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# POLÍMEROS INTELIGENTES PARA APLICACIONES EN SEGURIDAD Y CONTROL ALIMENTARIO

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***INFORMAN:***

El trabajo que describe esta memoria, titulada **"Polímeros inteligentes para aplicaciones en seguridad y control alimentario"** se ha realizado en los Departamentos de Química y de Biotecnología y Ciencia de los Alimentos de la Universidad de Burgos, bajo su dirección, por **Dña. Lara González Ceballos**, y autorizan su presentación para que sea calificada como **TESIS DOCTORAL**.

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# RESUMEN

Este trabajo describe el diseño de materiales poliméricos con diferentes aplicaciones en el campo de la alimentación. En primer lugar, de polímeros inteligentes como sensores colorimétricos para el análisis de diversas sustancias de interés en alimentación (aminas biógenas, polifenoles totales y nitritos). En segundo lugar, de materiales con potencial antimicrobiano, capaces de incrementar hasta en un 50% la vida útil de alimentos cárnicos envasados. Por último, de polímeros capaces de retener almidón y fenoles en su estructura, utilizados como parte del análisis de actividad diastásica en el caso del almidón y actividad glucosa oxidasa y catalasa en el caso de los fenoles. Todos los materiales desarrollados se han probado en matrices alimentarias, demostrando así su correcto funcionamiento y, por consiguiente, su potencial utilidad en la industria alimentaria.

# ABSTRACT

This work describes the design of polymeric materials, which are widely used in the food industry. Firstly, the use of smart polymers as colorimetric sensors for substances of interest such as biogenic amines, total polyphenols and nitrites is described. Secondly the antimicrobial capacity of a material is studied. This can increase the shelf-life of packaged meat foods by up to 50%. Finally, polymers able to keep starch and phenols in their structure are analyzed as part of the study of diastasic activity in the case of starch and glucose oxidase and catalase activity in the case of phenols. Every material developed, has been tested in food matrices in order to prove its effectiveness and therefore its potential use in the food industry

# CAPÍTULO 1

# Introducción general

Los polímeros son materiales que se encuentran integrados en prácticamente todos los ámbitos de la sociedad y que utilizamos diariamente. En los últimos 100 años, desde el descubrimiento del primer polímero íntegramente sintético, la ciencia y tecnología de polímeros ha avanzado enormemente, encontrando en la actualidad polímeros con aplicaciones avanzadas en biomedicina, envasado, transporte, seguridad civil y seguridad alimentaria entre muchas otras. En esta última aplicación es donde se centran los trabajos de esta memoria.

## 1.1 Antecedentes históricos de los polímeros

Un polímero se define como una macromolécula formada por la repetición de pequeñas unidades simples denominadas monómeros, los cuales se encuentran unidos entre sí mediante enlaces covalentes.[1,2]

Los polímeros son materiales que podemos encontrar en casi todos los ámbitos de la sociedad, integrados perfectamente en nuestra vida cotidiana y que nos permiten desarrollar nuestra actividad diaria tal y como la conocemos actualmente.

Los polímeros se pueden clasificar de muchas formas, atendiendo a su morfología, su cristalinidad, su comportamiento térmico o su método de obtención, entre otras características. Atendiendo a su origen, se pueden clasificar en dos grandes grupos; polímeros de origen natural y polímeros sintéticos. Entre los polímeros de origen natural encontramos biopolímeros como

el ácido desoxirribonucleico (ADN), el ácido ribonucleico (ARN) y otros como el algodón, el almidón, el látex o el caucho natural. El uso de los polímeros de origen natural se remonta hasta las primeras civilizaciones, momento en el cual algunos de ellos como el algodón ya se manipulaban con un objetivo concreto como la fabricación de prendas de ropa mediante técnicas de hilado.[3] Aunque los polímeros naturales forman parte del origen de la vida (ADN, proteínas, etc.), el término "polímero" y "polimérico" no fue acuñado hasta el año 1832 por Berzelius,[4] y no se presentó una teoría unificada de la estructura de los polímeros hasta el año 1920 por Hermann Staudinger,[5] quién propuso una estructura en forma de largas cadenas basada en la repetición de pequeñas unidades conectadas entre sí mediante enlaces covalentes.

Entre los polímeros sintéticos, podemos encontrar una gran variedad de ejemplos como el polietileno (PE), el poliestireno (PS), el polimetacrilato de metilo (PMMA), el polipropileno (PP) o el cloruro de polivinilo (PVC). La historia de los polímeros sintéticos es muy reciente y comenzó en el año 1907 con la comercialización del primer polímero íntegramente sintético; la baquelita. Este polímero fue sintetizado por el estadounidense Leo Beakland mediante un proceso de polimerización entre fenol y formaldehido y su uso como aislante eléctrico se extendió rápidamente.[6] Este descubrimiento supuso el punto de partida para la industria de los plásticos, ya que debido a las limitaciones que presentaba este nuevo polímero sintético, hizo que se promoviese la investigación en la síntesis de nuevos polímeros con diferentes propiedades para diversas aplicaciones.

Los polímeros tienen una gran importancia tanto en el ámbito tecnológico y científico como en el industrial, donde presentan diversas aplicaciones. Esto de se debe a que las propiedades físicas de un polímero dependen de su estructura química, por lo que sus aplicaciones son muy versátiles.[7] A su vez, esta estructura depende de la naturaleza de los monómeros que la integran, del tipo de enlaces establecidos entre ellos, así como de las fuerzas de interacción

que tienen lugar entre las cadenas del polímero. De esta forma se puede adaptar la estructura del polímero en función de la aplicación a la que está destinado.[8,9]

El desarrollo en el campo de la ciencia y tecnología de polímeros ha supuesto importantes avances en el diseño de materiales aplicables en campos muy diversos tales como automoción, biomedicina, envasado, embalaje, comunicaciones y transporte. Una de las aplicaciones más importantes de los polímeros ya que, aproximadamente, el 40 % de la producción mundial de estos materiales se destina a ella,[10] es la del envase y embalaje. Esta aplicación es especialmente importante en el campo de la alimentación, donde estos materiales pueden jugar un papel activo en el envasado de los productos mejorando alguna de sus propiedades y/o alargando su vida útil.

## 1.2. Polímeros en el campo de la alimentación

La cadena alimentaria se puede dividir en cuatro etapas: producción, industria alimentaria, comercialización o venta y consumidores. Los polímeros se encuentran presentes en todas ellas cumpliendo diversas funciones de gran relevancia. Entre estas funciones se pueden destacar las siguientes en función de la etapa en la cual nos encontremos (Figura 1.1).

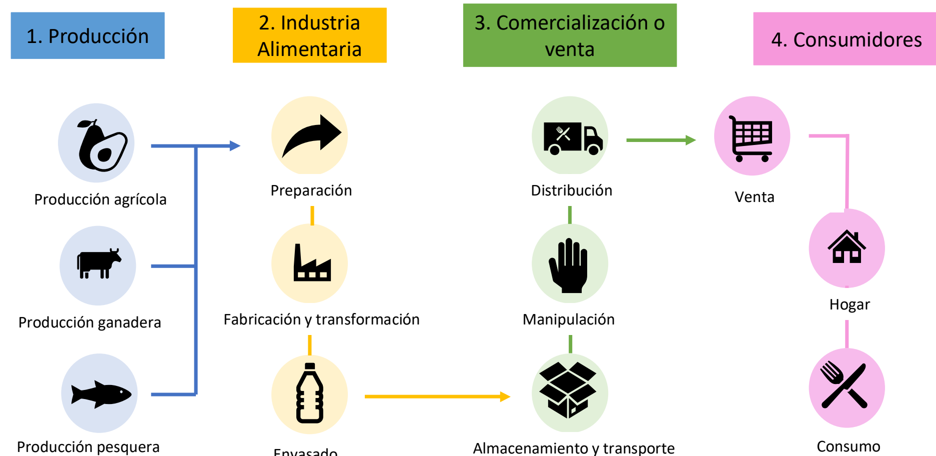


**Figura 1.1.** Etapas de la cadena alimentaria.

**Etapa de producción**. En la fase de producción, en concreto en la producción agrícola, se pueden encontrar polímeros absorbentes como el poliacrilato de sodio, cuya función es la de absorber agua para crear reservas de humedad en el suelo, garantizando así el suministro de esta para las plantas.[11] En la producción pesquera, se pueden encontrar polímeros sintéticos como el *nylon* o bien polímeros de origen natural como el algodón formando las redes de pesca. En la producción ganadera, destacan los plásticos destinados al ensilado de forrajes que son, generalmente, materiales multicapa formados por una combinación de resinas, metalocenos y polietileno de diferentes densidades.[12]

**Industria Alimentaria**. En esta etapa se engloba la preparación del alimento, su fabricación, transformación y su posterior envasado. Por tanto, la cantidad de materiales poliméricos utilizados es muy elevada. En la fabricación de alimentos se pueden utilizar estabilizantes, espesantes y gelificantes, los cuales son generalmente polímeros de origen natural como proteínas o polisacáridos derivados de hongos.[13] En la fase de fabricación y transformación del producto, el uso de un material polimérico u otro dependerá en gran medida del producto a fabricar. De forma muy general, en esta etapa se pueden encontrar numerosos polímeros formando parte de las fábricas de alimentación como, por ejemplo, resinas multicapa epoxi poliuretano para conformar los pavimentos industriales agroalimentarios y espuma de poliuretano rígido utilizado como aislante en neveras y congeladores. La fase de envasado de los alimentos es, sin duda, donde los materiales poliméricos toman una especial relevancia. Esto se debe a que, el envase de un producto en las últimas décadas ha pasado de ser una simple barrera mecánica, a tener un papel activo en la conservación y el incremento de la vida útil del producto que contiene. Actualmente, el tipo de polímero o polímeros utilizados para la fabricación del envase de un alimento dependerá del tipo de alimento que contenga. De forma muy general, se utilizan polímeros con buenas propiedades mecánicas, buenas propiedades barrera y generalmente transparentes. Los polímeros más utilizados para la fabricación de envases alimentarios son el polietileno (PE), polipropileno (PP) y el tereftalato de

polietileno (PET). En la actualidad, se trabaja en el diseño de envases que sean capaces de prolongar la vida útil de los productos que contienen.[14,15]

Un envase alimentario debe ser capaz de mantener la calidad y seguridad del producto, actuando como barrera mecánica, térmica, óptica y protegiendo al alimento frente a factores externos que puedan contribuir en su deterioro, como, por ejemplo, la proliferación de microorganismos patógenos. Actualmente, la investigación se encuentra orientada hacia la búsqueda de polímeros biodegradables más respetuosos con el medio ambiente y, a su vez, se ha introducido la idea del envasado activo, el cual tiene como objetivo principal alargar la vida útil del alimento que contiene. Este tipo de envases se fundamentan en la interacción entre el alimento, el propio envase y el entorno, elementos que interaccionan entre sí para prolongar la vida útil del producto y mantener o incluso mejorar la calidad y seguridad de este. Existen numerosos procesos que contribuyen al deterioro de los alimentos, algunos son de naturaleza física, otros son de naturaleza química, y otros son de naturaleza microbiológica y, mediante este tipo de envasado pueden ser modificados, con el objetivo de conseguir un incremento en la vida útil de los productos.[16]

**Comercialización o venta**. En esta etapa de la cadena alimentaria se incluyen los procesos de distribución, manipulación, transporte y almacenamiento. De forma muy general, en esta etapa, el uso de materiales poliméricos se centra en aplicaciones de embalaje y de aislamiento térmico en los transportes y en los almacenes. Para estos fines, los polímeros más habituales son el polietileno espumado y de diferentes densidades y espumas de poliuretano, entre otros.

**Consumidores.** Esta etapa incluye los procesos de compra de los alimentos y de consumo de los mismos. Por tanto, destacan el uso de polietileno que conforma las bolsas de los supermercados, los polímeros mencionados anteriormente que forman parte del envase de los alimentos, así como polímeros tales como poliestireno y policarbonatos que forman parte de los envases de plástico y microondas utilizados a nivel doméstico, entre otros.

Estos son solo algunos de los ejemplos del uso de diferentes polímeros en el campo de la alimentación. Durante el desarrollo de este trabajo, se ha investigado en el diseño y posible aplicación de diferentes tipos de polímeros; polímeros sensores, polímeros antimicrobianos y polímeros con otras aplicaciones en el control de calidad de alimentos.

## 1.3. Polímeros inteligentes. Polímeros sensores.

Un polímero inteligente (*smart polymer*) puede definirse como un polímero que responde a diferentes estímulos de su entorno (temperatura, pH, luz o presencia de analitos concretos, entre otros) mediante un mecanismo específico, generando una respuesta concreta.[17] Este tipo de polímeros se pueden clasificar en dos grandes grupos, los denominados polímeros reactivos (*responsive polymers*) y los denominados polímeros sensores (*sensory polymers*). La principal diferencia entre ambos radica en el tipo de respuesta generada por el polímero ante un estímulo concreto. En el caso de los polímeros reactivos, la respuesta generada es una acción concreta. Este tipo de polímeros se utilizan, por ejemplo, en el campo de la biomedicina para la liberación controlada de fármacos. Por otro lado, en el caso de los polímeros sensores la respuesta generada es una alerta concreta que puede traducirse en una información analítica de utilidad.[18]

El uso de los polímeros inteligentes se encuentra en la actualidad ampliamente extendido en multitud de campos. En el ámbito de la alimentación, destaca su expansión en aplicaciones como fabricación de envases orientados al incremento de la vida útil de diferentes alimentos,[19] así como en el reconocimiento e inmovilización de biomoléculas.[20]

Los alimentos pueden sufrir a lo largo de la cadena alimentaria contaminaciones por parte de multitud de sustancias como antibióticos o plaguicidas. También pueden generarse compuestos tóxicos para la salud del consumidor o compuestos que modifiquen las propiedades organolépticas del alimento. Resulta de gran importancia la detección de estos contaminantes con

el objeto de eliminarse, evitando así el potencial efecto negativo que éstos puedan ejercer sobre la salud del consumidor. Los polímeros, en concreto los denominados polímeros de impresión molecular, son una excelente opción para llevar a cabo este tipo de procesos. La impresión molecular es una técnica mediante la cual, partiendo de una matriz polimérica, se generan unos sitios de unión específicos de una molécula diana. Los polímeros utilizados en este tipo de técnicas presentan una gran estabilidad térmica y frente a distintos disolventes y cambios de pH, son reutilizables ya que pueden regenerarse y tienen un bajo coste.[21,22]

En cuanto al uso de polímeros inteligentes en envasado de alimentos, existen numerosos ejemplos. Uno de ellos es el uso de sales de poliacrilato de sodio como sistema de regulación de humedad en el interior del envase o bien, el uso de sensores poliméricos para monitorizar algún parámetro de calidad concreto.[23,24] Un sensor puede definirse como un dispositivo capaz de detectar una molécula concreta y, mediante un proceso de transducción, dar lugar a una señal cuantificable.

Los polímeros sensores presentan multitud de ventajas frente al uso de moléculas sensoras discretas, de pequeño tamaño. Por ejemplo, se pueden controlar la estructura y propiedades finales del sensor mediante la elección de los monómeros de partida que lo conforman. A su vez, se pueden modificar fácilmente tanto la forma de este tipo de polímeros como su hidrofobicidad, lo cual permite adaptar la forma del sensor en función a la aplicación final a la cual está destinado (detección de compuestos en fase gaseosa o en medios orgánicos, entre otras).[18]

La respuesta o señal generada por los polímeros sensores puede ser de diferente naturaleza (diferencia de potencial, cambio en el tamaño o forma, modificación de la conductividad eléctrica y cambio en la fluorescencia o en el color).[25,26,27,28,29] En función del tipo de señal generada, los sensores poliméricos se pueden clasificar en:

- *Sensores poliméricos piezoeléctricos*. La señal generada es una señal eléctrica cuando el material es sometido a fuerzas de compresión.[30]
- *Sensores poliméricos quimio-mecánicos*. La señal generada es un cambio en el tamaño, la forma y/o la estructura del material.[31]
- *Sensores poliméricos electroquímicos*. La señal generada es un cambio en la conductividad eléctrica del material.[32]
- *Sensores poliméricos fluorescentes*. La señal generada es un cambio en la fluorescencia del material.[33]
- *Sensores poliméricos colorimétricos*. La señal generada es un cambio en el color del material.[34]

En el campo de los sensores poliméricos, los estudios de esta tesis doctoral se centran en los sensores poliméricos colorimétricos. En este tipo de sensores, la señal generada es una variación cromática. En este aspecto, los sensores capaces de generar un cambio de color detectable a simple vista presentan un gran interés. A su vez, suponen una alternativa viable al uso de técnicas que requieren una gran inversión en equipos como la espectroscopía UV/Vis.

El cambio de color generado en cada sensor se puede cuantificar usando diferentes espacios de color. La definición digital del color se lleva a cabo dentro de un espacio de color, como puede ser el RGB (Figura 1.2), basado en la síntesis aditiva de los colores rojo, del inglés *red* (R), verde, del inglés *green* (G) y azul, del inglés *blue* (B).[35] El objetivo de utilizar un espacio de color para evaluar el cambio de color de un sensor es transformar un color digital en un valor numérico. Cada espacio de color tiene una forma diferente, y en cada uno de ellos se fija una coordenada la cual es un punto en ese espacio, asignando así un valor numérico a un color digital. Por ejemplo, mediante el espacio de color RGB se puede definir el color digital de una fotografía digital. Es decir, cada uno de los colores de una imagen digital se puede definir como una combinación de tres números, cada uno de ellos asociado a cada uno de los parámetros del espacio de color. En este espacio de color, cada parámetro oscila entre 0 y 255.

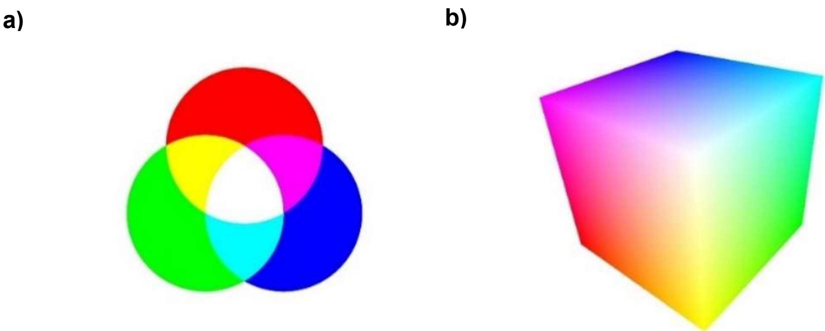


**Figura 1.2.** Espacio de color RGB: a) Modelo aditivo de colores rojo, verde y azul y b) representación tridimnesional en forma de cubo del modelo RGB.

El espacio de color RGB no es el único modelo de color, sino que existen otros similares como, por ejemplo, el espacio de color HSV (Figura 1.3), matiz, del inglés *Hue* (H), saturación, del inglés *saturation* (S) y brillo, del inglés *value* (V) o el espacio de color CIELAB (Figura 1.4) (Comisión Internacional de la Iluminación; del francés *Commission Internationale d'Éclairage*, LAB), entre otros.

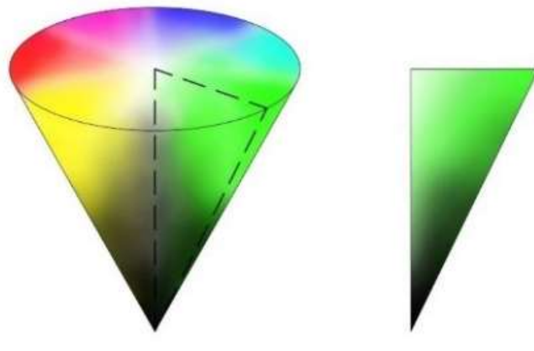

**Figura 1.3.** Cono de colores del espacio HSV.

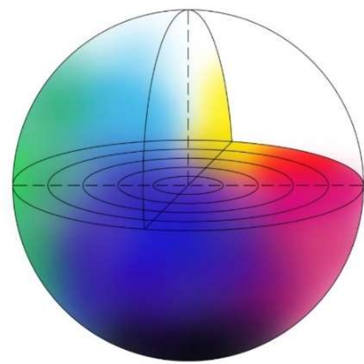

**Figura 1.4.** Modelo espacio de color CIELAB.

En el Grupo de Polímeros de la Universidad de Burgos se han diseñado numerosos sensores poliméricos colorimétricos. La aplicación de estos sensores se basa en la detección de una molécula de interés mediante un cambio de color del propio sensor. Este cambio de color se cuantifica mediante una técnica colorimétrica, como por ejemplo el seguimiento de los parámetros RGB de una fotografía tomada al sensor.[36,37,38]

La cuantificación del cambio de color mediante el análisis de los parámetros de color digital RGB, es la técnica que se ha denominado en el Grupo de Polímeros como “método RGB”. Este método utiliza fotografías digitales para llevar a cabo un análisis del color, y supone una alternativa viable a los métodos analíticos tradicionales.[39,40] De esta forma, se pueden representar curvas de calibrado que relacionen las variaciones de color del sensor con las variaciones de concentración de la molécula objeto de estudio. El proceso de detección y cuantificación de una molécula mediante el uso de este método aparece reflejado de forma esquemática en la Figura 1.5.

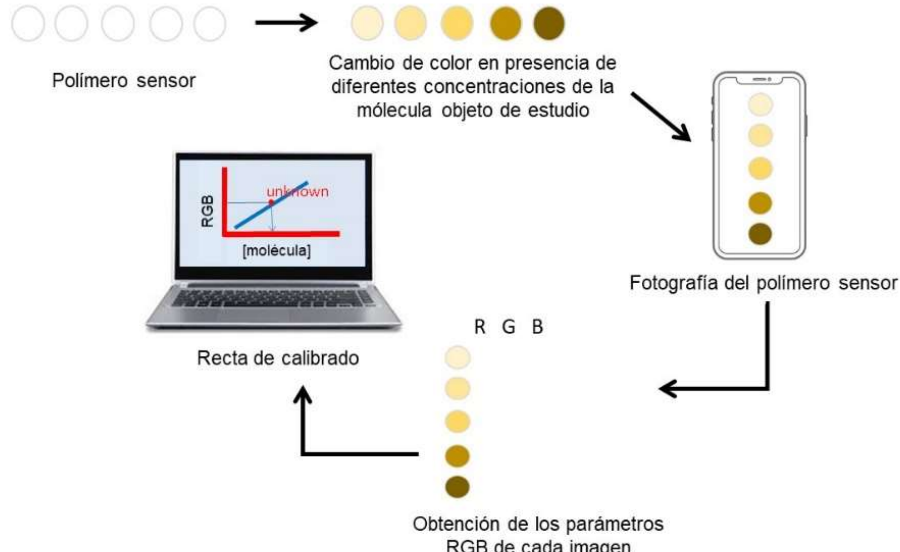


**Figura 1.5**. Esquema del proceso de detección y cuantificación de un analito de interés mediante el uso de sensores poliméricos colorimétricos.

Este método presenta numerosas ventajas, ya que cualquier persona no especializada puede llevar a cabo la medida de manera rápida y sencilla únicamente con un teléfono móvil.

En el desarrollo de esta tesis doctoral se ha empleado este método para la detección de diferentes moléculas de interés en alimentación. En la actualidad, esta técnica ha sido perfeccionada mediante el uso de una aplicación diseñada por el Grupo de Polímeros de la Universidad de Burgos en colaboración con la empresa burgalesa "Inforapps", *Colorimetric Titration*. Mediante el uso de esta aplicación para teléfonos móviles, se puede llevar a cabo el análisis completo de varios sensores de manera simultánea con una única fotografía, de manera rápida y sencilla.

Una de las hipótesis iniciales de este trabajo fue que los sensores poliméricos, en concreto los sensores poliméricos colorimétricos, pueden suponer una alternativa viable a los métodos de análisis tradicionales en materia de control de calidad de alimentos. Por lo tanto, el principal reto durante este

apartado de la presente memoria fue el desarrollo de sensores poliméricos colorimétricos para la medida de diferentes analitos de interés en alimentación, concretamente de aminas biógenas en muestras de pescado, polifenoles en muestras de miel y nitritos en muestras cárnicas procesadas.

## 1.4. Polímeros antimicrobianos

El envasado tradicional de alimentos únicamente se basa en el empleo de un recipiente que actúa como una barrera física para los alimentos que contiene. En la actualidad, los consumidores demandan alimentos con elevado valor nutricional, de alta calidad y seguridad alimentaria, largos tiempos de conservación y mínimo procesado. Por ello, el sector del envasado de alimentos ha evolucionado en las últimas décadas dando lugar a lo que se conoce como envasado activo, técnica donde el propio envase desempeña un papel activo en la calidad, seguridad, propiedades organolépticas y vida útil del alimento.[41,42] De esta forma, se consigue una disminución en la rotación de productos, los alimentos son más accesibles para zonas lejanas y se incrementa la vida útil del producto, reduciendo así del desperdicio de alimentos. Existen diferentes tipos de envases activos:

- *Envases orientados a cambios bruscos de temperatura*. Se trata de envases fabricados con diferentes polímeros que permiten el calentamiento y enfriamiento del alimento dentro del propio envase. Un ejemplo de este tipo de envases son los que contienen los alimentos de quinta gama listos para el consumo. Para este tipo de alimentos el envase desempeña un papel principal, ya que debe permitir el calentamiento o cocinado en el microondas u horno del alimento dentro del propio envase. Estos envases presentan una elevada resistencia térmica y física. Entre los polímeros más utilizados para su fabricación destacan el polietileno de alta densidad y el polipropileno.[43]

- *Envases de control de atmósfera de envasado*. Estos envases absorben o emiten sustancias que contribuyen a la mejora de diversas propiedades del alimento. Este tipo de envases pueden ser absorbentes o secuestrantes (*scavengers*) de oxígeno, etileno, vapor de agua y/o dióxido de carbono ($CO_2$), emisores de sustancias antimicrobianas como etanol y $CO_2$ o bien envases que regulen los niveles de humedad o presenten permeabilidad selectiva a diferentes gases.[44,45]
- *Envases orientados a modificar la composición del alimento*. Este tipo de envases pueden presentar propiedades antimicrobianas y antioxidantes, entre otras. La función del envase dependerá del elemento que se añada al mismo y esta adición se puede hacer mediante dos técnicas diferentes. En primer lugar, se puede introducir el elemento activo dentro del propio envase junto con el alimento. Por otro lado, el elemento activo se puede incorporar en la propia estructura del envase. En esta última técnica es en la que se centran todas las investigaciones actuales debido a la mayor aceptación por parte de los consumidores.[46,47]

En esta línea, la hipótesis inicial fue que se podría incrementar la vida útil de productos cárnicos, su calidad y seguridad utilizando un polímero que contiene una sustancia antimicrobiana en su estructura como parte de un envase activo. Por eso el principal reto planteado en este apartado de la presente memoria fue el diseño de un material polimérico con propiedades antimicrobianas, que forme parte de un envase activo interaccionando directamente con el alimento y que aumente la vida útil del producto que contiene.

## 1.5. Polímeros con aplicaciones en el control de calidad de alimentos

Los polímeros son materiales muy utilizados como soporte para diferentes moléculas de interés con diferentes apliaciones. Un ejemplo de este tipo de aplicaciones son los biosensores poliméricos, polímeros sobre los cuales se inmoviliza una molécula de origen biológico (enzimas, anticuerpos, aptámeros, etc.) para la detección de un analito concreto.[48,49]

En el ámbito biomédico, los polímeros inteligentes se utilizan para diversas aplicaciones como la liberación controlada de fármacos. En este caso, el polímero sirve como soporte para un fármaco, el cual se libera en su órgano o tejido diana mediante un estímulo concreto como puede ser un cambio de pH, cambios en la temperatura, exposición a radiación electromagnética o bien, la presencia de una molécula concreta como una enzima.[50]

En el campo de la alimentación, existen multitud de polímeros que se pueden utilizar para agilizar y simplificar análisis de medida de control de calidad de alimentos, reduciendo los largos tiempos de análisis y los costes derivados de los mismos.[51]

Por ello, la hipótesis principal en este caso fue que los polímeros se pueden utilizar para agilizar y simplificar análisis de medida de control de calidad en alimentos. Por ello, el principal reto planteado en este apartado de esta tesis doctoral, fue el diseño de diferentes polímeros con aplicaciones en el análisis de diferentes indicadores de calidad de alimentos.

## 1.6. Planteamiento de la investigación

### 1.6.1. Problemas detectados. Control de calidad en alimentación e incremento de la vida útil de alimentos envasados.

El control de calidad en alimentos resulta de vital importancia para garantizar la calidad del producto y proteger la salud del consumidor final. Este control se lleva a cabo mediante diversas técnicas que, en la mayoría de los casos, resultan tediosas, requieren de personal especializado y suponen un elevado coste económico y una gran inversión de tiempo.[52,53]

Para garantizar la calidad de un alimento, se deben evaluar diferentes parámetros en función a la matriz alimentaria estudiada, como pueden ser propiedades sensoriales (sabor y aroma, entre otros) o propiedades cuantificables (composición nutricional, carga microbiológica, presencia de contaminantes y otras propiedades). Existen normas y/o recomendaciones relativas a cada matriz alimentaria concreta, por lo que dependiendo del tipo de producto, se deben realizar unos u otros análisis. Estas normas y recomendaciones establecen los ingredientes permitidos, características físico-químicas que ha de tener un producto, normas de etiquetado, límites máximos legales o bien, recomendaciones para diversas sustancias como por ejemplo, concentración de ciertos aditivos así como criterios microbiológicos. De forma más específica, se han identificado siete problemas que corresponden a siete parámetros de calidad (aminas biógenas, polifenoles totales, actividad diastásica, actividad catalasa, actividad glucosa oxidasa, nitritos y carga microbiana) de especial relevancia en diferentes matrices alimentarias (pescado, miel, productos cárnicos cocidos y productos cárnicos frescos). A continuación, se plantea cada uno de los problemas en función a cada una de las matrices alimentarias mencionadas anteriormente:

**Pescado.** En el caso del pescado existe un parámetro de calidad muy importante, las aminas biógenas. Las aminas biógenas son compuestos nitrogenados generados por la descarboxilación oxidativa de ciertos

aminoácidos presentes en el pescado mediante enzimas descarboxilasas presentes en algunos microorganismos. Aunque existe una pequeña cantidad en muchos alimentos, una elevada concentración de estas, está estrechamente correlacionada con un incremento en el crecimiento de ciertos microorganismos. Este incremento en la concentración de microorganismos genera un deterioro en la calidad del producto y es una excelente medida de la frescura del alimento.[54,55] La ingesta de aminas biógenas puede provocar diversas alteraciones en la salud del consumidor tales como alteraciones gastrointestinales, hemodinámicas, cutáneas y neurológicas.[56,57] En cuanto al nivel de aminas biógenas permitido en alimentos derivados de la pesca, únicamente existe legislación relativa a los niveles de histamina,[58] que establece un máximo legal permitido para esta amina en diversas familias de productos pesqueros.

Actualmente no existe una forma rápida y sencilla de determinar la concentración de aminas biógenas en una matriz alimentaria, teniendo que recurrir a métodos de medida tradicionales como a la cromatografía líquida de alta eficacia (HPLC, acrónimo en inglés de *high performance liquid chromatography*).[59] Esta técnica, aunque resulta muy precisa, exacta y fiable, requiere de una gran inversión económica en equipos, uso por parte de personal especializado, largos tiempos de análisis y supone un coste elevado.

**Miel.** En el caso de la miel, existen numerosos parámetros de calidad de gran relevancia como el contenido en polifenoles totales, que se encuentra relacionado con la capacidad antioxidante de este alimento. A su vez, las actividades de diferentes enzimas como las diastasas, la glucosa oxidasa y la catalasa son importantes parámetros de calidad y están relacionadas con la calidad de la miel y el estado de conservación de la misma.[60] La actividad de las enzimas diastasas es un parámetro de calidad que se mide de forma rutinaria en mieles de todo el mundo. El resultado se expresa en la escala de Schade y aporta datos acerca del tiempo y condiciones de almacenamiento del producto, así como de si la miel ha sido sometida a procesos de calentamiento.[61] En resumen, la medida de este parámetro nos indica el nivel de frescura y posibles fraudes en

muestras de miel, pero el ensayo llevado a cabo para su medida resulta tedioso, costoso y requiere de personal especializado. A su vez, las actividades de las enzimas glucosa oxidasa y catalasa en muestras de miel, son parámetros de calidad importantes para este alimento por su relación con las actividades antioxidante y antimicrobiana de la miel, pero su determinación no suele llevarse a cabo de forma rutinaria debido a la elevada complejidad de los ensayos.

**Productos cárnicos cocidos**. En el caso de productos cárnicos cocidos, el nitrito potásico (E-249) y el nitrito de sodio (E-250) son aditivos ampliamente utilizados para evitar la proliferación de microorganismos, concretamente de esporas de *Clostridium botulinum*.[62] Este tipo de aditivos, a su vez, contribuyen junto con el nitrato sódico (E-251) y en nitrato potásico (E-252) al desarrollo del aroma, color y sabor característico de este tipo de productos. En el caso de los nitritos, la legislación establece en 150 mg/kg de producto final la cantidad máxima permitida de nitritos que se puede añadir durante el proceso de producción del alimento.[63] Existen normativas,[64] que regulan la cantidad máxima residual que deben contener algunos productos cárnicos, ya que el consumo de nitritos está relacionado con el cáncer de estómago y diversas enfermedades gastrointestinales.[65] Al igual que en los casos anteriores, el método convencional de medida de la concentración de este aditivo en alimentos resulta complejo, presenta un elevado coste económico y de tiempo y requiere de personal especializado.

**Productos cárnicos frescos.** Además de los inconvenientes mencionados anteriormente para la medida de diferentes parámetros de calidad de diversas matrices alimentarias, el incremento en la seguridad, calidad y vida útil de los alimentos envasados, más concretamente de los alimentos cárnicos frescos, resulta un tema de gran interés en la actualidad. Los consumidores demandan alimentos seguros, de elevada calidad y larga vida útil. Fruto de estas demandas, se han desarrollado envases activos y, más concretamente, envases que incrementen la vida útil del producto que contienen mediante la adición de sustancias antimicrobianas. Este tipo de envases, en general, se basa en la

dispersión de una sustancia antimicrobiana, por lo que existe migración de dicha sustancia al alimento, pudiendo afectar a la actividad antimicrobiana de la sustancia añadida y a las propiedades organolépticas del alimento.

Actualmente, la investigación en este campo se encuentra orientada hacia la fabricación de envases que contengan sustancias antimicrobianas ancladas de forma covalente al mismo y no de forma dispersa. De esta forma, se podrían disminuir los problemas asociados a la adición de antimicrobianos de forma libre, como la volatilidad del compuesto, inestabilidad química y la migración al alimento. Así, se puede incrementar la vida útil del producto, la selectividad y eficacia del compuesto antimicrobiano y reducir el impacto medioambiental.

### 1.6.2. Soluciones propuestas. Uso de polímeros sensores, polímeros antimicrobianos y materiales poliméricos con aplicaciones en el control de calidad de alimentos.

En primer lugar, relativo a los análisis de control de calidad de alimentos (pescado, miel y productos cárnicos cocidos), debido a los inconvenientes que presentan los métodos de medida tradicionales para el análisis de las sustancias de interés detalladas en el apartado anterior, se hace patente la necesidad de diseñar métodos más rápidos, sencillos y asequibles. En esta línea, se plantean dos soluciones. En primer lugar, se propone el diseño de sensores poliméricos colorimétricos para la detección y cuantificación de diferentes sustancias de interés (aminas biógenas, índice de polifenoles totales, y nitritos) relacionadas con calidad del pescado, la miel y los productos cárnicos cocidos, respectivamente. El uso de sensores químicos convencionales presenta varios inconvenientes que impiden que se extienda su uso como método de medida alternativo a los métodos tradicionales de control de calidad. Esto se debe a que, estos sensores, suelen ser moléculas orgánicas u organometálicas de bajo peso molecular que son poco estables frente a la luz, presentan baja resistencia térmica y baja solubilidad en agua y tienen tendencia a la migración. Por el contrario, el diseño de sensores poliméricos permite suprimir o mitigar gran parte

de estos inconvenientes debido a que se puede seleccionar la matriz polimérica más adecuada para la aplicación requerida, controlando así parámetros como la hidrofilia/hidrofobia, transparencia/opacidad, forma, entre otras.[66]

En segundo lugar, se propone el diseño de materiales poliméricos con capacidad de retener de forma selectiva determinadas sustancias en su estructura (almidón y fenoles), cuyo objetivo principal es agilizar y abaratar los análisis de medida de diversos parámetros de calidad (actividad enzimática de las enzimas diastasas, catalasa y glucosa oxidasa) de la miel.

