Universidad Miguel Hernández
Escuela Politécnica Superior de Orihuela
Departamento de Tecnología Agroalimentaria

Caracterización y aplicación de aceites esenciales de especias y aguas de lavado obtenidas como co-producto del proceso de obtención de fibra de cítricos como inhibidores naturales en productos cárnicos

Memoria realizada para optar al titulo de Doctor, presentada por

D. Manuel Viuda Martos

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TESIS DOCTORAL

Presentada por:

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Universidad Miguel Hernández
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CERTIFICA:

Que la Tesis Doctoral Titulada “Caracterización y aplicación de aceites esenciales de especias y aguas de lavado obtenidas como co-producto del proceso de obtención de fibra de cítricos como inhibidores naturales en productos cárnicos” de la que es autor el Ingeniero Agrónomo y Licenciado en Ciencia y Tecnología de Alimentos **Manuel Viuda Martos** ha sido realizada bajo la dirección de la Dra. Juana Fernández López y el Dr. José Angel Pérez Alvarez, la cual considero conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición publica.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a treinta de marzo de dos mil diez.

Fdo.: Dr. José Ramón Díaz Sánchez

Dña. Juana Fernández López, Dra. Veterinaria y Titular de Universidad del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández,

D. José Angel Pérez Alvarez, Dr. Ingeniero Agrónomo y Titular de Universidad del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández,

CERTIFICAN:

Que la Tesis Doctoral Titulada "Caracterización y aplicación de aceites esenciales de especias y aguas de lavado obtenidas como co-producto del proceso de obtención de fibra de cítricos como inhibidores naturales en productos cárnicos" llevada a cabo por el Ingeniero Agrónomo y Licenciado en Ciencia y Tecnología de Alimentos **Manuel Viuda Martos** ha sido realizada bajo nuestra dirección y autorizamos a que sea presentada para optar a la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que conste a los efectos oportunos se firma el presente certificado en Orihuela a treinta de marzo de dos mil diez.

Fdo.: Dra. Juana Fernández López Fdo.: Dr. José Angel Pérez Alvarez

Esta Tesis Doctoral ha sido realizada dentro del programa de becas pre-doctorales de la Caja del Mediterráneo (CAM)

“Lo que hacemos solo para nosotros
muere con nosotros.
Lo que hacemos para los demás
y para el mundo permanece
y es inmortal”

Albert Pike
(1809-1891)

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A mis Padres por
darme la oportunidad
que ellos no disfrutaron

A mis hermanos
José Antonio y Diana

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ESTRUCTURA DE LA TESIS

1.- ESTRUCTURA DE LA TESIS

Para la realización de la presente Tesis Doctoral se ha seguido una metodología basada en la publicación de artículos tanto de investigación como bibliográficos. Con esta Tesis se pretende obtener el titulo de Doctor con mención Europea, para ello en la redacción de la misma, se ha seguido la normativa vigente de la Universidad Miguel Hernández, concretamente el articulo 1.2 donde se indica: "Que parte de la Tesis Doctoral, al menos el resumen y las conclusiones, se haya redactado y presentado en una de las lenguas oficiales de la Unión Europea, distinta a alguna de las lenguas oficiales en España". En este caso se ha redactado y será expuesta en Ingles.

La estructura de esta tesis consta de una breve introducción (capítulo segundo) en la que se incluye una revisión bibliográfica sobre los alimentos funcionales en general. También incluye una revisión sobre la composición y múltiples propiedades, tanto tecnológicas como funcionales, de las especias y su posible uso como ingredientes para funcionalizar alimentos y sobre los co-productos cítricos como potenciales ingredientes funcionales en los procesos de elaboración de alimentos.

En el capítulo tercero se describen los objetivos propuestos y en el capítulo cuarto se presenta un resumen de los materiales y métodos utilizados para poder entender los distintos procesos de elaboración que se han llevado a cabo, los métodos de conservación aplicados y las determinaciones analíticas practicadas.

Seguidamente, en el capitulo quinto se recoge un resumen global de los resultados más relevantes obtenidos en los diferentes estudios realizados.

En el capitulo sexto se recogen las conclusiones de todos los estudios que forman parte de la presente tesis, mientras que el capítulo séptimo corresponde a la bibliografía consultada.

En el último capítulo de la presente tesis se incluyen las publicaciones que componen la base de la misma. El primer grupo de publicaciones se centra en la caracterización de los dos potenciales "ingredientes funcionales" seleccionados: los aceites esenciales de especias y las aguas de lavado (co-productos del proceso de obtención de fibra de naranja). Forman parte de este primer grupo 6 publicaciones: la primera, en la revista *Acta Chimica Slovenica* donde se identifica la composición

química, mediante cromatografía de gases y espectrometría de masas, de los aceites esenciales de orégano, tomillo y romero; la segunda, en la revista *Journal of Food Safety*, sobre la actividad antifúngica de dichos aceites esenciales; la tercera, en la revista *International Journal of Food Science and Technology* sobre la actividad antibacteriana de dichos aceites esenciales. La cuarta, en la revista *Flavour and Fragance Journal*, sobre la capacidad antioxidante de los aceites esenciales; la quinta, última referente a los aceites esenciales de especias, es un artículo de revisión publicado en la revista *Critical Reviews in Food Science and Food Safety* referente a las múltiples propiedades funcionales de las especias en general y de los aceites esenciales en particular; la última publicación perteneciente a este primer grupo se incluyó en la revista *Journal of Food Processing and Preservation* donde se recoge la caracterización fisicoquímica del segundo ingrediente funcional sometido a estudio, las aguas de lavado (co-producto del proceso de obtención de fibra de naranja).

El segundo grupo de publicaciones incluye 4 artículos donde se recogen los resultados de la aplicación de estos dos ingredientes funcionales en un producto cárnico cocido tipo mortadela. Las dos primeras publicaciones se centran en estudiar el efecto que dichos ingredientes funcionales tienen sobre las propiedades químicas, físico-químicas y sensoriales de la mortadela. Estos 2 trabajos han sido publicados en las revistas *Innovative Food Science and Emerging Technologies* y *LWT-Food Science and Technology*. Mientras que las dos últimas publicaciones se refieren al efecto de dichos ingredientes funcionales sobre la vida útil del producto cárnico. De estas 2 publicaciones, una lo está como capítulo de libro en el libro "*Sustainability of the Agri-Food Chain*" editado por la Royal Society of Chemistry, y la última publicación está en proceso de revisión en la revista *Food Research Internacional*.

De forma complementaria a estas publicaciones, se presentan una serie de comunicaciones (orales y escritas) sobre resultados de esta tesis, presentados a Congresos Internacionales.

INTRODUCCION

2.- INTRODUCCION

2.1.-Alimentos funcionales.

La evolución de los hábitos alimenticios ha ido variando a través del tiempo, pero siempre con el criterio básico de mantener la salud. En los últimos años existe un interés creciente, tanto por parte de los consumidores como de las industrias de alimentos y de los investigadores, en cómo los alimentos pueden ayudar a mantener la salud de nuestro organismo.

Cada vez está más aceptado el papel que la dieta tiene en la prevención y tratamiento de numerosas enfermedades. Los conceptos básicos de la nutrición están experimentando un cambio significativo (Menrad, 2003).

En la actualidad, el concepto clásico de "nutrición adecuada", es decir, aquella que aporta a través de los alimentos los nutrientes (hidratos de carbono, proteínas, grasas, vitaminas y minerales) suficientes para satisfacer las necesidades orgánicas particulares, tiende a ser sustituido por el de "nutrición óptima", que incluye, además de la definición anterior, la potencialidad de los alimentos para promocionar la salud, mejorar el bienestar y reducir el riesgo de desarrollar enfermedades (Perez-Alvarez, 2008). En este ámbito aparecen los alimentos funcionales, nutracéuticos, alimentos diseñados, alimentos terapéuticos, superalimentos o alimentos medicinales (Nagai e Inoue, 2004).

En los últimos años gran parte de la población se ha concienciado sobre la importancia de alimentarse correctamente, lo que ha generado un mercado de nuevos productos con beneficios adicionales, estos productos son los llamados alimentos funcionales (Pérez-Alvarez, 2008). Algunos son bajos en calorías, otros tienen menor cantidad de grasa, algunos adicionados con vitaminas, minerales, fibra, etc., por ello aportándolos en la dieta, permiten conseguir un estado de salud óptimo promoviendo el estado de bienestar y posiblemente reduciendo el riesgo de enfermedad.

Actualmente los alimentos no sólo son adquiridos para satisfacer la sensación de hambre y proporcionar los nutrientes necesarios al organismo, sino que también se consumen con el fin de prevenir enfermedades relacionadas con la nutrición y mejorar el bienestar físico y mental de los consumidores (Roberfroid, 2000). Éstos creen cada

vez más que los alimentos influyen directamente en su salud, por ello el consumo de alimentos funcionales ha ido en crecimiento en las últimas décadas, disminuyendo el coste de asistencia médica de la población envejecida.

Los alimentos funcionales se presentan en todas las categorías de alimentos, sin embargo, estos productos no están distribuidos en todos los segmentos del mercado, ya que la producción y el comercio de estos productos son bastante complejos, caros y arriesgados, y para ello es necesario que exista una demanda por parte del consumidor reconocida (Siro *et al.*, 2008).

En Europa, el consumo de alimentos con efectos beneficiosos para la salud aumenta continuamente, de acuerdo con Fitzpatrick (2003), el mercado de los alimentos funcionales sufre un incremento anual del 7 al 10%. Sin embargo, los europeos son en general mucho más críticos con los nuevos productos y tecnologías comparados con los consumidores americanos, ya que éstos se preocupan más por la seguridad de los nuevos alimentos. Este mercado es heterogéneo, presentando grandes diferencias regionales en cuanto al consumo y la aceptación de alimentos funcionales. En general, el interés de los consumidores en los alimentos funcionales en los países tanto del centro como del norte de Europa es más alto que en países del Mediterráneo, donde los consumidores consideran que los alimentos naturales y frescos son mejores para la salud (Siro *et al.*, 2008).

En España, el mercado de alimentos funcionales en 2006 representó aproximadamente el 17 % del total del mercado de alimentos; además se prevé que el consumo de estos alimentos alcance para 2020 el 40 %, aproximadamente. Entre 2000 y 2005 hubo un crecimiento de más del 50 %. En general, la actitud de los consumidores hacia los alimentos funcionales es positiva, lo que representa una tendencia sostenible en un mercado en crecimiento (Siro *et al.*, 2008).

2.1.1.- Definición de alimento funcional

No existe una definición única de alimento funcional ya que son muchos los contextos en los que dicho concepto entra en juego entre ellos los avances tecnológicos, la comercialización de alimentos y las normas de regulación de los mismos (Palou *et al.*, 2003).

Para Doyon y Labrecque (2008) en cualquier definición de alimento funcional se

deben de identificar cuatro conceptos claves como son:

i) Beneficios para la salud. El concepto de beneficio para la salud es clave en cualquier definición de alimento funcional. Estos beneficios para la salud podrían clasificarse como la mejora en la función de un determinado objetivo diana o como la reducción del riesgo de padecer determinadas enfermedades.

ii) La naturaleza de los alimentos. La naturaleza de los alimentos también parece ser un concepto importante. Un alimento funcional debe ser o debería parecerse a un alimento tradicional, aunque en su proceso de elaboración haya sufrido transformaciones como enriquecimiento, fortificación, adición o eliminación de componentes.

iii) Nivel de la función. El punto de partida es que todos los alimentos tienen funciones de nutrición; lo que hace que un alimento tenga propiedades funcionales son los beneficios, fisiológicos o psicológicos que provocan, más allá de sus funciones nutricionales básicas.

iv) Patrón de consumo. Este concepto establece que un alimento funcional debe ser parte de una dieta normal o ajustada a un patrón de consumo normal, en una situación geográfica específica y/o dentro de un determinado contexto cultural. Por lo tanto, un alimento que puede ser considerado funcional en un país no necesariamente tiene que ser considerado en otro.

Atendiendo a estos conceptos claves, la definición de alimento funcional, es compleja ya que puede abarcar distintos aspectos, ya que por ejemplo se podría decir que son aquellos que son obtenidos por cualquier procedimiento, con la característica particular de que alguno de sus componentes, sea o no nutriente, afecte a funciones diana del organismo, de manera especifica y positiva, y promueva un efecto fisiológico o psicológico mas allá de su valor nutritivo tradicional.

Como se ha mencionado anteriormente, el efecto positivo de un alimento funcional puede ser tanto su contribución al mantenimiento del estado de la salud y bienestar como a la reducción del riesgo de padecer una determinada enfermedad (Pérez-Álvarez *et al.*, 2003). El alimento funcional será en apariencia similar a un alimento convencional o puede ser un alimento convencional, que sea consumido como parte de una dieta usual y que se haya demostrado que tenga beneficios fisiológicos.

Las posibilidades de optimización de componentes dirigidas al desarrollo de alimento funcionales estarán básicamente fundamentadas en (Roberfroid 2000; Astiasaran *et al.*, 2003):

- Limitar (*L*) la presencia de un componente con efectos negativos. Tales compuestos pueden estar naturalmente presentes en el alimento o ser formados durante su procesado, conservación, etc.
- Incrementar (*I*) la concentración de un compuesto (nutriente o no nutriente), naturalmente presente en el alimento, con efectos beneficiosos. Este incremento puede ser "naturalmente" inducido o producido mediante procesos de reformulación.
- Remplazar (*L*+*I*) un componente, generalmente un macronutriente cuya ingestión es habitualmente excesiva y por ello causa efectos negativos, por un componente con efectos beneficiosos.
- Adicionar un componente que normalmente no se encuentra en la mayoría de los alimentos y que no es necesariamente un macronutriente o un micronutriente, pero que para el cual se haya encontrado un efecto beneficioso.
- Modificar la naturaleza de uno o más componentes con el propósito de mejorar los efectos beneficiosos para la salud.
- Aumentar la biodisponibilidad o estabilidad de un componente del cual se conoce su efecto funcional o reduce el riesgo de enfermedades potenciales.
- Combinaciones de las posibilidades descritas anteriormente.

Con lo anteriormente mencionado aparece una ultima definición de alimentos funcionales realizada por Doyon y Labrecque (2008) en donde se define alimento funcional como aquel alimento que es, o que presenta una gran similitud con un alimento convencional, que forma parte de una dieta estándar y que se consume de forma regular y en cantidades normales, habiendo demostrado también la capacidad de reducir el riesgo de desarrollar enfermedades crónicas específicas, o que beneficia diversas funciones diana del organismo a las que afecta, más allá de sus funciones nutricionales básicas.

2.1.2.- Desarrollo de alimentos funcionales

Como se ha mencionado anteriormente el desarrollo de productos o alimentos funcionales se basa en la posibilidad de potenciar la presencia de compuestos beneficiosos y/o limitar o eliminar la de aquellos otros con efectos negativos.

Para ello, se puede actuar a diversos niveles: a nivel genético, controlando la alimentación del animal o bien en los procesos de elaboración de alimentos (Roberfroid 2000; Siro *et al.*, 2008).

En la actualidad, existe un gran número de ingredientes o compuestos bioactivos como son los ácidos grasos ω-3 (Hjaltason y Haraldsson 2006), vitaminas (Baro *et al.*, 2003), probióticos (Salem *et al.*, 2006), prebióticos (Brink *et al.*, 2005), fitoquímicos (Wolfs *et al.*, 2006), péptidos bioactivos (Thoma-Worringer *et al.*, 2006), fibra (Fernández-López *et al.*, 2009), etc. a los que se asocia potenciales propiedades funcionales. Estos ingredientes funcionales ya han sido incorporados en una gran cantidad de alimentos (figura 1) que actualmente se pueden encontrar en el mercado.

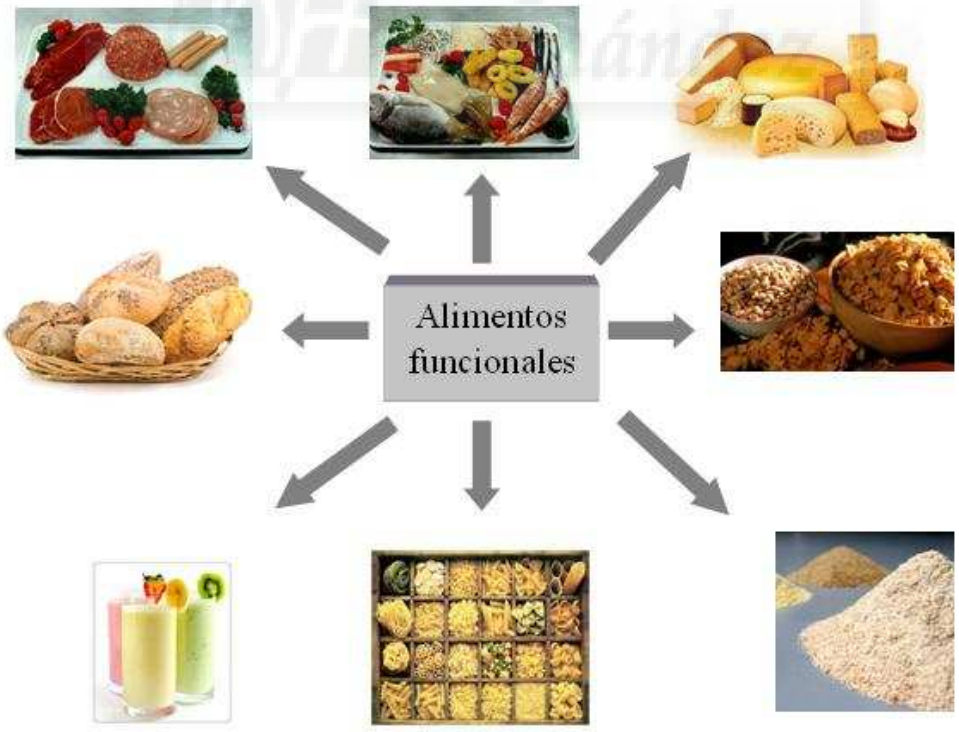

Figura 1. Alimentos enriquecidos con ingredientes funcionales.

De todo ello existe una gran cantidad de información disponible, lo que obliga a una adecuada búsqueda y selección en base a unos objetivos previamente establecidos. El desarrollo de un alimento funcional incluye (Roberfroid, 2002; Cercaci et al., 2006):

- Seleccionar uno o varios compuestos con potencial actividad funcional.
- Demostrar científicamente la funcionalidad o el efecto beneficioso.
- Identificar la biodisponibilidad y cantidad necesaria para el efecto beneficioso.
- Determinar la estabilidad del compuesto funcional a diversos tratamientos tecnológicos.
- Conocer si presenta efectos contrapuestos.
- Determinar la relación beneficio/riesgo que presenta.
- Definir la posible complementación con otros ingredientes.
- Identificar biomarcadores adecuados
- Conocer las aplicaciones, limitaciones y viabilidad tecnológica que puede presentar.

2.1.3.- Características de los alimentos funcionales

Los alimentos funcionales se diferencian claramente de otros productos farmacéuticos o parafarmacéuticos en función de la naturaleza del beneficio que ejercen y del modo de administración. Para que un alimento pueda ser clasificado como funcional debe cumplir una serie de características:

- El alimento debe ejercer un efecto positivo sobre la salud o sobre una función fisiológica.
- Los beneficios nutricionales y saludables de los alimentos o de los ingredientes específicos deben fundamentarse en una sólida base científica.
- La cantidad apropiada de ingesta diaria del alimento o del ingrediente debe ser establecida por expertos.
- El alimento, o el ingrediente no debe resultar nocivo si se ingiere por encima de la ingesta recomendada.
- El ingrediente de estar caracterizado por:

- Sus propiedades físicas y químicas, valoradas a través de métodos analíticos detallados.
- Su presencia cualitativa y cuantitativa en el alimento.

- El ingrediente no debe reducir el valor nutritivo del alimento.
- El alimento debe ser administrado como tal, de una manera convencional, nunca en forma de tabletas, capsulas o polvos.
- El ingrediente debe ser un compuesto natural.

De un modo convencional, se pueden describir tres condiciones que definen el carácter funcional de un alimento:

1) Ha de responder a las características de un alimento, es decir, debe tratarse de un sistema físico-químico más o menos complejo, integrado por ingredientes que se encuentran en la naturaleza.
2) Siempre debe ser consumido formando parte de la elaboración de los platos que integran los menús de las dietas alimentarias.
3) El alimento debe ejercer, una vez ingerido, un efecto positivo sobre una determinada función fisiológica (Sánchez-Muñiz 2004).

2.2.-Las especias como ingredientes funcionales

El uso de las especias y las hierbas aromáticas se conoce desde la antigüedad donde eran utilizadas, fundamentalmente, como conservantes, saborizantes y colorantes. Mas allá de estos usos, las especias también han sido objeto de estudio debido a las múltiples propiedades que presentan para “mejorar” la salud, ya que estas sustancias han sido y son pieza fundamental en la medicina tradicional de muchos países, lo que ha despertado el interés de diversas industrias como la química, la farmacéutica y la industria alimentaría, por ellas.

Actualmente se trata de conocer cómo el consumo de especias y hierbas aromáticas puede contribuir a mejorar la salud del consumidor. Se ha demostrado en estudios *in vitro* e *in vivo* los efectos que estas sustancias ejercen como antioxidantes, antimicrobianos, estimulantes digestivos, efectos hipolipidémicos, actividad antiinflamatoria, actividad antiviral, anticarcinogénico, etc. Estos efectos fisiológicos

beneficiosos también podrían poseer una posible aplicación preventiva en una gran variedad de patologías.

2.2.1.-Composición

Tanto las especias como las hierbas aromáticas pueden ser utilizadas en fresco, desecadas, enteras, troceadas o molidas y debido a sus características en cuanto a color, aroma y/o flavor son utilizadas en la preparación de alimentos y bebidas (Díaz-Maroto et al. 2002). En su composición se pueden encontrar proteínas, fibra, azúcares, aceites esenciales, minerales y pigmentos además de compuestos bioactivos como ácidos fenólicos, flavonoides, esteroles y cumarinas, (Susheela, 2000). Muchas de las propiedades funcionales que presentan las especias están asociadas con la presencia, tipo y contenido de sus compuestos de naturaleza fenólica, aunque dicha composición dependerá de diversos factores como parte de la planta utilizada, estado vegetativo, condiciones medioambientales, tipo de recolección, etc. (Cosentino *et al.,* 1999).

Uno de los componentes presentes en las especias, y que debido a su composición, fundamentalmente, en terpenos, monoterpenos y sesquiterpenos (hidrocarburos, alcoholes, cetonas, etc. que pueden ser acíclicos, monocíclicos, bicíclicos, tricíclicos), también es responsable de muchas de las propiedades funcionales que presentan las especias, son los aceites esenciales (AEs). En términos generales los AEs están compuestos por más de setenta componentes (Russo *et al.,* 1998). Los componentes mayoritarios pueden constituir por encima del 85% de la composición del AE mientras que otros componentes se encuentran en forma de trazas (Bauer *et al.*, 2001). Es muy importante el papel que juegan estos compuestos minoritarios ya que existen evidencias de que estos componentes contribuyen de manera significativa a las propiedades funcionales que el AE pueda presentar debido al posible sinergismo entre distintos componentes (Burt, 2004).

2.2.1.1.-Compuestos fenólicos

Uno de los principales responsables de la mayoría de las propiedades funcionales que presentan muchos alimentos, entre los que se encuentran las hierbas aromáticas y las especias, son los compuestos fenólicos, en cualquiera de sus formas, ya sean fenoles simples, flavonas, flavanonas, flavanoles, flavonoles, antocianinas, etc. Existen numerosos estudios que ponen de manifiesto las propiedades funcionales de los

compuestos fenólicos y más concretamente de los flavonoides, como la capacidad antioxidante (Li *et al.*, 2009), capacidad antibacteriana (Babajide *et al.*, 2008), capacidad antiviral (Fritz *et al.*, 2007), capacidad antiinflamatoria (Lameira *et al.*, 2008), efectos cardioprotectores (Celle *et al.*, 2004), propiedades anticarcinogénicas (Pergola *et al.*, 2006) e inhibidores de la agregación plaquetaria (Arct y Pytkowska, 2008).

2.2.1.2.- Terpenos

Los terpenos son el componente fundamental de los aceites esenciales. Con el nombre de terpenos se conoce a un grupo importante de componentes vegetales que tienen un origen biosintético común. Todos, aunque con estructuras químicas muy distintas, proceden de la condensación, en número variable, de unidades isoprénicas (Yoshikawa *et al.*, 1996). Entre sus distintas formas se pueden encontrar monoterpenos, diterpenos y sesquiterpenos y presentan la característica de ser muy volátiles. Estas sustancias también han demostrado poseer múltiples propiedades funcionales como capacidad antioxidante (Milan *et al.*, 2008) y capacidad antimicrobiana (Tatsadjieu *et al.*, 2010).

2.2.2.- Propiedades Antioxidantes

En los últimos años son muchos los ingredientes y co-productos de las industrias agroalimentarias que han sido estudiados como fuente de antioxidantes naturales. Entre estos compuestos se encuentran las especias que ya han demostrado ser eficaces como ralentizadores del proceso de oxidación lipídica (Fernández-López *et al.*, 2003).

La oxidación lipídica constituye una de las principales causas de deterioro de los alimentos y se produce durante el almacenamiento de materias primas, su procesado y el almacenamiento de los productos finales (Tepe *et al.*, 2005). Dicha oxidación conlleva una pérdida significativa del valor nutricional del alimento, ya que existe una pérdida de vitaminas y ácidos grasos esenciales, además de una perdida de calidad sensorial, ya que se producen cambios en el color, la textura y el sabor, lo que da como resultado una disminución de la vida útil del alimento y el rechazo por parte del consumidor (Fernández-López *et al.*, 2007).

Para evitar este deterioro, la industria de alimentos utiliza, en sus formulaciones, antioxidantes sintéticos. Sin embargo, debido a su naturaleza sintética, han sido

cuestionados a menudo por su seguridad y su eficiencia. Por esta razón existe un creciente interés en cómo, sustancias de origen vegetal, con potencial actividad antioxidante, pueden ser utilizados como aditivos naturales, para reemplazar a los antioxidantes sintéticos, cuyo uso es cada vez más restringido, debido a los efectos secundarios que presentan (Zheng y Wang, 2001).

La actividad antioxidante de las especias, de los AEs y de sus componentes ha sido objeto de múltiples estudios (Mata *et al.*, 2007, Milan *et al.*, 2008; Bozin *et al.*, 2008; Li *et al.*, 2009), aunque gran parte de estos ensayos se hayan realizado *in vitro*.

El mecanismo de acción que provoca esta actividad antioxidante no está todavía esclarecido. Existen diversos mecanismos de acción para dicha capacidad antioxidante: el secuestro de radicales libres, la donación de hidrógenos, la quelación de iones metálicos o incluso pueden actuar como sustrato de radicales como el superóxido o el hidroxil (Al-Mamary *et al.*, 2002). La determinación de la capacidad antioxidante de las especias y sus derivados en alimentos es un tema que actualmente está teniendo un importante auge por parte de los investigadores así como por parte de la industria agroalimentaria.

2.2.3.- Propiedades Antimicrobianas

Muchas de las propiedades organolépticas y sensoriales de los alimentos disminuyen con el paso del tiempo, debido a una gran cantidad de factores (temperatura, luz, la acción del oxígeno), sin embargo, el más importante es el deterioro causado por los microorganismos.

La adición de agentes antimicrobianos a los alimentos ha sido un método particularmente efectivo para controlar la contaminación microbiana (Kabuki *et al.*, 2000). Los antimicrobianos utilizados en la alimentación son compuestos añadidos o presentes en los alimentos de forma natural y que actúan disminuyendo o inhibiendo el crecimiento de los microorganismos (Davidson, 1997).

En la actualidad la industria alimentaria está sometida a una gran presión por parte de los consumidores debido al uso de conservantes sintéticos, que previenen el crecimiento microbiano causante del deterioro de alimentos, por lo que existe una tendencia al uso cada vez más habitual de sustancias naturales que puedan actuar como agentes antimicrobianos sustituyendo así, a los componentes químicos utilizados en la

actualidad (Deba *et al.*, 2008).

Entre estos antimicrobianos naturales se encuentran las especias y sus derivados, como los AE. La principal ventaja de los AE, y que hace que su uso en la alimentación pueda extenderse y ser utilizado en cualquier sistema alimentario, es su clasificación como GRAS (Generally Recognized As Safe) (Kabara, 1991), Teniendo en cuenta que se debe determinar la eficacia máxima del mismo y que además no provoque en el alimento cambios apreciables en sus propiedades organolépticas.

Se ha demostrado la actividad antimicrobiana de los AE sobre gran cantidad de cepas microbianas entre las que se encuentran *Staphylococcus* spp., *Lactobacillus* spp., *Enterobacteriaceae*, así como sobre ciertos mohos.

Los posibles modos de acción de los constituyentes de las especias se han mostrado en diversos estudios (Davidson y Naidu, 2000; Davidson, 2001) sin embargo, el mecanismo concreto todavía no ha sido completamente dilucidado. Prindle y Wright (1997) mencionan que el efecto de los compuestos fenólicos es dependiente de la concentración. A bajas concentraciones los compuestos fenólicos afectan a la actividad enzimática, especialmente a los enzimas asociados a la producción de energía, mientras que con grandes concentraciones se produce la desnaturalización de proteínas. El efecto de los compuestos fenólicos y terpenos sobre el crecimiento y la producción de toxinas podría ser el resultado de la afinidad de dichos compuestos para alterar la permeabilidad de la pared celular microbiana, lo que provoca una pérdida de macromoléculas, también podrían interactuar con las proteínas presentes en la membrana celular, provocando la deformación de la estructura y como consecuencia se produce una pérdida de funcionalidad.

La composición, estructura, así como los grupos funcionales de los compuestos que forman los AE desempeñan un papel importante en la determinación de su actividad antimicrobiana. Es un hecho comprobado que la concentración de aceite esencial a aplicar a un alimento es mucho mayor, del orden del 1-3%, que la cantidad adicionada cuando se realiza en ensayo *in vitro*, para obtener los mismos resultados de actividad antimicrobiana. Èsta es una de las razones por la que su uso en el sector alimentario está limitado, ya que el empleo de altas concentraciones provoca cambios organolépticos en el alimento y disminuye la aceptabilidad del mismo por parte del consumidor (Lis-

Balchin y Deans, 1997).

La interacción entre los grupos fenólicos de los AE y las proteínas, lípidos y aldehídos de los alimentos puede explicar, por lo menos parcialmente, la reducción en los efectos antimicrobianos. Se ha propuesto que los extractos derivados de las especias sean utilizados como agentes antimicrobianos formando parte de un sistema de tecnología de barreras que implique la preservación del alimento por la acción múltiple y simultánea de una serie de compuestos y/o acciones (Nychas y Skandamis, 2003). El desarrollo de los sistemas antimicrobianos multicomponentes para los alimentos requiere una comprensión muy exacta de los mecanismos de acción de los agentes específicos para poder centrar así la atención en combinaciones potencialmente eficaces (Holley y Gill, 2004).

El uso conjunto de aceites esenciales con otros factores pueden facilitar su utilización como agentes antimicrobianos sin afectar a las características organolépticas del producto, así, la utilización de pH bajos, bajas temperaturas o bajos niveles de oxígeno, permiten reducir las concentraciones de AE a utilizar (Skandamis y Nychas 2000).

2.2.4.- Propiedades Antiinflamatorias

La aplicación tópica de las especias y de los extractos procedentes de éstas, está muy extendido para el alivio de diversos dolores como son el dolor de espalda, reumatismo, erupciones cutáneas y procesos inflamatorios en general (Ramadan, 2007).

Existen diversos estudios donde se demuestra la capacidad antiinflamatoria de diversas especias (Pérez-Fons et al., 2006). Esta actividad antiinflamatoria se debe fundamentalmente a la presencia de flavonoides que inhiben el desarrollo de inflamaciones provocadas por una amplia variedad de agentes (Mani *et al.*, 2006). Entre estos flavonoides destaca especialmente la galangina presente en *Lippia graveolens* (Lin *et al.*, 2007), este compuesto es capaz de inhibir la actividad ciclo-oxigenasa (COX) y lipo-oxigenasa disminuyendo la acción de la poligalacturonasa y reduciendo la expresión de la isoforma inducible de la ciclo-oxigenasa (COX-2) (Raso *et al.*, 2001).

Para Srinivasan (2005) la actividad anti-inflamatoria de las especias se debe a la acción de sus compuestos activos que inhiben la formación de metabolitos como la prostaglandina E_2 (PgE_2) o leucotrienos o que inhiben la formación de enzimas

lisosomales por parte de los macrófagos, como son la hialuronidasa, colagenasa y elastasa. Poeckel *et al.,* (2008) describen la actividad antiinflamatoria del ácido carnósico y el carnosol, compuestos de naturaleza fenólica presentes en especias como el romero o la salvia, a través de la inhibición de la formación de compuestos pro-inflamatorios como los leucotrienos. Volate *et al.,* (2005) mencionan que compuestos como la quercetina, curcumina y silimarina presentes en las especias tienen los mismos efectos anti-inflamatorios que la indometacina (medicamento anti-inflamatorio no esteroideo).

2.2.5.- Propiedades Antivirales

Otra de las propiedades funcionales que presentan las especias y las hierbas aromáticas es la actividad antiviral. Benencia y Courreges (2000) describen que el eugenol, componente principal del aceite esencial del clavo (*Eugenia caryophyllus* L), presenta actividad antiviral frente a los virus del herpes simple tipo 1 y tipo 2 (HSV-1 y HSV-2) impidiendo la replicación de dichos virus tanto en ensayos *in vitro* como en modelos con ratones. Este mismo compuesto, el eugenol, fue analizado por Tragoolpua y Jatisatenr (2007) frente a los virus del herpes simple tipo 1 y tipo 2 (HSV-1 y HSV-2) llegando a la conclusión que este compuesto impedía la replicación de dichos virus. Schnitzler *et al.,* (2008) analizaron el efecto del aceite esencial de melisa (*Melissa officinalis*) frente a los virus del herpes simple tipo 1 y tipo 2 (HSV-1 y HSV-2) en ensayos *in vitro*, informado que este aceite presenta un importante actividad antiviral siempre y cuando se adicione antes de que el virus haya penetrado en la célula.

Koch *et al.* (2008) analizaron el efecto de los aceites esenciales de diversas hierbas aromaticas y especias como son el hisopo (*Hyssopus officinalis*), tomillo (*Thymus vulgaris* L.), anis (*Pimpinella anisum*), jengibre (*Zingiber officinale*), camomila (*Chamaemelum nobile*) y sándalo (*Santalum álbum*) frente al virus del herpes simple tipo 2 (HSV-2), indicando que todos los aceites esenciales analizados presentaban actividad antiviral, siendo su posible mecanismo de acción por la interacción con el desarrollo viral.

2.2.6.-Propiedades anticarcinogénicas

Muchos de los componentes no nutritivos presentes en las frutas y las verduras así como, en las hierbas aromáticas y especias, son conocidos por su actividad

potencial como agentes quimioprotectores frente al cáncer. Entre los mecanismos de acción por los cuales estos compuestos actúan como agentes protectores frente al cáncer se podrían distinguir los siguientes (Tanaka y Sugie, 2008).

- Inhibición de los enzimas de fase I o bloqueo de la formación del carcinógeno.
- Inducción de los enzimas de decodificación de fase II
- Secuestro de los agentes reactivos sobre el ADN
- Modulación de hormonas homeostásicas.
- Supresión de la proliferación de hiper-células inducidas por el carcinógeno.
- Inducción de la apoptosis.
- Depresión de la angiogénesis del tumor.
- Inhibición de ciertas expresiones fenotípicas de células preneoplásicas y neoplásicas

Son muchos los estudios donde se pone de manifiesto las propiedades anticarcinogénicas de las hierbas aromáticas y las especias (Tsai *et al.*, 2007; Bonaccorsi *et al.*, 2008; Ramos *et al.*, 2008). Entre las distintas especias con potencial actividad anticarcinogénica cabria destacar: el romero (*Rosmarinus officinalis*) y sus componentes, acido ursólico, carnosol, etc.; la cúrcuma (*Curcuma longa*) y su principal componente, la curcumina; y la pimienta (*Piper nigrum*) con su componente principal, la capsaicina.

2.2.7.-Estimulante digestivo

El uso de especias en los alimentos favorece la posterior digestión de los mismos ya que éstas provocan una serie de efectos beneficiosos sobre los mecanismos encargados del proceso de la digestión. La presencia de especias en los alimentos provoca un aumento en la secreción salivar y de los jugos gástricos lo que ayuda a una mejor digestión (Tapsell *et al.*, 2006). Además, provoca un aumento en la secreción biliar con una mayor concentración de ácidos biliares los cuales desempeñan un papel fundamental en la digestión y absorción de las grasas (Bhat *et al.*, 1984). Las especias también ejercen su acción sobre el páncreas aumentando la secreción de enzimas

digestivos como la lipasa, amilasa, tripsina y quimotripsina los cuales ejercen una labor muy importante en la digestión de los alimentos (Tapsell *et al.*, 2006).

2.2.8.-Influencia en la hipolipidemia

Los agentes hipolipidémicos son un grupo diverso de fármacos que se utilizan en el tratamiento de hiperlipidemias. Una investigación reciente (Srinivasan, 2005) muestra que las especias resultan ser eficaces como agentes hipocolesterolémicos. Aparte de los efectos hipocolesterolémicos también ejercen efectos beneficiosos en el metabolismo general de los lípidos en distintas condiciones de lipidemia (Nalini *et al.*, 2006).

Existen evidencias científicas de que la oxidación por radicales libres de las lipoproteínas de baja densidad (LDL) juega un papel muy importante en el desarrollo de la placa de ateroma (Cannon, 2007). En este sentido se ha comprobado que compuestos presentes en el mirto (*Myrtus communis* L.) protegen del daño de oxidación de las LDL, además de ejercer un efecto protector sobre los ácidos grasos poliinsaturados y el colesterol (Rosa *et al.*, 2008).

Según los estudios realizados por Dhandapani *et al.*, (2002) el tratamiento, en ratas, con comino (*Cuminunm cyminum*) provoca una reducción significativa de los niveles de colesterol, tanto a nivel plasmático como en los tejidos, además de reducir los niveles de fosfolípidos, ácidos grasos libres y triglicéridos. Así mismo Kempaiah y Srinivasan (2006) comprobaron que la inclusión de curcumina (0,2%) o capsaicina (0.015%), componentes mayoritarios de la curcuma y de la pimienta, respectivamente, en la dieta de ratas con altos niveles de colesterol provocaba un descenso en los niveles de triglicéridos y colesterol plasmático. Estudios que se ven corroborados por Baum *et al.*, (2007) donde se comprueba que la curcumina provoca un descenso en los niveles de colesterol sérico.

En otro estudio Manjunatha y Srinivasan (2006) analizaron estos dos mismos compuestos (curcumina y capsaicina) en ratas, conjuntamente y por separado, y en ambos casos se producía una inhibición de la oxidación de las LDL demostrando también un efecto de sinergia entre ambos compuestos.

El mecanismo de acción por el cual se produce este efecto hipocolesterolémicos parece ser que está relacionado con el aumento de los ácidos biliares responsables del metabolismo de los lípidos (Sambaiah y Srinivasan, 1991) a través de la activación de

enzimas hepáticos como la colesterol-7α-dihidroxilasa (Srinivasan y Sambaiah 1991). También se ha comprobado que el uso de especias en los alimentos puede provocar un descenso de la absorción intestinal de los lípidos, acompañado de una mayor excreción fecal del colesterol (Srinivasan y Srinivasan, 1995).

2.3.-Co-productos agroalimentarios como ingredientes funcionales

En la actualidad, la industria de alimentos está incrementando el uso de materias primas, tanto por la globalización de las mismas como por las nuevas tendencias de consumo. Esto ocasiona que las industrias agroalimentarias tengan cada día más problemas con el manejo y aprovechamiento de los subproductos, término que se tiene a utilizar cada día menos ya que se pretende introducir el de co-productos.

El término de co-producto no tiene, cara al consumidor, el aspecto tan negativo de la palabra subproducto, ya que lo asocia directamente "a productos de desecho". Sin embargo, un co-producto puede tener un valor económico importante si se le procesa adecuadamente para la obtención de sustancias con carácter bioactivo, aspecto de gran importancia en la actualidad, ya que estos compuestos pueden contribuir a mejorar el estado físico y mental del consumidor (Pérez-Alvarez *et al.*, 2002). Ya es habitual encontrarse con alimentos enriquecidos con estos compuestos bioactivos, como por ejemplo los polifenoles del té, las isoflavonas, los ácidos grasos omega 3, etc. A estos "nuevos alimentos" se les denomina alimentos funcionales.

En muchos casos, las fuentes de obtención de estos compuestos biactivos son los co-productos, ya que en la mayoría de los casos se encuentran firmemente "unidos" a las estructuras de los alimentos.

En los últimos años las industrias de alimentos están empezando a interesarse por sus co-productos y están invirtiendo en investigación y desarrollo, ya sea con medios propios o con el apoyo de las distintas Administraciones. De hecho, se tiende a aplicar e integrar los sistemas productivos para reducir el consumo de agua (aspecto de suma importancia para las industrias alimentarias localizadas en la Cuenca Mediterránea), hacer un uso eficiente de la energía, reducir el consumo de agentes de limpieza y desinfectantes, entre otros, para así obtener un mayor beneficio económico para la empresa.

En España, las empresas agroalimentarias generan una gran cantidad de co-

productos. Diversos estudios señalan que estos co-productos presentan un gran potencial, ya que esta "nueva materia prima" presenta una gran cantidad de fibra y diversos compuestos bioactivos (fitoquímicos). Sin embargo, las pequeñas y medianas empresas, a la hora de plantearse el aprovechamiento de este co-producto se encuentran con la particularidad de que estas materias primas presentan una elevada carga microbiana, siendo poco estables durante su almacenamiento (van Heerden *et al.*, 2002). Para evitar o reducir al máximo posible su contaminación se han aplicado diversos tratamientos (reducción de la a_w, control del potencial de óxido-reducción, utilización de antagonistas microbianos, ajustes de pH, etc.) con el fin de evitar que su deterioro llegue a afectar la obtención de dichos compuestos bioactivos.

También se debe tener en cuenta que los co-productos son materias primas muy heterogéneas, ya que están formadas por distintos tejidos, cada uno con muy distinta composición química, lo que hace muy difícil controlar también su procesamiento. No obstante, hay un gran interés es su posible aprovechamiento. Prueba de ello es el hecho de que se estén diseñando e implementando sistemas "a medida" para el aprovechamiento de determinados componentes bioactivos. Además de la inversión que supone toda innovación, las pequeñas y medianas industrias se encuentran ante otro gran reto y es el de contar con personal cualificado, aspecto que las grandes empresas multinacionales tienen perfectamente cubierto.

2.3.1.- Co-productos de las industrias procesadoras de cítricos

Los co-productos de cítricos, son aquellos obtenidos directamente de las industrias procesadoras de zumos y de las piezas retiradas del mercado (Piquer *et al.*, 2006).

El co-producto por excelencia de la industria de los zumos cítricos está constituido fundamentalmente por tres fracciones: (i) pulpa que contiene residuos del zumo, (ii) las células que lo contienen y (iii) las cortezas. Así, el flavedo contiene compuestos con importantes aplicaciones en la industria de alimentos como son los aceites esenciales y los terpenos, de enorme aplicación en la industria química (Chafer *et al.*, 2000), por su parte los carotenoides del flavedo pueden ser utilizados como pigmentos naturales para la mejora de la coloración de los zumos simples y concentrados, mientras que el albedo contiene celulosa, pectinas y diversos flavonoides

(Alesón-Carbonell *et al.*, 2002).

La preparación de extractos ricos en fibra dietética y antioxidantes naturales a partir de los co-productos procedentes de la industria de frutas cítricas es una alternativa viable que ha permitido la obtención de un potencial ingrediente funcional con un futuro prometedor en varias industrias de alimentos como la cárnica y la láctea (Alesón-Carbonell *et al.*, 2002; García-Pérez *et al.*, 2003; Fernández-López *et al.*, 2005). La adición de este extracto rico en fibra y antioxidantes naturales en la industria cárnica cuenta con un aliciente más y es el de que su adición reduce la concentración de nitrito residual en el producto, con lo cual se podría reducir la formación de sustancias perjudiciales para la salud como son las nitrosaminas (Fernández-López *et al.*, 2007).

El procesamiento de estos co-productos de las industrias de zumos de frutas cítricas genera, a su vez, otros co-productos que pueden tener un alto valor en la industria de alimentos como es el agua utilizada para los lavados de los co-productos durante el proceso de obtención del extracto de cítricos rico en fibra dietética. Este proceso utiliza una gran cantidad de agua, aspecto de suma importancia tanto desde el punto de vista económico como medioambiental y más teniendo en cuenta las necesidades hídricas que actualmente requiere toda la zona Mediterránea, especialmente la Comunidad Valenciana y la Región de Murcia.

También hay que tener en cuenta que el agua procedente de estos lavados arrastra gran cantidad de sustancias con un alto valor añadido como son los polifenoles, especialmente flavonoides, de los cuales los cítricos son una fuente importante. El tipo y la concentración de flavonoides presentes en los cítricos depende de la variedad y especie; por ejemplo, la hesperidina, es el principal flavonoide de las naranjas (Mouly *et al.*, 1996) encontrándose así mismo en una alta concentración en la fibra procedente de las mismas (Fernández-López *et al.*, 2007). La importancia que tienen los flavonoides en la alimentación está relacionada con los estudios que demuestran que algunos presentan un efecto beneficioso sobre la salud del consumidor (Schieber *et al.*, 2001).

El futuro es prometedor cara al aprovechamiento de los co-productos de las industrias de zumos de frutas cítricas, sin embargo, se requiere de más esfuerzo por parte de la comunidad científica, en llevar a cabo una investigación más aplicada, del cambio de mentalidad de muchos de los industriales del sector que permita que no sigan

viendo a estos co-productos como un “subproducto” sin valor económico ni potencialidad así como, un apoyo más decidido por parte de la Administración Central y/o Autonómica para facilitar a estas industrias su desarrollo.

OBJETIVOS

3.- OBJETIVOS

3.1- Objetivos generales

El objetivo general del presente estudio ha sido evaluar el potencial tecnológico de los aceites esenciales de diferentes especias de la Dieta Mediterránea, y de un co-producto de la industria citrícola en el proceso de elaboración y vida útil de un sistema modelo cárnico.

Para alcanzar este objetivo general, se plantearon los siguientes objetivos particulares.

3.2.- Objetivos particulares

- Caracterizar químicamente los aceites esenciales de las especias de la Dieta Mediterránea seleccionadas.
- Determinar la capacidad de inhibición del crecimiento fúngico de dichos aceites esenciales, frente a cepas implicadas en el deterioro de alimentos.
- Determinar la capacidad antibacteriana de los aceites esenciales frente a cepas utilizadas como estárter en procesos de elaboración de alimentos y frente a cepas relacionadas con el deterioro de los mismos.
- Conocer la capacidad antioxidante de los aceites esenciales mediante la utilización de diferentes técnicas analíticas.
- Caracterizar química, físico-química y microbiológicamente las aguas de lavado obtenidas como co-producto del proceso de obtención de fibra de cítricos.
- Aplicar los aceites esenciales de especias y las aguas de lavado obtenidas como co-producto del proceso de obtención de fibra de cítricos, como ingredientes en un sistema modelo de pasta fina, determinando su efecto sobre las propiedades químicas, físico-químicas y sensoriales de dicho sistema modelo.
- Evaluar la vida útil de un sistema modelo de pasta fina elaborado con aceites esenciales de especias y aguas de lavado obtenidas como co-producto del proceso de obtención de fibra de cítricos, analizando el producto desde el punto de vista químico, físico-químico, microbiológico y sensorial.

MATERIALES Y METODOS

4.- MATERIALES Y METODOS

En este apartado se recoge un resumen de los ingredientes utilizados, de los procesos de elaboración aplicados y de los análisis efectuados. La información completa de toda esta metodología se ha desarrollado en los respectivos artículos publicados en revistas internacionales y que se adjuntan a esta memoria.

4.1.- Aceites esenciales de especias.

Los aceites esenciales utilizados en el presente estudio, tanto para la determinación de la composición química como para la determinación de las propiedades antioxidantes, antibacterianas y antifúngicas, fueron los siguientes: tomillo (*Thymus vulgaris* L.), romero (*Rosmarinus officinalis* L.), salvia (*Salvia officinalis* L.), orégano (*Oringanum vulgare* L.), clavo (*Syzygium aromaticum* L.) y comino (*Cuminum cyminum* L.). Todos los aceites fueron suministrados por Ravetllat Aromatics, (Barcelona, España) excepto el aceite esencial de comino que fue suministrado por Ventos, (Barcelona, España). Se decidió el empleo de aceites esenciales comerciales para evitar posibles variaciones en la composición y que fuese un producto estandarizado.

4.1.1.- Composición química de los aceites esenciales de especias

La determinación de la composición de química de los distintos aceites esenciales estudiados se realizó mediante cromatografía de gases/espectrometría de masas (CG/MS) en un cromatógrafo de gases Shimadzu GC-17A (Shimadzu Corporation, Tokio, Japón), acoplado a un detector selectivo de masas Shimadzu GCMS-QP5050A (Shimadzu Corporation, Tokio, Japón). La identificación de los compuestos se realizó mediante comparación de los tiempos de retención y el espectro de masas con los de los estándares previamente inyectados así como con la biblioteca Wiley 229 del sistema, los valores del índice de Kovats y valores presentes en la literatura científica.

4.1.2.- Determinación de la actividad antifúngica de los aceites esenciales

La actividad antifúngica de los aceites esenciales se ensayó frente a dos cepas de hongos que están relacionadas con el deterioro de alimentos como son *Aspergillus niger* y *Aspergillus flavus*. Para ensayar el efecto inhibidor se empleo el método de dilución

en agar, siguiendo las recomendaciones de Fraternale *et al.*, (2003). Las concentraciones analizadas fueron 0,011; 0,022; 0,033 y 0,044% para los aceites esenciales de tomillo, orégano y clavo, mientras que para los aceites esenciales de romero y salvia las concentraciones ensayadas fueron 0,11; 0,22; 0,33 y 0,44%. El hecho de utilizar una concentración mayor para los aceites esenciales de salvia y romero se debe a que en un ensayo previo utilizando las concentraciones de 0,011; 0,022; 0,033 y 0,044% no se apreciaba ningún tipo de inhibición en los hongos ensayados.

4.1.3- Determinación de la actividad antibacteriana de los aceites esenciales

Para la determinación de la actividad antibacteriana se seleccionaron cepas bacterianas utilizadas en la industria de alimentos como cultivos iniciadores como son: *Staphylococcus xylosus* CECT 237, *Staphylococcus carnosus* CECT 4491, *Lactobacillus sakei*, CECT 4808, *Lactobacillus curvatus* CECT 904, así como cepas bacterianas relacionadas con el deterioro de alimentos como son: *Enterobacter gergoviae* CECT 857 y *Enterobacter amnigenus* CECT 4078, suministradas todas ellas por la Colección Española de Cultivos Tipo. Para la determinación de la actividad antibacteriana se empleó el método de difusión de disco en agar siguiendo las recomendaciones de Tepe *et al.*, (2005). También se determinó el efecto de la concentración siguiendo el método descrito por Viuda-Martos *et al.*, (2005).

4.1.4.- Determinación de la actividad antioxidante de los aceites esenciales

La determinación de la actividad antioxidante *in vitro* se realizó siguiendo cinco diferentes métodos analíticos como son: el método del secuestro del radical 2,2'-difenil-1-picrilhidrazil (DPPH) siguiendo las recomendaciones de Brand-Williams *et al.*, (1995), el poder antioxidante de la reducción de hierro (FRAP) según el método descrito por Oyaizu, (1986); la determinación de la actividad antioxidante de las especies reactivas del ácido tiobarbitúrico (TBARS) siguiendo las indicaciones de Daker *et al.*, (2008); capacidad quelante del ion ferroso (FIC) según el método utilizado por Singh y Rajini (2004) y la determinación automática de la estabilidad oxidativa de las grasas (RANCIMAT).

4.2.- Agua de lavado procedente del proceso de obtención de fibra de cítricos.

4.2.1. Obtención del agua de lavado

En el proceso de obtención de la fibra de naranja se generan a su vez una serie de co-productos entre los que se encuentra el agua con la que se realizan los lavados. Para la obtención del agua de lavado se utilizó, como materia prima, el co-producto generado en el proceso de obtención de zumo de naranja mediante un sistema de extracción FMC, procedente de una industria de zumos de la Vega Baja.

Una vez recogido este co-producto, se trasladó a la Planta Piloto del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández, donde se procedió a su trituración durante 20 segundos en una picadora cutter vertical (Tecator 1094 Homogeneizer, Tekator, Hoganas, Suecia), con el fin de lograr un tamaño de partícula uniforme y así aumentar la superficie de contacto en el agua.

Una vez triturado, se procedió a realizar el lavado utilizando una proporción de 1 kg de co-producto por 1 L de agua, bajo agitación constante, durante 8 min. La temperatura del agua de lavado se mantuvo constante a 80 ºC, durante el proceso (Fernández-Ginés, 2005). Una vez finalizado el lavado se procedió al escurrido y prensado del producto con tela de nylon, a través de un tamiz de 0,710 mm. El agua obtenida del lavado del co-producto se almacenó a -21 ºC para proceder posteriormente a su análisis.

En la figura 2 se muestra el proceso de obtención del agua de lavado y de la fibra dietética de naranja.

