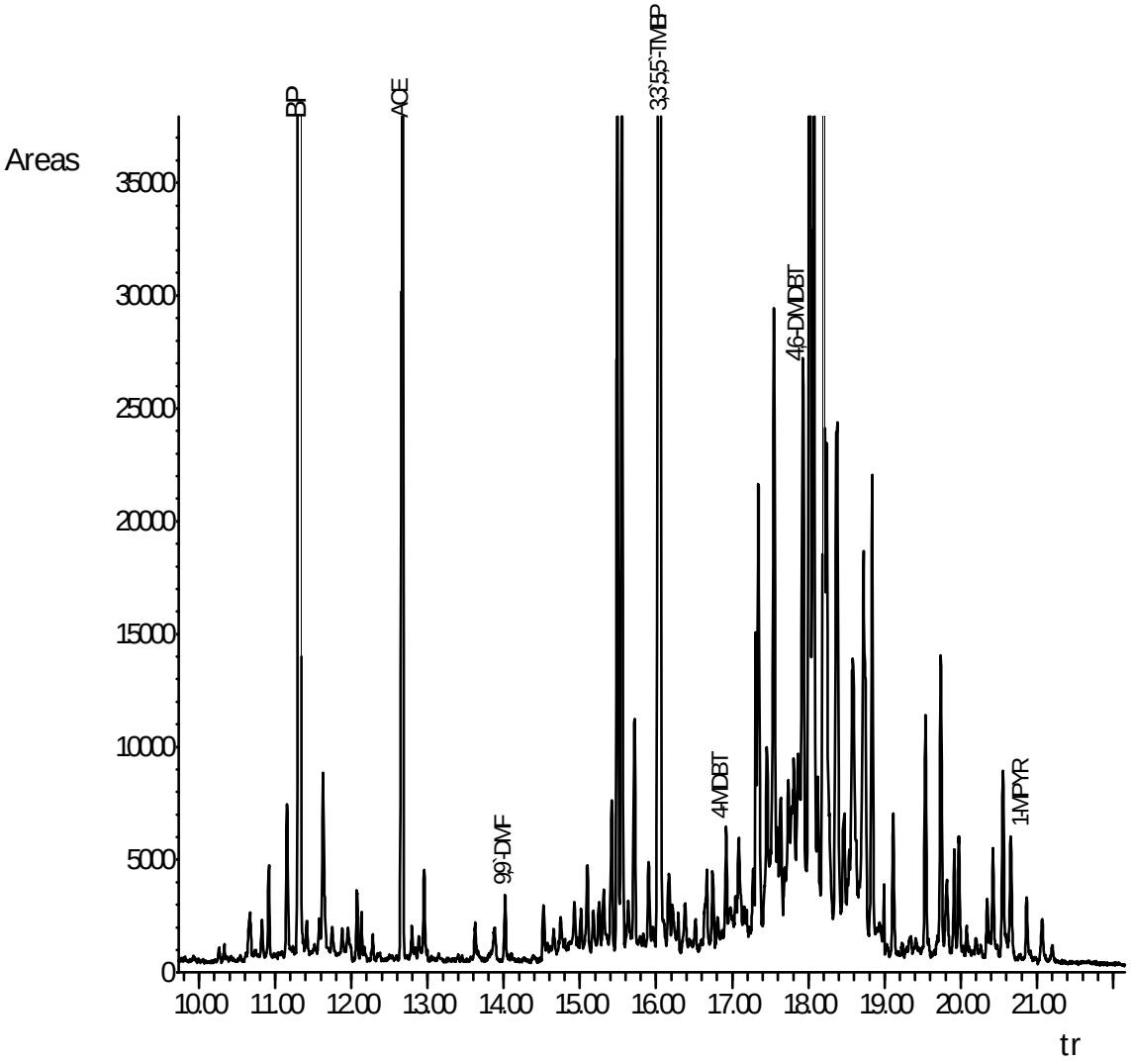


Figure 3. Chromatogram of the MOAH markers in the AD7 sample after the curing process analysed by HS-SPME-GC-MS.

Twelve of the proposed MOAH markers were identified in the adhesives that had a UCM. The identified compounds were: 2-MN, 1-MN, BP, 2,6-MN, ACE, 9,9'-DMF, 2,6-DIPN, 3,3',5,5'-TMB, 4-MBT, 4,6-DMBT, 3,6-DMP and 1-MPYR. There was no evidence of the presence of heavy compounds such as BNT, CHRY, PER and BbF in the samples.

For the quantitative study, only the five adhesives that presented the characteristic UCM of MOH and some of the MOAH markers were considered. The analytical method used was HS-SPME-GC-MS, and the analytical characteristics of the method are shown in Table 2. The method showed good linearity, with regression coefficients between 0.988 and 0.997 for the different calibration curves. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using the signal to noise (S/N) method. The S/N was established from the chromatograms of the analytes at low concentrations, close to noise. The limits of detection (LOD) and limit of quantification (LOQ) of the method, established as three times and 10 times S/N ratio respectively, were lower than 0.40 μg g^{-1} for all compounds and the relative standard deviation (RSD) had a value lower than 11%.

Table 3 shows the initial concentrations of MOAH markers in the five selected adhesives. The average concentration of the alkylated PAHs, 2,6-MN, 9,9'-DMF, 3,3',5,5'-TMB, 3,6-DMP and 1-MPYR was in the range of 0.46-31.3 μg g^{-1}; while the mean concentrations of the 2-MN and 1-MN isomers were between 0.77 and 1.37 μg g^{-1}. On the other hand, aromatic compounds with sulphur heteroatoms (4-MBT, 4,6-DMBT), commonly used in other studies to mark oil hydrocarbon contamination (Li et al., 2012; Yang et al., 2016), were detected in four of the samples (AD1, AD5, AD7 and AD8) at concentrations that ranged from 2.27 to 12.6 μg g^{-1}.

Table 2. Analytical parameters of the HS-SPME-GC-MS method.

Compounds	Linearity ($\mu g\ g^{-1}$)	R^2	Slope	LOD ($\mu g\ g^{-1}$)	LOQ ($\mu g\ g^{-1}$)	RSD (%)
2-MN	0.524-14.7	0.995	9.40E+02	0.113	0.377	10.1
1-MN	0.572-16.0	0.994	1.48E+03	0.103	0.345	5.2
BP	0.551-31.6	0.994	1.69E+03	0.050	0.167	3.4
2,6-DMN	0.526-25.1	0.995	1.68E+03	0.073	0.244	3.5
ACE	0.534-30.6	0.994	1.99E+03	0.014	0.048	3.0
9,9'-DMF	0.550-21.9	0.988	4.31E+03	0.030	0.099	2.2
2,6-DIPN	0.542-25.8	0.992	4.61E+03	0.012	0.040	5.6
3,3',5,5'-TMBP	0.54-31.0	0.997	3.25E+03	0.013	0.042	6.3
4-MDBT	0.538-25.5	0.991	1.95E+03	0.040	0.134	3.6
4,6-DMDBT	0.5321-14.9	0.995	1.99E+03	0.024	0.078	1.7
3,6-DMP	0.526-14.8	0.991	1.71E+03	0.038	0.125	8.9
1-MPYR	0.534-15.0	0.995	2.56E+03	0.022	0.074	3.4

The most abundant markers were BP and ACE. These two markers were identified in the five adhesives studied, in concentrations of 2.25-33.8 $\mu g\ g^{-1}$. The compound 2,6-DIPN was identified in two adhesives (AD6 and AD8); however, their concentrations were below the limit of quantification.

The PSA adhesive (AD7) was among the most contaminated samples, probably associated with resins or plasticizers used to improve the viscoelastic properties of the adhesive (O'Brien et al., 2007).

Table 3. Initial concentration of MOAH markers (mean ± SD) expressed as μg of compound per g of cured adhesive.

Compounds	MOAH markers initial concentration μg g^{-1} ± SD				
	AD1	AD5	AD6	AD7	AD8
2-MN	0.77 ± 0.01	nd	1.37 ± 0.08	nd	nd
1-MN	<LOQ	<LOQ	0.84 ± 0.07	<LOQ	1.03 ± 0.08
BP	7.39 ± 0.33	7.41 ± 0.38	5.21 ± 0.43	33.78 ± 0.66	3.38 ± 0.18
2,6-MN	nd	<LOQ	1.05 ± 0.09	1.29 ± 0.01	nd
ACE	2.74 ± 0.12	2.25 ± 0.08	28.70 ± 0.79	30.23 ± 0.94	2.65 ± 0.07
9,9-DMF	2.68 ± 0.11	<LOQ	<LOQ	0.85 ± 0.12	1.64 ± 0.03
2,6-DIPN	nd	nd	<LOQ	nd	<LOQ
3,3,5,5-TMB	2.32 ± 0.06	0.46 ± 0.02	1.23 ± 0.03	31.27 ± 2.62	nd
4-MBT	4.41 ± 0.16	2.27 ± 0.04	<LOQ	2.67 ± 0.27	8.69 ± 0.72
4,6-DMBT	2.57 ± 0.05	<LOQ	<LOQ	5.35 ± 0.44	12.58 ± 0.60
3,6-DMP	2.95 ± 0.29	2.00 ± 0.03	1.61 ± 0.15	nd	3.85 ± 0.05
1-MPYR	nd	3.77 ± 0.38	1.26 ± 0.03	2.10 ± 0.02	2.11 ± 0.12

nd: Compound not detected in the adhesive

6.3. Migration results

Before the migration studies, the analytical methodology was optimised to extract the analytes from the Tenax. Four extractions with n-hexane were carried out, but only the first three extracts contained significant amounts of analytes. Therefore, the optimal number of extractions was set at three. The extraction efficiency was evaluated by recovery tests. The results of these tests (see Table 4) show that except for the heaviest analytes (4,6-DMDBT, 3,6-DMP and 1-MPYR), the three extractions were sufficient to achieve recoveries higher than 91%.