El Grupo de Polímeros de la Universidad de Burgos tiene una amplia experiencia en el diseño de este tipo de sensores poliméricos con diferentes aplicaciones en diversos campos tales como:

- *Seguridad civil y ambiental*. En este campo, se han desarrollado numerosos sensores para la detección de moléculas de gran interés como el TNT.[67,68,69]
- *Biomedicina*. En este campo destaca la aportación del grupo en el diseño de sensores para monitorización de heridas crónicas y fibrosis quística.[70,71]
- *Seguridad alimentaria*. Destaca la aportación en el diseño de sensores poliméricos utilizados como método de medida rápido, sencillo y de bajo coste de varios parámetros relacionados con la calidad de diversas matrices alimentarias como, por ejemplo, polifenoles totales en vinos,[72] o mercurio en muestras de pescado.[73]

Por otro lado, el Grupo de Nutrición y Bromatología de la Universidad de Burgos posee una amplia experiencia en la puesta a punto de métodos analíticos y análisis de alimentos. Por tanto, y fruto de la experiencia de ambos grupos, este trabajo se centra en el diseño de sensores poliméricos colorimétricos para la detección de especies de interés en el control de calidad alimentaria, concretamente, de aminas biógenas en muestras de pescado, polifenoles totales y actividad antioxidante en muestras de miel y nitritos en muestras de carne procesada. A su vez, se presentan otro tipo de materiales poliméricos con

propiedades avanzadas utilizados como parte del análisis de actividad diastásica, glucosa oxidasa y catalasa en muestras miel. Estos sensores y materiales poliméricos minimizan costes y tiempos de análisis y pueden ser utilizados por personal no especializado.

Relativo a los materiales poliméricos con propiedades antimicrobianas para el desarrollo de envases activos, la adición de forma dispersa de las propias sustancias antimicrobianas presenta numerosas desventajas como, por ejemplo, posible migración al alimento, con la pérdida de calidad que eso conlleva en el producto y la pérdida de actividad antimicrobiana. Debido a esto, se hace patente la necesidad de desarrollar alternativas capaces de alargar la vida útil de los alimentos sin alterar sus propiedades y sin perder la capacidad antimicrobiana de la sustancia adicionada.

El Grupo de Polímeros presenta una gran destreza en el anclaje covalente de multitud de especies a una matriz polimérica, tratándose de la metodología habitual de trabajo utilizada en el grupo mientras que el grupo de Tecnología de Alimentos de la Universidad de Burgos, presenta una amplia experiencia en el estudio de vida útil de diferentes productos cárnicos,[74,75] así como en el incremento de la vida útil de este tipo de productos mediante el uso de diferentes sustancias con potencial capacidad antimicrobiana.[76,77] Trabajando de forma conjunta, la investigación se encaminó al diseño de un material polimérico con propiedades antimicrobianas para alargar la vida útil de productos cárnicos envasados. Este material se encuentra formado por una matriz polimérica a la que se ha anclado de forma covalente vainillina (un aceite esencial), evitando así su migración y los posibles efectos indeseables que esto puede ocasionar.

En esta memoria se presenta el trabajo realizado relacionado en el ámbito de sistemas sensores y materiales poliméricos dirigidos a la mejora de los análisis de calidad de alimentos, así como el trabajo realizado en el ámbito del envasado de alimentos dirigido al diseño de envases activos que prolonguen la vida útil del alimento.

## 1.7. Objetivos

El objetivo principal de este trabajo ha sido desarrollar:

1) Diferentes sensores poliméricos colorimétricos para la detección y cuantificación de parámetros de especial interés en el control de calidad de alimentos de una forma rápida y sencilla.

2) Nuevos materiales poliméricos:

2.1. Con actividad antimicrobiana para su incorporación en envases alimentarios, de modo que contribuyan a la prolongación de la vida útil del alimento que contienen.

2.2. Con capacidad de retención de determinadas sustancias en su estructura, de modo que se optimice el análisis de parámetros de calidad de la miel, haciéndolo más rápido y sencillo.

Los objetivos específicos han sido el diseño de:

1) Sensores poliméricos colorimétricos para la detección y cuantificación de aminas biógenas en muestras de pescado, polifenoles totales en muestras de miel y nitritos en muestras de carne procesada.

2) Un material polimérico con un resto de azo-derivado de un aceite esencial como componente absorbente con propiedades antimicrobianas. El objeto de este elemento es doble, aumentar la vida útil del producto y actuar como absorbente en las bandejas de carne, reteniendo el exudado generado por este tipo de productos.

3) Matrices poliméricas capaces de retener almidón y fenoles en su estructura con fin de ser incorporadas para la detección y cuantificación de diversos parámetros de calidad en mieles tales como actividad diastásica, actividad catalasa y actividad glucosa oxidasa.

## 1.8. Estructura de la Memoria

El trabajo realizado para la consecución de los objetivos planteados se presenta en esta memoria dividido en cuatro capítulos, consistiendo el primero en esta introducción, en la que se explica de manera general el contexto y objetivos del trabajo llevado a cabo.

Los capítulos siguientes explican la metodología y los trabajos realizados para la consecución de dichos objetivos. En el Capítulo 2 se detalla la síntesis y caracterización de sensores poliméricos para la detección de parámetros de interés en el control de calidad de alimentos. En este capítulo se describen tres sensores poliméricos diferentes. El primero está orientado a la detección de aminas biógenas en muestras de pescado. Consiste en una poliamida aromática con subunidades sensoras (grupos bromonaftilamida), llevándose a cabo la detección mediante una reacción de sustitución aromática nucleófila del haluro entre la subunidad sensora y las aminas biógenas presentes en el medio, por formación de acrilaminas coloreadas. El segundo está orientado a la detección de polifenoles totales y actividad antioxidante en mieles. En este caso la detección se lleva a cabo mediante la reacción con una subunidad sensora (sal de diazonio), anclada a la matriz polimérica de forma covalente. El tercero está orientado a la detección de nitritos en muestras de carnes procesadas. Esta reacción se lleva a cabo mediante un acoplamiento diazoico. A su vez, se presentan los métodos convencionales de medida para cada analito concreto y se comparan con el método de medida llevado a cabo mediante el uso del polímero sensor. Los procedimientos experimentales, resultados, discusión y conclusiones se recogen en los artículos científicos enviados, aceptados y/o publicados.

En el Capítulo 3, se presenta el desarrollo de un material polimérico azo-derivado con aceites esenciales, concretamente vainillina, anclada covalentemente a la matriz polimérica. Se presenta como un material con doble funcionalidad; como absorbente de humedad en la industria del envasado en productos cárnicos, y como polímero antimicrobiano capaz de prolongar la vida útil de una matriz alimentaria cuando se añade como parte del envase de ésta. Los procedimientos experimentales, resultados, discusión y conclusiones se encuentran recogidos en los artículos científicos enviados, aceptados y/o publicados.

El Capítulo 4 se centra en el estudio de matrices poliméricas diversas con aplicaciones avanzadas en el control de calidad de alimentos. Concretamente, se utiliza una matriz polimérica capaz de retener el sustrato (almidón) de las enzimas diastasas de miel, facilitando así su análisis. A su vez, se utiliza una matriz polimérica capaz de retener polifenoles de forma específica en su estructura. De esta forma, se consigue eliminar los interferentes de la medida de la actividad enzimática de las enzimas catalasa y glucosa oxidasa en miel, facilitando así su análisis. Además, se presentan los métodos convencionales de medida para cada analito concreto y se comparan con el método de medida llevado a cabo mediante el uso del material polimérico. Los procedimientos experimentales, resultados, discusión y conclusiones se aportan en los artículos científicos enviados, aceptados y/o publicados.

Por último, se recogen las conclusiones extraídas en el desarrollo del trabajo, se analiza el cumplimiento de los objetivos fijados y se plantean perspectivas futuras.

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# CAPÍTULO 2

# Polímeros sensores para la detección de analitos de interés en el campo de la alimentación

Existen numerosos parámetros de calidad en diferentes matrices alimentarias que se analizan de forma rutinaria. Los métodos de análisis convencionales son fiables, pero resultan tediosos y costosos. En este sentido y dentro de los objetivos del proyecto de esta tesis, se han preparado tres sensores poliméricos colorimétricos para la detección y cuantificación rápida y sencilla de tres parámetros de calidad en alimentos: aminas biógenas, polifenoles y nitritos.

## 2.1. Introducción

Tal y como aparece reflejado en el Capítulo 1, uno de los objetivos principales de esta memoria consiste en desarrollar polímeros sensores con potencial aplicación en el control de calidad de alimentos. En este capítulo, se presentan tres trabajos de investigación que se han realizado en relación con este objetivo. En los tres trabajos se presentan sensores poliméricos colorimétricos. En primer lugar, se describe un sensor polimérico capaz de detectar aminas biógenas en medio gaseoso para pescados. En segundo lugar, un sensor polimérico capaz de detectar polifenoles en medio acuoso para mieles y, en tercer lugar, un sensor polimérico capaz de detectar nitritos en productos cárnicos procesados al colocarlo en contacto directo con el alimento.

## 2.2. Detección de aminas biógenas

Las aminas biógenas son compuestos nitrogenados de bajo peso molecular generados, principalmente, por descarboxilación de aminoácidos o por aminación y transaminación de aldehídos y cetonas.[1]

Este tipo de compuestos se encuentra de manera natural en los seres vivos, ya que intervienen en procesos fisiológicos esenciales para ellos. Por ejemplo, en plantas, la putrescina y la espermidina ejercen un papel muy importante como respuesta al estrés y, en animales, la histamina actúa como neurotransmisor.[2,3]

En los alimentos, las reacciones de descarboxilación de aminoácidos se llevan a cabo por enzimas presentes en diversos microorganismos, por lo que son indicativo de contaminación microbiana. Las aminas biógenas más significativas y de mayor importancia en alimentación son la histamina, putrescina, cadaverina, tiramina y triptamina. Las diferentes aminas biógenas, así como los aminoácidos de los cuales provienen aparecen reflejados en la Tabla 2.1.

**Tabla 2.1.** Aminas biógenas y aminoácidos de los que provienen.

| Aminoácido | Amina biógena |
|---|---|
| Histidina | Histamina |
| Arginina | Putrescina |
| Lisina | Cadaverina |
| Tirosina | Tiramina |
| Triptófano | Triptamina |
| Metionina | Espermidina/Espermina |

Para que se produzca la reacción de descarboxilación enzimática que genera como producto aminas biógenas, se requiere de la presencia de aminoácidos libres y microrganismos, así como un medio propicio para su crecimiento.

Los productos derivados de la pesca son los alimentos más susceptibles de contener elevadas concentraciones de aminas biógenas. Esto se debe a que contienen una elevada concentración de aminoácidos libres y, a su vez, son alimentos perecederos, por lo que presentan un gran riesgo de contaminación microbiana. Este tipo de contaminación depende, en gran medida, de la especie de pescado, siendo los de la familia *Scombridae* los más susceptibles. A su vez, elevadas concentraciones de aminas biógenas también están estrechamente relacionadas con las condiciones higiénicas durante todo el procesado, desde la captura hasta la distribución. Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO),[4] la concentración de aminas biógenas en un pescado recién capturado es prácticamente despreciable pero el proceso de descomposición de este comienza inmediatamente después de la captura. Es por esto por lo que una concentración de aminas biógenas elevada en los pescados es un excelente indicador de la frescura de este tipo de productos.

Por otra parte, las concentraciones altas de aminas biógenas en alimentos pueden ser tóxicas para los consumidores. A su vez, hay que tener en cuenta que las aminas biógenas pueden reaccionar con los nitritos dando lugar a nitrosaminas, que son compuestos cancerígenos.[5] La intoxicación más habitual relacionada con las aminas biógenas se produce por la ingestión de elevadas concentraciones de histamina, dando lugar a diversos síntomas de diferente gravedad como pueden ser hipotensión, edema, espasmos bronquiales, dolor de cabeza, picor y enrojecimiento, así como problemas gastrointestinales.[6]

Aunque solo existe legislación relativa a los niveles de histamina máximos permitidos en alimentos procedentes de la pesca,[7] concentraciones elevadas de otras aminas biógenas como putrescina y cadaverina pueden potenciar los efectos derivados de una intoxicación por histamina. A su vez, concentraciones elevadas de estas dos aminas biógenas se han correlacionado en diversos estudios con el deterioro de productos pesqueros como en carpas o atunes.[8] Concretamente, mediante estos estudios se ha concluido que las carpas de buena calidad contienen hasta 10 mg/kg de putrescina, las de calidad aceptable hasta 20 mg/kg, y las que contenían más de 20 mg de putrescina por cada kg de producto se encontraban en proceso de descomposición. Además, otras investigaciones establecen unos niveles de 0,5 mg/kg de putrescina como límite de aceptación para el atún enlatado.[9] Estos estudios plasman la correlación entre la concentración de aminas biógenas y la calidad de este tipo de alimentos.

La presencia de elevadas concentraciones de aminas biógenas también puede dar lugar a la formación de nitrosaminas, que son compuestos cancerígenos que han demostrado ser promotores de varios tipos de cáncer en humanos, destacando el cáncer gástrico. Las nitrosaminas se forman, generalmente, mediante reacciones entre aminas secundarias y terciarias y nitritos.[10] En este aspecto, aminas primarias como la putrescina o la cadaverina pueden sufrir reacciones de ciclación cuando se calientan, dando lugar a aminas secundarias como la pirrolidina y la piperidina, que pueden reaccionar con nitritos dando lugar a nitrosaminas.[11] Los nitritos involucrados en este tipo de reacciones no se encuentran de forma natural en los productos pesqueros, sino que se pueden añadir en forma de nitrito sódico como impureza de sales de salado, o bien pueden formarse por la reducción de nitratos a nitritos vía bacteriana.

Una vez contextualizado el problema relativo a la presencia de elevadas concentraciones de aminas biógenas en productos procedentes de la pesca, se tratará brevemente de las diferentes formas de determinar su concentración. El método tradicional de medida más utilizado es la cromatrografía líquida de alta eficacia (HPLC).[12] Dentro de esta técnica existen multitud de opciones, siendo la metodología de referencia la que implica una derivatización post-columna con *o*-ftaldehído o espectrofluorimetría. Existen otros métodos de análisis tales como ensayos ELISA (acrónimo inglés correspondiente a *Enzyme-Linked Immunosorbent Assay*),[13] en los cuales se emplean anticuerpos para la detección de las especies diana. Estos métodos se basan en procedimientos largos y costosos que requieren una gran inversión económica y de tiempo y, a su vez, tienen que llevarse a cabo por personal especializado. Debido a la importancia en la medida de la concentración de aminas biógenas en productos procedentes de la pesca, y a los inconvenientes que presentan los métodos de análisis convencionales, se diseñó un sensor polimérico colorimétrico para la detección y cuantificación de manera sencilla y directa de aminas biógenas. El estudio completo se publicó en la revista *Sensors and Actuators B: Chemical*.[14]

## 2.3. Análsisis de polifenoles totales en mieles

Los polifenoles son sustancias que poseen más de un grupo fenol y se pueden clasificar de diferentes maneras. Una de ellas es atendiendo al número de anillos fenólicos así como a los componentes estructurales que unen dichos anillos, diferenciándose así en ácidos fenólicos (ácidos hidroxicinámicos y ácidos hidroxibenzoicos), flavonoides, estilbenos y lignanos (Tabla 2.2).[15]

**Tabla 2.2.** Clasificación de los polifenoles y estructura química.

| | | |
|---|---|---|
| Ácidos fenólicos | Ácidos hidroxicinámicos | $R_1$=OH<br>$R_1$=$R_2$=OH<br>$R_1$=$OCH_3$, $R_2$=OH |
| | Ácidos hidroxibenzoicos | $R_1$=$R_2$=OH, $R_3$=H<br>$R_1$=$R_2$=$R_3$=OH |
| Flavonoides | | |
| Estilbenos | | HO, OH, OH |
| Lignanos | | $H_3CO$, HO, $CH_2OH$, $CH_2OH$, $OCH_3$, OH |

Los polifenoles se encuentran en muchos alimentos como productos hortofructícolas, cereales, café, té, cacao y miel. Este trabajo se orienta a la determinación de polifenoles totales en este último alimento; la miel.

Los polifenoles presentan una elevada capacidad antioxidante, por lo que su ingesta es potencialmente beneficiosa para los consumidores. Existen evidencias de que el consumo de polifenoles contribuye a la prevención de enfermedades cardiovasculares, osteoporosis y cáncer.[16] Estas evidencias se encuentras apoyadas por la Organización Mundial de la Salud,[17] organismo que considera posible que los polifenoles ejerzan un papel protector frente a enfermedades cardiovasculares. Actualmente los estudios se centran en las potenciales ventajas que los polifenoles puedan ejercer en la prevención de enfermedades neurodegenerativas y diabetes *mellitus*. En 2004 se celebró en Vichy (Francia), la primera conferencia internacional acerca de polifenoles y salud. En este congreso se presentaron más de 350 estudios provenientes de científicos de más de 30 países, lo que refleja la gran importancia que se está dando a este tema.[18]

Debido a las potenciales ventajas para la salud que genera el consumo de polifenoles por su capacidad antioxidante, el análisis de estos compuestos resulta de enorme interés. Por ello, un alimento con actividad antioxidante presenta un gran valor añadido, de modo que el contenido en polifenoles totales es un parámetro de calidad en numerosas matrices alimentarias. Algunas mieles han mostrado actividades antioxidantes comparables con las de algunos alimentos hortofructícolas.[19] En la miel existen multitud de polifenoles entre los cuales destacan quercetina, ácido cafeico, crisina, galangina, acacetina, quercetina, kaempferol, pinocembrina, y apigenina (Tabla 2.3).

**Tabla 2.3.** Estructura química de los principales polifenoles de la miel.

| Polifenol | Estructura química |
| --- | --- |
| Ácido cafeico | |
| Crisina | |
| Galangnina | |
| Quercetina | |
| Acacetina | |
| Kaempferol | |
| Pinocembrina | |
| Apigenina | |

A su vez, el estudio de forma individualizada de cada polifenol concreto puede servir para identificar el tipo de miel y su origen botánico.[20]

Para la detección y cuantificación de polifenoles en alimentos existen numerosos métodos analíticos entre los cuales destacan:

- *Métodos espectrofotométricos*. En este grupo sobresale el método de Folin-Ciocalteu, donde los polifenoles presentes en una muestra reaccionan con el reactivo de Folin dando lugar a una coloración azul medible espectrofotométricamente a una longitud de onda de 765 nm.[21]
- *Métodos cromatográficos*. Son métodos muy utilizados debido a su gran precisión, exactitud y sensibilidad. Entre estos métodos destacan la cromatografía líquida de alta eficacia (HPLC),[22] la cromatografía en capa fina (TLC, acrónimo en inglés de *thin-layer chromatography*),[23] y la cromatografía de gases (GC, acrónimo en inglés de *gas chromatography*).[24]
- *Métodos enzimáticos*. Se fundamentan en una reacción de redox catalizada por una enzima, generalmente entre la enzima peroxidasa y los polifenoles de la muestra. Esta reacción genera radicales fenoxi, que reaccionan con sustratos aromáticos dando lugar a aductos coloreados.[25]

Para la determinación de la capacidad antioxidante de un alimento existen numerosos análisis entre los que se encuentran:[26]

- *Métodos basados en la utilización de radicales libres*. En este grupo destacan los métodos de análisis TEAC, acrónimo en inglés de*Trolox Equivalent Antioxidant Capacity*, ORAC, acrónimo en inglés de *Oxygen Radical Absorbance Capacity*, TRAP, acrónimo en inglés de *Total radical trapping power* y FRAP, acrónimo en inglés de *Ferric ion Reducing Antioxidant Power*.

- *Métodos enzimáticos*. Destaca la determinación de la actividad de las enzimas superóxido dismutasa eritrocitaria, enzima glutatión peroxidasa eritrocitaria, glutatión reductasa y/o catalasa.
- *Métodos cromatográficos*. Son métodos muy utilizados para la determinación de polifenoles en diversas matrices alimentarias. Destaca el uso de HPLC en combinación con diferentes detectores, como por ejemplo, detector de masas o detector UV-Vis.
- *Métodos electroquímicos.* Voltametría, amperometría, entre otros.

Estos métodos, tanto los orientados a la cuantificación de polifenoles antioxidantes como los orientados a la determinación de actividad antioxidante, se basan en procedimientos largos y costosos que requieren una gran inversión económica y de tiempo, y en ocasiones deben realizarse por parte de personal especializado. Debido a estos inconvenientes, se diseñó un sensor polimérico para la detección y cuantificación de manera sencilla y directa de polifenoles en mieles y para la determinación de su actividad antioxidante. Se escogió un polímero sensor colorimétrico, intuitivo y fácil de utilizar por personal no especializado. Además, los datos obtenidos mediante este sensor se correlacionaron con los obtenidos mediante métodos de medida convenciones. Los métodos de medida de referencia utilizados fueron el método de Folin-Ciocalteu para la cuantificación de polifenoles y el método TEAC para la determinación de la actividad antioxidante. El estudio completo se publicó en la revista *Food Chemistry*.[27]

## 2.4. Detección de nitritos en muestras cárnicas procesadas

En el campo de la alimentación, se conoce bajo el nombre de nitritos a las sales de nitrito, principalmente nitrito de sodio y nitrito de potasio. Los nitritos se pueden encontrar de forma natural en algunos alimentos, como vegetales o en agua, debido a la acción de bacterias nitrificantes, que generan nitritos en una etapa intermedia en la formación de nitratos como sales de nitrato, principalmente nitrato de sodio y nitrato de potasio. Además, los nitritos son compuestos muy utilizados como aditivos en alimentación y aparecen en el etiquetado de alimentos como nitrito de sodio (E-250) y nitrito de potasio (E-249) (Figura 2.1). Estas sales de nitrito se suelen utilizar en combinación con sales de nitrato y son aditivos alimentarios regulados y autorizados por la Unión Europea. Por tanto, los nitritos pueden encontrarse en los alimentos debido a su adición como aditivos, o bien fruto de reacciones de reducción de nitratos.

**a)** $NaNO_2$ **b)** $KNO_2$

**c)**



**Figura 2.1.** Fórmula y estructura de los nitritos: **a)** nitrito de sódico; **b)** nitrito potásico; y c) formas resonantes e hibrido de resonancia.

Los nitritos se utilizan como aditivos, principalmente, en alimentos cárnicos procesados como embutidos curados o cocidos. En este tipo de productos actúan alargando su vida útil, ya que impiden el crecimiento de microorganismos patógenos, concretamente de *Clostridium botulinum*.[28] Los nitritos también actúan como potentes agentes antioxidantes, retrasando el enranciamiento de los alimentos. A su vez, en combinación con los nitratos actúan como potenciadores de las características organolépticas tales como sabor, color y aroma.[29]

Los nitratos no son tóxicos para los mamíferos, pero los nitritos están clasificados en el grupo 2A del Centro Internacional de Investigaciones sobre el Cáncer (IARC, por sus siglas en inglés International *Agency for Research on Cancer*), es decir, se trata de compuestos posiblemente cancerígenos para el ser humano. Esto se debe a que los nitritos, mediante diferentes mecanismos, pueden dar lugar a *N*-nitroso compuestos carcinógenos. A su vez, los nitritos presentan toxicidad por sí mismos, ya que su ingesta puede provocar reacciones alérgicas, pueden actuar como agentes vasodilatadores o, en casos extremos, pueden provocar situaciones de metahemoglobinemia debido a que promueven la formación de metahemoglobina a partir de oxihemoglobina.[30]

Relativo a los *N*-nitroso compuestos, las nitrosaminas formadas por reacciones de nitrosación a partir de compuestos derivados de los nitritos suponen las sustancias más peligrosas para la salud del consumidor. Esta reacción tiene lugar entre los nitritos y sus derivados, los cuales reaccionan con aminas secundarias dando lugar a nitrosaminas.[31] Las nitrosaminas son compuestos teratógenos, mutágenos y carcinógenos, altamente peligrosos para la salud humana.[32]

La formación de nitrosaminas mediante reacciones de nitrosación a partir de derivados de nitritos puede tener lugar de forma endógena u exógena. De forma endógena, esta reacción se puede llevar a cabo en el organismo del consumidor de forma microbiológica mediante bacterias o bien, de forma química en el estómago, debido a las condiciones de pH. De forma exógena, las nitrosaminas pueden formarse en los alimentos debido a la presencia de los precursores necesarios junto con unas condiciones óptimas de pH y humedad. A su vez, diferentes factores como envasado con mallas de goma, tratamientos de fritura y cocinado a elevadas temperaturas, así como el ahumado favorecen las reacciones de nitrosación.

La legislación europea establece límites máximos de nitritos que se pueden añadir durante el procesado de alimentos (150 mg/kg) así como límites máximos residuales (entre 50 y 175 mg/kg) los cuales varían en función del producto.[33] Esto se debe a los potenciales problemas para salud derivados del uso de este tipo de aditivos mencionados anteriormente. En esta línea, la tendencia actual a nivel mundial es la de rebajar lo máximo posible la adición de nitritos y nitratos a los alimentos, debido a la formación de nitrosaminas y a los efectos nocivos para la salud que derivan de su ingesta.[34]

Los métodos tradicionales más utilizados para la cuantificación de nitritos en alimentos son los métodos cromatográficos como el HPLC,[35] métodos espectrofotométricos,[36] quimioluminiscencia,[37] o bien métodos electroforéticos.[38] Todos ellos se basan en procedimientos largos y costosos que requieren una gran inversión económica y de tiempo y, a su vez, son métodos que tienen que llevarse a cabo habitualmente por personal especializado.

Con el fin de solventar los problemas derivados del análisis de nitritos en muestras de carne procesada utilizando métodos de análisis convencionales, se diseñó un sensor polimérico para la detección y cuantificación de manera sencilla y directa de nitritos. Los resultados obtenidos mediante este sensor se correlacionaron con los resultados obtenidos relativos a la concentración de nitritos en las muestras alimentarias medidos con un método de referencia espectrofotométrico. El estudio completo se publicó en la revista *ACS Applied Materials & Interfaces*.

## 2.5 Referencias


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## 2.6. Artículos publicados

A continuación, se describen los resultados obtenidos a través de la transcripción íntegra de los tres trabajos publicados:

- *Functional aromatic polyamides for the preparation of coated fibres as smart labels for the visual detection of biogenic amine vapours and fish spoilage*
- *A simple one-pot determination of both total phenolic content and antioxidant activity of honey by polymer chemosensors.*
- *Rapid and inexpensive detection of nitrites in processed meat using a colorimetric polymer sensor and a mobile app.*

*Functional aromatic polyamides for the preparation of coated fibres as smart labels for the visual detection of biogenic amine vapours and fish spoilage*

# Functional aromatic polyamides for the preparation of coated fibres as smart labels for the visual detection of biogenic amine vapours and fish spoilage

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## Abstract

We have prepared high-performance functional aromatic polyamides with sensory pendant groups toward amines. These polymers are colourless. The pendant groups have bromonaphthalimide motifs that react with amines rendering coloured arylamines by nucleophilic aromatic substitution of the halide. Thus, these materials are colorimetric sensors toward amines. In the interest of saving costs, while having a sensory material with a high specific surface, cotton fabrics were coated, in order to render coated fibres as smart colorimetric labels toward amines. Moreover, as fish spoilage by microorganism increases the biogenic amines in food, we have applied the smart labels to visually follow the food spoilage. Also, a picture taken to the films allowed to obtain, in seconds, the digital colour definition parameters (RGB) that were correlated with the results of expensive and time-consuming conventional techniques used to obtain relevant food quality data, such as total amine concentration (treatment of the sample and HPLC), colony-forming unit (microbiological assays), total volatile basic nitrogen (TVB-N), and organoleptic test (sensory test). The smart labels are inexpensive, granting in seconds, the visual qualitative evaluation of the food quality, or even quantitative, and they comply with the European food contact materials regulation.

*Sensors and Actuators, B: Chemical* **2020**, 304, 127249

**Keywords:** Amine sensors; biogenic amines; polymer chemosensors; smart labels; fish spoilage

## 1. Introduction

The detection and quantification of chemical species are one of the main topics of chemistry. These tasks are undertaken by skilled personnel using sophisticated methods and techniques developed by scientists and technicians, such as titrimetry, electroanalytical methods, spectroscopy, chromatography, gravimetry, microscopy, and so on [1]. In recent years there has been an increase in the interest in chemical sensors, or chemosensors, as alternative analysis techniques for the in-situ, inexpensive and rapid determination of chemical species, especially in environmental, biomedical, security and food control applications [2,3,4,5,6,7,8,9,10,11]. A chemosensor, or probe, is a molecule that interacts selectively with target species with a concomitant variation of at least one microscopical property that can be detected and correlated with the target species concentration in the so-called transduction or response.

When the response is a variation of the colour of the system, we have chromogenic chemosensors, and the detection can be carried out visually, even by untrained personnel [12,13,14,15]. Moreover, a simple picture of the chemosensory system can be used to precisely quantify the concentration of the target species [16,17].

However, chromogenic probes are usually organic molecules that can only be exploited in organic solution or, at most, in organic/water solutions. We have overcome this drawback by preparing polymeric chemosensors that have a number of advantages compared with conventional organic probes: a) hydrophobic organic sensory motifs can be anchored to hydrophilic polymer structures to render water-soluble polymers, for sensing water resources and environmental samples; b) solid sensory films can be prepared for sensing not only in water but also for sensing target gases and vapours; c) the anchored sensory motifs cannot migrate to the measuring media and can be easily recovered; and d) the polymer backbone can be chemically designed for tuning the response of the sensory motifs in terms of selectivity, sensitivity and response times.

Accordingly, we have prepared sensory high-performance aromatic polyamides [18,19] as solid colorimetric chemosensors of biogenic amine vapours as intelligent labels for visually following the food spoilage. Biogenic amines are a group of nitrogenous compounds with low-molecular-weight, generated in food by decarboxylation of free amino acids, through the metabolism of different microorganisms grown in the process through which fish gets spoiled [20]. Thus, the amount of biogenic amines and the microbial counts are a good indicator of the fish freshness and quality [21,22]. Another good quality indicator is total volatile basic nitrogen (TVB-N) [23,24], along with the subjective organoleptic study carried out by sensory evaluation.

## 2. Experimental section

### *2.1. Materials*

All of the materials and solvents are commercially available and were used as received unless otherwise indicated: N-bromosuccinimide (99%, Sigma-Aldrich), dimethylformamide (DMF, 99%, Sigma-Aldrich), acenaphthene (97%, Alfa Aesar), ethanol (Absolute, VWR), glacial acetic acid (100%, VWR), sodium dichromate dihydrate (≥99.5%, Sigma-Aldrich), 5-aminoisophthalic acid (≥98%, VWR), sodium acetate (≥99.5%, Fluka), thionyl chloride (SOCl2) (≥99%, VWR), heptane (≥99%, VWR), aniline (>99%, Alfa Aesar), piperazine (99%, Sigma-Aldrich), morpholine (≥99%, Sigma-Aldrich), β--ethylendiamine (99%, Alfa Aesar), 1-butylamine (99%, Alfa Aesar), β-phenethylamine (99%, Sigma-Aldrich), tyramine (99%, Sigma-Aldrich), triptamine (98%, Aldrich), putrescine (>98%, Alfa Aesar), cadaverine (≥97%, Fluka), spermine (97%, Alfa Aesar), spermidine (≥99%, Sigma-Aldrich), histamine (97%, Acros Organics), trimethylamine (40% solution in water, VWR). m-Phenylenediamine (MPD) is commercially available (>99%, Sigma-Aldrich) and was purified by double vacuum sublimation and stored under a nitrogen atmosphere. Isophthaloyl dichloride (>99%, Sigma-Aldrich), was purified by double crystallization from dry heptane. N,N-dimethylacetamide (DMAc) (99%, Sigma-Aldrich) was vacuum-distilled over phosphorous pentoxide twice and then stored over 4 Å molecular sieves.

For the HPLC analysis the following materials were used as received: sodium hydroxide (VWR, 99%), benzoyl chloride (99%, Sigma-Aldrich), sodium chloride (99.5%, Sigma-Aldrich), hydrochloric acid (37%, VWR) diethyl ether (99.9%, VWR), methanol (HPLC grade, 99.8%, VWR), cadaverine hydrochloride (98%, Sigma-Aldrich), tyramine hydrochloride (98%, Sigma-Aldrich), histamine dihydrochloride (99%, Sigma-Aldrich), putrescine hydrochloride (99%, Sigma-Aldrich), trichloroacetic acid (99.5%, PanReac)

For the microbiological analysis, Plate Count Agar (PCA), (Scharlau) and Ringer solution (OXOID) were used.

For the total volatile basic nitrogen (TVB-N) determination, magnesium oxide (90%, Merckmilipore), boric acid (≥99.5%, Sigma-Aldrich), trichloroacetic acid (≥99.5%, PanReac) and hydrochloric acid (37%, VWR) were used as received.

*2.2. Instrumentation and measurements*

Nuclear magnetic resonance spectra (1H and 13C NMR) were recorded in deuterated dimethylsulfoxide (DMSO-d6) as the solvent, with a Varian Inova 400 spectrometer operating at 399.92 and 100.57 MHz, respectively.

Infrared spectra (FT-IR) were recorded with a FT/IR-4200 FT-IR Jasco Spectrometer with an ATR-PRO410-S single reflection accessory.

High-resolution electron impact mass spectra (EI-HRMS) were obtained at 70 eV on a 6460 Triple Quad (Agilent)mass spectrometer.

Thermogravimetric analysis (TGA) data were recorded for a 5 mg sample under a nitrogen or synthetic air atmosphere on a TA Instrument Q50 TGA analyzer using the next procedure: First, the polyamides were heated from RT to 100 ºC at 10 ºC·min-1 and kept at that temperature for 5 min to eliminate the moisture content, then, the polyamides were heated at 10 ºC min-1 to 800 ºC to complete the TGA analysis. The limiting oxygen index (LOI) was estimated using the experimental Van Krevelen equation: LOI = 17.5 + 0.4 CR, where CR is the char yield weight percentage at 800 ºC obtained from the TGA measurements under a nitrogen atmosphere.

The polymer solubility was determined with 10 mg of the polymer together with 1 mL of a solvent and stirring for 24 h at 20 ºC. The polymer was considered soluble at room temperature if a homogenous solution was obtained. If the polymer was insoluble at room temperature, the mixture was heated to reflux for 2 h, and considered soluble on heating if a homogeneous solution was obtained this way. Otherwise, the polymer was considered insoluble.

Water sorption was calculated gravimetrically. 200 mg of sample was dried for 24 h at 60 ºC in an oven with phosphorous pentoxide, and then placed in a closed box at 20 ºC with a saturated aqueous solution of $NaNO_2$ to provide a relative humidity of 65 %. The samples were weighed periodically for 8 days until a constant weight was obtained.

The intrinsic viscosity [η] was calculated by measuring the inherent viscosities, $\eta_{inh}$, of the aramids at different polymer concentrations (0.5, 0.3, 0.1, 0.05 and 0.025 $g{\cdot}dL^{-1}$) with a Ubbelohde viscometer using DMAc with 5% LiCl as the solvent at 25 ºC ± 0.1 ºC and extrapolating to zero concentration. The number average molecular weight (*Mw*) of the polyamides was measured using the Mark-Houwink-Sakurada equation, $[\eta] = k\ Mw^{\alpha}$, where the values of the constant k for polymer solutions in 5% LiCl DMAc at 25 ºC are 0.00037 dL $g^{-1}$ and 0.74, respectively [25].

The food spoilage analysis carried out with the sensory label, the HPLC measurements, the microbiological assays and the volatile total basic nitrogen (TVB-N) were performed with 2 Kg of fish (*Trachurus trachurus*) bought in a local supermarket. The skin and bones were removed, and the fish were mixed and homogenized. For the sensorial analysis, two entire fish samples of *Trachurus trachurus* were chosen and stored at room temperature while the study was performed.

The biogenic amines determination with the smart labels was carried out by taking a digital photo of the labels inside a closed vial containing biogenic amines or fish, in the presence of a colour reference (in our case a small piece of red tape). This reference was used to normalize the colour response with a conventional image

processing software, which reduces the errors associated with the different illumination conditions in different photos. The photographs were taken with a Samsung S8 smartphone and the B (blue) parameter of the RGB (red, green and blue) colour model was obtained with Photoshop Software to automatically average the data over an 11 x 11 (121) pixel area.