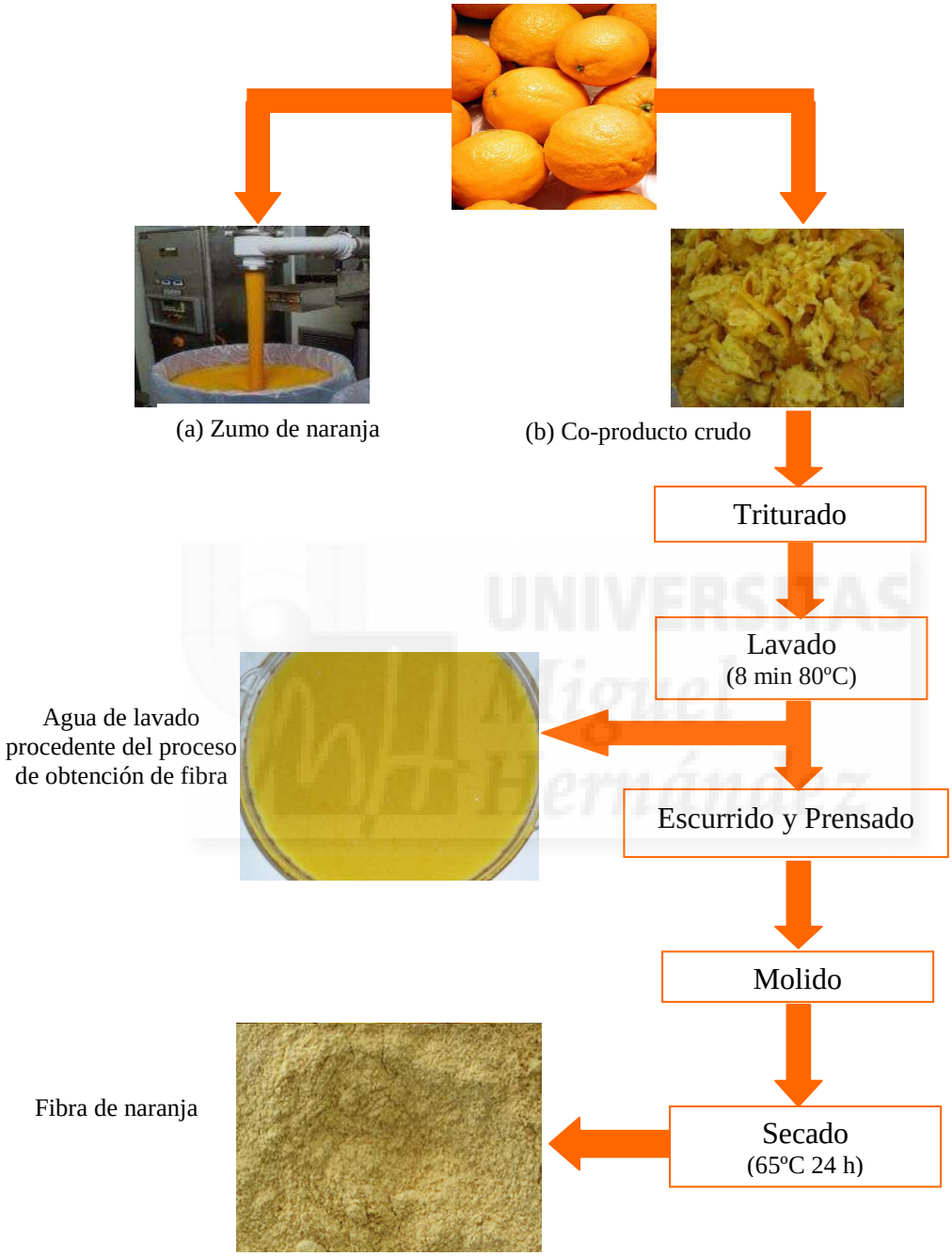

Figura 2. Diagrama de flujo del proceso de obtención del agua de lavado y fibra de naranja.

4.2.2- Caracterización del agua de lavado.

4.2.2.1.- pH

Para la determinación del pH en el agua de lavado se empleó un equipo Crison micro pH meter 2001 con un electrodo para alimentos líquidos GLP 21 (Crison Instrument, S.A Alella, Barcelona).

4.2.2.2.- Sólidos Solubles

La determinación de los sólidos solubles del agua de lavado se realizó mediante un refractómetro digital, modelo DR-101 (Cosecta S.A. Barcelona España).

4.2.2.3.- Color

Las determinaciones de color se realizaron utilizando un colorímetro Minolta CR-300 (Minolta Camera Co. Osaka, Japón), con iluminante D_{65}, y el observador 10º al que se le acopló un soporte para la medición de líquidos CR-A70 (Minolta Camera Co. Osaka, Japón). Las muestras objeto de estudio se introdujeron en tubos de cristal de baja reflectancia.

4.2.2.4.- Determinación de compuestos fenólicos

La cuantificación e identificación de compuestos fenólicos se realizó mediante cromatografía liquida de alta resolución (HPLC) en un cromatógrafo Hewlett Packard HP-1100 (Woldbronn, Alemania) siguiendo las indicaciones de Benavente *et al.*, (1999). Los productos de naturaleza fenólica se identificaron mediante comparación del tiempo de retención y su correspondiente espectro de absorción visible-ultravioleta (V/UV) con tiempo de retención y espectro de absorción de los compuestos utilizados como estándares: ácido cafeico, ácido ferúlico, ácido *p*-cumarico, eriocitrina, neoeriocitrina, narirutina, neohesperidina, hesperidina, diosmina, poncirina, hesperetina y neodiosmina todos ellos fueron suministrados por Extrasynthese (Genay, Francia).

4.2.2.5.- Determinación de ácidos orgánicos y azúcares.

El contenido en ácidos orgánicos y azúcares de las aguas de lavado se realizó mediante cromatografía liquida de alta resolución (HPLC) en un cromatógrafo Hewlett Packard HP-1100 (Woldbronn, Alemania) siguiendo las indicaciones de Dougthy (1995). Los ácidos orgánicos y azúcares se identificaron mediante comparación del

tiempo de retención con tiempo de retención del estándar previamente analizado. Como estándares de ácidos orgánicos se utilizaron: ácido L-ascórbico, ácido málico, ácido cítrico, ácido oxálico, ácido acético, ácido láctico y ácido succínico. Todos ellos fueron suministrados por Sigma (Poole, Dorset, Reino Unido). Como estándares de azúcares se utilizaron: glucosa, fructosa y sacarosa. Todos ellos fueron suministrados por Sigma (Poole, Dorset, Reino Unido).

4.3.- Sistema modelo de embutido de pasta fina tipo mortadela

4.3.1. Proceso de elaboración

Las materias primas magras y grasas procedentes de carne de porcino se obtuvieron de un matadero homologado. Las materias primas cárnicas fueron trasladadas en recipientes herméticos y protegidos de la luz para evitar alteraciones en el color de las muestras, manteniendo éstas a temperaturas de refrigeración, hasta su utilización en los laboratorios del Departamento de Tecnología de Alimentos de la Escuela Politécnica Superior de Orihuela.

El sistema modelo de pasta fina se realizó siguiendo la siguiente formulación (en base cárnica): 50% de magro de cerdo, 50% de panceta, 15% de agua (hielo), 3% de fécula de patata, 2,5% de sal, (p/p), 300mg/kg de tripolifosfato de sodio, 500 mg/kg de ascorbato de sodio, 150 mg/kg de nitrito de sodio y especias (0,01% de pimienta negra, 0,005% de nuez moscada y 0,2% de ajo en polvo). Esta formulación se consideró como la fórmula control.

Para evaluar la influencia de la adición de agua de lavado procedente del proceso de obtención de fibra de naranja y de los aceites esenciales de orégano, tomillo y romero, el contenido en agua de la formula control fue remplazado por agua de lavado en diferentes concentraciones y los aceites esenciales se adicionaron directamente obteniendo las distintas formulaciones especificadas en la tabla 1.

Tabla 1. Formulación de los distintos ensayos realizados en sistemas modelo de embutido de pasta fina tipo mortadela, adicionada con aguas de lavado y aceites esenciales de tomillo, romero y orégano.

Formulación	Agua de lavado (%)	Aceite esencial de tomillo (%)	Aceite esencial de romero (%)	Aceite esencial de orégano (%)
Control	0	0	0	0
CFWW5	5	0	0	0
CFWW10	10	0	0	0
TEO200	0	0,02	0	0
CFWW10-TEO200	5	0,02	0	0
CFWW10-TEO200	10	0,02	0	0
REO200	0	0	0,02	0
CFWW5-REO200	5	0	0,02	0
CFWW10-REO200	10	0	0,02	0
OEO200	0	0	0	0,02
CFWW5-OEO200	5	0	0	0,02
CFWW10-OEO200	10	0	0	0,02

Las distintas formulaciones a estudiar se prepararon en la Planta Piloto de alimentos que el grupo de Industrialización de Productos de Origen Animal dispone en la Universidad Miguel Hernández (Campus Orihuela) siguiendo las recomendaciones de Sayas-Barberá *et al.* (2002). En la figura 3 se puede observar el diagrama de flujo del proceso de elaboración del producto cárnico cocido tipo mortadela.

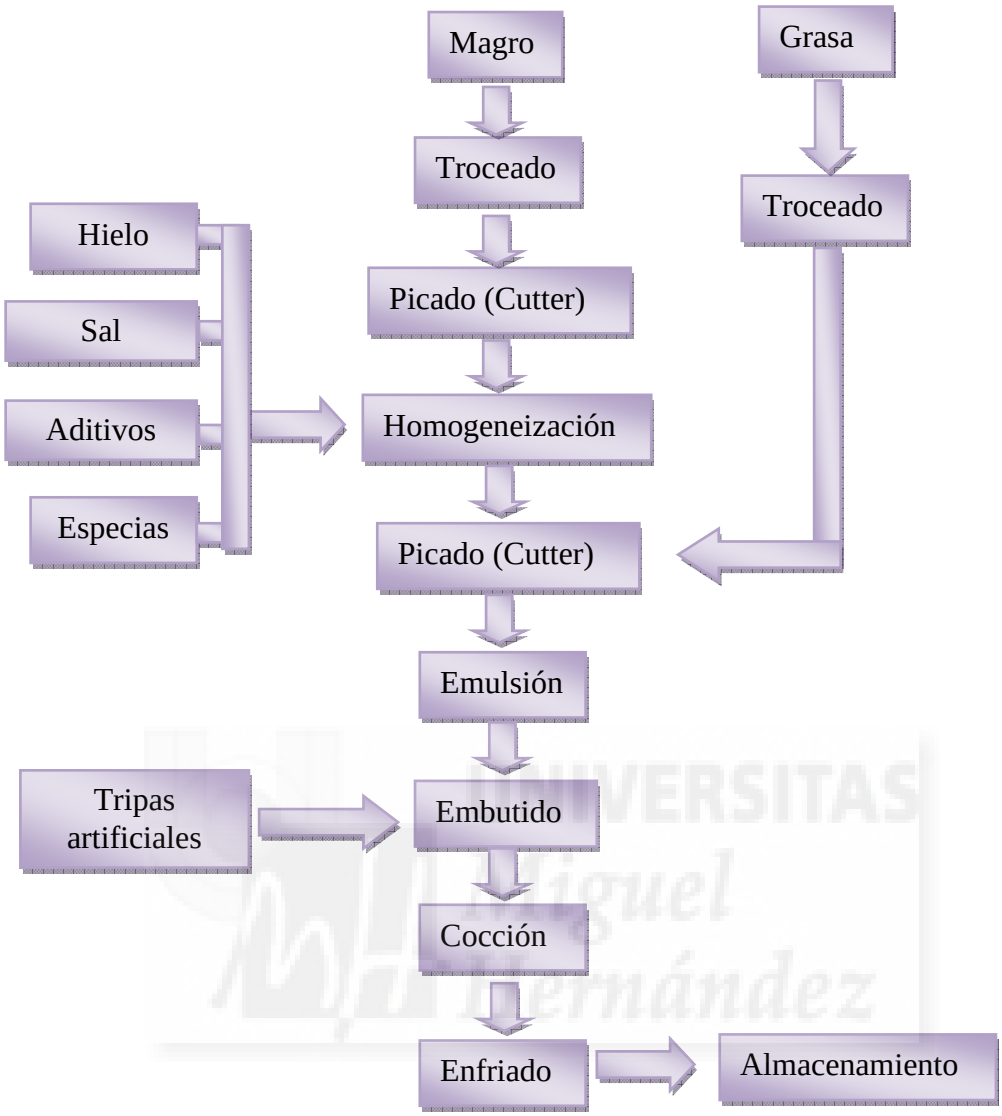

Figura 3. Diagrama de flujo del proceso de elaboración de la mortadela.

El magro de cerdo mantenido a una temperatura de 4 ± 1 ºC se introdujo en una cutter (Tecator 1094 Homogeneizer, Tekator, Hoganas, Suecia) junto con la sal y se homogeneizó la mezcla durante 30 s. Posteriormente, se adicionaron el resto de aditivos e ingredientes a excepción de la grasa y se homogeneizaron durante otros 30 s. Tras esta homogeneización se adicionó la panceta, previamente troceada en cubos de 10 cm de lado, y se procedió a la homogeneización durante 2 min.

Transcurrido este tiempo la mezcla se embutió en porciones de 200 g en tripa artificial Fibran-Pack (Fibran, Gerona, España) de 55 mm de diámetro; dichas tripas se remojaron durante 30 min antes de ser utilizadas. Una vez remojadas fueron grapadas en los extremos con una grapadora de embutidos y se introdujeron en un baño de agua a

100 ºC para su cocción. El proceso de cocción finalizó cuando los embutidos alcanzaron la temperatura de 70 ± 2ºC en el centro geométrico. Una vez alcanzada la temperatura se sacaron las mortadelas del baño y se enfriaron a temperatura de 8 ± 2ºC durante 15 min. Posteriormente, los embutidos se almacenaron en condiciones de refrigeración (4 ± 1ºC) durante 24h.

4.3.2- Caracterización del producto cárnico.

4.3.2.1.- Determinaciones Físico-Químicas.

4.3.2.1.1.- Color.

Las determinaciones de color se efectuaron de acuerdo a las normas de la Asociación Americana de la Carne (Hunt *et al.,* 1991). Dichas determinaciones se realizaron mediante un colorímetro Minolta CM-2002 (Minolta Camera Co., Osaka, Japón) con iluminante D_{65}, y el observador 10º. En todas las determinaciones de color se interpusieron cristales de baja reflectancia Minolta CR-A51/1829-752 (Minolta Co., Osaka, Japón) entre las muestras y el equipo (Hunt *et al.,* 1991).

4.3.2.1.2.- pH.

El pH de cada una de las unidades de muestras se determinó bajo las directrices del Ministerio de Agricultura Pesca y Alimentación (1994), mediante disolución acuosa en una proporción 1:10 de producto y agua destilada. Las lecturas de pH se efectuaron con un equipo Crison modelo 507 (Crison, Barcelona, España) equipado con un electrodo de membrana Crison nº 52-32 (Crison, Barcelona, España).

4.3.2.1.3.- Actividad de agua.

La actividad de agua de las distintas muestras se determinó mediante un equipo Novasina modelo Sprint TH-500 (Pfäffikon, Suiza). Las muestras se analizaron a una temperatura de trabajo de 25 ºC.

4.3.2.1.4.- Análisis de Textura.

El ensayo utilizado para evaluar la textura instrumental de las muestras fue el Análisis de Perfil de Textura (TPA) siguiendo las directrices de Bourne (1978). El ensayo se basa en comprimir una porción de alimento con una sonda cilíndrica en dos ciclos consecutivos, imitando la acción de la masticación. De esta compresión se extrae

la curva fuerza-tiempo, de la cual se obtienen una serie de parámetros relacionados como son la dureza, masticabilidad, gomosidad, adhesividad, elasticidad y cohesividad.

La dureza, se define como la fuerza máxima obtenida en el primer ciclo de compresión.

La cohesividad, es la relación entre el área positiva obtenida durante la segunda compresión y el área positiva obtenida durante la primera.

La adhesividad, es el área negativa obtenida tras el primer ciclo de compresión, representando la fuerza necesaria para separar la superficie compresora de la muestra, después de haberla comprimido por primera vez.

La elasticidad viene determinada por la altura que la muestra recupera entre el final de la primera compresión y el principio de la segunda.

La gomosidad viene determinada por el producto de la dureza por la cohesividad.

La masticabilidad es el producto de la gomosidad por la elasticidad.

Para realizar esta determinación se utilizó un texturómetro TAXT2 Texture Analyser (GU7 1YL, Londres, Inglaterra), equipado con una sonda cilíndrica (plato de compresión) de 100 mm de diámetro (P100), la velocidad de la sonda durante la prueba fue de 5 mm/s y el grado de compresión ejercida fue de un 70%.

Para su análisis las muestras fueron cortadas en cubos de 1 cm, y se dejaron atemperar a la temperatura de ensayo (4±1ºC) durante 2 horas, en la cámara a 3-5 ºC hasta la hora de su análisis.

4.3.2.2.- Determinaciones Químicas.

4.3.2.2.1.- Humedad.

Las determinaciones de humedad se efectuaron siguiendo las directrices de la AOAC (1995). El valor de humedad se expresa como % (g de agua/100 g de producto).

4.3.2.2.2.- Cenizas.

La determinación de cenizas se realizó siguiendo las directrices de la AOAC (1995) utilizando una mufla Hobersal modelo 12-PR/300 “PAD” (Hobersal S.A. Barcelona, España). Los resultados se expresan como % (g de cenizas/100 g de

producto).

4.3.2.2.3.- Proteínas.

La determinación de la cantidad de proteínas presente en la mortadela se realizó utilizando el método Kjeldhal. En primer lugar las muestras son introducidas en un digestor, Büchi Digestion Unit modelo 426, para posteriormente ser introducidas en un equipo de destilación Büchi Destillation Unit modelo B-316 (Büchi, Suiza). Los resultados se expresan como % (g de proteinas/100 g de producto).

4.3.2.2.4.- Grasas.

La determinación de grasa en la mortadela se realizó siguiendo las directrices de la AOAC (1995) utilizando un extractor tipo Soxhlet J.P. Selecta modelo 60003286 (J.P. Selecta, Barcelona, España). Los resultados se expresan como % (g de grasa/100 g de producto).

4.3.2.2.5.- Concentración de nitrito residual.

La determinación de nitrito residual presente en la mortadela se realizó a través de la reacción de Griess-Ilosvay siguiendo las directrices de la Norma ISO/DIS 2918 (1975). El espectrofotómetro utilizado para la medición de absorbancias fue un Unicam Heλios modelo Gamma (γ) (Unicam, Reino Unido). Los datos obtenidos se expresaron como mg $NaNO_2$/kg muestra.

4.3.2.2.6.- Análisis del acido 2-Tiobarbitúrico (TBA).

La determinación de la oxidación lipídica se realizó mediante el test del acido 2-tiobarbiturico siguiendo las directrices descritas por Buege y Aut (1978). Los resultados se expresaron como mg Malonaldehido/kg de muestra.

4.3.2.2.7.- Extracción y cuantificación de compuestos fenólicos.

La extracción de los compuestos fenólicos en las muestras de mortadela se realizó tomando 2 g de muestra a la que se adicionó 6 mL de dimetilsulfoxido (Sharlau, Barcelona, España) permaneciendo en agitación durante 120 min en un baño de ultrasonidos Selecta (Selecta S.A. Barcelona, España). Los diferentes extractos fueron pasados a través de un filtro de nylon de 0,45 µm (Sharlau, Barcelona, España) y se almacenaron a -21 ºC hasta su análisis.

La cuantificación de los compuestos fenólicos se realizó mediante cromatografía líquida de alta resolución (HPLC) en un cromatógrafo Hewlett Packard HP-1100 (Woldbronn, Alemania) siguiendo las indicaciones de Benavente *et al.*, (1999). Los productos de naturaleza fenólica se identificaron mediante comparación del tiempo de retención y su correspondiente espectro de absorción visible-ultravioleta (V/UV) con tiempo de retención y espectro de absorción de los compuestos utilizados como standards: ácido cafeico, ácido ferúlico, ácido *p*-cumarico, eriocitrina, neoeriocitrina, narirutina, neohesperidina, hesperidina, diosmina, poncirina, hesperetina y neodiosmina todos ellos fueron suministrados por Extrasynthese (Genay, Francia).

4.3.2.3.- Análisis microbiológico.

Para el recuento microbiológico, se tomaron 25 g de muestra que se homogeneizó con 225 mL de agua de peptona al 1,5% en un Stomacher 400 (Colworth, Londres, Reino Unido) durante 2 minutos. Los medios de cultivo utilizados y las condiciones de incubación se detallan a continuación:

- Recuento total de aerobios mesófilos en agar de recuento en placa (PCA) a 35 ºC durante 48 horas.
- Recuento total de aerobios psicrófilos en agar de recuento en placa (PCA) a 7 ºC durante 7 días.
- Recuento total de bacterias ácido lácticas en una doble capa de MRS (Man, Rogosa and Sharpe Agar) a 35 ºC durante 48 horas.
- Recuento total de enterobacterias en Agar Glucosa Vilis Rojo Violeta (VRBG) a 35 ºC durante 48 horas.

4.3.2.4. Análisis sensorial.

Para la evaluación sensorial se utilizaron 30 jueces, no entrenados (estudiantes de la Universidad Miguel Hernández) seleccionados en base a su experiencia previa en el consumo de productos cárnicos de pasta fina. Los atributos medidos fueron los siguientes: para la evaluación externa se determinó aspecto, tono, brillo, intensidad de color, homogeneidad, y olor; para el sabor se determinó el sabor ácido, salado y carácter graso; para la textura se determinó la dureza y jugosidad y finalmente se evaluó la percepción global del producto.

Para la determinación de dicha evaluación sensorial se llevo a cabo un análisis cuantitativo descriptivo QDA (IFT, 1981). La evaluación sensorial se realizó en el Laboratorio de Análisis Sensorial de la Universidad Miguel Hernández (Escuela Politécnica Superior de Orihuela, Departamento de Tecnología Agroalimentaria) de acuerdo con los estándares internacionales (ASTM 1986; ISO 1988).

Las muestras fueron cortadas en trozos de 1,5 x 2 cm y presentadas a los jueces para su evaluación. Los atributos sensoriales se midieron en una escala desestructurada con descriptores tanto al principio como al final.

4.3.3. Análisis de la vida útil.

Para la evaluación de la vida útil de las mortadelas se seleccionó la concentración del 5% de agua de lavado, ya que fue la concentración más valorada en los análisis sensoriales realizados durante la caracterización de las mortadelas. La concentración de aceites esenciales de especias permaneció constante (0,02%).

Para realizar dicha determinación se cortaron lonchas de aproximadamente 1,5 cm de espesor de las distintas muestras envasándolas en bandejas de polietileno bajo tres diferentes métodos: expuestas al aire, bajo atmosfera modificada (80% N_2 y 20% CO_2) y a vacio. Las bandejas fueron selladas con una lámina de poliamina con las siguientes características: permeabilidad al vapor de agua 1,1 $g/m^2/24$ h a 23 °C, permeabilidad al nitrógeno 10 $cm^3/m^2/24$ h a 23 °C, permeabilidad al dióxido de carbono 140 $cm^3/m^2/24$ h a 23 °C, y permeabilidad al oxígeno 30 $cm^3/m^2/24$ h a 23 °C (Fibran, Girona, España).

Las bandejas fueron almacenadas en una cabina en condiciones de refrigeración 4 ± 1 °C simulando las condiciones de un supermercado. La cabina estaba sometida a una iluminación forzada por una lámpara OSRAM (1000 Lux). Para minimizar las posibles diferencias de temperatura e iluminación de las muestras en la cabina las posiciones de éstas eran cambiadas cada 24 horas. Una vez bajo las condiciones de conservación, se fueron tomando muestras a los 0, 6, 12, 18 y 24 días, para establecer el tiempo máximo de conservación.

Para este estudio las determinaciones realizadas fueron: TBA, color, pH, contenido en compuestos fenólicos, textura, análisis sensorial y microbiológico. Todas estas determinaciones quedan descritas en los distintos puntos tratados en este capítulo.

4.4. Metodología estadística

La metodología estadística se diseñó para cada uno de los estudios a analizar. La totalidad de los análisis se realizaron mediante el paquete estadístico Statgraphics Plus para Windows versión 5.1 (Statical Graphics Corp., Rockville, USA) utilizando el programa Analisis of Variance.

Para la determinación de las media y la desviación estándar se siguieron métodos estadísticos convencionales. El análisis estadístico empleado en cada ensayo fue la aplicación de un análisis de la varianza (ANOVA) de uno, dos o tres factores, dependiendo del ensayo realizado.

Para estudiar entre que variables de los factores principales las diferencias fueron estadísticamente significativas se realizaron contrastes entre las medias aplicando el test de Tukey siguiendo las recomendaciones de Afifi y Azen (1979).

RESULTADOS Y DISCUSION

5.- RESULTS AND DISCUSSION

This chapter resumes the main results and discussion referring to the different works carried out, the complete versions of which have been published in international journals, copies of which are attached to this report.

5.1.- Chemical composition of the spice essential oils

The objective of this work was to determine the chemical composition, using gas chromatography/mass spectrometry (GC/MS), of six essential oils widely used in the Mediterranean basin: oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), cumin (*Cuminum cyminum* L.) and clove (*Syzygium aromaticum* L.). This work was published in *Acta Chimica Slovenica* (2007, *54*, 921-926).

Table 2 shows the number of compounds identified and the principal components of the six essential oils analysed

Table 2. Principal components identified in the essential oils analysed.

Essential oil	Identified compounds	Main components	% área
Oregano	32	Carvacrol	61.21
		p-cimene	15.12
		γ-terpinene	4.80
Thyme	52	4-Terpinenol	13.15
		γ-terpinene	9.21
		cis-sabinene hydrate	7.65
Rosmary	39	β-pinene	12.75
		α-pinene	36.42
		Camphor	15.65
Sage	37	Camphor	24.95
		1,8-cineole	24.75
		Camphene	7.63
Cumin	26	γ-pinene	27.40
		p-cymene	20.49
		Cuminal	20.39
Clove	5	Eugenol	85.50
		β-cariophylene	10.54
		α-humulene	3.12

The six essential oils analysed show a widely varying composition. Some were found in all the oils, some were found in high concentrations in a few oils, while some were only identified in one particular essential oil.

This great variability compared with the data available in the literature can be attributed to factors such as the climatic conditions, soil, geographical situation, vegetative state of the plant, the part of the plant used to obtain the oil and the method used to do so.

5.2.- Determination of antifungal activity of the essential oils

The objective of this work was to determine the effectiveness of the essential oils from oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), clove (*Syzygium aromaticum* L.), rosemary (*Rosmarinus officinalis* L) and sage (*Salvia officinalis* L) as natural inhibitors of some moulds related with food spoilage, such as *Aspergillus niger* and *Aspergillus flavus*. This work was published in *Journal of Food Safety* (2007, *27*, 91-101).

All the essential oils (EOs) at all the concentrations assayed, except rosemary and sage, inhibited the growth of both moulds (see Figure 4).

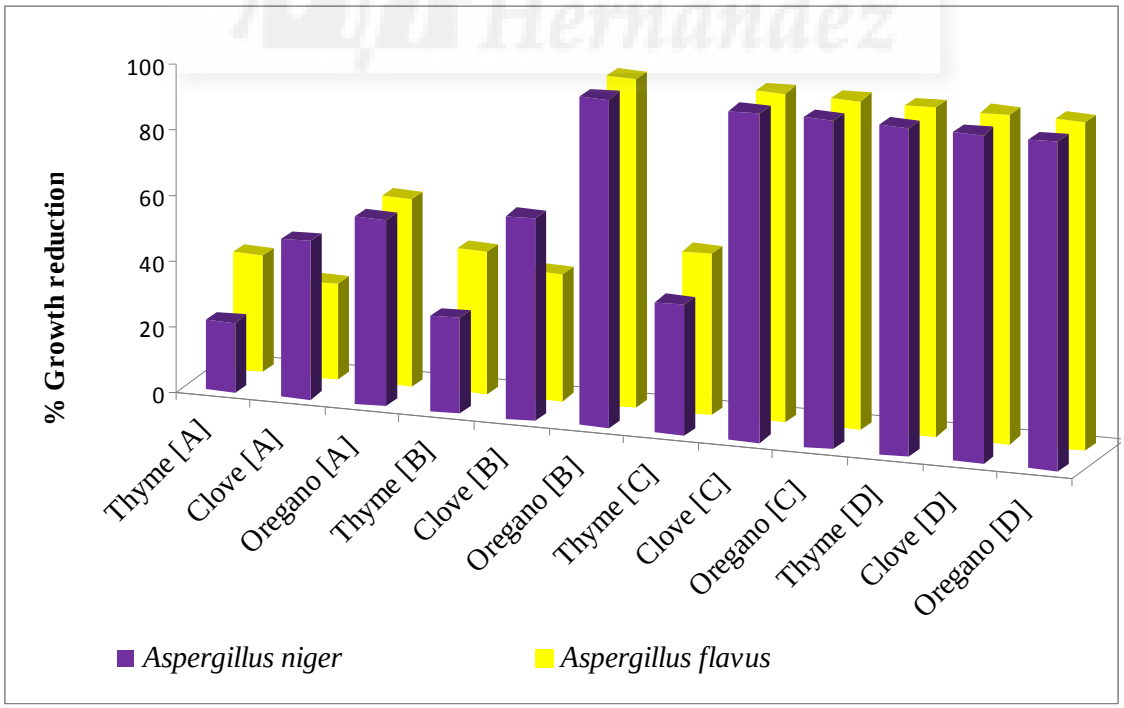

Figure 4. % growth reduction of *Aspergillus niger* and *Aspergillus flavus* by the EOs of thyme, oregano and clove at different concentrations: [A] 0.011%; [B] 0.022%; [C] 0.033%; [D] 0.044%.

Of the EOs analysed, thyme EO was the only one to inhibit the growth of both moulds in a dose-dependent manner. Of note was the greater efficacy of thyme EO against *Aspergillus flavus* compared with *Aspergillus niger*. Oregano EO had the highest inhibitory power since, at 0.022% it totally inhibited the growth of both moulds, followed by clove EO, which managed to do the same at 0.033%.

When it was seen that the EOs of rosemary and sage had no inhibitory effect at these concentrations, their concentration was increased ten-fold. Figure 5 shows the inhibition of *Aspergillus niger* obtained in the presence of different concentrations of rosemary EO.

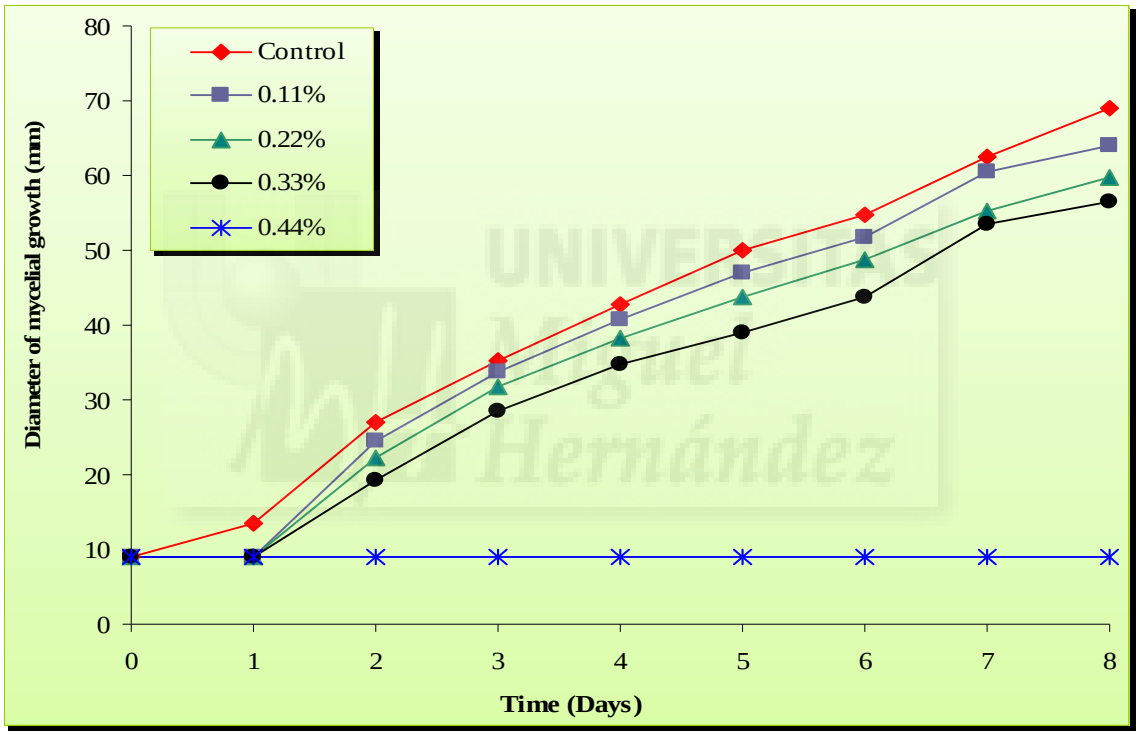

Figure 5. Antifungal activity of rosemary (*Rosmarinus officinalis* L.) essential oil at different concentrations (0.11%, 0.22%, 0.33% and 0.44%) on the mould *Aspergillus niger*.

It can be seen that a reduction of mycelial growth of 7.13% was obtained at a concentration of 0.11%. When this concentration was doubled to 0.22% a growth reduction of 13.31% was obtained. Finally when an EO concentration of 0.33% was used, the reduction in mycelial growth reached 18.06%. As occurred with sage EO, a concentration of 0.44% totally inhibited growth of the mould.

Figure 6 shows the inhibition of *Aspergillus niger* obtained in the presence of different concentrations of sage EO. The addition of 0.11% sage EO brought about a reduction of 16.58%; double this concentration (0.22%), led to an 18.50% inhibition, while the addition of 0.33 % reduced the mycelial growth by 24.87%. The use of 0.44 % led to total inhibition.

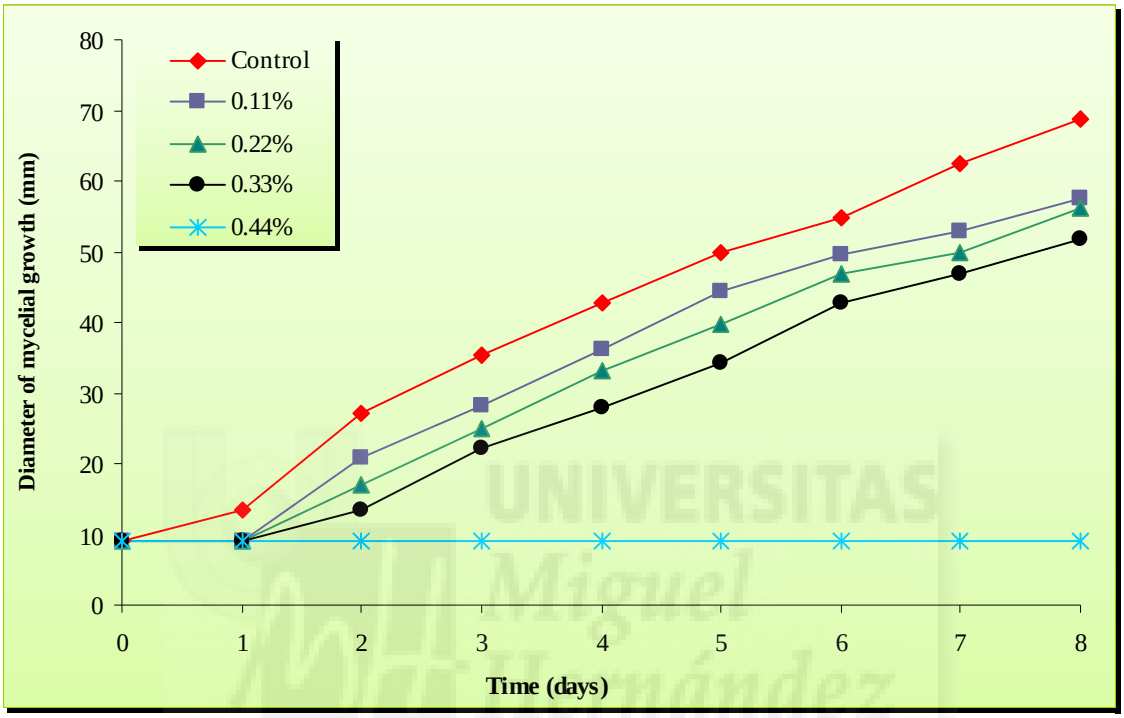

Figure 6. Antifungal activity of sage (*Salvia officinalis*) essential oil at different concentrations (0.11%, 0.22%, 0.33% and 0.44%) on the mould *Aspergillus niger*.

When the reduction in the mycelial growth in *A. niger* was analysed, sage EO was seen to have a stronger inhibition capacity than rosemary. As can be seen from Figure 7 the inhibition of *Aspergillus flavus* growth in the presence of different concentrations of sage EO was 6.89%, 11.50% y 17.80% for concentrations of 0.11, 0.22 y 0.33%, respectively. A concentration of 0.44% totally inhibited growth of the mould.

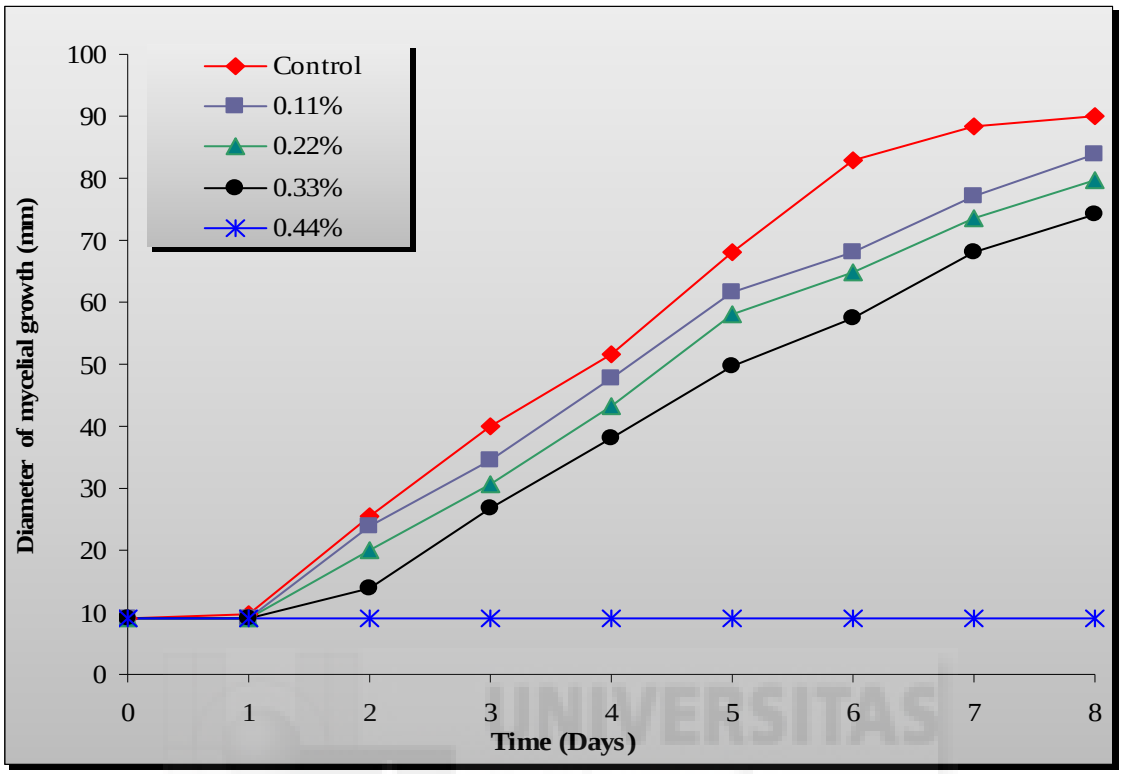

Figure 7. Antifungal activity of sage (*Salvia officinalis*) essential oil at different concentrations (0.11%, 0.22%, 0.33% and 0.44%) on the mould *Aspergillus flavus*.

Figure 8 shows the inhibition of *Aspergillus flavus* observed in the presence of different concentrations of rosemary EO. The addition of 0.11% sage EO brought about a reduction of 4.67%; double this concentration (0.22%), led to a 10.63% inhibition, while the addition of 0.33 % reduced growth by 17.66%. The use of 0.44 % led to total inhibition.

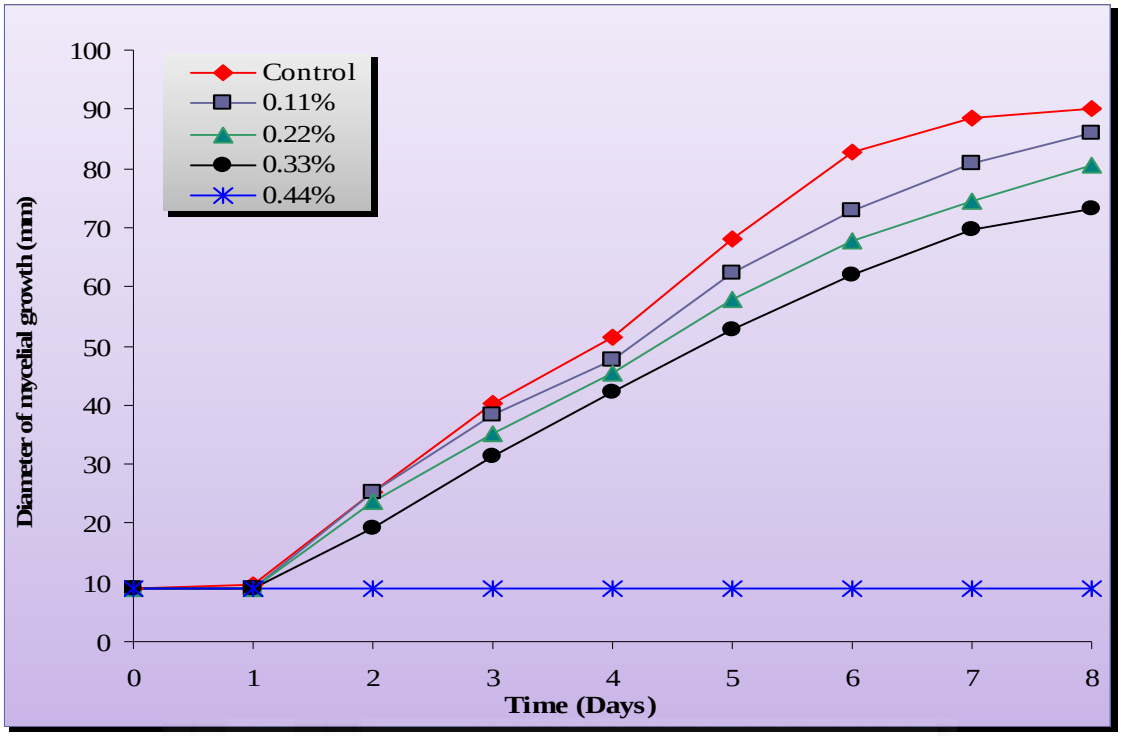

Figure 8. Antifungal activity of rosemary (*Rosmarinus officinalis* L) essential oil at different concentrations (0.11%, 0.22%, 0.33% and 0.44%) on the mould *Aspergillus flavus*.

EOs are a complex mixture of volatile compounds that present, among other properties, antifungal activity since they reduce or totally inhibit fungal growth, normally, in a concentration-dependent way (Sharma and Tripathi 2008). This antifungal activity of EOs can be largely attributed to their principal components, although the possibility of synergism or antagonism with some minor components cannot be ruled out (Deba *et al.*, 2007).

Several studies have described possible mechanisms for this antifungal activity. For example, it is commonly accepted that it is the toxic effects that the components of the EOs have on the functionality and structure of the cell membrane that is responsible for such antifungal activity (Sikkema *et al.*, 1995). For Omidbeygi *et al.* (2007), the components of EOs cross the cell membrane and interact with enzymes and proteins of the membrane, provoking a flow of protons towards the exterior, which in turn provokes changes in the cells and brings about their death. Lucini *et al*, (2006) indicates that the inhibition of mycelial growth is brought about by the action of the monoterpenes present in the EOs. These components lead to an increase in the peroxides hydroxyl, alcoxyl and alcoperoxyl, which provoke cell death. For Sharma and Tripathi (2008)

EOs act in the mycelium hyphae, provoking an exit of protons from the cytoplasm and loss of cell wall integrity and rigidity in the hyphae and the subsequent collapse of the leads to mycelium death.

5.3.- Determination of antibacterial activity of essential oils

The objective of this study was to determine the effectiveness of the essential oils from oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), clove (*Syzygium aromaticum* L.), rosemary (*Rosmarinus officinalis* L) and sage (*Salvia officinalis* L) on the growth of several bacteria, some of which are used in the food industry as starter cultures (*Lactobacillus curvatus, Lactobacillus sakei, Staphylococcus carnosus* and *Staphylococcus xylosus*) and some related with food spoilage (*Enterobacter gergoviae* and *Enterobacter amnigenus*. This work was published in *International Journal of Food Science and Technology* (2008, *43*, 526-531).

All the EOs studied showed antibacterial activity. In the case of the bacteria used as starter cultures (Figure 9), oregano EO showed the greatest inhibition against their growth, with diameters of inhibition ranging from 35.29 mm in the case of *Staphylococcus xylosus* to 45.20 mm in the case of *Lactobacillus curvatus*.

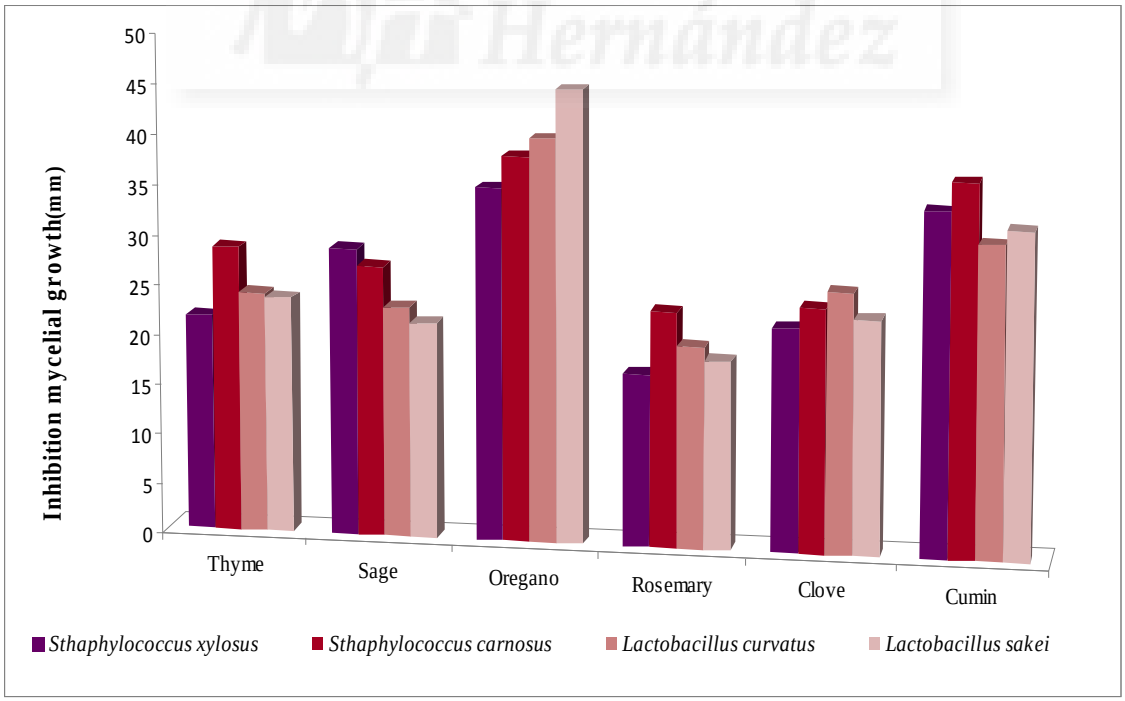

Figure 9. Antibacterial activity of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L), cumin (*Cuminum cyminum* L) and clove (*Syzygium aromaticum* L) essential oils on bacteria used as starter cultures in the food industry

The next most effective oil was cumin EO, which showed an inhibition halo similar but slightly below that of oregano: 31.23 mm for *Lactobacillus sakei* and 37.22 mm for *Staphylococcus carnosus.*

The lowest degree of inhibition was that presented by rosemary EO, while the others showed very similar values amongst themselves.

In the case of bacteria associated with food spoilage (Figure 10) *Enterobacter gergoviae* was more sensitive strain to all the EOs analysed than *Enterobacter amnigenus*, except in the case of oregano EO. Thyme was the most effective EO against *Enterobacter gergoviae*. In the case of the bacterium *Enterobacter amnigenus* oregano EO was the strongest inhibitor. Rosemary EO was again the least effective inhibitor of this type of bacterium.

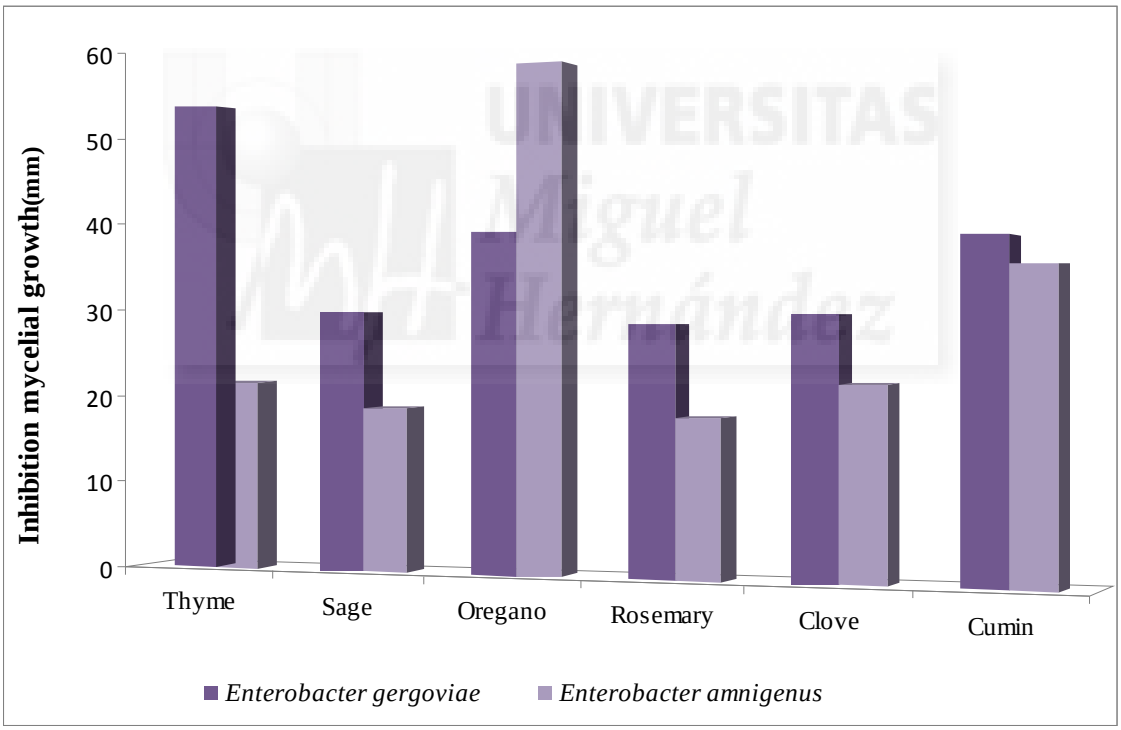

Figure 10. Antibacterial activity of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L), cumin (*Cuminum cyminum* L) and clove (*Syzygium aromaticum* L) essential oils on bacteria responsible of food spoilage.

The action mechanism through which bacterial growth is inhibited has not been widely studied (Lambert *et al.*, 2001). Bearing in mind the large number of chemical components present in EOs, their antibacterial activity is very likely not due to a specific mechanism but to a the joint action of different mechanisms acting on a variety

of cell targets (Skandamis *et al.*, 2001; Carson *et al.*, 2002). Figure 11 shows the different mechanism proponed to this effect

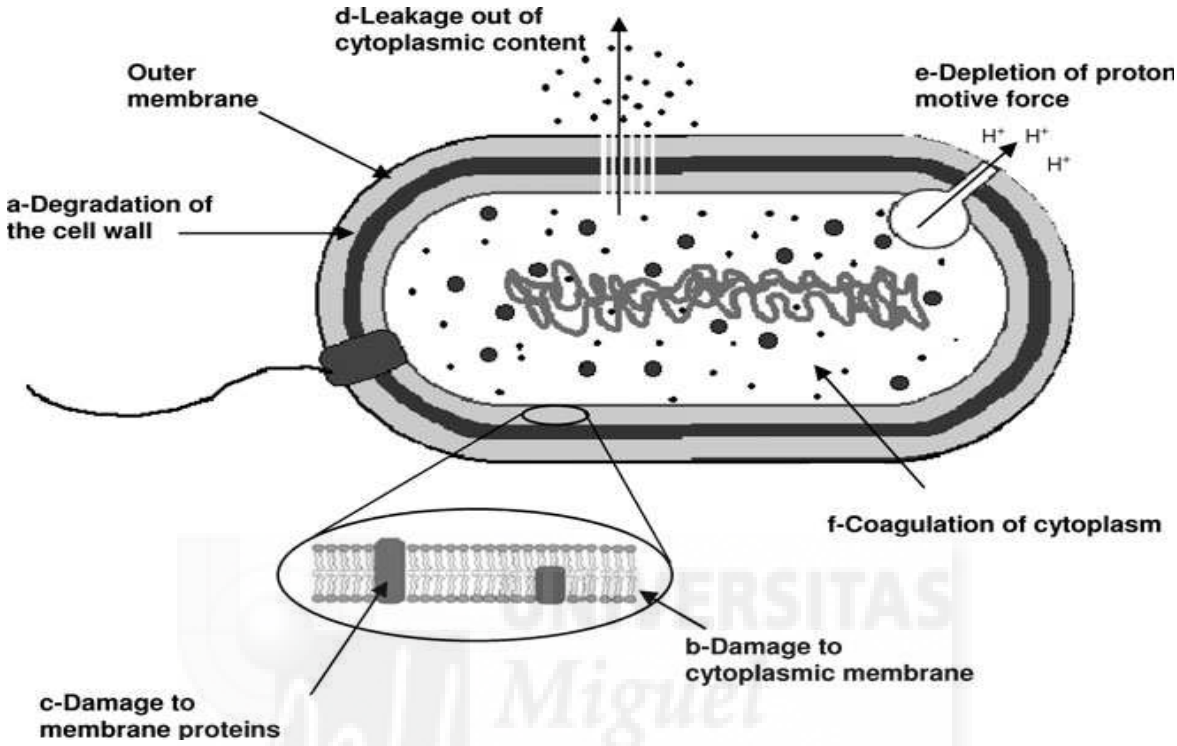

Figure 11. Action mechanisms proposed for essential oils and their components on bacterial cells. Source: Raybaudi-Massilia et al. (2009).

5.4.- Determination of antioxidant activity of EOs

We determined the phenol content and *in vitro* antioxidant activity of the essential oils from oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), clove (*Syzygium aromaticum* L.), rosemary (*Rosmarinus officinalis* L) and sage (*Salvia officinalis* L). The *in vitro* antioxidant activity was determined using five analytical methods: radical scavenging of 2,2′-Diphenyl-1-picrylhydrazyl (DPPH); the ferric reducing antioxidant power (FRAP); determination of the antioxidant activity of thiobarbituric acid-reactive substances (TBARS); ferric ion chelating capacity (FIC), and the automatic determination of the oxidative stability of (RANCIMAT). This work was published in *Flavour and Fragrance Journal of Food Safety* (2010, *25*, 13-19).

Figure 12 shows the phenol content, expressed as gallic acid equivalents (mg/L), of the EOs analyzed.

Clove EO had the highest phenol content (898.89±3.37 mg GAE/L), followed by oregano and thyme, which showed similar values to each other. Rosemary and sage EOs showed the lowest phenol contents.