Table 4. Recovery percentages obtained in the extraction of 23 μg of the marker compounds per g^{-1} of Tenax and their coefficients of variation (n = 3).

Compounds	Recovery (%)	RSD (%)
2MN	106.2	3.84
1MN	103.8	5.23
BP	102.1	3.35
2,6MN	100.5	3.52
ACE	100.7	2.97
9,9-DMF	99.2	2.24
2,6-DIPN	103.0	2.56
3,3,5,5-TMB	92.3	6.27
4-MBT	92.6	3.60
4,6-DMBT	85.9	1.69
3,6-DMP	83.3	1.90
1-MPYR	83.0	3.41

The migration values of MOAH markers in laminates are shown in Table 5 and are expressed as μg of compounds per dm2 of laminate. Migration percentage was also calculated based on the initial concentration, taking into account the grammage of the adhesive in the laminate. The percentage of migration was between 12 and 75%.

The most volatile compounds (2-MN, 1-MN and 2,6-MN) were not detected in the migration tests as they were likely lost during curing and laminate manufacturing processes. The compounds with the highest migration values were BP and ACE. Both compounds were detected in samples AD1, AD5, AD6 and AD7. The alkylated PAHs, 9,9'-DMF, 3,3',5,5'-TMB, 3,6-DMP, 1-MPYR and the compounds 4-MBT and 4,6-DMBT were detected in the migration samples from AD1, AD5, AD7 and AD8.

The migration of the MOAH fraction from the laminates was also determined by GC-FID after the extraction of Tenax. MOAH was separated from MOSH by SPE with silver impregnated silica. The quantification was carried out by dividing the chromatogram into MOAH sub-fractions, which were integrated following the procedure recommended by (Bratinova & Hoeskstra, 2019).

Table 5. Migration values of MOAH markers (mean ± SD) expressed as μg of compound per dm^2 of laminate and percentage of migration calculated based on the initial concentration of the markers in the adhesive.

Compounds	MOAH marker migration μg dm^{-2} ± SD /(%)					
	LOD	Laminate AD1	Laminate AD5	Laminate AD6	Laminate AD7	Laminate AD8
2-MN	0.354	nd	nd	nd	nd	nd
1-MN	0.323	nd	nd	nd	nd	nd
BP	0.156	4.23 ± 0.23 (56)	5.64 ± 0.11 (61)	3.23 ± 0.25 (50)	21.33 ± 1.95 (42)	<LOD
2,6-MN	0.229	nd	nd	nd	nd	nd
ACE	0.045	0.69 ± 0.01 (24)	1.01 ± 0.05 (36)	13.46 ± 1.48 (38)	8.52 ± 0.87 (19)	<LOD
9,9-DMF	0.093	nd	nd	nd	nd	1.48 ± 0.19 (75)
2,6-DIPN	0.038	nd	nd	nd	nd	nd
3,3,5,5-TMB	0.040	0.62 ± 0.04 (60)	nd	nd	12.22 ± 0.48 (21)	nd
4-MBT	0.126	1.54 ± 0.03 (34)	1.74 ± 0.08 (61)	nd	1.84 ± 0.06 (46)	1.69 ± 0.20 (16)
4,6-DMBT	0.074	1.61 ± 0.05 (61)	nd	nd	2.54 ± 0.17 (32)	1.77 ± 0.21 (12)
3,6-DMP	0.117	2.00 ± 0.06 (65)	nd	nd	nd	nd
1-MPYR	0.069	nd	1.56 ± 0.11 (33)	nd	1.06 ± 0.07 (33)	nd

The migration values of the MOAH sub-fractions are shown in Table 6. These values are expressed as μg MOAH per dm^2 of the laminate. MOAH was detected and quantified in the five adhesives studied. The limit of quantification of the method was below 2.2 μg dm^{-2}. The average migration of the C16-C25 MOAH sub-fraction reached values from 6.78 to 19.65 μg dm^{-2} of laminate and the concentration of the only C25-C35 sub-fraction detected was 5.39 μg dm^{-2}.

The highest levels of MOAH migration were found in the laminates of adhesives AD1, AD5 and AD7, which were also characterised by presenting the highest number of marker compounds during migration. Figure 4 shows the migration chromatogram of the AD5 adhesive laminate, which presents a MOAH hump in the range of the C16-C25

sub-fraction. All the laminates of the hot melt adhesives studied had a MOAH hump in this range. The AD1 adhesive, in addition to the C16-C25 hump, presented another MOAH hump in the range of the C25-C35 sub-fraction. In none of the laminates was MOAH found in the range C10-C16 or beyond C35.

Table 6. MOAH content in adhesive samples. The values were expressed as μg of the MOAH fraction per dm^{-2} of the laminate.

	MOAH fraction migrated (μg dm^{-2} ± SD)	
Sample	C16-C25	C25-C35
AD 1	19.65 ± 1.06	5.39 ± 0.78
AD 5	9.22 ± 0.32	nd
AD 6	7.48 ± 0.74	nd
AD 7	8.19 ± 0.67	nd
AD 8	6.78 ± 0.28	nd

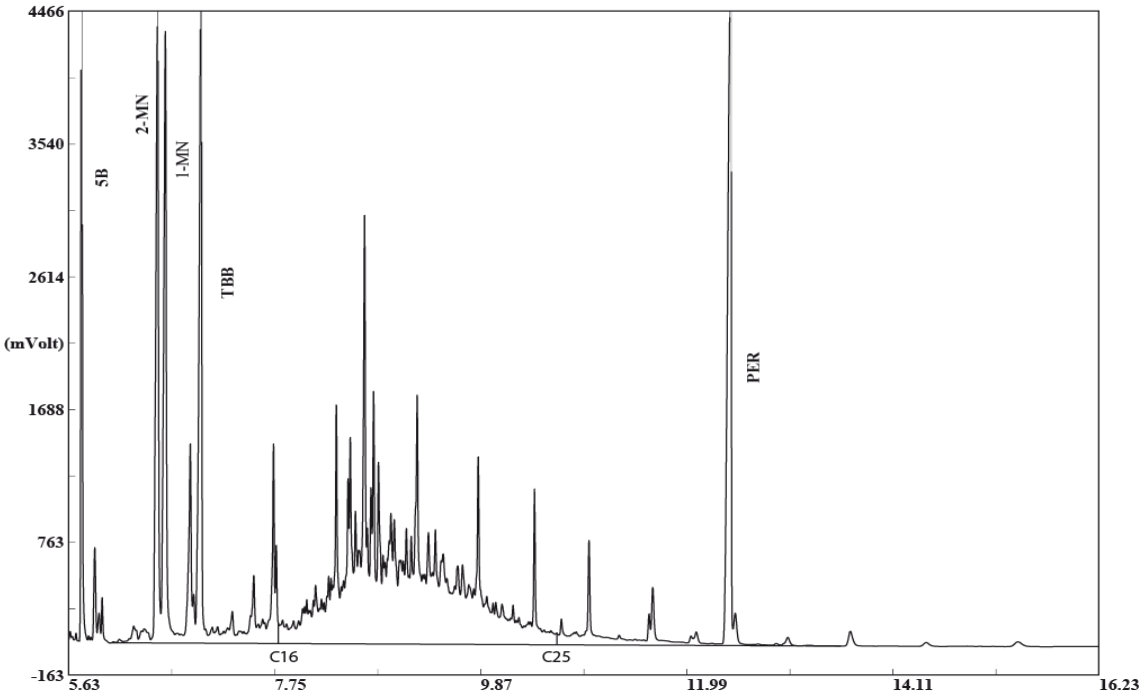


Figure 4. MOAH fraction chromatogram after migration of the AD 5 adhesive to Tenax analysed by GC-FID.

The MOAH C16-C25 sub-fraction belongs to the volatile range of mineral oils, characterized by migrating through the gas phase. The compounds used as chemical markers for MOAH are also within this volatility range (<C25), and their behaviour during migration is similar to the MOAH fraction. It is likely that the migration of the C25-C35 MOAH sub-fraction, heavier and less volatile than C16-C25, occurs by diffusion (Eicher et al, 2015). It is important to note that the cardboard used to manufacture the laminate was examined before the migration tests, and the results revealed that the board was free of MOAH. Therefore, the MOAH detected in the migration tests, like the MOAH markers, had the hot melt adhesive as their only source.

In summary, although the HS-SPME-GC-MS method showed a lower affinity for the higher molecular weight analytes under the tested conditions, the technique proved to be efficient for identifying and quantifying the higher volatile markers, which have chemical structures and similar behaviour as the MOAHs with the potential to migrate.

7. Conclusions

A method of HS-SPME-GC-MS has been developed in order to identify and quantify MOAHs markers in eight hotmelt adhesives used in food contact materials. Both their initial concentrations and migration tests were determined. This method allowed us to obtain a limit of detection between 0.012-0.11μg g^{-1} and good linearity and reproducibility, being a good alternative method suitable for the rapid, direct and reliable detection of MOAH markers from solid samples, without the need for previous treatments.

Five of the adhesives studied contained MOAHs, and the most abundant markers were BP, ACE and 3,3',5,5'-TMB. The compound with the highest percentage of migration was 9,9-DMF. Identifying specific MOAH markers is important to rule out or confirm the presence of MOAH in contaminated samples. According to the results of this investigation, hot melt adhesives based on EVA (ethylene/vinyl acetate) and PSA

represent a possible source of MOAH contamination in food; and eight of the evaluated compounds (BP, ACE, 9,9'-DMF, 3,3',5,5'-TMB, 3,6-DMP, 1-MPYR, 4-MBT and 4,6-DMBT) were suitable for marking MOAH contamination of adhesives.