The HPLC results for the different samples were obtained using an HPLC VARIAN ProStar system, with a 150 x 4.6 mm Microsorb-MW 100-5 C18 column, and UV detection at 230 nm. Water (solvent A) and methanol (solvent B) were used as mobile phases. The solvent gradient started with 60% A-40% B, reaching 35% A-65% B at 6 min, 20% A-80% B at 7 min, 15% A85% B at 12 min, and 50% A50% B at 15 min, followed by the return to the initial conditions. Before the derivatization, the fish samples were shaken using a vortex mixer; VWR, centrifuged twice using a MEGAFUGE 16R Centrifuge, Thermo SCIENTIFIC and filtered with a filter paper Whatman® No.4 or a syringe filter (Puradisc™, Whatman™ 0.45 μm, VWR).

For the microbiological assay, 25 g of skinless and boneless fish (Trachurus trachurus) were aseptically taken and were homogenized with 225 mL of Ringer solution using a stomacher (STOMACHER 4000, LABORATORY BLENDER).

For the total volatile basic nitrogen (TVB-N) analysis, fish samples were homogenised by using a manual homogenizer and centrifugated using a MEGAFUGE 16R Centrifuge, Thermo SCIENTIFIC. A common steam distillation assembly was used.

In the analyses, 3-8 replicates were performed (except for HPLC that was just one measurement due to the time needed), and the errors were calculated once the dispersion of each series of data was determined. In the sensorial analysis were the measurements were performed by 14 people, the standard deviation or mean squared error was calculated.

*2.3. Synthesis of sensory polymers*

The sensory aromatic polyamides are prepared following the conventional way from two commercial monomers, *m*-phenylenediamine (***c***) and isophthaloyl dichloride (***b***), together with a synthesized aromatic diacid dichloride having the amine sensory motif (***a***) as pendant structure (See Scheme 1A and 1C, and Section S4 of the Supplementary information -SI-). The ratios of constitutional repeating units of polymer **P1** and **P2** were calculated with $^{1}H$ NMR (see SI, Section S4), and agreed with the monomer feed ratio, considering the error of the measuring technique (calculated X:Y ratio was 0.1:0.9 for **P1** and 0.45:0.55 for **P2**).

**Scheme 1.** Synthesis of: a) monomer; b) aromatic polyamide model and its general reaction with amines; and c) aromatic polyamides.

Before the preparation of polyamides, the viability of the polyamide synthesis was checked by previously preparing a polyamide model (**M1**) using polymerization conditions (SI, Section S2.). The characterization of **M1** allows us

to discard by-products that could impair the preparation of the polymers. This step is important because aromatic polyamides are high-performance materials characterized by outstanding thermal and mechanical properties, and any by-reactions would impair these performances. Also, they are highly insoluble and not so easy to characterize. Moreover, **M1** also allows for the testing of the sensory mechanism. Thus, colourless organic solutions of **M1** react with amines rendering coloured arylamines by nucleophilic aromatic substitution of bromine (tested amines: piperazine, morpholine, ethylenediamine or 1-butylamine). The synthetic conditions and the analysis and properties of monomers, model and polymers are reported in the supplementary information (SI, Section S3-S6).

From the viewpoint of the polymeric materials, the prepared aromatic polyamides are thermally resistant polymers with degradation temperatures, in terms of 5% and 10% weight loss calculated by thermogravimetry, of around 430 and 475 ºC, respectively (SI, Section S6). They are soluble only in aprotic polar solvents, such as *N*-methyl-2-pyrrolidone, *N,N*-dimethylacetamide, *N,N*-dimethylformamide or dimethyl sulfoxide (SI, Sections S5). The average molecular mass of **P1** and **P2** was $M_w = 6.4\times10^4$ and $M_w = 4.5\times10^4$, respectively, and the intrinsic viscosities $[\eta] = 1.18$ dL·g$^{-1}$ and $[\eta] = 0.95$ dL·g$^{-1}$, respectively.

## 3. Results and discussion

### *3.1. Sensing of biogenic amines vapours*

According to literature, bromonaphthalimides react with amines rendering coloured arylamines by nucleophilic aromatic substitution of the halide [26-28]. Thus, the chemical reaction and the evolution of colour can be used to detect amines. Accordingly, we thought that polymers with bromonaphthalimides moieties could be worth to prepare sensory material and we designed aromatic polyamides containing these moieties. However, previous to the sensory polymer preparation we checked the validity of the idea by synthesizing a model molecule of the sensory polymer (**M1**), that renders the coloured arylamines, as anticipated, upon contact with amines (**M2**) (Scheme 1), thus validating the idea

and the mechanism described in the literature. The reaction of **M1** with amines to render **M2** is observed inmediately, even at room temperature, though it was carried out at higher temperature (SI, section S3) for obtaining high purity chemical for chemical characterization.

As expected by the behaviour of **M1**, the colourless sensory polymers **P1** and **P2** turned reddish in atmospheres containing ethylenediamine, putrescine and cadaverine, among other amines. Thus, we decided to prepare coated cotton fibres as sensory labels to amine vapours ($\mathbf{F_{P1}}$ and $\mathbf{F_{P2}}$ stand for the coated fibres prepared with polymers **P1** and **P2**, respectively) (see SI, Section S7, for preparation methodology). The coated fibres have advantages over sensory films. Thus, the thinner the coating the smaller the quantities of sensory polymers used and the lower response time of the sensor (because of the higher specific surface). In addition, migration tests were performed for $\mathbf{F_{P1}}$ and the results followed the restriction for the overall migration limit (<10 mg/dm$^2$) as defined in Commission Regulation (EU) No 10/2011 for food contact materials (See SI, Section S8). The response of the sensory material $\mathbf{F_{P1}}$ towards commercial biogenic amines is depicted in Figure 1. A photograph taken to the smart coated fibres allow for the individual titration of the amines, as shown as an example for putrescine in Figure 2, where it is shown the representation of B (blue component of the colour definition of the smart fibres) *vs* putrescine vapour concentration (titration curves for other amines are shown in SI, Section S9.

1 2 3 4 5 6 7 8 9 10 11 12 13

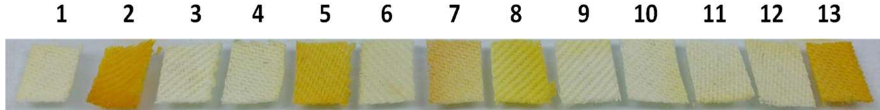

**Figure 1.** Response of coated fibre $\mathbf{F_{P1}}$ to the presence of amines (20 mg of amines in the bottom of a 14 ml sealed vial at 20 ºC, the fibre on the top of the vial without contacting the amines): 1. Blank; 2. β-ethylenediamine; 3. Spermine, 4. Spermidine; 5. Cadaverine; 6. Histamine; 7. Trimethylamine; 8. Morpholine; 9. Piperazine; 10. Tryptamine; 11. Tyramine; 12. β-phenylenediamine; 13. Putrescine

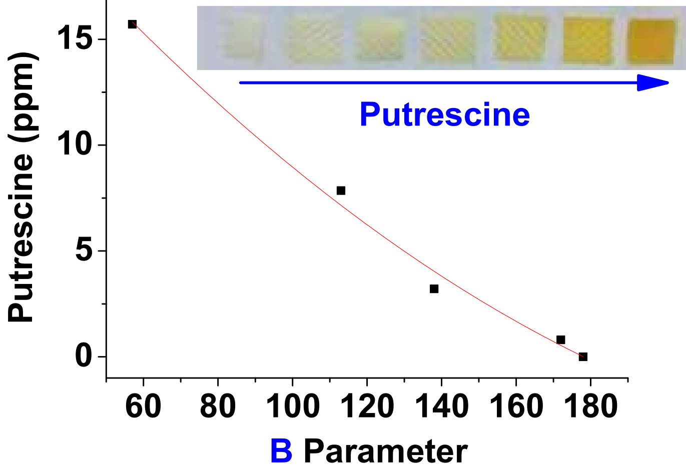


**Figure 2.** Response of coated fibre $\mathbf{F_{P1}}$ to the presence of putrescine (different amounts of putrescine in the bottom of a sealed vial at 20 ºC with the fibre on the top of the vial without contacting the putrescine).

*3.2. Sensing of biogenic amines vapours from fish spoilage*

Once demonstrated that the smart coated fibres colorimetric sense the presence of amine vapours, and known that the spoilage of food, and specifically fish, is accompanied by the concomitant generation of biogenic amines [29], the deterioration of fish meat was followed with the colour development of the $\mathbf{F_{P2}}$ by two means: a) visually, and b) analysing the B component digital colour definition (RGB parameters) of pictures taken to the materials with smartphones. For this purpose, the fish Atlantic horse mackerel (*Trachurus trachurus*) was maintained at a constant temperature (20 ºC) and samples were taken along time for 3 days (see Table 1). The methodology followed is described in SI, Section S10.

*3.3. Comparison with conventional tests for fish quality control*

The colour evolution, in terms of the digital definition of the colour of a picture taken with a smartphone (B parameter), was compared with well-established techniques [30-33], such as HPLC, microbiological assay, total volatile basic nitrogen (TVB-N), and sensory test (Figure 3). The concentration of amines (histamine, putrescine, cadaverine and tyramine) was determined by HPLC [34]. Similarly, the concentration amount of aerobic mesophilic microorganisms and the TVB-N were calculated [35]. Also, the subjective sensory test was performed [36]. These are conventional tests for fish quality control carried out systematically by highly-skilled personnel with expensive equipment or are time-consuming, or both. The methodology followed for HPLC calibration and measurements and for the other techniques is conventional and it is described in the SI (Sections S11-S14).

**Table 1.** Evolution of $\mathbf{F_{P2}}$ sensory labels along time in a vial containing fish meat (Atlantic horse mackerel).

| Time (minutes) | B parameter | Sensory label |
|---|---|---|
| 0 | 137±0.50 | |
| 1520 | 121±0.83 | |
| 2960 | 118±0.90 | |
| 4400 | 108±0.87 | |

Thus, a simple picture taken to a smart label $\mathbf{F_{P2}}$ inside a package of fish, without contacting it, can be used to estimate the total amine concentration (treatment of the sample and HPLC), colony-forming unit (microbiological assays), total volatile basic nitrogen (TVB-N), and organoleptic test (sensory test). These are four relevant parameters usually measured for food control (see the correlation curves in SI, Sections S11-S11). Moreover, the colour can be used to visually predict the quality, in terms of the chemical parameters commented.

Each label has an estimated cost of production of 0.02 euros, so it can be considered inexpensive, especially compared to the cost of analysis of the microbiological assay, HPCL, or total volatile basic nitrogen. Also, it is interesting to consider that the analysis is carried out at a given time just taking a picture, without spending time and without the need of highly trained personnel.

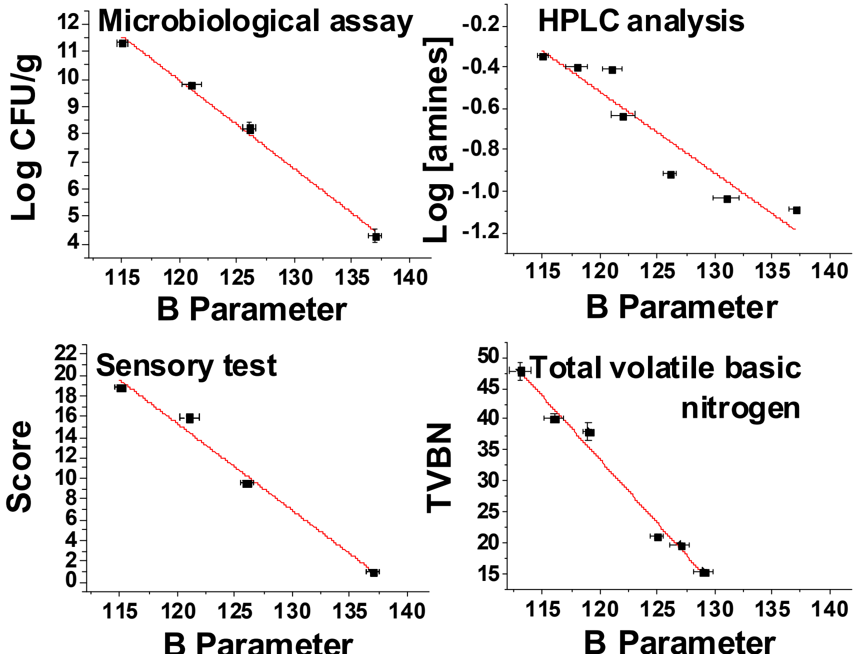


**Figure 3.** Correlation of the B parameter (blue component of the RGB parameters that define the digital colour) of pictures taken along time (3 days) to $\mathbf{F}_{P2}$ in an atmosphere containing fish meat with microbiological results, total amine content determined by HPLC, total volatile basic nitrogen and sensory test.

## 4. Conclusions

In summary, we have demonstrated that cotton fibres coated with high-performance sensory polymers can be used as costless colorimetric smart labels for the following of fish spoilage. The labels change their colour in the presence of biogenic amines in the atmosphere without being in contact with the fish meat (the amines are produced mainly by microorganisms along the spoilage process).

This colour can be used to visually detect the freshness of the fish, or even the label can be photographed with a smartphone and its colour definition used to calculate within seconds relevant analytical data Otherwise, this information could only be obtained by trained personnel with time-consuming and expensive techniques, such as total amine concentration (treatment of the sample and HPLC), colony-forming unit (microbiological assays), total volatile basic nitrogen (TVB-N), and organoleptic test (sensory test).

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*A simple one-pot determination of both total phenolic content and antioxidant activity of honey by polymer chemosensors*

# A simple one-pot determination of both total phenolic content and antioxidant activity of honey by polymer chemosensors

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## Abstract

We have developed a new method for the rapid (2h) and inexpensive (materials cost<0.02 €/sample) "2-in-1" determination of the total phenolic content (**TPC**) and the antioxidant activity (**AOX**) in honey samples. The method is based on hydrophilic colorimetric films with diazonium groups, which react with phenols rendering highly colored azo groups. The ***TPC*** of the sample is correlated to its trolox equivalent antioxidant capacity (***TEAC***). The intensity of the color allows us to determine both ***TPC*** and ***TEAC*** of the sample by the analysis of a picture taken with a smartphone that is analysed by the use of the color-definition-parameters (RGB). The controlled light conditions and the systematic use of the same camera avoid the periodical calibration of the system improving the efficiency of the method. Thus, it is a simple method carried out by non-specialized personnel and it involves much lower money and time investment compared to traditional methods.

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**Keywords**: Honey; sensor; total phenolic content; antioxidant activity; TEAC; polymer; polyphenols.

## 1. Introduction

Honey is a product made by bees from flower nectar or honeydew mixed with bees' secretions after maturing in honeycombs (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). It is used as a natural sweetener. Honey contains more than 181 compounds, being the monosaccharides fructose and glucose the most abundant ones. The proportion of each sugar is different, depending on the honey type (Ball, 2007). Honey has also low quantities of other compounds, such as proteins, enzymes, vitamins, amino acids, minerals, trace elements, aromatic substances, and polyphenols (Bogdanov, 2016). Currently, honey polyphenols are of growing interest because they help authenticate honey, and notoriously contribute to antioxidant capacity, antimicrobial activity (Alvarez-Suarez et al., 2010; Baltrušayt, Venskmonis, & Čeksteryte, 2007; Estevinho, Pereira, Moreira, Dias, & Pereira, 2008; Rao, Krishnan, Salleh, & Gan, 2016), as well as other potentially beneficial effects.

In 2017, the world production of honey was around 1,800,000 t, being more than 700,000 t intended for international trade with an estimated export value of 2,364 million US$ (FAOSTAT, 2014). Spain ranked in the 4th worldwide position, leading the EU honey production with 29,393.2 t (Ministerio de Agricultura Pesca y Alimentación, 2019). All these data show the commercial significance of honey, so that researching on new rapid and low cost methods for its authentication and quality control is of utmost importance.

Apart from the compulsory parameters of legal regulations (Thrasyvoulou et al., 2018), nowadays, most laboratories that analyze honeys determine both total phenolic content (**TPC**) and antioxidant activity (**AOX**), because the results of these parameters can contribute to improve honey commercialization. The most common method to analyze **TPC** is the spectroscopic assay using the Folin-Ciocalteu reagent (Singleton & Rossi, 1965), that is still time consuming despite having been successively modified and improved. As for the measurement of **AOX**, one of the most employed

procedures is the spectrophotometric method known as trolox equivalent antioxidant capacity (***TEAC***), using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (***ABTS***) as the radical source (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999), that is also time-consuming, because the ***ABTS*** radical formation takes 16 hours. Other methods as GS/MS or HPLC with different detectors require a large expenditure of money and time, as well as specialized personnel (Quintero-Lira, Ángeles Santos, Aguirre-Álvarez, Reyes-Munguía, Almaraz-Buendía, & Campos-Montiel, 2017). For all this, a quick, easy, and simple method for the double detection of **TPC** and **AOX** of honey would be a breakthrough in this field.

Chemical sensors (or chemosensors) are rapid detection methods, based on the interaction of a receptor molecule with target species, which generates a quantifiable change in a macroscopic property of the material. In the case of a change of color (chromogenic sensor), the response can be visually for a semi-quantitative detection but can be also easily registered and analyzed with a smartphone and a PC respectively, by using the digital color definition parameters (RGB) (Vallejos, Muñoz, Ibeas, Serna, García, & García, 2013). The use of chemosensors for honey control is increasingly being used, mainly concerning honey sugars (Isa et al., 2017; Revenga-Parra et al., 2020), and antibiotics' residues detection (Bougrini et al., 2016; Cervera-Chiner et al., 2020).

The purpose of this work is to develop sensory colorimetric films for the rapid and low cost dual determination of the **TPC** and the **AOX** in honey samples, so that the analysis can be carried out by non-specialized personnel, quickly, and without using dangerous reagents, organic solvents, or personal protective equipment (PPE).

## 2. Material and methods

*2.1. Samples*

This study was carried out with eight representative honeys harvested in 2019 in Castilla-León, a Spanish area located in the northern Iberian Plateau that held the highest number (3,827) of apicultural undertakings in Spain in 2018, representing 16% of the total apicultural undertakings of this country (Ministerio de Agricultura, Pesca y Alimentación, 2019). Honeys' botanical origins were determined by both melissopalinology (Louveaux, Maurizio, & Vorwohl, 1978; Terradillos, Muniategui, Sancho, Huidobro, & Simal-Lozano, 1994; Von Der Ohe, Persano Oddo, Piana, Morlot, & Martin, 2004), and sensory analyses (Marcazzan, Mucignat-Caretta, Marina Marchese, & Piana, 2018; Persano Oddo & Piro, 2004; Piana, Persano Oddo, Bentabol, Bruneau, Bogdanov, & Guyot Declerck, 2004), there being 1 ling heather (Calluna vulgaris (L.) Hull) honey (sample 1), 2 honeydew honeys (samples 2 and 3) and 5 multifloral honeys (samples 4-8). The sediment of the samples showed that the most important secondary pollen types were Leguminosae type Trifolium spp., Leguminosae type Genista spp., Rosaceae type Rubus spp. and Compositae type Helianthus annuus L. Additional information about honey samples in ESI S1.

*2.2. Materials*

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: sodium hydroxide (VWR, 99 %), 1-vinyl-2-pyrrolidone (**VP**) (Aldrich, ≥ 98%), methylmethacrylate (**MMA**) (Aldrich, 99%), 4-aminostyrene (**$SNH_2$**) (Aldrich, 97%), sodium nitrite (VWR, 99.5%), m-cresol (AlfaAesar, > 99 %), Folin-Ciocalteu 2 N reagent solution (Aldrich, 99%), hydrochloric acid (Aldrich, 37 %), gallic acid (**GA**) (Aldrich, 97.5-102.5 %), sodium carbonate (Aldrich, 99.5 %), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (***ABTS***) (Aldrich, ≥ 98 %, HPLC), potassium persulfate (Aldrich, ≥ 99%), (±)-

6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) (Aldrich, 97%). Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallized twice from methanol.

For the ***Folin-Ciocalteu*** and ***TEAC*** methods, honeys were treated according to the following procedure: 5 g honey was dissolved in 10 mL of distilled water. Then, the solution was transferred to a 50 mL flask, completed with distilled water, and filtered through a Whatman nº1 paper. The resulting solution was labeled as **HOx**. For the method of sensory colorimetric films, 5 g honey was dissolved in 45 ml NaOH aqueous solution (0.1M). The mixture was shaken and homogenized for 10 minutes and then, it was filtered through a Whatman nº1 paper. The resulting solution was labeled as **B-HOx**. More information about honey samples in ESI S1.

*2.3. Preparation of the sensory polymeric films*

The starting film was prepared by bulk radical polymerization of three commercial monomers: **VP**, **MMA**, and **$SNH_2$** in a molar feed ratio of 49.5/49.5/1 (**VP/MMA/$SNH_2$**) using 1% mol of AIBN as radical thermal initiator. The polymerization was carried out at 60 ºC, overnight, in a mold comprised between two silanized glasses (100 µm thick), in an oxygen-free atmosphere. The film was removed from the mold and 8 mm diameter discs were cut with a punch and dipped into an acid solution of $NaNO_2$ (10 mL of water, 1 mL of HCl 37 %, and 40 mg of $NaNO_2$) at RT for 90 min.. Stable sensory films with pendant benzenediazonium salt motifs were easily prepared by this way.(Bustamante et al., 2019).

*2.4. Instrumentation and methods*

UV/Vis spectra were recorded using a Hitachi U-3900 UV/Vis spectrophotometer. The RGB analysis was carried out by taking a picture of the polymeric discs, with a Samsung Galaxy S8 smartphone and a retro-illumination homemade lightbox previously developed,(Vallejos, Reglero, García, & García, 2017, stereolithography archive available as supporting data) essential to always

reproduce the same light and distance conditions (camera specifications: 1/3.6″ 8Mp sensor-1.22 μm pixel size, f/1.7-aperture lens, 25 mm-equivalent focal length, autofocus). The sample tubes used for the preparation of the different honey solutions (**HOx**) shaking, were carried out using a vortex shaker (VWR).

The reference method for the determination of **TPC** of honey was the so-called ***Folin-Ciocalteu*** assay, and it was performed according to the methodology described by Sancho et al. (2016), based on Singleton & Rossi (1965) procedure. To carry it out, 0.5 mL of **HOx** was mixed with 2.5 mL of ***Folin-Ciocalteu*** 0.2 N reagent solution. The resulting mixture was homogenized using a tubes agitator (Vortex shaker, VWR) and then, the samples were left at room temperature for 5 minutes and 2 mL of sodium carbonate (75 g/L) was added and the mixture was homogenized again. Finally, the tubes were incubated for two hours in darkness at room temperature and the absorbance of samples was recorded at 760 nm using a Hitachi U-3900 UV/Vis spectrophotometer. More information about this method in **ESI S3.**

The chosen reference method for determining the antioxidant activity was the ***TEAC*** method (Re et al., 1999), modified for our purpose (Sancho et al., 2016), based on the inhibition of ***ABTS*** by honey components. To carry out this assay, 10 μl of **HOx** was mixed with 1490 μl of ***ABTS*** radical (***ABTS*** radical is generated by mixing an aqueous solution of ***ABTS*** (7 mM), and $K_2S_2O_8$ (2.45 mM) aqueous solution. The resulting solution was stirred in darkness for 16-18 h at room temperature. Secondly, 1490 μl of ***ABTS*** reagent was mixed with 10 μl of standard trolox aqueous solutions ranging from 0 to 3 mM, and the absorbance at 734 nm was recorded after 6 minutes ($A_6$) by triplicate. The inhibition percentage of the sample was obtained by the following equation ($\mathbf{100*(A_b-A_6)/A_b}$), where $\mathbf{A_b}$ is the absorbance (734 nm) of the sample used as blank and $\mathbf{A_6}$ the absorbance of the mixture of **ABTS** and **Trolox** sample after 6 minutes mixed). The resulting mixture was homogenized and then, the samples were incubated for 6 minutes in darkness at room temperature and the absorbance of

samples was recorded at 734 nm using a Hitachi U-3900 UV/Vis spectrophotometer. More information about this method in **ESI S4.**

For the analysis with the sensory colorimetric films, 8 mm diameter discs were directly dipped for 2 hours in 10 ml of **B-HOx** at room temperature, without a further experimental procedure. The discs were removed from the solution and washed 3 times with NaOH 0.1M for 15 minutes for finally taking the photographs in triplicate. RGB parameters of the pictures were analyzed using GIMP 2 free image software, and it was found that the B (blue) parameter is the only significant variable, the only one that brings relevant information. In the same way, a titration of the sensory colorimetric films with gallic acid (**GA**) was carried out, by dipping 8 mm diameter discs in basic aqueous solutions (NaOH 0.1M) of **GA** ranging from 50 to 260 mg/L.

## 3. Results and discussion

The sensory polymers that the study is about have been already tested and characterized as sensors for different kinds of phenols, as phenols used as fungicides and pesticides (Bustamante et al., 2019), or polyphenols in wines (Vallejos, Moreno, Ibeas, Muñoz, García, & García, 2019). In this case, we have oriented the developed materials for a real need, improving the existing reference methods for the determination of **TPC** and **AOX** in terms of time, money, and simplicity. For that, honeys were firstly analyzed with the reference methods, and secondly with the proposed one.

### *3.1. Titration of sensory colorimetric films with GA using the sensory colorimetric films*

**GA** is the phenol used as a standard in ***Folin-Ciocalteu*** reference method. In our previous works (Bustamante et al., 2019), this sensor has been tested with various phenols, but not with **GA**, so we found mandatory to confirm the sensory behavior of the sensory colorimetric films with this phenol. However, this is only a verification test, which does not take in account the matrix effect of honey.

The titration with **GA** was performed by dipping 8 mm sensory discs in aqueous basic solutions (NaOH, 0.1 M) of **GA** in concentrations ranging from 50 to 260 mg/L (**Fig. 1**) following the procedure and washing process described in the experimental part. The color development of the sensory films from grey to brow-yellowish could be visually broadly correlated with the **GA** concentration. The titration curve was built correlating the blue (B) parameters of the digital images of the films with the concentration of **GA**, as depicted in **Fig. 1** (RGB parameters of the digital photographs are shown in **ESI, Section S2**). This Figure shows a linear ($R^2$ = 0.994) correlation pointing out to the sensory films as tools to detect and measure the concentration of **GA** in water environments. Therefore, and based on this result, the next step was to measure the **TPC** in honey samples with the sensory polymeric films comparing, at the same time, the results obtained with ***Folin-Ciocalteu*** method.

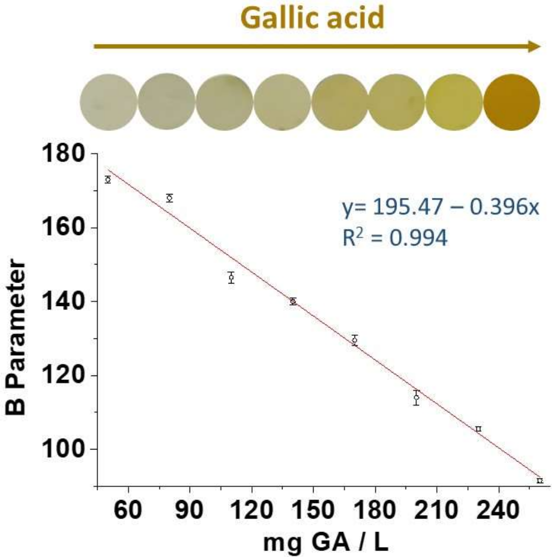



**Figure 1.** Top: Pictures were taken to the 8 mm diameter sensory films after dipping for 2h in NaOH 0.1M solutions of GA in different concentrations; from 50 to 260 mg/L. Graph: Graphical representation of the blue parameter (B) of the RGB parameters vs the concentration of GA. The fitted linear equation is showed within the graph.

*3.2. Determination of the total phenolic content (TPC) of honeys by Folin-Ciocalteu method*

This spectrophotometric method measures the absorption of a blue complex formed through a redox reaction between a sample´s phenols and the ***Folin-Ciocalteu*** reagent, as compared with a **GA** standard (Gülçin, Şat, Beydemir, Elmastaş, & Küfrevioğlu, 2004). The **TPC** of honeys is expressed as mg of **GA** per 100 grams of honey. **Table 1** shows the results for the measured honey samples, and the corresponding titration curve with **GA** can be found in **ESI S3.**

**Table 1.** Total phenolic content of the measured honeys by the ***Folin-Ciocalteu*** method. The table shows the average of the 3 measurements of each honey sample.

| Total phenolic content (mg GA / 100 g honey) | |
|---|---|
| Honey-1 | 106.71 ± 4.87 |
| Honey-2 | 37.44 ± 3.25 |
| Honey-3 | 120.90 ± 5.18 |
| Honey-4 | 46.85 ± 3.47 |
| Honey-5 | 30.16 ± 3.09 |
| Honey-6 | 53.91 ± 3.63 |
| Honey-7 | 93.68 ± 4.56 |
| Honey-8 | 35.72 ± 3.21 |

*3.3. Determination of antioxidant activity by **TEAC** assay*

The method follows the suppression of the characteristic long-wave absorption band of the ***ABTS*** radical in presence of hydrogen-donating antioxidants. This spectrophotometric assay measures the relative ability of antioxidants to scavenge the ***ABTS*** radical generated in aqueous phase, as compared with a trolox (water-soluble vitamin E analog) standard (Dykes, Rooney, Waniska, & Rooney, 2005), thus, the antioxidant activity is usually expressed as μmol of

trolox per 100 grams honey. **Table 2** shows the results for the measured honeys (fresh weight), and the corresponding titration curve with trolox can be found in **ESI S4.**

**Table 2**. Antioxidant activity of the measured honey by the **TEAC** method. The table shows the average of 3 measurements of each honey, and the obtained antioxidant activity expressed as μmol of trolox / 100 g honey.

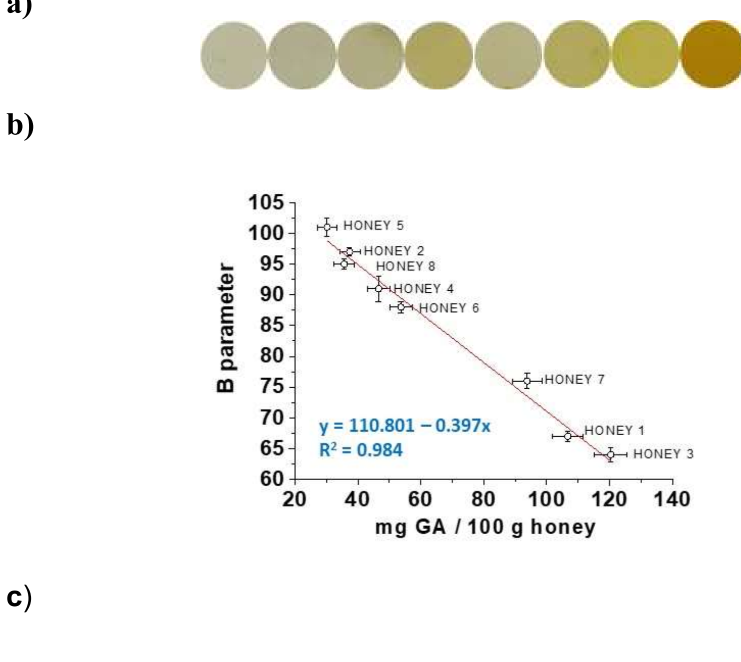
| **Antioxidant activity (μmol trolox / 100 g honey)** | |
|---|---|
| **Honey-1** | 729.33 ± 116.02 |
| **Honey-2** | 459.53 ± 104.81 |
| **Honey-3** | 727.39 ± 115.94 |
| **Honey-4** | 485.84 ± 105.90 |
| **Honey-5** | 395.83 ± 102.16 |
| **Honey-6** | 470.66 ± 105.27 |
| **Honey-7** | 615.07 ± 111.27 |
| **Honey-8** | 447.48 ± 104.30 |

*3.4. Correlation study between Folin-Ciocalteu and TEAC methods with the sensory colorimetric films*

The proposed method of the sensory colorimetric films is based on the color change produced by the formed highly colored azo groups between a sample´s phenols and the diazonium salt motifs of the discs. The measured experimental variable is the blue parameter of the RGB parameters (see **ESI S5**),and is represented *vs* the obtained data from ***Folin-Ciocalteu*** and ***TEAC*** methods.The correlation between methods is clearly observed in **Fig. 2**, and the initial proposal to determine both the **TPC** and the **AOX** with a single analysis is confirmed, just by dipping the sensory colorimetric films for 2 hours in **B-HO$_{(1\text{-}8)}$** solutions at room temperature. This new method saves time, reagents, and money in comparison, not only with ***Folin-Ciocalteu*** or ***TEAC*** methods but also with others which

present the same drawbacks. **Table 3** shows a brief comparison of all these methods with our proposed one, in terms of required equipment, response time, visual response (Y/N), and low-cost nature (Y/N).

**a)**




**b)**

**c)**

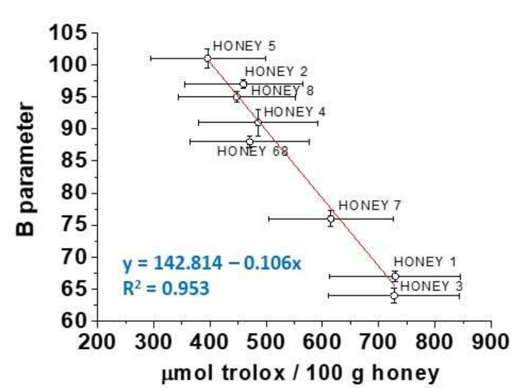



**Figure 2. a)** Sensory colorimetric films after dipping in 10 ml of **B-HO$_{(1\text{-}8)}$** solutions for 2 hours at room temperature; **b)** Correlation between total phenolic content obtained by ***Folin-Ciocalteu*** method and B parameter of the sensory colorimetric films (B parameter); **c)** Correlation between the antioxidant activity obtained by ***TEAC*** method and B parameter of the sensory colorimetric films.

**Table 3.** Comparison between conventional methods and sensory colorimetric film for the determination of the total phenolic content (**TPC**)and antioxidant activity.

| Methodology | Equipment | Measured property | Response time | Visual response | Low-Cost | References |
|---|---|---|---|---|---|---|
| TPC | Spectrophotometer. Vortex shaker. | Polyphenol content | ≈3.5 h | No | No | (Alvarez-Suarez et al., 2010) |
| *TEAC* method | Spectrophotometer, Vortex shaker. | Antioxidant activity | ≈3 h | No | No | (Alvarez-Suarez et al., 2010; Moniruzzaman, Khalil, Sulaiman, & Gan, 2012) |
| High-performance liquid chromatography (HPLC) | HPLC | Polyphenol content | ≈5 h | No | No | (Pyrzynska & Biesaga, 2009) |
| Gas chromatography (GC) | Gas chromatograph | Polyphenol content | ≈12 h | No | No | (Alvarez-Suarez et al., 2010; Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2006) |
| Capilar electrophoresis (CE) | Power source, capillaries, detector. | Polyphenol content | ≈4 h | No | No | (Andrade, Ferreres, Gil, & Tomás-Barberán, 1997; Gómez-Caravaca et al., 2006) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ultra performance liquid chromatography (UPLC) | Chromatographic system | Polyphenol content | ≈5 h. | No | No | (Alvarez-Suarez et al., 2010; Muñoz Jáuregui, Ortíz Ureta, Blanco Blasco, Castañeda Castañeda, Alvarado Yarasca, & Ruiz Quiroz, 2014; Trautvetter, Koelling-speer, Speer, Trautvetter, Koelling-speer, & Speer, 2009) |
| FRAP assay | Spectrophotometer | Antioxidant activity | ≈1.5 h | No | No | (Aljadi & Kamaruddin, 2004; Alvarez-Suarez et al., 2010; Lachman, Orsák, Hejtmánkářová, 2010; Moniruzzaman et al., 2012; Muñoz Jáuregui et al., 2014) |
| DPPH assay | Spectrophotometer, Vortex shaker. | Antioxidant activity | ≈1.5 h | No | No | (Akbulut, Özcan, & Çoklar, 2009; Moniruzzaman et al., 2012; Muñoz Jáuregui et al., 2014; Trautvetter et al., 2009) |
| 2-desoxi-D-ribose assay | Spectrophotometer, Water bath | Antioxidant activity | ≈3.5 h | No | No | (*Repositorio Institucional* Universidad Distrital - RIUD: Estudio Cromatográfico por HPLC-UV, Cuantificación de Fenoles, Flavonoides y Evaluación de la Capacidad Antioxidante en Miel de Abejas>, n.d.; Trautvetter et al., 2009) |
| Superoxide anion assay | Spectrophotometer, Vortex shaker. | Antioxidant activity | ≈30 min | No | No | (Lachman et al., 2010; Trautvetter et al., 2009) |
| Polymeric sensor | Smartphone, PC, Vortex shaker. | Polyphenol content | 2 h | Yes | Yes | (Vallejos et al., 2019) |
| Sensory colorimetric films | Smartphone, PC, Vortex shaker. | Antioxidant activity & Polyphenol content | 2 h | Yes | Yes | This Work |

*3.5. Proof of concept. Determination of TPC and AOX with sensory colorimetric films*

Once demonstrated the correlation between the reference methods and the proposed method, is possible to calculate the **TPC** and the **AOX** of all honeys only by substituting B parameter in the fitted equations showed in **Fig. 2**. As summary, **Table 4** shows the obtained results of **TPC** and **AOX**, both by reference methods and the proposed one.