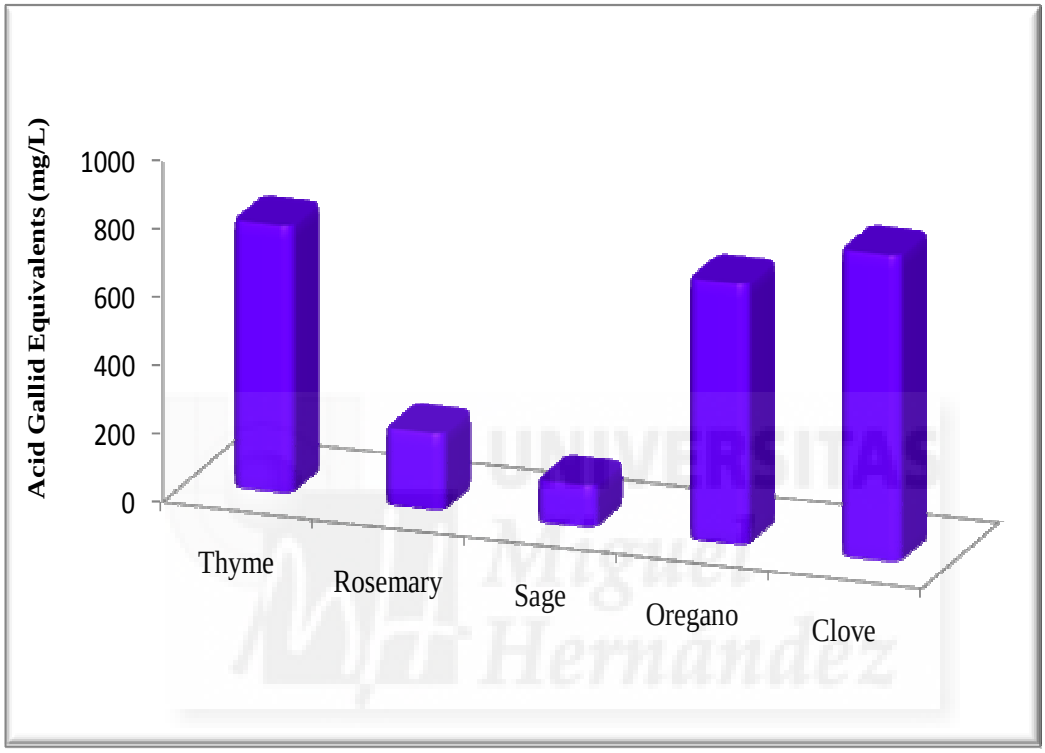

Figure 12. Total phenol content expressed as gallic acid equivalents (mg/L) in the EOs of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L) and clove (*Syzygium aromaticum* L).

Figure 13 shows the antioxidant activity of the essential oils of thyme, sage, oregano, rosemary and clove at different concentrations, as determined by the DPHH method. Note that the degree of oxidation inhibition was concentration dependent in all cases, except in the case of clove, which showed the highest degree of inhibition, but with hardly any difference between the concentrations analysed.

The next most powerful antioxidant was thyme, which clearly shows its concentration-dependence, rosemary EO had the lowest antioxidant power.

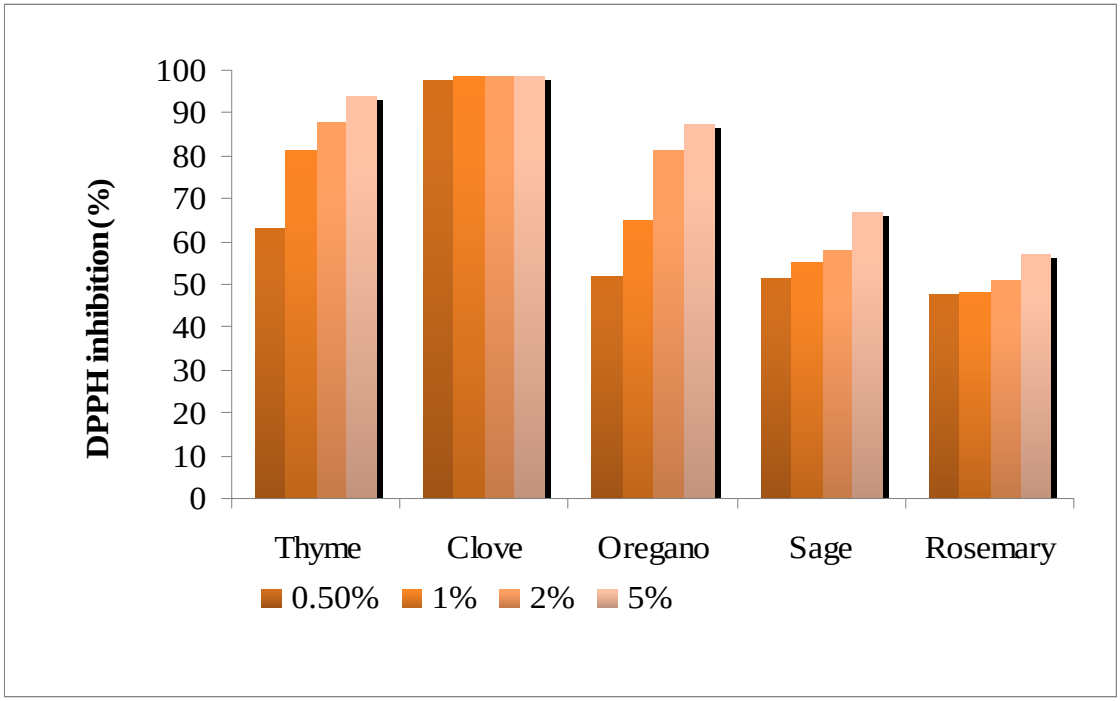

Figure 13. Antioxidant activity of different concentrations of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L) and clove (*Syzygium aromaticum* L) essential oils as determined by the DPPH method.

Note the high correlation (R^2=0.985) between the degree of oxidation inhibition (determined by the DPPH method) and the phenol content for all the EOs except sage.

Figure 14 shows the antioxidant activity of the essential oils of thyme, sage, oregano, rosemary and clove at different concentrations, as determined by the FIC method. As in the case of the DPPH method, the antioxidant power was concentration-dependent.

In this case, rosemary and sage (at 5%) were the EOs that showed the greatest metal chelating capacity (76.06 and 72.29 %, respectively). Oregano EO (at all the concentrations assayed) showed the lowest values.

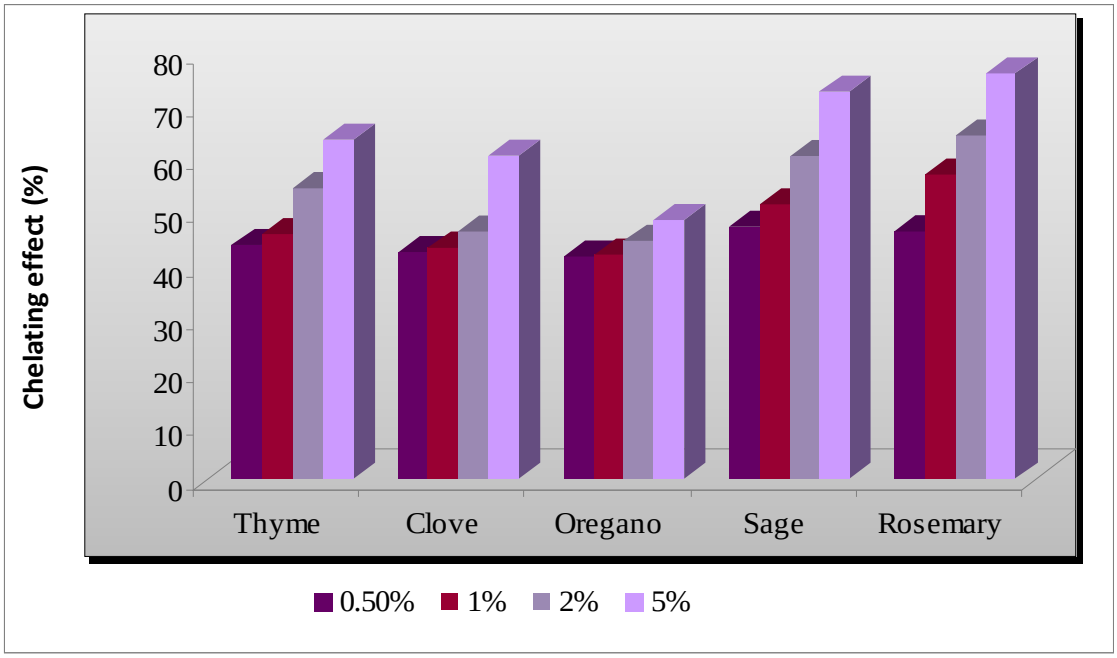

Figure 14. Antioxidant activity of different concentrations of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L) and clove (*Syzygium aromaticum* L) essential oils as determined by the FIC method.

Analysis of metal ion-chelating properties showed that all the essential oils studied were capable of chelating iron (II) and did so in a concentration-dependent manner. The metal chelating power is of great interest in the food industry, where the ions belonging to transition metals can catalyse the decomposition of hydroperoxides, contributing to lipid oxidation – the main source of food spoilage.

Figure 15 shows the antioxidant activity of the essential oils of thyme, sage, oregano, rosemary and clove at different concentrations, as determined by the FRAP method. Once again, the antioxidant activity of the EOs is seen to be concentration dependent.

As in the DPPH method, clove EO, at all concentrations, had the highest antioxidant power with values of 1.27 and 1.47 mM trolox/L. Sage and rosemary EOs had the lowest activities in this respect.

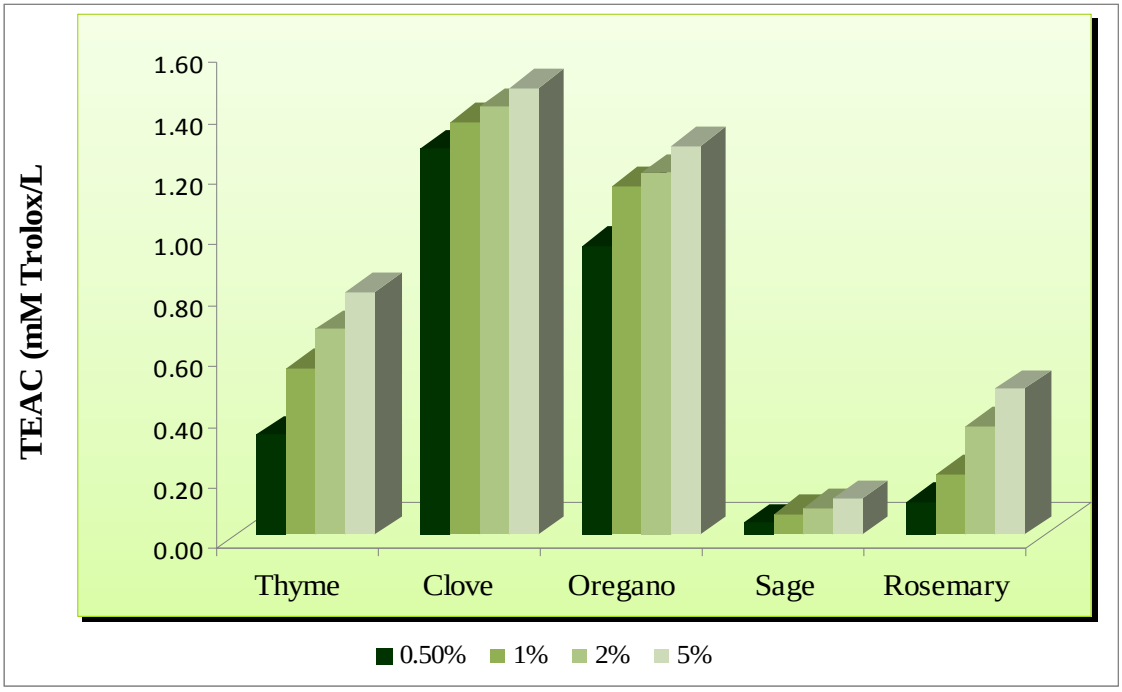

Figure 15. Antioxidant activity of different concentrations of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L) and clove (*Syzygium aromaticum* L) essential oils as determined by the FRAP method.

Figure 16 shows the antioxidant activity of the essential oils of thyme, sage, oregano, rosemary and clove at different concentrations, as determined by the TBARS method. Thyme EO showed the highest degree of free radical formation at the highest concentration assayed (5%).

At concentrations of 0.5, 1 and 2%, oregano EO had the highest antioxidant potential, while rosemary and sage had the lowest antioxidant power.

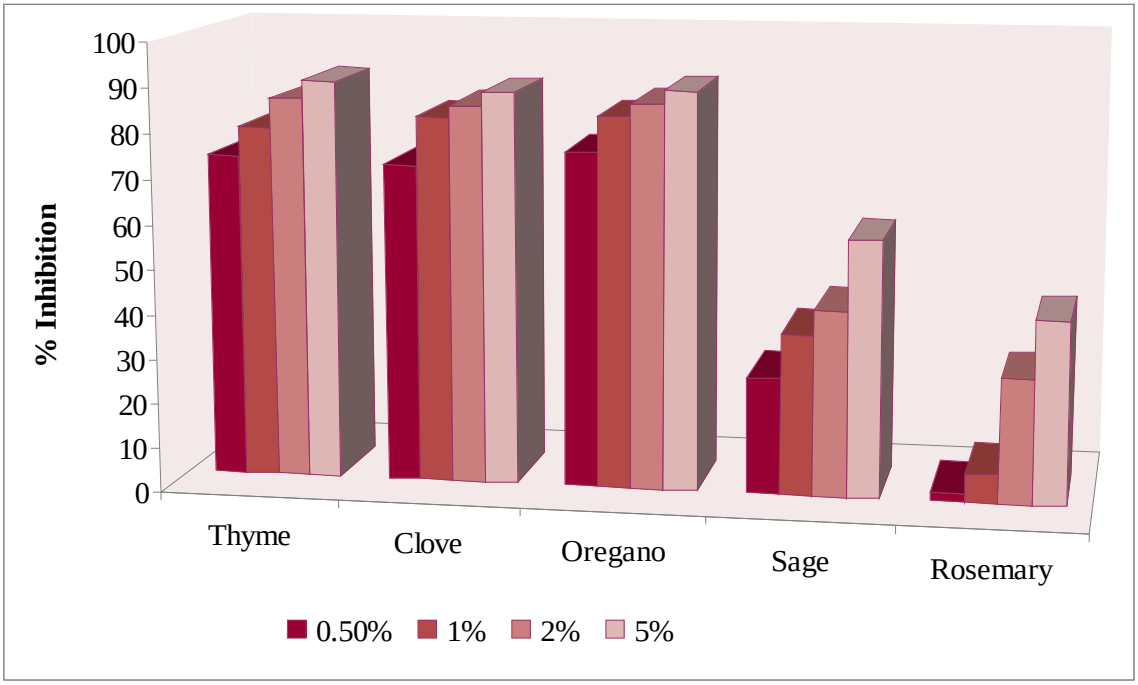

Figure 16. Antioxidant activity of different concentrations of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L) and clove (*Syzygium aromaticum* L) essential oils as determined by the TBARS method.

Figure 17 shows the antioxidant activity of the essential oils of thyme, sage, oregano, rosemary and clove at different concentrations, as determined by the RANCIMAT method.

According to this method the essential oils analysed showed antioxidant activity ranging from 1.05 to 1.67 at 5%, values which fell as the concentration assayed fell. Note that at low concentrations both sage and rosemary had a pro-oxidant effect since the antioxidant activity was less than 1. The pro-oxidant effect of phenolic compounds has been widely described (Galati *et al.*, 2002; Cirico and Omaye, 2006), although this effect is reduced at high concentrations or in certain environmental conditions (Di Majo *et al.*, 2005).

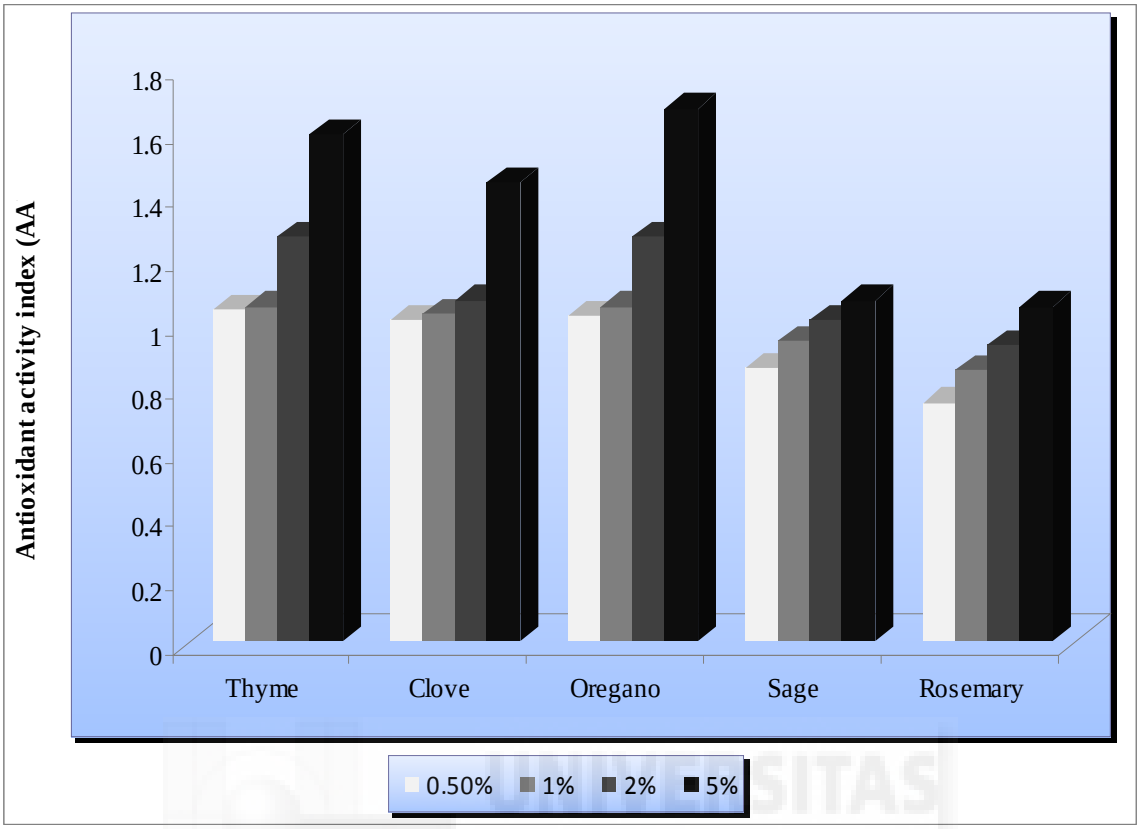

Figure 17. Antioxidant activity of different concentrations of oregano (*Origanum vulgare* L), thyme (*Thymus vulgaris* L), rosemary (*Rosmarinus officinalis* L), sage (*Salvia officinalis* L) and clove (*Syzygium aromaticum* L) essential oils as determined by the RANCIMAT method.

Although the antioxidant activity of spice EOs has been widely studied, their action mechanisms are still not understood completely. Several suggestions have been proposed in this respect, including the sequestering of free radicals, hydrogen atom donation, metal ion chelation and acting as substrate for different radicals such as superoxide and hydroxyl (Sebranek *et al.*, 2005).

5.5.- Characterisation of citrus fibre washing waters from the processes involved in obtaining fibre

The aim was to characterise physico-chemically and microbiologically the citrus fibre washing waters obtained as co-products after obtaining the fibre from oranges, by determining the pH, soluble solids, colour, residual nitrite reduction and total antioxidant activity, together with the content and concentration of phenolic compounds, organic acids and sugars. This work was published in *Journal of Food Processing and Preservation.*

Table 3 shows the pH, ºBrix and colorimetric parameters of citrus fibre washing water left after obtaining orange fibre

Table 3. pH, ºBrix and colour coordinates (Lightness (L*), redness (a*, ± red-green), yellowness (b*, ±yellow-blue) and the psychophysical parameter, Croma (C*)) of citrus fibre washing waters.

Sample	pH	ºBrix	Color			
			L*	a*	b*	C*
citrus fibre washing water	4.56±0.02	7.16±0.02	56.44±0.03	-6.83±0.03	34.03±0.05	34.71±0.06

Table 4 shows the mean values of the enterobacteria, coliform and aerobic mesophilic found in samples of citrus fibre washing waters. As can be seen, no enterobacteria or coliformes were detected, while the mean values of the mesophilic counts could be considered acceptable in all the samples analysed. Such low values would be related with the very lo pH values shown by the samples, which would hinder the growth of microorganisms.

Table 4. Levels of enterobacteria, coliform and aerobic mesophilic in citrus fibre washing water samples.

Sample	Enterobacteria log (cfu/g)	Coliform log (cfu/g)	Aerobic mesophilic log (cfu/g)
citrus fibre washing water	No detected	No detected	2.17±0.14

The chromatographic analysis of the citrus fibre waste water detected several peaks corresponding to phenolic compounds (see Figure 18, which also depicts their concentrations). Among them hydroxycinnamic acids such as caffeic, *p*-coumaric and ferulic acids. Also identified were the flavanones 7-*O*-neohesperidoside such as neohesperidin together with the flavanones 7-*O*-rutinoside such as eriocitrin, hesperidin and narirutin.

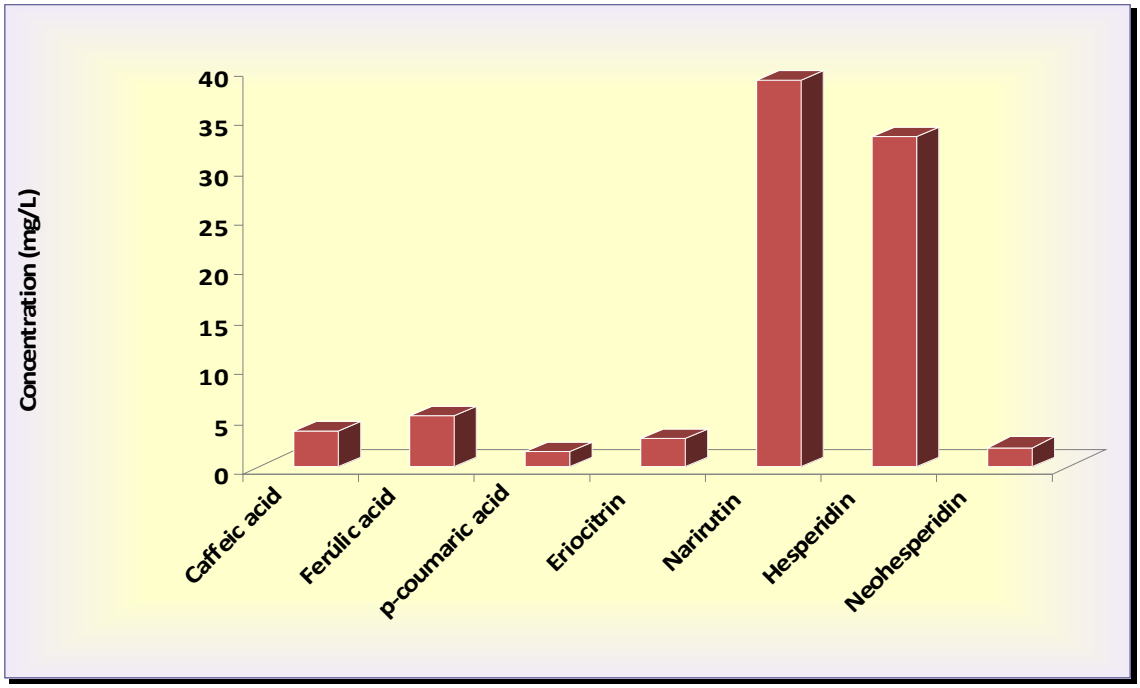

Figure 18. Type and concentration of phenolic compounds found in citrus fibre washing water

The principal peak was that of narirutin with a concentration of 38.91 mg/L; the next largest peak corresponded to hesperidin with a concentration of 33.91 mg/L.

A mean number of five organic acids were identified in the citrus fibre washing water samples: oxalic citric, ascorbic, succinic and acetic acids. Succinic acid being present in the highest concentrations (see Figure19).

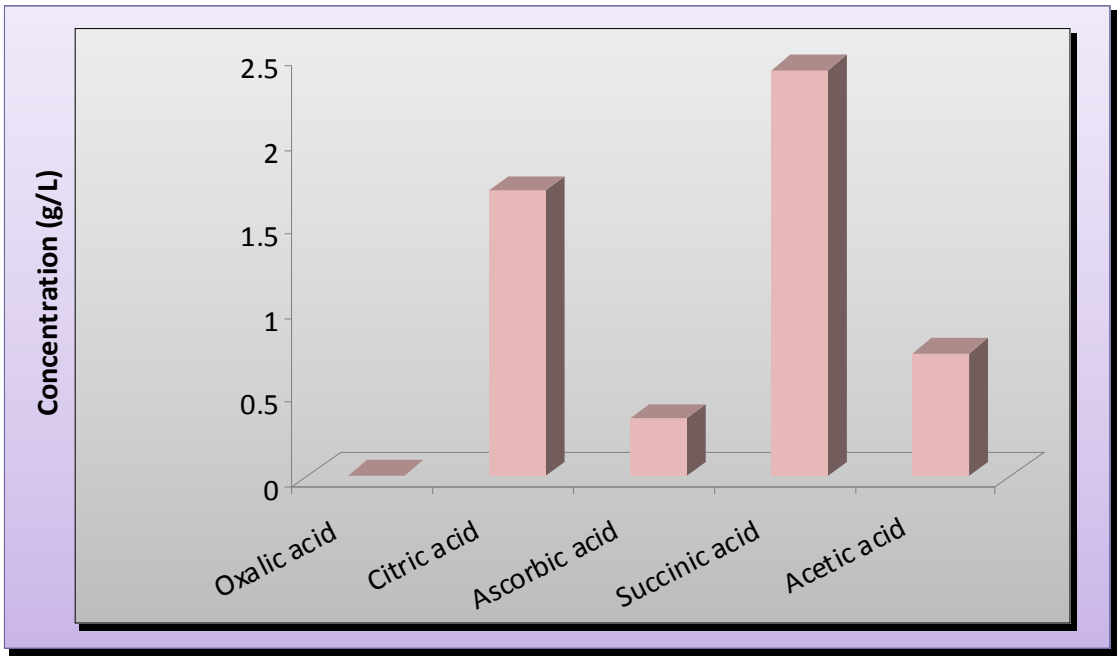

Figure 19. Mean concentration (g/L) of the organic acids found in the citrus fibre washing water.

As regards the sugars found in the citrus fibre washing water simples, the three principal ones were glucose; fructose and sucrose (see Figure 20). The same sugars are the principal sugars present in citric pulp and therefore in juices and extracts of the same. Glucose showed the highest concentration.

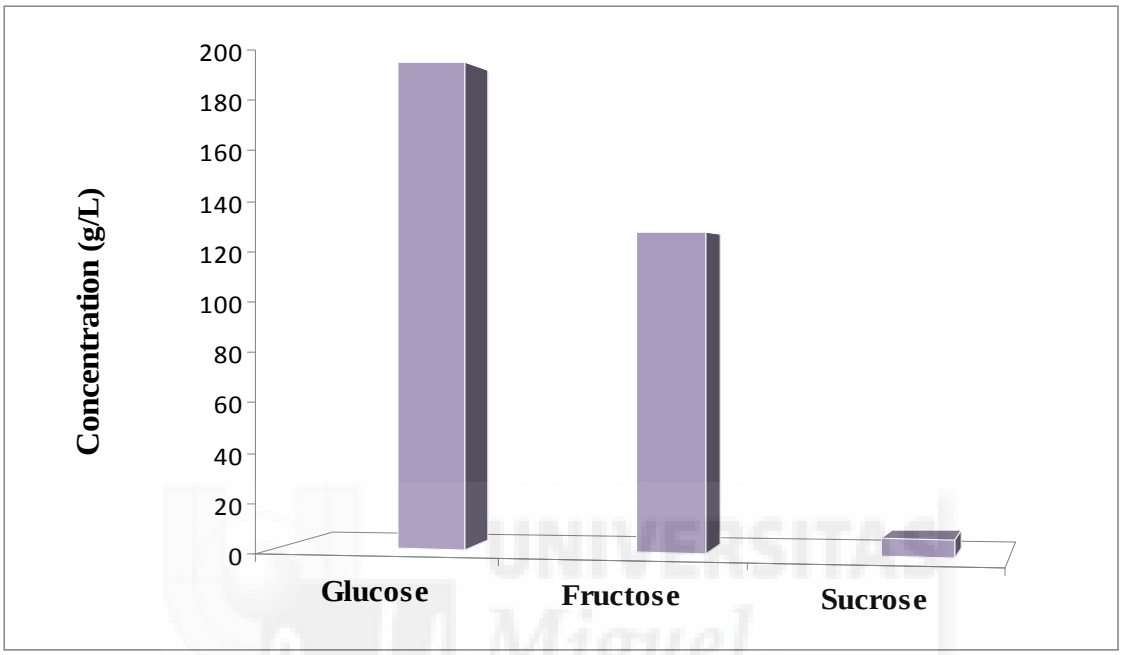

Figure 20. Mean concentration (g/L) of the sugars found in the citrus fibre washing water.

5.6.- Effect of adding citrus fibre washing water and thyme, rosemary and oregano essential oils on the chemical, physico-chemical and sensory characteristics of a cooked meat product type *mortadella*

We studied the effect of adding different concentrations of citrus fibre washing water (CFWW) (5%-10%), obtained as a co-product during the extraction of dietetic fibre, and/or (TEO) (0.02%) and/or rosemary (REO) (0.02%) and/or oregano (OEO) (0.02%) essential oils on the chemical, physico-chemical and sensory characteristics of a cooked meat product type *mortadella*. One part of this work was published in *Innovative Food Science and Emerging Technologies* (2009, *10*, 655-660), the other one was published in *LWT-Food Science and Nutrition* (2010, *43*, 958-963).

5.6.1.- Chemical properties

All the formulations assayed showed lower moisture and fat values than the control simple ($p<0.05$). The lower moisture content could be due to the fact that the citrus fibre washing water used instead of water contained 16 ºBrix dissolved soluble

solids. As regards the ash content, the values were higher than the corresponding control values ($p<0.05$) while the protein content was unaffected ($p>0.05$) by the addition of water and the spice EOs

As regards residual nitrite levels, the addition of CFWW and/or spice essential oils (thyme, oregano and/or rosemary) lead to a significant ($p<0.05$) decrease. Figure 21 shows the percentage reduction obtained. Note that as the concentration of the co-products increased, the residual nitrite levels fell in a concentration-dependent way. Mainly responsible for this effect was the citrus fibre washing water because when the EOs were added alone, the reduction was less pronounced. The synergism was confirmed by the observation that the reduction in residual nitrite levels obtained by adding both the citrus fibre washing water and EOs together was greater than the sum of the reductions obtained by adding citrus fibre washing water and EOs separately.

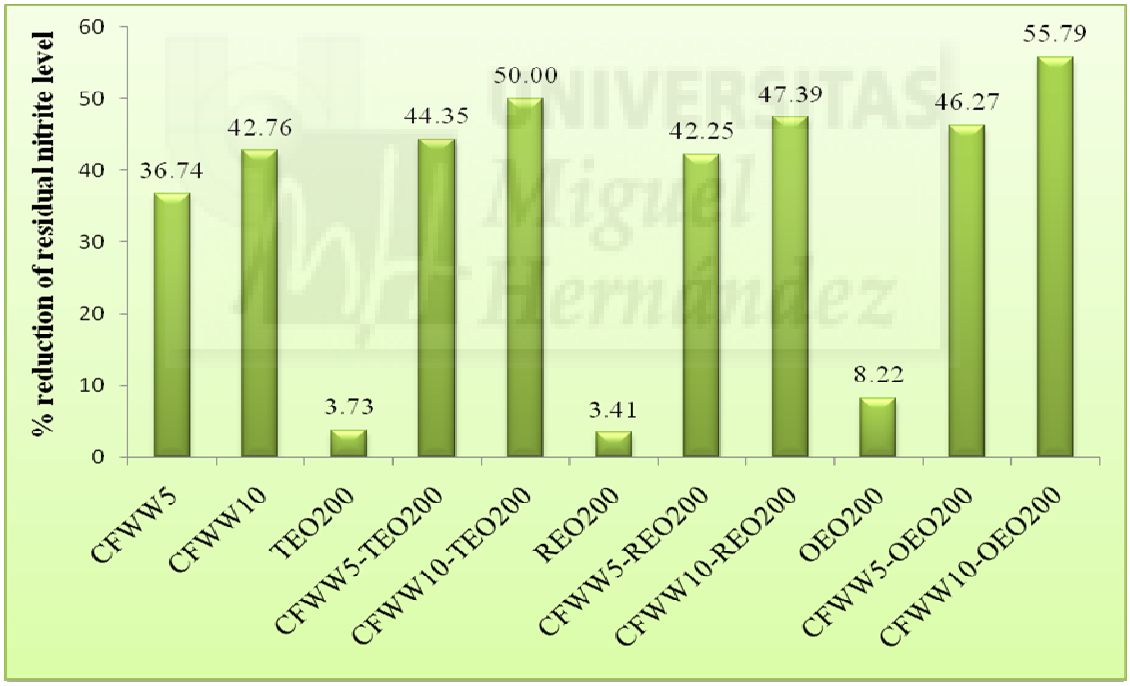

Figure 21. Percentages of reduction of residual nitrite in a cooked meat product to which citrus fibre washing water and/or spice essential oils (thyme, rosemary, oregano) were added. See Table 1 for identification of the formulas.

In the case of citrus fibre washing water and thyme EO, for the CFWW5-TEO200 sample, the reduction obtained was 9.58% higher than expected, while for CFWW10-TEO200 the reduction was 7.55% higher than expected. In the case of rosemary, the CFWW5-REO200 combination achieved a reduction that was 5.23% higher than expected, while CFWW10-REO200 achieved a reduction 2.64% higher than

expected. Finally in the citrus fibre washing water/oregano combinations CFWW5-OEO200 reduced the levels 2.91% more than expected and CFWW10-OEO200 9.43%.

This decrease in the residual nitrite levels would be due to the reactivity of the nitrite with the different bio-active compounds, mainly terpenes, polyphenols and flavonoids present in the citrus fibre washing water and different EOs.

Several studies have mentioned the reactivity of nitrite with phenolic-type compounds (Santhosh *et al.*, 2005; Balzer *et al.*, 2007), while others have mentioned more specific reactions with phenolic acids such as caffeic or ferulic acids (Krishnaswamy, 2001; Garrote *et al.*, 2004), both of which are present in the citrus fibre washing water, as can be seen from Figure 18. As will be seen below, these phenolic acids are not detected in the meat product to which the citrus fibre washing water is added, a loss which can be attributed to the reaction of these compounds with the components of the meat matrix, among them nitrite.

5.6.2.-Physico-chemical properties

In the case of colour, the addition of citrus fibre washing water and/or EOs of spices (thyme, oregano, rosemary) did not produce significant effects ($p>0.05$) compared with the control in any of the coordinates (L*, a*, b*) analysed, probably because both the citrus fibre washing water and EOs were perfectly integrated in the structural matrix of the meat emulsion, although there may have been some effect within the matrix on the part of the bioactive compounds dissolved therein.

All the samples showed mean values of L* (65.10±0.64), a* (8.90±0.25) and b* (7.31±0.26)

The addition of citrus fibre washing water and/or EOs of spices (thyme, oregano, rosemary) produced a slight decrease ($p<0.05$) in water activity compared with the control. The values obtained showed that the final products could be classified as having a medium moisture level, meaning that they would be stable at ambient temperatures.

The pH fell slightly ($p>0.05$) in the samples treated with fibre washing water and/or EOs of spices (thyme, oregano, rosemary) compared with the control, showing a mean value of 6.22±0.03.

Regarding texture assay (TPA). None of the texture parameters analysed (hardness, cohesiveness, springiness and chewiness) was affected ($p>0.05$) by the addition of citrus fibre washing water and/or EOs, the mean values being 1430.92±1.43 g for hardness, 0.52±0.00 for cohesiveness, 3.28±0.02 mm for springiness and 2445.44±25.20 g x mm for chewiness.

5.6.3.-Lipid oxidation

The lipid oxidation values, as determined by the TBA method, showed a decrease ($p<0.05$) in the degree of oxidation in all the formulas containing citrus fibre washing water and/or spice EOs compared with the control (see Figure 22 for the percentage decrease)

As occurred with the percentage of reduction in residual nitrite, the reduction in lipid oxidation, increase as the concentration of added co-product increased. In this case, though, the main agents responsible for the reduction were the essential oils, since their addition led to a greater decrease than was observed in the case of citrus fibre washing water.

Indeed, in this case, there was an antagonistic effect between the citrus fibre washing water and the EOs, since the reduction obtained when both were added jointly was less than the total of the reductions obtained when they were added separately. For example, in the case of samples to which citrus fibre washing water and thyme EO was added the reduction in lipid oxidation was 19.58% less than expected for CFWW5-TEO200, while for CFWW10-TEO200 a reduction of 26.95% below the expected value was obtained.

When the antagonistic effect of citrus fibre washing water and rosemary EO was analysed, the reductions were 30.01% and 24.96% less than expected in the case of CFWW5-REO200 and CFWW10-REO200, respectively.

Finally, when citrus fibre washing water and oregano EO were added, the reduction was 24.97% less than expected for CFWW5-OEO200 and 6.94% less than expected for CFWW10-OEO200.

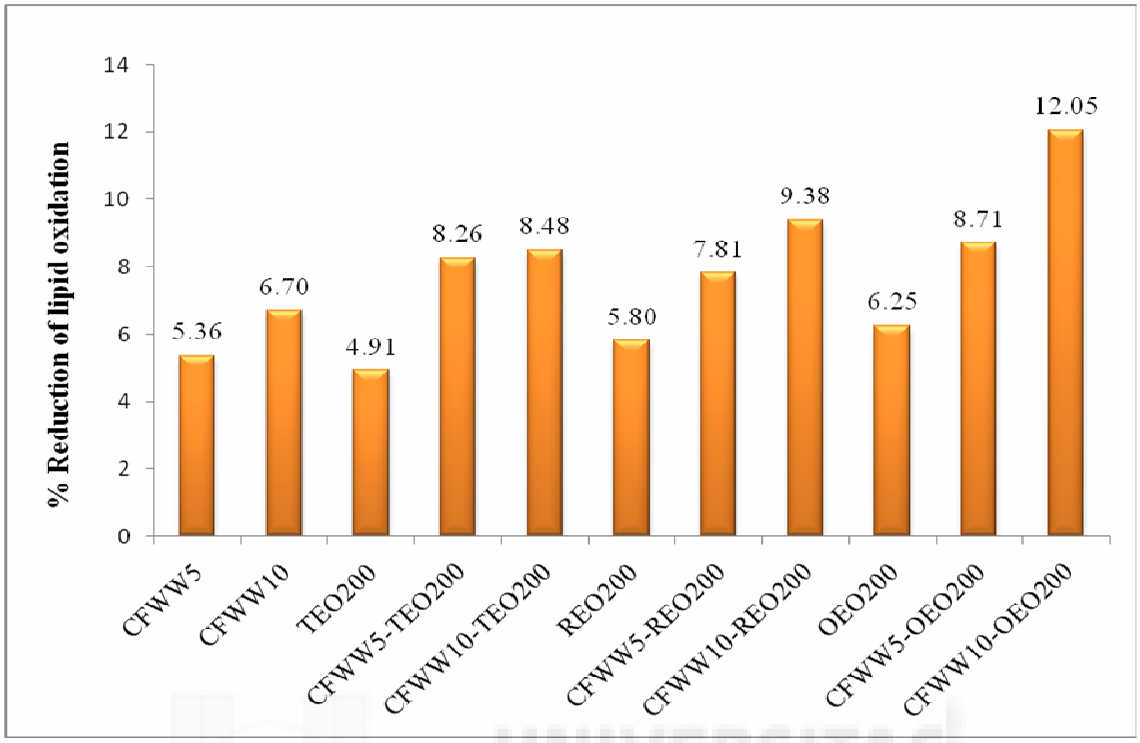

Figure 22. Percentage of reduction in the degree of oxidation recorded in a cooked meat product to which citrus fibre washing water and/or essential oils of thyme, rosemary and oregano were added.

Many of the functional properties of both citrus fibre washing water and essential oils are associated with the presence of phenolic and terpenic compounds they contain. Phenolic compounds and flavonoids have been seen to possess substantial antioxidant activity, mainly due to the redox properties of the hydroxyl groups in the phenolic ring and the structural relations between the different parts of its chemical structure (Balasundram *et al.*, 2006).

The antioxidant activity of spices, since it is the result of phenolic compounds, is influenced by many factors (Burda and Oleszek, 2001), such as:

- The system or matrix in which they are incorporated (interactions between components, temperature, pH and the concentration of the components).
- Their hydrophobic/hydrophilic character.
- The localisation and number of hydroxyl groups in the aromatic ring.

The antioxidant activity of by-products from the "orange industry" has been widely demonstrated (Fernández-Gines *et al.*, 2004; Fernández-López et *al.*, 2007) and is basically due to the fruit's composition in which phenolic compounds predominate.

The components of essential oils such as oregano, thyme and rosemary also possess antioxidant activity (Youdim et *al.*, 2002; Kulisic *et al.*, 2004, Fasseas *et al.*, 2008).

However, the exact mechanism that provokes this antioxidant activity on the part of the compounds is still unclear, although various mechanisms have been proposed, among them free radical sequestration, hydrogen donation, metallic ion chelation or even acting as substrates for radicals such as superoxide and hydroxyl (Al-Mamary *et al.*, 2002; Amarowicz *et al.*, 2004). These bioactive compounds with antioxidant properties can also interfere with oxidation propagation reactions (Russo *et al.*, 2000), and inhibit the enzymatic systems involved in initiation reactions (You *et al.*, 1999).

5.6.4.-Phenolic compounds

Table 5 shows the flavonoids present in the different formulations. In the samples to which citrus fibre washing water was added the only phenolic compounds identified were narirutin and hesperidin. The concentration of these compounds in the samples depended on the percentage of citrus fibre washing water added. For example, with 5% added citrus fibre washing water, the concentration of hesperidin ranging 10.18 to 10.44 µg/g ($p>0.05$), while at 10% the concentrations varied from 22.61 to 22.68 µg/g sample ($p>0.05$).

Table 5. Concentration of phenolic compounds (hesperidin and narirutin) in a cooked meat product to which citrus fibre washing water and spice (thyme, rosemary, oregano) essential oils were added.

Formulation	Hesperidin (µg/g sample)	Narirutin (µg/g sample)
CFWW5	10.44	5.75
CFWW10	22.61	12.20
CFWW5-TEO200	10.18	5.71
CFWW10-TEO200	22.68	12.21
CFWW5-REO200	10.34	5.90
CFWW10-REO200	22.63	12.31
CFWW5-OEO200	10.36	5.85
CFWW10-OEO200	22.56	12.29

Narirutin showed lower concentrations varying ($p>0.05$) between 5.75 and 5.90 µg/g in samples with 5% added citrus fibre washing water to 12.20 and 12.29 µg/g in the formulations containing 10% added citrus fibre washing water.

The most abundant compounds in citrus fruit are glycosylated flavones and polymethoxyflavones (Kawaii *et al*., 1999), while the major components in oranges are hesperidin, narirutin and didimin (Peterson *et al*. 2006). Of these, only hesperidin and narirutin were identified in our samples. The rest of the phenolic compounds identified in the citrus fibre washing water, including caffeic, *p*-coumaric and ferulic acids, eriocitrin and neohesperidin (Figure 18), and the major phenolic compounds of essential oils (carvacrol and *p*-cymene on oregano EO, 4-terpinenol and γ-terpinene in thyme EO and β-pinene y α-pinene on rosemary EO) were not detected in any sample.

These must have been lost in the different stages of the elaboration process (i) I the technological processing or (ii) in reactions with the components present in the samples, such as nitrite, as mentioned above, or intervening in oxidation inhibition reactions.

5.6.5.-Sensory evaluation

Figure 23 shows the results of the sensory analysis for the control samples and those to which citrus fibre washing water at 5 or 10% had been added with or without the essential oils of rosemary, thyme and oregano. In general terms, the control sample had the highest score, while the sample with 10% citrus fibre washing water and oregano EO scored lowest. As regards shine, there were differences between the results of the panel and those measured instrumentally, the panellists observing greater lightness in the control, while the lightness measured instrumentally were similar for all the formulations.

As regards odor, the strong spicy aroma was not disliked by the panellists and the formulations containing the different EOs were not marked much differently from the others. As regards the acid taste, the salty taste and fatty characteristics were marked similarly in all the samples. In the case of fatness, the sensory analysis coincided with the chemical analysis as, as mentioned, no differences were detected between the different formulations. The sensory and chemical analyses also agree as regard hardness.

The samples added with 5% citrus fibre washing water and 0.02% thyme or oregano or rosemary EOs (CFWW5+OEO200; CFWW5+TEO200 and CFWW5+REO200, respectively) were the most appreciated by the panellists.

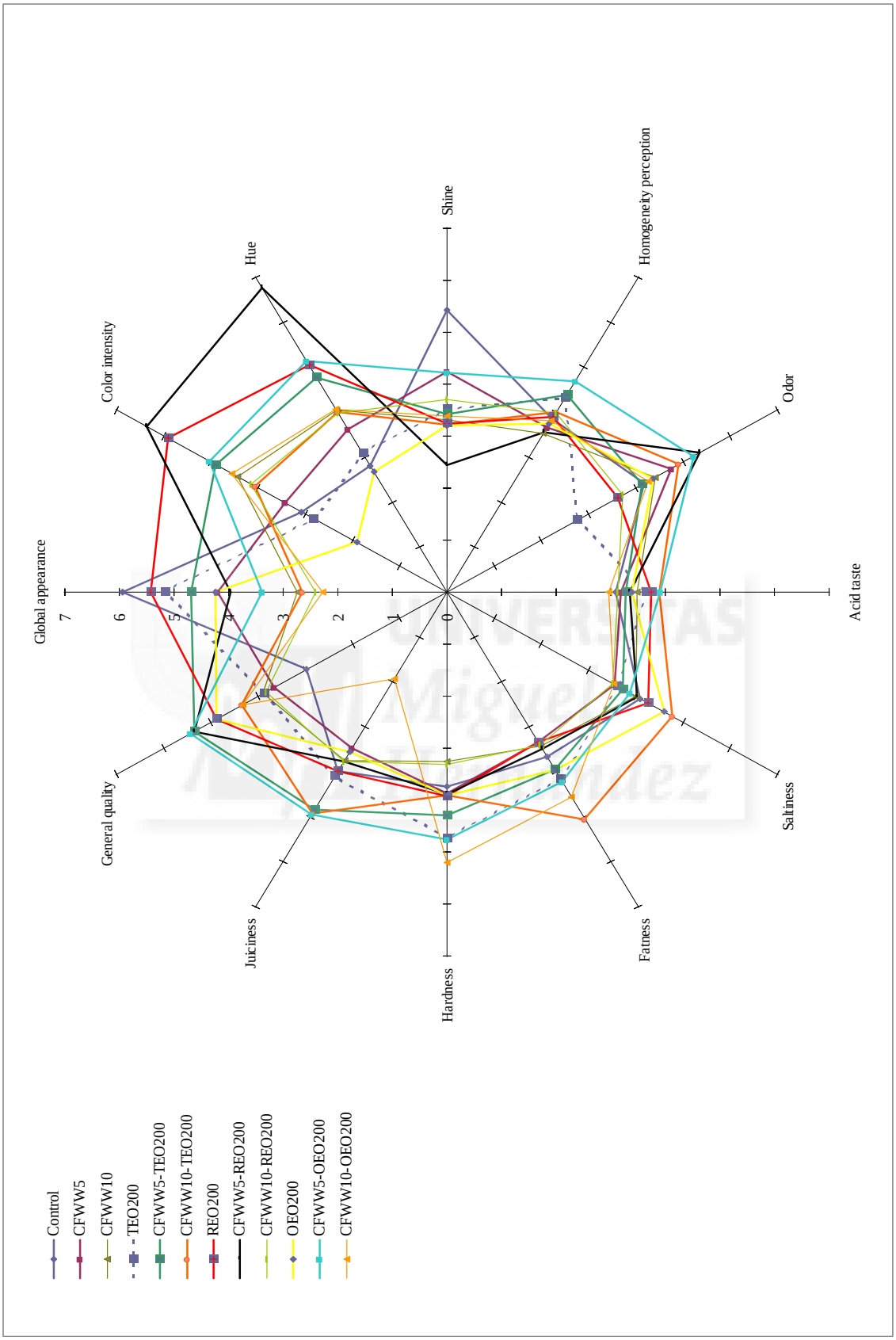

Figure 23: Results of the quantitative descriptive analysis of the different formulations analysed to which citrus fibre washing water and thyme, rosemary and oregano essential oils had been added.

5.7.-Effect of packaging conditions on the shelf life of a cooked meat product type *mortadella* added with citrus fibre washing water and/or oregano, thyme or rosemary essential oils.

The aim of this work was to study the effect of (i) the addition of citrus fiber washing water (CFWW) and/or rosemary essential oil (REO) and/or thyme essential oil (TEO) and/or oregano essential oil (OEO); (ii) the packaging conditions and (iii) storage time on the physico-chemical, microbiological and sensory characteristics of a cooked meat product type *mortadellas*. One part of this work is under review in the journal *Food Research International*. The other one was published as chapter in the book *Total Food. Sustainability of the Agri-Food chain*.

5.7.1.-Physico-chemical properties

As regards colour, lightness (L*) was not affected ($p>0.05$) by any of the factors studied: packaging, treatment or time. The values of L* varied from 64.65; 64.63; 64.23 and 64.29 in the control, CFWW+TEO, CFWW+REO and CFWW+OEO, respectively at the beginning of storage (day 0) to 65.71-65.83 (depending on packaging) in the control; 65.34-65.40 (depending on packaging) for CFWW+TEO; 65.28-65.36 (depending on packaging) for CFWW+REO and 65.33-65.44 (depending on packaging) for CFWW+OEO at the end of the study.

Packaging had no significant effect ($p>0.05$) on the red-green coordinate (a*), either, although the time of storage did ($p<0.05$) in all the treatments and all types of packaging assayed. In this case the initial values of 9.33, 9.20, 9.10 and 9.21 in the control, CFWW+TEO, CFWW+REO and CFWW+OEO, respectively, fell to 6.06-6.21 (depending on packaging) in the control, 6.22-6.69 (depending on packaging) in CFWW+TEO, 6.12-6.62 (depending on packaging) in CFWW+REO and 6.17-6.49 (depending on packaging) in CFWW+OEO.

The yellow-blue coordinate (b*) was unaffected ($p>0.05$) packaging type and storage, but, contrary to that observed for a*, the storage time provoked a significant ($p<0.05$) increase for all the treatments and packaging types. The values of b* varied from 7.27; 7.31; 7.23 and 7.23 in the control, CFWW+TEO, CFWW+REO and CFWW+OEO, respectively at the beginning of storage (day 0) to 8.65-8.74 (depending on packaging) in the control, 8.48-8.55 (depending on packaging) in CFWW+TEO

8.34-8.46 (depending on packaging) in CFWW+REO and 8.59-8.62 (depending on packaging) in CFWW+OEO at the end of the time studied.

Of the different parameter analysed for the texture profile (hardness, cohesiveness, springiness and chewiness), none was significantly affected ($p>0.05$) by any of the three factors studied: packaging, treatment and storage time.

As regards the pH, the type of packaging and treatment had no significant effect ($p>0.05$) on this parameter in any of the groups of sample analysed although storage time had the greatest effect: in the control, CFWW+TEO, CFWW+REO and CFWW+OEO samples, the pH fell from 6.30; 6.26, 6.28 and 6.29, respectively, at the beginning of storage (day 0) to 6.15-6.16 (depending on packaging) in the control, 6.17-6.18 (depending on packaging) in CFWW+TEO, 6.16 (depending on packaging) in CFWW+REO and 6.15-6.17 (depending on packaging) in CFWW+OEO at the end of the assay (day 24). The continuous reduction is probably due to the gradual growth of lactic bacteria, which generate lactic acid, which, in turn, is responsible for the reduction in pH.

5.7.2.-Lipid oxidation

Figure 24 shows the effect of packaging conditions, the addition of citrus fibre washing water and spice EOs and the storage time on lipid oxidation in the *mortadellas* studied. At the beginning of storage (day 0), the samples with added citrus fibre washing water and spice EOs showed a lower degree of oxidation than the control samples, although the differences were not significant ($p>0.05$).

After 6 days of storage, the lowest oxidation values were observed in the vacuum packed CFWW+TEO, CFWW+REO and CFWW+OEO samples (5.32, 5.30 and 5.28 mg MAD/kg sample, respectively).

After 12 days of storage, the same samples continued to show the lowest oxidation values ($P<0.05$), although there was no significant difference ($p>0.05$) between the control and the CFWW+TEO sample packed in modified atmosphere.

At the end of the period studied (24 days) the vacuum packed CFWW+TEO, CFWW+REO and CFWW+OEO samples still shoed the lowest degree of oxidation ($P<0.05$), while those packed in air showed the highest degree.

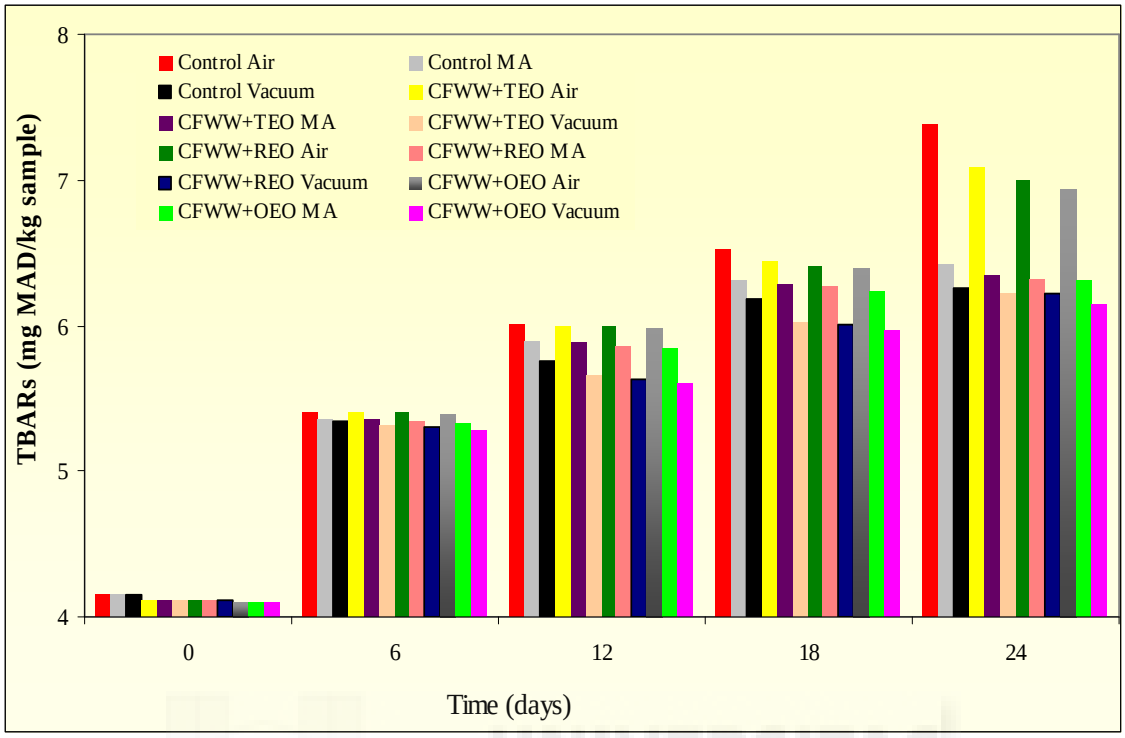

Figure 24: Evolution of TBARS in a cooked meat product with added citrus fibre washing water and thyme or rosemary or oregano essential oils, packed in air, modified atmosphere or vacuum and exposed to light for 24 hours.

According to the data, packaging had a very clear effect, the samples packed in air (one of the principal agents responsible for oxidation) showing the highest degree of oxidation. The combined effect of vacuum packaging and the bioactive compounds present in citrus fibre washing water and spice EOs, basically phenolic acids and flavonoids (as mentioned in section 5.6.4.) was responsible for the lesser degree of oxidation observed in these samples.