Finally, the fraction of MOAH migrated was determined by GC-FID. MOAH was characterised by eluting mainly in the C16-C25 range in concentrations between 6.78-19.65 μg dm^{-2} of laminate. The adhesives with the highest migration of MOAH also showed a higher number of markers. GC-FID and HS-SPME-GC-MS can be considered as complementary tools to quantify and check the MOAH contamination, respectively.

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Capítulo 4

Migration of toxic mineral oil aromatic hydrocarbons (MOAH) from cardboard containers to dry food and prediction tool.

1. Resumen
2. Objetivos
3. Esquema de trabajo
4. Introduction
5. Materials and methods
6. Results and discussion
7. Conclusions
8. References

1. Resumen

En esta sección se estudia la migración de hidrocarburos aromáticos de aceite mineral (MOAH) desde envases primario de cartón a alimentos secos, utilizando dieciséis hidrocarburos aromáticos como sustancias modelo, cubriendo una amplia gama de masas moleculares y estructuras químicas. Los experimentos de migración se realizaron utilizando óxido de polifenileno modificado como simulante alimentario y dos alimentos secos: cuscús y polenta. Las pruebas de migración se realizaron para simular el almacenamiento a temperatura ambiente durante períodos prolongados y envases de alimentos calientes como el peor escenario. Se aplicaron algoritmos de análisis multivariante para correlacionar y agrupar la migración de sustancias modelo, y se construyó un modelo de regresión de mínimos cuadrados parciales (PLSR) para predecir la migración en el peor de los casos. Los resultados mostraron fuertes correlaciones en los patrones de migración de las sustancias modelo, basadas en su volatilidad, matriz alimentaria, tiempo de migración y temperatura. Se observó un comportamiento diferente entre la migración de las sustancias modelo más volátiles y las más pesadas.

2. Objetivos

El objetivo principal de este capítulo fue estudiar el comportamiento migratorio de MOAH desde envases primarios de cartón a un simulante alimenticio (Tenax) y dos alimentos secos (cuscús y polenta), en las peores condiciones de tiempo y temperatura. Para ello, se utilizaron dieciséis compuestos modelos que cubrían el rango volátil de MOAH.

Los objetivos específicos fueron:

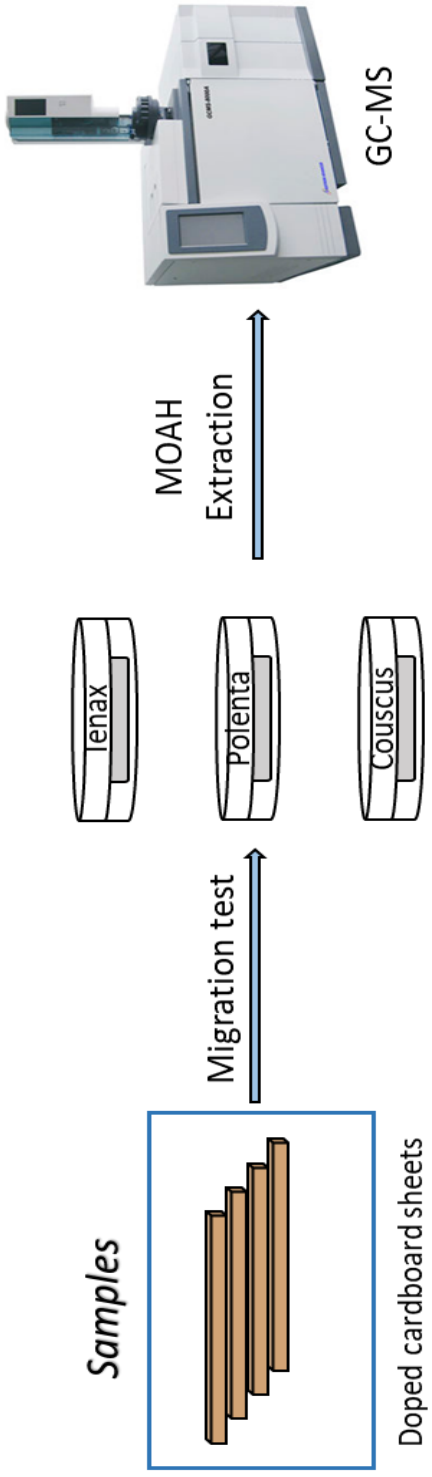
- Contrastación de la migración de MOAH desde el cartón a los alimentos secos con la migración al simulante alimentario.
- Análisis de las diferencias en el comportamiento migratorio de tres sub-fracciones MOAH (C10-C16, C16-C25 y C25-C35).
- Uso de la estadística multivariada para comparar y predecir la migración de MOAH.

Las tareas llevadas a cabo para lograr los objetivos fueron las siguientes:

- Selección de sustancias modelo que representen a MOAH en una amplia gama de masas moleculares y estructuras químicas.
- Clasificación de las sustancias modelos en las tres subfraciones MOAH estudiadas (C10-C16, C16-C25 y C25-C35).
- Aplicación de métodos de extracción y análisis de MOAH en el cartón empleado para verificar la ausencia de MOAH.
- Extracción y análisis de las sustancias modelo retenidas en el cartón después de las etapas de enriquecimiento y evaporación del solvente.
- Extracción y análisis de las sustancias modelos remanentes en el cartón después de la migración.

- Realización de ensayos de migración y determinación de las sustancias modelos migradas.
- Aplicación de técnicas de análisis multivariadas (HCA, PCA y PLSR) para examinar el grado de asociación de las sustancias modelo e identificar posibles patrones de migración.

3. Esquema de trabajo



Esquema 1. Diseño experimental del Capítulo 1.

4. Introduction

Paper and cardboard are frequently used as primary, secondary and tertiary food packaging materials; they are intended to contain, protect, transport and store food products. However, several scientific studies have shown that cardboard and paper packaging can be contaminated with mineral oil hydrocarbons (MOH), which could migrate from packaging to food in significant quantities (Biedermann & Grob, 2010; Buist et al., 2020; Dima, Verzera, & Grob, 2011; Lorenzini et al., 2010; Vollmer et al., 2011).

MOHs are complex mixtures of hydrocarbons that come mainly from petroleum; they are divided into mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). MOSH has been associated with the formation of hepatic microgranulomas; however, due to the low incidence of lipogranulomas in the population, they are not considered toxicologically dangerous (Bevan et al., 2020). On the other hand, the MOAH fraction has been considered the most dangerous for human health since the presence of three or more aromatic rings in these compounds can present genotoxic and carcinogenic activity (Carrillo et al., 2019; EFSA, 2012). This fraction is mainly composed of alkylated mono or polycyclic aromatic hydrocarbons and also contains, to a lesser extent, non-alkylated aromatic hydrocarbons and aromatic compounds with heteroatoms, mostly sulphur (EFSA, 2012).

There are several sources of MOAH: mineral oil-based offset inks used to decorate the surface of cardboard, recycled paper fibres, lubricants, waxes, adhesives, and processing aids used during the manufacture of packaging (Biedermann & Grob, 2010; Laine, Pitkänen et al., 2016).

Migration from cardboard or paper depends on the structure of the material, the concentration of the migrant and its chemical-physical properties, the storage periods, environmental conditions, and the food type (Arvanitoyannis & Kotsanopoulos, 2014; Poça et al., 2011). The movement of migrants through the porous structure of cardboard or paper occurs through adsorption/desorption processes, which are limited by the

chemical nature of the cellulosic fibre and the migrant (Poças et al., 2011). The gaseous phase is the main route by which mineral oils are transferred to food, and therefore the volatile fraction is the most important one (Biedermann & Grob, 2012; Fiselier & Grob, 2011). Nevertheless, migration by direct contact is also relevant and has to be taken into account (Barp et al., 2015; Eicher et al., 2015; Pack et al., 2020). There are no quantitative measures for the toxic potency of individual MOAH, but according to EFSA, they could be one or two orders of magnitude higher than that of MOSH. For this reason, EFSA considers that the intake values of MOAH that are supposed to come from migration from paper and cardboard (between 0.008 and 0.022 mg/kg bw/day for children and around 0.004 and 0.011 mg/kg bw/day for adults) could be a health concern (Buist et al., 2020; EFSA 2012).

The migration study of MOAH from cardboard or paper to food is an arduous task. In addition to the complex chemical and physical processes that occur during migration, the MOAHs are made up of numerous isomers covering a wide range of volatility. Another challenging task is to find food packaging that contains different MOAHs at high enough concentration levels. To simplify this problem, some researchers have used surrogate substances to model the migration of MOHs from paper and study the barrier properties of different materials against MOHs (Ewender, Franz, & Welle, 2012; Guazzotti et al., 2015; Laine et al., 2016). Recently, Fengler and Gruber (2020) used surrogates to investigate MOSHs and MOAHs migration from paper packaging to two food simulants, Tenax and Sorb-Star (Fengler & Gruber, 2020).

There is no standardised method to test the migration of MOAH from cardboard materials. Although some authors maintain that Tenax (modified polyphenylene oxide) overestimates migration, it continues to be the simulant recommended to assess migration from cardboard (AENOR, 2004) and plastic materials (European Commission, 2011) intended to be in contact with dry food. Furthermore, the EU regulation 10/2011 suggests simulating the worst conditions of time and temperature during migration tests and increasing temperature to accelerate the migration in food stored for long periods (European Commission, 2011).