**Table 4**. Total phenolic content and antioxidant activity of honeys measured both the reference methods (***Folin-Ciocalteu*** and ***TEAC***) and the proposed one (sensory colorimetric films).

| | Total phenolic content | | Antioxidant activity | |
|---|---|---|---|---|
| | ***Folin-Ciocalteu*** method (mg GA / 100 g honey) | *Sensory colorimetric films* (mg GA / 100 g honey) | ***TEAC*** method (μmol trolox / 100 g honey) | *Sensory colorimetric films* (μmol trolox / 100 g honey) |
| **Honey 1** | 106.71 | 114.78 | 729.33 | 722.96 |
| **Honey 2** | 37.44 | 33.11 | 459.53 | 428.56 |
| **Honey 3** | 120.90 | 122.95 | 727.39 | 752.40 |
| **Honey 4** | 46.85 | 49.44 | 485.84 | 487.44 |
| **Honey 5** | 30.16 | 22.22 | 395.83 | 389.30 |
| **Honey 6** | 53.91 | 57.61 | 470.66 | 516.88 |
| **Honey 7** | 93.68 | 90.28 | 615.07 | 634.64 |
| **Honey 8** | 35.72 | 38.55 | 447.48 | 448.18 |

## 4. Conclusions

Chemical sensors, or chemosensors, have great potential in the field of *in-situ*, fast, and low-cost analysis. Among chemical sensors, polymeric sensors have advantages of lack of migration of the sensor subunits, manageability, and possibility of working in solid-state. We have studied a sensor with diazonium moieties pendant to the main acrylic chains that can be used as a colorimetric chemosensor for the quantification of **TPC** and the determination of **AOX** on

honey samples. The color of the sensors changes according to the samples' polyphenols concentration. This sensor allows us to determine the **TPC** and the **AOX** of different honey samples in a single measurement, in 2h (see **ESI S6**) , by only taking a photograph to the sensory colorimetric films after immersion in honey samples, avoiding high-cost methodologies that require expensive equipment, trained personnel and long analysis time. This sensor has great potential in the quality control of honey samples reducing costs derived from this type of analysis, deeply diminishing the environmental impact of the measurements and greatly speeding up the analysis time.

## CRediT authorship contribution statement

Lara González-Ceballos: Investigation, Writing - original draft. Maria del Mar Cavia: Conceptualization, Methodology. Miguel A. Fernández-Muiño: Conceptualization, Methodology. Sandra M. Osés: Conceptualization, Methodology, Investigation. M. Teresa Sancho: Conceptualization, Methodology, Writing - original draft. Saturnino Ibeas: Methodology, Investigation, Validation. Félix C. García: Conceptualization, Methodology. José M. García: Project administra-tion, Methodology, Funding acquisition. Saúl Vallejos: Conceptualization, Methodology, Writing - original draft.

## Declaration of Competing Interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128300

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*Easy nitrite analysis of processed meat with colorimetric polymer sensors and a smartphone app.*

# Easy nitrite analysis of processed meat with colorimetric polymer sensors and a smartphone app

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## Abstract

We have developed an *in-situ* methodology for determining nitrite concentration in processed meats that can also be used by unskilled personnel. It is based on a novel colorimetric film-shaped sensory polymer that changes its color upon contacting the meat, and a new mobile app that automatically calculates the manufacturing and residual nitrite concentration by only taking digital photographs of sensory films and analyzing digital color parameters. The film-shaped polymer sensor detects nitrite anions by an azo-coupling reaction since they activate this reaction between two of the four monomers that the copolymer is based on. The sensory polymer is complemented with an app, which analyses the color in two different digital color spaces (RGB and HSV) and performs a set of 32 data fittings representing the concentration of nitrites *vs.* 8 different variables, finally providing the nitrite concentration of the test samples using the best fitting curve. The calculated concentration of nitrites correlates with a validated method (ISO 2918: 1975) usually used to determine nitrites, and no statistically significant difference between these methods and our proposed one has been found in our study (26 meat samples, 8 prepared and 18 commercial). Our method represents a great advance in terms of analysis time, simplicity, and orientation to use by average citizens.

*ACS Applied Materials & Interfaces*.

**Keywords**: Polymer chemosensory films, sensors, nitrites in meat, colorimetry, RGB, HSV, colour space.

## 1. Introduction

The interest of society in having a balanced and healthy diet has increased remarkably in the last two decades,.[1–4] As a direct consequence, there is a need to detect and control different chemical compounds added to processed food, such as processed meat.[5]

One of the most worrying additives is nitrites, commonly used as preservatives in food products such as processed meat,[6–9] and which provide a characteristic pinkish color and fresh meat flavor to products, e.g., york ham or mortadella (see **Figure 1**). However, nitrites are directly related to gastrointestinal tumors, stomach cancer, and the so-called blue baby syndrome.[6,8,10] The World Health Organization (WHO) has set a fatal nitrite dose of 3 mg $L^{-1}$ (65.2 μM) in drinking water. In human body, concentrations from 33 to 250 mg of nitrite per kg of body weight are lethal, and from 0.4 to 200 mg/kg of body weight are enough to cause methaemoglobinaemia.[11]

With this background, there is a high interest in controlling nitrite amount in foodstuffs, especially by the final consumer,[12–14] since due to nitrite´s reactivity with hemoglobin and other products, the residual level of nitrites varies over time.[15,16] In general, actual methods for determining the nitrite concentration are not oriented to the use by average citizens, are tedious, require reagents manipulation by specialized personnel, and use laboratory equipment (such as fluorimeters or spectrophotometers).[6,17–19] Most relevant advances in this field described in the bibliography depict devices based on light-emitting diodes and photodiodes.[20,21] Despite being increasingly simple systems, they are still dependent on a battery, an electrical circuit, etc., so their application in real-life is not obvious, especially in food control, food safety, or food packaging. Electrochemical systems are also a family of powerful analytical techniques but present also the same disadvantages.[22]

This work describes a sensory polymer sensitive to nitrites,[23] as a colorimetric sensory film (POLYSEN) for the visual detection of nitrites by simply putting it in contact with the food surface for 15 min and then immersing it in a basic solution. Going further to visual detection, color changes registered with a smartphone digital camera are an increasingly proven tool to quantify species of interest,[24–26] including nitrites.[27,28] However, we have realized that the end-user must analyze and often interpret the results. Again, this is a barrier for most citizens, so we have boosted the usability of our nitrite quantification system with the development of an application for Android and iOS "Colorimetric Titration" (freely available from App Store and Google Play),[29,30] which automatically auto-calibrates the measurement, analyses the data, and finally outputs a nitrite concentration result to the user.

In short, the novelty of our proposal for analyzing nitrites in meat lies in the combination of the following characteristics: (1) a costless novel sensory material based on a high amount of a hydrophilic monomer (*N*-vinyl-2-pyrrolidone, mol 45%), which ensures the absorbent properties of the material, and a hydrophobic monomer (methylmethacrylate, mol 45%), which ensures that the material can withstand handling by non-specialized personnel; (2) easy experimental procedure by only touching and revealing; (3) self-calibrated measurement; (4) a novel smartphone application that allows the *in-situ* measurement that can be easily used by the average persons (**Figure 1**).

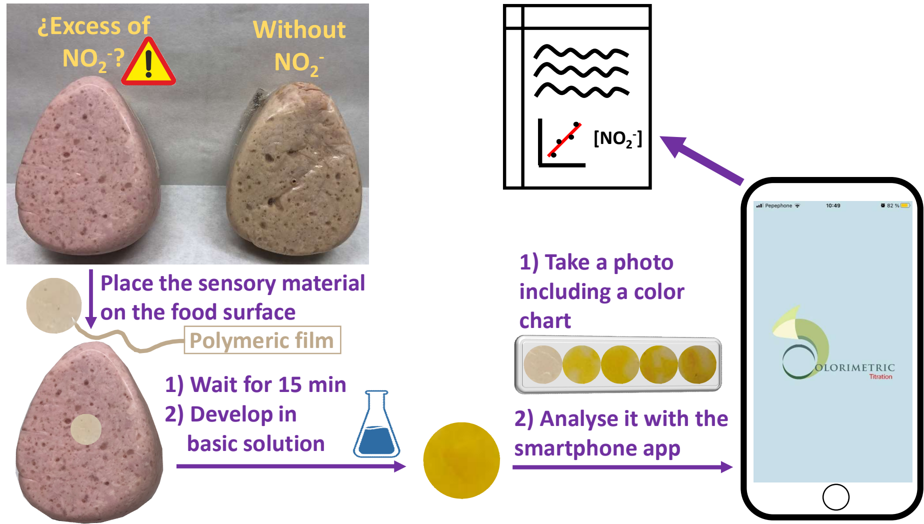


**Figure 1**. Proposed methodology for the one-pot determination of the nitrite concentration in food samples. The new method is based on a film-shaped polymeric film, and is powered by the smartphone application Colorimetric Titration.

## 2. Material and Methods

### *2.1. Meat Samples*

This methodology was initially validated with cooked pork shoulder samples prepared on a pilot plant. Once the procedure was optimized, commercial samples of different products containing varying percentages of meat pork were also tested.

#### *2.1.1. Prepared meat samples*

A total of 8 representative samples of cooked pork shoulder (5 samples to calibrate the system, and 3 test samples, termed as T1, T2, and T3) were prepared. The nitrite concentrations added in the manufacturing were 1, 37.5, 112.5, 150, and 300 mg/kg. Additionally, replicates of 300 (T1), 37.5 (T2), and 1 mg/kg (T3) were prepared as test samples.

For the elaboration of meat products in the pilot plant, pork shoulder trimmings were used. The fat was removed from the trimmings to obtain lean meat and was minced with a 15 mm plate in a meat grinder. A total of 8 kg of meat mass was made by vacuum mixing lean meat as the starting mix with the composition depicted in **Table S1 (ESI-Section S2)**. Following, this mass was split into 8 batches of 1 kg, and different amounts of sodium nitrite, sodium chloride, carrageenan, starch, and water were added according to **Table S2 (ESI-Section S2)** and all mixed together. Each batch's meat mass was vacuum-sealed in a heat-shrinkable plastic bag, clipped, and after a short dipping in hot water, was put in stainless steel molds before cooking in a meat oven at 80° C till the product reached 72°C in the center of the meat piece. Once cooked, products were kept for 24 hours in refrigeration before unmoulding. This process, especially the mixing, was optimized to obtain very homogeneous samples regarding composition and surface texture.

*2.1.2. Commercial meat samples*

Once the method was optimized with the prepared samples, 18 different meat products were purchased to carry out a proof of concept. The products were purchased in two different supermarkets in north Spain (additional information in **Section S7** in the SI**)**.

*2.2. Materials*

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (MMA) (Merk, 99%), *N*-vinylpyrrolidone (VP) (Acros Organic, 99%), 4-aminostyrene ($SNH_2$) (Aldrich, 99%), 3-aminophenol (Aldrich, 99%), methacrylic anhydride (Aldrich, 99%), hexane (Aldrich, 99%), hydrochloric acid (VWR-Prolabo, 37%), sodium hydroxide (VWR-Prolabo, 99%), sodium nitrite (Applichem Panreac, 99%), ethanol (Aldrich, 96%), zinc acetate dihydrate (Aldrich, 99.5%), acetic acid glacial (Aldrich, 100%), potassium hexacyanoferrate (II) trihydrate (Aldrich, >98.5%), sulfanilic acid (Aldrich, 99%), 1-naphthylamine

(Merck, 99%). Azo-bis-isobutyronitrile (AIBN, Aldrich, 98%) was recrystallized twice from methanol.

*2.3. Instrumentation*

Digital pictures were taken to the sensors (8 mm diameter discs) with an iPhone 8 smartphone (Apple Inc., Cupertino, CA, USA) and processed with the developed APP. For the proof of concept (Section 3.4), an additional smartphone was used (Samsung Galaxy Note 20 Ultra) to demonstrate the methodology´s versatility and the not dependence on a specific smartphone. The app can be freely downloaded from the App Store and Google Play.[29,30] UV/Vis spectra were recorded using a Hitachi U-3900 UV/Vis spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) with an ATR-PRO410-S single reflection accessory. High-resolution electron-impact mass spectrometry (EI-HRMS) was carried out on a spectrometer (Micromass AutoSpec Waters mass, Micromass Holdings Ltd., Cary, North Carolina) using an ionization energy of 70 eV and a mass resolving power >10000. $^{1}H$ and $^{13}C\{^{1}H\}$ NMR NMR spectra (Avance III HD spectrometer, Bruker Corporation, Billerica, Massachusetts, USA) were recorded at 300 MHz for 1H, and 75 MHz for 13C, using deuterated solvents like dimethyl sulfoxide (DMSO-d6) or deuterated chloroform (CDCl3) at 25°C.

*2.4. Design and synthesis of the Film shaped polymeric sensor (POLYSEN)*

The polymeric material was designed as a film with gel behavior.[31] In previous works,[32] we have shown that this type of sensory material can react effectively with targets in the solid state. This property makes this format ideal for the proposed application, in which the film is simply left on the food´s surface, without the need to carry out the detection using a solvent.

The sensory film was prepared by bulk radical polymerization of three commercial monomers (VP, MMA, and $SNH_2$) and one synthetic monomer (*N*-(3-hydroxyphenyl)methacrylamide, HPMA) in a molar feed ratio of 45/45/5/5

(VP/MMA/SNH2/HPMA) using 1% weight of AIBN as radical thermal initiator. The synthesis and characterization of HPMA are depicted in **ESI-Section S3**. In an oxygen-free atmosphere, the polymerization was carried out overnight at 60°C, in a mold comprised of two silanized glasses (100 µm thick). The sensory film was removed from the mold, washed three times with methanol, dipped in HCl (4%) for 30 minutes, and thoroughly washed with water to eliminate the excess of acid. This acid treatment allows the formation of nitrosyl cation in the presence of nitrites. Sensory discs, $\phi$ 8mm, were punched from POLYSEN. The complete characterization of the material is depicted in **ESI-Section S4**.

*2.5 Methods*

*2.5.1. ISO 2918:1975 Reference method for the detection of nitrite anions*

In this standardized method, the food sample is extracted by stirring in hot ethanol for at least 1 hour and treated with *Carrez Reagents.* The solution is shaken and let stand for 10 min, and finally centrifuged for 5 min at 2000 r.p.m. to separate the fat and evaporate the liquid to a final volume of 200 mL.

After decolorization with activated charcoal and the addition of the colorimetric reagent (sulfanilic acid and α-naphthylamine), the solution is allowed to stand in the dark for 15 min at room temperature. Finally, the absorbance is measured at 520 nm wavelength and the analysis time for 1 meat sample was around 150 min.

All samples, both prepared and commercial ones, were tested with this method. The 8 prepared meat samples were measured 9 days after their preparation, and the commercial samples were bought and measured on December 10$^{th}$ and 14$^{th}$, 2021.

The calibration curve was carried out in the same way, using prepared aqueous sodium nitrite solutions at different concentrations. More information about the standardized procedure can be found on standard ISO 2918:1975 and **Section S1** in the SI.

### *2.5.2. POLYSEN + APP method for the detection of nitrite anions*

The proposed method is based on three main steps: (1) the preparation of the color chart; (2) the taking of the digital photograph, including the test sample and the color chart; and (3) the measurement using the APP.

Firstly, one POLYSEN disc was placed on the surface of each prepared meat sample (5 prepared meat samples as calibration samples) with different nitrite concentrations for 15 minutes at room temperature. After that, the POLYSEN discs were dipped in an aqueous NaOH 0.1 mol/L solution for 1 minute at room temperature. POLYSEN discs were plasticized as a color chart, which is, in this case, specifically designed for cooked pork shoulder samples, and stable along time (at least for 1 year). The calibration meat samples must always be measured in parallel with the reference method, but this step is mandatory only once and is expected to be carried out by the manufacturer of POLYSEN in a real situation, not by the end-user.

Secondly, the test samples were measured in the same way, i.e., by placing them on the meat surface for 15 min and revealed in NaOH. The color chart and the test samples were photographed together with an iPhone 8 (see **Figure 2**). This step is expected to be carried out by the end-user in a real situation.

Thirdly, the taken photo was analyzed with the APP. The proposed method is based on taking a joint photo of the calibration color chart (prepared by the manufacturer) and the test POLYSEN discs (tested by the end-user). In this way, the system self-calibrates in each measurement, since the conditions for image acquisition are exactly the same for both the calibration color chart and the test POLYSEN discs. This fact makes the method not dependent on the smartphone model, the lighting conditions, or the distance to the object.

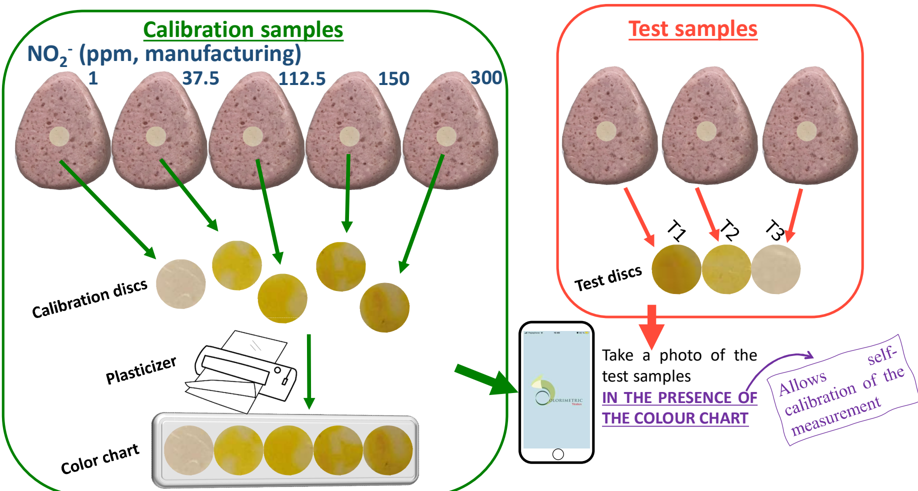


**Figure 2**. Image of different POLYSEN discs (5 titration POLYSENs and 3 test POLYSENs) after being in food contact for 15 min, and then developed in aqueous NaOH 0.1 mol/L solution for 1 min. Then, the titration samples are plasticized as a color chart, and finally, it is photographed together with the test samples.

Once the photograph is taken, the application first asks for the number of discs used for the calibration color chart and the number of test POLYSEN discs (**Figure 3a**). After that, the user has to adapt the circular selector to the size and position of each disc of the calibration color chart and then enter the concentration values which were initially added by the manufacturer (**Figure 3b**). Finally, the user must adapt the circular selector to the test POLYSEN discs. At this point, the application reads the different digital color parameters (red R, green G, blue B, hue H, saturation S, and value V). Additionally, by using the read RGB parameters, the principal components of RGB,[33] and ΔRGB,[34] are also calculated. In total, 8 different variables. The application performs the graphical representation of the entered concentration against the 8 variables (**Figure 3c**), builds the graphs also with the logarithm of the concentration, and performs linear and quadratic fits. In total, the application makes 32 fits, and the fitted curve with the highest $R^2$ coefficient is considered the best option; thus, it appears in the first position. This part of the work was performed with an iPhone 8, a calibration color chart of 5 points and 3 test POLYSEN discs. Taking no care of the illumination

(standard lighting conditions), the nitrite concentration versus green (G) parameter of digital color reaches the highest $R^2$ coefficient. Thus, this is the fitted curve equation the app uses to calculate concentrations of the test samples, as shown in **Figure 3d**. For more information about the use of the application, see **ESI-Section S5**.

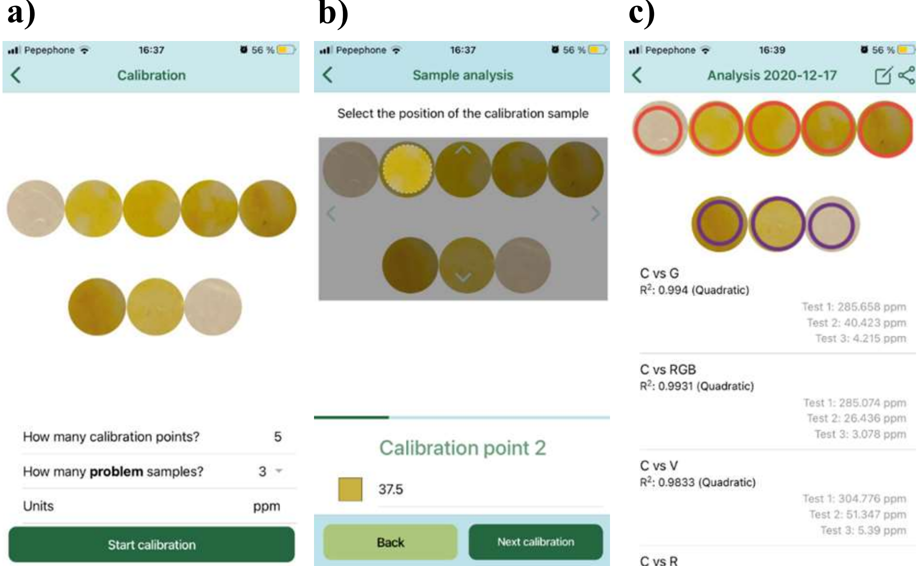


**Figure 3.** a) Screen showed in the APP after the photo is uploaded, asking for the number of titration and test samples. b) Screen showed in the APP, in which the user has to adapt the circular selector to each POLYSEN disc and enter the known concentration value. In the case of test samples, the user has only to adapt the circular selector. c) Screen showed in the APP after performing 16 linear and 16 quadratic fits of concentration (and the logarithm of concentration) versus different digital color parameters. The fit with the highest $R^2$ parameter is chosen as the best option and is shown in the first position. d) Final screen shown by the APP after clicking on each fit. The APP shows the graph with the titration (blue) and test (yellow) points, as well as the calculated nitrite concentrations for the test samples.

After these considerations, it is worth mentioning that the photograph containing the same calibration color chart, with the same test POLYSEN disc, but made in other light conditions (such as in a dark room, using the camera flash), or taken with another smartphone model, will indeed show different colors, and therefore different parameters. However, as far as the end-user is concerned, it does not imply any problem because the app will simply calculate the

concentration of the test sample with another equation that fits better in those lighting conditions, that smartphone model, etc.

*2.6. Migration tests*

The migration study was carried out as described in Commission Regulation (EU) no. 10/2011 (and amendments) relating to plastic materials and articles oriented to food contact applications.

The standard test methods were carried out at 100 °C with acetic acid, ethanol, and olive oil (*EN 1186-3:202, 2002; UNE-EN 1186-1:2002, 2002)*.[35,36]

*2.7. Limits of detection (LOD) and quantification (LOQ)*

The limit of detection (LOD) and the limit of quantification (LOQ) of our sensory system was calculated by the following equations: LOD=3.3 x SD/s and LOQ=10 x SD/s, where "SD" is the standard deviation of the blank sample and "s" is the slope of a calibration curve in the region of low analyte content (below 21 ppm; more information in **the Section S6** in the SI).

## 3. Results and discussion

*3.1. Nitrite detection principle*

Nitrite detection is based on the widely known and studied azo coupling, both in solution and in the solid phase.[31,37] This irreversible reaction is activated by nitrite anions and is based on 3 stages, as shown in **Figure 4**. First, nitrite anions react with the hydrochloric acid contained in the material and form the highly reactive nitrosyl cation. In the second stage, nitrosyl cations react with the aniline units provided by the monomer $SNH_2$, resulting in the formation of a benzenediazonium salt derivative. Immediately, this benzenediazonium salt derivative reacts with the phenol units provided by the HPMA monomer, allowing the formation of the yellow-red azo compound. This straightforward concept, combined with the digital color analysis performed with the APP, results in a simple method for the detection of nitrites with a LOD of 0.85 ppm and a LOQ of 2.57 ppm.

Since the hydroxyl group of monomer HPMA activates both ortho and para positions, the azo-coupling reaction could occur in 3 different positions, being the most favored one the *p*-OH position. In fact, the synthesis and characterization of very similar compounds (valid as models) have already been published, both in *p*-OH and *o*-OH positions.[38,39]

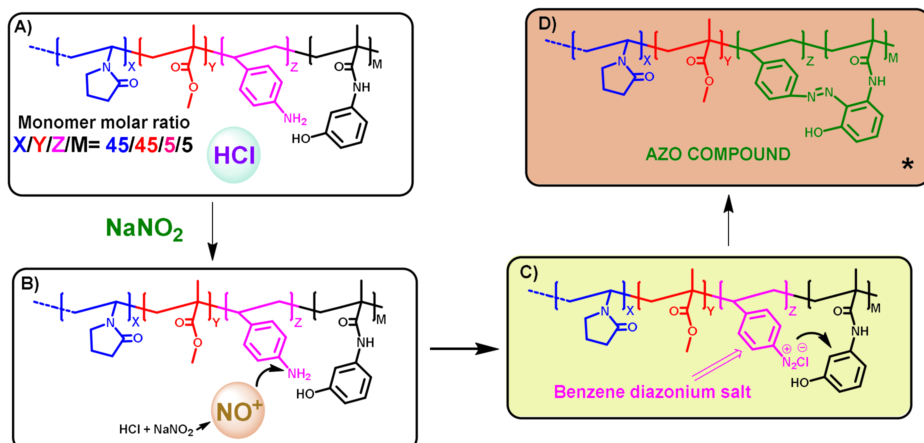


**Figure 4.** Film-shaped polymeric sensor's (POLYSEN) chemical structures in the different stages of the detection process: A) Starting material. B) Nitrosyl cation formation inside the film. C) Benzenediazonium salt formation. D) Azo compound formation. *The formation of the azo compound is also possible in p-OH position, but only the o-OH substitution is shown for clarity.

Although only 10 mol % of the monomeric units are reactive in the presence of nitrites, the rest of the material has a very high relevance, especially concerning stability over time, handling, and hydrophilicity. As shown in previous works, this type of material allows stabilizing compounds as reactive as benzene diazonium salts, allowing these sensors to be used months after their manufacture without losing activity.[31] In addition, the molecular recognition of the sensory motifs towards the target is improved in this type of solid material compared to the same recognition in liquid solutions, due to the protective effect exerted by the polymeric environment.[40] Finally, the combination of hydrophilic and hydrophobic monomers allows working on 100 % aqueous media, keeping good manageability/handling even in the swelled state.

*3.2. Migration study*

The nitrite detection implies the material contact the surface of the foodstuff, as plastics in everyday packaged meat products. Thus, the material was tested under the same regulations for ensuring the non-migration of any substance (toxic or not toxic) to the foodstuff. The results show that POLYSEN complies with the restriction for the overall migration limit (<10 mg $dm^{-2}$) as defined in the mentioned European regulation. Specifically, migration results obtained for POLYSEN in 3 % acetic acid, 10 % ethanol, and olive oil were 0.7, 5.9, and 4.8 mg $dm^{-2}$, respectively.

*3.3. Validation of the sensory polymer with prepared meat samples*

The results of nitrite concentration calculated by both the reference and POLYSEN + APP methods are shown in **Table 1**.

**Table 1**. Obtained data for nitrite concentration of test samples calculated with the reference method (ISO 2918:1975) and the POLYSEN + APP method. Nitrite concentration data from reference and POLYSEN+APP methods are means of ± standard deviation of 2 and 3 replicates, respectively.

| | **Residual [$NO_2^-$] (mg/kg)** | |
|---|---|---|
| **Test Samples** | **ISO 2918:1975 (Reference method)** | **POLYSEN +APP method** |
| **T1** | 108.62 ± 0.12 | 108.01 ± 4.32 |
| **T2** | 10.59 ± 0.16 | 13.12 ± 1.91 |
| **T3** | 3.57 ± 0.08 | 2.90 ± 0.27 |

For the proposed method, the application was run with the photograph containing the calibration color chart and test POLYSEN discs shown in **Figure 2**. While the manufacturing nitrite concentrations of the prepared meat samples were 1, 37.5, 112.5, 150, and 300 ppm, the obtained residual concentrations were 3.57 ± 0.08, 10.59 ± 0.16, 45.23 ± 0.31, 54.16 ± 0.01, 108.62 ± 0.12 ppm, respectively. Thus, we used these residual values since we think they are more

realistic. All nitrite values are reduced after manufacturing due to the reactivity of nitrites with different compounds, such as hemoglobin, except for the sample made with 1 ppm of nitrites, which increases to 3.57 ppm. Our interpretation is that it is the basal nitrite concentration, since the value obtained from a blank sample provided a very similar result.

The best fit resulting from the analysis was the $[NO_2^-]$ *vs.* G parameter (green), obtaining a $R^2$ coefficient of 0.992. Fits with other parameters could also be good, and it is the end-user who chooses the best, or the most appropriate, or simply the one with the higher $R^2$ coefficient value. For that, the APP can export all results as a “CSV” file that can be found in **ESI-APP-DATA.** Once selected the best fit, the application calculates the nitrite concentration of the test samples (**Table 1)**.

As shown in **Table 1**, the proposed POLYSEN + APP method gives very similar values to the reference method, i.e., the proposed method could replace the reference method for quantifying nitrite in food samples. A statistical analysis also supports these results (see **Section S8** in the SI), in which no statistically significant differences have been found between reference method and POLYSEN + APP method.

### *3.4. Proof of concept. Working with commercial samples*

We describe a new method for detecting nitrite anions aimed to be used as home analysis by non-skilled citizens. Thus, we believe a real example would be clarifying for the reader. Therefore, we pose a fictitious situation (based on completely real measurements) in which a manufacturer of polymeric materials starts manufacturing the "POLYSEN KIT".

- Case study A

This case study was carried out with the iPhone 8 of subject A, and a color chart containing 7 points, which was prepared according to section 2.5.2, but in this case with 7 commercial samples of different products (**Figures 5a and 5b**).

Subject A took a small portion of the product he wanted to analyze and left a POLYSEN disk on its surface for 15 min. After developing the disc in basic water, he took a joint photo of the color chart and the test disc, and analyzed it with the Colorimetric Titration app (**Figure 5c**).

The best fit result was the one that appeared first (concentration *vs.* G), and the nitrite concentration result was 18.3 mg of nitrites per kg (**Figure 5d**).

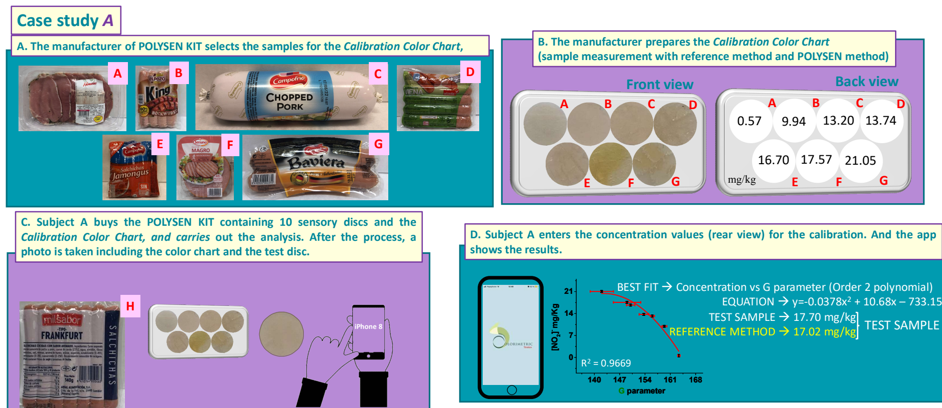


**Figure 5**. Case study A of the proof of concept. a) choice of samples for the preparation of the calibration color chart; b) calibration color chart, front and back view; c) example of measurement of a problem sample with an iPhone 8 and a POLYSEN disk; d) results of the test sample obtained by the app Colorimetric Titration (the concentration value obtained by the reference method is also displayed).

- Case study B

This case study was done with a different smartphone and completely different commercial products. Specifically, the color chart was made with 9 commercial products (**Figure 6a and 6b**).

Subject B followed the same procedure as subject A, but the joint photo of the color chart and test disc was taken under different light conditions, with another smartphone model (Samsung Galaxy Note20 Ultra 5G), and at a different distance to the object.

After the analysis with the app (**Figure 6c**), subject B reached the best fit when representing [$NO_2^-$] versus "H" parameter (from HSV color space: Hue, Saturation, Value). The nitrite concentration result was 6.3 mg of nitrites per kg (**Figure 6d**).

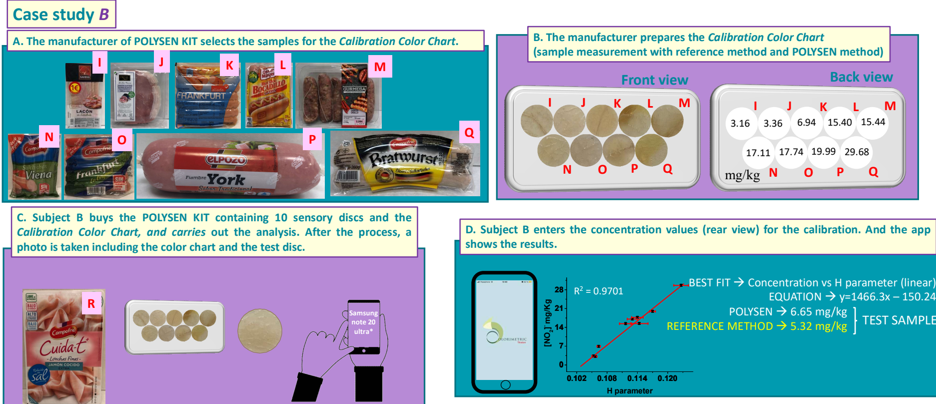


**Figure 6.** Case study B of the proof of concept. a) choice of samples for the preparation of the calibration color chart; b) calibration color chart, front and back view; c) example of measurement of a problem sample with an iPhone 8 and a POLYSEN disk; d) results of the test sample obtained by the app Colorimetric Titration (the concentration value obtained by the reference method is also displayed).

To draw solid conclusions about the results of this proof of concept, we measured the nitrite concentration of the two test samples of the two case studies with the reference method. As shown in **Figures 5d** and **6d**, the results are very similar to those obtained with the POLYSEN KIT. Specifically, the nitrite concentrations obtained with the reference method were 17.0 and 5.3 mg/kg for case studies A and B, respectively.

Additionally, a statistical analysis of the results was performed (**ESI-Section S7**), and no significant differences were found between both methods.

## 4. Conclusions

A new sensory polymeric film-shaped material has been developed to detect nitrite in meat products through a color change. The procedure is as simple as leaving the material on the meat surface, waiting for 15 minutes, and developing it in aqueous NaOH (1M). This research work is designed so that unskilled citizens could use this sensor since the developed application for smartphones quickly analyses the color change and gives a nitrite concentration value. One of the weak points when using photograph-based analysis has been overcome: the conditions in which the photographs are taken. First, the calibration POLYSEN discs must be plasticized once the calibration samples have been measured to obtain a calibration color chart stable over time. Second, the photograph of the test samples must always be taken in the presence of the color chart. In this way, this robust methodology eliminates the interferences that different lighting conditions or the use of different smartphones could cause. In other words, the system self-calibrates for each measurement. This study is intended as a proof of concept in which it has been demonstrated that the methodology is practical and works. The methodology has been optimized with 8 prepared cooked pork shoulder samples, but additionally, a proof of concept with 18 commercial meat products has been carried out. This new procedure based on POLYSEN+APP has great potential in the quality control of meat, reducing analysis times and costs.