The solubility of flavonoids in oils and fats is low and the role that these compounds play in oxidation is not significant. However, in the case of emulsions they actively contribute to diminishing the degree of oxidation (Zhou, *et al.*, 2005). Flavonoids act more or less as antioxidants in accordance with their chemical structure (Choe and Mind, 2009), concentration, temperature, light, type of substrate, the physical state of the system in which they are found and any interaction with the components of the system itself (Yanishlieva-Maslarova, 2001). Another factor intervening in their acting as antioxidants is the presence in the system of any other substance with this capacity, but a different action mechanism (Decker, 2002).

5.7.3.-Phenolic compounds

In all the simples analysed, except the control samples, the only phenolic compounds identified were narirutin and hesperidin, the latter in greater concentrations. In the samples with added citrus fibre washing water and spice EOs both time and the type of packaging affected the concentration of these two compounds in the resulting meat product.

At day 0, there were no statistically significant ($p>0.05$) differences in the levels of hesperidin and narirutin between the samples with added citrus fibre washing water and spice EOs for any of the packaging methods used (air, MAP and vacuum).

When the effect of time was analysed, the concentration of both hesperidin and narirutin fell as storage progressed, and statistically significant differences ($p<0.05$) were observed between the levels recorded on days 6, 12, 18 and 24, falling in the case of hesperedin from 10.44, 10.36 and 10.39 µg/g sample for CFWW+TEO, CFWW+REO and CFWW+OEO to 8.54-9.01; 8.49-8.98 and 8.51-9.04 (depending on the packaging) at the end of the assay. In the case of narirutin, the initial values of 5.77, 5.81 and 5.85 µg/g simple in CFWW+TEO, CFWW+REO and CFWW+OEO, fell 3.86-4.12; 3.91-4.16 and 3.80-4.18, (depending on the packaging) at the end of the assay.

With regard to the type of packaging, the samples exposed to air showed lower values of hesperidin and narirutin than those packed in MAP or vacuum. The samples packed in the last mentioned packaging showed the highest levels of both compounds, probably due to the degree of oxidation and the protective effect of the flavonoids. In the air-packed samples, polyphenols would have reacted more rapidly against the free radicals produced - hence their reduction and lower concentrations. This would corroborate the data obtained for the hesperidin concentrations observed in MAP and vacuum packaging, where a correlation between the degree of oxidation and the concentration of flavonoids was once again evident.

5.7.4.-Sensory analysis

Vacuum packed CFWW+TEO was best evaluated ($P<0.05$) for its overall appearance by the panellists, while the control samples packed in air or MAP obtained the worst results in this respect ($P<0.05$). Scores for the acid taste, salty taste and fatty character were very similar ($P>0.05$) for all the samples analysed, regardless of the type

of packaging used. Despite the strong spicy aroma, this was not found unpleasant by the panellists, who marked the formulations containing the different EOs very similarly (P>0.05) to the rest of the samples. Vacuum packed samples containing citrus fibre washing water and/or thyme, rosemary or oregano were best marked by the panellists, with scores of 4.84, 4.93 and 4.76, respectively.

5.7.5.-Micobiological analysis

Enterobacteria or psycotrophic bacteria were not detected in any of the control samples or those with added citrus fibre washing water and/or thyme, oregano or rosemary EO in any of the storage conditions (air, MAP, vacuum) at any time of the storage period (24 days), probably due to three reasons: (i) the effectiveness of the heat treatment, (ii) the aseptic slicing process and (iii) the presence of the sodium chloride contained in the products

Figures 25 and 26 show the effect of adding citrus fibre washing water and spice EOs, the packaging conditions and storage time on the growth of aerobic bacteria and lactic acid.

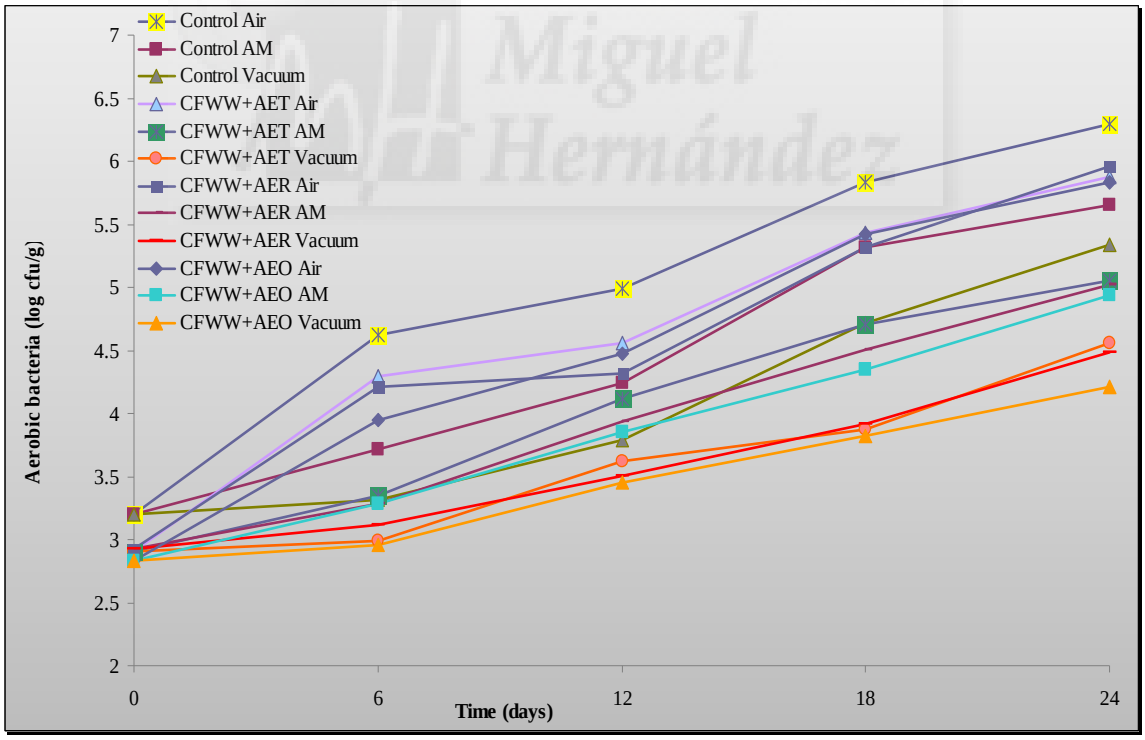

Figure 25. Evolution of aerobic bacteria counts in a cooked meat product, added with citrus fibre washing water and thyme, rosemary or oregano essential oils, packed in air, modified atmosphere or vacuum and exposed to light for 24 hours.

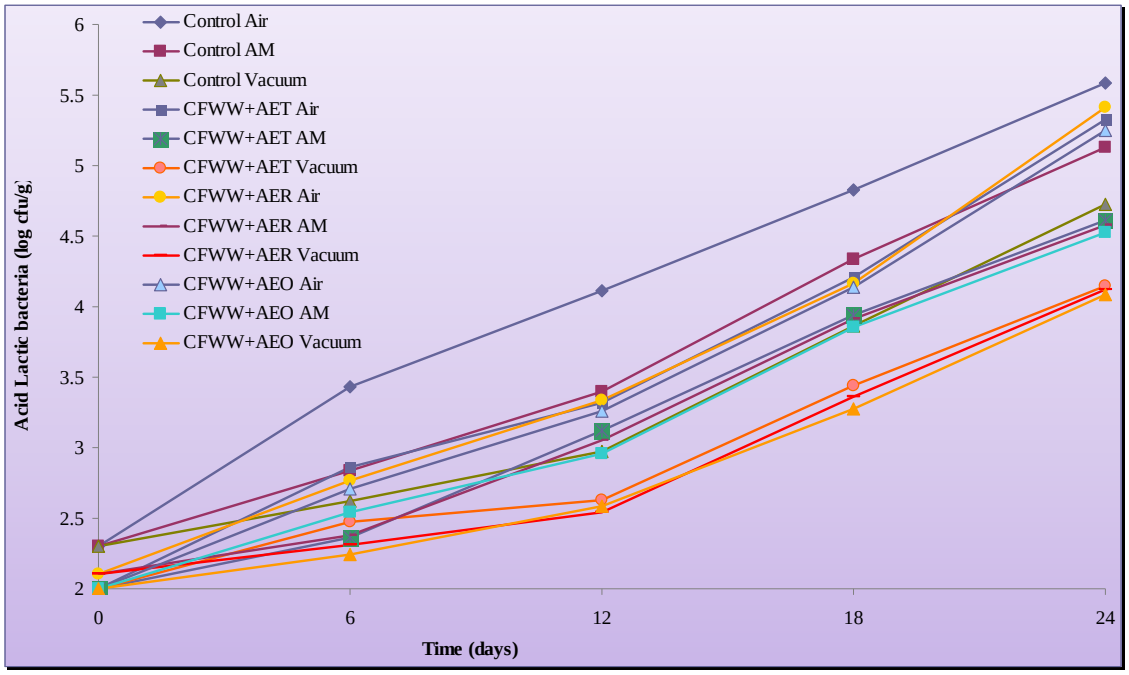

Figure 26. Evolution of lactic acid bacteria counts in a cooked meat product, added with citrus fibre washing water and thyme, rosemary or oregano essential oils, packed in air, modified atmosphere or vacuum and exposed to light for 24 hours.

At the beginning of storage time (day 0), the samples with added CFWW+TEO, CFWW+REO or CFWW+OEO showed lower lactic acid bacteria and aerobic bacteria counts ($p<0.05$) than the control sample in all three types of packaging, with no significant difference ($p>0.05$) between them.

After 12 days of storage, all the vacuum packed samples, both control and those with added CFWW+TEO, CFWW+REO or CFWW+OEO showing the lowest levels of lactic acid and aerobic bacteria growth.

At the end of the study (day 24) the vacuum packed CFWW+TEO, CFWW+REO and CFWW+OEO samples showed the lowest levels ($p<0.05$) lactic acid and aerobic bacteria growth, again with no significant differences between them ($p>0.05$), while the control samples packed in air showed the highest counts of lactic acid and aerobic bacteria.

The combination of 0.87 to 0.89 water activity, a pH of around 6, heat treatment and subsequent storage at 4ºC seems to be sufficient to obtain microbiologically stable cooked products suitable for storing for up to 24 days. In all the samples and in all the packaging conditions, the total aerobic and lactic bacteria counts at the end of 24 days storage were below those considered necessary to cause degradation in this type of product.

CONCLUSIONES

6.- CONCLUSIONS

1. The six spices studied show widely varying compositions. In five of them (oregano, thyme, sage, rosemary and cumin), between 26 and 52 different compounds were identified, while only five were identified in clove. The compounds can be grouped into two large groups, the first mainly composed of terpenes and terpenoids (carvacrol, camphor, γ-terpinene, β-pinene and α-pinene) and the other by aromatic and aliphatic constituents (eugenol).
2. Of the six spices studied, the EOs of sage and rosemary showed the lowest *in vitro* antifungal activity against *Aspergillus niger* and *Aspergillus flavus,* concentrations ten-fold those needed for the other EOs being necessary to obtain similar results in this respect. The EOs of oregano and clove were the best inhibitors of both moulds, and both could be used as antifungal agents in the food industry to replace synthetic ones.
3. All the spice EOs tested showed antibacterial activity *in vitro* both against bacteria used as starter cultures in food manufacture (*Lactobacillus curvatus, Lactobacillus sakei, Staphylococcus carnosus* and *Staphylococcus xylosus),* and against those involved in food spoilage (*Enterobacter gergoviae* and *Enterobacter amnigenus).* Oregano EO was the strongest inhibitor of both bacteria groups and rosemary EO the weakest. However, for spice EOs can be used in the food industry, it should be borne in mind that its efficiency is reduced in complex food matrices,
4. All the EOs assayed had antioxidant activity. Clove and oregano showed the highest antioxidant capacity for the five methods used to evaluate the same (H^+ donating capacity, metal chelating capacity, iron reducing capacity, peroxidation inhibition and reduction in lipid oxidation induction time). Sage EO had the lowest antioxidant effect. All the EOs could be used in the food industry as long as they do not negatively affect the sensory characteristics of the products in which they are incorporated.
5. The citrus fibre washing water obtained as by-product during the process to obtain fibre can be considered a potential ingredient in the manufacture of meat products, mainly because the bioactive compounds they contain (principally the flavonoids narirutin and hesperidin) reduce the levels of residual nitrite and oxidation.

6. The use of such citrus fibre washing waters and the EOs of rosemary, oregano and thyme in the manufacture of *mortadella* type cooked products has a synergistic effect in reducing the residual nitrite levels but has an antagonistic effect on oxidation processes. Despite this, the incorporation of these ingredients diminishes oxidation of the meat products. In our assay, the chemical and physico-chemical properties of the resulting meat products did not differ from those of the control products, but scored better in the sensory analysis.
7. The incorporation of the above mentioned products and compounds in *mortadella* type cooked products, together with vacuum packaging prolongs their shelf-life. This combination of factors seems to be sufficient to obtain microbiologically stable cooked meat products which can be stored for up to 24 days.

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7.- BIBLIOGRAFIA

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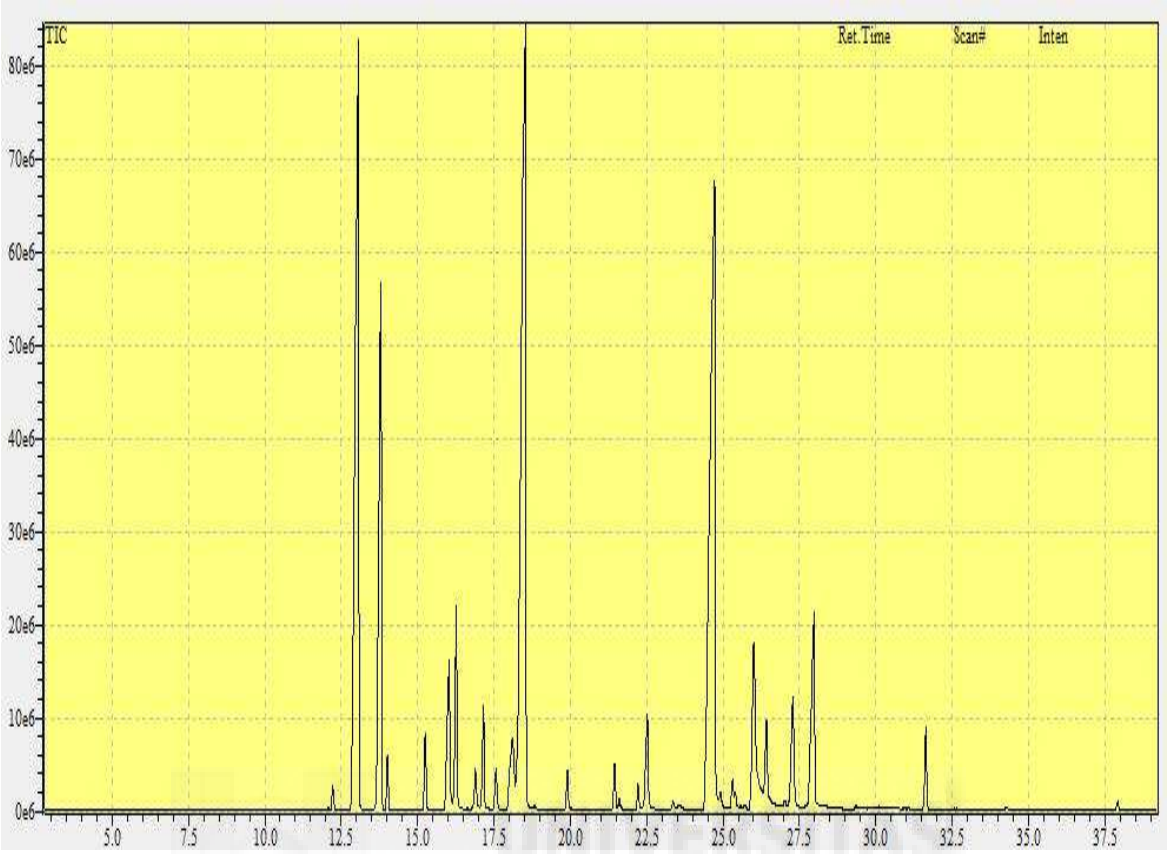

TITULO:	Chemical Composition of the Essential Oils Obtained From Some Spices Widely Used in Mediterranean Region
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Chemical Composition of the Essential Oils Obtained From Some Spices Widely Used in Mediterranean Region

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Abstract

Spices are widely used in the countries of Southern Europe and North Africa where they play a central role in the Mediterranean diet. Spices are used for their flavour and aroma and also for the sensations that they produce; they can be used as colouring as for their nutritional and antioxidant properties.

The aim of this work was to determine the chemical composition of the essential oil of six spices widely used in the Murcia Region (Spain): oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), cumin (*Cuminum cyminum*) and clove (*Syzygium aromaticum* L). Essential oils were chemically analysed and identified by GC-MS.

The principal components of sage essential oil were camphor (24.95%), 1,8-cineole (24.75%) and camphene (7.63%). Major oil components of oregano included carvacrol (61.21%) and p-cymene (15.12%). The essential oil of thyme was characterized by a high content of terpinen-4-ol (13.15%), γ-terpinene (9.21%) and cis-sabinene hydrate (7.65%). The predominant compounds in clove essential oil were eugenol (85.5%), β-caryophyllene (10.54%) and α-humulene (3.12%) while β-pinene (12.75%), α-pinene (36.42%) and camphor (15.65%) were the main constituents of rosemary essential oil. Cumin essential oil was manly composed of γ-pinene (27.4%), p-cymene (20.49%) and cuminal (20.39%).

Keywords: Essential oil, spices, chemical composition, GC/MS.

1. Introduction

Spice production in Mediterranean countries is approximately 38 million tonnes per year, with Turkey being the highest producer. Other countries, too, show impressive production figures but are normally dedicated to one specific spice; for example, in Spain, paprika is the most important spice crop.[1]

Spices are aromatic plant products which are frequently used to enhance food palatability. Most spices were originally indigenous to the tropics; for instance, cinnamon, pepper, clove and nutmeg. However, Mediterranean countries have also provided a number of aromatic seeds (coriander, mustard) and other spices such as bay leaf, thyme and oregano.[2] At present, about 44000 ha are dedicated to the production of spices in countries bordering the Mediterranean Sea.

Many essential oils and extracts obtained from spices and plants have recently gained in interest both or the general population and for the scientific community.[1] Many plants are used for different purposes, for example, in the food, drugs and perfumery sectors. Several researchers have shown interest in biologically active compounds isolated from plants and spices for eliminating pathogenic microorganisms because of the resistance that many microorganisms have built up to antibiotics.[3]

Culinary spices and herbs contain a wide variety of active phytochemicals (including flavonoids, terpenes, polyphenols, curcumins, coumarins) and may fulfil more that one function in any food to which they are added.[4] Spices also contain fibre, proteins, sugars, cations and pigments (carotenoids, chlorophylls, etc.). Phenolic compounds, as are vanillin, gallic acid, caffeic acid, etc. are involved in olfactory, taste and tactile

sensations and volatile compounds such as essentials oils.[5]

The aim of this work was to determine the chemical composition of the essentials oils from several spices widely used in Mediterranean countries: oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), cumin (*Cuminum cyminum*) and clove (*Syzygium aromaticum*).

2. Experimental

2. 1. Essential Oils

The essential oils of thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), clove (*Syzygium aromaticum* L.), rosemary (*Rosmarinus officinalis* L.), cumin (*Cuminum ciminum* L.*)* and oregano (*Origanum vulgare* L.) were obtanined by steam distillation, and were purchased from Ravetllat Aromatics, (Barcelona, Spain). The following authentic compounds were employed as standards in the gas-chromatography analyses: camphene, p-cymene, eugenol, (+)-limonene, α-pinene, β-pinene, α-terpinene, 1,8-cineole, thymol, linalool and carvacrol (Extrasynthese, Lyons, France).

2. 2. Analysis Conditions

2. 2. 1. Gas Chromatography Analysis(GC)

The essentials oils were analysed using a Shimadzu GC-17A equipped with FID detector and HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 250 and 270 °C, respectively. Oven temperature was kept at 50 °C for 3 min, then gradually raised to 240 °C at 3 °C/min. Helium was the carrier gas, at a flow rate of 0.8 mL/min. Diluted samples (1/10 acetone, v/v) of 0.2 μL were injected manually in the split mode (split ratio 1/44). Quantitative data were obtained electronically from FID area data without using correction factors. All the tests were performed in triplicate.

2. 2. 2. Gas Chromatography/mass Spectrometry Analysis (GC/MS)

Analysis of the essentials oils was performed using a Shimadzu GC-17A equipped with a Shimadzu GCMS-QP5050A mass selective detector and a HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). For GC/MS detection, an electron ionization system with an ionization energy of 70 eV was used. Helium at a flow rate of 0.8 mL/min was used as carrier gas. Injector and MS transfer line temperatures were set at 250 and 270 °C, respectively. Oven program temperatures was the same as for the GC analysis. Diluted samples (1/10 acetone, v/v) of 0.2 μL were injected automatically in the split mode (split ratio 1/44). The components were identified by comparing their relative retention times and mass spectra with those of standards (for the main components), Wiley 229 library data of the GC/MS system, Kovats Index and literature data.[6] All the tests were performed in triplicate.

3. Results and Discussion

3. 1. Chemical Composition of the Essential Oil

The chemical composition of the essential oil of *Thymus vulgaris* L., *Salvia officinalis* L., *Syzygium aromaticum* L., *Rosmarinus officinalis* L., *Cuminum cyminum* L. and *Origanum vulgare* L. was studied. The main constituents of each oil, their relative percentage of the total chromatogram area, Kovats index and retention times are summarized in Table 1.

The six essential oils analyzed varied greatly in composition. Some components were common to several oils, but were present in large amounts in only a few oils, whereas other components were found just in one oil. For example, α-pinene was present in all the essential oils except clove, but its percentage only exceeded 35% in rosemary. In the essential oil of thyme, 52 compounds were identified, representing 91.4% of the total oil, the major constituents being terpinen-4-ol (13.15%), γ-terpinene (9.21%), cis-sabinene hydrate (7.65%), linalool (7.12%) and p-cymene (5.75%). Tomaino et al.[7] reported that the major constituents of thyme essential oil were thymol (45.3%), p-cymene (26.1%) and linalool (6.17%). Several early studies on *Thymus* spices suggested that the main components of the oils were terpinen-4-ol, γ-terpinene, p-cymene in *T. baeticus*,[8] carvacrol, γ-terpinene and p-cymene in *T. revolutus*,[9] 1,8 cineole and linalool in *T. mastichina*,[10] p-cymene and carvacrol in *T. capitatus*,[11] thymol and p-cymene in *T. daenensis* and thymol, carvacrol and *p*-cymene in *T. kotschyanus*,[12] thymol, carvacrol and *p*-cymene in *T. spathulifolius*.[13]

This great variability and diversity observed, in the chemical composition of the essential oils of *Thymus* species and subspecies can be attributed to climatic and soil variations, stage of the vegetative cycle, seasonal variation, etc.[14] In some cases, two different varieties may provide the same essential oil yield and quality, even though the plants are morphologically different.[15] Some studies have reported that thyme essential oil possesses a high level of the phenolic precursors, p-cymene and γ-terpinene, probably due to its early flowering time.[16]

When the essential oil of oregano was analyzed by GC-MS 32 compounds were identified, representing 88.5% of the total oil, the major constituent being carvacrol (61.21%). Other important compounds were p-cymene (15.12%) and γ-terpinene (4.80%). Sezik et al.[17] investigated the essential oil composition of four subspecies of

Table 1. Constituents of sage, thyme, rosemary, cumin, clove and oregano and their relative percentages of total chromatogram area, Kovats Index and retention time.

Compounds	KI[a]	Essentials oils					
		Sage (% area)	Thyme (% area)	Rosemary (% area)	Cumin (% area)	Clove (% area)	Oregano (% area)
α-thujene	928	Tr.	2.10	0.15	0.30	–	0.07
α-pinene	936	6.75	5.16	36.42	0.76	–	2.34
camphene	951	7.63	1.30	11.08	Tr.	–	0.30
sabinene	974	0.09	1.66	–	0.26	–	–
β-pinene	977	5.19	0.65	3.67	12.75	–	0.44
β-myrcene	992	1.58	2.69	2.19	0.67	–	1.45
α-terpinene	1019	–	4.05	Tr.	0.16	–	0.56
p-cymene	1026	1.60	5.79	2.14	20.49	–	15.12
limonene	1031	–	5.09	–	0.56	–	1.41
1,8-cineole	1034	24.74	2.93	12.02	0.23	–	1.11
γ-terpinene	1060	1.04	9.21	0.18	27.44	–	4.80
cis-sabinene hydrate	1070	–	7.65	Tr.	Tr.	–	–
terpinolene	1089	0.67	1.56	0.27	0.08	–	3.63
linalool	1104	1.84	7.12	0.66	0.09	–	–
1-terpineol	1125	0.14	0.95	–	–	–	0.08
dihydrocarveol	1144	–	0.89	–	–	–	Tr.
verbenol	1148	–	1.15	–	–	–	–
camphor	1151	24.95	–	15.05	–	–	–
isoborneol	1162	1.11	–	0.44	–	–	0.08
borneol	1172	2.29	4.07	4.00	–	–	0.58
terpinen-4-ol	1181	0.19	13.15	0.38	0.43	–	–
α-terpineol	1195	3.60	5.84	1.14	0.44	–	0.15
verbenone	1211	Tr.	5.69	0.12	–	–	–
cuminal	1226	–	–	–	20.39	–	–
bornyl acetate	1288	3.38	0.38	2.13	–	–	0.31
2-caren-10-al	1289	–	–	–	7.85	–	–
carbicol	1293	–	–	–	4.35	–	–
thymol	1296	–	2.27	–	–	–	0.48
carvacrol	1304	–	0.13	–	Tr.	–	61.21
α-terpinyl acetate	1353	5.95	0.84	0.07	–	–	Tr.
eugenol	1370	–	–	–	–	85.85	–
β-caryophyllene	1426	1.80	0.71	1.64	–	10.54	2.62
α-humulene	1460	Tr.	Tr.	0.16	–	3.12	0.24
cyclogermacrene	1501	–	0.13	–	–	–	–
δ-cadinene	1528	–	0.35	0.09	–	0.29	Tr.
SEM		0.76	0.65	0.35	0.24	0.12	0.46

–: Not detected. Tr: Trace (Area ≤ 0.06%). [a]: Kovats Index in DB-5 column in reference to n-alkanes (C_8–C_{32})
SEM: Standard Error of the means

Origanum vulgare, subsp. *hirtum* (Link) Ietswaart, subsp. *gracile* (C. Koch) Ietswaart, subsp. *vulgare* and subsp. *viride* (Boiss.) Hayek, all of which grow wild in Turkey, and identified more than 80 constituents. *Oringanum vulgare* subsp. *hirtum* was rich in carvacrol (70.47%), while subsp. *gracile* contained β-caryophyllene (17.54%) and germacrene-D (12.75%) as dominant components. The major components of subsp. *viride* were terpinen-4-ol (16.82%) and germacrene-D (15.87%) and of subsp. *vulgare* terpinen-4-ol + β-caryophyllene (20.94%) and germacrene-D (17.80%). In *Origanum calcaratum* and *Origanum scabrum* essential oils, 22 and 28 components, respectively, were identified by Demetzos et al.[18] The major components of *Origanum calcaratum* were thymol (42.8%), p-cymene (18.1%), carvacrol (12.9%), γ-terpinene (9.6%) and isocaryophyllene (4.7%), while carvacrol (66.7%) was predominant in *Origanum scabrum* oil, which also contained *p*-cymene (7.8%), γ-terpinene (3.6%) and caryophyllene oxide (2.1%). Kokkini et al.[19] reported that the four major components of the essential oil of *Origanum vulgare* subsp. *hirtum* from autumn-collected plants were γ-terpinene, 0.6–3.6%; p-cymene, 17.3–51.3%; thymol, 0.2–42.8%; and carvacrol 1.7–69.6%. The essential oils of oregano spp., *Origanum vulgare* L. subsp. *viridulum* (Martin-Donos) Nyman from Greece, and *Origanum libanoticum* Boiss. and *O.*

syriacum L. from Lebanon were investigated by Arnold et al.[20] the first contained thymol (61.0–69.1%), the second contained methyl thymol (32.8%) and the last oil contained carvacrol (88.3%) as the major flavour compounds. Veres et al.[21] investigated the composition of *Origanum vulgare* subsp. *hirtum* oil and found it to contain carvacrol (76.4%), γ-terpinene (6.6%), thymol (0.23%), and p-cymene (4.7%) as the main constituents while the major compounds in *Origanum vulgare* subsp. *vulgare* oil were p-cymene (22.3%), caryophyllene oxide (10.2%), sabinene (7.9%), γ-terpinene (5.1%), thymol (0.34%) and spathulenol (4.8%).

GC-MS analyses of sage essential oils identified 37 constituents, representing 90.0% of the total oil. The main components were camphor (24.95%), 1,8-cineole (24.75%) and camphene (7.63%). There are many reports on the chemical composition of the oils isolated from the plants belonging to the genus *Salvia*,[22,23] most of which indicate that 1,8-cineole and borneol are the main constituents. These variations in the essential oil composition might have arisen from several differences (climatic, seasonal, geographical, geological).[22]

Asllani[24] investigated the composition of essential oils obtained from wild Albanian sage, from detected about 30 were identified. The major components identified were α-thujene (12.2–49.3%), β-thujene (3.1–10.5%), camphor (13.7–37.8%) and 1,8-cineole (3.9–23.4%). These results agree with the results presented here, except that α-thujene and β-thujene were not detected. Pino et al.[25] analysed the essential oil of sage (*Salvia officinalis* subsp. *altissima*) grown in Cuba. Among the 43 compounds identified, germacrene-D (32.9%), β-caryophyllene (31.8%) and caryophyllene oxide (23.2%) were the major constituents. Lorenzo et al.[26] investigated essential oils obtained by steam distillation from the leaves and inflorescences of *Salvia sclarea* plants cultivated in Uruguay identifying 27 components. The essential oil was found to contain high levels of linalool (8–22%), linalyl acetate (39–48%) germacrene-D (8–20%) and β-caryophyllene (3–5%). Lima et al.[27] investigated the essential oil of sage isolated from the air-dried aerial parts of the plants, and identified more than 50 compounds. The major compounds were cis-thujene (17.4%), α-humulene (13.3%), 1,8-cineole (12.7%), caryophyllene (8.5%) and borneol (8.3%). When Vera et al.[28] analysed the steam-distilled essential oil from the flowering parts of sage grown on Reunion Island, it was found to contain 51 compounds, the major ones being α-thujene (43.3–45.5%) and β-thujene (8.4–8.8%), camphor (15.9–16.2%) and 1,8-cineole (5.8–8.3%). Carruba et al.[29] reported that they were qualitative and quantitative di-fferences between the essential oils from the inflorescences and leaves, the former being characterized by a high content of linalool (26–29%) and linalyl acetate (35–53%) with germacrene-D as the main compound (68–69%). The inflorescences at full flowering stage were richer in linalool, α-terpineol and germacrene-D, but showed a lower content in linalyl acetate compared with those collected at early stags. Latifeh and Mehdi[30] reported, the development stage did not influence the oil composition of leaves. The quality and quantity of the compounds in different parts of the plant were not the same (e.g. α-thujene and β-thujene contents were lowest in the leaves collected at the flowering stage, 1.2% and 3%, respectively). The quantities of camphor (2.9%), 1,8-cineole (2%), α-thujene (6.4%) and β-thujene (1.6%) in the essential oil of aerial parts of the plant were lower than the international standards (33%, 10%, 16% and 2%, respectively).

In clove essential oil, five compounds were identified as representing 98.5% of the total. The predominant compounds were eugenol (85.5%), β-caryophyllene (10.54%), α-humulene (3.12%), δ-cadinene (0.29%) and caryophyllene oxide (0.20%). Raina et al.[31] found 16 compounds, the main components being eugenol (94%) and β-caryophyllene (2.9%), while Zapata and Meireles[32] reported that eugenol (58.62%) eugenyl acetate (19.58%), β-caryophyllene (19.87%) and α-humulene (1.60%) were the major constituents. Kwang and Shibamoto[33] reported that eugenol and eugenyl acetate were the major constituents of clove essential oils made from buds which agree with the results obtained in this study, except in the case of eugenyl acetate, which was not found. The yield and quality of the essential oil depends on the part of the plant used, the predominant compounds in essential oil from clove stems being eugenol (83–92%), β-caryophyllene (4–12%), eugenyl acetate (0.5–4%), while the predominant compounds in the essential oil obtained from leaves are eugenol (80–92%), β-caryophyllene (4–17%) and eugenyl acetate (0.2–4%). The predominant compounds in the essential oil of clove buds are eugenol (75–87%), β-caryophyllene (2–7%) and eugenyl acetate (8–15%).[34]

Turning on attention to rosemary, 39 components were identified in the essential oil, representing 89.5% of the total, the major constituents being, α-pinene (36.42%), camphor (15.65%), 1,8-cineole (12.02%) and camphene (11.08%). Dellacassa et al.[35] investigated the composition of the essential oils obtained from the leaves of *Rosmarinus officinalis* cultivars growing in different areas of Uruguay and southern Brazil. Finding that the former were rich in α-pinene (37.8–46.2%) and 1,8-cineole (13.4–13.8%), while the essential oil from cultivated Brazilian plants contained α-pinene (32.2%) and 1,8-cineole (14.7%), and that from wild Brazilian plants contained α-pinene (12.4%), myrcene (22.7%) and 1,8-cineole (15.3%). The essential oil from the fresh leaves of *Rosmarinus officinalis* L. grown in Rio de Janeiro, Brazil, was isolated and analysed by Porte et al.[36] 45 constituents were identified, the major constituents of the oil being camphor (26.0%), 1,8-cineole (22.1%), myrcene (12.4%) and α-pinene (11.5%). That the geographical location of where the plant grows can also contribute to the content and quality of essential oil was confirmed by Guillem et al.[37] who reported that rosemary essential oil

appeared to be more complex and richer in flavour notes than other previously studied Spanish rosemary oils, and had intermediate proportions of α-pinene and 1,8-cineole compared with rosemary essential oils from different geographical origins, but higher proportions of camphor, verbenone and linalool. Tomei et al.[38] investigated the essential oils from flowers and leaves of *Rosmarinus officinalis* (collected from the wild in southern Spain), and found the main components to be camphor (32.33%), 1,8-cineole (14.41%) and α-pinene (11.56%). The essential oils of *Rosmarinus officinalis* from Spain, were analysed by Chalchat el al.[39] who found them to be rich in α-pinene (24.7%), 1,8-cineole (21.8%), and camphor (18.9%), although they also contained some borneol (4.5%), findings that are in agreement with the results presented here. Soliman et al.[40] carried out a comparative study of the essential oils prepared from the fresh leaves of plants collected from Siani and Giza. Analysis of the oils by GC-MS led to the identification of 43 components in the sample from Siani, with verbenone (12.3%), camphor (11.3%), bornyl acetate (7.6%) and limonene (7.1%) being the major constituents. 37 components were identified in the sample from Giza but in this case camphor (14.9%), α-pinene (9.3%) and 1,8-cineole (9.0%) were the main constituents. Boutekedjiret et al.[41] investigated the essential oil from flowering aerial parts of *Rosmarinus officinalis* collected in Algeria. More than 90% of the components were identified, with 1,8-cineole (52.4%) and camphor (12.6%) being the major components. Benhabiles et al.[42] also investigated the essential oil, from flowering aerial parts of *Rosmarinus officinalis* collected in Algeria observing the major components to be camphor, borneol, α-terpineol, bornyl acetate, β-caryophyllene, δ-cadinene, muurolene and α-humulene.

The effect of harvest time on oil production and chemical composition is very important. The highest oil yields were recorded during the fruiting period (summer), while the lowest concentrations of camphor and maximum concentrations of α-pinene were observed in winter. Concentration of 1,8-cineole was proximally constant throughout the year, though other oil constituent levels varied randomly with the plant life cycle. It is suggested that seasonal and geographical variations in the content of the most representative components help in the quality control of rosemary oils and, consequently, for deducing the best period for processing.[43]

GC-MS analyses of cumin essential oils identified 26 constituents, representing the 80.0% of the total oil. The main components of cumin essential oil were γ-pinene (27.4%), p-cymene (20.49%), cuminal (20.39%) and β-pinene (12.75%). In a study carried out by Ia-cobellis et al.[44] p-mentha-1,4-dien-7-al, cuminal, γ-pinene and β-pinene were seen to be the major constituents of cumin oil. These findings agree with those results presented here, except for p-mentha-1,4-dien-7-al, which was not found, and p-cymene, which represented 20.49% of the total. The composition of cumin essential oil of Turkish origin was investigated by Baser[45] who found it to be characterized by high amounts of cuminal, p-mentha-1,4-dien-7-al, γ-pinene, β-pinene, perilla aldehyde and p-mentha-1,3-dien-7-al. Anon[46] reported that the main constituents of Egyptian cumin essential oil were cuminal, β-pinene, γ-pinene, p-mentha-1,3-dien-7-al, p-mentha-1,4-dien-7-al and p-cymene. Atta et al.[47] investigated the composition of cumin seeds obtained from di-fferent localities in Turkey with Cuminal (19.6–27.0%), p-mentha-1,3-dien-7-al (4.3–12.3%), p-mentha-1,4-dien-7-al (24.5–44.9%), γ-terpinene (7.1–14.1%), p-cymene (4.6–12.0%) and β-pinene (2.9–8.9%) identified as the major components.

4. Conclusions

The major components of oregano essential oil, included carvacrol (61.21%) and p-cymene (15.12%). Sage essential oil mainly contained camphor (24.95%) and 1,8-cineole (24.75%). The essential oil from thyme is characterized by a high content of terpinen-4-ol (13.15%) and γ-terpinene (9.21%). The predominant compounds in clove essential oil are eugenol (85.5%) and β-caryophyllene (10.54%). The main constituents of rosemary essential oil are β-pinene (12.75%) and α-pinene (36.42%), and the major constituents of cumin essential oil are γ-pinene (27.4%) and p-cymene (20.49%).

There is then, great variability in the chemical composition of essential oils obtained from spices. Such variability depends on several factors including climatic, season, geographical location, geology, part of the plant and the method used to obtain the essential oil.

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Povzetek

Začimbe so v državah južne Evrope in severne Afrike široko v uporabi zaradi okusa, arome in barve. Cilj tega dela je bila določitev kemijske sestave eteričnih olj šestih dišavnic in začimbnic iz španske regije Murcia: origano (*Origanum vulgare*), timijan (*Thymus vulgaris*), rožmarin (*Rosmarinus officinalis*), žajbelj (*Salvia officinalis*), kumina (*Cuminum cyminum*) in nageljnove žbice (*Syzygium aromaticum*). Analize smo opravili z uporabo GC-MS.

Glavne sestavine eteričnega olja žajblja so bile kafra (24,95 %), 1,8-cineol (24,75 %) in kamfen (7,63 %); origana: karvakrol (61,21 %) in *p*-kimen (15,12 %); timijana: terpinen-4-ol (13,15 %), γ-terpinen (9,21 %) in cis-sabinen hidrat (7,65 %); nageljnovih žbic: eugenol (85,5 %), β-kariofilen (10,54 %) in α-humulen (3,12 %); rožmarina: β-pinen (12,75 %), α-pinen (36,42 %) in kafra (15,65 %), ter kumine: γ-pinen (27,4 %), *p*-kimen (20,49 %) in kuminal (20,39 %).

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ANTIFUNGAL ACTIVITIES OF THYME, CLOVE AND OREGANO ESSENTIAL OILS

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ABSTRACT

*The antifungal potential of essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) was determined. To establish this antifungal potential, two molds related to food spoilage,* Aspergillus niger *and* Aspergillus flavus, *were selected. The agar dilution method was employed for the determination of antifungal activities. The three essential oils analyzed presented inhibitory effects on both molds tested. Oregano essential oil showed the highest inhibition of mold growth, followed by clove and thyme.* Aspergillus flavus *was more sensitive to thyme essential oil than* A. niger. *Clove essential oil was a stronger inhibitor against* A. niger *than against* A. flavus.

PRACTICAL APPLICATIONS

The use of essential oils from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) as antifungal agents will be suitable for applications on the food industry. They can be used as growth inhibitors of *Aspergillus niger* and *Aspergillus flavus*, two of the more important molds of foodborne diseases and/or food spoilage. The main reason for their suitability is their natural origin, which consumers find comforting and which is beneficial for the environment, and the very low risk that pathogens will develop resistance to the mixture of components that make up the oils with

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their apparent diversity of antifungal mechanisms. These beneficial characteristics could increase food safety and shelf life.

INTRODUCTION

In many traditional products (meats, fish and dairy products) from the Mediterranean basin, the use of spices is common, whether in the elaboration of food or in its preservation. Products such as pepper sausage, where pepper may be incorporated in the meat or added to the surface, and cheese which includes a variety of spices, are widely consumed. In both cases, sensorial factors (taste and smell) are of greater concern than other factors (technological, shelf life, etc.).

Furthermore, in this type of "homemade" or traditional product, the use of chemical agents is frowned upon by consumers. However, as any other type of food, these traditional products are exposed to chemical and/or microbiological alteration and subsequent adverse economic consequences for the manufacturer.

Another serious problem about the use of certain chemical agents used in the protection against such alterations is the development of resistance to them by the fungi concerned. Furthermore, the application of higher concentrations of chemicals in an attempt to overcome this problem increases the risk of high-level toxic residues in the products (Daferera *et al*. 2003).

A variety of microorganisms can lead to food spoilage in the food industry. So far, many pathogenic molds, such as *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp., have been reported as the causal agents of foodborne diseases and/or food spoilage (Betts *et al*. 1999). Recently, there has been considerable interest expressed in extracts and essential oils from aromatic plants with antimicrobial activities for controlling pathogens and toxin-producing microorganisms in foods (Soliman and Badeea 2002; Tepe *et al.* 2005).

Numerous studies have documented the antifungal properties of plant essential oils (Bouchra *et al*. 2003; Daferera *et al*. 2003; Sokmen *et al*. 2004). These properties are caused by many active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarins and curcumines (Tepe *et al.* 2005). Because of health and economic considerations, the search for antifungal agents is extensive (Paster *et al*. 1995). Natural plant extracts may provide an alternative way to protect foods or feeds from fungal contamination.

The specific objective of this work was to determine the effectiveness of using the essentials oils from oregano (*O. vulgare* L.), thyme (*T. vulgaris* L.) and clove (*S. aromaticum* L.) in combating the growth of some molds related to food spoilage, namely *A. niger* and *A. flavus*.

MATERIALS AND METHODS

Essential Oils

The essential oil of thyme (*T. vulgaris*), ref. F71180L, was obtained by steam distillation from leaves, stem and flowers; its density at 20C was 0.944 g/mL, the refraction index at 20C was 1.507, while the boiling point was higher than 100C. Clove (*S. aromaticum*), essential oil ref. F08568L, was obtained by steam extraction from the fruit; its density at 20C was 1.093 g/mL, and the refraction index at 20C was 1.478, while the boiling point was higher than 70C. Oregano (*O. vulgare*), essential oil ref. F70900L, was obtained by steam extraction from flowers; its density at 20C was 0.938 g/mL, and the refraction index at 20C was 1.509 while its boiling point was higher than 100C. All essential oils were purchased from Ravetllat Aromatics (Barcelona, Spain).

Antifungal Activity

Microbial Strains. The essentials oils were individually tested against a panel of molds, consisting of *A. niger* CECT 2091 and *A. flavus* CECT 2685. Both species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia.

Agar Dilution Method. The food pathogenic fungi were tested by the agar dilution method (Fraternale *et al*. 2003), with some modifications, in the appropriate culture medium (potato dextrose agar; Oxoid, Basingstoke, Hampshire, England). The oils tested were added to the culture medium at a temperature of 40–45C, and then poured into petri dishes (10 cm in diameter). Concentrations of 2, 4, 6 and 8 μL essential oils/18 mL culture medium (0.11, 0.22, 0.33 and 0.44 μL/mL culture medium, respectively) were tested for thyme, clove and oregano. The molds were inoculated as soon as the medium had solidified. A disk (9 mm in diameter; Schlinder & Schuell, Dassel, Germany) of mycelial material, taken from the edge of 5-day-old fungi cultures, was placed at the center of each petri dish. The petri dish with the inoculum was then placed to incubate at 25C. The efficacy of treatment was evaluated each day during 8 days by measuring the diameter of the fungus colonized. The values were expressed in millimeters diameter per day. All tests were performed in triplicate.

Statistical Analysis

Each parameter was tested in triplicate. Conventional statistical methods were used to calculated means and standard deviations. Statistical analysis

one-way (analysis of variance [ANOVA]) was applied to the data to determine differences ($P < 0.05$). To ascertain significant differences between the levels of the main factor, Tukey's test was applied between means (Afifi and Azen 1979). ANOVAs were made with the following factor: time (9 levels; 0, 1, 2, 3, 4, 5, 6, 7 and 8 days) for each concentration and essential oil. Another statistical ANOVA (one-way ANOVA) was applied using the following factor: essential oils: (3 levels; thyme, clove and oregano). Statistical data analysis was undertaken using the statistical package Statgraphics plus 2.0.

RESULTS AND DISCUSSION

The antifungal activities of the essential oils of thyme, clove and oregano on the molds *A. niger* and *A. flavus* are shown in Tables 1 and 2, respectively. Table 3 shows the values of percent growth reductions of the same essentials oils at day 8.

In the case of *A. niger*, it can be seen that thyme essential oil reduced fungal growth when used at 2 µL/18 mL culture medium, although the total inhibition was only attached when 8 µL was used.

The total inhibition was also obtained with the 8 and 6 µL/18 mL culture medium concentrations of clove essential oil, while the 4 and 2 µL concentrations achieved lower reductions, although both reduction values were higher than those achieved with thyme essential oil.

When oregano essential oil was used, the 2 µL concentration reduced mycelium growth, total inhibition being achieved in this case with the 4, 6 and 8 µL/18 mL culture medium concentrations.

Oregano essential oil, then, provided the highest degree of inhibition of *A. niger* growth because at the lowest concentration, it was a more potent inhibitor of growth than the other essential oils studied. Only clove at the 4 µL concentration showed a slightly higher degree than oregano at 2 µL. The next essential oil in order of its power to inhibit *A. niger* growth was clove, thyme being the poorest inhibitor.

In the case of *A. flavus*, thyme essential oil reduced mycelial growth at 2, 4 and 6 µL. Inhibition was total at 8 µL. These percent reduction values are higher than those obtained with *A. niger*, meaning that *A. flavus* was more sensitive to thyme essential oil than *A. niger.*

With clove, mycelial growth was completely inhibited at 6 and 8 µL, as it was in the case of *A. niger*. Clove essential oil was a stronger inhibitor of *A. niger* than of *A. flavus.*

In the case of the essential oil of oregano, growth was reduced at 2 µL (by 57%) and totally so by all the higher concentrations. Such growth reduction values showed similar values to those obtained with *A. niger*.

TABLE 1.
ANTIFUNGAL ACTIVITY OF THYME, CLOVE AND OREGANO ESSENTIAL OILS USING AGAR DILUTION METHOD UPON *ASPERGILLUS NIGER*

	Diameter (mean and SD $n = 3$) of mycelial growth (mm) including disk diameter of 9 mm								
	0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
Control	9.0 ± 0.0	13.46 ± 0.23	27.11 ± 0.26	35.32 ± 0.16	42.77 ± 0.29	49.97 ± 0.22	54.67 ± 0.28	62.51 ± 0.33	68.88 ± 0.30
Thyme 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	14.15 ± 0.10^{bL}	24.05 ± 0.22^{cL}	27.85 ± 0.20^{dL}	35.32 ± 0.26^{eL}	43.25 ± 0.35^{fL}	49.61 ± 0.20^{gL}	54.31 ± 0.22^{hL}
Thyme 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	12.31 ± 0.10^{bA}	17.52 ± 0.26^{cA}	23.38 ± 0.30^{dA}	31.92 ± 0.27^{eA}	39.86 ± 0.41^{fA}	44.61 ± 0.25^{gA}	48.94 ± 0.44^{hA}
Thyme 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	11.02 ± 0.12^{bX}	14.31 ± 0.32^{cX}	18.63 ± 0.16^{dX}	24.15 ± 0.26^{eX}	29.94 ± 0.39^{fX}	36.72 ± 0.17^{gX}	41.53 ± 0.24^{hX}
Thyme 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Clove 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	10.07 ± 0.05^{bM}	13.68 ± 0.09^{cM}	16.55 ± 0.22^{dM}	21.98 ± 0.26^{eM}	27.21 ± 0.19^{fM}	31.77 ± 0.18^{gM}	35.81 ± 0.19^{hM}
Clove 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aB}	9.00 ± 0.00^{aB}	13.18 ± 0.24^{dB}	17.31 ± 0.26^{eB}	21.02 ± 0.34^{fB}	23.38 ± 0.24^{gB}	26.51 ± 0.22^{hB}
Clove 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}
Clove 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Oregano 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	10.56 ± 0.13^{bN}	11.89 ± 0.19^{cN}	14.86 ± 0.28^{dN}	18.21 ± 0.26^{eN}	21.98 ± 0.32^{fN}	25.78 ± 0.22^{gN}	29.74 ± 0.27^{hN}
Oregano 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aB}	9.00 ± 0.00^{aB}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}
Oregano 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}
Oregano 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}

Values followed by the same small letter within the same line are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (L–N) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (A–C) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (X–Z) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (R–T) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.

TABLE 2.
ANTIFUNGAL ACTIVITY OF THYME, CLOVE AND OREGANO ESSENTIAL OILS USING AGAR DILUTION METHOD UPON *ASPERGILLUS FLAVUS*

	Diameter (mean and SD $n = 3$) of mycelial growth (mm) including disk diameter of 9 mm								
	0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
Control	9.00 ± 0.00	9.67 ± 0.20	25.39 ± 0.38	40.12 ± 0.31	51.53 ± 0.34	67.93 ± 0.42	82.76 ± 0.25	88,43 ± 0.16	90.00 ± 0.00
Thyme 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	15.73 ± 0.36^{bL}	20.04 ± 0.38^{cL}	26.89 ± 0.41^{dL}	34.81 ± 0.30^{eL}	41.01 ± 0.42^{fL}	50.42 ± 0.49^{gL}	57.94 ± 0.43^{hL}
Thyme 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	13.96 ± 0.52^{bA}	17.89 ± 0.45^{cA}	23.54 ± 0.33^{dA}	30.61 ± 0.40^{eA}	36.94 ± 0.40^{fA}	44.62 ± 0.25^{gA}	51.04 ± 0.36^{hA}
Thyme 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	11.63 ± 0.11^{bX}	14.72 ± 0.28^{cX}	18.93 ± 0.45^{dX}	24.91 ± 0.21^{eX}	31.14 ± 0.30^{fX}	37.82 ± 0.31^{gX}	45.82 ± 0.25^{hX}
Thyme 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Clove 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	23.4 ± 0.41^{bM}	28.27 ± 0.36^{cM}	38.05 ± 0.29^{dM}	45.82 ± 0.27^{eM}	51.00 ± 0.36^{fM}	56.91 ± 0.41^{gM}	63.83 ± 0.28^{hM}
Clove 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	18.39 ± 0.32^{bB}	25.46 ± 0.30^{cB}	34.15 ± 0.24^{dB}	41.78 ± 0.53^{eB}	45.94 ± 0.28^{fB}	49.63 ± 0.27^{gB}	55.23 ± 0.28^{hB}
Clove 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	$9.00 \pm .00^{aY}$	9.00 ± 0.00^{aY}
Clove 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Oregano 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	11.01 ± 0.08^{bN}	15.65 ± 0.21^{cN}	19.67 ± 0.13^{dN}	24.53 ± 0.33^{eN}	29.31 ± 0.38^{fN}	33.51 ± 0.39^{gN}	38.72 ± 0.16^{hN}
Oregano 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}
Oregano 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	90.0 ± 0.00^{aY}	9.00 ± 0.00^{aY}
Oregano 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}

Values followed by the same small letter within the same line are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (L–N) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (A–C) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (X–Z) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (R–T) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.

TABLE 3.
REDUCTION PERCENTAGE VALUES OF THYME, CLOVE AND OREGANO ESSENTIAL OILS UPON THE GROWTH OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS NIGER*

% Growth reduction

	Concentration (µL/18 mL)	Thyme	Clove	Oregano
A. niger	2	21.2	48.0	56.8
	4	29.0	61.5	100.0
	6	39.7	100.0	100.0
	8	100.0	100.0	100.0
A. flavus	2	35.6	29.1	57.0
	4	43.3	38.6	100.0
	6	49.1	100.0	100.0
	8	100.0	100.0	100.0

Sokmen *et al*. (2004) demonstrated the capacity of thyme essential oil at 10 µL to inhibit the growth of molds such as *Alternaria* spp., *A. flavus*, *Fusarium* spp. and *Penicillium* spp. This antifungal capacity of thyme essential oil has also been demonstrated by Montes and Carvajal (1998) and Basilico and Basilico (1999) on fungi such as *A. flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus fumigatus* and *Fusarium* spp. Other authors, too, including El-Maraghy (1995) and Inouye *et al*. (2000), confirmed these results. According to Soliman and Badeea (2002), this antifungal effect might be caused by the β-pinene content of thyme essential oil because it can reach values of 29.9–37.6%.

Paster *et al*. (1995) demonstrated the antifungal activity of oregano essential oil at concentrations of 2 and 2.5 µL/L on the mycelium and spores of *A. niger*, *A. flavus* and *A. ochraceus*, findings that agree with those of other authors, including Baratta *et al*. (1998) and Bouchra *et al*. (2003), who also showed that the antifungal activity of oregano essential oil on *A. niger* is much stronger than other essential oils such those of rosemary or sage. It is well established (Arnold *et al*. 2000; Veres *et al*. 2003) that carvacol is the major component of oregano, and many authors (Sokovic *et al*. 2002; Lopez *et al*. 2005) have attributed the antifungal properties of oregano to this compound. Its action mechanism has not been firmly established, although interaction with the cell membrane of the pathogen is thought to be likely (Veres *et al*. 2003).

The other essential oil to show a high antifungal capacity on the molds *A. niger* and *A. flavus* was clove, and other authors such as Chalfoun *et al*. (2004) and Kong *et al*. (2004) working with molds of the genera *Aspergillus* spp. and *Penicillium* spp. corroborate these findings. Some authors (Kong *et al*. 2004; Chami *et al.* 2005) have attributed this inhibitory capacity to eugenol, the major component of clove essential oil.

Possible modes of action of essential oil constituents (phenolic and terpenes) have been reported in different reviews (Davidson 2001). However, the mechanisms have not been completely elucidated. Prindle and Wright (1977) mentioned that the effect of phenolic compounds is concentration dependent. At low concentrations, phenols affect enzyme activity, especially of those enzymes associated with energy production; at greater concentrations, they cause protein denaturation. The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds to alter microbial cell permeability, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in their structure and functionality (Fung *et al*. 1977). Lis-Balchin and Deans (1997) reported that strong antimicrobial activity could be correlated with essential oils containing a high percentage of monoterpenes, eugenol, cinnamic aldehyde and thymol. Conner and Beuchat (1984) suggested that the antimicrobial activity of the essential oils of herbs and spices or their constituents such as thymol, carvacrol, eugenol, etc., could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds. Nychas (1995) indicated that phenolic compounds could denature the enzymes responsible for spore germination or interfere with the amino acids involved in germination. Once the phenolic compounds have crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity. Davidson (2001) reported that the exact cause–effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined, although it seems that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material functionality.