The main objective of this research was to study the migration behaviour of MOAHs from cardboard packaging materials to Tenax, as a food simulant, and to two different dry food samples, couscous and polenta. For this purpose, cardboard samples were previously fortified with 16 model substances that represent MOAH in a wide range of molecular masses and chemical structures. The use of model substances would provide more detailed information on the processes that occur during the migration of MOAH and facilitates the construction of a PLSR model to predict MOAH migration.

5. Materials and methods

5.1. Reagents and samples

Analytical standards: 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2–MN), biphenyl (BP), 2,6-dimethylnaphthalene (2,6-DMN), acenaphthene (ACE), 2,6-diisopropylnaphthalene (2,6-DIPN), 3,3',5,5'-tetramethylbiphenyl (3,3',5,5'-TMBP), 4-methyldibenzothiophene (4-MDBT), 4,6-dimethyldibenzothiophene (4,6-DMDBT), 1-methylpyrene (1-MPYR), benzo(b)naphtha(1,2-d)thiophene (BNT), chrysene (CHRY), benzo(b)fluoranthene (BbF), perylene (PER); and the standard mixture of saturated alkanes (C7-C40) of 1000 μg mL^{-1} for each component in n-hexane were purchased from Sigma-Aldrich (Madrid, Spain). While 9,9'-dimethylfluorene (9,9-DMF) was supplied by Tokyo Chemical Industry CO., LTD, and 3,6-dimethylphenanthrene (3,6-DMP) was obtained from Dr Ehrenstorfer (Augsburg, Germany). Acetone, n-hexane, and ethanol absolute for HPLC were from Panreac (Barcelona, Spain). Tenax TA 60/100 mesh was supplied by Supelco (Bellefonte, USA).

The cardboard used in the migration tests had a thickness of 1 mm and a grammage of 412 g m^{-2} and came from a container for dry food obtained in a Spanish supermarket. The dry foods evaluated, corn grits (polenta) and wheat semolina (couscous) of medium grain, were also bought in local commerce. Both the cardboard and the dry foods were previously analysed to verify that they were free of MOAH.

5.2. Model substances

Sixteen aromatic hydrocarbons were selected as model substances for the study. They were selected because they represent MOAH in a wide range of volatility, molecular weight, boiling points, and molecular structure. These compounds included alkylated and non-alkylated aromatic hydrocarbons, biphenyls and heterocyclic aromatic sulphur compounds, ranging from142 to 252 Da (see Table 1), all were associated to the presence of MOAH in various types of minerals oils and proposed as MOAH markers (Jaén et al., 2021).

Another selection criteria were based on the probability of studying the behaviour of the MOAH sub-fractions during migration. MOAH chromatograms are usually divided into sub-fractions, defined according to the retention time of a mixture of n-alkanes, analysed under the same chromatographic conditions as MOAH (Bratinova & Hoeskstra, 2019; Gruber et al., 2019). Selected substances elute in the range of MOAH sub-fractions noted for their potential to migrate C10 to C16 (1-MN, 2-MN, BP, 2,6-DMN, ACE, 9,9'-DMF); C16 to C25 (2,6-DIPN, 3,3', 5,5'-TMBP, 4-MDBT, 4,6-DMDBT, 3,6-DMP, 1-MPYR, BNT) and C25-C35 (CHRY, BbF and PER). The location of the model substances in the different sub-fractions was carried out as indicated by Bratinova & Hoeskstra (Bratinova & Hoeskstra, 2019).

5.3. Spiking procedure for the cardboard

Before the migration assays, cardboard sheets with an area of 0.08 dm^2 were prepared. These sheets were spiked with 50 µL of a standard solution of 350 mg L^{-1} of the model substances. The standard solution was evenly distributed with a syringe throughout the cardboard sheet. After that, the spiked cardboard sheet was left dry at room temperature for one hour, and subsequently, migration tests were carried out with dry food samples and Tenax.

5.4. Migration test

The migration tests were carried out with two dry foods (polenta and couscous) and a food simulant (Tenax). Before the migration tests, Tenax was purified by Soxhlet extraction with acetone for 6 h and subsequently dried in the oven at 160 °C for 6 h (AENOR, 2004). The dry food samples were subjected to extraction with n-hexane and analysis by GC-MS to verify that they were not contaminated with MOAH.

The time and temperature conditions for the migration tests were selected based on Regulation EU/10/2011 for plastics (European Commission, 2011) and considering the intended uses of cardboard materials in the worst conditions. These conditions were: 2 h at 70 ºC in the case of hot food containers; and 10 days at 60 ºC to simulate storage at room temperature for long periods. In addition, migration at 60 ºC was also evaluated at 3 and 6 days in order to evaluate the migration kinetics.

The cardboard sheets spiked with the model substances were placed in direct contact with the dry food and the food simulant. For the tests with Tenax, 0.32 g of the food simulant were used according to the 4 g dm^{-2} ratio established by UNE-EN-14338 (AENOR, 2004). However, in the case of dry food, it was necessary to use 0.65 g to completely cover the surface of the cardboard and form a uniform layer. Finally, the whole was wrapped in aluminium foil, placed in a glass Petri dish of 6 cm in diameter and placed in the oven under the migration conditions. Three replicates and a blank were prepared for each assay.

5.5. Extraction of model substances from cardboard

To know the concentration of the model substances retained in the cardboard after the spiked and solvent evaporation stages, 7 spiked cardboard sheets were extracted following the conventional procedures for extracting mineral oils from cardboard and paper samples (BfR& KLZH, 2012; Lorenzini et al., 2010; Vollmer et al., 2011) with slight modifications. The procedure was as follows: the cartons enriched with the model substances were dried for one hour at room temperature and afterwards cut into small

pieces with a border of approximately 0.5 cm and placed in a 20 mL glass vial. Subsequently, 15 mL of n-hexane/ethanol mixture (1: 1) were added to each vial and placed in an ultrasonic bath for 2 h. Then, the extract was decanted into another vial, and 5 mL of water were added to separate the n-hexane from the ethanol. The supernatant was separated and concentrated under a gentle stream of nitrogen gas to 1 mL.

The same procedure was used to analyse the cardboard samples after migration and determine the concentration of the analytes remaining in the cardboard.

5.6. Extraction of migrated model substances from dry foods and food simulants

After migration, dry food, as well as Tenax samples, were transferred to 20 mL vials. Subsequently, 4 mL of n-hexane were added to each vial, and they were placed in an ultrasonic bath at room temperature for 1 h. After this time, the n-hexane extract was transferred to a clean vial, and the sample was subjected to two consecutive extractions following the same procedure. The extracts collected from each replica were pooled in a vial and concentrated under a gentle stream of nitrogen gas at 40 ºC to 1 mL.

5.7. GC-MS

For the analysis, a gas chromatograph 7820A GC coupled to a single quadrupole mass spectrometer 5977B detector from Agilent Technologies (Santa Clara, CA, USA) was used. It was equipped with an electron ionisation (EI) ion source and a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). A chromatographic column HP-5MS of 30 m length x 25 mm inner diameter x 0.25 μm film thickness from Agilent was used. Helium was used as carrier gas at a constant flow of 1 mL min^{-1}. The injection was performed at 270 ºC in splitless mode, injection volume was 1 μL. The oven temperature program was as follows: initially 40 °C for 5 min, 10 °C min^{-1} to 300 °C and held at 300 °C for 10 min. MS analysis was carried out in SIM mode with 7 min solvent delay.

5.8. Statistical analysis

Unscrambler X10.3 software (Camo Analytics) was used for Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA), and Partial Least Squares Regression (PLSR).

For the PCA and HCA execution, the migration data were organised in a matrix with 16 variables (model substances) and 12 samples (migration tests). The PLSR model was built based on five predictor variables (boiling point, molecular mass, log P, solubility and migration time), and the response variable was the percentage of migration of the model substances in Tenax, polenta and couscous at 60 ºC. Except for the migration time, the values of the predictor variables were taken from ChemSpider database.

6. Results and discussion

6.1. Model substances in the cardboard

Once the samples were analysed and it was confirmed that they were free of MOAHs, the cardboards were spiked with the model substances. The concentration of the model substances adsorbed by the donor cardboard and their physical properties are shown in Table 1. The aim of the study was to study the migration behaviour of different MOAHs, in a wide range of polarities and chemical structures, from cardboard to Tenax and two different dry foods, under different migration conditions. Real samples would not contain so many MOAHs and, in addition, concentrations would be very low, making more difficult the analysis. For this reason, it was considered more interesting to spike the cardboards with a great variety of MOAHs that could reflect the migration behaviour of MOAHs with different structures, volatility or molecular weights.

A high-concentration solution of the model substances (350 mg L^{-1}) was used to spike the donor cardboard. The reason was to ensure that, despite the possible loss of

analytes due to volatilisation during the spiking analytes, drying or solvent evaporation, the amount adsorbed on the cardboard was high enough to provide reliable results.

In the event that the donor cardboard had adsorbed all the model substances, the expected concentration would be approximately 0.18 mg dm^{-2}. However, after evaporation of the solvent, the concentration detected ranged between 0.05 and 0.18 mg dm^{-2} with RSD values between 2 and 10% (see Table 1). The loss by evaporation of volatile substances during the cardboard spiking and drying process was evident. Substances with a low boiling point were the most affected. The heaviest substances had the highest concentrations in the donor cardboard. This concentration (Table 1) was considered the initial concentration of the model substances in the cardboard for future calculations.

Table 1. Physical properties of the substances used to model MOAH migration and the concentration (mg dm^{-2} ± RSD) adsorbed by the donor cardboard.