## Author Contributions

Marta Guembe-García: Carried out laboratory research related to the polymeric material and the app, wrote the draft of the manuscript and contributed to the contextualization of the work. Lara González-Ceballos: Carried out laboratory research related to the methodology described in the standard ISO 2918:1975, and collaborated on the first draft of the manuscript. Ana Arnaiz: Carried out statistical data analysis, collaborated on creating and designing Figures, and reviewed & edited the final manuscript. Miguel A. Fernández-Muiño: Carried out the first conceptualization of the work, and established the methodology related to the reference method. M. Teresa Sancho: Carried out the first conceptualization methodology related to the reference method, and wrote different experimental sections on the manuscript. Sandra M. Osés: Carried out laboratory research and reviewed/edited the final manuscript. Saturnino Ibeas: Carried out data analysis oriented to the validation of the proposed methodology, collaborated in the development of the software and reviewed the final version of the manuscript. Jordi Rovira: Provided mandatory resources for the work, such as the pilot plant for the production of packaged meat, established the methodology, and fine-tuned the meat production method. Beatriz Melero: Carried out laboratory research related to the pilot plant, contextualized the work with formulations used in the industry for the production of meat and collaborated in the writing of the supplementary material of the work. Cesar Represa: Carried out the main part of the informatics, and fine-tuned the application to adapt to the needs of the job, collaborated on revision tasks. Jose M. García: Participated in the global conceptualization, in the review of the final article, and sought financing for development. Saúl Vallejos: Participated in the global conceptualization, in the writing of the Original draft, in the review and edition of the final article, and sought financing for development.

## Supporting Information

Reference method used for the determination of the residual concentration of nitrite in meat samples (based on standard ISO 2918:1975); recipe for the manufacturing of cooked pork shoulder; synthesis and characterization of *N*-(3-hydroxyphenyl)methacrylamide (HPMA); characterization of the film-shaped polymeric sensor (POLYSEN); user manual for the smartphone APP "Colorimetric Titration"; statistical comparison between the reference and POLYSEN + APP methods; commercial samples; brief data (excel file).

## Conflicts of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

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# CAPÍTULO 3

# Polímeros con propiedades antimicrobianas en el campo del envasado activo de alimentos

El envasado de alimentos ha dejado de representar únicamente una barrera física, para dar lugar al concepto de envasado activo, donde el alimento interacciona con el propio envase y el entorno, mejorando su calidad y seguridad. El envasado activo antimicrobiano representa el tipo de envasado activo más común en la industria cárnica. Las demandas actuales en este sector se basan en la búsqueda de sustancias de origen natural, como por ejemplo los aceites esenciales, que prolonguen la vida útil del alimento. Este capítulo describe la síntesis de un polímero con potencial antimicrobiano capaz de prolongar la vida útil de alimentos de origen cárnico en un 50 %. Este material está basado en la unión covalente entre la matriz polimérica y un aceite esencial, la vainillina.

## 3.1. Introducción

Tal y como aparece reflejado en el Capítulo 1, uno de los objetivos principales de esta memoria consiste en el desarrollo de un polímero con propiedades antimicrobianas para aumentar la vida útil de productos cárnicos envasados. Este polímero se añade como parte de un envase para dar lugar a un envasado activo con doble función, prolongar la vida útil del producto y actuar como absorbente del exudado de la carne. En este capítulo se presentan un trabajo de investigación y una patente que se han basado en este objetivo.

El envasado activo es un tipo de envasado que tiene como objetivo alargar la vida útil del alimento que contiene. Este tipo de envasado se fundamenta en la interacción entre el alimento, el propio envase y el entorno, elementos que interaccionan entre sí para prolongar la vida útil del producto y mantener o incluso mejorar su calidad y seguridad. Existen diferentes tipos de envasado activo, entre los que se encuentra el envasado activo antimicrobiano, que se basa en la adición de una o varias sustancias antimicrobianas dentro del propio de envase, de tal forma que se inhiba o se retrase el crecimiento microbiano, lo que se traduce en un incremento en la duración del producto.[1] Las sustancias antimicrobianas se pueden añadir de forma dispersa dentro del propio envase o bien se pueden inmovilizar en este. La técnica de inmovilización presenta una importante ventaja frente a la adición de forma dispersa, ya que no existe migración de la sustancia al alimento.

La contaminación microbiana en los productos cárnicos tiene lugar principalmente en la superficie del alimento. Debido a esto, tradicionalmente se han utilizado diferentes aditivos y/o conservantes que se añaden directamente a la superficie del alimento con el fin de incrementar su vida útil. Otra fuente de contaminación microbiana importante son los absorbentes de humedad que se encuentran en las bandejas de productos cárnicos. Estos materiales tienen la función de retener el exudado del alimento, mejorando así la apariencia el producto y evitando un rechazo por parte del consumidor. Por otra parte, representan una importante fuente de contaminación debido a que los líquidos que retienen son un potente caldo de cultivo para una inmensa variedad de microorganismos.[2] Debido a esto, los aditivos y/o conservantes también se pueden añadir en este tipo de materiales. Entre estas sustancias con potencial poder antimicrobiano destacan el etanol, el dióxido de carbono, los antibióticos y las sustancias de origen natural, como los aceites esenciales.

Las demandas actuales se basan en el uso de sustancias antimicrobianas de origen natural, aceptadas para su uso en alimentación, de amplio espectro y que sean efectivas a bajas concentraciones. A su vez, se

requieren sustancias que no alteren las propiedades organolépticas del alimento y que presenten un bajo coste, de tal forma que no se encarezca demasiado el producto final. Entre las potenciales sustancias antimicrobianas que cumplen con estos requisitos se encuentran, entre otros, los aceites esenciales.[3]

Según la Organización Internacional de Normalización (ISO, por sus siglas en inglés *International Organization for Standardization*), un aceite esencial se define como "el producto obtenido a partir de una materia prima natural de origen vegetal, mediante destilación por arrastre de vapor, por procesos mecánicos a partir del epicarpio de los frutos cítricos u otros productos, o bien mediante destilación seca tras una separación física de la fase acuosa en el caso de que la hubiere". Tal y como se ha comentado, los aceites esenciales presentan propiedades antimicrobianas y antioxidantes, por lo que en la industria del envasado de alimentos se pueden utilizar para incrementar la vida útil de los productos. En contrapartida, estos compuestos son volátiles y muy inestables. Cuando entran en contacto con los alimentos se comienzan a degradar y, a su vez, alteran las propiedades organolépticas del alimento debido que presentan un fuerte aroma y muchas de ellas son sustancias coloreadas. Fruto de estos inconvenientes, se están desarrollando nuevas tecnologías que permitan aprovechar las propiedades beneficiosas de los aceites esenciales en el envasado de los alimentos, minimizando los efectos negativos que presenta su adición de forma directa.[4] Entre estas nuevas técnicas destaca su encapsulación en liposomas o bien su incorporación en nanopartículas sólidas de naturaleza lipídica.[5] A su vez, existen numerosos estudios en los cuales los aceites esenciales se añaden como parte de un filme utilizado como recubrimiento de un alimento o bien formando parte del propio envase.[6] Estos filmes, generalmente, son materiales poliméricos a base de quitosano que se les añaden aceites esenciales y que son capaces de prologar la vida útil de alimentos.[7,8]

En este trabajo se diseñó un polímero con un aceite esencial, en concreto el 4-hidroxi-3-metoxibenzaldehído (vainillina), anclado covalentemente a la matriz polimérica. De esta forma, se consiguió preparar un polímero con

propiedades antimicrobianas que reúne las ventajas que presenta el uso de aceites esenciales para el incremento de vida útil de los alimentos, pero evitando la modificación de las propiedades organolépticas de los mismos, así como el resto de inconvenientes mencionados anteriormente. El estudio completo se publicó en la revista *Food Packaging and Shelf Life*,[9] y el material se encuentra patentado (Figura 3.1., Número de solicitud: P202130734).[10]

MINISTERIO DE INDUSTRIA, ENERGIA Y TURISMO

Oficina Española de Patentes y Marcas

**Justificante de presentación electrónica de solicitud de patente**

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

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| Número de solicitud: | P202130734 | |
| Fecha de recepción: | 28 julio 2021, 12:26 (CEST) | |
| Oficina receptora: | OEPM Madrid | |
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| Solicitante: | Universidad de Burgos | |
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| País: | ES | |
| Título: | POLÍMEROS ANTISÉPTICOS CON GRUPOS FENÓLICOS | |
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**Figura 3.1.** Justificante de presentación electrónica de solicitud de patente. Número de solicitud: P202130734.

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## 3.3. Artículos publicados

A continuación, se describen los resultados obtenidos a través de la transcripción íntegra del trabajo publicado:

- *Metal-free organic polymer for the preparation of a reusable antimicrobial material with real-life application as absorbent food pad.*

*Metal-free organic polymer for the preparation of a reusable antimicrobial material with real-life application as absorbent food pad*

# Metal-free organic polymer for the preparation of a reusable antimicrobial material with real-life application as absorbent food pad

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## Abstract

There is a strong need to reduce food waste while maintaining the quality of packaged food. Thus, we have prepared a new fully organic and metal-free antimicrobial polymer, with the aim of increasing both the shelf life and safety of packaged meat. This antimicrobial polymer is based on widely available commercial acrylic monomers with covalently linked vanillin motifs, which are naturally occurring essential oils with antimicrobial characteristics. The film-shaped antibacterial polymeric material shows antibacterial activity for *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* with an R parameter of up to 3.18, 3.37 and 2.00 and inhibition % of up to 99.95%, 99.96%, and 99.02%, respectively. To show the potential of these materials, we conducted a proof of concept experiment in which the antimicrobial polymer film was used as an absorbent food pad. The results show that the use of the antimicrobial polymer film can increase the shelf life of a packaged meat product by 50 %. Since the antimicrobial activity is based on a covalently anchored group, there is no antimicrobial agent diffusion, and the antimicrobial activity persists beyond the first use because it is easily washable and reusable for at least 10 cycles.

*Food Packaging and Shelf Life* **2022**, 33, 100910

**Keywords**: Antimicrobial polymers; vanillin; azo-coupling; recyclable polymers; reusable polymers; packaged meat; food quality; shelf life; absorbent pads; food packaging.

## 1. Introduction

A biocide is defined in European legislation as a chemical substance or microorganism intended to destroy, deter, render harmless or exert a control effect on any harmful organism. In Europe, biocides are divided into different types of products, depending on their intended use, as stated in the Biocidal Products Regulation (BPR) of the EU No. 528/2012 (*European commission, 2012*): "Biocidal products are necessary for the control of organisms that are harmful to human or animal health and for the control of organisms that cause damage to natural or manufactured materials". However, it is necessary to consider some of the disadvantages of the actual antimicrobial and biocidal products. This need is stressed today due to the current pandemic, where the continuous cleaning of surfaces to eliminate SARS-CoV-2 has become mandatory (Mallakpour et al., 2021).

Antimicrobial products can pose risks to humans, animals, and the environment as a result of their intrinsic properties and associated use patterns (Sharma et al., 2021). Our first objective in this work was to prepare inherently antimicrobial materials that do not cause the migration of antimicrobial agents towards living beings, eliminating the dangers referred to in the aforementioned regulations.

Although there are some reviews concerning antimicrobial polymers as advanced materials, only a few are relevant in the field (Borjihan & Dong, 2020; Krishnamoorthy et al., 2014; Olmos et al., 2021). In fact, these reviews are mostly related to the antimicrobial activity exhibited by hybrid structures containing metals (Braun et al., 2020; Otoni et al., 2016; Shao et al., 2015; Tang et al., 2013; Wang et al., 2014), which makes these studies interesting from a scientific viewpoint but irrelevant to real-life applications due to both their high production

costs and the health and environmental concerns related to the use of metal cations (Carraher & Roner, 2014; Ding et al., 2019; Fernández et al., 2009; Luo et al., 2021; Tamayo et al., 2016; Zhang et al., 2014). The advantages of the antimicrobial activity of polymers over conventional antimicrobial agents against microorganisms (such as bacteria, fungi, and protozoans) include their nonvolatility, chemical stability, nontoxicity (difficulty in permeating through the skin of animals), ability to prolong product shelf life, increased efficiency, and selectivity while minimizing their environmental impact (Norrrahim et al., 2021; Sharma et al., 2021)

Although the preparation of materials with antibacterial properties is a topic that is booming (Cheng et al., 2021; Liu et al., 2020; Su et al., 2020), there is an urgent need to develop a scalable synthetic strategy involving different kinds of polymers whose potency against specific microorganisms is accompanied by less hazardous effects (Otoni et al., 2016). The second objective of our work involves more than just these advantages, proposing fully organic antimicrobial polymers (i.e., metal-free) that have a biocompatible main chain and pendant chemically anchored vanillin derivative motifs. At this point, the use of vanillin is significant because it is a natural product that is also a widely used and accepted flavouring food additive (*U.S. Food and Drug Administration, 2021*).

The preparation of new polymers with antimicrobial activities requires the synthesis, purification, and characterization of new compounds to be used as monomers, comonomers, or simple reagents for dispersion into the polymer matrix. This process is complex and time-consuming, and the resulting materials are not economically viable, so our third objective was to prepare these materials in a simple and easily scalable way for future applications. For this reason, we propose an antimicrobial polymer based on the antimicrobial activity of phenols (Park et al., 2001), specifically essential oils containing phenol structures (Fiore et al., 2021; Gañán & Brignole, 2011; He et al., 2021; Javed et al., 2011; Reyes et al., 2021; Syafiq et al., 2020), and even more specifically vanillin (Cerrutti et al., 1997; Fitzgerald et al., 2004). Furthermore, we designed a very easily

prepared film-shaped polymer that is 100 % based on inexpensive and widely available commercial monomers (such as *N*-vinyl-2-pyrrolidone, methyl methacrylate, and 4-aminostyrene) and can be easily modified by simply dipping the dense membrane in two solutions. 4-Aminostyrene acts as a functional motif in the polymer's main chain to anchor the vanillin motifs (phenol derivative) through an azo-coupling reaction (Bustamante et al., 2019). The proportion of this anchoring monomer is very low (mol 1 %), so the polymers can be easily scaled up for industrial production (i.e., there is no need for costly modification of the current production processes for prepared goods that contain polymers), and, at the same time, are safe for the environment and living beings.

Additionally, in this work, we have carried out a proof of concept experiment for the designed polymers as absorbents for packaged meat products (Han et al., 2018) to demonstrate the possible applications of these new polymers in one of the expected real-life scenarios. The current common absorbents are single-use products composed of silica gel or cellulose coated in plastic (Han et al., 2018) and generally do not have antimicrobial functionality or their antimicrobial activity ceases when all antimicrobial agents are delivered (Oral et al., 2009). These pads are not accepted for recycling or composting in the United States at this time, so they pose an environmental problem (Gaspar et al., 2019). Furthermore, their reusability is economically and environmentally impossible due to their porous nature. Microorganisms, such as bacteria, highly contaminate the absorbent pad, which necessitates the use of advanced washing processes in such a way that it is not profitable.

In short, the novelty of our proposal lies in the combination of the following characteristics: (1) the dense nature of the material allows it to be washable and reusable; (2) the antimicrobial agent is covalently anchored to the polymer chains, so the polymers do not lose antimicrobial activity after use and do not deliver any substance; (3) metal-free composition; (4) easy preparation procedure; (5) materials contain a high amount of a hydrophilic monomer (*N*-vinyl-2-pyrrolidone, mol 49.5 %), which ensures the absorbent properties of the material, and a

hydrophobic monomer (methylmethacrylate, mol 49.5 %), which ensures that the material can withstand washing processes.

## 2. Experimental

### *2.1. Materials*

All materials and solvents used were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (**MMA**, 99%, Merk, Darmstadt, Germany), *N*-vinyl-2-pyrrolidone (**VP,** 99%, Acros Organics, Geel, Belgium), 4-aminostyrene (**SNH$_2$**, 98%, TCI, Zwijndrecht, Belgium), ethylene glycol dimethacrylate (97.5%, Merk, Darmstadt, Germany), hydrochloric acid (37%, VWR International, Leuven, Belgium), sodium hydroxide (99%, VWR International, Leuven, Belgium), peracetic acid (35%, Acros Organics, Geel, Belgium), sodium nitrite (99%, Applichem Panreac, Darmstadt, Germany), pork meat (Carrefour, Burgos, Spain), streptomycin thallous acetate agar (OXOID LTD, Basingstoke, England), STA selective supplement (OXOID LTD, Basingstoke, England), MRS agar (OXOID LTD, Basingstoke, England), *Pseudomonas* agar base (OXOID LTD, Basingstoke, England), ringer solution (OXOID LTD, Basingstoke, England), plate count agar (PCA, Condalab, Torrejón de Ardoz, Spain), *Listeria* agar base (Scharlau, Barcelona, Spain), glycerol (VWR International, Leuven, Belgium), tryptone bile x-glucuronide agar (TBX, Scharlau, Barcelona, Spain), Baird Parker agar base (Condalab, Torrejón de Ardoz, Spain), violet red bile lactose agar (VRBL, VWR International, Leuven, Belgium), brain heart infusion (BHI, OXOID LTD, Basingstoke, England), *Listeria* chromogenic agar base Acc. Ottaviani & Agosti (ALOA, OXOID LTD, Basingstoke, England), tellurite egg yolk emulsion (Condalab, Torrejón de Ardoz, Spain), *Staphyloccocus aureus* ATCC 29923 (American Type Culture Collection, Manassas, USA), *Escherichia coli* CECT 50 (Colección Española de Cultivos Tipo, Valencia, Spain), *Listeria monocytogenes* ILSI 9 (University of Burgos, Burgos, Spain), propidium iodide (1 mg/ml solution in water, Invitrogen, Waltham, USA), and stock solution of phosphate buffered

saline (PBS 10X, Merk, Darmstadt, Germany). Azo-bis-isobutyronitrile (AIBN, 98%, Merk, Darmstadt, Germany) was recrystallized twice from methanol.

*2.2. Instrumentation*

Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) equipped with an ATR-PRO410-S single reflection accessory. High-resolution electron-impact mass spectrometry (EI-HRMS) was carried out using a spectrometer (Micromass AutoSpec Waters mass, Micromass Holdings Ltd., Cary, USA) with an ionization energy of 70 eV and a mass resolving power >10 000. $^{1}H$ and $^{13}C\{^{1}H\}$ NMR spectra (Avance III HD spectrometer, Bruker Corporation, Billerica, USA) were recorded at 300 MHz for $^{1}H$ and 75 MHz for $^{13}C$ using deuterated solvents such as dimethyl sulfoxide (DMSO-$d_6$) or deuterated chloroform ($CDCl_3$) at 25 °C.

The thermal and mechanical properties of the material were measured using thermogravimetric analysis, with 10-15 mg of the sample exposed to synthetic air and a nitrogen atmosphere at a heating rate of 10 °C/min (Q50 TGA analyser, TA Instruments, New Castle, USA); differential scanning calorimetry, with 10-15 mg of the sample exposed to a nitrogen atmosphere at a heating rate of 10 °C/min (Q200 DSC analyser, TA Instruments, New Castle, USA); and the tensile properties were analysed with 5 × 30 × 0.103 mm samples tested at 5 mm/min, dried at 60 °C for 1 hour, with an inter-jaw distance of 9.44 mm (EZ Test Compact Table-Top Universal Tester, Shimadzu, Kyoto, Japan).

The weight percentage of the water taken up by the films upon soaking in pure water at 20 °C until equilibrium (water-swelling percentage, WSP) was obtained from the weight of a dry sample film (ωd) and its water-swelled weight (ωs) using the following expression: $WSP = 100 \times [(\omega s \times \omega d)/\omega d]$.

AFM-RAMAN spectra were recorded with an Alpha300R – Alpha300A AFM (WITec, Ulm, Germany) using laser radiation at wavelengths of 532 nm and 785 nm at 100 × magnification. RAMAN spectra were obtained at room temperature at 5 μm intervals on the Z-axis (thickness) to check the homogeneity

of the material. Scanning electron microscopy (SEM) experiments were carried out for the material's surface and the cross-section using an electron microscope (FEI Quanta 600, FELMI-ZFE, Graz, Austria). Films were dried in air, fractured and gold sputtered in vacuum to assure the electrical conductivity of the films.

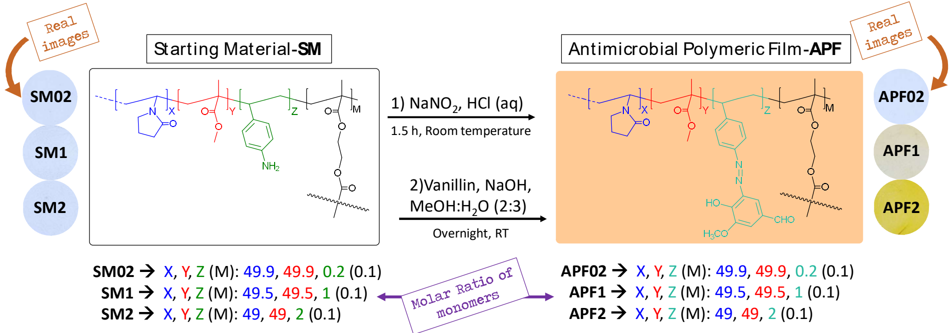
Images of bacterial cells stained with propidium iodide (PI) were acquired using a microscope (CTR6000, Leica Microsystems, Wetzlar, Germany) equipped with an N21 (red) filter cube using the following settings: excitation wavelengths of 515-560 nm and an emission wavelength of 590 nm. Meat packaging was carried out with a modified atmosphere packaging (MAP) machine (Efabind, Murcia, Spain) coupled with an OxyBaby 6.0 gas analyser (Witt-Gasetechnik GmbH & Co KG, Witten, Germany).

The powder X-ray diffraction (PXRD) patterns were obtained using a diffractometer (D8 Discover Davinci design, Bruker Corporation, Billerica, Massachusetts, USA) operating at 40 kV with a Cu(Kα) radiation source and a scan step time of 2 s. Each spectrum was acquired from 5° to 70° using a step size of 0.05° (2θ). The wavelength of the X-ray radiation was 1.54060 nm with an intensity of 30 mA.

*2.3. Antimicrobial polymeric film preparation*

The starting material was prepared by the bulk radical polymerization of three commercial monomers (**VP**, **MMA**, and **SNH$_2$**) in different molar ratios with 0.1 % mol of a crosslinker (ethylene glycol dimethacrylate) and 1 % by weight of AIBN as a radical thermal initiator (**see Scheme 1**). The polymerization was carried out at 60 °C overnight in a mould (width, length, and thickness of 90×120×0.1 mm, respectively) comprised of two silanized glasses in an oxygen-free atmosphere. The films were removed from the mould and washed once with methanol and twice with water. The films were then dipped in a sodium nitrite acid solution (125 mL water, 12 mL HCl (37%), 0.5 g $NaNO_2$) for 90 minutes into which a benzene diazonium salt was formed. Finally, the films were dipped into a basic vanillin solution (60 mL NaOH 1 M, 40 mL methanol, 1 g vanillin) to facilitate an azo-coupling reaction. The obtained antimicrobial polymeric films were washed

exhaustively with basic water and water, dried at 60 °C, and then sterilized with a UV lamp (365 nm) for 24 hours.




**Scheme 1**. Preparation of the antimicrobial polymer films from the starting materials. The scheme shows the molar ratios used for each polymer, and real images (adapted as circular shapes) of the films taken with a camera to illustrate the colours of the materials.

Given the low proportion of aniline groups in the starting materials (0.2, 1 and 2 mol %), as well as the low ratio of groups derived from vanillin in the antimicrobial polymeric films, we prepared an additional polymer with a higher molar ratio of vanillin motifs (mol 10 %) to enable characterization by FT-IR spectroscopy.

*2.4. Bacterial strains and inoculum preparation*

In this study, *Staphylococcus aureus* ATCC 29923, *Escherichia coli* CECT, and different strains of *Listeria monocytogenes* were used: *Listeria monocytogenes* ILSI 29 (International Life Sciences Institute North America), *Listeria monocytogenes* ILSI 9, and *Listeria monocytogenes* C6 1449 isolated from a mincing machine in a poultry processing plant (Department of Biotechnology and Food Science Collection, Burgos, Spain). *Staphylococcus aureus* ATCC 29923 was grown onto plate count agar (Oxoid), *Escherichia coli* on tryptone bile X-glucuronide agar (Scharlau), and *Listeria* strains on agar *Listeria* ALOA (Oxoid). One colony of each strain was transferred into BHI broth (Oxoid) and grown at 37 °C for 24 hours to achieve a viable cell population of 8 log CFU/mL. Decimal dilutions were performed in Ringer solution (Oxoid) to achieve the proper inoculum concentration (5 log CFU/mL).

*2.5. Experimental procedure used for the evaluation of antimicrobial capacity and efficacy*

The study aimed to evaluate the antimicrobial capacity of the prepared material against different bacteria, such as *E. coli, S. aureus, and L. monocytogenes*, following established procedures (*ISO Standard 22196:2011*, *JIS Standard Z 2801:2010+A:2012*).

The tested materials were cut into square shapes (4 x 4 cm). After sterilization with ethanol (70 %), 300 µL of the bacterial inoculum *(E. coli, S. aureus, or L. monocytogenes*) was streaked onto the square pieces, and the inoculum was covered with another square piece (4 x 4 cm) of sterile polyethylene. The samples were incubated for 24-48 hours at 35 °C ± 1 °C, and the bacteria were collected by sterile homogenization with 10 mL of culture broth (Ringer solution). One hundred microlitres of the homogenate was taken, inoculated in the corresponding culture medium, and allowed to incubate for the necessary time for each bacterium (**Fig. 1**). The count of viable bacteria was performed by counting the colonies on the incubated plates and comparing this with the initial inoculum concentration. The antibacterial activities of the antimicrobial polymeric films were compared to the starting materials and calculated using the final counts of viable bacteria that were incubated with the antimicrobial polymeric films. Both the inhibition % and R parameter were calculated using the following formulae:

- Inhibition (%) = 100 - ((Final count (CFU/$cm^2$) * 100) / (Initial count (CFU/$cm^2$))
- R (antibacterial activity) = Ut – At
- Ut = average of the logarithmic number of viable bacteria after inoculation on starting materials after 24 hours.

At = average of the logarithmic number of viable bacteria after inoculation on antimicrobial polymeric films after 24 hours.

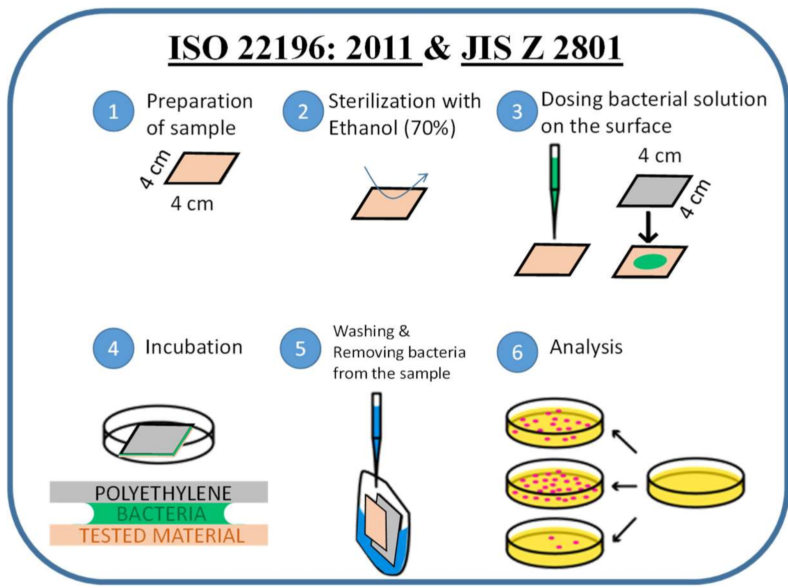



**Figure 1.** Graphical abstract of the experimental procedure according to the ISO 22196: 2011 & JIS Z 2801 standards.

*2.6. Migration study*

The migration study was *carried out both qualitatively (Kirby-Bauer method) and quantitatively* (*European Commission, 2011)*. For the former, an 8 mm diameter disc of starting material **SM1** was first immersed in a vanillin solution (3 mL NaOH 1 M, 2 mL MeOH and 50 mg vanillin) to obtain a material with dispersed vanillin, i.e., noncovalent binding). The material was incubated at 37 °C for 24 hours in petri dishes containing TBX culture medium with *E. coli.* The same procedure was followed for the antimicrobial polymer film **APF1**. For the latter, migration was studied as described in EU No. 10/2011 (*European Commission, 2011)* and amendments, which relates to plastic materials and articles oriented towards food contact applications. The standard test methods were carried out at high temperatures (100 °C or reflux temperature) with acetic acid, ethanol and olive oil (*UNE-EN 1186-1, 2002 and UNE-EN 1186-3, 2002).*

*2.7. Determination of the bacterial cell membrane integrity*

The bacterial cell membrane integrity was studied by using propidium iodide (PI) and further optical microscopy analysis. Bacteria (*E. coli* and *S. aureus*) were collected from the surface of the materials used in Section 3.5 after 24 hours of contact with starting material **SM1** and antimicrobial polymeric film **APF1** using 1X phosphate-buffered saline (PBS). Cells were diluted 1/100 with 1X PBS and incubated for 5 minutes at room temperature with 5 µg/ml PI. As a negative control, bacterial cells were grown at 37 °C for 24 hours, and, as a positive control, bacteria were treated with heat at 80 °C for 4 min.

*2.8. Preparation of meat packages for the proof of concept*

All packages were prepared the same day and with the same piece of meat to minimize experimental errors. First, 200 - 220 g of fresh pork loin meat was placed in hermetically sealed packages with a modified atmosphere (75% $N_2$ - 25% $CO_2$). In total, 21 meat packages were prepared, 7 **CONTROLs**, 7 with starting material **SM1**, and 7 with antimicrobial polymeric film **APF1**, and these packages were stored at 4 °C.

Samples were removed vertically with a scalpel so that the sample could be visualized as a sandwich that contained the outer Part 1 (in contact with the atmosphere), the internal part of the meat, and the outer Part 2 (in contact with **APF1**). The microbiota for these samples were evaluated at different times (0, 2, 4, 7, 9, 11, and 15 days) and for each type of packaging. This evaluation was carried out by using conventional experimental procedures in microbiology (Pascual Anderson & Calderón y Pascual, 2000; *ISO Standard 7218:2007*; *ISO Standard 6887-1:2017*).

*2.9. APF1 washing process*

We carried out different antimicrobial polymeric film **APF1** washing procedures to ensure that this process is cheap as possible for future applications and to achieve an environmentally sustainable washing process. Specifically, we carried out four different washing processes (WP) for the antimicrobial polymeric film **APF1** used in the proof of concept described in Section 3.7 after 15 days.

- WP-A: Antimicrobial polymeric film **APF1** was dipped in water, which was renewed four times every 30 minutes, and finally dried with a sterile gauze.
- WP-B: After WPA, antimicrobial polymeric film **APF1** was dipped in ethanol, which was renewed four times every 30 minutes. The material was finally rinsed with water.
- WP-C: After WPB, antimicrobial polymeric film **APF1** was exposed to 365 nm UV radiation for 15 minutes.
- WP-D: After WPA, antimicrobial polymeric film **APF1** was dipped in an aqueous peracetic acid solution (5%) for 30 minutes at 55 °C. The material was finally rinsed with water.

Washed materials were incubated in peptone water at 25 °C (since it is a high nutrient culture broth) overnight with orbital shaking in such a way that any residual bacteria on the material surface could grow. Then, 100 µL of the culture broth was taken and distributed on PCA culture media and incubated in aerobic and anaerobic conditions at 30 °C before the final bacterial count. These culture media are general and nonspecific, and they give an idea of the general microbial population.

### *2.10. Statistical analyses*

Statistical analyses were performed using GraphPad Prism v8. First, the normality and homoscedasticity of the data were analysed. When the data fulfilled both assumptions, one-way or two-way ANOVA followed by Tukey’s multiple comparisons test ($p<0.05$) was run. When the data fulfilled the assumption of normality but not homoscedasticity, Welch's ANOVA followed by Unpaired t with Welch's correction ($p<0.05$) was performed. When the data did not fulfil the assumption of normality, a parametric Kruskal–Wallis test ($p<0.05$) was performed followed by the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate.

## 3. Results and discussion

### *3.1. Chemical characterization of the materials*

As a crosslinked material, the characterization of the newly formed motifs is impossible due to the requirement of polymer solubilisation, so we studied the polymer with the higher molar ratio of vanillin motifs (mol 10%) by FT-IR spectroscopy. Thus, this film is valid as a model for understanding the changes in the material's chemical structure measured using FT-IR (**Figure S1**, **ESI-Section 1**). Bands at 2270 $cm^{-1}$ and 1577 $cm^{-1}$ confirm the formation of both the benzene diazonium salt and azo compound in the polymer (Bustamante et al., 2019).

Our material is a 100 μm thick dense membrane for which we have made chemical changes, so it is necessary to show that the material is homogeneous after the reaction, i.e., that the reaction does not occur only in the outer part but also in the inner part of the material. RAMAN spectra confirmed the homogeneity of the material since no difference in the data was observed at different depths, as shown in **Figure S1** (**ESI-Section 1)**.

*3.2. Physical characterization of the materials*

Scanning electron microscopy (SEM) tests were performed for the starting materials and antimicrobial polymeric films. The results all showed the same dense structure, which is crucial to prevent the bacteria from entering the material and to demonstrate not only the antimicrobial effect of the antimicrobial polymeric film surface but also that such films are easily cleanable, washable and reusable.

Additionally, PXRD measurements for the starting materials and antimicrobial polymeric films were carried out (**Figure S1**, **ESI-Section 1**). These measurements show typical amorphous haloes, which give the mean distance between the polymer chains and allow for the elucidation of the effect of the azo-coupling reaction on the mean distance ($\langle R \rangle = 5/8(\lambda/\sin \theta_{max})$). In other words, the higher the $\theta_{max}$, the lower the distance between the chains. This angle is increased from starting material **SM02** to **SM2**, meaning that <R> is decreased with increasing content of amino groups, indicating an increase in the interchain interactions promoted by this polar group; whereas <R> is increased from antimicrobial polymeric film **APF02** to **APF2**, showing the influence of the bulky lateral groups in increasing the distance between polymer chains.

*3.3. Thermal and mechanical characterization*

We consider a material to be manageable when it has suitable properties and can be easily manually manipulated without special care. In our case, given that one of the possible applications of the prepared material is as an absorbent food pad, this manageability must also be adequate when the material exists in a swollen state. Therefore, we optimized the monomer and crosslinker molar ratios to obtain materials with not only an appropriate WSP (see **Table 1**) but also good manageability.

The mechanical properties of the prepared materials were more objectively studied, and the resulting Young's modulus was determined to be 730 MPa. These data confirm the manageability that was visually observed upon film

handling. The dimensions of the samples were chosen based on our experience with generic polyacrylic materials (Guembe-García et al., 2020, 2021).

Additionally, we carried out TGA and DSC experiments with all starting materials and antimicrobial polymeric films. **Table 1** shows $T_5$ and $T_{10}$ (temperatures at which 5 % and 10 % weight loss is observed, respectively) obtained from the thermogravimetric analysis and the glass transition temperature ($T$g) obtained from DSC measurement. Both the TGA and DSC patterns are shown in **Figure S1** (**ESI-Section 1**).

**Table 1.** Water swelling percentage (WSP), glass transition temperature ($T$g), $T_5$ and $T_{10}$ for different starting materials and antimicrobial polymer films.

| | SM02 | SM1 | SM2 | APF02 | APF1 | APF2 |
|---|---|---|---|---|---|---|
| $T_5$ (°C) | 335 | 332 | 334 | 327 | 329 | 330 |
| $T_{10}$ (°C) | 349 | 347 | 350 | 343 | 345 | 346 |
| $T$g (°C) | 140 | 143 | 145 | 139 | 144 | 143 |
| *WSP* | 35 | 50 | 35 | 40 | 65 | 45 |

*3.4. Migration tests*

First, as one of the potential applications of these materials is related to food packaging, we checked that no migration of any substance occurs through a study of the antimicrobial polymeric film **APF1** and starting material **SM1** by the Kirby-Bauer method (Dunkelberg, 1981). This qualitative and simple test allows for the examination of the anchoring of the antimicrobial substances that we have covalently anchored to the material, i.e., to determine if these substances are anchored, or, on the contrary, simply remain dispersed in the material. Over time, the vanillin contained in the material migrates to the solution, and a halo is observed around the disc, as shown in **Figure 2**. However, when the same assay was performed with the antimicrobial polymeric film **APF1**, no halo was observed, confirming the lack of migration of the antimicrobial substances towards the culture media.