Several studies have attempted to determine the efficacy of extracts from selected plants as antimicrobial and antifungal agents (Lopez *et al*. 2000). Some studies have shown that specific essential oils and phenolic compounds can control the growth rate and spore germination time of spoilage fungi (Hope *et al*. 2003).

The antifungal activity of essential oils is mainly attributable to their major components although the possibility of other phenomena, such as synergy or antagonism with minor components, must also be borne in mind (Daferera *et al*. 2003).

CONCLUSIONS

From the reported results, it can be concluded that the tested essential oils exhibited, *in vitro*, broad spectrum of antifungal activity against *A. niger* and

A. flavus. So, these essential oils can be used as antifungal agents, being the main reason for their suitability, their natural origin, which consumers find comforting and which is beneficial for the environment, and the very low risk that pathogens will develop resistance to the mixture of components that make up the oils with their apparent diversity of antifungal mechanisms.

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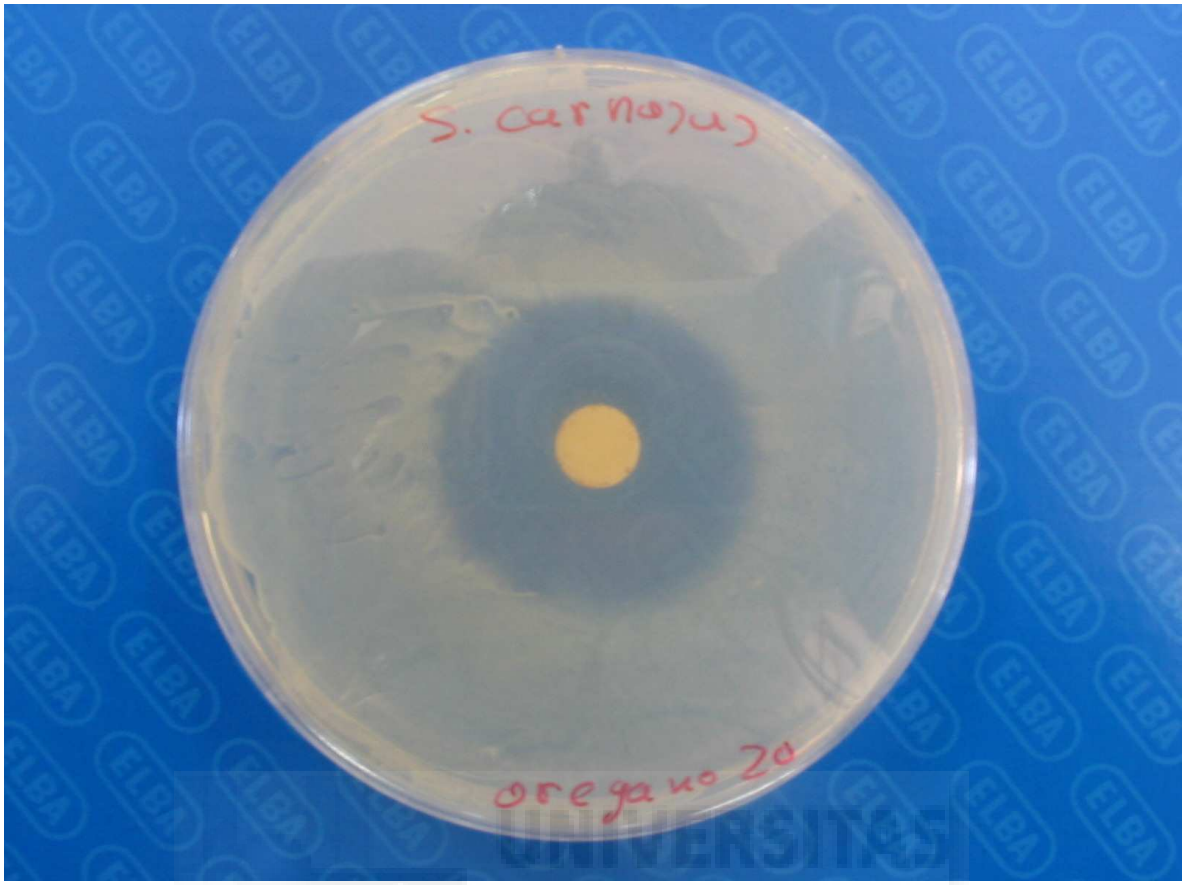

TITULO:	Antibacterial activity of different essential oils obtained from spices widely used in Mediterranean diet
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Antibacterial activity of different essential oils obtained from spices widely used in Mediterranean diet

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Summary Raw and processed foods are open to contamination during their production, sale and distribution. At present, therefore, a wide variety of chemical preservatives are used throughout the food industry to prevent the growth of food spoiling bacteria. However health and economic considerations have led to a search for alternatives, such as essentials oils that can safely be used as substitutes for fungicides and bactericides to partially or completely inhibit the growth of fungi and bacteria. The aim of this work was to determine the effectiveness of the essentials oils from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), cumin (*Cuminum cyminum*) and clove (*Syzygium aromaticum*) on the growth of some bacteria commonly used in the food industry, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus* or related to food spoilage *Enterobacter gergoviae*, *Enterobacter amnigenus*. The agar disc diffusion method was used to determine the antibacterial activities of the oils. All six essential oils analysed had an inhibitory effect on the six tested bacteria. Oregano essential oil showed the highest inhibition effect followed by cumin and clove.

Keywords Antibacterial, essential oil, rosemary, sage, thyme.

Introduction

Raw and processed foods are open to contamination during their production, sale and distribution (Deak and Beuchar, 1996). At present, therefore, a wide variety of chemical preservatives are used throughout the food industry to prevent the growth of food spoiling bacterias (Davidson, 2001). However, owing to the economical impact of spoiled foods and consumers' growing concerns over the safety of foods containing synthetic chemicals, much attention has been paid to naturally derived compounds or natural compounds (Alzoreky & Nakahara, 2003).

Essential oils and extracts obtained from many plants have recently gained in popularity and excited scientific interest (Sokmen *et al*., 2004; Tepe *et al*., 2005). However, progress in the application of spice-derived compounds as antimicrobial agents in food products has been slow. The major problems include accurate identification of the active components and the apparent requirement for concentrations that halter the sensory qualities of the food (Nychas & Skandamis, 2003; Roller & Board, 2003). Researchers are interested in biologically active compounds isolated from plant species for eliminating pathogenic micro-organims because of the resistance that micro-organims have built up against antibiotics (Essawi & Srour, 2000). For health and economic considerations, research has been directed at finding some essentials oils that could safely be used as substitutes for fungicides and bactericides to partially or completely inhibit the growth of fungi and bacteria (Soliman & Badeea, 2002). The development of multi-component antimicrobial systems for food products requires a greater understanding of the mechanisms of action of specific agents so that attention can be focused on potentially effective combinations (Gill & Holley, 2004). To resolve the problem of high concentrations, it has been proposed that spice-derived compounds should be utilized in a system of antimicrobial agents in a form of hurdle technology (Nychas & Skandamis, 2003; Roller & Board, 2003).

More than 1340 plants are known to be potential sources of antimicrobial compounds but few have been studied scientifically (Wilkins & Board, 1989). Over 30 000 different components isolated from plant oils compounds containing phenol groups are used in the food industry (Meeker & Linke, 1988). Prindle &

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Wright (1977) mentioned that the effect of the phenolic compounds present in spice essential oils is concentration-dependent. At low concentrations, phenols affected enzyme activity, especially of those enzymes associated with energy production, while at greater concentrations, they caused protein denaturation. Several studies have examined the effect on fungi of compounds isolated from essential oils extracted from plants in the search for natural fungicides and a number of these oil constituents have been shown to be inhibitory (Pitt & Hocking, 1997; Betts *et al*., 1999).

The specific objectives of this work was to determine the effectiveness of the essential oils from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), cumin (*Cuminum cyminum*) and clove (*Syzygium aromaticum*) on the growth of some bacteria usually used in food industry as starter culture, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus* and related to food spoilage *Enterobacter gergoviae* and *Enterobacter amnigenus*.

Materials and methods

Essential oils

The essential oil of thyme (*Thymus vulgaris* L.), ref. F71180L, was obtained by steam distillation from leaves, stem and flowers; its density at 20 °C is 0.944 g mL^{-1}, the refraction index at 20 °C is 1.507, while the boiling point is higher than 100 °C. Clove (*Syzygium aromaticum* L.), essential oil ref. F08568L, was obtained by steam extraction from the fruit; its density at 20 °C is 1.093 g mL^{-1}, the refraction index at 20 °C is 1.478, while the boiling point is higher than 70 °C. Oregano (*Oringanum vulgare* L.), essential oil ref. F70900L, was obtained by steam extraction from flowers; its density at 20 °C is 0.938 g mL^{-1}, the refraction index at 20 °C is 1.509 and its boiling point is higher than 100 °C. Cumin (*Cuminum cyminum* L.) essential oil was obtained by steam distillation from seeds, its density at 20 °C is 0.915 g mL^{-1} and the refraction index at 20 °C is 1.503 while the boiling point is 53 °C. Sage (*Salvia officinalis*L.) essential oil ref. F71070L, was obtained by steam distillation from leaves and flowers; its density at 20 °C is 0.915 g mL^{-1} and the refraction index at 20 °C is 1.467, while the boiling point is lower than 100 °C. The essential oil of rosemary (*Rosmarinus officinali* L) ref. F71371R, was obtained by steam distillation of the entire plant; its density at 20 °C is 0.909 g mL^{-1} and the refraction index at 20 °C is 1.467 while the boiling point is 52 °C. Essential oils of thyme, oregano, sage, rosemary and clove were purchased from Ravetllat Aromatics (Barcelona, Spain). Essential oil of cumin was purchased from Ventos (Barcelona, Spain).

Antimicrobial activity

Microbial strains

The essentials oils were individually tested against a panel of bacteria: *Staphylococcus xylosus* CECT 237, *Staphylococcus carnosus* CECT 4491, *Lactobacillus sakei*, CECT 4808, *Lactobacillus curvatus* CECT 904, *Enterobacter gergoviae* CECT 857 and *Enterobacter amnigenus* CECT 4078. All these species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia.

Agar disc diffusion method

The agar disc diffusion method described by Tepe *et al*. (2005) with some modifications was used to determine the antibacterial capacity of the essential oils. Briefly, a suspension (0.1 mL of 10^6 CFU mL^{-1}) of each microorganism was spread on the solid medium plates (Nutrient Agar I; Oxoid, Basingstoke, Hampshire, England) in the case of *S. xylosus*, *S. carnosus*, *E. gergoviae* and *E. amnigenus*; de Mann Rogosa Sharpe (MRS) agar (Sharlau, Barcelona, Spain) for *L. sakei* and *L. curvatus*). Filter paper discs, 9 mm in diameter (Schlinder & Schuell, Dassel, Germany) were impregnated with 40 μL of the oil and placed on the inoculated plates; these plates were incubated at 37 °C for 48 h in the case of *Staphylococcus* spp. and *Enterobacter* spp. and at 30 °C for 48 h in the case of *Lactobacillus* spp. The diameters of the inhibition zones were measured in millimetres. All tests were performed in triplicate.

Determination of concentration effect

The concentration effect (CE) was studied for to ascertain which doses of essential oil had an inhibitory effect on bacterial growth in the disc diffusion assay. The culture techniques used were those described in the previous paragraph (Agar disc diffusion method), but adding 40, 20, 10, 4 and 2 μL of essential oil which meant doses of 100%, 50%, 25%, 10% and 5% of the initial volume (Viuda *et al*., 2005). All tests were performed in triplicate.

Statistical analysis

Each parameter was tested in triplicate. Conventional statistical methods were used to calculate means and standard deviations, while ANOVA was applied to the data to determine differences ($P < 0.05$). To ascertain significant differences between the levels of the main factor, Tukey's test was applied between means (Afifi & Azen, 1979). ANOVA was made with the following factors: doses (five levels; 40, 20, 10, 4 and 2 μL) for each essential oil. Statistical data analysis was undertaken using the statistical package Statgraphics plus 2.0

Results and discussion

Antibacterial activity

The *in vitro* antibacterial activities of thyme, sage, cumin, rosemary, clove and oregano essential oils against the micro-organisms and their activity potentials were qualitatively and quantitatively assessed for the presence or absence of inhibition zones (Table 1).

The essential oils of thyme, sage, rosemary, oregano, cumin and clove showed inhibitory effects ($P < 0.05$) on the six tested bacteria. The agar disc diffusion method indicated that oregano essential oil showed the highest ($P < 0.05$) antibacterial activity against the six bacteria tested, with inhibition zones ranging from 35.29 mm on *S. xylosus* to 57.90 mm on *E. amnigenus*. In the case of *E. gergoviae*, thyme was the most ($P < 0.05$) potent inhibitor. The next most ($P < 0.05$) effective essential oil in this respect was cumin, which showed inhibition zones between 31.23 mm on *L. sakei* and 38.17 mm on *E. gergoviae*. Rosemary essential oil performed the worst ($P < 0.05$) in the inhibition assays with all six bacteria, while the other oils showed similar antibacterial activities.

The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds (Conner, 1993). Chemical analysis of these oils have shown that the principal active compounds of these oils are principally carvacrol, thymol, citral, eugenol, 1–8 cineole, limonene, pinene, linalool and their precursors (Viuda *et al*., 2006). Differences in the antimicrobial activity should be attributed to their chemical composition and relative proportions of the individual constituents in the essential oils. Several authors (Arnold *et al*., 2000; Veres *et al*., 2003) have claimed that the major component of oregano essential oil is carvacrol, and the antimicrobial activity of this compound has been confirmed on bacteria such as *Escherichia coli*, *Salmonella typhimurum*, *Listeria monocytogenes* (Kim *et al*., 1995; Cosentino *et al*., 1999), *Staphylococcus aureus* (Cosentino *et al*., 1999; Lambert *et al*., 2001) and *Bacillus cereus* (Cosentino *et al*., 1999; Ultee *et al*., 2000).

The inhibition action mechanism has not been studied in great detail (Lambert *et al*., 2001). Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not because of one specific mechanism but that there are several targets in the cell (Skandamis *et al*., 2001; Carson *et al*., 2002). Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted (Burt, 2004).

An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Sikkema *et al*., 1995). The leakage of ions and other cell contents can then occur (Lambert *et al*., 2001; Carson *et al*., 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer & Hugo, 1991).

The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds to alter microbial cell permeability, leading to the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in structure and functionality (Fung *et al*., 1977). Lis-Balchin & Deans (1997) reported that strong antimicrobial activity was associated with essential oils containing a high percentage of monoterpenes, eugenol, cinnamic aldehyde and thymol. Davidson (2001) reported that the exact cause-effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined, but that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material.

Components of essential oils also appear to act on cell proteins embedded in the cytoplasmic membrane (Knobloch *et al*., 1989). Most studies investigating the action of whole essential oils against food spoilage organisms and food-borne pathogens agree that, in general, essential oils are slightly more active against

Table 1 Antimicrobial activity of thyme, sage, cumin, rosemary, clove and oregano essential oils using disc diffusion method

	Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 9 mm					
Essential oil	***Staphylococcus xylosus***	***Staphylococcus carnosus***	***Enterobacter gergoviae***	***Enterobacter amnigenus***	***Lactobacillus sakei***	***Lactobacillus curvatus***
Thyme	21.60 ± 0.78	28.57 ± 0.81	53.85 ± 1.28	21.61 ± 0.86	24.05 ± 0.86	23.64 ± 1.17
Sage	28.76 ± 1.04	27.08 ± 0.94	29.68 ± 0.75	18.77 ± 1.07	23.05 ± 0.49	21.55 ± 0.95
Oregano	35.29 ± 0.88	38.47 ± 1.16	38.92 ± 0.53	57.90 ± 0.95	40.29 ± 1.05	45.20 ± 1.64
Rosemary	17.23 ± 0.91	23.53 ± 0.79	28.47 ± 1.67	18.07 ± 0.83	20.17 ± 0.79	18.82 ± 0.73
Clove	22.37 ± 0.59	24.39 ± 0.88	29.5 ± 0.71	21.96 ± 0.91	26.03 ± 1.12	23.45 ± 0.91
Cumin	34.34 ± 1.23	37.22 ± 1.21	38.17 ± 0.78	35.04 ± 1.01	31.23 ± 0.52	32.65 ± 0.83

gram-positive than gram-negative bacteria (Cosentino *et al*., 1999; Ruberto *et al*., 2000; Cimanga *et al*., 2002; Harpaz *et al*., 2003; Karaman *et al*., 2003). However, these results show that spice essential oil did not possess any selective antimicrobial activity on the basis of the cell wall differences of bacteria. These results are in accordance with those described by Sokmen *et al*. (2004), who affirmed that the essential oils of spices show no selectivity as regards the cell walls of bacteria.

Determination of CE

The CE values for the bacterial strains can be seen in Table 2. The essential oils of oregano, cumin and clove showed inhibitory effects ($P < 0.05$) on all six tested bacteria in all added doses.

The inhibitory effect of each oil was seen to be proportional to its doses. The disks impregnated with 4 and 2 μL of essential oils of sage and rosemary and the disk impregnated with 2 μL of essential oil of thyme did not have inhibitory effects ($P > 0.05$) on any of the six tested bacteria. The discs impregnated with 10 μL of sage essential oil had no inhibitory effect ($P > 0.05$) on *S. carnosus* or *E. gergoviae*.

As regards thyme essential oil, significant differences ($P < 0.05$) were found between the 10%, 25%, 50% and 100% doses in the case of *S. xylosus*, *S. carnosus*, *E. gergoviae* and *L. sakei*. In the case of *E. amnigenus*, differences were not significantly different ($P > 0.05$) between 10% and 25%, but were ($P < 0.05$) between the 50% and 100% doses. The same was true in the case of *L. curvatus*.

Table 2 The concentration effect of thyme, sage, cumin, rosemary, clove and oregano essential oils

		Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 9 mm					
Essential oil	**Doses* (%)**	***Staphylococcus xylosus***	***Staphylococcus carnosus***	***Enterobacter gergoviae***	***Enterobacter amnigenus***	***Lactobacillus sakei***	***Lactobacillus curvatus***
Thyme	5	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	10	11.30 ± 0.04^{a}	13.51 ± 0.45^{a}	11.68 ± 0.51^{a}	12.27 ± 0.56^{a}	11.04 ± 0.41^{a}	10.78 ± 0.93^{a}
	25	17.08 ± 0.62^{b}	19.84 ± 0.41^{b}	21.95 ± 0.34^{b}	13.21 ± 0.71^{a}	13.92 ± 0.86^{b}	12.98 ± 0.51^{a}
	50	19.24 ± 0.62^{c}	23.51 ± 0.81^{c}	37.56 ± 0.71^{c}	17.55 ± 1.04^{b}	16.32 ± 0.53^{c}	17.03 ± 0.68^{b}
	100	21.60 ± 0.78^{d}	28.57 ± 0.81^{d}	53.85 ± 1.28^{d}	21.61 ± 0.86^{c}	24.05 ± 0.86^{d}	23.64 ± 1.17^{c}
Sage	5	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	10	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	25	11.41 ± 0.83^{a}	N.A.	N.A.	11.44 ± 0.81^{a}	13.01 ± 0.32^{a}	12.42 ± 0.51^{a}
	50	20.98 ± 0.69^{b}	21.87 ± 1.03^{a}	22.28 ± 1.10^{a}	16.14 ± 0.69^{b}	18.32 ± 0.51^{b}	17.21 ± 0.55^{b}
	100	28.76 ± 1.04^{c}	27.08 ± 0.94^{b}	29.68 ± 0.75^{b}	18.77 ± 1.07^{c}	23.05 ± 0.49^{c}	21.55 ± 0.95^{c}
Oregano	5	20.08 ± 0.35^{a}	13.56 ± 0.66^{a}	25.88 ± 0.41^{a}	25.91 ± 0.74^{a}	18.09 ± 0.87^{a}	21.31 ± 0.83^{a}
	10	25.09 ± 1.06^{b}	15.61 ± 0.19^{b}	30.77 ± 0.77^{b}	29.86 ± 0.68^{b}	20.77 ± 1.04^{b}	25.99 ± 0.42^{b}
	25	31.56 ± 0.82^{c}	22.89 ± 1.00^{c}	33.33 ± 0.91^{c}	33.46 ± 0.85^{c}	26.52 ± 0.54^{c}	31.06 ± 0.87^{c}
	50	32.10 ± 0.06^{c}	33.73 ± 0.76^{d}	37.44 ± 0.51^{d}	36.52 ± 0.51^{d}	34.81 ± 0.75^{d}	36.12 ± 1.05^{d}
	100	35.29 ± 0.88^{d}	38.47 ± 1.16^{e}	38.92 ± 0.53^{e}	57.90 ± 0.95^{e}	40.29 ± 1.05^{e}	45.20 ± 1.64^{e}
Rosemary	5	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	10	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	25	10.88 ± 0.03^{a}	12.51 ± 0.87^{a}	11.69 ± 0.55^{a}	11.75 ± 0.58^{a}	12.32 ± 0.77^{a}	11.94 ± 0.29^{a}
	50	15.81 ± 0.21^{b}	17.26 ± 0.61^{b}	21.19 ± 0.39^{b}	12.93 ± 0.71^{b}	16.45 ± 0.50^{b}	15.61 ± 0.74^{b}
	100	17.23 ± 0.91^{c}	23.53 ± 0.79^{c}	28.47 ± 1.67^{c}	18.07 ± 0.83^{c}	20.17 ± 0.79^{c}	18.82 ± 0.73^{c}
Clove	5	11.37 ± 0.09^{a}	12.78 ± 0.14^{a}	18.13 ± 0.48^{a}	11.33 ± 0.87^{a}	12.94 ± 1.00^{a}	12.73 ± 0.36^{a}
	10	13.22 ± 0.15^{b}	18.26 ± 0.59^{b}	19.67 ± 0.71^{b}	12.80 ± 0.48^{b}	14.04 ± 0.73^{a}	13.44 ± 0.72^{a}
	25	14.77 ± 0.43^{c}	21.03 ± 0.39^{c}	23.89 ± 0.62^{c}	13.32 ± 0.87^{c}	16.81 ± 0.59^{b}	15.25 ± 0.53^{b}
	50	17.74 ± 0.52^{d}	23.79 ± 0.73^{d}	25.12 ± 0.87^{d}	18.38 ± 1.04^{d}	19.11 ± 0.87^{c}	17.97 ± 0.86^{c}
	100	22.37 ± 0.59^{e}	24.39 ± 0.88^{e}	29.5 ± 0.71^{e}	21.96 ± 0.91^{e}	26.03 ± 1.12^{d}	23.45 ± 0.91^{d}
Cumin	5	11.06 ± 0.09^{a}	18.55 ± 0.35^{a}	12.93 ± 0.12^{a}	11.30 ± 0.59^{a}	12.54 ± 0.67^{a}	13.76 ± 0.78^{a}
	10	21.91 ± 0.21^{b}	27.78 ± 1.04^{b}	21.75 ± 0.46^{b}	14.02 ± 0.71^{b}	16.03 ± 0.29^{b}	17.04 ± 1.02^{b}
	25	25.51 ± 0.33^{c}	33.58 ± 0.23^{c}	27.59 ± 0.69^{c}	22.25 ± 0.56^{c}	21.91 ± 82^{c}	23.19 ± 0.54^{c}
	50	32.00 ± 0.39^{d}	34.89 ± 0.27^{d}	33.03 ± 0.7^{d}	31.07 ± 0.42^{d}	27.46 ± 0.92^{d}	29.51 ± 0.94^{d}
	100	34.34 ± 1.23^{e}	37.22 ± 1.21^{e}	38.17 ± 0.78^{e}	35.04 ± 1.01^{e}	31.23 ± 0.52^{e}	32.65 ± 0.83^{e}

*Doses of essential oil referred to initial volume (40 μL).

$^{a-e}$For the same essential oil, values followed by different letters within the same column are significantly different ($P < 0.05$) according to Tukey's multiple range test.

N.A., non-active.

As regards sage essential oil, significant differences existed ($P < 0.05$) between the 25%, 50% and 100% doses in the case of *S. carnosus* and *E. gergoviae*, and between 10%, 25%, 50% and 100% in the case of *E. amnigenus*, *S. xylosus*, *L. curvatus* and *L. sakei*.

When oregano essential oil was used on *S. carnosus*, *E. gergoviae*, *E. amnigenus*, *L. curvatus* and *L. sakei*, there were significant differences ($P < 0.05$) between all the concentrations assayed, while on *S. xylosus* the differences were not significantly different ($P > 0.05$) between the 25% and 50% doses.

As regards rosemary, significant differences ($P < 0.05$) were observed between the 25%, 50%and 100% doses for all six bacteria analysed.

When clove essential oil was analysed statistically, significant differences ($P < 0.05$) existed between all five doses used on all six bacteria. The same was true for cumin essential oil.

As can be seen, these essential oils showed antibacterial activity not only against food spoilage microbiota but also against microbiota used in food processing (fermentation process). This is very important because a lot of food elaboration process include a fermentation stage. If some essentials oils are going to be used in this type of foods, the antibacterial activity against the microorganism used in the fermentation stage must be checked.

It can be concluded that these essential oils (from oregano, thyme, rosemary, sage, cumin and clove) posses *in vitro* antibacterial activity against *L. curvatus*, *L. sakei*, *S. carnosus*, *S. xylosus*, *E. gergoviae* and *E. ammigenus*, although, the effects of thyme, rosemary and sage essential oils are dose-dependent. However, if essential oils were to be more widely applied as antibacterials in foods, it must be taken into account that the antibacterial efficiency is diminished when they are added to more complex materials (such as food products) and the organoleptic impact would be important and also that issues of safety and toxicity will need to be addressed.

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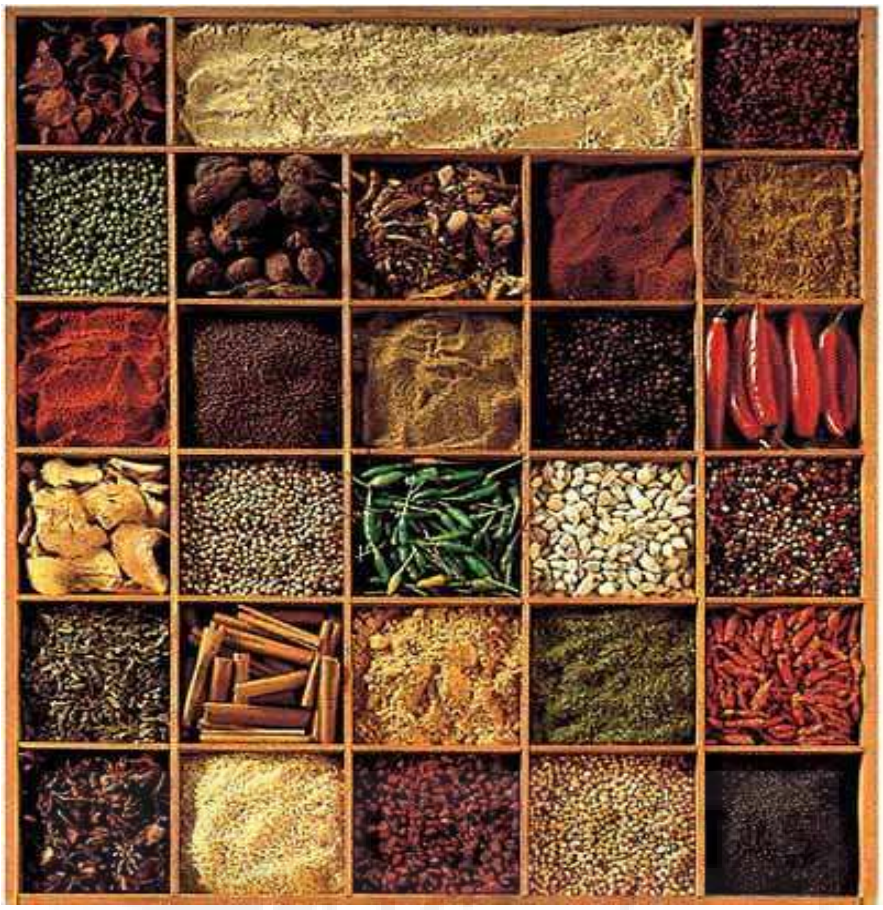

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Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet

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ABSTRACT: The oxidative degradation of lipids is one of the main factors limiting the shelf-life of food products. In recent years, several undesirable disorders have been detected as side-effects of using commonly used synthetic antioxidants. Apart from their use as aroma additives in food, essential oils from aromatic plants have shown potential for use in small amounts in fat-containing food systems to prevent or delay some types of chemical deterioration that occur during storage. Using a multiple-method approach, the antioxidant activity of the essentials oils from several spices widely used in Mediterranean countries was tested: oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and clove (*Syzygium aromaticum*). Their total phenolic compound content was also determined. The clove essential oil had the highest amount of total phenols (898.89 mg/l GAE) and showed the highest percentage inhibition of DPPH radical (98.74%) and the highest FRAP value (1.47 TEAC). The thyme essential oil produced the highest percentage inhibition of TBARS (89.84%). All the essential oils studied were capable of chelating iron(II), the rosemary essential oil producing the highest effect (76.06%) in this respect. The oregano essential oil had the highest antioxidant activity index in the Rancimat test.

Keywords: spices; essential oils; antioxidant; FRAP; DPPH; FIC; TBARS; Rancimat

Introduction

Lipid oxidation, which occurs during the storage of raw materials, processing, heat treatment and subsequent storage of final products, is one of the main causes of rancidity in food products.[1] This process is initiated by exposure to the enzyme lypoxygenase, heat, ionizing radiation, light, metal ions and metalloprotein catalysts.[2] Such oxidation leads to a significant loss of a food's nutritional value, since it involves a loss of vitamins and essential fatty acids. It also affects the food's sensory quality – changes in colour, texture and taste – which shortens its shelf-life and can result in rejection on the part of consumers.[3] In the food industry, the rate of auto-oxidation is reduced by freezing, refrigeration, packaging under inert gas in the absence of oxygen and vacuum packaging.[2] In cases where these methods are neither economic nor practical from the nutritional and technological points of view, it is highly desirable to control oxidation by the addition of antioxidants, inhibitory substances that do not reduce food quality.[4] Antioxidants also have an important role in preventing a variety of lifestyle-related diseases and ageing because these, too, are closely related to active oxygen and lipid peroxidation.[5] However, the results of a great deal of research into the antioxidant properties of different substances have not identified any antioxidant that may be active in all food products. Such a characteristic results from many factors, e.g. stability at the time of processing, mixing ability and activity in different lipid systems, and the need to fulfil legal requirements.[6]

The most widely used synthetic antioxidants in food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl galate (PG) and tetrary butyl hydroquinone (TBHQ).[7] However, due to their unstable nature, most of these are heat-sensitive and volatile in steam;[8] they have also been the cause of concern about their safety and efficiency.[9] Consequently, there has been a growing interest in searching for and using natural antioxidants, for three principal reasons:[10] (a) numerous clinical and epidemiological studies have demonstrated that the consumption of fruits and vegetables is associated with reduced risks of developing chronic diseases such as cancer, cardiovascular disorders and diabetes; (b) safety considerations regarding the potential harmful effects of the chronic consumption of synthetic antioxidants in foods and beverages; and (c) the public's perception that natural and dietary antioxidants are safer than synthetic analogues. The result has been an increased interest in spices, aromatic and medicinal plants as sources of natural antioxidants to replace synthetic antioxidants.

Many sources of antioxidants of a plant origin have been studied in recent years.[11] Among these, many aromatic plants and spices have been shown to be effective in retarding the process of lipid peroxidation.[12] One component present in aromatic plants and spices, and which may act as a natural antioxidant, is the corresponding essential oil. In general terms, essential oils are composed of >70 components, principally polyphenols,

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terpenes, monoterpenes and sesquiterpenes,[12] some of which may represent more than 85% of the total content. Nowadays, essential oils and their components are gaining increasing attention because of their relatively safe status, their wide acceptance by consumers, and the possibility of their exploitation for potential multi-purpose functional uses.[13]

The aim of this study was to determine the total phenol content (TPC) and the antioxidant activity of the essentials oils from several spices widely used in Mediterranean countries: oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and clove (*Syzygium aromaticum*). Five different test systems were used: 2,2′-diphenyl-1-picrylhydrazyl (DPPH); the ferric reducing antioxidant power (FRAP); the thiobarbituric acid reactive species (TBARS) test; the determination of ferrous ion-chelating ability (FIC); and the Rancimat method.

Experimental

Chemicals

Ascorbic acid, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferrozine, Folin–Ciocalteu's reagent, gallic acid, iron(III) chloride, iron(II) chloride, trichloroacetic acid (TCA) and Trolox were from Sigma Chemical Company (Germany). Dibasic potassium phosphate, 2-thiobarbituric acid (TBA), sodium carbonate and dibasic sodium phosphate were from Merck (Darmstadt, Germany). Potassium hexacyanoferrate was from Fluka BioChemika (Germany). The solvent used for preparing standard solutions was methanol of HPLC ultra-gradient grade, supplied by Merck.

Essential Oils

The essential oil of thyme (*Thymus vulgaris* L.; ref. F71180L) was obtained by steam distillation from leaves, stems and flowers; its density at 20°C is 0.944 g/ml. Clove (*Syzygium aromaticum* L.; ref. F08568L) essential oil was obtained by steam extraction from the fruit; its density at 20°C is 1.093 g/ml. Oregano (*Oringanum vulgare* L.; ref. F70900L) essential oil was obtained by steam extraction from flowers; its density at 20°C is 0.938 g/ml. Sage (*Salvia officinalis* L.; ref. F71070L) essential oil was obtained by steam distillation from leaves and flowers; its density at 20°C is 0.915 g/ml. The essential oil of rosemary (*Rosmarinus officinalis* L.; ref. F71371R) was obtained by steam distillation of the entire plant; its density at 20°C is 0.909 g/ml. Essential oils of thyme, oregano, sage, rosemary and clove were purchased from Ravetllat Aromatics (Barcelona, Spain).

Total Phenol Content

The total phenol content (TPC) was determined using Folin–Ciocalteu's reagent.[14] A volume of 0.3 ml of a methanolic solution of essential oils (EOs; 50 g/l) was introduced into test tubes followed by 2.5 ml Folin–Ciocalteu's reagent (diluted 10 times with water) and 2 ml sodium carbonate (7.5% w/v). The tubes were vortexed, covered with parafilm and incubated at 50°C for 5 min. Absorption at 760 nm was measured with a HP 8451 spectrophotometer (Hewlett-Packard, Cambridge, UK) and compared to a gallic acid calibration curve. The results were expressed as mg gallic acid equivalents (GAE)/l sample. Each assay was carried out in triplicate.

Determination of Antioxidant Activity Using the 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

The antioxidant activity of oregano, thyme, sage, rosemary and clove essential oils was measured in terms of hydrogen-donating or radical scavenging ability, using the stable radical DPPH.[15] A volume of 50 µl of a methanolic stock solution of EOs of four different concentrations (50, 20, 10 and 5 g/l) was put into a cuvette, and 2 ml 6×10^{-5} M methanolic solution of DPPH was added. Ascorbic acid and butylated hydroxytoluene (BHT; in the same concentration) were used as references. The mixtures were well shaken in a vortex (2500 rpm) for 1 min and then placed in a dark room. The decrease in absorbance at 517 nm was determined using a HP 8451 spectrophotometer (Hewlett-Packard) after 1 h for all samples. Methanol was used to zero the spectrophotometer. Absorbance of the radical without antioxidant (control) was measured daily. The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC_{50}) was calculated graphically. Inhibition (%) was plotted against the sample concentration in the reaction system. The percentage inhibition of the DPPH radical was calculated according to the formula of Yen and Duh:[16]

$$\%I = \left[\left(A_B - A_S \right) / A_B \right] \times 100$$

where I = DPPH inhibition (%), A_B = absorbance of control sample ($t = 0$ h) and A_S = absorbance of a tested sample at the end of the reaction ($t = 1$ h).

Ferric Reducing Antioxidant Power

The ferric reducing power (FRAP) of the essential oils was determined by using the potassium ferricyanide–ferric chloride method.[17] Different dilutions (1 ml aliquots) of EOs (50, 20, 10 and 5 g/l) were each added to 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixtures were incubated at 50°C for 20 min, after which 2.5 ml trichloroacetic acid (10%) was added. An aliquot of the mixture (2.5 ml) was taken and mixed with 2.5 ml water and 0.5 ml 1% $FeCl_3$. The absorbance at 700 nm was measured after allowing the solution to stand for 30 min. The FRAP of a sample is estimated in terms of Trolox equivalent antioxidant capacity (TEAC) in mM/l Trolox. Each assay was carried out in triplicate.

Thiobarbituric Acid Reactive Species Test

The method of Daker *et al.*[2] was modified to determine the thiobarbituric acid reactive substance (TBARS), a secondary product of lipid peroxidation. For this, 0.1 ml of different dilutions of EOs (50, 20, 10 and 5 g/l) was added to mixture that contained 1 ml fowl egg yolk emulsified with 0.1 M phosphate buffer, pH 7.4, to obtain a final concentration of 25 g/l and 100 µl 1 mM Fe^{2+}. The mixture was incubated at 37°C for 1 h, after which it was treated with 0.5 ml freshly prepared 15% trichloroacetic acid (TCA) and 1 ml 1% thiobarbituric acid (TBA). The reaction tubes were kept in a boiling water bath for 10 min. Upon cooling with ice, the tubes were centrifuged at $3500 \times g$ for 10 min to remove precipitated protein. The formation of TBARS was measured by removing 100 µl supernatant and measuring the absorbance at 532 nm. The control was buffered egg yolk with Fe^{2+} only. BHT and ascorbic acid were used as the standards. The percentage inhibition ratio was calculated from the following equation:

$$\%\,\text{inhibition} = \left[\left(A_{\text{Control}} - A_{\text{Sample}} \right) / A_{\text{Control}} \right] \times 100$$

where A_{Control} refers to the absorbance of the control and A_{Sample} is the absorbance of the sample.

To determine the concentration needed to achieve 50% inhibition of phospholipids oxidation in egg yolk (EC_{50}), the percentage of lipid peroxidation inhibition was plotted against essential oil concentration. Each assay was carried out in triplicate.

Ferrous Ion-chelating Ability Assay

The ferrous ion-chelating (FIC) assay was carried out according to the method of Singh and Rajini,[18] with some modifications. Solutions of 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine were diluted 20 times. Briefly, an aliquot (1 ml) of different concentrations of EOs (50, 20, 10 and 5 g/l) was

mixed with 1 ml $FeCl_2 \cdot 4H_2O$. After 5 min incubation, the reaction was initiated by the addition of ferrozine (1 ml). The mixture was shaken vigorously and after a further 10 min incubation period the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe^{+2} complex formation was calculated by using the formula:

$$\text{Chelating effect}\,(\%) = \left[(1 - A_S)/A_B\right] \times 100$$

where A_B = absorbance of control sample (the control contains $FeCl_2$ and ferrozine, complex formation molecules) and A_S = absorbance of a tested sample.

Rancimat Assay

A Rancimat 743 (Methrohm, Switzerland) was used to determine the antioxidant lipid activity of oregano, thyme, sage, rosemary and clove essential oils. The Rancimat worked on the following principle. A solution (100 µl) of different concentrations of EOs (50, 20, 10 and 5 g/l) was added to the lard (2.5 g), previously melted, giving a final concentration of 0.2%, 0.08%, 0.04% and 0.02% of EOs in the reacting system. The lard with and without added antioxidant was heated at 120°C and an air flow of 20 l/h was constantly blown into the mixture. The end of the induction period (IP) was characterized by the sudden increase of water conductivity, due to the dissociation of volatile carboxylic acids.[19] The antioxidant activity index (AAI) was calculated from the measured induction times, according to the following formula by Forster *et al.*:[20]

$$\text{AAI} = \text{induction period of lard with antioxidant} / \text{induction period of pure lard}$$

An antioxidant activity index >1 indicates inhibition of the lipid oxidation; the higher the value, the better the antioxidant activity.[21]

Statistical Analysis

Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. Analysis of variance (ANOVA) was applied to the data to determine differences ($p < 0.05$). To discover where there were significant differences between the levels of the main factor, contrasts (Tukey's test) between means were made.[22] For the antioxidant activity, ANOVAs with two factors (essential oil and concentration) were applied for each parameter. The statistical analyses were made using Statgraphics 5.1 for Windows. A correlation between total phenols and antioxidant capacity was made using the function CORREL from Microsoft Excel software.

Results and Discussion

The chemical compositions of the essential oils used in this work were previously determined by Viuda-Martos *et al.*[23] In the essential oil of thyme, the major constituents were terpinen-4-ol (13.1%), γ-terpinene (9.2%), *cis*-sabinene hydrate (7.6%), linalool (7.1%) and *p*-cymene (5.7%). In oregano essential oil the major constituent was carvacrol (61.2%). In sage essential oil the main components were camphor (25.0%), 1,8-cineole (24.7%) and camphene (7.6%). In clove essential oil the predominant compounds were eugenol (85.5%) and β-caryophyllene (10.5%). In the essential oil of rosemary the major constituents were α-pinene (36.4%), camphor (15.6%) and 1,8-cineole (12.0%).

Total Phenol Content

The total phenolic compound (TPC) content of the thyme, oregano, rosemary, clove and sage essential oils are presented in Table 1. In the clove EO a high content of total phenols (898.89 mg GAE/l) was obtained. Gülçin *et al.*[24] also demonstrated that clove extracts have a high phenol content. Thyme and oregano EOs were seen to be a less rich source of total phenols (783.81 and 763.97 mg GAE/l, respectively), while rosemary and sage EOs showed the lowest amount of total phenols.

Table 1. The total phenols content (TPC) of thyme, oregano, clove, sage and rosemary essential oil

Essential oil	Total phenols GAE*(mg/l)
Clove	898.89 ± 3.37^a
Thyme	783.81 ± 2.24^b
Oregano	763.97 ± 1.12^c
Rosemary	225.08 ± 6.73^d
Sage	122.98 ± 0.22^e

*Gallic acid equivalent.
Values followed by the same letter within the same column are not significantly different ($p > 0.05$) according to Tukey's multiple range test.

The phenolic compounds content could be used as an important indicator of the antioxidant capacity, which may be used as a preliminary screen for essential oils when intended as natural sources of antioxidants in functional foods.[25] Many authors[26–27] have described the potential antioxidant properties of polyphenols. These compounds act as antioxidants by donation of a hydrogen atom, as an acceptor of free radicals, by interrupting chain oxidation reactions or by chelating metals.[28]

Antioxidant Activities

The antioxidant activities of spice essential oils have been widely demonstrated,[29] although the mechanism of such activity is not fully understood. Several explanations have been provided, among them the following: the sequestration of free radicals; hydrogen donation; metallic ion chelation; or even acting as substrate for radicals such as superoxide or hydroxyl.[30] These bioactive compounds with antioxidant properties also interfere with propagation reactions[31] and inhibit the enzymatic systems involved in initiation reactions.[32] The activities of essential oils such as antioxidants depend not only on their structural features but also on many other factors, such as concentration, temperature, light, type of substrate and physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists.[33] The use of simplified model systems, which mimic the main features of a given food system, or antioxidant assays for quantifying the antioxidant action can be very helpful in clarifying the action of potential antioxidants.[34]

DPPH Assay

The DPPH free radical does not require any special preparation and is considered a simple and very fast method for determining antioxidant activity. In contrast, DPPH can only be dissolved in organic media, especially in ethanol, which is an important limitation when interpreting the role of hydrophilic antioxidants.[35]

The radical scavenging capacity of the spice EOs was tested using the 'stable' free radical, DPPH. Table 2 shows the effective

Table 2. Antioxidant activity of thyme, oregano, clove, sage and rosemary essential oil at different concentrations (A = 5 g/l, B = 10 g/l, C = 20 g/l, D = 50 g/l), measured by the DPPH method

	DPPH Inhibition (%)				
	A	B	C	D	IC_{50}*
Thyme	62.87 ± 0.03^{aA}	81.30 ± 0.04^{bA}	88.08 ± 0.02^{cA}	93.94 ± 0.02^{dA}	1.10
Oregano	51.79 ± 0.01^{aB}	64.85 ± 0.00^{bB}	81.17 ± 0.00^{cB}	87.19 ± 0.00^{dB}	3.90
Clove	97.85 ± 0.00^{aC}	98.40 ± 0.00^{bC}	98.45 ± 0.00^{cC}	98.74 ± 0.00^{dC}	0.38
Sage	51.17 ± 0.01^{aD}	55.22 ± 0.00^{bD}	58.01 ± 0.01^{cD}	66.59 ± 0.03^{dD}	4.20
Rosemary	47.54 ± 0.00^{aE}	48.35 ± 0.00^{bE}	50.76 ± 0.02^{cE}	56.95 ± 0.01^{dE}	17.00
Ascorbic acid	96.61 ± 0.01^{aF}	97.73 ± 0.00^{bF}	97.86 ± 0.00^{cF}	97.92 ± 0.01^{dF}	0.42
BHT	95.93 ± 0.00^{aG}	96.85 ± 0.01^{bG}	97.23 ± 0.00^{cG}	97.69 ± 0.00^{dG}	0.53

* IC_{50}, concentration (g/l) for a 50% inhibition.
Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) according to Tukey's multiple range test.
Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's multiple range test.

Table 3. Antioxidant activity of thyme, oregano, clove, sage and rosemary essential oil at different concentrations (A = 5 g/l, B = 10 g/l, C = 20 g/l, D = 50 g/l), measured by TBARS assay

	TBARS Inhibition (%)				
	A	B	C	D	EC_{50}*
Thyme	72.75 ± 0.30^{aA}	79.18 ± 0.71^{bA}	85.77 ± 0.10^{cA}	89.84 ± 0.20^{dA}	0.090
Oregano	75.46 ± 0.30^{aB}	83.55 ± 0.40^{bB}	86.34 ± 0.51^{cB}	89.27 ± 0.00^{dB}	0.021
Clove	71.53 ± 0.40^{aC}	82.33 ± 0.10^{bC}	85.05 ± 1.11^{cD}	88.13 ± 0.20^{dC}	0.023
Sage	26.25 ± 0.51^{aD}	36.19 ± 0.40^{bD}	41.70 ± 0.10^{cE}	57.58 ± 0.91^{dD}	35.56
Rosemary	2.00 ± 0.20^{aE}	6.58 ± 0.40^{bE}	28.04 ± 0.40^{cF}	41.20 ± 0.20^{dE}	52.55
Ascorbic acid	35.41 ± 0.30^{aF}	70.17 ± 0.30^{bF}	84.19 ± 0.30^{cG}	88.13 ± 0.20^{dC}	7.98
BHT	81.83 ± 0.20^{aG}	85.84 ± 0.20^{bG}	89.48 ± 0.10^{cH}	92.78 ± 0.10^{dF}	0.001

* EC_{50}, concentration (g/l) for a 50% inhibition.
Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) according to Tukey's multiple range test.
Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's multiple range test.

concentrations of each EO required to scavenge DPPH radical and the scavenging values as inhibition (%). It can be seen that the essential oils analysed exhibited varying degrees of scavenging capacities. Clove essential oil showed the strongest ($p < 0.05$) radical scavenging effect (98.74 ± 0.00%) at 50 mg/ml, which is higher than that observed for the positive controls, BHT and ascorbic acid (97.69 ± 0.00% and 97.92 ± 0.01%, respectively). This activity was followed by the thyme EO (93.94 ± 0.02%) and oregano EO (87.19 ± 0.00%). Sage and rosemary EOs showed the lowest scavenging activity ($p < 0.05$).

The DPPH assay measures the ability of the extract to donate hydrogen to the DPPH radical, resulting in bleaching of the DPPH solution. The greater the bleaching action, the higher the antioxidant activity, which is reflected in a lower IC_{50}.[36] The values of IC_{50} were in the order: clove < ascorbic acid < BHT < thyme < oregano < sage < rosemary. It is interesting to note that for all the concentrations and EOs studied, excepted for sage essential oil, there was a linear correlation between TPC and the percentage inhibition of DPPH ($y = 0.061x + 42.86$; $R^2 = 0.985$). Djeridane *et al.*[37] and Katalinic *et al.*[38] demonstrated a linear correlation between the total phenolic compound content and antioxidant capacity.

TBARS Assay

The TBARS assay is sensitive, requires small sample amounts and provides reproducible results. This method is preferable for obtaining useful data in an environment similar to the real-life situation and allows testing of both lipophilic and hydrophilic substances.[12]

The thyme, clove, oregano, rosemary and sage essential oils were examined for their ability to act as radical scavenging agents and compared with the ability of ascorbic acid and BHT. Thyme essential oil showed the highest inhibition (89.84 ± 0.20%; $p < 0.05$) of all the essential oils analysed (Table 3).

Sage and rosemary essential oils showed the lowest inhibition values (57.58 ± 0.90% and 41.20 ± 0.20%, respectively). Thyme, oregano and clove essential oils were better radical scavenging agents than ascorbic acid, while BHT showed the highest radical scavenging activity of all.

The EC_{50} values ranged from 0.001 to 52.55 mg/ml and the lipid peroxidation inhibitory potency decreased in the order: rosemary > sage > ascorbic acid > thyme > clove > oregano > BHT.

FRAP Assay

The FRAP method is a simple, very rapid, inexpensive and reproducible method, which can be applied to the assay of antioxidants in plasma or botanicals.[39] Table 4 shows the ferric reducing capacity obtained using the FRAP assay. A concentration-dependent ferric reducing capacity was found for all the essential oils studied. Clove essential oil, at all the concentrations analysed, showed the highest ($p < 0.05$) ferric reducing capacity in terms of Trolox concentrations, followed by oregano essential oil. Sage and rosemary essential oil had little ferric reducing capacity compared with clove and oregano essential oil.

With further data analysis, a significant linear correlation ($y = 0.001x + 0.006$; $R^2 = 0.984$) between FRAP values and total phenolic contents of the EOs analysed was observed, except for the thyme essential oil.

Numerous studies have demonstrated the antioxidant activity of rosemary and sage;[40,41] however, in this study rosemary and sage essential oils showed the lowest values of percentage inhibition of DPPH, TBARS and FRAP. A possible explanation could be that the antioxidant effect is due to several non-volatile compounds, such as carnosol, quercetine, rosmarinic acid and caffeic acid, none of which was found in the chemical composition of these essential oils.[23]

Ferrous Ion-chelating Assay

One of the possible mechanisms of the antioxidative action is the chelation of transition metals. Transition metal ions can stimulate lipid peroxidation at two ways: (a) participating in the generation of initiating species; and (b) accelerating peroxidation, decomposing lipid hydroperoxides into other components which are able to abstract hydrogen, perpetuating the chain of reaction of lipid peroxidation.[42]

Analysis of metal ion-chelating properties showed that all the essential oils studied were capable of chelating iron (II) and did so in a concentration-dependent manner (Table 5). At all

Table 4. Antioxidant activity of thyme, oregano, clove, sage and rosemary essential oil at different concentrations (A = 5 g/l, B = 10 g/l, C = 20 g/l, D = 50 g/l), measured by the FRAP method

	FRAP TEAC* (mM Trolox/l)			
	A	B	C	D
Thyme	0.33 ± 0.00^{aA}	0.55 ± 0.01^{bA}	0.68 ± 0.00^{cA}	0.80 ± 0.00^{dA}
Oregano	0.95 ± 0.03^{aB}	1.15 ± 0.01^{bB}	1.19 ± 0.01^{cB}	1.28 ± 0.00^{dB}
Clove	1.27 ± 0.01^{aC}	1.36 ± 0.00^{bC}	1.41 ± 0.00^{cC}	1.47 ± 0.00^{dC}
Sage	0.04 ± 0.00^{aD}	0.07 ± 0.00^{bD}	0.09 ± 0.00^{cD}	0.12 ± 0.00^{dD}
Rosemary	0.11 ± 0.00^{aE}	0.20 ± 0.00^{bE}	0.36 ± 0.00^{cE}	0.48 ± 0.00^{dE}
Ascorbic acid	0.82 ± 0.01^{aF}	1.00 ± 0.00^{bF}	1.15 ± 0.00^{cF}	1.35 ± 0.01^{dF}
BHT	0.80 ± 0.00^{aG}	0.97 ± 0.01^{bG}	1.13 ± 0.00^{cG}	1.31 ± 0.00^{dG}

*TEAC, Trolox equivalent antioxidant capacity.
Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) according to Tukey's multiple range test.
Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's multiple range test.

Table 5. Antioxidant activity of thyme, oregano, clove, sage and rosemary essential oil at different concentrations (A = 5 g/l, B = 10 g/l, C = 20 g/l, D = 50 g/l), measured by the ferrous iron chelating assay

	FIC Chelating effect (%)				
	A	B	C	D	*EC_{50}
Thyme	43.51 ± 0.33^{aA}	45.74 ± 0.87^{bA}	54.49 ± 0.43^{cA}	63.62 ± 0.11^{dA}	17.32
Oregano	41.44 ± 0.22^{aB}	41.83 ± 0.11^{bB}	44.59 ± 0.33^{cB}	48.20 ± 0.43^{dB}	59.35
Clove	42.52 ± 0.22^{aC}	43.13 ± 0.43^{aC}	46.28 ± 0.54^{cC}	60.04 ± 0.33^{dC}	25.79
Sage	47.28 ± 0.65^{aD}	51.56 ± 0.33^{bD}	60.48 ± 0.43^{cD}	72.29 ± 0.65^{dD}	7.16
Rosemary	46.28 ± 0.54^{aD}	56.87 ± 0.33^{bE}	64.24 ± 0.54^{cE}	76.06 ± 0.54^{dE}	4.76
Ascorbic acid	25.79 ± 0.21^{aE}	26.85 ± 0.14^{bF}	30.85 ± 0.32^{cF}	36.07 ± 0.29^{dF}	104.55
BHT	35.92 ± 0.17^{aF}	39.45 ± 0.21^{bG}	42.52 ± 0.27^{cG}	44.67 ± 0.18^{dG}	65.15

*EC_{50}, concentration (g/l) for a 50% chelating effect.
Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) according to Tukey's multiple range test.
Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's multiple range test.

Table 6. Antioxidant activity of thyme, oregano, clove, sage and rosemary essential oil at different concentrations (A = 5 g/l, B = 10 g/l, C = 20 g/l, D = 50 g/l) measured by the Rancimat method

	Rancimat Antioxidant activity index (AAI)			
	A	B	C	D
Thyme	1.04 ± 0.01^{aA}	1.05 ± 0.01^{aA}	1.27 ± 0.04^{bA}	1.59 ± 0.01^{cA}
Oregano	1.02 ± 0.03^{aA}	1.05 ± 0.02^{aA}	1.27 ± 0.03^{bA}	1.67 ± 0.03^{cB}
Clove	1.01 ± 0.01^{aA}	1.03 ± 0.01^{aA}	1.07 ± 0.01^{bB}	1.44 ± 0.01^{cC}
Sage	0.86 ± 0.05^{aB}	0.94 ± 0.01^{bB}	1.01 ± 0.01^{cC}	1.07 ± 0.01^{dD}
Rosemary	0.75 ± 0.05^{aC}	0.85 ± 0.02^{bC}	0.93 ± 0.01^{cD}	1.05 ± 0.01^{dD}
Ascorbic acid	1.02 ± 0.03^{aA}	1.07 ± 0.00^{bD}	1.13 ± 0.03^{cE}	1.44 ± 0.01^{dC}
BHT	1.23 ± 0.04^{aD}	1.65 ± 0.02^{bE}	2.04 ± 0.03^{cF}	2.42 ± 0.14^{dE}

Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) according to Tukey's multiple range test.
Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's multiple range test.

concentrations (5, 10, 20 and 50 g/l), rosemary and sage essential oils showed the highest values ($p < 0.05$) for chelating iron(II) (76.06 ± 0.54% and 72.29 ± 0.65%, respectively). Oregano essential oils at all the concentrations showed the lowest values of metal chelation. The main component of oregano essential oil is carvacrol,[23] a mono-hydroxylated compound unable to form a complex with Fe^{+2}.[43] All the essential oils studied were better chelators of iron(II) than ascorbic acid and BHT.