Compound	Molecular formula	MM (Da)	Log P	Boiling point (ºC)	Donor cardboard (mg dm^{-2} ± RSD)
2-methylnaphthalene	$C_{11}H_{10}$	142.197	3.91	239.9 ± 3.0	0.05 ± 4.38
1-methylnaphthalene	$C_{11}H_{10}$	142.197	3.91	242.8 ± 3.0	0.05 ± 4.94
Biphenyl	$C_{12}H_{10}$	154.208	3.98	258.0 ± 7.0	0.07 ± 5.38
2,6-dimethylnaphtalene	$C_{12}H_{12}$	156.224	4.37	264.4 ± 10.0	0.07 ± 5.91
Acenaphtene	$C_{12}H_{10}$	154.208	4.19	279.0 ± 0.0	0.08 ± 4.12
9,9'-dimethylfluorene	$C_{15}H_{14}$	194.272	5.20	300.8 ± 12.0	0.09 ± 8.54
2,6-diisopropylnaphtalene	$C_{16}H_{20}$	212.330	6.13	305.8 ± 12.0	0.11 ± 5.38
3,3',5,5'-tetramethylbiphenyl	$C_{16}H_{18}$	210.314	5.82	300.9 ± 37.0	0.11 ± 5.98
4-methyldibenzotiophene	$C_{13}H_{10}S$	198.283	4.84	349.0 ± 11.0	0.12 ± 9.72
4,6-dimethyldibenzotiophene	$C_{14}H_{12}S$	212.310	5.30	364.9 ± 11.0	0.13 ± 5.99
3,6-dimethylphenanthrene	$C_{16}H_{14}$	206.282	5.60	363.0 ± 0.0	0.14 ± 2.19
1-methylpyrene	$C_{17}H_{12}$	216.277	5.63	387.4 ± 9.0	0.15 ± 8.54
Benzo(b)naphtho(2,1-d)tiophene	$C_{16}H_{10}S$	234.316	5.61	434.3 ± 14.0	0.16 ± 8.00
Chrysene	$C_{18}H_{12}$	228.288	5.91	448.0 ± 0.0	0.18 ± 6.84
Benzo(b)fluoranthene	$C_{20}H_{12}$	252.309	6.40	467.5 ± 12.0	0.17 ± 6.36
Perylene	$C_{20}H_{12}$	252.309	6.40	467.5 ± 12.0	0.17 ± 5.05

6.2. Migration in the food simulant (Tenax)

For the purpose of this discussion, it should be remembered that the model substances represent three MOAH sub-fractions. Based on the elution range of the n-alkanes (Bratinova & Hoeskstra, 2019; Gruber et al., 2019), the most volatile substances in this study, 1-MN, 2-MN, BP, 2,6-DMN, ACE, 9,9-DMF, were located in C10-C16 fraction; those of intermediate volatility, 2,6-DIPN, 3,3',5,5'-TMBP, 4-MDBT, 4,6-DMDBT, 3,6-DMP, 1-MPYR, BNT, were eluted in the range C16-C25; and the heaviest ones, CHRY, BbF and PER, in the range C25-C35.

It is also important to note that the migration results of the model substances in Tenax and dry foods are expressed as a percentage related to the concentration detected in the spiked cardboard after the drying step and solvent volatilisation (see Table 1).

The migration results of the model substances in Tenax are presented in Figure 1 A. As it can be seen, the migration percentage for the most volatile substances in the elution range C10-C16 was greater than 94%; besides, these substances reached their maximum migration on the third day. The substances with retention time between C16-C25 had a more heterogeneous behaviour with migrations ranging from 40-98%; the percentage of migration of this fraction decreases as a function of the model substances' molecular weight. Conversely, the heaviest compounds (eluted between C25-C35) with a slower migration rate reached equilibrium on the sixth day and migrated below 45%.

The migration of model substances decreased when increasing carbon number, and the heaviest compounds needed more time to reach the equilibrium. When dry food is in direct contact with cardboard, migration occurs through two mechanisms, gas phase and diffusion (Poças et al., 2011). In other words, model substances are transferred to dry foods both by gas and by diffusion depending on their volatility. The main mechanism of migration of volatile substances is through the gas phase; these compounds evaporate and then condense on the Tenax (Biedermann & Grob, 2012), and their migration is faster, while the heavier compounds migrate mainly by diffusion and reach equilibrium more slowly.

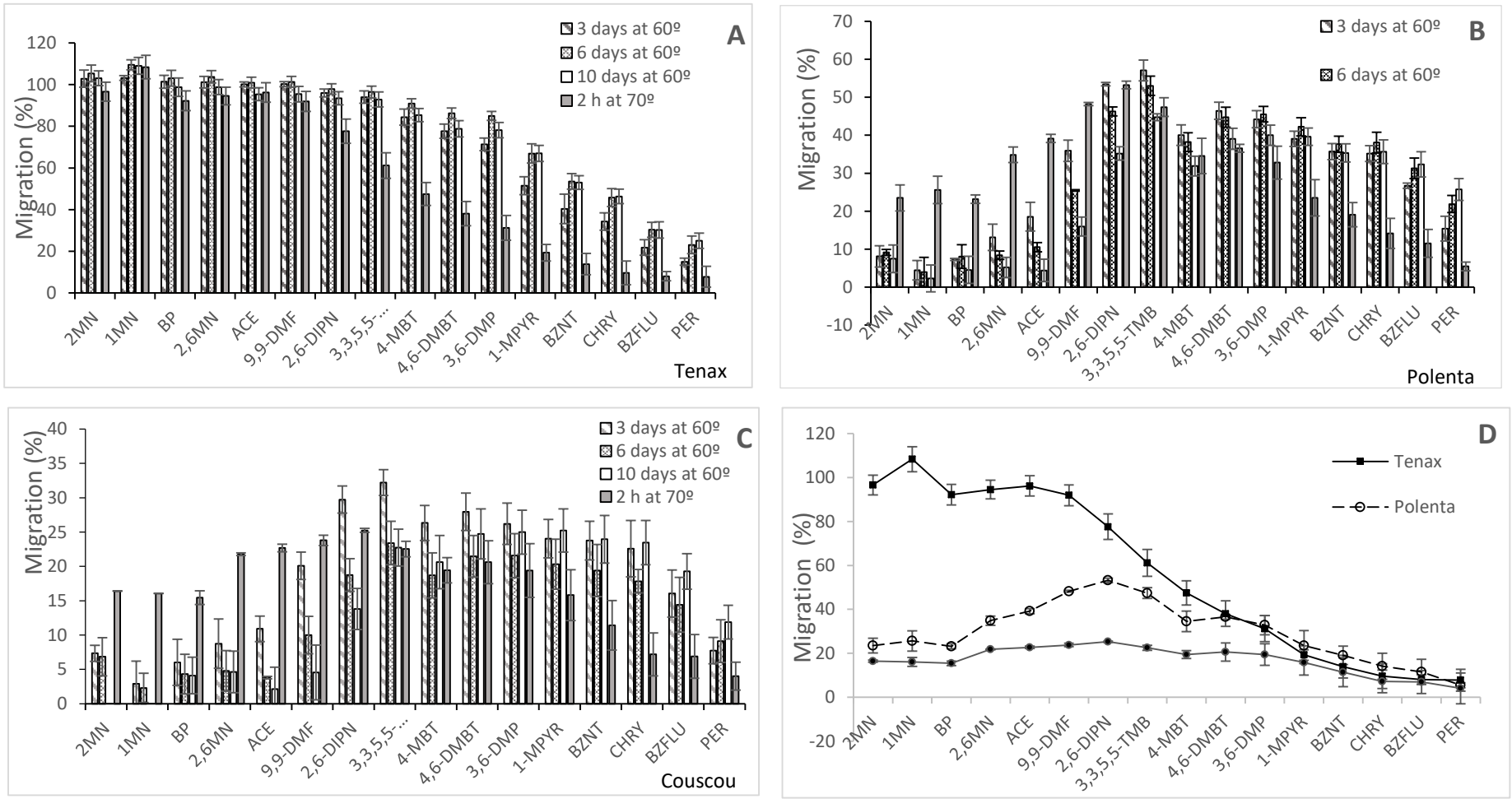


Figure 1. Migration of model substances at 60ºC in different storage periods (3, 6 and 10 days) to Tenax (A), Polenta (B) and Cuscus (C). Migration of model substances at 70º for 2 h to Tenax, polenta and couscous (D). MOAH sub-fractions: C10 to C16 (1-MN, 2-MN, BP, 2,6-DMN, ACE, 9,9-DMF), C16 to C25 (2,6-DIPN, 3,3 ',5,5'-TMBP, 4-MDBT, 4, 6-DMDBT, 3,6-DMP, 1-MPYR, BNT) and C25-C35 (CHRY, BbF and PER).