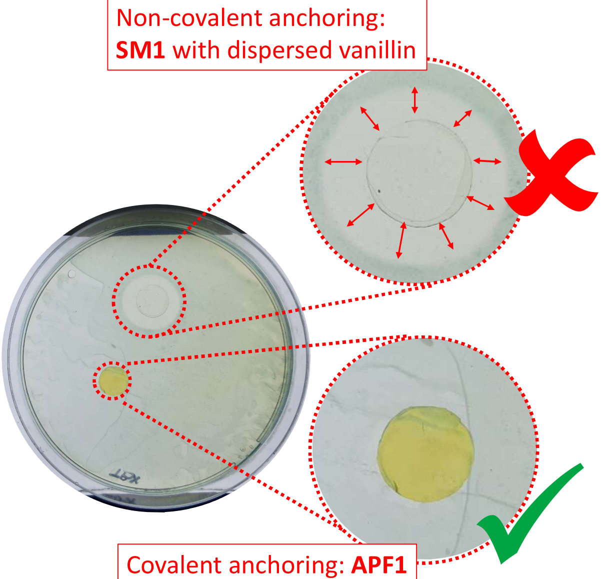



**Figure 2.** Migration test for both covalently and noncovalently anchored vanillin based on the Kirby-Bauer method. The presence of a halo in the test for the starting material **SM1** with dispersed vanillin indicates the migration of vanillin towards the media.

Additionally, a migration study following the EU No. 10/2011 regulations (*European Commission, 2011)* and amendments shows that the antimicrobial polymeric film **APF1** complies with the restriction for the overall migration limit (<10 mg dm$^{-2}$), as defined in the aforementioned European regulation. Specifically, the migration results obtained for antimicrobial polymeric film **APF1** in 3% acetic acid, 10% ethanol and olive oil were 0.7, 5.9, and 4.8 mg dm$^{-2}$, respectively.

The chemical formulation of the antimicrobial polymeric film **APF1** mainly comprises N-vinyl-2-pyrrolidone and methylmethacrylate. Regarding the former, both the monomer (*N*-vinyl-2-pyrrolidone) and polymer (polyvinylpyrrolidone) are authorized food contact materials (FCMs), with FCM numbers of 376 and 552, respectively, according to EU No. 10/2011. Regarding the latter, crosslinked methyl methacrylate copolymers are authorized food contact materials (FCM

number: 664). Moreover, methyl methacrylate copolymers with maximum level percentages for other polymers are also authorized FCMs with FCM numbers of 865 to 869. However, due to the material's smart antimicrobial behaviour, the EU No. 450/2009 regulation (*European Commission, 2009)* for active and intelligent materials and articles that are intended to come into contact with food is applied, so the material should be further optimized prior to commercialization.

*3.5. Antimicrobial capacity and efficacy*

The assays were performed with *E. coli* (gram-negative) and *S. aureus* (gram-positive) for the different antimicrobial polymeric films. **Table 2** shows the results obtained for the initial/final count of bacteria, inhibition percentage, and antibacterial efficiency of the material expressed as "R", which should be higher than 2, as expressed in the standards.

**Table 2.** Evaluation of the antimicrobial capacity and efficacy of **APFs** (ISO 22196:2011; JIS Z 2801:2010+A: 2012).

| | | Antimicrobial polymeric film | | |
|---|---|---|---|---|
| | | APF02 | APF1 | APF2 |
| *E. coli* | Initial count (log CFU/cm$^2$) | 5.46 ± 0.00 b | 6.41 ± 0.00 a | 5.46 ± 0.00 b |
| | Final count (log CFU/cm$^2$) | 4.85 ± 0.02 c | 3.58 ± 0.02 d | 2.19 ± 0.11 e |
| | Inhibition (%) | 75.86 ± 0.69 a | 99.85 ± 0.01 b | 99.95 ± 0.02 b |
| | Antibacterial activity (R) | 0.55 ± 0.03 a | 2.77 ± 0.03 b | 3.18 ± 0.08 c |
| *S. aureus* | Initial count (log CFU/cm$^2$) | 5.41 ± 0.00 b | 6.56 ± 0.00 a | 5.41 ± 0.00 b |
| | Final count (log CFU/cm$^2$) | 4.77 ± 0.02 c | 3.44 ± 0.04 d | 1.97 ± 0.03 e |
| | Inhibition (%) | 77.05 ± 0.80 a | 99.92 ± 0.01 b | 99.96 ± 0.01 b |
| | Antibacterial activity (R) | 0.58 ± 0.03 a | 3.06 ± 0.03 b | 3.37 ± 0.02 c |

*CECT (Colección Española de Cultivos Tipo); ATCC (American Type Culture Collection), ILSI (International Life Sciences Institute North America). Data are the means of the ± SD for three replicates. Different letters indicate significant differences within the bacterial counts ($p<0.05$, Two-way ANOVA followed by Tukey's multiple comparisons test), inhibition and antibacterial activity ($p<0.05$, One-way ANOVA followed by Tukey's multiple comparisons test) data.

As explained in the standards, antimicrobial polymeric film **APF02** cannot be considered an antimicrobial material since its R parameter is lower than 2, and it is worth noting that antimicrobial polymeric film **APF2** contains twice the amount of 4-aminostyrene, which is the most expensive reagent used in the polymer synthesis. Thus, we selected antimicrobial polymeric film **APF1** as the polymer with the best antimicrobial activity/cost ratio, and we chose it as an absorbent food pad for the proof of concept experiment discussed below. Due to this application, we also analysed this material's antimicrobial capacity with *Listeria monocytogenes*, since these bacteria can cause very severe problems in the food industry (*European Commission, 2005; EFSA and ECDC, 2019)*. For this assay, the antimicrobial polymeric film **APF1** showed 99.05 ± 0.06 % inhibition and an R parameter of 2.00 ± 0.01.

*3.6. Study of the antimicrobial effect on bacterial cells*

The objective of this study carried out with propidium iodide (PI) was to characterize the effect of antimicrobial polymeric films on bacterial cells. PI is a nonmembrane permeable intercalating agent that binds DNA. Therefore, PI cannot penetrate healthy cells, resulting in low fluorescence, but can penetrate cells with damaged membranes and bind DNA, leading to a 20- to 30-fold increase in the fluorescence intensity of the cells.

As shown in **Figure 3**, the fluorescence enhancement observed in the bacterial cells in contact with antimicrobial polymeric film **APF1** demonstrates that the cell membrane integrity is compromised. These results agree with previous studies in which the antimicrobial effect of vanillin was produced by destabilization of cell membrane integrity, resulting in a loss of pH homeostasis and ionic balance (Fitzgerald et al., 2004; Saibabu et al., 2020). Therefore, the

covalent immobilization of vanillin does not alter the mode of action of this essential oil.

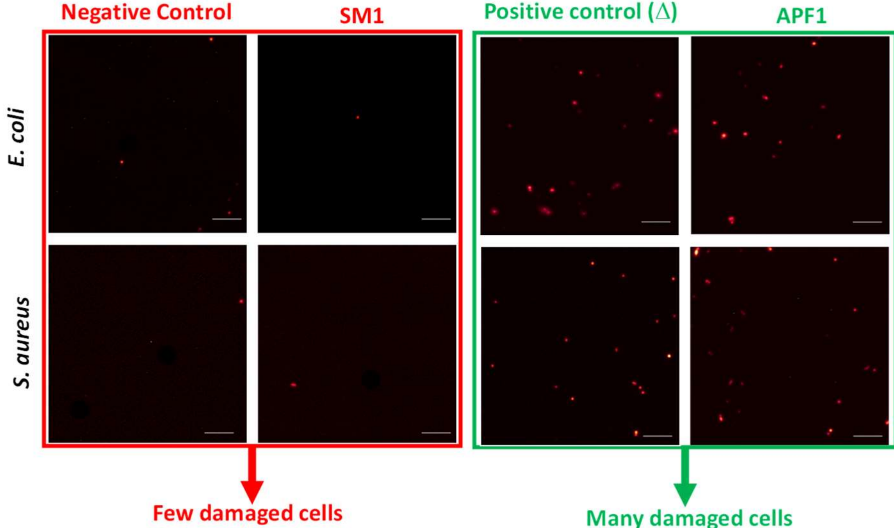



**Figure 3.** Fluorescence images of *E. coli* and *S. aureus* cells after treatment with propidium iodide. Microscope images of the control and starting material **SM1** show a very low number of damaged cells (red points), while many damaged cells (red points) can be observed both in the antimicrobial polymeric film **APF1** and heated samples. Scale bars in the images correspond to 25 μm.

### *3.7. Proof of concept: Antimicrobial polymeric films as absorbent food pads with biocidal activity*

In this work, we wanted to demonstrate the potential of these new materials with a proof of concept experiment that is pertinent to real life applications. Undoubtedly, these materials will be tested soon as surface coatings, sanitary material coatings, fibre coatings, etc. However, the proof of concept experiment herein involves the use of the prepared material as an absorbent food pad. For this, we chose packaged pork loin steaks as an example and prepared 3 types of packaging: without an absorbent food pad (**CONTROL**), with starting material **SM1**, and with the antimicrobial polymeric film **APF1**.

All data can be found in **ESI-Section 2**, but as a summary, as shown in **Figure 4,** we show the meat packages with starting material **SM1** and antimicrobial polymeric film **APF1** on Day 15 and the inhibition % for both materials at different times.

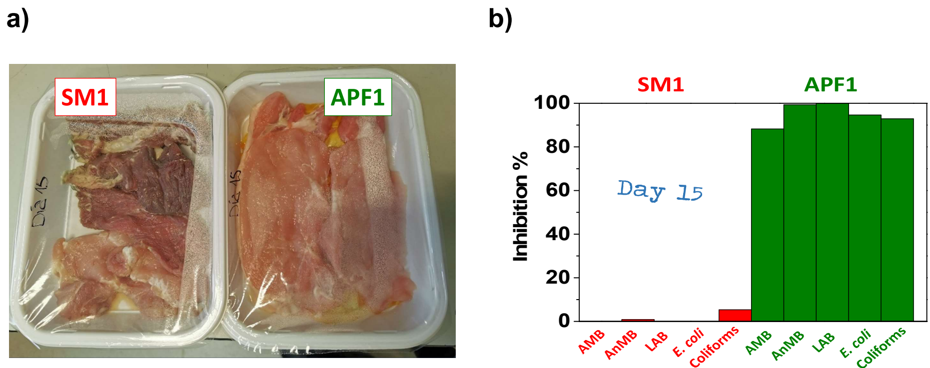



**Figure 4.** (**a**) Image of packaged meat after 15 days with starting material **SM1** and antimicrobial polymeric film **APF1**. (**b**) Inhibition % for both the starting material **SM1** and antimicrobial polymeric film **APF1** at Day 15 for the studied bacteria: aerobic mesophilic bacteria (AMB), anaerobic mesophilic bacteria (AnMB), lactic acid bacteria (LAB), *E. coli*, and coliforms.

The analysed samples contain the outer Part 1 (in contact with the 75 % $N_2$ – 25 % $CO_2$ modified atmosphere), the internal part of the meat, and the outer Part 2 (in contact with **APF1**). Considering that bacterial proliferation occurs mainly in the external parts of meat (outer Parts 1 and 2) to colonize the internal part, our interpretation is that **APF1** exerts great bacterial inhibition in outer Part 2 but never in outer Part 1 because no antimicrobial substance is released. However, as shown by the experimental results, the antibacterial effect exerted on outer Part 2 greatly impacts the bacterial proliferation for the entire sample, probably due to the combination of the antimicrobial effect with the gel behaviour of **APF1**, which absorbs meat exudates, further delaying the formation of bacterial colonies.

As shown in **Figure 4a**, after 15 days, the appearance of the meat is remarkably changes, which correlates with the inhibition % results obtained for the studied bacteria. However, the most important result for these assays is the estimation of the shelf life for the packaged pork meat. Thus, the shelf life of the packaged meat both without packaging food pads (**CONTROL**) and using starting material **SM1** is estimated to be 10 days (considering that meat is not suitable for consumption when the count of total aerobic mesophilic bacteria is greater than 7 log CFU/g, as indicated in the literature (Chouliara et al., 2007; ICMSF, 2005; Kanatt et al., 2013; Mouafo et al., 2020). Nevertheless, by using our antimicrobial polymeric film **APF1** as the packaging food pad, the shelf life is increased to 15 days. In other words, the shelf life of the same product is increased by 50%.

A few months later, the proof of concept was repeated, following exactly the same steps. Despite different bacterial counts on Day 0, the same results for increased shelf-life were obtained. The data for this second proof of concept are shown in **ESI-Section S2**.

The obtained results have enormous impact, since the extension of the expiration date reduces food waste, generates social and environmental benefits, and increases competitiveness, profit, and employment for the companies that market this packaging.

*3.8. Reusing antimicrobial polymeric film APF1*

First, we studied the antimicrobial polymeric film **APF1** used in Section 3.7 by SEM after 15 days in contact with pork meat. **Figure 5** shows SEM images of the surface and cross-section of the material. This preliminary characterization is crucial to understanding the antimicrobial mechanism for antimicrobial polymeric films, since the dense structure of the material makes it impossible for bacteria to enter the material's interior. Therefore, antimicrobial activity occurs only via contact with the material´s surface. Additionally, this dense structure makes the washing (reusability) of the material much easier, totally effective, and more pertinent to real-life applications since bacteria only contaminate the exterior of

the antimicrobial polymeric films; thus, we different washing processes were developed.

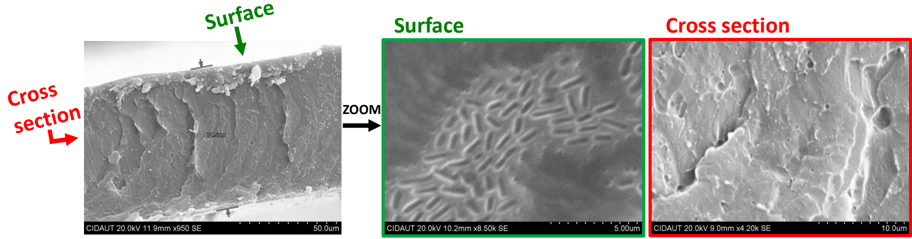



**Figure 5**. SEM images of antimicrobial polymeric film **APF1**: material´s surface containing bacteria (green); cross-section after fracture showing no bacteria contamination in the material's interior (red).

Second, the antimicrobial polymeric film **APF1** was "used & washed" up to 10 times. The subsequent antimicrobial activity was studied with *E. coli* as described in Section 3.5, and the washing process was carried out by the simplest method described in Section 2.8 (washing process WP-A) since the final bacterial count for all the WPs was 0 CFU/cm$^2$. This means that all the washing processes were effective, even for the simple process using only water. The inhibition % and the “R” parameter for the original material were determined to be 99.85 and 2.77, respectively. After 8 “use & wash” cycles, no statistically significant difference was observed in these data, as shown in **Table S5** (**ESI-Section S3**). In addition, for the case of the material after 10 “use & wash” cycles, the inhibition % and the “R” parameter were determined to be 99.76 and 2.52, respectively, so the material maintained high antimicrobial activity. This fact is a direct consequence of the covalent anchoring of vanillin motifs to the polymer. According to these results, the antimicrobial polymeric film **APF1** is easily washable, and, therefore, reusable (full data for all “use & wash” cycles can be found in **ESI-Section 3**). **Table 3** depicts a selection of polymeric materials used in food packaging as antimicrobial materials, with which we compare our development in terms of antimicrobial agent, antimicrobial activity, and especially in terms of material reusability, since we have not found any publications reporting this advantage.

**Table 3.** Comparison of the data for different antimicrobial materials

| Material | Antimicrobial agent | Antimicrobial activity (Inhibition % or CFU/g reduction) | Reusable (Y/N) | Ref. |
|---|---|---|---|---|
| Poly(ethylene oxide) | Metal-free. Tea tree oil and beta-cyclodextrin | 99.99% inhibition against *E. coli.* | - | (Cui et al., 2018; Limjaroen et al., 2016) |
| Polyethylene | Metal-free. Carvacrol-HNT | 85% inhibition against *Aeromonas hydrophila.* 48% against aerobic count. | - | (Alkan Tas et al., 2019) |
| - Poly(ethylene-co-vinyl acetate)<br>- Linear Low Density Polyethylene<br>- Polypropylene | Metal-free. Isobutyl-4-hydroxybenzoate | 6 log CFU/g reduction (LLDPE)<br>4 CFU/g reduction (EVA)<br>1 CFU/g reduction (PP) against *S. aureus* | - | (Cottaz et al., 2019) |
| Polylactide films | Metallic. Nanostructured aluminium-doped zinc oxide (AZO) | [60% - 99%] against viable *E. coli* | - | (Valerini et al., 2018) |
| Poly(lactic acid) | Metal-free. Lauroyl arginate | 3.43 log CFU/g reduction against *E. coli* | - | (Li et al., 2021) |
| Polylactide films | Metallic. Bimetallic Ag–Cu Nanoparticles and Essential Oils | 3 log CFU/ml reduction against *S. typhimurium* counts and 1 for *C. jejuni*. | - | (Ahmed et al., 2018) |

| | | | | |
|---|---|---|---|---|
| Chitosan | Metal-free. Graphitic carbon nitride | 99.8% inhibition against *E. coli*<br>99.9% inhibition against *S. aureus*. | - | (Ni et al., 2021) |
| Nanocellulose films | Metallic. Dextran coated silver nanoparticles | 99.9% inhibition against *E. coli* viable bacteria and 83.3-97.8 against *S. aureus* viable bacteria. | - | (Lazić et al., 2020) |
| Polylactic acid | Metal-free. Nisin | - 4 log CFU/mL reduction against *E. coli*<br>- 2 log CFU/mL reduction against *S. enteriditis*. | - | (Jin & Zhang, 2008) |
| Polyethylene terephthalate | Metallic. Silver nanoparticles | Ag concentrations of 0.05 mg/L reduced *E. coli* by $10^5$ CFU/mL<br>Ag concentrations of 0.015 mg/L reduced *E. coli* by $10^2$ CFU/mL | - | (Braun et al., 2021) |
| N-vinyl-2-pyrrolidone & methylmethacrylate copolymer | Metal-free. Vanillin derivative | 99.95% inhibition against *E. coli*<br>99.96% inhibition against *S. aureus*. | At least 10 times | This work |

## 4. Conclusions

There is a strong need to increase the safety and shelf life of packaged food to maintain food quality and reduce food waste. Within this frame, we designed and prepared an innocuous polymeric material with antimicrobial characteristics and exploited it to increase the shelf life of packaged meat products by 50 %. The antimicrobial polymer is prepared with commercially available monomers having pendant vanillin motifs, with this phenol-based essential oil acting as an antimicrobial agent. The polymer can be prepared as an absorbent food pad for packaged meat and has the following characteristics: a) it is safe, with no migration into the meat; b) antimicrobial activity occurs without the need for microorganisms to assimilate the antimicrobial agent; and c) the environmental impact of the developed film and its products is very low, since they can be reused at least 10 times by simply washing with water. The methodology used can be extended to the study of other types of phenol derivative-based essential oils, such as eugenol, thymol, and carvacrol. Additionally, the polymer nature of the antimicrobial material and its persistent antimicrobial characteristic envisage its use in other fields, such as antimicrobial coatings, fibres, nanofibres, and composites.

## CRediT authorship contribution statement

Lara González-Ceballos: Methodology, Investigation, Writing – original draft. José Carlos Guirado-Moreno: Methodology, Investigation. Marta Guembe-García: Methodology, Investigation. Jordi Rovira: Resources, Conceptualization, Methodology, Beatriz Melero: Conceptualization, Writing – review & editing, Visualization. Ana Arnaiz: Methodology, Investigation. Ana María Díez: Conceptualization, Methodology, Writing – review & editing. Jose M. García: Resources, Conceptualization, Methodology, Writing – review & editing, Funding acquisition. Saúl Vallejos: Investigation, Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Supervision.

## Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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# CAPÍTULO 4

## Determinación de parámetros de calidad en miel mediante el uso de materiales poliméricos

Una etapa fundamental en la industria alimentaria es el control de calidad de alimentos, cuyo objetivo final es la protección del consumidor, garantizando que se cumplan los requisitos de calidad e inocuidad establecidos en la legislación vigente. El control de calidad se lleva a cabo mediante diferentes técnicas físico-químicas o microbiológicas, las cuales suponen un coste económico y de tiempo muy importante. Este capítulo describe la síntesis y aplicación de dos materiales poliméricos en el control de calidad de la miel, simplificando, abaratando y reduciendo el tiempo de análisis de tres parámetros de calidad de esta matriz alimentaria. En eprimer lugar, se presenta un material capaz de retener almidón y ser utilizado en la medida de la actividad diastásica en muestras de miel. En segundo lugar, se describe una matriz polimérica con grupos borónicos capaz de retener polifenoles derivados del catecol mediante la formación de ésteres borónicos. La eliminación de estos compuestos supone una fase previa imprescindible para la medida de la actividad de dos enzimas en la miel, la catalasa y la glucosa oxidasa.

## 4.1. Introducción

Tal y como aparece reflejado en el Capítulo 1, uno de los principales objetivos de esta memoria es desarrollar nuevos materiales poliméricos para analizar parámetros de calidad de diferentes matrices alimentarias de una forma más rápida, sencilla y económica. En primer lugar, se planterará el problema que se afronta en este capítulo, y alrededor del cual gira todo el trabajo experimental de esta sección, es decir, la búsqueda de métodos alternativos de análisis de parámetros de calidad de la miel.

La miel es un fluido viscoso producido por la abeja *Apis mellifera* y se utiliza como edulcorante en alimentación. Entre su composición se encuentran más de 180 sustancias, entre las que destacan diferentes azúcares, como la glucosa, la fructosa o la maltosa, representando aproximadamente el 80 % de la composición total de la miel. Entre el resto de los compuestos se encuentran agua y otros componentes minoritarios como sales minerales, vitaminas, elementos traza, aminoácidos y polifenoles.[1,2] La proporción de cada uno de estos componentes está determinada por el tipo y el origen botánico de la miel.

La miel es un producto con una gran importancia comercial, siendo la producción mundial en el año 2019 de 1.800.000 toneladas.[3] España es el primer país productor de Europa (con un censo de colmenas en 2019 superior a 3.000.000, representando un 16% del total de las colmenas en Europa) y el cuarto a nivel mundial, con una producción estimada de 29.400 toneladas.[4,5]

Según datos de la Organización de Consumidores y Usuarios (OCU),[6] la miel es el tercer producto más adulterado del mundo, lo cual hace especialmente necesario el hecho de disponer de métodos de análisis de diferentes parámetros de calidad de la forma más rápida y sencilla posible.

En la actualidad, muchos de los métodos analíticos aplicados a la miel son tediosos, requieren de personal especializado y de una gran inversión en equipos e infraestructuras, así como un coste en tiempo elevado.

Existen numerosos parámetros de calidad en la miel, entre los que se encuentran la medida de la actividad de tres tipos de enzimas, las enzimas diastasas, la enzima catalasa y la enzima glucosa oxidasa.

### 4.1.1. Actividad diastásica

La actividad de las enzimas diastasas o actividad diastásica se encuentra entre los parámetros de calidad analizados de forma rutinaria. Esta medida permite conocer si el almacenamiento de la miel se ha llevado a cabo de forma correcta, si la miel ha sido sometida a procesos de calentamiento, así como el grado de frescura de la misma. Las enzimas diastasas (α-amilasas) son enzimas que se encuentran de forma natural en la miel, provenientes de las abejas y cuya función es la de hidrolizar carbohidratos complejos hasta carbohidratos simples. La medida de este parámetro se encuentra legalmente regulada según la escala de Schade.

Existen dos métodos de análisis de la actividad diastásica en mieles; el método de Schade,[7] y el método de Phadebas,[8] siendo el primero el más utilizado. El método de Schade se basa en el análisis de la evolución de una solución de almidón normalizada por la acción de las enzimas diastasas utilizando una disolución de yodo-yoduro como indicador. Se trata de un método tedioso, que requiere el empleo de un almidón con un contenido en agua determinado y un índice de azul entre 0,5 y 0,55, por lo que se requiere de estandarización previa del almidón. El índice de azul hace referencia a la coloración del engrudo de almidón con una solución de yodo-yoduro en medio ácido, medida tras 60 minutos de reacción a una longitud de onda de 660 nm. El índice de azul de un almidón no viene indicado por el fabricante, y es un punto crítico del análisis, ya que es muy complicado encontrar un almidón que cumpla con esta característica concreta. Este método requiere de personal especializado, una gran cantidad de reactivos, equipos e instalaciones, así como un coste de tiempo importante, ya que este análisis dura como mínimo 45 minutos (incluyendo la preparación de la muestra y normalización del almidón), pudiendo llegar a las 8 horas, dependiendo del tipo de miel.

En este trabajo se diseñó un polímero en forma de filme que sirve como soporte para el almacenamiento de almidón marcado con un colorante azul. Este material polimérico actúa como recipiente de dicho almidón, por lo que cuando se pone en contacto con una muestra de miel, las enzimas diastasas lo hidrolizan hasta azúcares reductores (glucosa), que se liberan a la disolución. De esta forma, se puede determinar la actividad diastásica de la miel de forma indirecta mediante la cuantificación de azúcares reductores en la disolución utilizando el método de Fehling, ya que el almidón al encontrarse en el interior del polímero y no en la disolución no interfiere en la medida. La principal ventaja que presenta este método frente al método de Schade es que no requiere estandarización previa del almidón, además de suponer un ahorro importante de costes y de tiempo. El estudio completo se publicó en la revista *Food Chemistry*.[9]

#### 4.1.2. Actividad catalasa y glucosa oxidasa

Las enzimas catalasa y glucosa oxidasa son dos enzimas presentes en la miel y su actividad se encuentra correlacionada con la actividad antioxidante y antibacteriana, respectivamente, de esta matriz alimentaria. En la miel de Manuka el efecto antibacteriano deriva en gran medida a la acción del metilglioxal.[10] Sin embargo, en la mayor parte de las mieles, su acción antibacteriana se debe a dos mecanismos diferentes. En primer lugar, a la acción del peróxido de hidrógeno ($H_2O_2$) generado por la reacción catalizada por parte de la glucosa-oxidasa.[11] En segundo lugar, al efecto osmótico de los azúcares.[12,13] El peróxido de hidrógeno se considera como el componente con mayor capacidad antibacteriana de la miel, y su concentración se encuentra regulada por la actividad de las enzimas catalasa y glucosa oxidasa.

La catalasa es una enzima que regula la actividad de la enzima glucosa-oxidasa, controlando así el equilibrio de $H_2O_2$.[14] Esta enzima cataliza la reacción de descomposición del $H_2O_2$ hasta agua ($H_2O$) y oxígeno ($O_2$).

La glucosa oxidasa es una enzima que presenta un gran interés ya que cataliza la oxidación de la glucosa hasta ácido glucónico y $H_2O_2$. El ácido glucónico es el ácido mayoritario en la miel, y se encuentra en equilibrio con la

gluconolactona, generando una acidez que protege al néctar frente a procesos de fermentación. A su vez, el $H_2O_2$ formado durante la reacción catalizada por esta enzima, protege a la miel frente a microorganismos patógenos, es decir, presenta acción antibacteriana.

Es importante destacar que la actividad de ambas enzimas disminuye con el tiempo de almacenamiento, por lo que la medida de su actividad es un parámetro de calidad muy importante de la miel.[15]

El método de medida de la actividad de ambas enzimas más utilizado en la actualidad se basa en un análisis espectrofotométrico capaz de cuantificar el $H_2O_2$. Este método requiere de una etapa previa de purificación de la miel, necesaria para eliminar los interferentes de dicha medida.[16] Hasta el momento no se ha encontrado en la bibliografía ningún estudio científico que determine cuáles son estos interferentes.

Esta etapa de purificación consiste en un proceso de diálisis de la miel mediante el uso de membranas comerciales. Se trata de un proceso tedioso, que requiere un elevado consumo de reactivos (6 litros de tampón fosfato de pH 7 para cada una de las muestras a analizar) y de tiempo, 24 horas para cada una de las muestras. A su vez, supone un coste elevado, ya que es necesario mantener las muestras en refrigeración a 4 °C durante 24 horas, así como el uso de una membrana comercial para dializar cada una de las muestras. A su vez, este proceso de diálisis no está estudiado y es capaz de retirar numerosos compuestos de la miel como azúcares y polifenoles.

En este trabajo se estudiaron cuáles son los interferentes en la medida de ambas enzimas y se diseñó una membrana polimérica capaz de retenerlos de forma específica, eliminándolos de la disolución. De esta forma, se consiguió reducir el tiempo del ensayo desde 24 horas hasta 90 minutos y el consumo de tampón fosfato desde 6 litros hasta 25 mililitros. A su vez, no se requiere refrigeración.

El estudio completo se encuentra enviado a la revista *Food Chemistry*, con identificador FOODCHEM-D-22-05766 y el material se encuentra patentado (Figura 4.1; Número de solicitud: P202230376).

**Justificante de presentación electrónica de solicitud de patente**

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

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|---|---|---|
| Número de solicitud: | P202230376 | |
| Fecha de recepción: | 26 abril 2022, 12:30 (CEST) | |
| Oficina receptora: | OEPM Madrid | |
| Su referencia: | P-08851 | |
| Solicitante: | Universidad de Burgos | |
| Número de solicitantes: | 2 | |
| País: | ES | |
| Título: | EXTRACCIÓN O SEPARACIÓN SELECTIVA DE FENOLES CON POLÍMEROS QUE COMPRENDEN GRUPOS BORÓNICOS | |
| Documentos enviados: | Descripcion.pdf (27 p.) | package-data.xml |
| | Reivindicaciones.pdf (7 p.) | es-request.xml |
| | Resumen.pdf (1 p.) | application-body.xml |
| | Dibujos.pdf (10 p.) | es-fee-sheet.xml |
| | OLF-ARCHIVE-1.zip | feesheet.pdf |
| | FEERCPT-1.pdf (1 p.) | request.pdf |
| | FEERCPT-2.pdf (1 p.) | |
| Enviados por: | CN=Manuel Illescas 12612 | |
| Fecha y hora de recepción: | 26 abril 2022, 12:30 (CEST) | |

**Figura 4.1.** Justificante de presentación electrónica de solicitud de patente. Número de solicitud: P202230376.

## 4.2. Referencias

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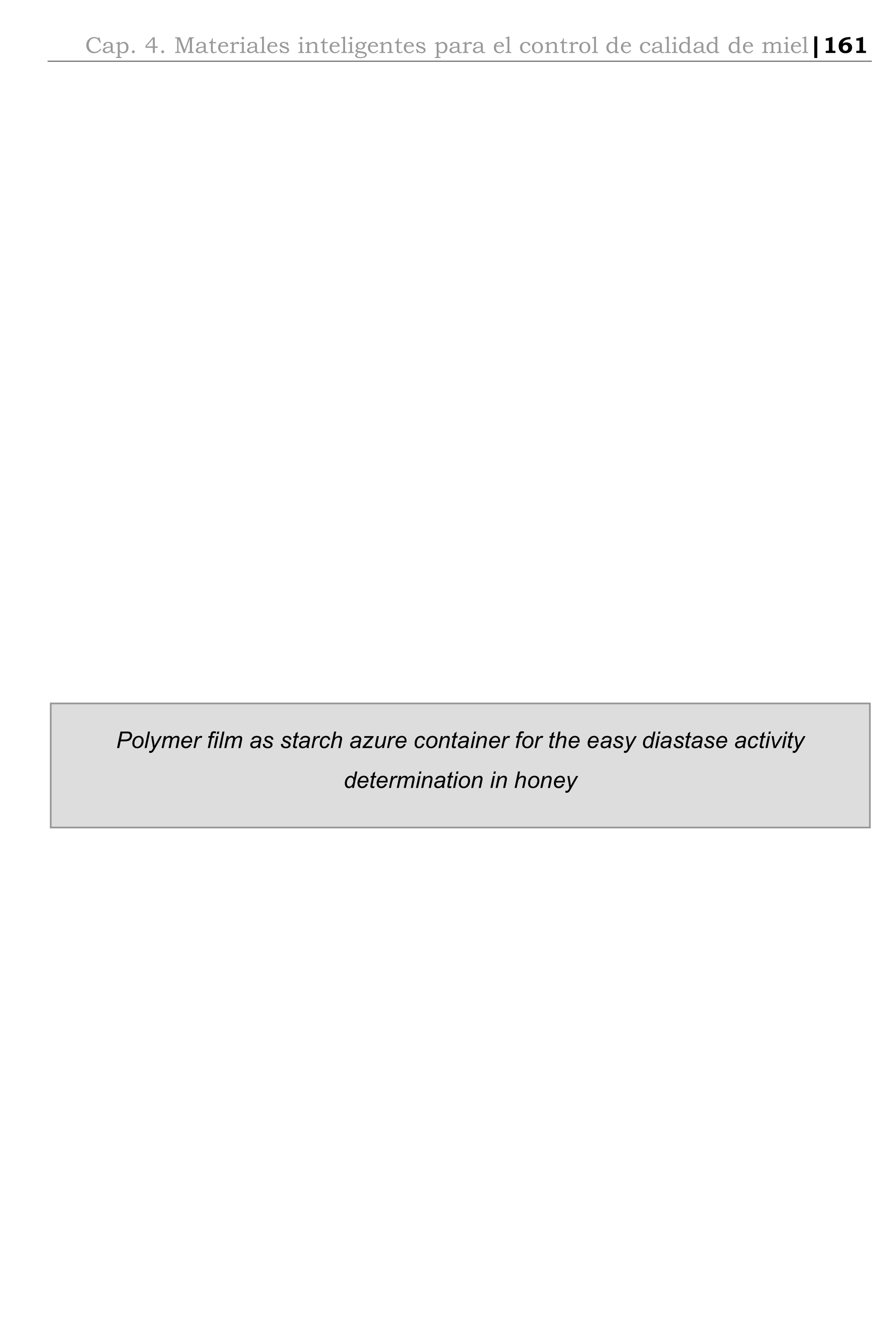
## 4.3. Artículos publicados y/o enviados

A continuación, se describen los resultados obtenidos a través de la transcripción íntegra de los trabajos publicados y/o enviados:

- *Polymer film as starch azure container for the easy diastase activity determination in honey*.
- *Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer*.



*Polymer film as starch azure container for the easy diastase activity determination in honey*

# Polymer film as starch azure container for the easy diastase activity determination in honey

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## Abstract

A new original application for a polyacrylic film based on the monomers 2-(dimethylamino)ethyl methacrylate (**NNDA**), 2-hydroxyethyl acrylate (**2HEA**) and methylmethacrylate (**MMA**) as a starch azure container has been set up for a simple determination of honey diastase activity. The proposed method is based on the correlation of reducing sugars generated during the enzymatic process with the Schade reference assay. The polyacrylic film is charged with starch azure acting as a container for this substance; thus, the starch does not interfere in the measurement of reducing sugars, so that the diastase activity is easily calculated. The method has been contrasted with Schade method, showing good correlation and differences under 0.4% between methods in some honey samples. The polyacrylic film has great potential for the routine honey diastase activity assessment in small laboratories, dramatically reducing analysis time and cost.

*Food Chemistry* **2021**, 355, 129629

**Keywords**: Honey; diastase activity; starch azure; polymer; film.

## 1. Introduction

Honey is a sweet food made by bees, whose composition includes a variety of compounds (Bogdanov, 2017) among them sugars, organic acids, minerals, vitamins, amino acids, polyphenols, substances responsible for aroma and flavours and enzymes (diastase, invertase, glucose oxidase, catalase, and others). This study focuses on honey diastases that are currently enzymes of paramount importance in honey control (Thrasyvoulou et al., 2018).

Diastase hydrolyses starch into dextrin and finally into reducing sugars (**RS**), in this case, glucose. The activity of this enzyme in honey is one of the indicators of honey quality since it is related to ageing, as well as to inadequate processing and storage procedures. Honey usually does not fulfil the conditions for commercialisation after being overheated, reheated or/and stored for a long time (Pasias et al., 2017; Tosi et al., 2008), because diastase activity has dropped below the legal limit (OJEC, 2001). Thus, a fast method to accurately analyse the diastase activity is currently of utmost importance.

Nowadays, the most used methods for the determination of the diastase activity in honey are two, namely Schade and Phadebas assays (S. Bogdanov, 2009; Stoldt et al., 2019) However, the first is tedious and time-consuming, and the latter is expensive for a significant number of analytical determinations and has not yet been broadly accepted as a reference method.