Rosemary and sage essential oils showed the lowest EC_{50} values (4.76 and 7.16 g/l, respectively). Therefore it can be said that all the essential oils showed mild chelating activity, which is of great significance because the chelation of transition metals is of great potential interest in the food industry, where the transition of metal ions, by catalysing the interaction and decomposition of hydroperoxides, contributes to lipid oxidation, which is the main source of degradation of food products.[44]

Rancimat Test

The Rancimat test is a very easy and inexpensive method, which requires small sample volumes and achieves reproducible results. Although this technique has been questioned, it is commonly used in the food industry and governmental analytical laboratories.[45] Table 6 gives the related antioxidant activity index (AAI) of lard with the essential oils added. The higher the induction period of the lard with the essential oils added, compared with the control (pure lard), the better the antioxidant activity of that compound.[46] The antioxidant activity index, as determined by the Rancimat method, decreased in the order: BHT > oregano > thyme > ascorbic acid > clove > sage > rosemary. According to this method, oregano, thyme, clove, sage and rosemary EOs showed antioxidant activity (AAI = 1.05–1.67), but less than the activity of synthetic antioxidants (BHT, AAI = 2.42). At the maximum concentration (5%), oregano essential oil showed the highest ($p < 0.05$) antioxidant activity index (1.67) of all the essential oils analysed. At a concentration of 2% no differences were found ($p > 0.05$) between oregano and thyme EOs. At concentrations of 0.5% and 1% no differences were found ($p > 0.05$) between oregano, thyme and clove essential oils. At all concentrations (0.5%, 1%, 2% and 5%) sage and rosemary essential oils showed the lowest ($p < 0.05$) antioxidant activity indices.

Conclusions

The results obtained using five different methods to evaluate the antioxidant activity (DPPH, FRAP, TBARS, FIC and Rancimat) showed that oregano, clove, thyme, sage and rosemary essential oils can be considered good sources of natural compounds with significant antioxidant activity, which can be attributed to the high percentage of the main constituents or to synergy among the different oil constituents.

It is very difficult to assess the antioxidant activity of a product on the basis of a single method. Antioxidant activity assessment may require a combination of different methods, and the results obtained in this study confirm the difficulty of comparing the results of the many different methods used to test antioxidant activities.

The correct estimation of the antioxidant activity of a given essential oil requires the evaluation of its optimal concentration. On the other hand, the differences found in the different methodologies may, to a certain extent, be explained by the relative amounts of minor compounds in the oils, which may play a major role in the final oil antioxidant effect.

In conclusion, the antioxidant power measured depends on the chosen method, the concentration and the nature and physicochemical properties of the studied antioxidant.

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SPICES AS FUNCTIONAL FOODS: A REVIEW

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ABSTRACT

Spices and aromatic herbs have been used since antiquity as preservatives, colorants and flavour enhancers. Spices, which have long been the basis of traditional medicine in many countries, have also been the subject of study, particularly by the chemical, pharmaceutical and food industries, because of their potential use to improve health. Both *in vitro* and *in vivo* studies have demonstrated how these substances act as antioxidants, digestive stimulants and hypolipidemics and show antibacterial, anti-inflammatory, antiviral and anti-cancerigenic activities. These beneficial physiological effects may also have possible preventative applications in a variety of pathologies. The aim of this revision is to present an overview of the potential of spices and aromatic herbs as functional foods.

Keywords: flavonoids, antioxidant, antibacterial, hipolipidemic, antiinflammatory, anticancerigenic.

INTRODUCTION

The first documented uses of spices date back to 5000 BC when the Sumerians were known to use thyme for its beneficial effects. In 2000 BC a precursor of curry was used in India, while Egyptian papyruses from 1555 BC mention the use of coriander, fennel, juniper, cumin, garlic and thyme (Bellamy *et al.*, 1992), and dried mint dating from 1000 BC has been found in pyramids from the same country (Block, 1986). The use of spices was common practice in Ancient Greece and Rome, although their much more extensive use began with Charlemagne. The journeys of Marco Polo (XIII century) and the European colonisation of America, Africa and Asia (XV-XVIII centuries) spread their use even further a field.

Spices are an important part of human nutrition and have a place in all the cultures of the world. The literature describes how they impart flavour and reduce the need for salt and fatty condiments, improve digestion and provide the organism with extra antioxidants that prevent the appearance of physiological and metabolic alterations (Perez-Alvarez *et al.*, 2002).

Recent years have seen increased interest on the part of consumers, researchers and the food industry into how food products can help maintain the health of the organism, while the role which diet plays in the prevention and treatment of many illnesses has become widely accepted. Basic concepts of nutrition are undergoing significant change, while the classical concept of "adequate nutrition", that is, the provision of nutrients (carbohydrates, proteins, fats, vitamins and minerals) is slowly being replaced by the concept of "optimal nutrition", which, besides the components mentioned above, includes the potentiality of foods to promote health, improve wellbeing (Perez-Alvarez, 2007) and reduce the risk of developing disease. Hence, the appearance of terms likes functional foods, designed or therapeutic foods, superfoods or medicinal foods (Nagai and Inoue, 2004).

From the functional food point of view, perhaps we should first look at the way in which aromatic herbs and spices are used in the diet. There is no one definition of the term functional food, which is used in many contexts, including as technological advances, food marketing and food regulatory norms (Palou *et al.*, 2003). Indeed, the concept of functional food is complex and may refer to many possible aspects, including food obtained by any process, whose particular characteristic is that one or more of its components, whether or not that component is itself a nutrient, affects the target function of the organism in a specific and positive way, promoting a physiological or psychological effect beyond the merely nutritional. The positive effect of a functional food may include the maintenance of health or wellbeing, or a reduction in the risk of suffering a given illness (Pérez-Álvarez *et al.*, 2003). Functional food may be obtained by modifying one or more of the ingredients, or by eliminating the same (Pérez-Álvarez *et al.*, 2003). Aromatic herbs and spices conform to this definition in several ways, although the establishment of any function would involve identifying the bioactive components of these products to help specify their possible beneficial effects on health.

Health claims are science-based, and are related to health benefits of the products helping consumers to prevent disease and improve their health through sound dietary decisions using enhanced nutrition information (FDA, 2003).

Consumers repeatedly express interest and belief in foods that promote health. Numerous studies show that consumers find value in claims (Wansink, 2003; Ippolito and Mathios, 1993)

Industry should take into account that the use of functional ingredients is related to the health of consumers. The science therefore must be scientifically and extensive proved. For this reason the "spice health claims" are very expensive and need a lot of investment.

The specifications for spice health claims are still relatively unclear and so industry could claim that there is a certain amount of inherited cultural knowledge and little scientific evidence. Moreover, it is important to note that some rejections related to spice healthy aspects are not a rejection of the ingredient or product, but just that the scientific evidence submitted is incomplete.

Human clinical trials are also important, and the value of such trials for the food industry is undeniable, but all too often nutrients are pulled out of context, following the same methodology as used for the testing of drugs. Hence in many cultures the use of spices to cure pathologies is important and may well serve a base on which to rest scientific evidence in support of health benefits.

However, spices as a whole food do not normally form part of the usual food chain, randomised clinical trials are the best of the best because such compounds can be tested and retested successfully.

CHEMICAL COMPOSITION OF SPICES

The first distinction to be made is that between a culinary herb and a spice. In general, the leaves of a plant used in cooking are denominated culinary herbs, while any other part of the plant is known as a spice. Spices can be leaf (e.g. bay leaf), buds (clove), bark (cinnamon), root (ginger), berries (grains of pepper), seeds (cumin) or even the stigma of the flower (saffron).

Both spices and herbs can be used fresh, dried, whole, chopped or ground and, due to their colour, aroma and/or flavour characteristics are used in the preparation of foods and drinks (Díaz-Maroto *et al.*, 2002). In their composition can be found proteins, fibre, sugars, essential oils, minerals and pigments (Viuda *et al.*, 2007a), besides bioactive compounds such as phenolic acids, flavonoids, sterols and cumarins (Susheela, 2000). Many of the functional properties presented by spices are associated with the presence, type and concentration of phenolic compounds, although the exact composition will depend on several factors, such as the part of the plant used, its vegetative state, environmental conditions, harvesting technique, etc. (Cosentino *et al.*, 1999). Table 1 shows the main compounds of a phenolic nature in several spices.

Other compounds present in spices are the essential oils (EOs), which, due to their content in terpenes, monoterpenes and sesquiterpenes (as hydrocarbons, alcohols, ketones, etc., which may be acyclic, monocyclic, bicyclics, tricyclics), are responsible for many of their functional properties. These, in general terms, are composed of more than seventy components (Russo *et al.*, 1999), some of which may represent more than 85% of the total content, while others may only be present in trace amounts (Bauer *et al.*, 2001). However, the role played by these minor compounds is very important since evidence suggests that they may contribute significantly to the functional properties of EOs, in which they sometimes act synergically. Table 2 shows the major components of several spices.

As with the phenolic compounds, the composition, type and concentration of the EOs is influenced by climate, harvesting time, part of the plant used, extraction method, etc. (Faleiro *et al.*, 2002). For example, Kokkini et al., (1997) described the four major components of *Origanum vulgare* subsp. *Hirtum* harvested in autumn as: carvacrol (1.7-69.6%), thymol, (0.2-42.8%), *p*-cymene, (17.3-51.3%) and γ-terpinene (0.6-3.6%) while Veres *et al.*, (2003) analysing the same variety harvested at another time of

the year and in a different geographical area found the major components to be carvacrol (76.4%), γ-terpinene (6.6%), *p*-cymene (4.7%) and thymol (0.23%).

Phenolic compounds

One of the main compounds responsible for most of the functional properties of many foods, among them herbs and spices, are phenolic compounds in any of their forms, whether simple phenols, flavones, flavanones, flavanols, flavonols, anthocyanins, etc. Many studies have pointed to the functional properties of phenolic compounds and, more specifically, flavonoids, which include their antioxidant (Bozin *et al.*, 2008; Li *et al.*, 2009), antibacterial (Adedapo *et al.*, 2008; Babajide *et al.*, 2008), antiviral (Tait *et al.*, 2006; Fritz *et al.*, 2007) and anti-inflammatory (Lin *et al.*, 2008a; Lameira *et al.*, 2008) capacities; their cardioprotective (Moon *et al.*, 2003; Celle *et al.*, 2004) and anticarcinogenic (Pergola *et al.*, 2006; Russo *et al.*, 2006) effects, and their ability to act as inhibitors of platelet aggregation (Weng *et al.*, 2006; Arct and Pytkowska, 2008).

Phenolic compounds are to be found in most plants and in many cases they contribute to their colour and taste (Belitz and Grosh, 1997). Chemically, phenols can be defined as substances that possess an aromatic ring bound to one or more hydrogenated substituents, including their functional derivates (Marin *et al.*, 2001). The simplest phenols are liquid or solid with a low fusion point and high boiling point since they form hydrogen bonds. They are colourless, unless they present a group capable of giving them colour. However, they are easily oxidised, which is why they frequently appear to be coloured (Marín *et al.*, 2001).

Terpenes

The name given to a substantial group of vegetal components with a common biosynthetic origin, are the fundamental component of essential oils. Despite their very different chemical structures, all result from the condensation of isoprenic units (Yoshikawa *et al.*, 1996). Among their different forms are monoterpenes, diterpenes and sesquiterpenes. They are extremely volatile and have been demonstrated to possess multiple functional properties, including antioxidant (Mata *et al.*, 2007, Milan *et al.*, 2008), antimicrobial (Viuda *et al.*, 2007b; Viuda *et al.*, 2007c) and antiviral (Schnitzler *et al.*, 2008; Koch *et al.*, 2008) capacities.

ANTIOXIDANT PROPERTIES

In recent years many ingredients and by-products of the agro-food industry have been studied as possible sources of natural antioxidants. Among such compounds are spices, which have shown their ability to slow down the process of lipidic oxidation (Fernández-López *et al.*, 2003). This process is one of the principal causes of food spoilage and may occur during the storage of raw materials, processing or the storage of the final product (Tepe *et al.*, 2005). Such oxidation leads to a significant loss of a food's nutritional value since it involves a loss of vitamins and essential fatty acids. It also affects the food's sensorial quality – changes in colour, texture and taste – which shortens its shelf life and can result in rejection on the part of consumers (Fernández-López *et al.*, 2007). To avoid such spoilage the food industry has resorted to synthetic antioxidant formulations, some of the most commonly used being butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate (PG) (Valencia *et al.*, 2007). However, because of their synthetic origin, their safety and efficacy are frequently questioned. The result has been a growing interest in substances of a vegetal origin that show antioxidant potential for

use as natural additives to replace the synthetic antioxidants, whose use is increasingly restricted due to the secondary effects they may produce (Zheng and Wang, 2001).

The antioxidant activity of spices, essential oils and their components have been the subject of many studies (Mata *et al.*, 2007, Milan *et al.*, 2008; Bozin *et al.*, 2008; Li *et al.*, 2009). Many of the assays related with these studies have been conducted *in vitro*.

The action mechanism set in motion by the antioxidant activity of these compounds is still not clearly understood. Among possible mechanisms are the scavenging of free radicals, hydrogen donation, the chelating of metallic ions or the capacity to act as substrate of radicals such as superoxide or hydroxyl (Van Aecker *et al.*, 1996; Al-Mamary *et al.*, 2002).

These bioactive compounds with their antioxidant properties may also interfere with propagation reactions (Cotelle *et al.*, 1996; Russo *et al.*, 2000), or inhibit the enzymatic systems involved in initiation reactions (Hoult *et al.*, 1994; You *et al.*, 1999). Differences in the antioxidant activities of flavonoids depend on their different molecular structures, especially as regards the degree of hydroxylation and methylation of the compounds (Mayer *et al.*, 1998).

The determination of the antioxidant capacity of spices and their derivates in foods is being given greater importance by researchers and those involved in the agro-food industry. Table 3 provides a list of studies into the antioxidant capacity of several spices and their derivates.

ANTIBACTERIAL PROPERTIES

Many organoleptic and sensorial properties of foods diminish with time. There are many factors involved (temperature, light, oxygen, etc.) but the most important is undoubtedly the action of microorganisms. Many techniques have been used, some since antiquity, to preserve foods from microorganic attack, including refrigeration, freezing, water activity reduction, the restriction of nutrients, acidification, modified atmosphere packaging, fermentation or the addition of antimicrobial compounds. To these may be added new technologies such as high pressure, electric pulses, nanotechnology and irradiation (Viuda *et al.*, 2008).

The addition of microbial agents to foods is a particularly effective method for controlling microbial contamination (Kabuki *et al.*, 2000). The agents used in food are compounds that are added or found naturally therein and which act by inhibiting the growth of the microorganisms (Davidson, 1997). As stated above, the food industry is under pressure from consumers to replace synthetic preservatives by natural ones and spices tend to be the main source of these (Deba *et al.*, 2007).

Among these natural antimicrobials are spices and their derivates such as essential oils (EOs). The main advantage of using EOs and which makes their use in the food industry so widespread is that they are classified as GRAS (Generally Recognized As Safe) (Kabara, 1991), although it must be borne in mind that their maximum efficacy must be determined and they should not provoke appreciable changes in the organoleptic properties of the foods to which they are added.

Antimicrobial compounds in food, whether or not processed, can increase the shelf life of the same by reducing the growth of microorganisms or by reducing their viability (Beuchat and Golden, 1987). Originally, spices and the EOs were added to foods in order to modify or potentiate a given taste, and only indirectly to increase the food's useful life by reducing the microbial load or inhibiting their growth and multiplication (Kim *et al.*, 2001).

The antimicrobial properties of EOs have been demonstrated against a large number of microbial strains, among them *Staphylococcus* spp., *Lactobacillus* spp. and *Enterobacteriaceae*, as well as certain moulds (e.g. *Aspegillus* spp.) (Viuda-Martos *et al.*, 2007b; Viuda-Martos *et al.*, 2007c).

Generally, the composition, structure and functional groups of EOs play an important role in determining their antimicrobial capacity. Compounds containing phenolic groups are responsible for these antimicrobial properties, although other compounds, too, present the same properties (Dorman and Deans, 2000).

Possible action modes of the constituents of spices have been proposed (Sofos, 1998; Davidson and Naidu, 2000; Davidson, 2001), although no specific mechanism has been confirmed. Prindle and Wright (1997) claimed that the effect of phenolic compounds is dose-dependent. At low concentrations, the phenolic compounds affect the enzymatic activity, especially of those associated to energy production, while high concentrations result in protein denaturalisation. The effect of phenolic compounds and terpenes on the growth and production of toxins may be the result of their propensity to alter the permeability of the microbe cell wall, leading to the loss of macromolecules; they might also interact with the proteins present in the cell wall, deforming its structure and leading to a loss of functionality (Fung *et al.*, 1977).

The composition, structure and functional groups of EOs play an important role in determining their antimicrobial capacity. It has been demonstrated that the concentration of an EO to be added to a foodstuff is greater (1-3%) than the quantity added to in vitro assays to obtain the same level of antimicrobial activity, which is one of the reasons that their use in the food sector is limited, since high concentrations lead to organopleptic alterations, reducing consumer acceptability (Lis-Balchin and Deans, 1997).

The interaction between the phenolic groups of EOs and proteins, lipids and aldehydes may partially explain the reduction of antimicrobial effects. It has been suggested that the extracts derived from spices be used as antimicrobial agents forming part of a technological barrier system that involves the preservation of food by the multiple and simultaneous action of a series of compounds and/or actions (Nychas and Skandamis, 2003).

The development of multicomponent antimicrobial systems for foods will depend on totally understanding the action mechanisms of specific agents to be able to seek potential effective combinations (Holley and Gill, 2004). The joint use of essential oils, along with other factors, will facilitate their use as antimicrobial agents without affecting the organoleptic characteristics of the product concerned: for example, of low pH values, low temperatures or low oxygen levels will permit lower concentrations of EOs to be used (Skandamis and Nychas 2000).

Several factors may increase microbial resistance to EOs: for example, the greater availability of nutrients in foods than in the culture media used in *in vitro* tests, which may permit bacteria to repair damaged cells more quickly (Burt, 2004). Not only intrinsic characteristics of the food such as the proportion of fatty acids, water content, protein content, presence of antioxidants, pH salt, etc. maybe relevant in this respect, but also extrinsic factors, such as temperature, vacuum packaging, gas, air, the particular characteristics of the mocroorganisms in question, may also influence bacterial sensitivity (Tassou *et al.*, 1995).

In general, the susceptibility of bacteria to the antimicrobial effect of EOs increases as the pH of the food, the storage temperature and quantity of oxygen in the packaging decrease (Skandamis and Nychas 2000). At low pH, the hydrophobicity of EOs increases, facilitating their solubility in the membrane lipids and hence helping their antimicrobial action (Juven *et al.*, 1994).

It is accepted that high levels of fat and/or protein in foods protect bacteria against the action of EOs. For example, if the EO dissolves in the lipid phase of the food there will be less available to act on the bacteria present in the aqueous phase (Mejlholm and Dalgaard, 2002), while carbohydrates do not appear to offer the bacteria as much protection against the action of EOs (Shelef *et al.*, 1984). On the other hand, a high concentration of water and/or salt facilitates the action of EOs (Skandamis and Nychas 2000). Table 4 lists how certain spices and their derivates have been used as antimicrobial agents in different foods.

ANTI-INFLAMMATORY ACTIVITY

The topical application of spices and extracts is common practice for alleviating a variety of discomforts such as backache, rheumatism, skin rashes and inflammatory processes in general (Ramadan, 2007). The inflammatory process is triggered by several chemical and/or biological aspects that include pro-inflammatory enzymes and cytokines, low molecular weight compounds such as eicosanoids or the enzymatic degradation of tissues (Dao *et al.*, 2004). Several studies (Cho, *et al.*, 2004) have related cyclooxigenase-2 (COX-2) with the inflammatory process. This enzyme is an isoform of cyclooxigenase (COX) which is responsible for catalysing araquidonic acid to prostaglandin. The other isoform is cyclooxigenase-1 (COX-1) which regulates homeostasis processes (Dao *et al.*, 2004).

Many studies have demonstrated the anti-inflammatory capacity of spices (Fang *et al.*, 2005; Pérez-Fons *et al.*, 2006). This capacity is basically due to the presence of flavonoids that inhibit the development of inflammation provoked by a variety of agents (Mani *et al.*, 2006). Among these flavonoids, galangin, which is present in *Lippia graveolens* (Lin *et al.*, 2007), is capable of inhibiting cyclooxigenase (COX) and lipooxygenase, weakening the action of polygalacturonase and reducing the expression of the inducible isoform of ciclooxigenasa (COX-2) (Raso *et al.*, 2001). For Houghton *et al.*, (1995), the anti-inflammatory activity arises from the inhibition of eicosanoic acid and of the lipidic peroxidation of the cell membrane through the inhibition of cyclooxigenase and 5-lipooxigenase enzymatic systems.

For Srinvasan (2005), the anti-inflammatory activity of spices is due to the action of its active compounds that inhibit the formation of metabolites such as prostaglandin E_2 (PgE_2) or leukocytes or which inhibit the formation of liposomal enzymes by macrophages such as collagenase and elastase. Poeckel *et al.*, (2008) describes the anti-inflammatory activity of carnosic acid and carnosol, compounds of a phenolic nature occurring in spices such as rosemary and sage, which act through the formation of pro-inflammatory compounds such as leukotrienes. Volate *et al.*, (2005) suggested that the compounds present in spices such as quercetin, curcumin and silymarin have the same anti-inflammatory effects as indometacin (a non-steroidal drug).

INHIBITION OF PLATELET AGGREGATION

Thrombosis resulting from platelet aggregation plays a very important role in cardiovascular diseases. Spices and their extracts, being rich in natural antioxidants, may exercise a degree of

antiplatelet activity (Suneetha and Krishnakantha 2005). Duarte *et al.,* (2001a) thought that the flavonoids present in spices may diminish platelet aggregation, although high doses are necessary. The action mechanisms of flavonoids in this process could be related with their inhibition of the enzyme phosphodiesterase (Beretz *et al.,* 1986), the inhibition of cyclooxigenase, with the consequent depression of thromboxane A2 synthesis (Tzeng *et al.,* 1992), or inhibition of the metabolism of araquidonic acid (Duarte *et al.,* 2001a). For Heemskerk and Sage (1994), the mechanism through which platelet aggregation occurs is related with the levels of calcium: increased calcium levels in the cytosol provoke platelet aggregation due to the action of certain enzymes that are not fully functional at low calcium concentrations.

Platelet aggregation may be inhibited through the action of different components in spices or their extracts with the membrane bi-layer. This would result in swelling of the membranes and therefore affect the mobilisation of calcium. In turn, this would block the platelet aggregation mechanism since the enzymes necessary for aggregation would lack stimulation (Suneetha and Krishnakantha 2005).

For Lee *et al.,* (2006), the platelet inhibiting effect of carnasol, a flavonoid present in herbs and spices of the genus *Laminaceae*, is mediated by the inhibition of the thromboxane A2 receptor and mobilisation of the cytosol calcium. For Manach *et al.,* (1996), the extent of inhibition depends on the type of induction and structure of the flavonoid, so that kaempherol and quercetin would inhibit aggregation by inducing araquidonic acid. Chang and Hsu (1992) describe how luteolin and chlorogenic acid impede the platelet aggregation induced by collagen and ADP.

ANTIVIRAL ACTIVITY

Another functional property of spices and herbs is their antiviral activity. Aruoma *et al.,* (1996) showed that carnosol and carnosic acid, the two main components of rosemary, show inhibitory activity over the human immunodeficiency virus (HIV). Similarly, Critchfield *et al.,* (1996) claimed that apigenin, a flavonoid characteristic of several spices (thyme, sage, oregano, rosemary), inhibited HIV-1 in latent infection models through a mechanism that probably included inhibition of viral transcription. Bedoya *et al.,* (2002) described how hydroalcoholic extracts of hyssop (*Hysopp officinalis)* had inhibitory effect on HIV-1.

Several studies have described the antiviral activity of curcumin (Joe *et al.,* 2004 Lin and Lee 2006). In studies carried out by Kutluay *et al.,* (2008), curcumin was seen to inhibit the activity of histone acetyltransferase, thus impeding the genic promotion of the type 1 herpes simple virus (HSV-1). Si *et al.,* (2007) described how curcumin acts against the replication of coxsackievirus by disrupting the ubiquitin-proteasome system (UPS).

The antiviral effect of essential oils, fundamental components of spices, has also been demonstrated. For example, Benencia and Courreges (2000) demonstrated, both *in vitro* and in a mouse model, that eugenol, the principal component of clove bud (*Eugenia caryophyllus*) essential oil presented antiviral activity against the types 1 and 2 herpes simple viruses (HSV-1 and HSV-2). The same compound was investigated by Tragoolpua and Jatisatenr (2007), who found that it impeded the replication of HSV-I and HSV-2. When Schnitzler *et al.,* (2008) analysed the *in vitro* effect of the EO of melissa (*Melissa officinalis*) against HSV-1 and HSV-2, they found that it showed strong antiviral activity as long as it was added after the virus had penetrated the cell. In an analysis of the effect of the EOs of several herbs and

spices against HSV-2, Koch *et al.*, (2008) found that hyssop (*Hyssopus officinalis*), thyme (*Thymus vulgaris* L.), anis (*Pimpinella anisum*), ginger (*Zingiber officinale*), camomile (*Chamaemelum nobile*) and sandalwood (*Santalum álbum*) all showed antiviral activity, possibly through their interaction with the development of the virus.

ANTICARCINOGENIC PROPERTIES

Many of the non-nutritive components of fruit and vegetables, and of herbs and spices, are known to possess potential activity as chemoprotective agents against cancer. Among the action mechanisms proposed for these compounds are (Tanaka *et al.*, 2008):

- Inhibiting of the phase I enzymes or blocking carcinogen formation.
- Induction of phase II (detoxification) enzymes.
- Scavenging DNA reactive agents.
- Modulation of homeostatic hormones.
- Suppression hyper-cells proliferation induced by carcinogen.
- Induction of apoptosis.
- Depression of tumour angiogenesis.
- Inhibition of phenotypic expressions of preneoplastic and neoplastic cells.

Many studies have discussed the anticarcinogenic properties of aromatic herbs and spices (Aggarwal *et al.*, 2007; Tsai *et al.*, 2007; Bonaccorsi *et al.* 2008; Ramos *et al.*, 2008; Sanchez *et al.*, 2008). Particularly mentioned in this respect has been rosemary and its components ursolic acid, carnosol, etc., turmeric and its principal component curcumin, and pepper (principal component capsaicin).

Tsai *et al.*, (2007) demonstrated that 500 µg/mL of rosemary suppressed the production of NO, which is responsible for oxidative stress in many diseases, including cancer. In studies using hamsters, Slamenová *et al.*, (2002) showed that the application of 30 µg/mL of a rosemary extract protected DNA from oxidative damage, the action mechanism being related with the capacity of rosemary to sequester OH^- radicals and singlet oxygen ($^1O_2$). A component of rosemary, carnosol, was studied by Dörrie *et al.*, (2001) and was seen to present anticarcinogenic activity against several cancerigenic lines, the authors suggesting that cell apoptosis would cause the loss of DNA, externalisation of the phosphatidylserine cell membrane and depolarisation the mitochondrial membrane. Prior to this, Singletary *et al.*, (1996) showed that the application of carnosol prevented the damage to DNA caused by 7,12-dimethylbenz[*a*]anthracene (DMBA) which provoked the formation of tumours in rat mammary glands. A similar study was carried out by Sancheti and Goyal (2006) in which the authors indicated that an extract from rosemary leaves lengthened the latency period of tumours and reduced the incidence of malign tumours induced by DMBA in rats. Another component of rosemary, ursolic acid, has also been to show anticarcinogenic activity. Bonaccorsi *et al.*, (2008) indicated that this acid inhibited reverse transcriptase activity of tumoural cells, which is related with the control of neoplastic cell proliferation and differentiation. Ramos *et al.*, (2008) indicated that ursolic acid acted as an anticarcinogenic agent through cell mediated mechanisms.

Another spice widely studied for its anticarcinogenic effects is pepper. Chow *et al.*, (2007) indicated that the principal component, capsaicin, promotes the apoptosis of carcinogenic cells, corroborating the findings of Baba *et al.*, (2006) concerning tumoral cells, although the mechanism was unclear. Sánchez *et*

al., (2008) demonstrated the antiproliferative effect induced by capsaicin in prostate cancer cells (PC-3) through a stress-related mechanism.

Turmeric (*Curcuma longa*), too, has been seen to have anticarcinogenic properties, especially its main component curcumin. In laboratory studies, Tayyen *et al.*, (2006) demonstrated that turmeric significantly reduced cancer of the colon, while Anand *et al.*, (2008) demonstrated its effect against several types of cancer, including leukaemia, breast, ovarian and gastrointestinal cancer, as a result of its capacity to induce apoptosis or inhibit metastasis and angiogenesis. Lin *et al.*, (2008b) also cited its ability to induce apoptosis in cancerous lung cells. Aggarwal *et al.*, (2007) observed that curcumin exhibited similar activity to recently discovered tumoral necrosis blocking factors (Humira, Remicade and Enbrel), blockers of vascular endothelial cell growth factor (Avastin) and human epidermal growth factor receptor blockers (Erbitux, Erlotinib and Geftinib).

DIGESTIVE STIMULANTS

The use of spices in foods favours their subsequent digestion because of the beneficial effects they have on the digestive process. They increase the secretion of saliva and gastric juices (Tapsell *et al.*, 2006), increase the concentration of biliary acids which play a fundamental role in the digestion and absorption of fatty acids (Bhat *et al.*, 1984) and increase the secretion of digestive enzymes in the pancreas, such as lipase, amylase, trypsin and chemotrypsin which also play an important role in digestion (Tapsell *et al.*, 2006).

ANTI-ULCEROUS PROPERTIES

Another of the functional properties of both herbs and spices is the protection they offer against ulcers (Borrelli and Izzo 2000; Kakub, 2007), a capacity due fundamentally to the presence of phenolic compounds, especially flavonoids (Batista *et al.*, 2004; Hiruma-Lima *et al.*, 2006).

The way in which these compounds act seems to vary greatly. Vilegas *et al.*, (1999) mention that flavonoids provoke an increase in prostaglandins, which have an inhibitory effect on acidic secretions and thus prevent the formation of ulcers. Speroni and Ferri (1993) suggest that flavonoids increase the prostaglandin content of the gastric mucosa, protecting it from the formation of ulcers. Other authors suggest that ulcerous lesions are related with oxygen reactive species. Flavonoids inhibit lipidic peroxidation by substantially increasing the activity of glutathione peroxidase (Martin *et al.*, 1998; Duarte *et al.*, 2001b).

Some studies, such as those of Leite *et al.*, (2001), point to the anti-ulcerous capacity of flavonoids like kaempherol and quercetin, compounds that are present in several spices. Another theory proposed by Borrelli and Izzo (2000), Osadebe and Okoye (2003), among others is that the anti-ulcerous capacity is due to the joint action of flavonoids with other substances, such as terpenes, sterols, tannins, carbohydrates, glycosides, saponins and traces of alkaloids, which, together with flavonoids are to be found in herbs and spices.

ANTI-DIABETIC PROPERTIES

Diabetes is the most common metabolic disease in the world and is still increasing. International Diabetes Federation, mentioned that 194 million people had diabetes in 2003, which will increase to 333 million by 2025 (Sicree *et al.*, 2003) According to the World Heath Organisation, it is the third most important disease after cardiovascular and oenological disorders. One of the ways to control diabetes

mellitus is through the diet and it is here that herbs and spices can play a part. Indeed numerous studies have described their ant-diabetic activity (Khan *et al.*, 2003; Anderson *et al.*, 2004; Srinivasan 2005; Büyückbalci and El-Nehir, 2008).

For example, cinnamon (*Cinnamomum zeylanicum*) is by far the most valued spice for improving diabetes. In *in vitro* experiments, Khan *et al.,* (1990) showed that it has a potentiating effect on insulin due to the high concentrations of chrome, a known insulin potentiator (Anderson, 1997). According to Khan *et al.,* (2003), the ingestion of 1-6 g of cinnamon in the diet decreases serum glucose levels after 40 days. Cao *et al.*, (2007) demonstrated that cinnamon extracts increase levels of insulin beta and transporter 4 glucose in diabetic rats, thus decreasing the serum levels of glucose. Babu *et al.,* (2007) indicated that cinnamaldehyde, a component of cinnamon, has a hypoglucemic effect on diabetic rats. Extracts of cinnamon activate glucogen synthase, increase glucose uptake, inhibit glucogen synthase kinase-3 and activate insulin receptor kinase and inhibit dephosphorylation of the insulin receptor, leading to maximal phosphorylation of the insulin receptor (Jarvill-Taylor *et al.,* 2001). All of these effects would lead to increased insulin sensitivity.

Another species showing potential anti-diabetic activity is turmeric (*Curcuma longa*). Srinivasan (2005) stated that the daily intake of curcumin (colouring principle of turmeric) not only reduced the fasting blood sugar level, but also lowered the dosage of insulin needed for normoglycaemia. Arun and Nalini (2002) reported that curcumin reduced blood glucose in alloxan-induced diabetic rats and suggested that curcumin had a better hypoglycemic effect than turmeric although the mechanism of the anti-diabetic action was left unclear. Fujiwara *et al.,* (2008) reported that the anti-diabetic effects of curcumin are partly due to a reduction in hepatic glucose production caused by activation of AMP kinase and inhibition of hepatic glucose-6-phosphatase activity and phosphoenolpyruvate carboxykinase activity. For Nishiyama *et al.,* (2005) the anti-diabetic effect of turmeric is due to the presence of curcuminoids and sesquiterpenoids, which also have a synergic effect.

Fenugreek, too, shows anti-diabetic activity. Sharma *et al.,* (1996a) envisaged a hypoglycaemic effect in a human trial involving 60 non-insulin-dependent diabetes mellitus patients. Fenugreek whole seed powder was given at 25 g/day through the diet in two doses over a period of 24 weeks. Fasting blood glucose was lowered and glucose tolerance improved. Sugar excretion was significantly reduced and insulin levels were also diminished. Eidi *et al.,* (2007) analysed the effect of oral administering fenugreek extracts on diabetic mice, observing a reduction in serum levels of glucose. The anti-diabetic effect was similar to those presented by glibenclamida an anti-diabetic drug. In the opinion of Acharya *et al.,* (2008), the anti-diabetic effect of the seeds and leaves of this spice are due to the presence of steroidal sapogenins and mucilaginous fibres.

INFLUENCE ON HYPOLIPIDEMIA

Hypolipidemic agents are a diverse group of drugs used to treat hyperlipidemia. They are used to reduce the total cholesterol concentration in blood, LDL cholesterol and triglycerides, especially in patients with a high risk of cardiovascular problems. Srinivasan (2005) demonstrated that spices can act as effective hypocholesterolemics, although they also have beneficial effects on the general lipid metabolism in different conditions of lipidemia (Nalini *et al.,* 2006).

There are scientific evidences that free radical oxidation of the low density lipoproteins (LDL) plays an important role in the development of atheroma plaque (Cannon, 2007). Compounds present in myrtle (*Myrtus communis* L.) have been shown to significantly preserved protect LDL from oxidative damage and showed remarkable protective effect on the reduction of polyunsaturated fatty acids and cholesterol, inhibiting the increase of their oxidative products (Rosa *et al.*, 2008).

In a study using rats, Dhandapani *et al.*, (2002) showed that treatment with cumin (*Cuminunm cyminum*) significantly reduces cholesterol levels at both plasma and tissue levels, besides reducing phospholipid, free fatty acid and triglyceride levels. Similarly, Kempaiah and Srinivasan (2006) showed that the inclusion of cumin (0.2%) or capsaicin (0.015%), the major components of turmeric and pepper, respectively, in the diet of rats with high cholesterol levels lowers plasmatic levels of triglycerides and cholesterol. These studies were corroborated by Baum *et al.*, (2007), who indicated that curcumin lowers serum cholesterol levels. In another study, Manjunatha and Srinivasan (2006) analysed curcumin and capsaicin separately and together in rats, finding that in both cases LDL oxidation was inhibited and that they acted synergically. Al-Habori *et al.*, (1998) showed that LDL and triglyceride levels were reduced in rabbits given fenugreek (*Trigonella foenum graecum*), an effect attributed to the presence of saponins, fibre and, especially, a trigonellin alkaloid (Al-Habori and Raman, 1998).

The mechanism through which the mentioned hypocholesterolemic effect is produced seems to be related with the billiary acids responsible for the metabolism of lipids (Sambaiah and Srinivasan, 1991) through the activation of hepatic enzymes such as cholesterol-7α-hydrosylase (Srinivasan and Sambaiah 1991). It has also been demonstrated that spices in foods can reduce the intestinal absorption of lipids, accompanied by a greater excretion of cholesterol (Srinivasan and Srinivasan, 1995).

CONCLUSIONS

Besides the role that they play in imparting taste and flavour to the food we eat, the long list of beneficial physiological effects that spices and herbs have on health suggests that they might well be considered as essential (and natural) components of our diet. Every spice has its own potentially beneficial property and there is the possibility, too, that they might have synergic effects, increasing their culinary and health-related potential. However, despite this multiplicity of potentially beneficial activity that herbs and spices possess, more in-depth information is required concerning how exactly exposure to their components achieves a specific molecular response. Only when such information is available will it be possible to define strategies of intervention to obtain maximum benefits from herbs and spices without exposure to undesirable side effects.

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TABLES

Table 1. Principal phenolic-type compounds present in spices

Common name	Scientific name	Major phenolic compound	Reference
Rosemary	*Rosmarinus officinalis*	Rosmarinic acid carnosol Ursolic acid Carnosic acid	Almela *et al.*, (2006)
Thyme	*Thymus vulgaris*	Chlorogenic acid Apigenin-7-*O*-glucoside Rosmarinic acid Luteolin-7-*O*-glucósido	(Brantner *et al.*, 2005)
Sage	*Salvia officinalis*	Carnosic acid carnosol Luteolin-7-*O*-glucoside Rosmarinic acid	Lima *et al.*, (2007); Poeckel *et al.*, (2008)
Oregano	*Origanum vulgare*	Ferulic acid Rosmarinic acid Caffeic acid	Exarchou *et al.*, (2002)
Chili pepper	*Capsicum annuum*	Luteolin Capasaicin Quercetin	Lee *et al.*, (1995)
Ginger	*Zingiber officinale*	6-shogaol 10-gingerol 8-gingerol 6-gingerol	Ghayur *et al.*, (2005)
Turmeric	*Curcuma longa*	Caffeic acid p-coumaric acid Ferulic acid Syringic acid	Suhaj, (2006)
Dill	*Anethum graveolens*	Quercetina Kaempherol Isorhamnetin	Justesen and Knuthesen (2001)
Parsley	*Petroselinum crispum*	Caffeic acid p-coumaric acido Ferulic acid Galic acid	Muchuweti *et al.*, (2007)
St John's wort	*Hypericum perforatum*	Kaempherol Quercetin Caffeic acid p-coumaric acid Ferulic acid	Wojdyło *et al.*, (2007)

Table 2. Major components and approximate composition of the essential oils of some spices

Common name	Scientific name	Major components	Approximate % composition	References
Sage	*Salvia officinalis*	Camphor 1,8-cineole	38% 24%	Asllani, (2000)
Rosemary	*Rosmarinus officinalis*	α-pinene 1,8-cineole Camphor	2-46% 3-89% 2-14%	Dellacasa *et al.*, (1999); Daferera *et al.*, (2003)
Clove	*Syzygium aromaticum*	Eugenol Eugenyl acetate	58-94% Traces-20%	Raina *et al.*, (2001); Viuda et al (2007a)
Peppermint	*Mentha piperita*	1,8-cineole Piperitone	18.38% 4.56%	Ka *et al.*, (2005)
Dill	*Anethum graveolens*	Carvone Limonene	55.2% 16.6%	Singh *et al.*, (2005)
Ginger	*Zingiber officinale*	Geranial zingerone	24.2% 14.2%	Menon *et al.*, (2007)
Cumin	*Cuminum cyminum*	γ-terpinene Cuminal *P*-cymene	15-30% 20-36% 18-20.5%	Jirovetz *et al.*, (2005); Viuda *et al.*, (2007a)
Oregano	*Origanum vulgare*	Carvacrol Thymol *P*-cymene	Trace-88% Trace-43% Trace-52%	Veres *et al.*, (2003); Arnold *et al.*, (2000)
Cinnamon	Cinnamomum zeylanicum	Cinnamaldehyde *β*-Caryophyllene α-terpineol	77.1% 6% 4%	Marongiu *et al.*, (2007)
Thyme	*Thymus vulgaris*	Thymol *P*-cymene Carvacrol	10-64% 10-56% 2-11%	Cosentino *et al.*, (1999); Juliano *et al.*, (2000)

Table 3. Overview of studies testing the antioxidant activity of some spices and essential oils in food

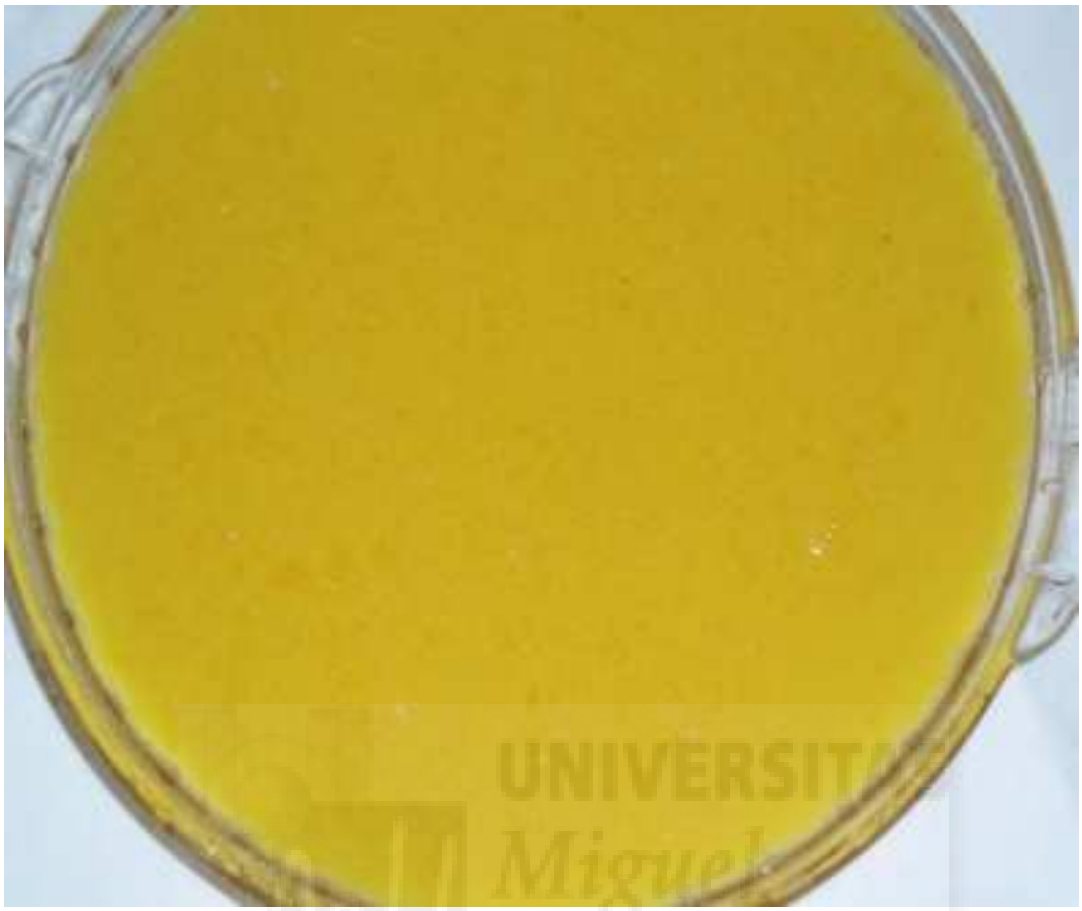
Food group	Food	Spice	Concentration applied	Combination with another preservation method	Reference
Meat	Cured pork fat	Extract of rosemary	0.3%.		Iriarte, *et al.*, (1992)
	Meat balls turkey mince	Sage	1%		Karpinska, *et al.*, (2001)
	dehydrated chicken meat	Extract of rosemary	1%		Nissen, *et al.*, (2000)
	beef mince	Extract of rosemary	0.25%.	Irradiation (1-4 kGy) wrapped in PVC film	Formanek, *et al.*, (2003)
	Patties from muscle of cattle	Extract of rosemary	0.2-0.25%	Wrapped in MAP	Formanek, *et al.*, (2001)
	Meat balls poultry mince	Extract of rosemary	1-1.5%	frozen storage	Karpinska, *et al.*, (2000)
	beef steaks	oleoresin rosemary	0.05 and 0.1%	sodium tripolyphosphate	Stoick, *et al.*, (1989)
	fresh, minced chicken	Rosemary	0.1%		O'Sullivan, *et al.*, (2004a)
	pork sausage	rosemary extract	2.5%	Refrigerated	Sebranek, *et al.*, (2005)
	cooked chicken patties	Tea catechins	0.01%		O'Sullivan, *et al.*, (2004a)
	beef, skinless lean pork, and cured pork back fat	Rosemary powder	0.1%		Korimova, *et al.*, (2003)
	pork patties	Rosemary	0.1%		McCarthy, *et al.*, (2001)
	beef patties	oregano	0.5%.	MAP	Sanchez, *et al.*, (2003)
	pork patties	Catechins	0.25%		McCarthy *et al.*, (2001)
	chicken fat	Sage, rosemary	0.5%		Saricoban and Ozcan, (2004)
	Bologna sausage	Thyme essential oil	0.02%		Viuda *et al.*, (2008a)
	Bologna sausage	Oregano essential oil	0.02%		Viuda *et al.*, (2008b)
	Bologna sausage	Oregano essential oil	0.02%		Viuda *et al.*, (2008c)
	pork patties	Sage	0.05%		McCarthy *et al.*, (2001)
	chicken nuggets	Sage	0.1%	Salt (0.75%)	O'Sullivan, *et al.*, (2004b)
	raw beef steaks	oleoresin rosemary	0.10%	sodium tripolyphosphate	Stoick, *et al.*, (1991)
	buffalo Ghee meat	methanolic extracts of black cumin	0.12%		Emara and Abdel-Kader, (2004)
	lard	Black cumin extract	0.4%		Al-Ismail, (2002)
	Goat meat for fermented sausage	Rosemary	0.05%		Nassu *et al.*, (2003)
	lamb meat	Potato peel extract	0.04%	irradiated	Kanatt *et al.*, (2005)
	Minced beef	Oregano oil	0.5-1%		Skandamis and Nychas, (2001)
	Beef muscle	Oregano oil	1.0%		Oussalah *et al.*, (2004)
Fish	raw fish	clove, rosemary, mace,	0.1%		Liu, *et al.*, (1996)

		oregano, thyme and sage			
	mackerel oil	Dried oregano	1%		Tsimidou, *et al.*, (1995)
	fish oil	Sage	5%		Weng, *et al.*, (1998)
	sardine oil	Rosemary	0.02%	alpha-tocopherol (0.05%)	Wada and Fang, (1992)
	Anchovy lipids	rosemary extract	0.1%	Tocopherol (0.1%) + Vacuum packaging	Young *et al.*, (2000)
	crushed bonito meat	Rosemary	0.02%	alpha-tocopherol (0.05%)	Wada and Fang (1992)
	Catfish cooked	clove, rosemary, oregano, sage and thyme	0.1%		Liu *et al.*, (1996)
Oil	refined olive oil	Oregano	5%		Martinez-Tome *et al.*, (2001)
	rice bran oil	rosemary, sage, oregano, ginger and thyme	2%		Chae *et al.*, (2000)
	refined olive oil	Rosemary	5%		Martinez-Tome *et al.*, (2001)
	maize oil	Black cumin extracted with diethyl ether	0.4%,		Al-Ismail, (2002)
	sunflower oil	thyme, rosemary and sage	2%		Beddows *et al.*, (2000)
Others	salad	marjoram	1.5%		Ninfali, *et al.*, (2005)
	biscuits	Cumin	0.225%		Badei *et al.*, (2000)

Table 4. Overview of studies testing the antibacterial activity of some spices and essential oils in food

Food group	Food	Spice	Concentration applied	Bacterial species	Combination with another preservation method	References
Meat	Raw chicken meat mince	cinnamon extracts	0.8%	*Aeromonas hydrophila*		Yadav *et al.*, (2004)
	Cooked chicken sausage	Mustard oil	0.1%	*Escherichia coli*		Lemay *et al.*, (2002)
	Fresh beef fillets	Oregano oil	0.8%	*Salmonella typhimurium*	vacuum or MAP	Skandamis *et al.*, (2002a)
	Hotdogs	Clove oil	1 %	*Listeria monocytogenes*		Singh *et al.*, (2003).
	Beef meat balls	Rosemary oil	0.1% (w/w)	*Listeria spp.; Brochothrix spp.; Lactobacillus spp.; Leuconostoc spp.*		Fernandez-Lopez *et al.*, (2005)
	Minced beef	Oregano oil	0.05-0.1%	Natural flora	MAP	Skandamis and Nychas, (2001)
	Beef fillets	Oregano oil	0.8%	*Listeria monocytogenes*	vacuum or MAP	Tsigarida *et al.*, (2000)
	Pork liver sausage	Rosemary oil	1%	*Listeria monocytogenes*		Pandit and Shelef, (1994)
	Chicken frankfurters	Clove oil	1-2%	*Listeria monocytogenes*		Mytle *et al.*, (2006)
	Beef fillets	Oregano, cranberry powders	ratio of 75% oregano to 25% cranberry	*Listeria monocytogenes*	T (4ºC)	Lin *et al.*, (2004)
	Minced pork	Oregano oil	0.1-0.2%	*Clostridium botulinum* spores	Vacuum packaging	Ismaiel and Pierson, (1990)
	Chicken noodles	Sage oil	0.2-0.5%	*Bacillus cereus; Staphylococus aureus*		Shelef *et al.*, (1984)
	Cooked pork	Coriander oil	1250 µg/cm^2	*Aeromonas hydrophila*		Stecchini *et al.*, (1993)
	Ham	Cilantro oil	0.1-6%	*Listeria monocytogenes*	Vacuum packaging	Gill *et al.*, (2002)
	Cooked chicken	Clove oil	2%	*Aeromonas hydrophila* *Listeria monocytogenes*		Hao *et al.*, (1998)
	Pâté	Mint oil	0.5-2%	*Listeria monocytogenes*		Tassou *et al.*, (1995)
	Beef	Oregano oil or clove oil	1%	*Listeria monocytogenes* Scott	T (4ºC)	Ting and Deibel, 1992)
	Cooked pork	Clove oil	500 µg/cm^2	*Aeromonas hydrophila*		Stecchini *et al.*, (1993)
Fish	Cod fillets	Oregano oil	0.05%	*Photobacterium phosphoreum*	Packaged in modified atmosphere	Mejlholm and Dalgaard, (2002)
	Mackerel broth	Clove oil	0.5%	*Enterobacter aerogenes*	2% NaCl	Wendakoon and Sakaguchi, (1993)

	Salmon fillets	Oregano oil	0.05%	*Photobacterium phosphoreum*		Mejlholm and Dalgaard, (2002)
	Cooked shrimps	Thyme oil	1.5%	*Pseudomonas putida*	Cinnamaldehyde	Ouattara *et al.*, (2001)
	Asian sea bass	Thyme oil	0.05%	*Natural flora*	Refrigerated	Harpaz *et al.*, (2003)
	fish roe salad	Mint oil	0.5-2.0%	*Salmonella enteritidis*	Storage T[a]	Tassou *et al.*, (1995)
Dairy	Yoghurt	Clove oil	0.005-0.5% in milk before fermentation	*Streptococcus thermophilus*		Bayoummi, (1992)
	mutton cheese	Cinnamon powder	6%	*Listeria monocytogenes*	T 30°C	Menon *et al.*, (2002)
	mozzarella cheese	Clove oil	0.5 - 1%	*Listeria monocytogenes*		Menon and Garg, (2001)
	soft cheese	ground cloves	1%	*Listeria monocytogenes*		Leuschner and Ielsch, (2003)
	full fat cheese	clove oil	1%	*Listeria monocytogenes*		Smith *et al.*, (2001)
	low fat cheese	bay, clove, cinnamon oil	1%	*Salmonella enteritidis*		Smith *et al.*, (2001)
Vegetables	Lettuce	Thyme oil	0.1-10 mL L^{-1}	*Escherichia coli* O157:H7		Singh *et al.*, (2002).
	aubergine salad	Oregano oil	1%	*Escherichia coli* O157:H7	Low pH	Skandamis *et al.*, (2002b)
	Lettuce	Thyme oil	1%	*Shigella sonnei; Shigella flexneri*	EO added to washing water	Bagamboula *et al.*, (2004)
	Carrots	Thyme oil	0.1-10 mL L^{-1}	*Escherichia coli* O157:H7	EO added to washing water	Singh *et al.*, (2002).
Fuits	Apple juice	Cinnamon	0.1%	*Listeria monocytogenes*	T 5ºC	Yuste and Fung, (2002)
	Melon	Carvacrol	1 mM in dipping solution	Natural flora		Roller and Seedhar, (2002)
	Kiwifruit	Carvacrol	1 mM in dipping solution	Natural flora		Roller and Seedhar, (2002)
Others	Spaghetti sauce	Thyme	1% (w/v)	*Shigella spp.*		Bagamboula *et al.*, (2003)
	Spaghetti sauce	Basil	1% (w/v)	*Shigella spp.*		Bagamboula *et al.*, (2003)



TITULO:	Physico-chemical characterisation of the orange juice waste water of a citrus by-product
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PHYSICO-CHEMICAL CHARACTERISATION OF THE ORANGE JUICE WASTE WATER OF A CITRUS BY-PRODUCT

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ABSTRACT

Large quantities of wastes are produced during the industrial transformation of citrus fruit, and these constitute a serious problem. However, instead of disposing of them, they can be used to obtain by-products rich in bioactive compounds. One such by-product is the orange juice waste water generated in the process of obtaining citrus fibre. The aim of this study was to characterise the physico-chemical and microbiological properties of the orange juice waste water of a citrus by-product by determining the pH, soluble solids, colour, reduction of residual nitrite and total antioxidant activity, along with the phenolic compounds, organic acids and sugars it contains. The orange juice waste water decreases the nitrite levels and shows a high capacity to reduce the formation of free radicals. Narirutin and hesperidin were the most concentrated phenolic compounds, while the main sugar was glucose. Five organic acids were identified, the main one being succinic acid.

PRACTICAL APPLICATIONS

The orange juice waste water can be suitable for applications on the food industry. It is an important source of phenolic compounds which antioxidant properties could be very appreciated in a big number of food processing to avoid its oxidation during processing but also during storage period. The effect on residual nitrite level could be very important in the elaboration process in where the nitrite was used, for example in meat products industry; the reduction of residual nitrite level could reduce the possibility to nitrosamine formation. Other important reason for their suitability is their natural origin, which consumers find comforting and which is beneficial for the environment.