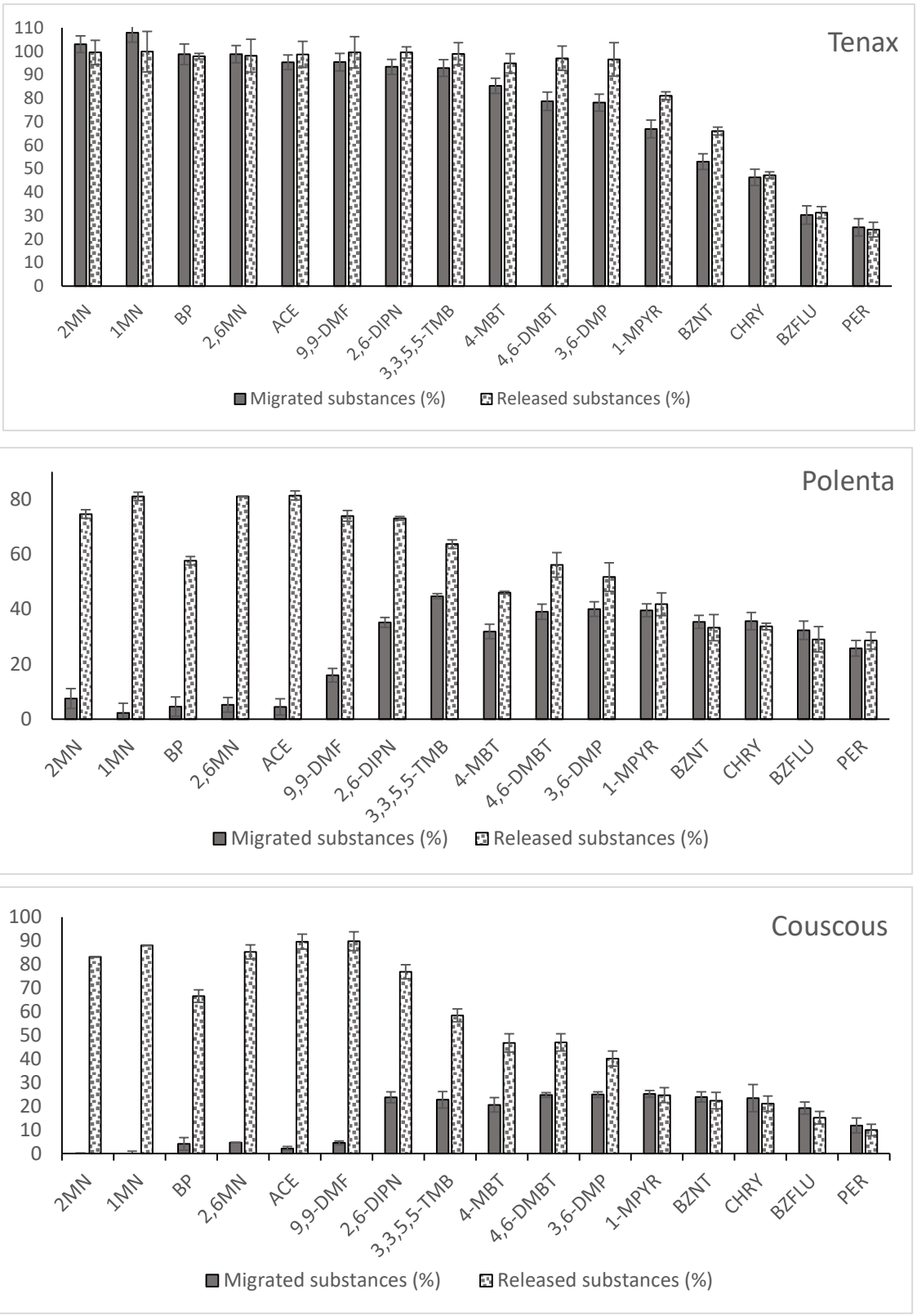


Figure 2. Comparison in percentage (%) of the model substances released by the donor cardboard during 10 days at 60 ºC and those retained by Tenax, polenta and couscous, respectively.

In addition, gradual losses in the percentage of migration the model substances of C16-C25 fraction were observed, which became more evident after 10 days of migration (Figure 1A). It can be assumed that these losses are linked to desorption processes in Tenax favoured by temperature and migration time, already reported by (Nerín, Contín, & Asensio, 2007). In order to confirm this hypothesis, the model substances retained in the donor cardboard were extracted and analysed after migration; the results obtained were used to calculate the total percentage of substances released by the donor cardboard. Figure 2 compares the percentage of model substances released by the donor cardboard at 60ºC for 10 days, with the percentage retained in the Tenax under the same conditions. The results showed that between 6 to 18% of the C16-C25 substances released by the cardboard were not found in the Tenax.

Figure 1A also shows the behaviour of the model substances when the spiked cardboard was subjected to temperature close to 70 °C for short periods (2 h). Under these conditions, the percentages of migration of the most volatile substances (C10-C16) were very similar to those reached in longer periods of migration; however, the heaviest substances that migrate mainly by diffusion presented migration percentages lower than those obtained under longer storage conditions. Thus confirming that the more volatile hydrocarbons migrate faster and that their transfer rate from cardboard to food decreases with volatility (Biedermann & Grob, 2012; Hauder, Benz et al., 2013).

6.3. Migration to dry foods

Figures 1B and 1C show the migration of model substances to polenta and couscous, respectively. As can be seen, the substances that model the C10-C16 fraction showed the lowest migration percentages and, in some cases, a tendency to decrease over time. In polenta, the migration of these substances was below 40%, while in couscous, it was below 25%. For some compounds such as 2-MN and 1-MN, no migration was observed in couscous after 10 days of migration. Probably the desorption phenomenon was more intense in the food matrix than in the Tenax.

On the other hand, the migration of C16-C25 fraction was below 60% in polenta and 35% in couscous. Most of these compounds reached their maximum migration after three days, observing that in some cases, these compounds also showed a gradual loss of their concentration over time, as it was previously observed in Tenax. The percentage of substances released by the cardboard was compared to the percentage of substances detected in polenta and couscous (see Figure 2), clearly observing that a significant percentage of the model substances C10-C16 and C16-C25 released by the cardboard during migration, were not found in dry food. Therefore, 0% migration (in the case of undetected analytes) does not mean that migration did not occur, but that the dry food did not retain the analytes, and 100% migration would mean that the dry food adsorbed all the analytes released by the cardboard.

In contrast, the substances used to model the C25-C35 fraction of MOAH migrated slowly, at lower concentrations (see Figures 1B and 1C) and did not show losses related to the desorption processes (see Figure 2). These results agree with the results found by Eicher et al. (2015), concerning the migration of hydrocarbons with high boiling points, by direct contact, which is lower and slower than that of volatile hydrocarbons; however, it is not negligible (Eicher et al., 2015), and can represent a significant source of contamination for dry food.

In general, the content of the model substances in polenta was higher than in couscous. This can be attributed to the fact that the couscous particles were larger in diameter than polenta and therefore had a smaller exposed surface area, thus reducing the adsorption capacity of the food. On the other hand, Tenax is a fine, porous solid with a greater exposed surface and greater adsorption capacity than the tested dry foods. Consequently, the migration of MOAHs C10-C16 and C16-C25 was faster, and the migration values were greater than in dry food. However, the heavier substances (fraction MOAH C25-C35) showed a migratory behaviour to dry foods quite similar to Tenax, especially to polenta. The migratory behaviour of these substances confirms the good properties of Tenax as a food simulant for dry food, since migration values below the specific migration limit in this simulant will guarantee the safety of consumers as

was also published by Triantafyllou et al. (Triantafyllou, Akrida-Demertzi, & Demertzis, 2007).

Figures 1B and 1C also show the migration of the model substances in polenta and couscous at 70º C for 2h. The migration values of the fraction C10-C16 were higher than those obtained in prolonged storage conditions at 60ºC, thus indicating that the temperature plays a more important role than the exposure time. In the case of the C16-C25 fraction, it is evident that the increase in temperature also influences the migration mechanism, which could make substances with intermediate volatility more accessible for being transferred through the gas phase (Lorenzini et al., 2010). While in the heavier compounds of the C16-C25 fraction (3,6-DMP, 1-MPYR, BZNT) and the C25-C35 fraction, migration decreased as the number of carbons increases in the model substances. Furthermore, compared to migration in long storage periods, migration percentage was lower, suggesting that the exposure time was more important than the temperature.

Finally, Figure 1D compares the migration of the model substances in Tenax, polenta and couscous at 70 ºC for 2 h. The migration of the most volatile substances (C10-C16) was significantly higher in Tenax than in dry foods; however, for substances of intermediate volatility (fraction C16-C25), the migratory difference between Tenax and dry foods decreased with the increasing weight of the model substances. For the heavier substances (fraction C25-C35), the behaviour in Tenax is similar to that of dry foods. These facts also confirm that Tenax is a good solid food simulant, as it overestimates the migration of most types of solid food, as expected from a safety point of view.

As the paper and board samples were spiked with MOAHs, risk assessment of these samples cannot be evaluated. Initial analysis of MOAHs in the samples before being spiked confirmed that in this case, none of the samples contained MOAHs, what confirms that these samples are safe concerning the MOAHs.

6.4. Multivariate statistical analysis

In order to study the degree of association of model substances and identify possible migration patterns, known statistical tools of multivariate analysis were used, such as principal component analysis (PCA), hierarchical cluster analysis (HCA) and Partial least squares regression (PLSR).

Based on the similarity of the experimental results obtained at 60 ºC and 70 ºC, HCA classified the model substances into two large groups of compounds (see dendrogram of Figure 3). The first group comprised the 6 most volatile substances (fraction MOAH C10-C16), and the second group included the 10 remaining substances. This classification was reinforced by the results observed in the model substances' correlation matrix (Table S1). The correlation matrix showed, with a significance below 0.05, that the most volatile compounds (C10-C16) had a positive and robust direct correlation among them, thus indicating that the migratory factors studied affect this type of substances in the same way and that its behaviour during migration was similar.