This work has been developed using Schade procedure as the reference assay, which is based on a previous starch standardisation, and requires partial shipments of starch samples by the supplier, verification of the appropriateness of the starch, and correction by the supplier (if applicable) until a valid batch (starch) is found. The validity of the batch depends on whether the absorbance of the blue complex that starch forms in the presence of lugol is within a narrow absorption range at a given wavelength (660 nm, absorbance between 0.5-0.55; see ESI S1), and once achieved, the user

usually must purchase the entire batch, which is costly. After checking that the starch fulfils every requirement, the experimental assay of Schade method can be carried out. The diastase activity of a honey sample is determined by adding the standardised starch. The decrease of the concentration of starch in the sample is monitored at different times, following by UV-Vis spectrophotometry a blue complex formed in the presence of lugol (more information in **ESI S2**). Using Schade method, the time for sample analysis is variable from 45 min to several hours, depending on the diastase activity of the honey, which makes the procedure tedious, and complicated when planning the analysis of a great number of samples. In fact, some authors (Sak-Bosnar & Sakač, 2012; Sakač & Sak-Bosnar, 2012) have already developed direct and alternative methods for the determination of diastasic activity based on a platinum redox sensor. Despite being a great advance, the study was based on only 10 kinds of honey, and the method requires the use of relatively expensive equipment, electrodes, and other instrumentation necessary for typical electrochemical analysis, so, these methods are not accessible for beekeeping cooperatives, and are not oriented to small laboratories.

In this research, we propose a new original application for a simple polyacrylic film based on 2-(dimethylamino)ethyl methacrylate (**NNDA**), 2-hydroxyethyl acrylate (**2HEA**) and methylmethacrylate (**MMA**) as a starch azure container for the easy determination of honey diastase activity. Our proposal is based on the correlation of our proposed method with the reference method. Additionally, our proposed method does not follow the decrease of the starch concentration, but the increase of reducing sugars concentration generated during the enzymatic process, i.e., the starch standardisation process is not necessary, which is a significant advantage.

The use of the polyacrylic film is mandatory for the quantification of **RS**, which is carried out by Fehling method (Fehling, 1849) (more information in **ESI S3**). Without the polyacrylic film, starch would interfere in the

measurement of reducing sugars, and the analysis would be impossible. However, the studied polyacrylic film can be charged with starch azure and can act as a container for this substance. In this way, the starch azure does not interfere in the measurement of reducing sugars, and the diastase activity can be calculated. The reason for using starch azure instead of conventional starch is the naked eye verification of the charging process with starch of the polyacrylic film. **Figure 1** shows a graphical summary of the proposed method.

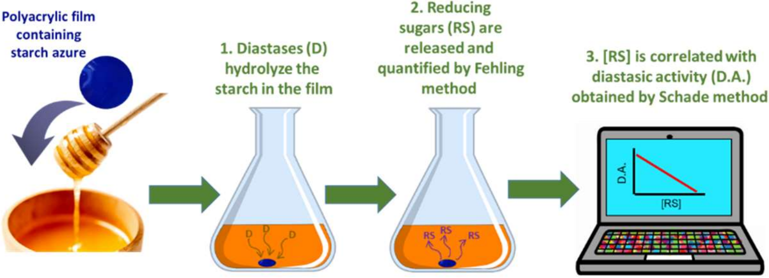


**Figure 1**. Graphical abstracts of the proposed method for the determination of the diastase activity of honey, by using a polyacrylic film containing starch azure.

## 2. Materials and methods

### *2.1.Honey Samples*

This study was carried out with 20 representative honeys harvested in 2019 in Castilla-León, a Spanish area located in the northern Iberian Plateau that held the highest number (3,827) of apicultural undertakings in Spain in 2018, representing 16% of the total apicultural undertakings of this country (Ministerio de Agricultura, 2019). Honeys' botanical origins were determined by both melissopalinology (Louveaux et al., 1978; Terradillos et al., 1994; Von Der Ohe et al., 2004), and sensory analyses (Marcazzan et al., 2018; Persano Oddo & Piro, 2004; Piana et al., 2004), there is 1 chestnut (Fagaceae type Castanea sativa Miller) honey (sample 1), 3 honeydew honeys (samples 2-4), 1 lavender (Lamiaceae type Lavandula latifolia Medik.) honey (sample 5), 5 ling heather (Ericaceae type Calluna vulgaris (L.) Hull) honeys (samples 6-10) and 10 multifloral honeys (samples 11-20). The sediment of the samples

showed that the most important secondary pollen types were Leguminosae type Trifolium spp., Leguminosae type Genista spp., Rosaceae type Rubus spp., Compositae type Helianthus annuus L. and Ericaceae type Erica spp. All honey samples were fresh, and were tested as soon as they were received.

*2.2. Materials*

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (**MMA**) (Aldrich, 99%), N,N-(dimethylamino)ethyl methacrylate (**NNDA**) (Aldrich, 98%), 2-hydroxyethyl acrylate (**2HEA**), (Aldrich, 96%), ethylene glycol dimethacrylate (E) (Aldrich, 98%), hydrochloric acid (VWR, 36%), methanol (Aldrich, 99.8%), starch azure (Aldrich), dimethyl sulfoxide (**DMSO**) (VWR, 99%), copper (II) sulfate pentahydrate (MERCK, ≥ 98%), potassium sodium tartrate tetrahydrate (Aldrich, 99%), sodium hydroxide (Aldrich, 97%), methylene blue (PANREAC), sodium chloride (Aldrich, 99%), sodium acetate (Aldrich, 99%), glacial acetic acid (≥99%), wheat starch (GUINAMA), potassium iodide (Aldrich, ≥99.99%), iodine (Aldrich, ≥99.99%). Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallised twice from methanol.

*2.3. Starch containing polyacrylic film*

The polyacrylic film was prepared by bulk radical polymerisation of three commercial monomers: **NNDA**, **2HEA** and **MMA**, in a molar feed ratio of 45/45/10 (**NNDA**/**2HEA**/**MMA**) using 1% weight of AIBN as radical thermal initiator, and 5% mol of E as the crosslinking agent. This mixture of monomers was chosen due to the excellent workability and controlled swelling of films, as we have optimised in previous works (González-Ceballos et al., 2020; Guembe-García, Peredo-Guzmán, et al., 2020; Guembe-García, Vallejos, et al., 2020). The polymerisation was carried out at 60 ºC, overnight, in a mould comprised between two silanised glasses (100 μm thick), in an oxygen-free atmosphere. The polyacrylic film was removed from the mould, washed three times with methanol, and dipped in HCl (4%). This treatment with HCl

protonates the tertiary amine group of **NNDA**, and improves the starch charging process. Finally, the polyacrylic film was washed several times with water until the pH of washing water was 7.

The starch azure charging process was carried out by dipping a 12 mm diameter disc of the polyacrylic film in 5 mL of a starch azure solution (2% in DMSO) for 24 hours. The charging process was finished until no colour evolution was visually observed in the polyacrylic film. Finally, the blue-coloured polyacrylic film was dractically dipped in water, and washed with water (3x10 mL). This radical media change from DMSO to water is decisive for the encapsulation of starch azure. The swelling percentage of the film in DMSO is 230%, which allows the entry of starch. However, in water, the swelling of the film is 110%, which generates a very rapid contraction of the volume of the film, which leaves the starch retained inside. **Figure 2** shows the chemical structure of the prepared polymer before and after immersion in starch azure solution, and a real image of the prepared material.

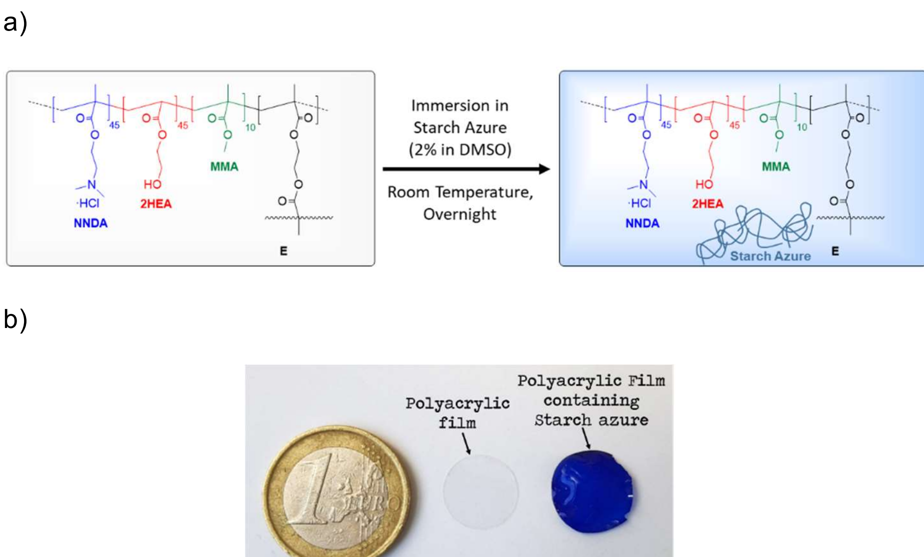


**Figure 2. a)** Chemical structure of the polyacrylic film before and after immersion in starch azure solution (2% in DMSO**). b)** Image of the polyacrylic film before and after immersion in starch azure solution (2% in DMSO).

The amount of starch azure in the polyacrylic film was estimated in a 14.8 wt%, and was calculated by thermogravimetry (more details in ESI S4).

*2.4. Instrumentation*

UV/Vis spectra were recorded using a Varian Cary 400 Bio UV/Vis spectrophotometer.

Samples were heated using a thermostatic bath Grant W28.

*2.5 Methods*

*2.5.1. After Schade method (Reference method, approved by the International Honey Commission, 2009, (S. Bogdanov, 2009)*

Diastase activity was measured in Schade units per gram of honey. One Schade unit is the amount of enzyme that will convert 0.01 grams of starch to the prescribed end in one hour at 40 °C under the test conditions.

Briefly, in this method, honey was dissolved in distilled water. Then, 5 mL pH 5.3 acetate buffer and 3 mL 2.9% (w/v) NaCl aqueous solution were added, transferring the solution to a 50 mL volumetric flask and making up to the mark. After 10 mL of the diluted sample was set at 40 ºC, 5 mL of starch solution was added, stirring the solution and then starting the timer. At periodic intervals, starting at 5 minutes, 1 mL aliquots were taken and mixed with lugol (a solution of molecular iodine (I2) and potassium iodide (KI) in distilled water). The formed blue colour was measured at 660 nm. The absorbance depends on the concentration of starch, which decreases with time due to diastase activity. The analysis continued until an absorbance of 0.235 or lower was obtained. Table 1 shows the diastase activities of measured honeys determined by this method, and the complete procedure is depicted in ESI S1.

2.5.2. *Polyacrylic films containing starch azure method (PAFSA method)*

The interaction of honey with **PAFSA** generates an increment in the concentration of **RS** through the hydrolysis of starch; thus, each honey was tested before and after the interaction with the **PAFSA** for determining the increment of the concentration of **RS**. For it, a stock solution of each honey was prepared dissolving 2 grams of honey in 100 mL of distilled water. After that, two different measurements of the concentration of RS were carried out: (1) Without **PAFSA**. 10 mL of this stock solution was diluted to 500 mL, added to the burette, and tested according to Fehling method, in which the concentration of reducing sugars is measured by titration using methylene blue as a colourimetric indicator (Bicudo de Almeida-Muradian et al., 2020; S. Bogdanov, 2009) The analysis was performed in triplicate for each honey. (2) With **PAFSA**: One disc of **PAFSA** was added to the stock solution (50 mL) and stirred for exactly 30 minutes. Note that three replicates with three different **PAFSA** discs were carried out for each honey. **PAFSA** discs of these replicates were prepared individually, in order to check the good reproducibility of the manufacturing method. After that, a 10 mL fraction was separated and diluted to 500 mL with water, added to the burette, and tested according to Fehling method. The difference in the concentration of **RS** (with **PAFSA** – without **PAFSA**) was correlated with the diastase activity calculated as described in section 2.5.1.

## 3. Results and discussion

*3.1. Correlation of methods*

In this work, we propose an easy, low cost and rapid method for the determination of diastase activity of honeys, as a substitute for the reference method, which is based on a tedious standardisation of starch samples.

The diastase activity of 20 different honeys was determined by the Shade method, and the concentration of the reducing sugars was calculated by PAFSA method. Results are shown in **Table 1**.

**Table 1.** Diastase activity (expressed in Schade units) and increment of reducing sugars concentration (%) of measured honeys. Table shows the average of 3 replicates with each method.

| Sample number | Botanical origin | Schade Method<br>Diastase activity (Schade units) | PAFSA Method<br>Δ [Reducing Sugars] (%) |
|---|---|---|---|
| 1 | Chestnut | 19.0 ± 0.3 | 2.90 ± 0.18 |
| 2 | Honeydew | 14.7 ± 0.5 | 3.48 ± 0.18 |
| 3 | Honeydew | 37.0 ± 0.1 | 1.10 ± 0.19 |
| 4 | Honeydew | 30.0 ± 0.1 | 1.90 ± 0.17 |
| 5 | Lavender | 9.8 ± 0.9 | 3.80 ± 0.15 |
| 6 | Ling heather | 14.1 ± 2.1 | 3.30 ± 0.20 |
| 7 | Ling heather | 19.4 ± 0.6 | 3.00 ± 0.19 |
| 8 | Ling heather | 10.1 ±1.1 | 3.80 ± 0.18 |
| 9 | Ling heather | 27.3 ± 4.9 | 1.90 ± 0.16 |
| 10 | Ling heather | 35.3 ± 0.1 | 1.30 ± 0.17 |
| 11 | Multifloral | 17.1 ± 2.8 | 3.00 ± 0.19 |
| 12 | Multifloral | 17.2 ± 2.2 | 3.10 ± 0.17 |
| 13 | Multifloral | 17.0 ± 2.8 | 3.10 ± 0.17 |
| 14 | Multifloral | 28.3 ± 3.2 | 2.20 ± 0.19 |
| 15 | Multifloral | 24.4 ± 1.9 | 2.40 ± 0.16 |
| 16 | Multifloral | 20.3 ±1.6 | 2.70 ± 0.19 |
| 17 | Multifloral | 16.6 ± 0.5 | 3.30 ± 0.17 |
| 18 | Multifloral | 27.0 ± 0.1 | 2.40 ± 0.16 |
| 19 | Multifloral | 22.1 ± 2.2 | 2.40 ± 0.17 |
| 20 | Multifloral | 28.6 ± 2.3 | 2.30 ± 0.17 |

**Figure 3** shows the good correlation between both methods, i.e., **PAFSA** method can replace the reference method for the determination of the diastase activity of honeys. These results are also supported by a statistical analysis, in which we have compared the standard deviations and the

averages of both methods, and we can affirm that there is no statistically significant difference neither between standard deviations nor between averages of the Schade and **PAFSA** methods.

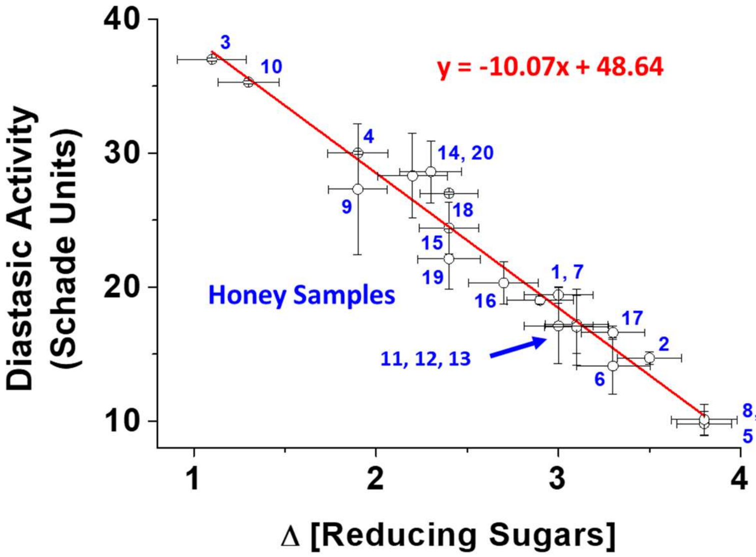


**Figure 3**. Graphical representation of the diastase activity obtained with Schade Method against the concentration of reducing sugars obtained with **PAFSA** method.

A typical procedure for a generic titration curve involves the preparation of several freshly prepared solutions with a pattern (target species), and the measurement of these solutions with the appropriate technique. Then, the food sample is prepared and measured in the same way, and the titration allows the determination of the target species. However, the food sample contains hundreds of compounds which could interfere in the measurement, and usually, an interference study is mandatory because the titration using aqueous solutions does not include all these compounds. In our case, this interference study was not necessary, since the titration was not carried out with prepared aqueous samples, but with real samples of honey, which contained all the compounds that are usually found in honey and which could interfere in the analysis.

Although the work has been carried out with honeys with different botanical origins, and also have a wide range of diastase activity (from 9.8 to 37 Schade units), to get a more accurate equation the procedure should be calibrated with a greater number of honey samples from different origins.

*3.2. Proof of concept*

The obtained equation for the linear fitting (**Figure 3**) allows the calculation of the diastase activity of honeys by **PAFSA** method. Each increment of reducing sugars concentration was introduced as "X" variable, and "Y" (diastase activity) was calculated for every honey. **Figure 4** shows the comparison of diastase activity determined both by reference and **PAFSA** methods (numerical values with calculated errors can be found in **ESI S5**).

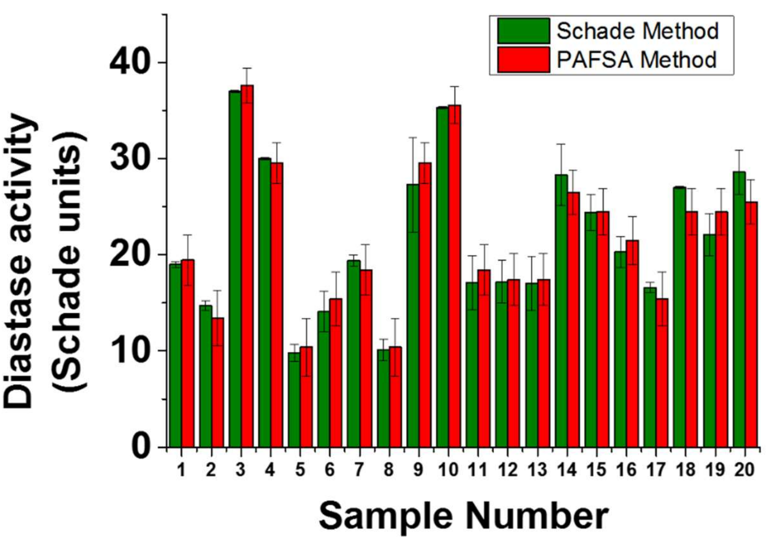


**Figure 4.** Comparison of diastase activity determined both by Schade and **PAFSA** methods.

The results obtained show that the **PAFSA** method provides very similar values to those of the Shade method for the diastase activity of the studied honeys. In the case of sample 15, the difference is less than 0.4%, although in other cases such as samples 19 and 20, the difference is greater,

around 10%. However, and as shown in Table 2, the advantages of the **PAFSA** method in terms of time and cost make it a real alternative to the reference method. For example, our method requires 30 min for every honey sample, and the time with Shade method can varies from 120 min to 180 min in the measured honeys, depending on the diastase activity.

**Table 2.** Comparative table of Shade and **PAFSA** methods

| Method | Time for 1 sample | Starch standardisation | Equipment |
|---|---|---|---|
| Schade | 2-3 hours | Yes | Spectrophotometer<br>Thermostatized bath<br>Hot-plate |
| PAFSA | 30 min | No | Thermostatized bath |

## 31.Conclusions

Diastase activity is a key parameter on honey analysis since it is related to the quality of honey and possible frauds. Schade assay is the most used method for the determination of the diastase activity in honeys, but it implies a very tedious search to find a proper starch, and usually takes a long time, particularly for honeys with low diastase activities. The use of a polyacrylic film as a starch azure container avoids the starch standardization process, and dramatically simplify the determination of the honey diastase activity. The polyacrylic film allows the measurement of the diastase activity through the quantification of reducing sugars produced during the enzymatic process. This simple methodology is not possible in the presence of starch in solution; thus, the function of the polyacrylic film as starch container is determinant. The polyacrylic film has great potential in the routine quality control of honey samples, effectively reducing analysis time and cost. The reproducibility of the manufacturing process of **PAFSA** is really high, and it can be easily used by technicians in small local laboratories and laboratories of beekeeping

cooperatives because only basic glassware equipment is necessary. This work supposes a proof of concept which should be optimised for each user, performing the calibration with a greater number of honey samples from different origins.

## CRediT authorship contribution statement

**Lara González-Ceballos:** Investigation, Writing - original draft. **Miguel A. Fernández-Muiño:** Conceptualization, Methodology. **Sandra M. Osés:** Conceptualization, Methodology, Investigation. **M. Teresa Sancho:** Writing - review & editing, Visualization. **Saturnino Ibeas:** Methodology, Investigation, Validation, Methodology, Investigation, Validation. **Jose Antonio Reglero Ruiz:** Investigation, Methodology. **Saúl Vallejos:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization.

## Declaration of Competing Interest

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

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*Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer*

# Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer

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## Abstract

Glucose oxidase (GOX) and catalase (CAT) regulate the amount of H2O2 in honey, by generating or consuming it, so they are related to the antibacterial and antioxidant activity of honey. However, their activities are hardly analysed, since the process requires a previous dialysis that is non-selective, very time-consuming (>24h), eco-unfriendly (>6L of buffer) and expensive. This research shows the design of a material that selectively removes the actual interferents. The film-shaped polymer is immersed for 90 minutes within a honey solution (12.5 mL of buffer), where it interacts exclusively with 1,2-dihydroxybenzenes, which we proved to be the real interferents (the polymeric material contains motifs derived from phenylboronic acid to interact with 1,2-diols). Polymeric chains favour condensation to occur exclusively with 1,2-dihydroxybenzenes, excluding monosaccharides. For the proposed objective, the interferents' removal using our designed polymer is selective, rapid, low cost (1.42 € per test), and ecofriendly (saves solvents and chemicals).

*Food Chemistry,* FOODCHEM-D-22-05766

**Keywords**: Catechol, 1,2-dihydroxybenzene, phenylboronic acid, glucose oxidase, catalase, honey.

## 1. Introduction

Polyphenols, reducing sugars, minerals, organic acids, vitamins, amino acids, substances responsible for aroma and flavours, or enzymes, are just some examples of the wide variety of compounds present in honey, a foodstuff produced by bees (White & Doner, 1980).

Behind China, the European Union is the second-largest producer of this natural sweetener, with around 600,000 beekeepers and 17 million hives producing 250,000 tons of honey each year. The consumption of this food is so high in Europe that around additional 200,000 tons must be imported annually (Duch Guillot, 2018).

Considering the conspicuous business, one of the priority objectives of the European Union is the quality control of the products produced inside and outside Europe through the characterization of the most relevant properties of honey, their antioxidant and antibacterial activities. The first is mainly due to the presence of polyphenols, while many authors consider that the most relevant antibacterial agent present in honey is hydrogen peroxide ($H_2O_2$). The concentration of this substance in honey is determined by the activity of two enzymes: glucose oxidase (GOX) and catalase (CAT). GOX generates $H_2O_2$ in the glucose oxidation process, whereas CAT breaks it down, generating water and oxygen. Additionally, the activity of these enzymes decreases with the storage time. Therefore, the measurement of the enzyme activity is one of honey's most important quality parameters (Huidobro et al., 2005; Schepartz & Subers, 1966).

In the last decades, the determination of GOX and CAT activities in honey samples has been carried out by different methods, most of which are based on $H_2O_2$ quantification before and after an incubation period. Dold & Witzenhausen (1955) have quantified GOX activity by a microbiological procedure using *Staphylococcus aureus*. The method was precise enough but tedious to be used for routine determinations. Several authors quantified $H_2O_2$ by titration (Kiermieier

& Köberlein, 1954; Salashinski, N A; Bazhenova, 1979). However, these methods were not suitable for the determination of GOX and CAT activities because titration reagents involved secondary reactions (Griebel & Heß, 1940; Schepartz & Subers, 1966). So far, the most used method to determine both GOX and CAT activities is based on spectrophotometric measurements of $H_2O_2$. Some assays determined GOX activity directly on honey solutions (Flanjak et al., 2016; Sahin et al., 2020; Jonathan W. White et al., 1963), but honey interferences made these procedures unreliable for analytical purposes (Sánchez Castro, 2000; Schepartz & Subers, 1964b). To properly determine honey's GOX and CAT activities spectrophotometrically, a purification step by dialysis proved to be mandatory in order to remove all interferents (Schepartz & Subers, 1966). Despite being used for decades, dialysis is a very tedious process that takes 24 h, consuming 6 litres of phosphate buffer at pH 7 for each honey sample. It is based on the use of commercial dialysis membranes, being a very abrupt procedure that allows different compounds to pass through the membrane, such as sugars (fructose, glucose, maltose, among others), polyphenols and other honey constituents. Nevertheless, neither the real interferent substances nor the actual necessity for all those compounds´ removal have been researched so far.

The aim of this work is to study and describe the real interferents for the determination of GOX and CAT activities in honey and, eventually, to develop a simple and easy alternative and good value-for-money method to remove all actual interferences for the reliable spectrophotometric measurement of both enzymatic activities in honey. The studied assay is based on the design of a polymeric material for the highly specific removal of those interferents, as graphically depicted in **Figure 1**. The proposed new strategy is carried out in 90 min and using only 25 mL of phosphate buffer (pH 7), leading to an improvement of 94% and 99.6% in the experimental time and buffer volume, respectively. Furthermore, the polymeric material is made with 100% commercially available monomers, which favours its future industrial scaling and its use in the food industry and research.

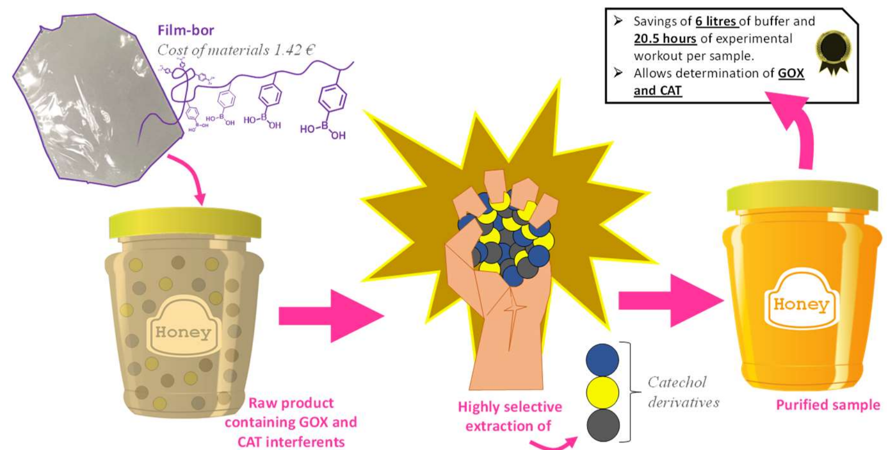


**Figure 1**. Graphical representation of the proposed methodology for honey purification and enzyme activity determination (GOX and CAT).

## 2. Materials and methods

### *2.1. Honey Samples*

This study was carried out with 29 representative kinds of honey harvested in 2019 in Castilla-León, a Spanish area located in the northern Iberian Plateau that held the highest number (3,827) of apicultural undertakings in Spain in 2018, representing 16% of the total apicultural activities of this country (Ministerio de Agricultura, 2019). Honeys' botanical origins were determined by both melissopalinology (Louveaux et al., 1978; Terradillos et al., 1994; Von Der Ohe et al., 2004), and sensory analyses (Marcazzan et al., 2018; Persano Oddo & Piro, 2004; Piana et al., 2004), there was 2 lavender (Lamiaceae type *Lavandula sp.*) honeys (samples 1 and 12), 3 forest honeys (samples 2, 10 and 22), 12 ling heather (Ericaceae type *Calluna vulgaris*) honeys (samples 3, 5, 6, 8, 9, 14, 15, 16, 20, 21 and 24), 7 multifloral honeys (samples 13, 23, 27, 28 and 29), 1 centaury flower (*Centaurea cyanus*) honey (sample 11), 1 holly (*Ilex aquifolium*) honey and 3 honeydew honeys (samples 19, 25 and 26). The sediment of the samples showed that the most important secondary pollen types were Leguminosae type *Trifolium* spp., Leguminosae type *Genista* spp., Rosaceae

type *Rubus* spp., Compositae type *Helianthus annuus* L. and Ericaceae type *Erica* spp. All honey samples were fresh and were tested as soon as they were received.

*2.2. Materials*

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (**MMA**) (Aldrich, 99%), *N,N*-dimethylaminoethyl metacrylate (**NNDA**) (Aldrich, 98%), 2-hydroxyethyl acrylate (**2HEA**), (Aldrich, 96%), ethylene glycol dimethacrylate (**E**) (Aldrich, 98%), hydrochloric acid (VWR, 37%), methanol (Aldrich, 99.8%), 4-vinylphenylboronic acid (**BOR**) (TCI, 95%), sodium dihydrogen phosphate (Merck, 99.99%), di-sodium hydrogen phosphate anhydrous (Merck, 99.99%), potassium phosphate monobasic (Aldrich, ≥99%), sodium phosphate dibasic dihydrate (Panreac, ≥99%), ammonium metavanadate (Aldrich, ≥99%), sulfuric acid (VWR, 95%), *o*-dianisidine dihydrochloride (Sigma, >99%), hydrogen peroxide (Aldrich, ≥30%), catechol (Aldrich, 99%), resorcinol (Merck, 99%), hydroquinone (Aldrich, 99%), quercetin hydrate (Aldrich, 95%), D-(+)-Glucose (Panreac, 99.99%), D-(-)-Fructose (Aldrich, 99%), D-(+)-Maltose (Sigma, 97.5%), sodium permanganate monohydrate (Aldrich, ≥95%), peroxidase from horseradish (Sigma Aldrich, EC 1.11.1.7), dialysis membranes (Aldrich, D6191), dimethylsulfoxide-$d_6$ (VWR, 99.9%), catalase from beef liver (EC 1.11.1.6) from Roche Diagnostics (Mannheim, Germany), glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) from Sigma Aldrich. Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallized twice from methanol.

*2.3. Preparation of the polymeric film containing phenylboronic acid groups (**Film-bor**)*

The polymeric polyacrylic film **Film-bor** was prepared by bulk radical polymerization of four commercial monomers: *N,N*-dimethylaminoethyl methacrylate (**NNDA**), 2-hydroxyethyl acrylate (**2HEA**), methyl methacrylate (**MMA**) and 4-vinylphenylboronic acid (**BOR**) in a molar feed ratio of 42.5/42.5/10/5 (**NNDA/2HEA/MMA/BOR**) using 1% weight of AIBN as radical

thermal initiator, and 5% mol of ethylene glycol dimethacrylate (**E**) as the crosslinking agent. This formulation was optimized for working with honey samples in previous works (González-Ceballos et al., 2021). The polymerization was carried out overnight at 60 ºC in a mould comprised between two silanised glasses (100 µm thick), in an oxygen-free atmosphere. **Film-bor** was removed from the mould, washed three times with methanol, and dipped in HCl (4%). The treatment with HCl protonates the tertiary amine group of **NNDA**, improving the water swelling percentage of the material. Finally, the **Film-bor** was washed several times with water until the pH of the washing water was 7. Complete characterization of the polymer can be found in Supplementary Material (SM)**, Section S1**. **Figure 2a** shows the chemical structure of **Film-bor** and a real image of the prepared material.

*2.4. Preparation of the linear polymer containing phenylboronic acid groups (**Linear-bor**)*

**Linear-bor** polymer was prepared by radical co-polymerization of the same commercially available monomers as **Film-bor**, and in the same molar ratio, i.e., **NNDA/2HEA/MMA/BOR** (42.5/42.5/10/5). First, 15.9 mmol of NNDA (2.5 g), 15.9 mmol of 2HEA (1.85 g), 3.7 mmol of MMA (374 mg), and 1.87 mmol of BOR (277 mg) were dissolved in DMF (18.7 mL), and the solution was added to a round-bottom pressure flask. Subsequently, radical thermal initiator AIBN (307 mg, 1.87 mmol) was added, the solution was sonicated for 10 min, and heated overnight at 60 °C, under a nitrogen atmosphere, and without stirring. The solution was then dropwise added to diethyl ether (150 mL) with magnetic stirring, yielding the desired product as a yellowish precipitate. Finally, polymers were purified in a Soxhlet apparatus with diethyl ether as washing solvent to eliminate DMF traces. Yield ≈ 65%. Complete polymer characterization can be found in the electronic supporting information (**SM-Section S1**). **Figure 2b** shows the chemical structure of **Linear-bor**, and a real image of the prepared polymer.

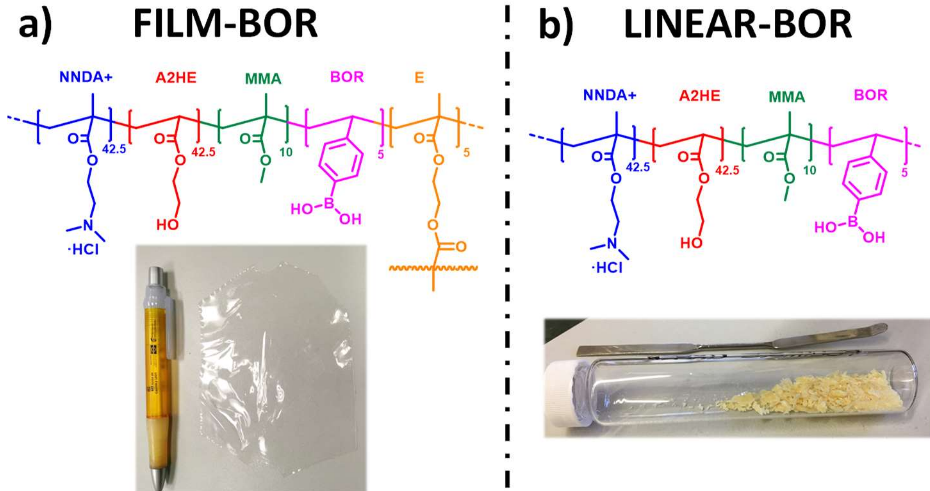


**Figure 2**. Chemical structures and real images of **a**) the membrane **Film-bor**, and **b**) the water-soluble linear polymer **Linear-bor**.

*2.5. Instrumentation*

Inductively coupled plasma mass spectrometry (ICP-MS) measurements were recorded on an Agilent 7500 ICP-MS spectrometer (Agilent, Santa Clara, USA). $^{1}H$ and $^{13}C\{^{1}H\}$ NMR spectra (Avance III HD spectrometer, Bruker Corporation, Billerica, Massachusetts, USA) were recorded at 300 MHz for $^{1}H$ and 75 MHz for $^{13}C$ using deuterated solvents such as dimethyl sulfoxide (DMSO-$d_6$) at 25 °C.

The weight percentage of water taken up by the films upon soaking in pure water at 20°C until reaching equilibrium (water-swelling percentage, WSP) was obtained from the weight of a dry sample film ($\omega_d$) and its water-swelled weight ($\omega_s$) using the following expression: $WSP=100\times[(\omega_s-\omega_d)/\omega_d]$. The powder X-ray diffraction (PXRD) patterns were obtained using a diffractometer (D8 Discover Davinci design, Bruker Corporation, Billerica, Massachusetts, USA) operating at 40 kV, using Cu(Kα) as the radiation source, a scan step size of 0.02°, and a scan step time of 2 s.

The polymers were thermally characterized by using thermogravimetric analysis (Q50 TGA analyzer, TA Instruments, New Castle, DE, USA) with 10–15 mg of sample under synthetic air and nitrogen atmosphere at 10°C·min$^{-1}$; differential scanning calorimetry, with 10–15 mg of the sample under a nitrogen atmosphere at a heating rate of 10°C min$^{-1}$ (Q200 DSC analyzer, TA Instruments, New Castle, DE, USA); and tensile properties analysis, with 5 × 9.44 × 0.103 mm samples tested at 5 mm min$^{-1}$ (EZ Test Compact Table-Top Universal Tester, Shimadzu Kyoto, Japan). Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) with an ATR-PRO410-S single reflection accessory.

Isothermal titration calorimetry (ITC) measurements were performed using a microcalorimeter (VP-ITC MicroCal Inc., Malvern, UK) equipped with two cells, one cell for sample (**Linear-bor**), at a final concentration of phenylboronic acid groups of 0.61 mM, and another one for reference, with volumes of 1.436 mL. The titrant syringe was filled with 280 µL solution (9 μM) of the tested compounds (catechol, resorcinol, hydroquinone, fructose, glucose, and maltose), which was step-by-step added to the sample cell (20 μL aliquots were added every 5 min). All the solutions were degassed for 10 min at 25.0 ± 0.1°C in a vacuum pump to avoid blistering in the syringe or in the calorimetric cells during the experiment. Calorimetric measurements were performed at 25.000 ± 0.001°C, with a constant stirring at 307 rpm in the sample cell.