Keywords: Orange juice waste water; flavonoids; DPPH, antioxidant activity; residual nitrite.

INTRODUCTION

Citrus fruits, especially mandarin, orange, lemon and grapefruit are the principal tropical and subtropical fruits, of which orange represents about 63% of the total (Fernández-López *et al.* 2004). Spain is the fifth orange-producing country in the world, after Brazil, USA, China and Mexico (Intercitrus, 2007), with an annual total of 5,105 million tonnes in the 2005-2006 season.

At present, supply greatly exceeds demand for fresh citrus products, which has led to the development of a flourishing processing industry, including the extraction of juice (Chafer *et al.* 2000). Once the juice has been extracted, the wastes that remain (pulp and molasses) are composed mainly of peel (flavedo and albedo), pulp and seeds (Braddock, 1999). These by-products represent 40-50% of the fruit and are a source of fibre (Fernández-López *et al.* 2004; Fernández-Gines *et al.* 2004), dried pulp, essential oils, D-limonene, pectin, seed oil, ascorbic acid and flavonoids (Ozaki *et al.* 2000; Siliha *et al.* 2000). The uses (animal feed, pectin extraction) for obtanining antioxidant fibre from these by-products are scarce (Park *et al.* 1996; Ros *et al.* 1998), and their disposal represents a problem. However, their composition has the potential to be used for other ends, for example to obtain dietary fibre. The extracts rich in dietary fibre and natural antioxidants from the by-products of the citrus processing industry is certainly viable and the extracts could be used as a functional ingredient in the meat (Aleson-Carbonell *et al.* 2005*;* Fernández-López *et al.* 2007) and dairy product (García-Pérez *et al.* 2006*;* Sendra *et al.* 2008) industries. The addition of such extract to meat products has the additional advantage of reducing the concentration of

residual nitrite in the products, thus attenuating the effect of potentially harmful substances such as nitrosamines (Fernández-López *et al.* 2007).

Residues of citrus juice production are mainly constituted by peels (albedo and flavedo) which are almost one-fourth of the whole fruit mass, seeds and fruit pulp remaining after juice and essential oil extraction (Braddock, 1999). It is composes principally by water, soluble sugars, fiber, organic acids, amino acids and proteins, minerals, oils and lipids, and also contains flavonoids and vitamins (Fernández-López *et al.*, 2004; Viuda-Martos *et al*., 2007). All of these components are found in different amounts depending on the fraction of the fruit (juice, albedo, flavedo, rag and pulp, and seeds) (Braddock, 1995), and their proportion in citrus juice residues depends on the juice extraction system used (Marín *et al*., 2002). Lario *et al.* (2004) determined some physicochemical properties of the raw residue from lemon juice industry (19.7% of dry matter, pH of 3.96, 0.96 for water activity, L* = 66.98, a* = -2.63 and b* = 27.44) all of which also depend on the chemical composition of the residue. It is therefore important to know exactly which compounds are present in the by-products generated by the food industry in general, and the citrus juice extraction industry in particular

The process of obtaining the fibre from citric fruits also generates a series of by-products, among them the washing water used since this process uses large quantities of water, which, besides having economic importance, is important from an environmental point of view.

One way of avoiding this problem would be to re-cycle the water to take advantage of the large quantity of potentially beneficial substances and to re-use the water, once depurated, which would represent a saving both in economic and environmental terms. The aim of this study, therefore, was to determine the physical-chemical (pH, soluble solids, colour) characteristics and chemical properties (residual nitrite reduction and total antioxidant activity), phenolic compounds, organic acids and sugar content and microbiological properties in the orange juice waste water generated in the reuse of a by-product of the citrus fruit processing industry.

MATERIAL AND METHODS

Obtaining the orange juice waste water

The material (peel, pieces of pulp and other vegetal remains) obtained as a by-product during juice extraction was triturated for 20 seconds in a vertical cutter (Tecator 1094 Homogeneizer, Tekator, Hoganas, Sweden) to obtain uniformly sized pieces and so increase the contact time during washing (1 L of water per kg of product). The mixture was stirred constantly and the water temperature was kept at 80º C during the 8 min that the washing process lasted (Fernández-Ginés 2005). After draining, the resultant mixture was passed through a 0.710 mm nylon mesh and the water was stored at -21º C until analysis.

Physical-chemical analysis

A Crison micro pH meter 2001 equipped with an electrode for liquid foods (GLP 21, Crison Instrument, S.A Alella, Barcelona) was used to determine the pH of the orange juice waste water. The measurement was repeated five times. The soluble solids in the orange juice waste water were determined by digital refractometer (DR-101, Cosecta S.A. Barcelona), again making five measurements. The CIELAB colour space was studied, determining the following color coordinates: lightness (L*), redness (a*, +/− red-green), yellowness (b*, +/− yellow-blue) and the psychophysical parameter, Croma. Colour

determinations were made using a Minolta CR-300 Colorimeter (Minolta Camera Co. Osaka, Japan), with illuminant D_{65} and 10º observer equipped with adapter for liquids samples CR-A70 (Minolta Camera Co. Osaka, Japan). For this, the samples were poured into low refractant glass vials and the measurements repeated five times.

Chemical analysis

To ascertain the capacity of the orange juice waste water to reduce the percentage of residual nitrite four amounts of sodium nitrite (200, 150, 100 and 50 ppm) were added to samples of orange juice waste water. The percentage of residual nitrite was determined at 0, 30, 60, 90, 120 and 180 min following the standard ISO/DIS 2918 (1975).

The radical scavenging capacity of waste water extracts was determined using DPPH· (2,2-diphenyl-1-picrylhydrazyl) as radical according to the method of Baltrusaityte *et al.* (2007) with some modifications. Waste water samples were centrifuged for 10 min at 11000 rpm. Supernatants (0.5, 1, 2, 4 mL) were placed in cuvettes and mixed with 4 mL of ethanol (extract solutions). Two millilitres of a 250 μm DPPH solution were mixed with 2 millilitres of extract solutions. The mixtures were well shaken in a Vortex (2500 rpm) for 1 min and then placed in a dark room. Absorbance at 515 nm was measured after 30 min incubation.

A blank sample contained the same amount of ethanol and DPPH solution. The measurements were performed in triplicate. The radical scavenging activity was calculated by the formula % $I = [(A_B - A_S)/A_B] \times 100$, where I = DPPH· inhibition %; A_B = absorption of blank sample; A_S = Absorption of a tested sample at the end of the reaction (t= 30 min). Ascorbic acid and Butylhydroxytoluene (BHT) (100mg/4mL ethanol) were used as reference.

Determination of polyphenolic compounds

Extraction of polyphenols

Orange juice waste water (50 mL) was extracted with ethyl acetate (3 x 50 mL). The organic phase was combined, dried over sodium sulphate, and filtered through a 0.45 μm membrane filter (Millipore Corporation, Bedford, USA). The mixture was transferred to a round-bottomed flask and the ethyl acetate was evaporated to dryness using a rotary evaporator R-205 (Büchi, Flawil, Switzerland) under reduced pressure(<100 mbar) at 40ºC. Five millilitres of dimethyl sulfoxide (DMSO) were added to the residue, and the mixture was well shaken by hand for 2 min. The solution was filtered through a 0.45 μm membrane filter before HPLC analysis.

HPLC analysis

The HPLC analysis was performed according to the method of Benavente et al. (1999) using a Hewlett Packard HP-1100 instrument (Woldbronn, Germany) equipped with a photodiode array detector and a C-18 column (Lichrospher, 250-4, Agilent) at 30ºC. Phenolic compounds were analysed in standard and sample solutions using a gradient elution at 1 mL/min with the following gradient programme (0–20 min 95–75% A, 20–40 min 75–50% A, 40–50 min 50–20% A, 50–60 min 20% A) with 2.5% acetic acid in water as solvent A and acetonitrile as solvent B. Phenolic compounds were identified by comparing retention times with photodiode array spectra, in the range 220–500 nm for standards (caffeic acid, ferulic acid, *p*-coumaric acid, eriocitrin, neoeriocitrin, narirutin, neohesperidin, hesperidin, diosmin, poncirin, hesperetin, neodiosmin) (Extrasynthese, Genay, France) and samples. The compounds were quantified

through calibration curves of standard compounds.

Organic Acid and Sugar Content.

Five millilitres of orange juice waste water was homogenized in 5 mL of distilled water and shaken vigorously for 5 min and then centrifuged at 11000 rpm for 10 min at 4°C. Two millilitres of the supernatant were filtered through a 0.45 µm Millipore filter (Millipore Corporation, Bedford, USA) and then 10 µL were injected into a Hewlett-Packard series 1100 HPLC according to the method of Dougthy (1995). The elution system consisted of 0.1% phosphoric acid running isocratically with a flow rate of 0.5 mL min^{-1}. The organic acids were eluted through a Supelco column (Supelcogel C-610H, 30 cm 7.8 mm, Supelco Park, Bellefonte, USA) and detected by absorbance at 210 nm. The standard curves of pure organic acids (L-ascorbic, malic, citric, oxalic, acetic, lactic and succinic acids) purchased from Sigma (Poole, Dorset, UK) were used for quantification. For sugar concentrations, the same HPLC, elution system, flow rate, and column were used. The sugars were detected by refractive index detector (RID). The standard curves of pure sugars (glucose, fructose, and sucrose) purchased from Sigma were used for quantification.

Microbiological analysis

A 10 g aliquot of sample was aseptically obtained. It was then homogeneized with 90 mL of sterile 1.5% peptone water in a Stomacher 400 (Colworth, London, UK) for 1.5 min. Aliquotes were serial diluted in peptone water and plated out following standard methodologies (Gerhardt *et al.* 1994).

Aerobic mesophilic bacteria were determined on 3M Petrifilm™ Aerobic Count Plate (3M España S.A., Madrid, Spain) incubated at 37ºC for 48h, enterobacteria were determined on 3M Petrifilm™ *Enterobacteriaceae* Count Plate incubated at 37ºC for 24h, Coliforms bacteria were determined on 3M Petrifilm™ Coliforms Count Plate incubated at 37ºC for 24h. Results were expressed as log CFU/g.

RESULTS AND DICUSSION

Physical-chemical analysis

The pH, ºBrix and colour values of the samples of orange juice waste water analysed are shown in Table 1. The pH ranged between 4.54 and 4.58, with a mean of 4.56, values that may have been due to the washing provoking the leaching of organic acids from the fruit. The main acids extracted in the washing process, according to Karadeniz (2004), were probably citric, malic and fumaric acids. The suspended soluble solids showed º Brix values ranging from 7.13 to 7.21 (mean 7.16). The lightness (L*) values ranged from 56.41 to 56.47 (mean 56.44), perhaps strongly influenced by the carotenes present in the by-product. Hyoung and Coates (2003) suggested that the modifications in the content and ratio between the different carotenes after the heat treatment, could be responsible for an increase in L* values. The coordinate a* (red-green) showed a mean value of -6.83. Lee and Coates (2002) attributed these values to the degradation of carotenes, especially lycopene and beta carotene, although, according to Rodrigo et al., (2004), lutein and violaxanthin may also be involved. For Hyoung and Coates (2003), luteoxanthin, cis-violaxanthin, antheraxanthin, isolutein, zeaxanthin and beta-cryptoxanthin are also present. The b* coordinate (yellow-blue) showed a mean value of 34.03, perhaps due to a high contribution of the pectins present in the orange juice waste water. The Croma values (mean 34.71) increase the grey component of colour and may (depending on the matrix in which it is incorporated) make the food less saturated.

Chemical analysis

The residual nitrite values correspond to the nitrite that has not reacted with any substance and which is therefore in available form (Fernández-López *et al.* 2007). Figure 1 shows how residual nitrite levels behaved during the analysis. As can be seen, at time 0 the nitrite level was already reduced by 20%, and was further reduced (40%) at 90 min, after which it remained constant. This reduction was probably caused by reaction with other components in the orange juice waste water, especially polyphenols. The reactivity of nitrite with phenolic compounds has been mentioned (Santhosh *et al.* 2005*;* Balzer *et al.* 2007), while Krishnaswamy (2001) and Garrote *et al*. (2004) describe how caffeic and ferulic acids protect against the nitrite level and prevent the formation of nitrosamines and nitrosamides in food and block the formation of carcinomas *in vivo*.

However, there are no references to how the orange juice waste water intervene in the reduction of nitrite, although other citrus products, such as orange fibre and lemon albedo, have been shown to reduce residual nitrite levels (Fernández-Ginés *et al.* 2003; Aleson-Carbonell *et al.* 2003; Fernández-López *et al.* 2004; Fernández-Ginés *et al.* 2004).

The DPPH radical scavenging capacity was used to ascertain the antioxidant potential of the orange juice waste water, as depicted in Figure 2. The capacity to inhibit the formation of radicals increases with the volume of orange juice waste water used. The use of 4 mL of orange juice waste water showed an equivalent activity to that provided by 0.1 g ascorbic acid or 0.1 g BHT (87.10%; 89.35% and 90.83% respectively). Several studies have pointed to the antioxidant activity of the phenolic and flavonoid compounds in citrus (Di Majo *et al.* 2005; Xu *et al.* 2008).

The antioxidant activity of the orange juice waste water, then, can be attributed to the phenolic compounds and flavonoids they contain, which act as radical scavengers due to their hydrogen-donating capacity. In this way, the radicals produced can be delocalized over the flavonoid structure (Burda and Oleszek, 2001).

According to Tripoli *et al.* (2007), the antioxidant capacity of flavonoids is linked to their particular chemical structure. For Bors *et al.* (1990), three structural groups are important when evaluating the antioxidant capacity of flavonoids: the ortho-dihydroxy structure of the B-ring, the double 2,3 bond in conjunction with the 4-oxo function and the hydroxyl groups in positions 3 (a) and 5 (b). Di Majo *et al.* (2005) agrees that it is the joint action of these three chemical and structural elements that is responsible for the antioxidant capacity, although the environment in which these compounds are found is also important in this respect. Thus, Finotti and Di Majo (2003) mention that all flavonoids show antioxidant activity in hydrophilic environments, but this activity is reduced in lipophilic environments, as is the case with neohesperidin, hesperetin and didymin, while other flavonoids, such as naringin, narirutin or naringenin, become pro-oxidant.

Phenolic composition

The chromatographic analysis of the orange juice waste water detected several peaks corresponding to phenolic compounds, among them hydroxycinnamic acids such as caffeic, *p*-coumaric and ferulic acids. Also identified were the flavanones 7-*O*-neohesperidoside such as neohesperidin and the flavanones 7-*O*-rutinoside such as eriocitrin, hesperidin and narirutin. According to Swatsitang et al. (2000), in citrics hydroxycinnamic acids and flavones are lower in concentration than flavanones. The main peak

corresponded to narirutin with a concentration of 38.91 mg/L, followed by hesperidin with 33.91 mg/L. The concentrations of the rest of the phenolic compounds are shown in Table 2.

The flavonoids and particularly the glycosylated flavanones and polymethoxyflavones are generally abundant in citrus fruits (Kawaii *et al.* 1999) and are to be found in the juice, flavedo and leaves, in proportions that differ among species (Gatusso *et al.* 2006). In orange, for example, the main flavonoids are hesperidin, narirutin y didimin (Leuzzi *et al.* 2000).

A large number of studies point to the beneficial effects of these compounds on health, including their protective role against cancer (Harris *et al.* 2007) cardiovascular diseases (Mazza 2007) and inflammatory, allergic and ulcerous disorders (Jung *et al.* 2007; Gorinstein *et al.* 2007; Lien *et al.* 2008). They have also been seen to possess antioxidant and anti-hypertensive properties (Ohtsuki *et al.* 2003; Hwang and Yen 2008). All these effects and benefits have led the pharmaceutical industry to consider the commercial production of these substances from citrics, especially hesperidin and diosmin and their promotion as medicaments, for example Daflon 500™, Variton™ and Elatec™.

Organic acid and sugar content

The efficiency of extraction process for organic acids was 54.21%. Five organic acids were identified in the orange juice waste water: oxalic, citric, ascorbic, succinic and acetic acids, succinic acid being the most prevalent (see Figure 3). Organic acids are the main compounds found in citrus fruit, where their nature and concentration depend on such factors as species, varieties and environmental conditions, including the climate, soil and irrigation practices (Marsh *et al.* 2003). However, no studies have been published on the organic acid content of the orange juice waste water involved in citrus transformation, although several authors have studied the matter in orange juice (Karadeniz 2004*;* Kirit *et al.* 2007), citric and malic acid being the main acids found.

As regards the sugars, the main ones found in orange juice waste water are glucose, fructose and sucrose, concentration in that order (see Figure 4). The efficiency of extraction process for sugars was 49.77%. The same sugars are the most prevalent in citrus pulp and, therefore, the juice and extracts obtained from it (Albertini *et al.* 2006). The fructose content depends on whether the fruit is acidic or not (Tzur *et al.* 1992), being lowest in acidic fruit and highest in non-acidic fruit (Albertini *et al.* 2006).

The sugar content of the orange juice waste water mean that this by-product could have a potential as ingredient in dry-cure meat products since they would be a source of carbon for the microbial flora present in this kind of product.

Microbiological analysis

Table 3 shows the mean enterobacteria, coliform and aerobic mesophilic content of the orange juice waste water. The mean microbiological counts for the first two showed no growth, probably because the samples had a very low pH (Parish *et al.* 2001). The last mentioned, too, remained practically constant and at tolerable levels in all the samples. According to Fernández et al. (Fernández *et al.* 1997), microbial counts during extraction are affected by the different stages through which the product passes. Thus, during washing the counts are reduced by several logarithmic units because of the high temperatures involved.

CONCLUSIONS

The orange juice waste water reduce the levels of nitrite by approximately 40%. They also show a high capacity to inhibit the formation of free radicals, according to the results of the DPPH analysis. Narirutin and hesperidin were the most concentrated phenolic compounds. The main sugars were glucose, fructose and sucrose, while, of the five organic acids identified, succinic was the most concentrated. The samples were free of enterobacteria, coliforms and mesophilic aerobes: Their physico-chemical properties mean that the orange juice waste water concerned could be potential ingredients for a number of food products.

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TABLES

Table 1. pH, ºBrix and colour of the samples of orange juice waste water analysed

			Color			
	pH	ºBrix	L*	a*	b*	C*
Means and StD	4.56±0.02	7.16±0.02	56.44±0.03	-6.83±0.03	34.03±0.05	34.71±0.06

Means and standard deviation of five replications

Table 2. Concentration of the phenolic compounds present in orange juice waste water.

Phenolic compound	**Concentration (mg/L)**
Caffeic acid	3.56±0.11
Ferulic Acid	5.15± 0.22
p-coumaric acid	1.48±0.07
Eriocitrin	2.89±0.16
Narirutin	38.91±0.12
Hesperidin	33.09±0.26
Neohesperidin	2.01±0.19

Means and standard deviation of three replications

Table 3. Enterobacteria, coliform and aerobic mesophilic levels in orange juice waste water.

Sample	**Enterobacteria log (UFC/g)**	**Coliforms log (UFC /g)**	**Aerobic mesophilic log (UFC /g)**
Orange juice Waste water	Not Detected	Not Detected	2.17±0.14

Means and standard deviation of three replications

FIGURES

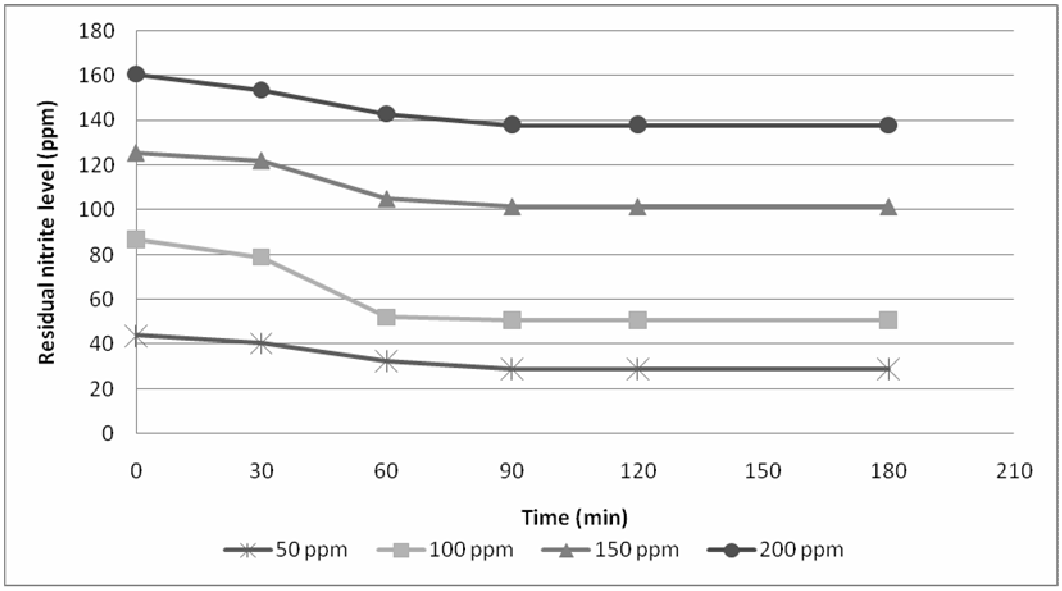

Figure1. Residual nitrite levels, on average, during the analysis.

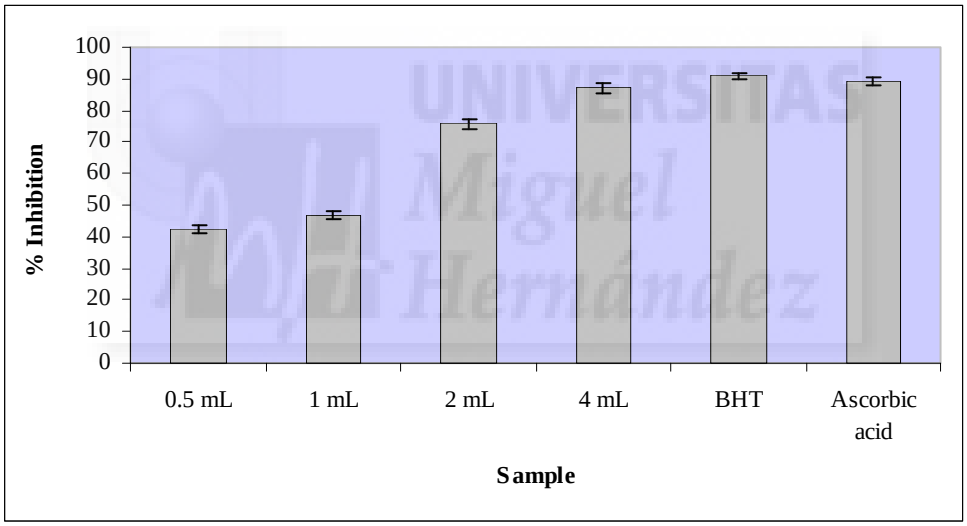

Figure 2. Radical scavenging activity of orange juice waste water samples, ascorbic acid and BHT in $DPPH^{\cdot+}$ reaction systems.

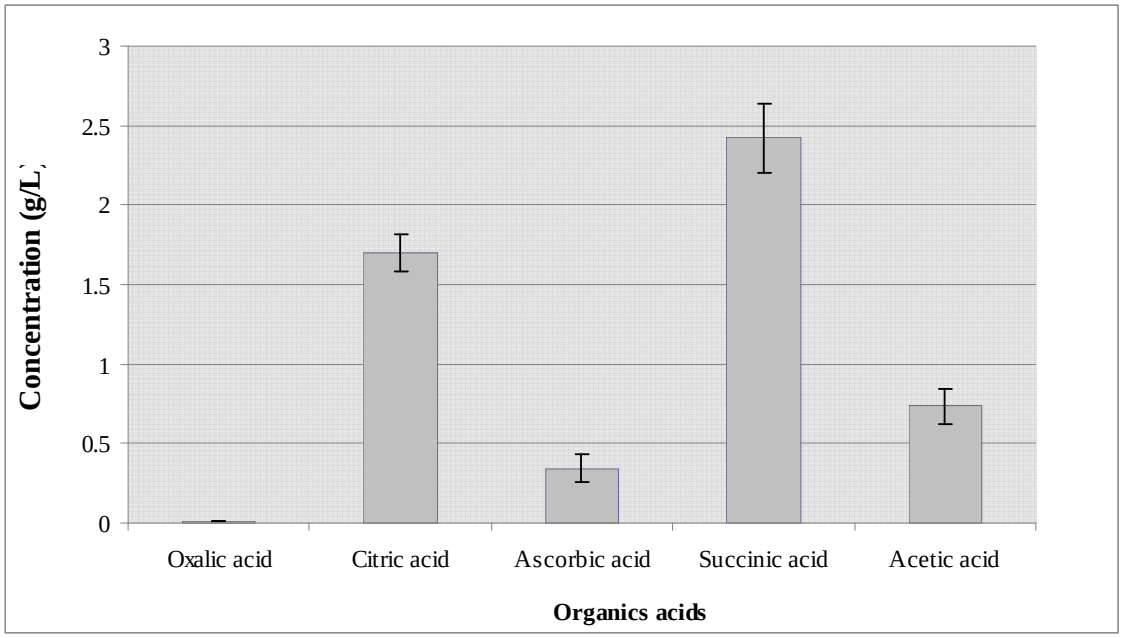

Figure 3. Concentration (g/L), on average, of the organic acids present in the orange juice waste water.

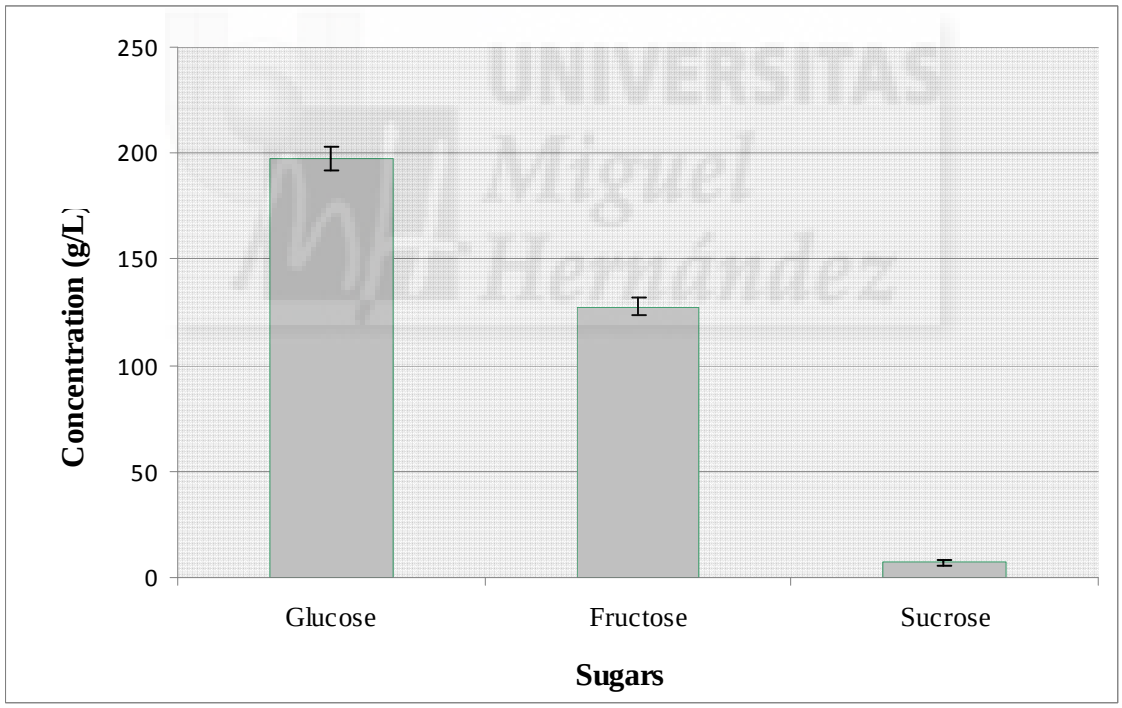

Figure 4. Concentration (g/L) of the sugars present in orange juice waste water.

TITULO:	Effect of adding citrus waste water, thyme and oregano essential oil on the chemical, physical and sensory characteristics of a bologna sausage
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Effect of adding citrus waste water, thyme and oregano essential oil on the chemical, physical and sensory characteristics of a bologna sausage

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ABSTRACT

The industrial transformation of citrus fruits generates large quantities of co-products rich in bioactive compounds that may well be suitable for other purposes. One such co-product is the water used in the process of obtaining fibre from orange. It was found that the addition of citrus waste water (5–10%) obtained as co-product during the extraction of dietary fibre and oregano or thyme essential oils (0.02%) to the bologna samples reduced the residual nitrite levels and the degree of lipid oxidation. The flavonoids hesperidin and narirutin were detected in all the samples, while those prepared with 5% of citrus waste water and 0.02% of either essential oil were the most highly valued from a sensory point of view.

Industrial relevance: The object of the present work was to study the effect of adding different concentrations (5–10%) of orange juice waste water obtained as co-product during the extraction of dietary fibre, and oregano or thyme essential oils (0.02%), on the chemical, physicochemical and sensory characteristics of a bologna-type sausage, to extend its shelf life. Addition of orange juice waste water and spices essential oil had a significant effect on shelf-life extension because of a reduction of the lipid oxidation degree.

1. Introduction

Citrus fruit, mainly mandarin, orange, lemon and grapefruit, is the main kind of tropical and sub-tropical fruit cultivated in the world (Fernández-López, Fernández-Ginés, Alesón-Carbonell, Sendra, Sayas-Barberá & Pérez-Alvarez, 2004), of which oranges account for approximately 63%. Spain, with production of 5105 million tonnes during the 2005–2006 season, is the world's fifth producer after Brazil, USA, China and Mexico (Intercitrus, 2007). Most of this fruit in Spain is destined to juice production, in a process which gives rise to a large quantity of co-products.

The composition of these co-products makes them suitable for other ends, such as obtaining dietary fibre (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008; Sendra et al., 2008), and, in turn, this gives rise to further useful co-products, among them the water used in the washing process. This process involves large volumes of water, which is a matter of both economic and environmental importance, especially considering that many citrus plantations are to be found in areas where water is scarce, as is the case, for example, with south east of Spain.

One way of minimising the consequences of this problem would be to re-use the water used in the above washing process. On the one hand, this water contains many compounds potentially beneficial both from a technological and health-related point of view and, on the other, once "treated", it could be re-used to reduce overall water consumption.

Research is currently being undertaken to improve techniques for extracting the bioactive compounds found in such co-products (Viuda-Martos, Fernández-López, Sayas Barberá, Pérez-Álvarez, & Sendra-Nadal, 2007) for use in the production of functional foods. Many of these bioactive compounds are of a polyphenolic nature and are eliminated during the elaboration of foodstuffs. It is therefore important to know exactly which polyphenolic compounds are present in the co-products generated by the food industry in general, and the citrus juice extraction industry in particular.

The aim of this study was to analyse the effect of adding different concentrations of the citrus waste water (CWW) used in the extraction of antioxidant fibre from oranges, together with 0.02% of thyme essential oil (TEO) or oregano essential oil (OEO), on the physical, chemical and sensory characteristics of a bologna-type sausage.

2. Materials and methods

2.1. Sausage manufacture

Bolognas were manufactured according to a traditional formula (only meat percentages add up to 100% while the percentages of others ingredients are related to meat): 50% lean pork meat, and 50% pork backfat; 15% water (in the form of ice, w/w), 3% potato starch (w/w),

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Table 1
Proportions of citrus waste water, oregano and thyme essential oil added to bologna sausages base mix to obtain the 7 formulations studied.

Formulation	Citrus waste water (%)	Oregano essential oil (%)	Thyme essential oil (%)
Formula 1	0	0	0
Formula 2	0	0.02	0
Formula 3	5	0.02	0
Formula 4	10	0.02	0
Formula 5	0	0	0.02
Formula 6	5	0	0.02
Formula 7	10	0	0.02

2.5% sodium chloride (w/w), 300 mg/kg sodium tripolyphosphate, 500 mg/kg sodium ascorbate, 150 mg/kg sodium nitrite, spices (0.01% black pepper, 0.005% nutmeg and 0.2% garlic powder). This original mixture was used as control sample.

To assess the influence of the concentration of CWW, and TEO and/or OEO, water content was replaced in the control formula by CWW in different concentrations and TEO and/or OEO were added to provide the seven formulations specified in Table 1. The pH of the citrus waste water ranged between 4.54 and 4.58 and the suspended soluble solids (as °Brix values) ranged from 16.18 to 16.21. No counts for enterobacteria and coliforms were found in CWW and mesophilic counts remained practically constant and at tolerable levels. The citrus waste water was obtained by the method described by Fernández-Ginés, Fernández-López, Sayas-Barberá, Sendra, and Pérez-Alvarez (2003) and oregano and thyme essential oils were supplied by Ravetllat Aromatics (Barcelona, Spain).

The products were prepared in a pilot plant according to industrial processing. Frozen raw material of animal origin, except pork backfat, was transferred to the cutter (Tecator 1094 Homogeneizer, Tekator, Höganäs, Sweden) with the sodium chloride to extract salt soluble proteins, after comminuting, the other ingredients and additives were added. Then, pork backfat, previously divided into cubes $10\times10\times10$ cm, was added and homogenized. This original mixture was split into 7 batches, to which CWW and OEO and/or TEO were added in different amounts to complete the 7 different formulas showed in Table 1.

The mixture was stuffed into artificial casing Fibran-Pack (Fibran, Girona, Spain) 100×150 mm long, clipped at both ends (Polyclip system/Niedecker, Germany) and cooked in a water bath. The sausages were kept in the bath until 72 °C was reached at the coldest point (geometric centre of the bologna sausage, which corresponds to the thickest part of the product). A thermocouple probe (Omega Engineering, Inc., Stamford, Conn., U.S.A.) positioned in the geometric centre of the bologna was used to monitor product temperature. When the endpoint temperature was achieved, the sausages were immediately chilled in ice. After reaching room temperature, the product was transferred to the lab in insulated boxes containing ice. The sausages (500 g) were stored at 4 °C until analyzed (24 h after). Processing was repeated three times with each formulation.

2.2. Chemical analysis

Moisture, ash, protein, and fat content were determined by AOAC (1995) methods. Moisture (g water/100 g sample) was determined by drying a 3 g sample at 105 °C to constant weight. Ash was performed at 550 °C for 2 h (g ash/100 g sample). Protein (g protein/100 g sample) was analyzed according to the Kjeldahl method. Factor 6.25 was used for conversion of nitrogen to crude protein. Fat (g fat/100 g sample) was calculated by weight loss after a 6-cycle extraction with petroleum ether in a Soxhlet apparatus.

Residual nitrite level (mg $NaNO_2$/kg sample) was determined in agreement with standards ISO/DIS 2918 (ISO, 1975).

2.3. Physical analysis

The CIE LAB colour space was studied following the procedure of Cassens et al. (1995). The following colour coordinates were determined: lightness (L*), redness (a*, +/− red–green), and yellowness (b*, +/− yellow–blue). Colour determinations were made, at 12 ± 2 °C by means of a Minolta CM-2002 (Minolta Camera Co., Osaka, Japan) spectrophotometer with illuminant D_{65}, 10° observer, 11 mm aperture of the instrument for illumination and 8 mm for measurement. American Meat Science Association guidelines for colour measurements were followed and spectrally pure glass (CRA51, Minolta Co., Osaka, Japan) was put between the samples and the equipment (Hunt et al., 1991).

pH was measured by blending a 5 g sample with 50 mL deionized water for 2 min. The pH of the resultant suspension was measured with a Crison pH meter (Model 507, Crison, Barcelona, Spain) equipped with a Crison combination electrode (Cat. nr 52, Crison, Barcelona, Spain).

Water activity was measured with a Novasina (SPRINT TH-500, Pfäffikon, Switzerland).

Texture profile analysis (TPA) was performed with a Texture Analyser TA-XT2 (Stable Micro Systems, Surrey, England). Bologna samples were removed from casing, cut into cubes ($1\times1\times1$ cm) and subjected to a 2-cycle compression test. All instrumental texture analyses were conducted on chilled (4 °C) samples. The samples were compressed to 70% original height through a 2-bite mechanism at a compression load of 25 kg, and a cross-head speed for 20 cm/min. The texture profile was determined as described by Bourne (1978).

2.4. Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) and DPPH method. The TBA method was determined following the recommendations of Buege and Aust (1978). TBARS values were calculated from a standard curve of malonaldehyde (MA) and expressed as mg MA/kg sample.

The radical scavenging capacity was determined using DPPH·(2,2-diphenyl-1-picrylhydrazyl) as radical according to the method of Baltrusaityte, Rimantas-Venskutonis, and Ceksteryte (2007) with some modifications. 100 mg of sample were placed in cuvettes and mixed with 4 mL of ethanol and it was vigorously shaken for 2 min (extract solutions). 2 mL of a 250 μm DPPH solution were mixed with 2 mL of extract solutions. The mixtures were well shaken in a Vortex (2500 rpm) for 1 min and then placed in a dark room. Absorbance at 515 nm was measured after 30 min incubation. A blank sample contained the same amount of ethanol and DPPH solution. The measurements were performed in triplicate. The radical scavenging activity was calculated by the formula% $I=[(\Delta_B-\Delta_S)/\Delta_B]\times100$, where I = DPPH inhibition%; Δ_B = absorbance of blank simple; Δ_S = Absorbance of a tested simple at the end of the reaction ($t=30$ min). The analysis was carried out in triplicate.

2.5. Polyphenolic compounds determination

2.5.1. Extraction of polyphenols

Samples (2 g) were weighed into a tube test and 6 mL of dimethylsulfoxide (DMSO) were added. The mixture was vigorously shaken for 2 min and left for 2 h in a Selecta ultrasonic water bath (Selecta S.A. Barcelona, Spain) without temperature control. Then, the mixture was filtered through an Albet nylon filter (Albet, Barcelona, Spain) of 45 μm before HPLC analysis.

2.5.2. HPLC analysis

The HPLC analysis was performed using a Hewlett Packard HP-1100 instrument (Woldbronn, Germany) equipped with a photodiode array detector and a C-18 column (Lichrospher, 250-4, Waters) at 30 °C. Phenolic compounds were analyzed in standard and sample

solutions using gradient elution at 1 mL/min with gradient program (0–20 min 95–75% A, 20–40 min 75–50% A, 40–50 min 50–20% A, 50–60 min 20% A) with 2.5% acetic acid in water as solvent A and acetonitrile as solvent B.

2.6. Sensory evaluation

Non-trained panellists (30) were recruited from the staff and students of the Miguel Hernández University, Alicante, Spain. Panellists were chosen on the basis of previous experience in consuming traditional bolognas. Furthermore, a preparatory session was held prior to testing, so that each panel could thoroughly discuss and clarify each attribute to be evaluated in bolognas. Testing was initiated after the panellists agreed on the specifications. A Quantitative Descriptive Analysis was carried out (IFT, 1981). All sensory work was carried out in the sensory laboratory at the University, which fulfils requirements according to the international standards (ASTM, 1986; ISO, 1988). During evaluation, the panellists were situated in private booths under incandescent/fluorescent light, with an intensity of approximately 350 lx. Rectangular pieces approximately 1.5 cm × 2 cm were cut from the centre of bologna slices and were served at room temperature (ASTM, 1988). Each panellist evaluated 3 replicates of all treatment groups; the sample presentation order was randomized for each panellist. Tap water was provided between samples to cleanse the palate. The sensory attributes were measured in unstructured scales with descriptors at both ends, no standards were provided. The attributes measured and their descriptors were as follows: for "external evaluation": global appearance (from conventional bologna appearance to unexpected appearance), colour (from extremely light to extremely dark), shine (from dull to bright), hue (from pale pink to brown), and homogeneity perception (from particulate to non-particles observed); for odour (from imperceptible to extremely intense); for "taste": acid taste, saltiness, and fatness (from imperceptible to extremely intense); and for "texture": hardness (from extremely soft to extremely tough), juiciness (from extremely dry to extremely moist). At the end of the test, panellists were asked to give a score for product quality of the product from 0 to 10.

2.7. Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations. Statistical analysis (ANOVA) was applied to the data to determine differences ($p<0.05$). To discover if there were significant differences between the levels of the main factor, contrasts (Tukey test) between means were made (Afifi & Azen, 1979). For the bolognas' characterization, ANOVAs with 2 factors (CWW: 0, 5, 10% and Essential oil: thyme and oregano) were applied for each parameter. The statistical analyses were made using Statgraphics 5.1 for Windows.

Table 2
Chemical composition of bolognas.

Formulation	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Nitrite (mg/kg)
Formula 1	65.62^{a}	2.14^{a}	13.11^{a}	21.62^{a}	21.42^{a}
Formula 2	63.52^{b}	2.55^{b}	13.11^{a}	21.50^{b}	19.66^{b}
Formula 3	62.85^{c}	2.65^{b}	13.08^{a}	21.47^{b}	11.51^{c}
Formula 4	61.83^{d}	2.74^{b}	13.12^{a}	21.42^{b}	9.47^{d}
Formula 5	63.58^{b}	2.59^{b}	13.10^{a}	21.49^{b}	20.62^{e}
Formula 6	62.86^{c}	2.60^{b}	13.12^{a}	21.48^{b}	11.92^{f}
Formula 7	61.46^{d}	2.75^{b}	13.11^{a}	21.51^{b}	10.71^{g}
SEM	0.09	0.029	0.014	0.022	0.032

For formulation details see Table 1. Values followed by the same letter in the same column showed no statistically significant differences ($p>0.05$) in Tukey's multiple range test.
SEM: Standard error of the means.

3. Results and discussion

3.1. Chemical analyses

Table 2 shows the results obtained for the chemical analysis of the different formulations studied. As can be seen the moisture content was lower in all the samples than in the control, with no statistically significant ($p>0.05$) differences between formulations 2 and 5, 3 and 6, and 4 and 7. This lower moisture content could have been due to the fact that the CWW introduced in the formulations instead of water would have contained dissolved soluble solids, 16°Brix (Viuda-Martos et al., 2008). The ash content was also significantly ($p<0.05$) higher in formulations 2, 3, 4, 5, 6 and 7, those containing 10% CWW and 0.02% TEO or 0.02% OEO showing the highest values (2.74% and 2.75% respectively). The protein and fat content showed no statistically significant ($p<0.05$) differences in any of the samples, while the addition of CWW and/or thyme or oregano EO led to a statistically significant ($p<0.05$) difference being observed in the level of residual nitrite: the higher the concentration of co-product added, the lower the nitrite concentration; i.e., the effect was dose-dependent. This drop in nitrite levels may have been due to the high reactivity of nitrite in the different bio-compounds present in the CWW (mainly polyphenols and flavonoids) and in both essential oils, especially terpenes. Several studies (Santhosh, Swarnam, & Ramadasan, 2005; Balzer, Rassaf, & Kelm, 2007) mention the reactivity of nitrite with phenolic compounds, while Krishnaswamy (2001) and Garrote, Cruz, Moure, Domínguez and Parajó (2004) mention that caffeic acid and ferulic acid offer strong protection against the nitrite ion.

3.2. Physical analyses

The addition of CWW and/or OEO or TEO had no effect ($p>0.05$) on the different colour parameters measured in the formulations. There were no changes in coordinates a* and b* in different formulas with respect to the control (formula 1), which means that neither contributed to the final colour of the product and nor did they produce alterations in the same. This would be due to the fact that CWW becomes part of the structural matrix of the emulsion, in which it is retained, provoking the disappearance, in colour terms, of all the dissolved pigments and bioactive compounds. The addition of other co-products of the citrus industry, such as fibre or albedo from lemon, did have an effect on the colour parameters (Fernández-Ginés et al., 2004; Alesón-Carbonell, Fernández-López, Pérez-Alvarez, & Kuri, 2005) due to the great size of the particles which could not form part of the matrix. As regards the water activity, the addition of the CWW and OEO or TEO causes this parameter to decrease below the control values. There were no statistically significant ($p>0.05$) differences between formulas 4 and 5, or between formulas 3 and 7, while formulas 2 and 6 did show statistically significant ($p<0.05$) differences from the rest of the formulas. The values obtained showed that these products contain an intermediate level of moisture, meaning that they will be stable at room temperature. As regards the pH, it fell slightly in the formulations to which CWW and/or EO had been added compared, with the control, with no statistically significant ($p>0.05$) differences between the values.

In the textural analysis of the resulting bolognas none of the parameters was affected ($p>0.05$) by the additional ingredients. As in the case of colour, the addition of the other citrus co-products affected the textural characteristics, especially de gumminess and chewiness (Fernández-Ginés et al., 2003).

Table 3
TBA values and DPPH levels of bolognas.

Formulation	TBA (mg malonaldehyde/kg sample)	DPPH (%)
Formula 1	4.38[a]	3.15[a]
Formula 2	4.20[b]	4.12[b]
Formula 3	4.09[c]	5.37[c]
Formula 4	3.94[d]	8.87[d]
Formula 5	4.26[b]	3.96[e]
Formula 6	4.11[c]	5.14[f]
Formula 7	4.10[c]	7.31[g]
SEM	0.023	0.02

For formulation details see Table 1. Values followed by the same letter in the same column showed no statistically significant differences ($p>0.05$) in Tukey's multiple range test.
SEM: Standard error of the means.

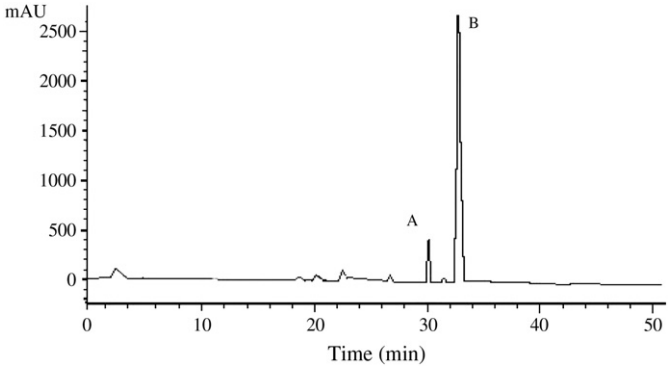

Fig. 1. Chromatographic profile of a bologna sample with CWW (5%) and TEO (0.02%) added. A: Narirutin; B: Hesperidin.

3.3. Lipid oxidation

Lipid oxidation was evaluated by determining the levels of TBARS and DPPH (Table 3). As regards TBARS, the addition of CWW and/or oregano or thyme EO decreased ($p<0.05$) this parameter in all the formulas with respect to the control. There were no statistically significant ($p>0.05$) differences between formulas 3, 6 and 7, or between formulas 2 and 5 ($p<0.05$).

In the case of DPPH, the addition of CWW and/or oregano or thyme EO increased the percentage of inhibition of the formation of radicals in all cases ($p<0.05$).

The antioxidant activity of co-products of the citrus industry has been widely demonstrated in the case of orange fibre (Fernández-Ginés et al., 2003, 2004; Fernández-López et al., 2007) and is mainly due to the phenolic compounds they contain. However, the action mechanism responsible for the antioxidant activity is not clear, although several mechanisms have been proposed. Among these, is the sequestration of free radicals, hydrogen donation, metallic ion chelation or even acting as substrate of radicals such as superoxide or hydroxyl (Al-Mamary, Al-Meeri, & Al-Habori, 2002). The activities of flavonoids such as antioxidants depend not only on their structural features but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists (Yanishlieva-Maslarova, 2001).

Finotti and Di Majo (2003) mention that all flavonoids show antioxidant activity in hydrophilic environments, but this activity is reduced in lipophilic environments, as is the case with neohesperidin, hesperetin and didymin, while other flavonoids, such as naringin, narirutin or naringenin, become pro-oxidant.

Furthermore, components of essential oils, including thyme and oregano EOs, show their own antioxidant activity (Youdim, Deans, & Finlayson, 2002; Kulisic, Radonic, Katalinic, & Milos, 2004). These bioactive compounds with antioxidant activities may also interfere with the propagation reactions (Russo et al., 2000), besides inhibiting the enzymatic systems involved in initiation reactions (You, Jong, & Kim, 1999).

Table 4
Flavonoids concentration of bolognas.

Formulation	Hesperidin (μg/g sample)	Narirutin (μg/g sample)
Formula 1	0.00[a]	0.00[a]
Formula 2	0.00[a]	0.00[a]
Formula 3	10.36[b]	5.85[b]
Formula 4	22.56[c]	12.29[c]
Formula 5	0.00[a]	0.00[a]
Formula 6	10.18[b]	5.71[b]
Formula 7	22.68[c]	12.21[c]
SEM	0.063	0.09

For formulation details see Table 1. Values followed by the same letter in the same column showed no statistically significant differences ($p>0.05$) in Tukey's multiple range test.
SEM: Standard error of the means.

3.4. Phenolic composition

Table 4 shows the flavonoid content of the different samples. In all of them, except that made from formulations 1 (control), 2 and 5, the only phenolic compounds identified were narirutin and hesperidin, the concentration of which depended on the percentage of CWW added. Thus, hesperidin values of 22.56 and 22.68 μg/g were observed for the samples with 10% CWW added and 10.36 and 10.18 μg/g for those with 5% CWW. In the case of narirutin the concentrations were lower: 12.29 and 12.21 μg/g for 10% CWW and 5.85 and 5.71 μg/g for 5% CWW.

Fig. 1 represents a chromatogram of one of the bologna samples to which 5% CWW and 0.02% OEO had been added. During processing of bologna sausage, most of the phenolic compounds found in CWW (such as caffeic, ferulic and *p*-cumaric acids) and all such compounds normally present in OEO and TEO are lost, may be due to heat treatment or reaction of these compounds with nitrites.

The flavonoids and particularly the glycosylated flavanones and polymethoxyflavones are generally abundant in citrus fruits (Kawaii, Tomo, Katase, Ogawa, & Yanou, 1999) and are found in the juice, flavedo and leaves, in proportions that differ between species (Gatusso et al., 2006). In orange, for example, the main flavonoids are hesperidin, narirutin and didimin (Leuzzi, Caristi, Panzera, & Licandro, 2000). Many studies have pointed to the beneficial effects of phenolic compounds on health, especially flavonoids, which help protect against pulmonary and cardiovascular diseases (Boots, Haenen, & Bast, 2008), cancer (Ashokkumar & Sudhandiran, 2008), besides having antioxidant and anti-hypertensive effects (Ohtsuki et al., 2003; Hwang & Yen, 2008). All these effects and benefits have led the pharmaceutical industry to consider the commercial production of these substances from citrics, especially hesperidin and diosmin and their promotion as medicaments, for example Daflon 500™, Variton™ and Elatec™.

3.5. Sensory analysis

Fig. 2 shows the results obtained for the sensorial evaluation of the different bolognas. Formula 1 showed the highest values for global appearance, while formulas 4 and 7 (10% CWW) were the worst. Formulas 3 and 6 showed the highest colour intensity, while formula 2 showed the lowest scores in this respect. Colour homogeneity was adjudged to be very similar in all the samples, although formula 3

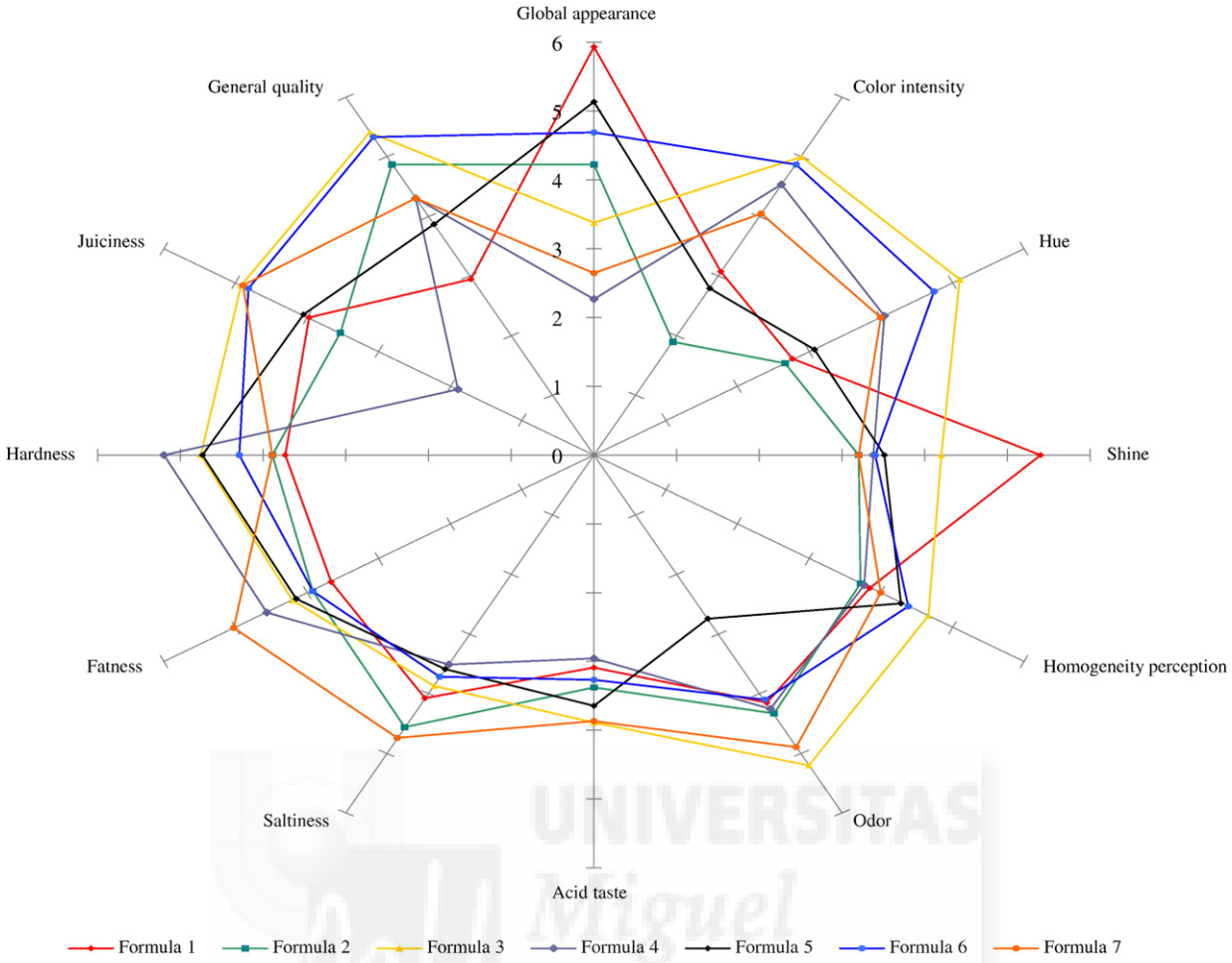

Fig. 2. Sensory evaluation: quantitative descriptive analysis carried out in the different bologna sausages. For formulation details see Table 1.

obtained the highest score. As regards lightness, differences arose between the results of the panellists and those obtained instrumentally, the panellists, the former judging formula 1 to have the highest value, while instrumental analysis found no difference between the different formulas.

A marked odour of spices was discernible in the bolognas containing the EOs, although this was not found disagreeable by the panellist, who evaluated all the samples equally, except for formula 5. Similar evaluations, too, were made with respect to acidic and salty taste, and fatty characteristics. This was also true in the case of hardness, the instrumental and sensory evaluations of this characteristic coinciding. The samples to which 5% CWW and 0.02% of either essential oil had been added were best appreciated by the panellists.