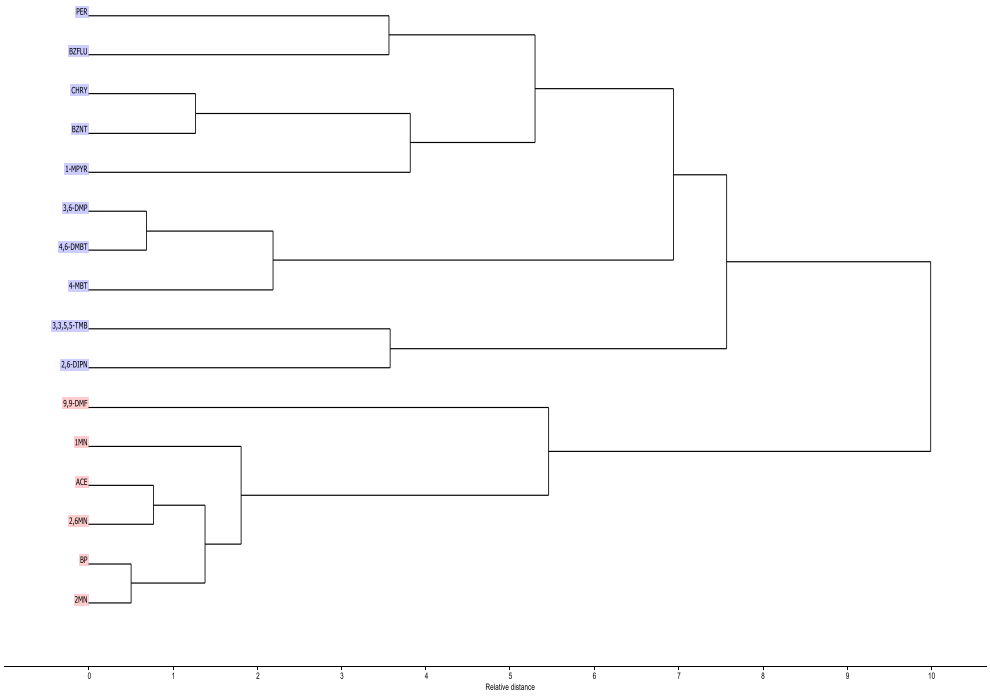


Figure 3. Dendrogram of the migration of model substances

Table 2. Correlation matrix of the migration of model substances.

	2MN	1MN	BP	2,6MN	ACE	9,9-DMF	2,6-DIPN	TMB	4-MBT	4,6-DMBT	3,6-DMP	1-MPYR	BZNT	CHRY	BZFLU	PER
2MN	1.0000															
1MN	0.9977	1.0000														
BP	0.9991	0.9972	1.0000													
2,6MN	0.9959	0.9965	0.9969	1.0000												
ACE	0.9900	0.9915	0.9906	0.9975	1.0000											
9,9-DMF	0.9774	0.9751	0.9768	0.9852	0.9917	1.0000										
2,6-DIPN	0.9297	0.9178	0.9279	0.9330	0.9409	0.9749	1.0000									
TMB	0.8713	0.8482	0.8712	0.8656	0.8660	0.9120	0.9750	1.0000								
4-MBT	0.9037	0.8804	0.9060	0.8959	0.8861	0.9145	0.9544	0.9814	1.0000							
4,6-DMBT	0.8162	0.7862	0.8187	0.8062	0.7951	0.8393	0.9151	0.9757	0.9840	1.0000						
3,6-DMP	0.7687	0.7360	0.7710	0.7542	0.7379	0.7839	0.8711	0.9497	0.9643	0.9940	1.0000					
1-MPYR	0.6236	0.5867	0.6245	0.5985	0.5734	0.6271	0.7436	0.8611	0.8786	0.9399	0.9700	1.0000				
BZNT	0.5004	0.4594	0.5013	0.4699	0.4438	0.5069	0.6464	0.7895	0.7997	0.8826	0.9235	0.9854	1.0000			
CHRY	0.3398	0.2962	0.3400	0.3018	0.2753	0.3471	0.5110	0.6794	0.6745	0.7803	0.8324	0.9325	0.9787	1.0000		
BZFLU	0.1267	0.0848	0.1242	0.0830	0.0573	0.1351	0.3217	0.5075	0.4760	0.6073	0.6726	0.8137	0.8870	0.9562	1.0000	
PER	0.2957	0.2605	0.2889	0.2450	0.2125	0.2709	0.4248	0.5816	0.5637	0.6643	0.7242	0.8456	0.8842	0.9239	0.9584	1.0000

The correlation for the rest of the substances showed considerable variability with positive correlation coefficients ranging from moderate to strong; these variations might result from the difference between the model substances' boiling points (Table 1). In contrast, the correlation between the most volatile compounds and the heaviest ones was low, showing different behaviours during migration.

Figure 4 shows the PCA score graph for the migration values of the model substances for all the test conditions. For the construction of the graph, two principal components (PC1 and PC2) were chosen because these two components explained 98% of the total variance (Figure 5), and PC1 retained 91% of the data variability. The tests with Tenax showed a markedly positive charge on PC1, associated with high migration values, especially for the most volatile compounds. Polenta and couscous migration samples were grouped on the left side of the chart, showing a more similar pattern. The chart also shows that migration test performed at 70 ºC had, in general, higher values on PC2 than migration test performed at 60 ºC, which is associated with higher migration values of the heaviest compounds at 60 ºC, probably because migration time in these experiments was longer.

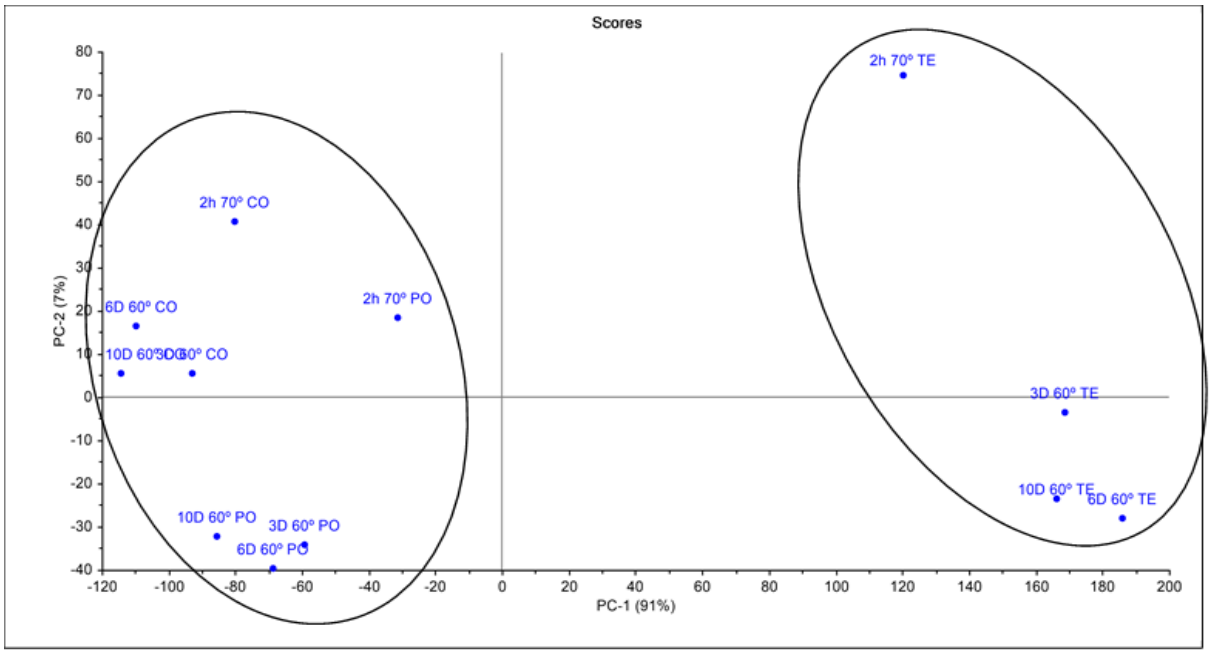


Figure 4. PCA score plot of model substance migration

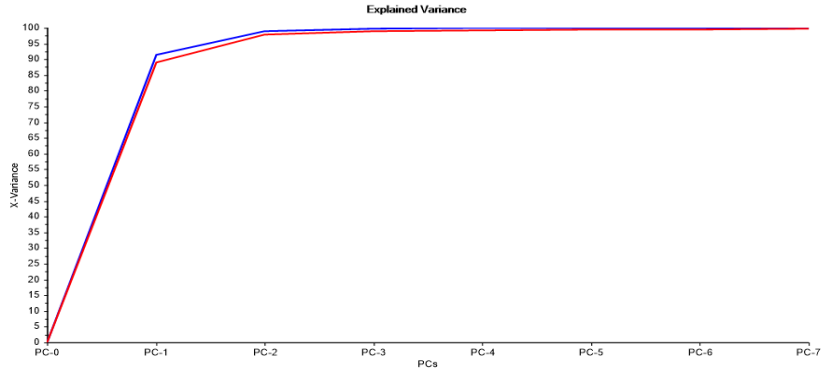


Figure 5. Plot of cumulative variance versus principal components

The possibility of predicting the migration of model substances in polenta, couscous and Tenax was explored by constructing PLSR models. The predictive capacity of the PLSR models was evaluated using the correlation coefficient (R2) and the root mean square error of estimation (RMSE). The results showed a large dispersion of the data and little linearity for polenta and couscous. In contrast, the PLSR model for Tenax (see Figure 3) shows good linearity with R2 values above 0.91 and RMSE less than 10%.

In other words, Tenax showed the best predictive capacity, and precise models could not be created to predict migration in polenta and couscous due to the variability of the data with respect to the storage period. Since the migration of the model substances in dry foods was lower than the migration in Tenax, the PLSR could be used to predict the migration of these compounds in the worst case.