The catechol concentration in aqueous solutions used in the permeation, kinetic and sorption isotherm analysis was determined via UV-Vis spectrophotometer measurements (UV-2600*i*, Shimadzu, Germany). Calibration line was performed for catechol in the analytical range 0-50 mg L$^{-1}$ by using the absorbance at $\lambda_{max}$ = 275 nm; a molar absorption coefficient equal to 48.6 L mol$^{-1}$ cm$^{-1}$ was obtained. The analysis was performed in duplicate, and the solutions were prepared in Milli-Q ultrapure water.

Enzyme interferents assays were performed using a Synergy HT microplate reader (BioTek®, Winooski, Vermont, USA), measuring absorbance at 452 nm.

The concentration of CAT, GOX, $H_2O_2$, and the compounds used as interferents was determined using a Hitachi U-3900 UV-Vis spectrophotometer (Tokyo, Japan).

The enzymatic (GOX and CAT) activities were measured using a CARY 400 Bio UV-Visible Spectrophotometer, measuring the absorbance, for both analyses, at 400 nm.

*2.6. Methods*

*2.6.1. Honey purification for the determination of the activity of GOX and CAT using Sigma Aldrich dialysis membranes D6191.*

The membranes had to be conditioned first, following the manufacturer's instructions. Therefore, the membrane was immersed in a beaker containing boiling water for 2 min, and the process was repeated for the same time in another beaker, avoiding the membrane cooling down between the first and second washes. Finally, the membrane was immersed in water at room temperature.

7.5 g of honey was dissolved in phosphate buffer 0.015M at pH 7 (4 mL) in a beaker. The lower end of the already conditioned membrane was closed with a clamp, and the solution was placed inside it using a funnel. The beaker was washed several times with buffer, using 8 additional mL, and finally, all portions were homogenized inside the membrane. After closing the upper end of the membrane with another clamp, it was dipped in a beaker containing 3 L of phosphate buffer 0.015 M and the system was kept at 4°C. The buffer was renewed (3 L) after 10 h, and the system was carefully homogenized every 6 h. After 22 h, the membrane was removed from the beaker and washed with the buffer solution. The content of the membrane was transferred to a 50 mL volumetric flask, and the same buffer was used to clean the interior of the membrane and make up to the mark.

*2.6.2. Honey purification for the determination of the activity of GOX and CAT using **Film-bor**.*

7.5 g of honey were homogenized with 12.5 mL of 0.015 M phosphate buffer and placed in a beaker with a film blanket. The system was gently stirred for 90 min at room temperature. Finally, the membrane was removed from the beaker, and the solution was transferred to a 50 mL volumetric flask and made up to the mark using the same buffer.

*2.6.3. Fundamentals on the determination of CAT activity in honey samples.*

The quantification of the CAT activity is based on the reaction between CAT and an excess of $H_2O_2$ (substrate); the non-reacted amount of $H_2O_2$ is then made to react with *o*-dianisidine and peroxidase, and the obtained coloured product is quantified by spectrophotometry, at 400 nm. Purified honey samples with methods depicted in sections 2.5.1 and 2.5.2 were tested following the detailed experimental procedure published by Huidobro et al. (2005) with modifications. More details about the experimental procedure can be found in **SM**-**Section S2**.

*2.6.4. Determination of GOX activity in honey samples.*

Contrarily to CAT, $H_2O_2$ is the reaction product (not the substrate) produced when GOX reacts with glucose. Therefore, the method for GOX activity determination is based on the addition of known amounts of glucose to the honey sample and the quantification of the generated $H_2O_2$. Similarly to CAT, the quantification of GOX activity is based on measuring the absorbance at 400 nm of the coloured product formed by reacting *o*-dianisidine and peroxidase with $H_2O_2$. Purified samples with methods depicted in sections 2.5.1 and 2.5.2 were tested based on the experimental procedure reported elsewhere (Schepartz & Subers, 2015). More details about the experimental procedure can be found in **SM**-**Section S3**.

*2.6.5. Interference study*

The study was carried out with catechol, resorcinol, hydroquinone, D-fructose, D-glucose, and maltose as possible interferents. Since catechol is a peroxidase substrate (García-Moreno et al., 1999; Huidobro et al., 2005), this study could not

be performed using the method described in sections 2.5.3. and 2.5.4. For this reason, we have used a modified version of the method described by Hadwan & Ali (2018). Inhibitory capacity of catechol, resorcinol, hydroquinone, glucose, fructose, and maltose was tested *in vitro* against commercial CAT and GOX using 96-well microplates. Basically, 10, 50, and 150 mg $L^{-1}$ of tested compounds were preincubated for 10 min with 20 ng of CAT or 1 h with 4 µg of GOX in a 50 mM phosphate buffer at pH 7. Subsequently, substrates were added at a final concentration of 4 mM ($H_2O_2$) or 80 mM (D-glucose) and incubated for 2 or 20 min at 37°C for CAT or GOX activity, respectively. Thereafter, the presence of $H_2O_2$ in the reaction was detected by the addition of ammonium metavanadate at a final concentration of 4 mM and 10 min of incubation at 28°C. Then, the absorbance was measured at 452 nm. System was calibrated with known amounts of $H_2O_2$ in a standard reaction mixture. Inhibitory activity of the analysed compounds was expressed as percentage of enzymatic activity relative to that in the absence of the tested compound. All assays were conducted by triplicate, and blanks were used to account for spontaneous hydrolysis of the substrates and unspecific reaction of the interferents with the rest of the reaction compounds.

*2.6.6. Permeation, kinetic and isotherm study of **Film-bor**.*

The permeation study was carried out with a system of horizontal communicating vessels (Valente et al., 2000). The studied material (**Film-bor**) was placed as a barrier in the channel between the two vessels, whose permeation area is 2 $cm^2$. Milli-Q ultrapure water was placed in vessel “A”, and an aqueous solution of catechol at 5000 mg $L^{-1}$ was placed in vessel “B”. The permeation process was studied by measuring the absorbance at 275 nm of the solution in vessel “A” over time.

Sorption analyses were performed at 25 °C, using a solid/liquid ratio of 50 mg of **Film-bor** (dried at 60°C for 2 h) per mL of solution. In detail, for kinetic sorption analysis, 0.15 g was dipped in 3 mL of an aqueous solution of catechol (15 mg $L^{-1}$) within 0–35 min. The mechanism was studied by fitting pseudo-first

order (PFO) and pseudo-second order (PSO) models to the experimental data. The non-linear equations are:

$$q_t = q_e(1 - e^{-k_1 t}) \quad \text{(eq. 1)}$$

$$q_t = \frac{k_2 q_e^2 t}{1 + k_2 q_e t} \quad \text{(eq. 2)}$$

where $q_t$ (mg g$^{-1}$) is the amount of sorbate at a defined interval of times (min) that at equilibrium state is defined as (mg g$^{-1}$). $k_1$ (min$^{-1}$) and $k_2$ (g mg$^{-1}$ min$^{-1}$) are the rate constants for PFO and PSO, respectively (William Kajjumba et al., 2019).

The goodness of fitting of the two models is compared by the Akaike information criterion (AIC) (eq. 3)

$$AIC = N ln\left(\frac{RSS}{N}\right) + 2k + \frac{2k(k+1)}{N-k-1} \; for \; \frac{N}{k} < 40 \quad \text{(eq. 4)}$$

where $N$ is the number of the experimental points, $RSS$ the residual sum of squares and $k$ the number of fitted parameters (Kingdom & Prins, 2016).

The isotherm analysis was performed using 0.1 g of **Film-bor** in 2 mL of aqueous catechol solutions at concentrations ranging from 0 to 500 mg L$^{-1}$. The batches were shaken in an incubator (ZWY-100H, Labwit) at constant temperature and 120 rpm. The Langmuir isotherm model was fitted to the experimental data to better understand the sorption process. The mathematical expression is described by equation 5.

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad \text{(eq. 5)}$$

where $q_m$ (mg g$^{-1}$) is the maximum sorption capacity per unit weight of sorbent, $C_e$ (mg L$^{-1}$) is the concentration of analyte at equilibrium state, and $K_L$ (L mg$^{-1}$) is the Langmuir constant.

The amount of sorbate per gram of sorbent at equilibrium state, $q_e$, and the removal efficiency of the sorbent, $RE\%$, were determined by the following equations:

$$q_e = \frac{C_0 - C_e}{m} \times V \quad \text{(eq. 6)}$$

$$RE\% = \frac{C_0 - C_e}{C_0} \qquad \text{(eq. 7)}$$

where $C_0$ (mg $L^{-1}$) is the initial concentration of catechol, *m* (g) is the mass of the sorbent, and *V* (L) is the volume of the solution.

*2.6.7 Statistical analyses*

The statistical analysis was performed using GraphPad Prism v8. Normality and homoscedasticity of the data were first analysed. When the data fulfilled both assumptions, Two-way ANOVA was conducted to analyze the enzyme activity inhibition with several compounds at different concentrations, and Tukey's multiple comparisons test ($p < 0.05$) was used. In addition, Two-way ANOVA was used to compare CAT and GOX activities in honey samples, comparing values within each row followed by Uncorrected Fisher's LSD test ($p < 0.05$).

## 3. Results and discussion

*3.1. Interference study*

The main purpose of this work was based on the study of the real interferents for the determination of honey's GOX and CAT activities. Many authors considered that the interferents were mainly reducing sugars (85% of honey´s total composition) (Sánchez Castro, 2000; Schepartz & Subers, 1964a). On the other hand, polyphenols such as catechol and its derivatives were identified in some publications as CAT interferents (Krych & Gebicka, 2013). Furthermore, some studies pointed out that far from being interferents, some reducing sugars as glucose increased the activity of CAT (Akbayirli & Akyilmaz, 2008). However, this matter is far from being clarified, due to the existing contradictory positions. Thus, one of our goals is to contribute to this clarification.

GOX and CAT activity assays have been performed with some of the major honey compounds, such as sugars and polyphenols, which were thought as the potential interferents of these enzymes. On the one hand, the decision for sugars was straightforward, as D-fructose, D-glucose, and maltose are the main honey carbohydrates. On the other hand, the decision on polyphenols was not

so clear since there are many different polyphenols in honey. Thus, we tackled this issue from the chemical point of view, classifying polyphenols as 1,2- 1,3- and 1,4-dihydroxybenzene derivatives. In this way, and always from a chemical viewpoint, all polyphenols were represented by catechol (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene) and hydroquinone (1,4-dihydroxybenzene) structures.

As shown in **Figure 3**, the 3 different types of dihydroxybenzenes (1,2-, 1,3-, and 1,4-) were clear interferents of CAT activity, unlike the 3 selected reducing sugars. On the other hand, all the interferents studied seemed to have the same inhibition effect on GOX activity, both polyphenols and reducing sugars. However, this inhibition was considerably lower, under 27% in all cases. These results were the basis for the design of the polymeric material.

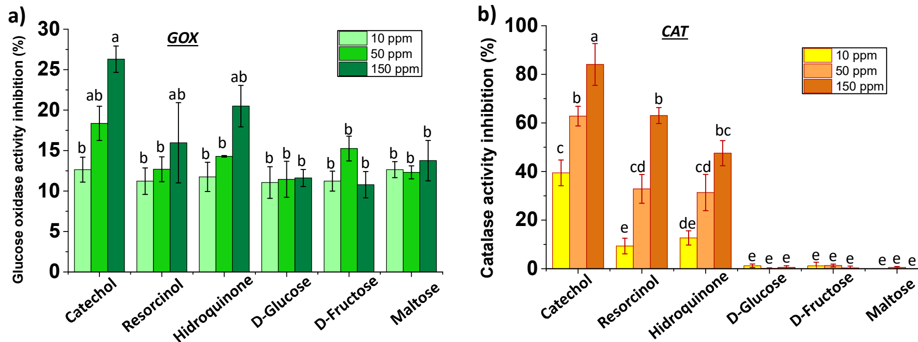


**Figure 3.** Inhibitory ability of catechol, resorcinol, hydroquinone, glucose, fructose and maltose against commercial CAT and GOX: a) percentage of GOX activity inhibition by catechol, resorcinol, hydroquinone, glucose, fructose and maltose at different concentrations (10, 50, 150 mg $L^{-1}$); and b) percentage of CAT activity inhibition by catechol, resorcinol, hydroquinone, glucose, fructose and maltose at different concentrations (10, 50, 150 mg $L^{-1}$). Data are mean ± SE of three independent replicates. Different letters indicate significant differences within each commercial enzyme activity inhibition ($p < 0.05$, two-way ANOVA followed by Tukey's multiple comparisons test).

*3.2. Design of the polymer structure for the extraction of polyphenols from honey samples*

As shown by the interference study, polyphenols are the real interferents for determining GOX and CAT activities in honey. The phenolic profile of honey is highly variable and depends largely on its botanical origin as well as on other factors such as geographical factors, among others (Yayinie et al., 2022). However, when talking about honey, we can assume that the vast majority of polyphenols contain 1,2-dihydroxybenzenes in their structures, mainly phenolic acids (gallic acid, caffeic acid and chlorogenic acid), and flavonoids (catechin, quercetin and luteolin) as studied by several authors and as depicted in **Figure 4a** (Cheung et al., 2019; Ciulu et al., 2016; Khalil et al., 2011; Pandey & Rizvi, 2009). Thus, we decided to synthesize a material able to extract these compounds in a very selective and effective manner. In this way, the honey sample would be as similar as possible to its natural state, but without these interferents.

Our first hypothesis was to prepare a polymeric material with phenylboronic pendant groups since the formation of boronic esters with catechol derivatives (Hagihara et al., 2008; Suzuki et al., 2020) and polyphenols in general (Hall, 2005) had been widely studied. However, phenylboronic acid derivatives were also studied as sensors for recognizing sugars (Matsumoto et al., 2009), and this could be somehow negative. Therefore, our second hypothesis was to modulate the reactivity of the polymeric material to react only with the 1,2-dihydroxybenzene derivatives. This fact could only be achieved with an appropriate design of the rest of the monomers used to prepare the material. In other words, the polymers should not only include receptors based on phenylboronic groups (5 mol%) but the rest of the monomers (95 mol%) should also be selected *ad hoc* for the proposed objective, i.e., to extract GOX and CAT activity interferents from honey.

After several tests with different formulations containing 5 mol% of phenylboronic pendant groups (see **SM-Section S4**), we concluded that the best

one was based on **NNDA**, **2HEA**, **MMA** and **BOR**. Additionally, the material was conditioned in an acidic medium (HCl, 4%) to protonate the **NNDA**´s tertiary amine groups and thus promoting the formation of boronic esters (Matsumoto et al., 2009). As a remark, we qualitatively carried out this preliminary study with catechol and quercetin, two of the most abundant 1,2-dihydroxybenzene derivatives in honey. As shown in **Figure 4b**, a drastic material colour change is observed in solutions of catechol (black) and quercetin (yellow). For the rest of the study, we only worked with catechol as the model for 1,2-dihydroxybenzene derivatives due to the poor water-solubility of quercetin.

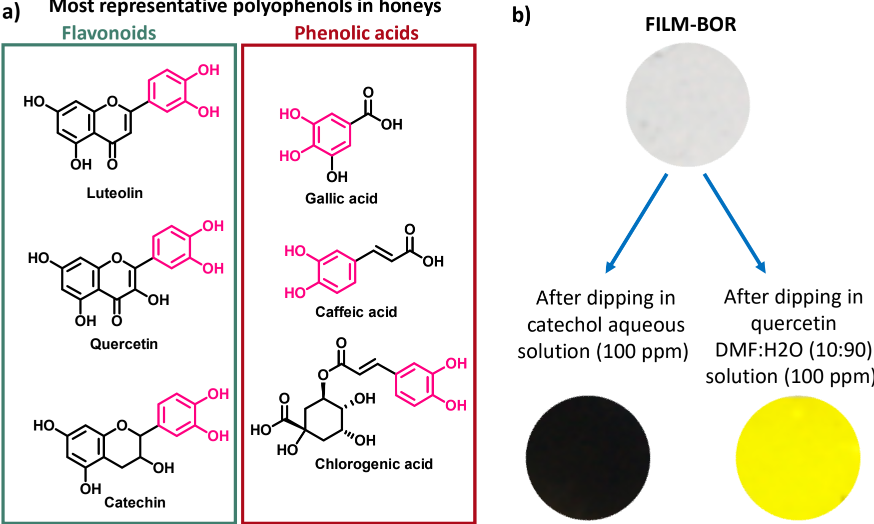


**Figure 4.** Interaction between **Film-bor** and 1,2-dihydroxybenzenes: a) Most representative polyphenols in honey, containing 1,2-dihydroxybenzene derivatives; b) qualitative experiment with catechol and quercetin. A 10 mm diameter disc of **Film-bor** was dipped in 100 ppm aqueous solutions of catechol and quercetin. For the latter, 10% of dimethylformamide had to be added to improve solubility.

Finally, it must be highlighted that the material cost to carry out one test is only 1.42 €, so the methodology is technically and economically viable.

*3.3. Isothermal titration calorimetry experiments (ITC).*

The key part of the ITC experiments was the choice of the concentrations of **Linear-bor** and the species studied. Since the greatest interference for the measurement of enzymatic activity was observed with catechol, these concentrations were adjusted using this compound as a model.

The concentration of catechol in honey can vary from 1.1 to 87.6 mg $L^{-1}$ depending on the type of honey (Silva et al., 2013), so we set 50 mg $L^{-1}$ of catechol, as well as other species, as the starting point for this study. It is worth noticing that the concentration of sugars in honey is much higher; however, we choose to work with the same concentrations for all carbohydrates to allow a reliable comparison and conclusions, as well.

ITC measurements are a powerful technique to provide information on the thermodynamic parameters, including the equilibrium constant ($K_{eq}$), the enthalpy ($\Delta$H) and the entropy ($\Delta$S) of interaction.

Regarding equilibrium constants, phenylboronic acid derivatives generally suffer condensation reactions with 1,2- and 1,3- diols, both in acid and alkaline media (Davis, 2001); however, as a general approach, equilibrium constants increase by increasing the pH (Springsteen & Wang, 2002). Specifically, with sugars, the reaction depends on the conformation of the monosaccharides and, therefore, on the spatial position of the -OH groups. The most favourable conformation for the formation of the phenylboronic ester was the *syn*-periplanar provided by the furanose structure (Elshaarani et al., 2018). Consequently, the equilibrium constants reported for fructose were much higher than for glucose, since the α and β tautomers of fructofuranose have abundances of 28% in honey samples (Mazzoni et al., 1997), while the abundance of the α and β tautomers of glucofuranose only reached up to 0.3 - 1.3% in aqueous solutions (Maple & Allerhand, 1987) and 0% in honey samples (Mazzoni et al., 1997).

At pH 7.4, the equilibrium constants found in the literature for interaction between boronic acid and D-fructose, D-glucose, and maltose are 160, 4.5 and 2.5 $M^{-1}$, respectively. However, the $K_{eq}$ for catechol is significantly higher, 830 $M^{-1}$ (Springsteen & Wang, 2002); this value was justified due to the high rigidity of 1,2-dihydroxybenzene derivatives, which facilitates the boronic ester formation.

Therefore, based on previously reported data, it is expected that our material might interact mainly with catechol and fructose and no (or less intense) interaction should occur for glucose, maltose, resorcinol and hydroquinone. **Figure 5a** shows the heat per injection for solutions of **Linear-bor** when adding selected carbohydrates or polyphenols. ITC detects only the reaction with catechol. The corresponding equilibrium constant for catechol containing solution is 1110 ± 104 $M^{-1}$ (individual graphs for each tested compound can be found in **SM-Section S5**). It can also be concluded that the interaction is exothermic ($\Delta H$ = 13.7 ±0.6 kJ $mol^{-1}$) and enthalpy-driven ($T\Delta S$ = 3.7 kJ $mol^{-1}$), which suggests a strong catechol-**Linear-bor** interaction, and the role of solvent can be essentially neglected.

However, it must be stressed that the selective behaviour towards catechol was considerably better than those initially expected and can be attributed to the environment generated by the polymer chains, which definitely favoured interaction with catechol, hindering interaction with sugars. Without the environment generated by polymer chains, the interaction with monosaccharides, especially with fructose, cannot be measured, essentially due to the low solubility of the monomer in water (Lorand & Edwards, 1959; Springsteen & Wang, 2002).

### *3.4. Permeation, kinetic, and isotherm study of **Film-bor**.*

In line with ITC experiments using **Linear-bor**, the permeation study showed a chemical interaction between **Film-bor** and catechol. As shown in **Figure 5b**, the time needed to reach a steady-state flux, which corresponds to the time-lag (q= 2.0 h), is significantly high, indicating that before permeating, the main process is an interaction between catechol and the **Linear-bor**, in which catechol is retained in the membrane, probably through a chemical condensation reaction.

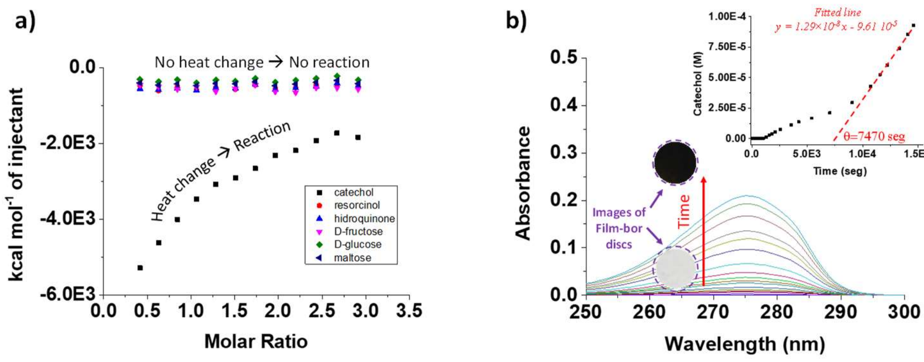


**Figure 5**. Interaction of **Linear-bor** and **Film-bor** with catechol: a) isothermal titration calorimetry experiments. Sample cell contained an aqueous solution of **Linear-bor** at a starting concentration of phenylboronic acid groups of 0.61 mM. Different experiments with 9 mM solutions of catechol, resorcinol, hydroquinone, fructose, glucose, and maltose were carried out by adding 20 µL aliquots every 5 min. Temperature: 25 °C; and b) **Film-bor** membrane permeation study with catechol at 25 °C. Membrane thickness = 100 µm. Area = 2 $cm^2$. Content of beaker A = 200 mL of MQ water. Content of beaker B = 200 mL of a 5000 mg $L^{-1}$ solution of catechol in Ultrapure water. The absorbance (at 275 nm) of beaker A was measured over time until a linear trend was achieved.

Considering the **Film-bor** membrane thickness (L = 100 μm), the permeation area (A = 2 cm$^2$), the cell volume (V = 200 mL), and the concentration of catechol in the donor cell ($C_{cat}$), the diffusion ($D$) and permeation ($P$) coefficients can be calculated by the following expressions:

$$D = L^2/6\theta \quad \text{(eq. 8)}$$

1. $$P = (V/A) \times m \times (L/C_{cat}) \quad \text{(eq. 9)}$$

where $m$ is the slope of the variation of concentration of catechol that permeates the membrane as a function of time, at the steady-state condition (see red dashed line in **Figure 5b**). Thus, we obtained a diffusion coefficient value of 2.2 × 10$^{-9}$ cm$^2$ s$^{-1}$, and a permeation coefficient value of 2.8 × 10$^{-7}$ cm$^2$ s$^{-1}$. As a negative control, we carried out the same experiment with resorcinol, obtaining very similar results for $P$ (5.25 × 10$^{-7}$ cm$^2$ s$^{-1}$), but one order of magnitude higher value for $D$ (1.72 × 10$^{-8}$ cm$^2$ s$^{-1}$). These results lead to a partition coefficient (K=P/D) for catechol equal to 128, 420% higher than that found for resorcinol (30). These values show a high affinity of catechol towards **Film-bor** membrane, in close agreement with the ITC results.

The kinetic sorption of catechol (15 mg L$^{-1}$) into **Film-bor** (**SM-Section S6, Figure S6a**) indicates that the equilibrium is reached in about 10 min. Considering that the sorption thickness is L/2, compared with that for the permeation experiments, such a time matches with the first step of the permeation analysis, i.e., at unsteady-state conditions (see **Figure 5b**). It can be hypothesized that such a behaviour can be justified by the saturation of the active sites inside the membrane – a prerequisite for achieving the steady-state flux. Surprisingly, the overall sorption kinetics data are better fitted by the PFO kinetic model (**SM-Section S6, Table S1**). This suggests a diffusion-controlled process once PFO model is comparable to a mass action rate for sorption seen as a transfer process (Vareda et al., 2016). For a deeper assessment on these results, the weight of data at short-range times (in this case, for $q_t/q_e$ < 0.85) for

the overall fitting was evaluated by using the Boyd equation (William Kajjumba et al., 2018), $B_t = mt + C$, where $B_t$ is defined as

$$B_t = 2\pi - \frac{\frac{\pi^2 q_t}{q_e}}{3} - 2\pi\sqrt{\left(1 - \frac{\frac{\pi q_t}{q_e}}{3}\right)} \qquad \text{(eq. 10)}$$

*m* is the slope, and *C* is a constant which defines the rate-limiting step. By fitting the Boyd equation to the experimental sorption data shown in **Figure S6a**, we obtained $C$=-0.133 (0.006), with a determination coefficient equal to 0.9939. This value clearly indicates that at short-range times a time lag governs the process, whilst for long-range times the process is mainly diffusional, in close agreement with the conclusions obtained above and the sorption isotherms as discussed further below.

Concerning the sorption isotherm, in **SM-Section S6, Figure S6b**, the Langmuir model perfectly fitted the experimental data indicating a homogeneous sorption system on active sites of the polymer. Once again, the results show that the boronic acid groups might act as active sites at the same energy for interacting with catechol. The favourable sorption process was also indicated by Webber-Chakkravorti constant, $0 < R_L < 1$ (**SM-Section S6 Table S1**). Finally, we must highlight that the **Film-bor** showed a maximum of *RE%* = 88% within 20 and 50 mg $L^{-1}$ (**SM-Section S6, Figure S6c**), which were real concentrations of polyphenols in honey samples, so, of outstanding importance for industrial application.

*3.5. Proof of concept. GOX and CAT activity determination of 29 honey samples.*

This study was carried out on 29 honey samples from different botanical origins. The activity of both enzymes was measured in the original honey samples (without purification), as a demonstration of the need for samples to be purified. Additionally, the same 29 samples were also characterized after a dialysis process using Sigma-Aldrich dialysis membranes D6191, and after a purification process using **Film-bor**. The results obtained are shown in **Table 1**.

**Table 1.** Activity results for GOX and CAT expressed as "μg $H_2O_2$ $g_{honey}^{-1}$" and "$g_{honey}^{-1}$ $min^{-1}$" respectively. The activity was measured in raw honeys, in dialyzed honeys using Sigma Aldrich dialysis membranes D6191 (Sigma), and in purified honeys using **Film-bor**. Data are means of two biological replicates ± standard deviation. Different letters indicate significant differences within the same row in GOX activity (upper case letters) or CAT activity (lower case letters). Two-way ANOVA followed by Uncorrected Fisher's LSD ($p < 0.05$) test was performed.

| Sample number | GOX activity (μg $H_2O_2$ $g_{honey}^{-1}$) | | | Catalase activity (×$10^{-3}$) ($g_{honey}^{-1}$ $min^{-1}$) | | |
|---|---|---|---|---|---|---|
| | As received | Sigma | Film–bor | As received | Sigma | Film–bor |
| 1 | nd | 2.1 ± 1.2 A | 7.7± 4.1 A | nd | 3.2 ± 0.5 a | 3.1 ± 2.1 a |
| 2 | 10.2 ± 5.1 A | 54.2 ± 50.4 A | 38.6 ± 33.5 A | 1.7 ± 0.2 a | 66.23 ± 0.4 c | 62.2 ± 2.2 b |
| 3 | 998.7 ± 53.6 A | 1121.8 ± 4.9 B | 1219.0 ± 75.2 B | 2.2 ± 0.3 a | 15.1 ± 0.4 b | 13.5 ± 1.1 b |
| 4 | 124.2 ± 2.1 A | 161.5 ± 5.3 B | nd | nd | 9.5 ± 0.6 a | 7.8 ± 1.9 a |
| 5 | 59.5 ± 3.4 A | 90.2 ± 36.5 A | 48.0 ± 3.0 A | nd | 11.9 ± 0.2 a | 11.4 ± 0.5 a |
| 6 | 215.7 ± 0.9 A | 264.5 ± 15.4 A | 170.0 ± 26.8 A | 36.6 ± 1.3 a | 76.1 ± 0.2 b | 71.45 ± 1.3 b |
| 7 | 214.3 ± 3.4 A | 330.2 ± 6.0 A | 336.0 ± 6.0 A | 3.5 ± 0.3 a | 30.3 ± 1.2 b | 28.6 ± 0.0 b |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8 | 711.3 ± 36.7 A | 903.2 ± 61.3 B | 914.4 ± 38.5 B | nd | 3.5 ± 0.2 a | 3.9 ± 0.6 a |
| 9 | 738.7 ± 4.8 A | 923.9 ± 44.4 B | 1105.6 ± 228.3 C | nd | 36.3 ± 3.4 b | 12.6 ± 1.3 a |
| 10 | 700.3 ± 8.2 A | 886.0 ± 27.7 B | 898.3 ± 2.8 B | 11.2 ± 0.9 a | 82.9 ± 1.2 b | 81.3 ± 2.5 b |
| 11 | 977.7 ± 51.9 A | 1032.7 ± 97.8 A | 1132.0 ± 1.9 B | 13.6 ± 3.3 b | 9.4 ± 3.4 a | 8.9 ± 3.4 a |
| 12 | 658.2 ± 3.0 A | 652.0 ± 66.4 A | 678.1 ± 39.4 A | nd | nd | 2.1 ± 0.0 |
| 13 | 386.1 ± 1.7 A | 513.4 ± 32.7 A | 498.1 ± 46.9 A | nd | nd | 2.3 ± 0.1 |
| 14 | 507.23 ± 76.6 A | 874.9 ± 220.9 B | 927.3 ± 122.7 B | nd | nd | nd |
| 15 | 29.0 ± 29.6 A | 615.9 ± 240.1 B | 655.1 ± 272.0 B | nd | 16.5 ± 0.5 a | 16.8 ± 0.3 a |
| 16 | 545.2 ± 111.4 A | 1181.7 ± 18.9 C | 1034.0 ± 106.3 B | 2.3 ± 0.3 a | 24.9 ± 1.1 b | 25.0 ± 0.7 b |
| 17 | 646.6 ± 64.2 B | 339.4 ± 110.8 A | 461.2 ± 62.8 A | nd | 6.0 ± 0.1 a | 6.2 ± 0.2 a |
| 18 | 504.5 ± 15.8 A | 790.12 ± 41.3 B | 749.8 ± 54.7 B | nd | nd | nd |
| 19 | 441.8 ± 149.1 A | 848.3 ± 38.9 B | 789.5 ± 35.0 B | 2.3 ± 0.6 a | 17.1 ± 1.2 c | 12.9 ± 2.5 b |

| | | | | | | |
|---|---|---|---|---|---|---|
| 20 | 478.4 ± 18.1 B | 600.8 ± 17.9 B | 236.6 ± 14.8 A | nd | 3.9 ± 0.5 a | 4.6 ± 1.1 a |
| 21 | 115.5 ± 19.0 A | 115.6 ± 27.7 A | 102.9 ± 7.9 A | nd | 5.8 ± 1.3 a | 4.5 ± 0.5 a |
| 22 | 364.4 ± 48.6 A | 657.6 ± 18.2 B | 664.9 ± 13.8 B | 14.3 ± 3.8 a | 25.12 ± 0.6 b | 27.23 ± 0.4 b |
| 23 | 73.0 ± 10.2 A | 196.8 ± 4.1 A | 204.4 ± 11.6 A | nd | nd | nd |
| 24 | 11.0 ± 5.3 A | 6.4 ± 0.3 A | 6.0 ± 0.9 A | nd | nd | nd |
| 25 | 488.5 ± 23.7 A | 459.0 ± 9.1 A | 697.4 ± 3.9 B | nd | 10.8 ± 0.6 a | 10.3 ± 0.1 a |
| 26 | 366.2 ± 9.1 A | 530.4 ± 1.5 B | 523.7 ± 9.1 B | 10.2 ± 1.8 a | 14.4 ± 0.5 b | 13.1 ± 0.6 ab |
| 27 | 484.7 ± 50.5 A | 590.6 ± 36.3 A | 612.8 ± 10.2 A | nd | nd | nd |
| 28 | Nd | 1494.3 ± 9.4 A | 1490.4 ± 6.9 A | nd | 4.3 ± 0.1 a | 4.2 ± 0.2 a |
| 29 | 186.8 ± 29.5 A | 421.8 ± 53.7 B | 387.4 ± 55.6 B | nd | 2.9 ± 0.2 a | 3.0 ± 0.2 a |

As shown in "As received" results, CAT activity could not be measured in many samples due to the interferents. This fact confirms the need to carry out a purification process in honey samples when determining GOX and CAT enzymatic activities.

There were no significant differences between the results of enzymatic activities obtained using the Sigma-Aldrich membrane and the **Film-bor** (commercial membrane vs **Film-bor**). Therefore, our proposed methodology using **Film-bor** is an excellent alternative to the actual procedure.

## 4. Conclusions

The actual interferents for reliably determining the honey's GOX and CAT activities are the catechol derivatives, so a film-shaped material extracts selectively this type of substances from honey samples has been designed, not compromising the rest of the original composition of the honey. The great achievement of this study has been to get the material to interact only with 1,2-dihydroxybenzenes, avoiding the reaction with other diols such as fructose, for which the role of the polymeric chains has been decisive. The results obtained with the proposed method are statistically equal to those obtained with the current purification method based on the use of a dialysis membrane, which postulates our material as a robust, simple, economical and ecofriendly alternative.

## Open Data

Open Data is available at https://riubu.ubu.es/handle/10259/5684 (Dataset of the work *Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer*).

## Conflicts of interest

The authors declare that they have no conflict of interest.

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# CONCLUSIONES

En base a los resultados obtenidos en este trabajo, se pueden establecer las siguientes concluisiones principales:

1) Los polímeros sensores colorimétricos son una herramienta muy útil, ya que nos han permitido analizar de forma rápida y sencilla diferentes sustancias de interés en control y calidad de alimentos (aminas biógenas en pescado, polifenoles totales en miel y nitritos en carne procesada).

2) Se han desarrollado tres polímeros con propiedades avanzadas:

    1. Un polímero con propiedades antimicrobianas, capaz de alargar la vida útil de productos cárnicos envasados hasta en un 50%.

    2. Dos polímeros diferentes capaces de retener sustancias de interés en su estructura, con el fin de agilizar y abaratar el análisis de control de calidad en mieles. El primero de ellos, con capacidad de retener almidón, ha sido utilizado de forma satisfactoria para el análisis de actividad diastásica en mieles. El segundo de ellos, capaz de retener polifenoles en su estructura, ha sido utilizado como alternativa viable al uso de membranas comerciales como parte del análisis de actividad glucosa oxidasa y catalasa en muestras de mieles.

En base e los resultados obtenidos en esta memoria, se ha demostrado el potencial de los polímeros para su uso en análisis de control de calidad de diferentes matrices alimentarias, de una forma más rápida, sencialla y económica. En el futuro, la investigación podría dirigirse hacia la búsqueda de nuevos materiales poliméricos con este tipo de aplicaciones en otras matrices alimentarias.

# MATERIAL SUPLEMENTARIO

- *Functional aromatic polyamides for the preparation of coated fibres as smart labels for the visual detection of biogenic amine vapours and fish spoilage* (pulsar para descargar)
- *A simple one-pot determination of both total phenolic content and antioxidant activity of honey by polymer Chemosensors* (pulsar para descargar)
- *Rapid and inexpensive detection of nitrites in processed meat using a colorimetric polymer sensor and a mobile app* (pulsar para descargar)
- *Metal-free organic polymer for the preparation of a reusable antimicrobial material with real-life application as absorbent food pad* (pulsar para descargar)
- *Polymer film as starch azure container for the easy diastase activity determination in honey* (pulsar para descargar)
- *Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer* (pulsar para descargar)