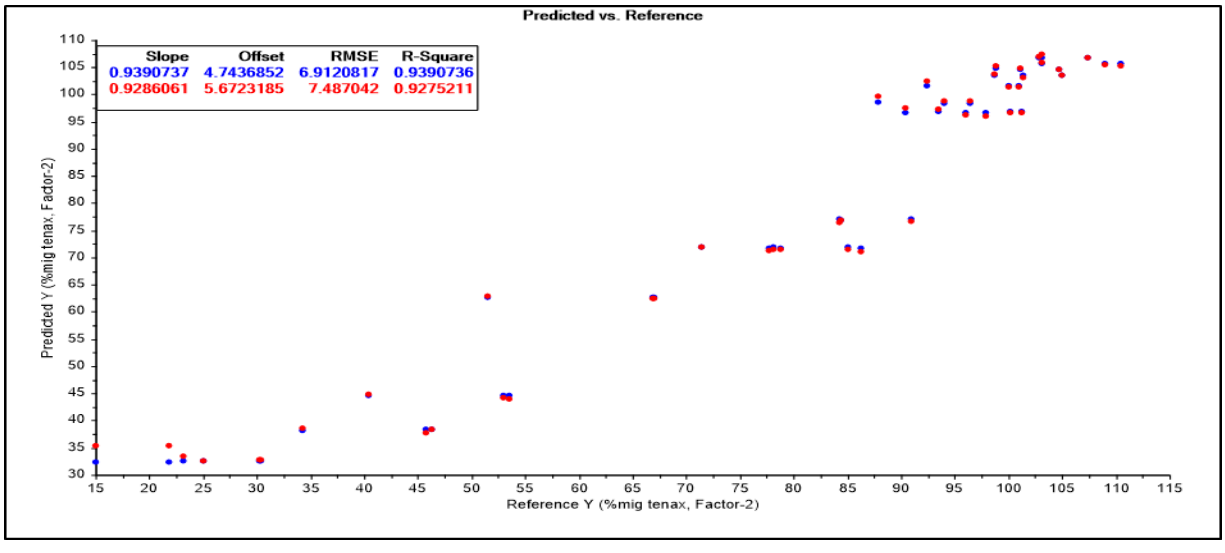


Figure 3. Predicted migration versus actual migration at 60ºC in Tenax.

7. Conclusion

The differences in the migratory behaviour of the MOAH model substances studied in this research were defined by their volatility, the chemical-physical nature of the food matrix and the migration conditions.

The most volatile substances migrated at a higher concentration and speed through the gas phase, being strongly adsorbed into Tenax, while in polenta and couscous, they were characterised by volatilisation and desorption phenomena. The heaviest substances migrated mainly by diffusion, and their behaviour was similar in both dry foods and Tenax.

The most volatile substances were characterised by presenting strong correlations between them and similar migratory behaviours. At the same time, the correlation of the substances with intermediate volatility was more variable; due to the influence of the migration temperature on the different migration mechanisms that these substances can suffer (diffusion and gas phase transference).

In general, the migration values of the model substances in Tenax were higher than in couscous and polenta, so it can be considered as the worst case in the simulation of migration to dry food. The PLSR model elaborated with the migration data in Tenax predicts migration values from the physicochemical properties of the model substances; this can be a very useful tool for future prediction experiments.

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SECCIÓN IV: Conclusiones Generales

CONCLUSIONES GENERALES

A continuación, se presentan las conclusiones más importantes de esta Tesis:

1. Las técnicas de análisis ASAP-QTOF-MS y APGC-QTOF-MS son herramientas útiles para la selección e identificación de marcadores específicos de MOAH presentes en el material de envasado de alimentos. La selectividad de estas técnicas ayuda a evitar posibles confusiones con la presencia de otro tipo de sustancias y permite una clara identificación de MOAH.

2. Utilizando la masa exacta de potenciales marcadores de MOAH se logró identificar 27 compuestos que podrían utilizarse como marcadores de MOAH en muestras contaminadas por aceites minerales. La presencia de la mayoría de estos marcadores se verificó en seis aceites minerales de diferentes orígenes. De los 27 compuestos se seleccionaron 16 como marcadores de una posible contaminación por aceite mineral. La detección de estos marcadores indica la presencia de trazas de contaminación MOAH en los envases de alimentos.

3. Se optimizaron métodos de tratamiento de muestras adecuados para la extracción de MOAH desde matrices de aceites minerales, rPET y materiales de cartón; y se desarrolló un método APGC-QTOF-MS con las mejores condiciones cromatográficas para el análisis de los marcadores en estudio. La aplicación de estos métodos permitió verificar la presencia de algunos marcadores en dichas muestras.

4. Para determinar los marcadores MOAH provenientes de tintas de impresión offset en envases alimentarios de cartón se optimizó un método SPE-GC-MS fuera de línea. Este método demostró ser de gran utilidad ya que su aplicación permitió la determinación de los marcadores MOAH con rapidez, alta sensibilidad, precisión y buenos porcentajes de recuperación.

5. Los marcadores MOAH determinados en las tintas de impresión offset fueron: 2-methylnaphthalene (2–MN), 1-methylnaphthalene (1-MN), biphenyl (BP), 2,6-dimethylnaphthalene (2,6-DMN), 9,9'-dimethylfluorene (9,9-DMF), 3,3',5,5'-tetramethylbiphenyl (3,3',5,5'-TMBP), 1-methylpyrene (1-MPYR), 4,6-dimethyldibenzothiophene (4,6-DMDBT), 3,6-dimethylphenanthrene (3,6-DMP), benzo(b)naphtha(1,2-d)thiophene (BNT). Todos los marcadores, con excepción del 9,9'-DMF se determinaron también en los envases contaminados con MOAH. De esta manera se demostró que estos compuestos pueden ser utilizados para confirmar la contaminación de envases alimentarios de cartón para alimentos con MOAH provenientes de tintas de impresión offset a base de aceite mineral.

6. Estos marcadores químicos junto con técnicas de análisis como la espectrometría de masas, son herramientas valiosas que pueden ser empleadas para complementar el análisis FID de la fracción MOAH, especialmente en laboratorios donde no se dispone de instrumentación analítica en serie para el estudio de MOAH.

7. Para determinar los marcadores MOAH presentes en adhesivos termofusibles se desarrolló y optimizó un método de HS-SPME-GC-MS. El método mostró buena sensibilidad, linealidad y reproducibilidad para la detección rápida, directa y fiable de los marcadores MOAH a partir de muestras sólidas de adhesivos termofusibles, sin necesidad de tratamientos previos. Este método también se aplicó al análisis de los marcadores MOAH después de la migración.

8. Los resultados revelaron que cinco de los adhesivos estudiados contenían MOAH, y que ocho de los compuestos evaluados (biphenyl (BP), acenaphthene (ACE), 9,9'-dimethylfluorene (9,9-DMF), 3,3',5,5'-tetramethylbiphenyl (3,3',5,5'-TMBP), 3,6-dimethylphenanthrene (3,6-DMP), 1-methylpyrene (1-MPYR), 4-methyldibenzothiophene (4-MDBT) y 4,6-dimethyldibenzothiophene (4,6-DMDBT) eran adecuados para marcar la contaminación MOAH proveniente de adhesivos. Esto demostró que la identificación de marcadores MOAH específicos es

importante para descartar o confirmar la fuente de contaminación y la presencia de MOAH.

9. Se aplicó la técnica GC-FID para cuantificar y caracterizar la fracción MOAH que migró de los adhesivos. Como resultado se encontró que la fracción MOAH proveniente de los adhesivos estudiados eluía en el rango C16-C25 y que los adhesivos con mayor contenido de MOAH también tenían un mayor número de marcadores. GC-FID y HS-SPME-GC-MS pueden considerarse herramientas complementarias para cuantificar y verificar la contaminación por MOAH.

10. El estudio de la migración de MOAH empleando sustancias modelo reveló que las diferencias en el comportamiento migratorio de las tres subfracciones MOAH estudiadas (C10-C16, C16-C25 y C25-C35) está ligado a su volatilidad, la naturaleza químico-física de la matriz alimentaria y las condiciones de migración. Las sustancias más volátiles (C10-C16) migraron principalmente a través de la fase gaseosa, con mayor rapidez y en porcentaje más altos, que las sustancias pesadas (C25-C35). Estas sustancias (C10-C16) además, fueron adsorbidas fuertemente en el Tenax, mientras que en la polenta y en el cuscús se caracterizaron por fenómenos de volatilización y desorción; y en el caso de las sustancias modelos de la sub-fracción C25-C35 la migración ocurrió principalmente por difusión, y su comportamiento fue similar tanto en alimentos secos como en Tenax. Por otro lado, las sustancias C16-C25 presentaron un comportamiento migratorio más variado.

11. La aplicación de técnicas de análisis multivariante a los resultados demostró que las sustancias que modelaron la sub-fracción C10-C16 estaban fuertemente correlacionadas entre ellas, exhibiendo comportamientos migratorios similares, lo mismo ocurrió con las sustancias de la sub-fracción C25-C35. Sin embargo, la correlación de las sustancias con volatilidad intermedia (C16-C25) fue más variable; esto se debe a la influencia de la temperatura de migración sobre los distintos mecanismos de migración de estas sustancias (difusión y transferencia de fase gaseosa).

12. Los porcentajes de migración de las sustancias modelo en Tenax fueron más altos que en el cuscús y la polenta, por lo que la migración al Tenax se considera como el peor de los casos. Por ello, el modelo PLSR elaborado con los datos de migración en Tenax puede ser una herramienta útil para predecir los valores de migración a partir de las propiedades fisicoquímicas de las sustancias modelo.

SECCIÓN V: Publicaciones

PUBLICACIONES

Jaén, J., Domeño, C., Alfaro, P., & Nerín, C. (2021). Atmospheric Solids Analysis Probe (ASAP) and Atmospheric Pressure Gas Chromatography (APGC) coupled to Quadrupole Time of Flight Mass Spectrometry (QTOF-MS) as alternative techniques to trace aromatic markers of mineral oils in food packaging. Talanta, 227(December 2020). https://doi.org/10.1016/j.talanta.2020.122079.

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