4. Conclusions

The results obtained in this study show that the use of citrus waste water and oregano or thyme essential oil as ingredients of the fine meat paste used to produce bologna-type sausage had no negative effect on any of the chemical or physical properties assessed. Their addition to cooked meat products is a viable alternative for increasing the oxidative stability of the samples, while reducing nitrite levels. This will contribute to reducing or eliminating the use of "non-natural" substances which are badly perceived by consumers, while increasing the added value of citric co-products and helping solve the problem of their accumulation.

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Effect of adding citrus fibre washing water and rosemary essential oil on the quality characteristics of a bologna sausage

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ABSTRACT

There is a growing demand of natural products in human diet, both due to the possible negative effects of synthetic food additives on human health and to the increased consumer perception for this problem in recent years. The aim of this work was to study the effect of adding rosemary essential oil (REO) (200 mg/kg) and different concentrations of citrus fibre washing water (CFWW) (50–100 g/kg), obtained as a co-product during the extraction of dietary fibre from citrus co-products, on the chemical, physical–chemical and sensorial characteristics of a bologna sausage. The moisture content and water activity fell in all the samples compared with the control values. The ash content in formulas added with CFWW and/or REO increased with respect to the control. The addition of CFWW and/or REO had no effect on CIE LAB parameters analysed, pH and textural properties. The samples analysed lowered the levels of residual nitrite and the extent of lipid oxidation. Analysis of the samples revealed the presence of the flavonoids, hesperidin and narirutin. Hesperidin concentrations were higher than narirutin. Sensorially, the most appreciated sample was the one containing 50 g/kg citrus fibre washing water and 200 mg/kg rosemary essential oil.

1. Introduction

The important role which diet plays in preventing and treating illnesses is widely accepted. The classical concept of "adequate nutrition" is tending to be replaced by the concept of "optimal nutrition", which includes the potential of food to promote health, improve general well-being and reduce the risk of developing certain illnesses (Bartrina, 2002). This is where functional foods play their part (Nagai & Inoue, 2004).

Consumer benefits from the consumption of so-called functional foods are potentially very wide-ranging, and are associated with different advantages to human health and quality of life (Frewer, Scholderer, & Lambert, 2003). Recent knowledge supports the hypothesis that, besides fulfilling nutritional needs, diet may modulate various functions in the body and may exhibit detrimental or beneficial roles in some diseases (Sarkar, 2007).

Everyday, the scientific literature contains new references to the beneficial effects of different ingredients and/or bioactive compounds with functional properties, and it is interesting that many of these functional ingredients are obtained from the co-products of the agro-food industry itself (Viuda-Martos et al., 2008a).

The preparation of extracts rich in dietary fibre and natural antioxidants from the co-products of the citrus processing industry could be used as a functional ingredient in, among others, the meat (Fernández-López et al., 2007) and dairy product (Sendra et al., 2008) industries. The process of obtaining the fibre from citric fruits also generates other co-products, among them the washing water used since this process uses large quantities of water, which, besides having economic importance, is important from an environmental point of view. One way of avoiding this problem would be to re-cycle the water to take advantage of the large quantity of potentially beneficial substances it contains and to re-use the water, once filtered, which would represent a saving both in economic and environmental terms.

The essential oils of spices could also be used as functional ingredients (Viuda-Martos, Fernández-López, & Pérez-Álvarez, 2008b). Numerous studies have documented the antioxidant, antimicrobial, antiviral, anti-inflammatory, anti-ulcerous, anti-carcinogenic properties of plant essential oils (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2007a; Bozin, Mimica-Dukic, Samojlik, Goran, & Igic, 2008). The determination of the antioxidant capacity of spices and their derivates in foods is being given greater importance by researchers and those involved

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in the agro-food industry. The antioxidant activity of spices, essential oils and their components in foods have been the subject of many studies (Oussalah, Caillet, Salmieri, Saucier, & Lacroix, 2004; Viuda-Martos et al., 2008a, 2008c)

The objective of this work was to study the effect of the addition of different concentrations of citrus fibre washing water (50 and 100 g/kg) and rosemary essential oil (200 mg/kg) on the physical, chemical and sensory characteristics of bologna sausage.

2. Material and methods

2.1. Sausage manufacture

Bolognas were manufactured according to a traditional formula: 500 g/kg lean pork meat, and 500 g/kg pork backfat; 150 g/kg water (in the form of ice), 30 g/kg potato starch, 25 g/kg sodium chloride, 300 mg/kg sodium tripolyphosphate, 500 mg/kg sodium ascorbate, 150 mg/kg sodium nitrite, spices (1 g/kg black pepper, 500 mg/kg nutmeg and 2 g/kg garlic powder). This original mixture was used as control sample.

To assess the influence of the concentration of citrus fibre washing water and rosemary essential oil, water content were replaced in the control formula by citrus fibre washing water in different concentrations and rosemary essential oil was added to provide the six formulations specified in Table 1. The citrus fibre washing water (CFWW) was obtained by the method described by Fernández-Ginés (2005). The material (peel, pieces of pulp and other vegetal remains), obtained as a by-product during juice extraction, was triturated for 20 s in a vertical cutter (Tecator 1094 Homogeneizer, Tekator, Hoganas, Sweden) to obtain uniformly sized pieces and so increase the contact time during washing (1 l of water per kg of product). The mixture was stirred constantly and the water temperature was kept at 80 °C during the 8 min that the washing process lasted. After draining, the resultant mixture was passed through a 0.710 mm nylon mesh and the water was stored at −21 °C until analysis. Rosemary essential oil (REO) was supplied by Ravetllat Aromatics (Barcelona, Spain). The chemical composition of rosemary essential oils and citrus fibre washing water used in this work was previously determined by Viuda-Martos, Ruiz-Navajas, Fernández-López, and Pérez-Álvarez (2007b) and Viuda-Martos, Fernández-López, Sayas-Barberá, Sendra, and Pérez-Alvarez (in press), respectively.

The products were prepared in a pilot plant according to industrial processing. Frozen raw material of animal origin, except pork backfat, was transferred to the cutter (Tecator 1094 Homogeneizer, Tekator, Höganäs, Sweden) with the sodium chloride to extract salt soluble proteins. After chopping, the other ingredients and additives were added. Then, pork backfat, previously divided into cubes 10 × 10 × 10 cm, were added. This original mixture was split into 6 batches, to which CFWW and/or REO were added in different amounts to complete the 6 different formulas shown in Table 1.

The mixture was stuffed into artificial casing Fibran-Pack (Fibran, Girona, Spain) 100 × 150 mm long, clipped at both ends (Polyclip system/Niedecker, Germany) and cooked in a water bath. The sausages were kept in the bath until the geometric centre of the bologna sausage, which corresponds to the thickest part of the product, reaches 72 °C. When the endpoint temperature was achieved, the sausages were immediately chilled in ice. After reaching room temperature, the product was transferred to the lab in insulated boxes containing ice. The sausages (500 g) were stored at 4 °C until analysed (24 h after). Processing was repeated three times with each formulation.

Table 1
Citrus fibre washing water (CFWW) and rosemary essential oil (REO) concentrations added to a bologna type cooked sausage.

Formulation	CFWW (g/kg)	REO (mg/kg)
C0R0	0	0
C50R0	50	0
C100R0	100	0
C0R200	0	200
C50R200	50	200
C100R200	100	200

2.2. Chemical analysis

Moisture, ash, protein, and fat, content were determined by AOAC (1995) methods. Residual nitrite level (mg $NaNO_2$/kg sample) was determined in agreement with standards ISO/DIS 2918 (ISO, 1975).

2.3. Physico-chemical analysis

The CIE LAB colour space was studied following the procedure of Cassens et al. (1995). The following colour coordinates were determined: lightness (L*), redness (a*, +/− red-green), and yellowness (b*, +/− yellow-blue). Colour determinations were made, at 12 ± 2 °C by means of a Minolta CM-2002 (Minolta Camera Co., Osaka, Japan) spectrophotometer with illuminant D_{65}, 10° observer, 11 mm aperture of the instrument for illumination and 8 mm for measurement. American Meat Science Association guidelines for colour measurements were followed and spectrally pure glass (CRA51, Minolta Co., Osaka, Japan) was put between the samples and the equipment (Hunt et al., 1991: pp. 1–12).

The pH was measured by blending a 5 g sample with 50 mL deionised water for 2 min The pH of the resultant suspension was measured with a Crison pH meter (Model 507, Crison, Barcelona, Spain) equipped with a Crison combination electrode (Cat. nr 52, Crison, Barcelona, Spain).

The water activity (aw) was measured at 25 °C by using an electric hygrometer NOVASINA TH200 (Novasina; Axair Ltd., Pfaeffikon, Switzerland).

Texture profile analysis (TPA) was performed with a Texture Analyser TA-XT2 (Stable Micro Systems, Surrey, England). Bologna samples were removed from casing, cut into cubes (1 × 1 × 1 cm) and subjected to a 2-cycle compression test. All instrumental texture analyses were conducted on chilled (4 °C) samples. The samples were compressed to 70% original height through a 2-bite mechanism at a compression load of 25 kg, and a cross-head speed for 20 cm/min. The texture profile was determined as described by Bourne (1978).

2.4. Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) and DPPH method. The TBA method was determined following the recommendations of Buege and Aust (1978). TBARS values were calculated from a standard curve of malonaldehyde (MA) and expressed as mg MAD/kg sample.

The radical scavenging capacity was determined using DPPH·(2,2-diphenyl-1-picrylhydrazyl) as radical according to the method of Baltrusaityte, Rimantas-Venskutonis, and Ceksteryte (2007) with some modifications. Samples (100 mg) were placed in cuvettes and mixed with 4 mL of ethanol and it was vigorously shaken for 2 min (extract solutions). Two millilitres of a 250 μmol/L DPPH solution were mixed with 2 mL of extract solutions. The mixtures were well shaken in a Vortex (2500 rpm) for 1 min and then placed in a dark room. Absorbance at 515 nm was measured after 30 min incubation. A blank sample contained the same

amount of ethanol and DPPH solution. The measurements were performed in triplicate. The radical scavenging activity was calculated by the formula % $I = [(\Delta_B - \Delta_S)/\Delta_B] \times 100$, where I = DPPH· inhibition %; Δ_B = absorbance of blank sample; Δ_S = Absorbance of a tested sample at the end of the reaction ($t = 30$ min).

2.5. Polyphenolic compounds determination

Samples (2 g) were weighed into a test tube and 6 mL of dimethylsulfoxide (DMSO) were added. The mixture was vigorously shaken for 2 min and left for 2 h in a Selecta ultrasonic water bath (Selecta S.A. Barcelona, Spain) without temperature control. Then, the mixture was filtered through an Albet nylon filter (Albet, Barcelona, Spain) of 45 μm before HPLC analysis. The HPLC analysis was performed using a Hewlett Packard HP-1100 instrument (Woldbronn, Germany) equipped with a photodiode array detector and a C-18 column (Lichrospher, 250-4, Waters). Phenolic compounds were analysed in standard and sample solutions using gradient elution at 1 mL/min with gradient program (0–20 min 95–75% A, 20–40 min 75–50% A, 40–50 min 50–20% A, 50–60 min 20% A) with 2.5 ml/100 ml acetic acid in water as solvent A and acetonitrile as solvent B.

2.6. Sensory evaluation

Panellists (15 men and 15women, aged 18–40 years) were recruited from the staff and students of the Miguel Hernández University, Alicante, Spain. Panellists were chosen on the basis of previous experience in consuming traditional bolognas. Furthermore, a preparatory session was held prior to testing, so that each panel could thoroughly discuss and clarify each attribute to be evaluated in bolognas. Testing was initiated after the panellists agreed on the specifications. A Quantitative Descriptive Analysis was carried out (IFT, 1981). All sensory work was carried out in the sensory laboratory at the University, which fulfils requirements according to the international standards (ASTM, 1986; ISO, 1988). During evaluation, the panellists were situated in private booths under incandescent/fluorescent light, with an intensity of approximately 350 lux. Rectangular pieces, approximately 1.5 cm × 2 cm, were cut from the centre of bologna slices and were served at room temperature (ASTM, 1988). Each panellist evaluated 3 replicates of all treatment groups; the sample presentation order was randomized for each panellist. Tap water was provided between samples to cleanse the palate. The sensory attributes were measured in unstructured scales with descriptors at both ends, no standards were provided. The attributes measured and their descriptors were as follows: for "external evaluation": global appearance (from conventional bologna appearance to unexpected appearance), colour (from extremely light to extremely dark), shine (from dull to bright), hue (from pale pink to brown), and homogeneity perception (from particulate to non particles observed); for odour (from imperceptible to extremely intense); for "taste": acid taste, saltiness, and fatness (from imperceptible to extremely intense); and for "texture": hardness (from extremely soft to extremely tough), juiciness (from extremely dry to extremely moist), At the end of the test, panellists were asked to give a score for product quality of the product from 0 to 10.

2.7. Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations. Statistical analysis (ANOVA) was applied to the data to determine differences ($P < 0.05$). To discover where there were significant differences between the levels of the main factor, contrasts (Tukey test) between means were made (Afifi & Azen, 1979). For the bolognas' characterization, ANOVAs with 2 factors (CFWW: 0, 50, 100 g/kg and REO: 0, 200 mg/kg) were applied for each parameter. The Statistical analyses were made using Statgraphics 5.1 for Windows.

3. Results and discussion

3.1. Chemical analyses

Table 2 shows the results obtained in the chemical analyses of the different formulations. As can be seen, the moisture content fell in all the samples compared with the control (C0R0) values, with no statistically significant differences ($p > 0.05$) between C50R0 sample and the C50R200 and C100R200 samples. Yet there were statistically significant differences ($p < 0.05$) between C100R0 and C0R200 samples and the rest of the samples. The ash content increased with respect to the control (C0R0), with no significant differences ($p > 0.05$) between C50R0, C100R0, C0R200 and C50R200 samples. The formula containing 100 g/kg CFWW and 200 mg/kg REO showed the highest ash content (29.0 g/kg). As regards the fat content, the results show no statistically significant differences ($p > 0.05$) between C50R0, C100R0, C0R200, C50R200 and C100R200 samples, although there were statistically significant differences ($p < 0.05$) between C100R0, C0R200, C100R200 samples and C0R0 sample. The protein content, on the other hand was not statistically significant different ($p > 0.05$) in any of the samples.

As regards the values of residual nitrite, the addition of CFWW and/or REO led to a significant ($p < 0.05$) decrease. Of note here was the synergistic effect between the CFWW and the REO, C50R200 and C100R200 samples led to reductions in this parameter of 42.25% and 47.38%, respectively compared with the control (C0R0), while the summed reductions provoked by C50R0 and C0R200 samples and C100R0 and C0R200 samples were 40.15% and 46.16%, respectively. These reductions in residual nitrite were probably due to the reaction of the different biocompounds present in both the CFWW, especially polyphenols and flavonoids and in both essential oils, especially terpenes. Several studies have described the reactivity of nitrites with polyphenols (Balzer, Rassaf, & Kelm, 2007; Santhosh, Swarnam, & Ramadasan, 2005) while Krishnaswamy (2001) and Garrote, Cruz, Moure, Domínguez, and Parajó (2004) reported that caffeic acid and ferulic acid offer strong protection against the nitrite ion.

3.2. Physico-chemical analyses

The addition of CFWW and/or REO had no effect ($p > 0.05$) on the pH, aw and colour parameters analysed. The addition of citrus,

Table 2
Chemical composition of bolognas formulated with different citrus fibre washing water and rosemary essential oil concentrations ($n = 3$).

Formulation	Moisture (g/kg)	Ash (g/kg)	Protein (g/kg)	Fat (g/kg)	Nitrite (mg/kg)
C0R0	656.21^{a}	21.42^{a}	131.13^{a}	216.23^{a}	21.42^{a}
C50R0	623.52^{b}	25.92^{b}	131.11^{a}	215.34^{ab}	13.55^{b}
C100R0	603.63^{c}	26.61^{b}	130.73^{a}	215.01^{b}	12.26^{c}
C0R200	634.61^{d}	26.43^{b}	131.07^{a}	214.56^{b}	20.69^{d}
C50R200	625.17^{b}	25.71^{b}	130.73^{a}	215.44^{ab}	12.37^{c}
C100R200	622.05^{b}	29.06^{c}	131.01^{a}	215.03^{b}	11.27^{e}
SEM	0.64	0.31	0.12	0.25	0.030

For formula denomination see Table 1. $^{a-e}$Values followed by the same letter in the same column didn't show statistically significant differences ($p > 0.05$) in Tukey's multiple range test.
SEM: Standard error of the means.

sugar cane and/or passion fruit fibre to meat products provoked changes in the L*, a* and b* colour coordinates, the intensity of the changes depending on the meat type (López-Vargas et al., 2008). However, the addition of CFWW had no such effect in bolognas sausage the mean values for the L*, a* and b* colour coordinates were 64.48, 9.22 and 7.90, respectively. There were no changes in the coordinates a* and b* in the different samples with respect to the control sample, which means that neither contributed to the final colour of the product and nor did they produce alterations in the same. This would be due to the fact that CFWW becomes part of the structural matrix of the emulsion, in which it is retained, provoking the disappearance, in colour terms, of all the dissolved bioactive pigments and compounds, unlike in the case of fibre, which, structurally, is composed of macromolecules that are rehydrated and remain outside the meat matrix, thus affecting the colour. As regards the pH, no significant differences ($p > 0.05$) arose from the addition of CFWW and/or REO with respect to the control the mean value was 6.22. The water activity followed a similar trend to the ash content; the mean value for this parameter was 0.87. The addition of the CFWW and REO causing this parameter to decrease below the formula control values, especially in C50R200 and C100R200 samples.

In textural analysis of the bolognas none of the parameters analysed were affected ($p > 0.05$) by the addition of CFWW and/or REO, unlike that which occurs when citrus fibre is added, in which case there is an increase in hardness while gumminess and chewiness decline (Fernández-Ginés, Fernández-López, Sayas-Barbera, Sendra, & Pérez-Alvarez, 2003). The means values for textural parameters analysed were: hardness 1430.99 g, Cohesiveness 0.52, springiness 3.26 mm, gumminess 747.69 g and chewiness 2439.74 g × mm.

3.3. Lipid oxidation

Lipid oxidation in the samples was evaluated by determining the levels of TBARS and DPPH, as depicted in Figs. 1 and 2, respectively. As regards TBARS (Fig. 1) the addition of CFWW and/or REO brought about a reduction ($p < 0.05$) in all the samples analysed with respect to the control. Note the lack of statistically significant differences ($p < 0.05$) between C0R200, C50R0 and C100R0 samples. C50R200 and C100R200 samples showed the lowest values ($p < 0.05$) for lipid oxidation (4.13 and 4.06 mg MA/kg sample, respectively). Of note here was the antagonistic effect between the CFWW and the REO, C50R200 and C100R200 samples

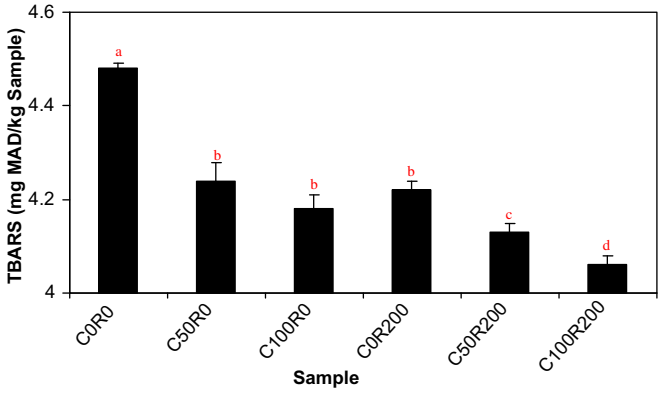

Fig. 1. Thiobarbituric acid reactive substances (TBARS) of the samples formulated with different citrus fibre washing water and rosemary essential oil concentrations ($n = 3$). For formula denomination see Table 1. [a–d]Values followed by the same letter in the same column didn't show statistically significant differences ($p > 0.05$) in Tukey's multiple range test.

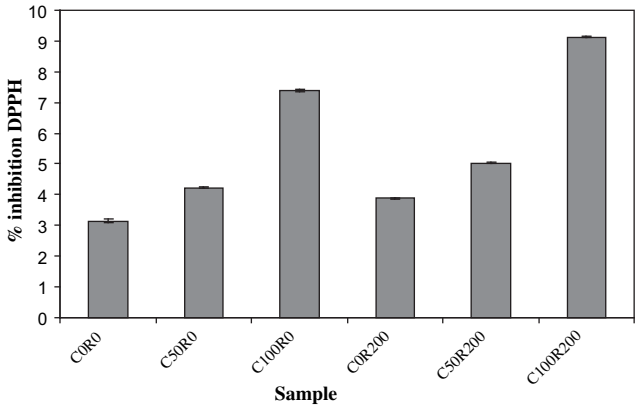

Fig. 2. Percentage of inhibition of DPPH·radical formation in the samples formulated with different citrus fibre washing water and rosemary essential oil concentrations ($n = 3$). For formula denominations see Table 1.

led to reductions in TBA values of 6.56% and 8.14%, respectively compared with the control (C0R0), while the summed reductions in TBA values provoked by C50R0 and C0R200 samples and C100R0 and C0R200 samples were 8.60% and 9.95%, respectively. As regards the DPPH values (Fig. 2), the addition of CFWW and/or REO increased the percentage by which radical formation was inhibited in all the samples with respect to the control.

The antioxidant activity of co-products from the industrial manipulation of oranges has been widely demonstrated (Fernández-Ginés et al., 2003; Fernández-López et al., 2007). Such activity is basically due to their composition, in which phenolic compounds and flavonoids predominate, since these sequester free radical as a result of their hydrogen-donating capacity.

Tripoli, La Guardia, Giammanco, Di Majo, and Giammanco (2007) reported that the antioxidant activity of flavonoids is linked to their particular chemical structure. For Bors, Hellers, Michel, and Saran (1990), three structural groups are important when evaluating the antioxidant capacity of flavonoids: the *O*-dihydroxy structure of the B-ring, the double 2,3 bond in conjunction with the 4-oxo function and the hydroxyl groups in positions 3 (a) and 5 (b). Di Majo et al. (2005) agree that it is the joint action of these three chemical and structural elements that is responsible for the antioxidant capacity, although the environment in which these compounds are found is also important in this respect. Thus, Finotti and Di Majo (2003) reported that all flavonoids show antioxidant activity in hydrophilic environments, but this activity is reduced in lipophilic environments, as is the case with neohesperidin, hesperetin and didymin, while other flavonoids, such as naringin, narirutin or naringenin, become pro-oxidant.

Table 3
Flavonoid concentration of bolognas formulated with different citrus fibre washing water and rosemary essential oil concentrations ($n = 3$).

Formulation	Hesperidin (μg/g sample)	Narirutin (μg/g sample)
C0R0	0.00[a]	0.00[a]
C50R0	10.44[b]	5.75[b]
C100R0	22.61[c]	12.20[c]
C0R200	0.00[a]	0.00[a]
C50R200	10.34[b]	5.90[b]
C100R200	22.63[c]	12.31[c]
SEM	0.26	0.13

For formula denomination see Table 1. [a–c]Values followed by the same letter in the same column didn't show statistically significant differences ($p > 0.05$) in Tukey's multiple range test.
SEM: Standard error of the means.

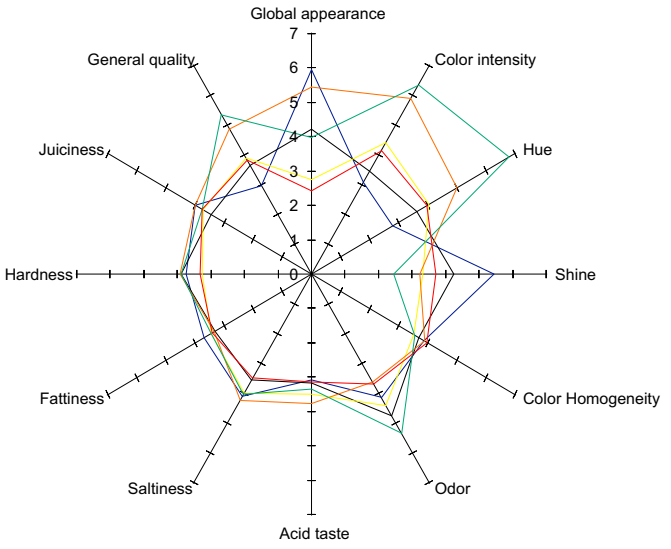

Fig. 3. Results of the sensory evaluation (quantitative descriptive analysis) carried out in the samples formulated with different citrus fibre washing water and rosemary essential oil concentrations. Global appearance: 0 (from conventional bologna appearance) to 10 (unexpected appearance); colour intensity: 0 (extremely light) to 10 (extremely dark), hue: 0 (pale pink) to 10 (brown); shine: 0 (dull) to 10 (bright); colour homogeneity: 0 (particulate) to 10 (non particles observed); odour, acid taste, saltiness and fattiness: 0 (imperceptible) to 10 (extremely intense); hardness: 0 (extremely soft) to 10 (extremely tough); juiciness: 0 (extremely dry) to 10 (extremely moist); general quality: 0 (poor quality) to 10 (high quality). —— C0R0; —— C50R0; —— C100R0; —— C0R200; —— C50R200; —— C100R200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

At the same time, the antioxidant effect on meat and fish products of the essential oils of spices in general and rosemary in particular is generally accepted. Rosemary essential oil shows antioxidant activity although the action mechanism of such activity is not fully understood (Sebranek, Sewalt, Robbins, & Houser, 2005). Several explanations have been provided, among them the following: the sequestration of free radicals, hydrogen donation, metallic ion chelation, or even acting as substrate for radicals like superoxide or hydroxyl (Al-Mamary, Al-Meeri, & Al-Habori, 2002). These bioactive compounds with antioxidant properties also interfere with propagation reactions (Russo et al., 2000), and inhibit the enzymatic systems involved in initiation reactions (You, Jong, & Kim, 1999). Differences in the antioxidant activities of flavonoids depend on the molecular structure, especially the degree of hydroxylation and methylation of the compounds (Mayer, Donovan, Pearson, Waterhouse, & Frankel, 1998).

3.4. Phenolic composition

Table 3 shows the flavonoid content of the different samples. In all of them, except C0R0 and C0R200 samples, the only phenolic compounds identified were narirutin and hesperidin, the concentration of which depended on the percentage of CFWW added. Thus, hesperidin values of 22.61 and 22.63 μg/g were observed for the samples with 100 g/kg CFWW added and 10.44 and 10.34 μg/g for those with 50 g/kg CFWW. In the case of narirutin the concentrations were lower: 12.20 and 12.31 μg/g for 100 g/kg CFWW added and 5.75 and 5.90 μg/g for 50 g/kg CFWW added. During processing most of the phenolic compounds found in OJWW (such as caffeic, ferulic and *p*-cumaric acids) are lost, as are all such compounds normally present in REO may be due to heat treatment or reaction of these compounds with nitrites.

Many studies have pointed to the beneficial effects of phenolic compounds on health, especially flavonoids, which help protect against cancer (Harris et al., 2007) cardiovascular diseases (Mazza, 2007) and inflammatory, allergic and ulcerous disorders (Jung, Choi, Nam, & Park, 2007; Lien, He, & Chuong, 2008), besides having antioxidant and anti-hypertensive effects (Hwang & Yen, 2008).

3.5. Sensorial analysis

Fig. 3 shows the results of a sensorial evaluation of the different samples assayed.

The C0R0 sample scored best for appearance, while C100R0 and C100R200 samples were least appreciated in this respect. As regards colour intensity, C0R200 and C50R200 samples showed the highest values, followed by C100R0 and C100R200 samples with hardly any difference between them. Colour homogeneity obtained similar values in all the samples analysed. As regards shine, differences arose between the appreciation of this property as measured by panellists and instrumental means, the panellists detecting greater shine in control (C0R0), while the instrumental measurement of L* showed similar values for all the samples.

Despite the marked odour of rosemary, this was not judged negatively by the panellists who scored the samples containing REO in a similar way or even higher than the rest of the samples (C50R200). Acid taste, saltiness and fattiness obtained similar values. In the case of fatness, the sensorial analysis coincided with the chemical analysis since, as mentioned above; there was no difference in the fat content of the different samples. Hardness values were also similar between panellists appreciation and instrumental measurements. The sample containing 50 g/kg CFWW and 200 mg/kg REO was best regarded by the panellists.

4. Conclusions

The addition of citrus fibre washing water and rosemary essential oil seems to be a viable alternative for manufacturing fine paste meat products, since they increase the acceptance rate of the products and their oxidative stability, while reducing the amounts of synthetic antioxidants necessary. In this way the "natural" image of the products is improved. Because they reduce residual nitrite levels they may also contribute to reducing the risk of potentially carcinogenic nitrosamines.

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TITULO:	Effect of packaging conditions on shelf-life of *mortadella* elaborated with citrus fibre washing water and thyme or rosemary essential oil.
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Effect of packaging conditions on shelf-life of *mortadella* elaborated with citrus fibre washing water and thyme or rosemary essential oil.

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ABSTRACT

The aim of this work was to study the effect of (i) the addition of citrus fibre washing water (CFWW) and rosemary essential oil (REO) or thyme essential oil (TEO) and (ii) storage conditions on the chemical, microbiological and sensorial properties of *mortadellas*, a bologna-type sausage. Experimental *mortadellas* contained 5% CFWW and 0.02% REO or 0.02% TEO. The resulting products were packed either in vacuum, modified atmosphere or air pouches and stored for 24 days. Lipid oxidation was assessed by the TBA method, while a quantitative descriptive analysis was carried out for sensory evaluation. Microbiological counts were also determined. CFWW+REO or CFWW+TEO samples stored in vacuum packaging showed the lowest TBA values. CFWW+REO samples stored in vacuum packaging showed the lowest counts of aerobic and lactic acid bacteria. The sensory evaluation provided similar scores for CFWW+REO and CFWW+TEO samples. The lowest scores were obtained for control *mortadella* stored in air packaging.

INTRODUCTION

The industrial transformation of fruits generates large quantities of co-products rich in bioactive compounds that may well be suitable for other purposes (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2009a). Some of these products have been recognized by several organizations (FDA and EFSA) as possessing proven health benefits. Depending on the availability of a suitable technology, these co-products can be converted into commercial products for use as raw materials for secondary processes (intermediate foods ingredients), as operating supplies, or as ingredients in new products (Sánchez-Zapata et al., 2009). From a technological and scientific point of view, many of these bioactive compounds present in the co-products have been shown to have a beneficial role on human health (Won et al., 2007; Englyst, Liu, & Englyst, 2007), improving the physiological functions of the organism and permitting the design and optimisation of foods that prevent or diminish the risk of certain chronic diseases (Ohr, 2007). It has been estimated that 5% of the foods consumed in Europe could be classified as functional (Pérez-Alvarez, 2008).

The addition of ingredients rich in bioactive compounds may have a technological purpose too: mainly the inhibition of lipid oxidation, a very important property especially in meat products. Natural and synthetic antioxidants have been commonly used to inhibit the development of oxidative reactions in meat products (Estevez & Cava, 2006). Materials derived from the plant kingdom, such as dried herbs and essential oils, have also been successfully used to reduce lipid oxidation in the same products (Estevez, Morcuende, Ventanas, & Cava, 2004).

The process of obtaining dietary fibre from orange juice co-products also generates a series of co-products, among them the washing water used since the process uses large quantities of water, an aspect which, besides being of economic importance, is important from an environmental point of view (Viuda-Martos, Fernández-López, Sayas-Barbera, Sendra, & Pérez-Alvarez, 2010a). One way of minimizing the consequences of this problem would be to re-use the water used in the above washing process, since it contains many compounds potentially beneficial both from a technological and health-related point of view and, once “treated”, it could be re-used to reduce overall water consumption (Viuda-Martos et al 2009a).

One component present in aromatic plants and spices, and which may act as a natural antioxidant, is the corresponding essential oil. In general terms, essential oils are composed of >70 components, principally polyphenols, terpenes, monoterpenes and sesquiterpenes, and are responsible for many of their functional (antioxidant, antibacterial, anti-inflammatory, anti-ulcerous, anti-carcinogenic, etc.) properties (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Alvarez, 2010b). Nowadays, essential oils and their components are gaining increasing attention because of their relatively safe status, their wide acceptance by consumers, and their potential multi-purpose functional uses (Ormancey, Sisalli, & Coutiere, 2001).

Spice essential oils, added at suitable concentrations, do not change the sensory properties (colour, taste or flavour) of the food matrix in which they are incorporated as supplement (Reglero et al., 2008).

The aim of this work was to study the effect of (i) the addition of citrus fibre washing water (CFWW) and rosemary essential oil (REO) or thyme essential oil (TEO) and (ii) storage conditions (air, modified atmosphere and vacuum packed) on the chemical, microbiological and sensorial properties of

mortadellas, a bologna-type sausage. Traditional formula was used as control sample and experimental *mortadellas* contained 5% CFWW and 0.02% REO or 0.02% TEO.

MATERIAL AND METHODS

Sausage manufacture

Mortadellas were manufactured according to a traditional formula (only the meat percentage adds up to 100% while the percentage of the other ingredients are related to the meat): 50% lean pork meat and 50% pork backfat; 15% water (ice, w/w), 3% potato starch (w/w), 2.5% sodium chloride (w/w), 300 mg/kg sodium tripolyphosphate, 500 mg/kg sodium ascorbate, 150 mg/kg sodium nitrite, spices (0.01% black pepper, 0.005% nutmeg and 0.2% garlic powder). This original mixture was used as control, while to assess the influence of the concentration of the citrus fibre washing water (CFWW), and thyme (TEO) or rosemary (REO) essential oil, the water content of the control formula was replaced in by CFWW (5%) and TEO or REO were added (0.02%).

The citrus fibre washing water was obtained by the method described by Fernández-Ginés, Fernández-López, Sayas-Barbera, Sendra and Pérez-Alvarez, (2003) and thyme and rosemary essential oils were supplied by Ravetllat Aromatics (Barcelona, Spain).

The products were prepared in a pilot plant of the IPOA research group, using industrial processing techniques. Frozen raw material of animal origin, except pork backfat, were transferred to the cutter (Tecator 1094 Homogeneizer, Tekator, Höganäs, Sweden) with the sodium chloride to extract salt-soluble proteins; after comminution, the other ingredients and additives were added. The pork backfat, previously divided into 10 × 10 × 10 cm cubes, was then added. After homogenization, the mixture was stuffed into Fibran-Pack (Fibran, Girona, Spain) artificial casing 100 × 150 mm long, clipped at both ends (Polyclip system/Niedecker, Germany) and cooked in a water bath. The mortadellas were kept in the bath until the coldest point reached 72 °C (geometric centre of each *mortadella*, which corresponds to the thickest part of the product). A thermocouple probe (Omega Engineering, Inc., Stamford, CT, USA) positioned in the geometric centre of the *mortadella* was used to monitor product temperature. When the endpoint temperature was achieved, the sausages were immediately chilled on ice. After reaching room temperature, the product was transferred to the laboratory in insulated boxes containing ice. The sausages (500 g each) were stored at 4 °C until analysis.

Storage conditions

Immediately after the *mortadella* manufacturing process had finished, some slices (1.5 cm thick) were aseptically taken from each sausage. The trays containing the slices were packed either in vacuum, modified atmosphere (MA) (80% N_2 and 20% CO_2) or air pouches made of polyethylene and polyamide laminate of 1.1 g/m^2/24 h water vapour permeability at 23 °C, 10 cm^3/m^2/24 h nitrogen permeability at 23 °C, 140 cm^3/m^2/24 h carbon dioxide permeability at 23 °C, and 30 cm^3/m^2/24 h oxygen permeability at 23 °C (Fibran, Girona, Spain). The pouches were heat-sealed and stored at 4 ± 1 °C in a cabinet simulating supermarket retail conditions. The cabinet was illuminated by a standard supermarket fluorescent lamp (OSRAM, Germany). All the samples were exposed to continuous light of 1000 lux at the surface, measuring the light intensity using a Lutron LX-102 luxometer (Taiwan). The positions of the samples in the cabinet were rotated every 24 h to minimize light intensity differences and possible temperature

variations at the surface of the *mortadellas*. The packs were stored for 24 d. Samples from each treatment and storage condition were taken at 0, 6, 12, 18, and 24 d (storage time) and analyzed on the same day.

Physico-chemical analysis

The CIE LAB colour space was studied following the procedure of Cassens et al., (1995). The following colour coordinates were determined: lightness (L*), redness (a*, ± red-green), and yellowness (b*, ± yellow-blue). Colour determinations were made, at 12 ± 2 °C by means of a Minolta CM-2002 (Minolta Camera Co., Osaka, Japan) spectrophotometer with illuminant D_{65}, 10° observer angle, 11 mm aperture for illumination and 8 mm for measurement. American Meat Science Association guidelines for colour measurements were followed and spectrally pure glass (CRA51, Minolta Co., Osaka, Japan) was placed between the samples and the equipment (Hunt et al., 1991).

The pH was measured by blending a 5 g sample with 50 mL deionised water for 2 min The pH of the resultant suspension was measured with a Crison pH meter (Model 507, Crison, Barcelona, Spain), equipped with a Crison combination electrode (Cat. nr 52, Crison, Barcelona, Spain).

Texture profile analysis (TPA) was performed with a TA-XT2 Texture Analyser (Stable Micro Systems, Surrey, England). *Mortadella* samples were removed from the casing, cut into cubes (1 × 1 × 1 cm) and subjected to a 2-cycle compression test. All instrumental texture analyses were conducted on chilled (4°C) samples. The samples were compressed to 70% original height through a 2-bite mechanism at a compression load of 25 kg, and a cross-head speed of 20 cm/min. The texture profile was determined as described by Bourne (1978).

Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) test following the recommendations of Buege and Aust (1978). TBARS values were calculated from a standard curve of malonaldehyde (MAD) and expressed as mg MAD/kg sample.

Determination of polyphenolic compounds

Extraction of polyphenols

Samples (2 g) were weighed into a tube test and 6 mL of dimethylsulfoxide (DMSO) were added. The mixture was vigorously shaken for 2 min and left for 2 h in an ultrasonic water bath without temperature control. Then, the mixture was filtered through an Albet nylon filter (Albet, Barcelona, Spain) of 45 μm diameter before HPLC analysis.

HPLC analysis

The HPLC analysis was performed using a Hewlett Packard HP-1100 instrument (Woldbronn, Germany) equipped with a photodiode array detector and a C-18 column (Lichrosphere, RP-18 250x4 mm, Agilent, Woldbronn, Germany) at T = 30ºC. Phenolic compounds were analyzed in standard and sample solutions using gradient elution at 1 mL/min with a gradient program of 0-20 min 95-75% A, 20-40 min 75-50% A, 40-50 min 50-20% A, 50-60 min 20% A. Using 2.5% acetic acid in water as solvent A and acetonitrile as solvent B.

Sensory evaluation

Non-trained panellists (15 men and 15women, aged 18-40 years) were recruited from the staff and students of the Miguel Hernández University, Alicante, Spain. Panellists were chosen on the basis of previous experience in consuming traditional *mortadellas*. Furthermore, a preparatory session was held

prior to testing, so that each panel could thoroughly discuss and clarify each attribute to be evaluated the products. Testing was initiated after the panellists agreed on the specifications. A Quantitative Descriptive Analysis was carried out (IFT, 1981). All sensory work was carried out in the sensory laboratory at the University, which fulfils requirements according to the international standards (ASTM 1986; ISO 1988). During evaluation, the panellists were situated in private booths under incandescent/fluorescent light, with an intensity of approximately 350 lux. Rectangular pieces of approximately 1.5 × 2 cm were cut from the centre of *mortadella* slices and were served at room temperature (ASTM 1988). Each panellist evaluated three replicates of all the treatment groups; the sample presentation order was randomized for each panellist. Unsalted crackers and room temperature water were provided between samples to cleanse the palate. The sensory attributes were measured on unstructured scales with descriptors at both ends; no standards were provided. The attributes measured and their descriptors were as follows: for "external evaluation": global appearance (from conventional *mortadella* appearance to unexpected appearance), colour (from extremely light to extremely dark), shine (from dull to bright), hue (from pale pink to brown), for "taste": acid taste, saltiness, and fatness (from imperceptible to extremely intense); and for "texture": hardness (from extremely soft to extremely tough), juiciness (from extremely dry to extremely moist). At the end of the test, panellists were asked to give a score for product quality from 0 to 10.

Microbiological analysis

Samples (25 g) were cut from the interior of the sausages with a sterile scalpel and forceps. Samples were then homogeneized with sterile 1.5% peptone water in a Stomacher 400 (Colworth, London, U.K.) for 1.5 min.

Total viable counts were determined on Plate Count Agar, *Enterobacteriaceae* using Violet Red Bile Glucose Agar (VRBGA) and Lactic acid bacteria (LAB) were counted on double layer MRS Agar at pH 5.6. In all cases, plates were incubated at 35 °C for 48 h. Psychrotrophic microbiota was determined on Plate Count Agar, and the plates were incubated at 7 °C for 10 d. Culture media were from Oxoid (Oxoid Unipath Ltd. Basingtoke, Hampshire, U.K.).

Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations. Statistical analysis (ANOVA) was applied to the data to determine differences ($P < 0.05$). To discover whether there were significant differences between the levels of the main factor, contrasts (Tukey test) between means were made (Afifi and Azen 1979). For the *mortadella* shelf-life determination, ANOVAs with three factors were applied for each parameter: storage time (0, 6, 12, 18 and 24), packaging conditions (vacuum, modified atmosphere and air) and treatments (control, CFWW+REO and CFWW+TEO). The Statistical analyses were made using Statgraphics 5.1 for Windows.

RESULTS AND DISCUSSION

Physico-chemical analysis

In meat quality, several factor affect consumer acceptability, thus in some meats, colour is the main attribute for consumers (Pérez-Alvarez & Fernández-López, 2006; Sánchez-Zapata et al., 2009). Colour is a very important parameter because brightness and particular shade of pink are expected in this type of product (García, Cáceres, & Selgas, 2007). Table 1 shows the effect on color coordinates, redness (a*)

and yellowness (b*) of adding CFWW+TEO or CFWW+REO to *mortadellas* stored in different packaging conditions.

As regards lightness (L*) neither the type of packaging, treatment or storage time had any effect (P>0.05) on this coordinate, the L* values ranging in the control, CFWW+TEO and CFWW+REO samples from 64.65, 64.632 and 64.23, respectively, at day zero to 65.71-65.83 (depending on the packaging) for control sample, 65.34-65.40 (depending on the packaging) for CFWW+TEO and 65.28-65.34 (depending on the packaging) CFWW+REO samples at the end of the experiment. The absence of any modification in this parameter may have been due to the protective effect of the packing film against oxygen, as mentioned by Fernández-Ginés et al. (2003).

For the red-green coordinate (a*) the type of packaging had no significant (P>0.05) effect in either the control samples or those with added CFWW+TEO or CFWW+REO. The storage time, however, did have an effect, and the a* value fell in the control (from 9.3 at the outset to 6.06-6.21 at the end of the experiment, depending on the packaging) in CFWW+TEO samples (from 9.20 to 6.22-6.69, depending on the packaging) and in CFWW+REO samples (from 9.10 to 6.12-6.62, depending on the packaging). This coordinate is affected by the structural integrity of the food, the content and disposition of the pigment (water or lipid-soluble) and surface water availability (Fernández-López, Sayas-Barberá, Navarro, Sendra, & Pérez-Álvarez, 2005). As regards the composition of the food, the water/oil relations of the product also play an important role. This coordinate, whether from a positive (red) or negative (green) point of view, may have a linear relationship with the concentration of pigment (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Alvarez, 2010c).

For the yellow-green coordinate (b*), the type of packaging (air, MA or vacuum) had no effect (P>0.05). In contrast, storage time (24 d) led to an increase (P<0.05) in this parameter both in the control samples, which increased from 7.27 at the outset to values ranging from 8.65 to 8.74 at the end of the experiment (depending on the packaging), in the CFWW+TEO samples from 7.31 at day zero, increasing to 8.48-8.61 at the end of the experiment (depending on the packaging) and in the samples with added CFWW+REO from 7.23 to 8.34-8.46 (depending on the packaging). The behaviour of b* depends to a great extent on the food matrix, and it is recognised that changes (pH, oxidation extent, water activity, etc.) in the matrix have a great influence on this coordinate in many foods (Cofrades, Serrano, Ayo, Solas, Carballo, & Jiménez-Colmenero, 2004).

As regards pH values, neither the type of packaging nor treatment had any statistically significant effect (P>0.05) on this parameter, while storage time was the most influential factor. In the control, CFWW+TEO and CFWW+REO samples, the pH decreased from 6.30, 6.26 and 6.28, respectively, at day zero until 6.15-6.16, 6.17-6.18 and 6.16, at the end of the experiment (depending on the packaging). The fall in pH coincided with the gradual growth of lactic bacteria in the samples which could be generating lactic acid, which would lead to the gradual decrease of pH. This agrees with the observations of Dykes, Cloete and Von Holy, (1991) who suggested that the decrease in pH during the storage of meat products is due to the action of lactic acid bacteria.

As regards the textural properties of the *mortadellas*, neither the type of packaging, treatment or time had any effect (P>0.05) on hardness, whose values in the control, CFWW+TEO and CFWW+REO samples ranged from 1417.40, 1424.04 and 1431.06 g, respectively, at day zero to 1427.90-1430.30 g

(depending on the packaging) in the control sample, 1424.65-1431.24 g (depending on the packaging) in CFWW+TEO and 1428.32-1430.74 g (depending on the packaging) in CFWW+REO at the end of the experiment. Cohesiveness was not affected (P>0.05) by CFWW+REO and CFWW+TEO addition, packaging or time. As regards springiness, CFWW+REO or CFWW+TEO addition and packaging had no effect (P>0.05). As time passed, the value of this parameter fell from 3.33 mm at day zero to 3.27-3.29 mm (depending on the packaging) on day 24 in the control samples, from 3.31 mm at day zero to 3.25-3.27 mm at the end of experiment, in the samples with added CFWW+TEO and from 3.30 mm at day zero to 3.26-3.27 mm (depending on the packaging) on day 24 in the CFWW+REO. As in the case of hardness, the addition of CFWW+TEO or CFWW+REO, packaging and time had no effect (P>0.05) on gumminess or chewiness.

Lipid oxidation

Table 2 shows the effect of adding citrus fibre washing water and spice essential oils, the packaging conditions and storage time on the lipid oxidation of *mortadella*. At day 0, the CFWW+TEO and CFWW+REO samples showed lower oxidation values than the control in all three types of packaging with no statistically significant differences (p>0.05) between either treatment. At day 6, the lowest oxidation values recorded were in the CFWW+TEO and CFWW+REO samples packed in vacuum (5.32 and 5.30 mg MAD/kg sample respectively), while no statistically significant differences (P>0.05) were observed between the air packed samples. At day 12, both CFWW+REO and CFWW+TEO samples packed in vacuum continued to show the lowest oxidation values (P<0.05), while no statistically significant differences (P<0.05) were observed between the MA packed CFWW+TEO sample and the MA packed control sample. At day 18, the CFWW+REO and CFWW+TEO samples packed in vacuum continued to show the lowest oxidation values (P<0.05), while air packed control sample showed the highest oxidation values. No statistically significant differences (P<0.05) were observed between, CFWW+TEO and CFWW+REO samples packed in MA. At the end of the experiment (24 days), the vacuum-packed CFWW+REO and CFWW +TEO treated samples showed the lowest degree of oxidation (P<0.05) of all the samples, regardless of packaging type, while both the air-packed control sample showed the highest values for this parameter.

Due to the high fat content, the comminuted nature of the raw materials and the thermal processing that such products undergo, they are prone to spoilage by lipid oxidation. The oxidative deterioration of lipid and proteins is a major concern for food technologists due to the loss of quality associated with these processes (Estevéz and Cava, 2006). Chopping and heating may catalyze the lipid oxidation because they disrupt the cellular protective compounds contained in cell membranes such as vitamin E, electron, and hydrogen donors (Keokamnerd, Acton, Han, & Dawson, 2008). The lower lipid oxidation values mentioned above obtained in CFWW+TEO and CFWW+REO would be due to the protective effect of the same, combined with the protective effect of packaging which prevents any contact with oxygen that is a major oxidizing agent. The agents responsible for the antioxidant activity in both citrus fibre washing water and thyme or rosemary essential oil are the bioactive compounds they contain, and mainly, polyphenols and tepenes.

The antioxidant activity of co-products obtained from the industrial manipulation of citrus fruit has

been widely demonstrated in meat products, whether cooked (Viuda-Martos et al., 2009a; Viuda-Martos et al., 2010c) or dry cured (Fernández-López, Viuda-Martos, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2007; Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008). Such activity is basically due to their composition, especially to phenolic compounds and flavonoids. The solubility of flavonoids in fats and oils is very low and their role in the oxidation of oil is not significant; however, they can contribute to decreasing the oxidation of fat in food emulsions (Zhou, Wu, Yang, & Liu, 2005). Thus, Finotti and Di Majo (2003) reported that all flavonoids show antioxidant activity in hydrophilic environments, but this activity is reduced in lipophilic environments, as is the case with neohesperidin, hesperetin and didymin, while other flavonoids, such as naringin, narirutin or naringenin, become pro-oxidant.

The antioxidant activity of spice essential oils in general and thyme, and rosemary in particular, is generally accepted (Kulisic, Radonic, Katalinic, & Milos, 2004; Viuda-Martos, Ruiz-Navajas, Sánchez-Zapata, Fernández-López, & Pérez-Alvarez, 2010d). Ruberto and Baratta (2000) reported that monoterpene hydrocarbons had a significant antioxidant protective effect, with several variants due to the different functional groups. Furthermore, some researchers show that some essential oils rich in nonphenolic compounds also have antioxidant potentials (El-Massry, El-Ghorab, & Farouk, 2002).

Phenolic compounds

Table 3 shows the flavonoid content of the samples analysed. In all of them, except the controls, the only phenolic compounds identified were narirutin and hesperidin, the latter in greater concentrations. This might see contradictory because in the flavonoid content of CFWW, the concentration of narirutin (38.91mg/L) is greater than that of hesperidin (33.09 mg/L) (Viuda-Martos et al. 2010a)

Hesperidin levels fell to a statistically significant extent ($P<0.05$) between day 0 and day 24, the type of packaging exercising a significant effect ($P<0.05$) in this respect. In the samples exposed to air the levels fell more than in the MA and vacuum-packed samples (from 10.44 and 10.36 µg/g of sample to 8.54 and 8.49 µg/g of sample for CFWW+TEO and CFWW+REO respectively). The case of narirutin was somewhat similar, its value falling as time progressed ($P<0.05$), particularly in air-packed samples (from 5.77 and 5.81 µg/g of sample to 3.86 and 3.91 µg/g of sample for CFWW+TEO and CFWW+REO, respectively).

This behaviour has been related with the degree of oxidation and the antioxidative protective effect of the flavonoid. These antioxidant activities have been widely demonstrated, although the mechanism of such activity is not fully understood. Several explanations have been provided, among them the following: the sequestration of free radicals; hydrogen donation; metallic ion chelation; or the flavonoids even acting as substrate for radicals such as superoxide or hydroxyl (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Sebranek, Sewalt, Robbins, & Houser, 2005).

Thus in air packed the polyphenols would have reacted more strongly with the free radicals produced, leading to their diminution and lower concentration (Viuda-Martos et al., 2010c). This idea was lent weight by the data obtained for the oxidation and concentration of hesperidin in the MA and vacuum packed samples, where there was, again, a correlation between the degree of oxidation and concentration of the flavonoid.

The rest of the phenolic compounds present in the citrus fibre washing water, including caffeic acid,

ferulic acid, *p*-coumaric acid or the flavonoids eriocitrin, or neohesperidin or terpenes present in thyme or rosemary essential oil such as carvacrol or thymol, were gradually lost during the elaboration process.

Sensory evaluation

Figure 1 show the results obtained for the sensory analysis carried out at the end (day 24) of the assay. The vacuum packed CFWW+TEO sample scored best for appearance, while the air and MA packed control samples were least appreciated in this respect. As regards colour intensity, vacuum packed CFWW+REO and CFWW+TEO samples showed the highest values. As regards shine, these were differences between the appreciation of this property as measured by panellists and instrumental means, the panellists detecting greater shine in vacuum packed CFWW+REO sample, while the instrumental measurement of L* showed similar values for all the samples.

In general, except for hue, juiciness and hardness, greater differences were seen between the air packed control samples for all the parameters considered, which were more poorly marked than the samples with added CFWW+REO and CFWW+TEO. The fat character, acid taste, salt taste and odour values were very similar in all cases. It should be noted that, despite the marked aroma of thyme or rosemary, this was not found unpleasant by the panellists, who valued the samples containing these essential oils almost equally with the controls. The best appreciated sample was the vacuum packed CFWW+REO sample, and the least acceptable the control sample exposed to air.

Microbiological analysis

In our experiment, no *enterobacteria* or psycotrophic bacteria were found in any of the treatments (control, CFWW+TEO and CFWW+REO), regardless of packaging method or time of storage, probably as a result of the effectiveness of the cooking and aseptic slicing process, together with the presence of sodium chloride in the products (Viuda-Martos, Ruiz-Navajas, Fernández-López, Sendra,E., Sayas-Barbera, & Pérez-Álvarez, 2009b). Figures 2a and 2b show the effect of treatments, packaging conditions and time on the growth of aerobic and lactic bacteria. On day 0, the CFWW+TEO and CFWW+REO samples showed lower lactic acid bacteria and aerobic bacteria growth values ($p<0.05$) than the control samples in all three types of packaging with no statistically significant differences ($p>0.05$) between the CFWW+TEO and CFWW+REO samples. On day 12, the vacuum packed control sample, CFWW+TEO and CFWW+REO vacuum packed samples showed the lowest ($p<0.05$) aerobic bacteria counts (3.79, 3.62 and 3.51 log ufc/g, respectively) and the lowest ($p<0.05$) lactic acid bacteria counts (2.97, 2.63 and 2.54 log ufc/g, respectively). The air packed control sample showed the highest ($p<0.05$) aerobic bacteria and lactic acid bacteria counts in all the samples. At the end of experiment (24 days) the vacuum packed CFWW+TEO and vacuum packed CFWW+REO samples showed the lowest aerobic bacteria and lactic acid bacteria counts ($p<0.05$), with no statistically significant differences ($p>0.05$) between them. The air packed control sample showed the highest ($p<0.05$) aerobic bacteria and lactic acid bacteria counts in both the control and treated samples.

The combination of a pH of around 6.0 and the heat treatment, as well as storage at a 4ºC, seems to be sufficient to produce a microbiologically stable product, which remains stable for at least 24 days of storage. In all the samples, the total aerobic bacteria and lactic acid counts at the end of the experiment were below those considered as representing a degraded product (high viscosity, off-colour, off-flavours). As in the case of lipid oxidation, the antimicrobial activity might be attributed to the bioactive compounds

present in both fibre and essential oils, especially polyphenols and terpenes.

CONCLUSIONS

The addition of citrus fibre washing water and thyme or rosemary essential oil is a technologically viable alternative in emulsified meat products, since they improve customer acceptance and have desirable effects as regards oxidative stability and reduced microbial growth, which contributes to prolonging their shelf-life and the "natural" image of the products is improved. Some polyphenolic compounds, such as flavonoids, are also introduced with the citrus fibre washing water and this may have a beneficial effect on human health since they have been linked with the prevention of various illnesses.

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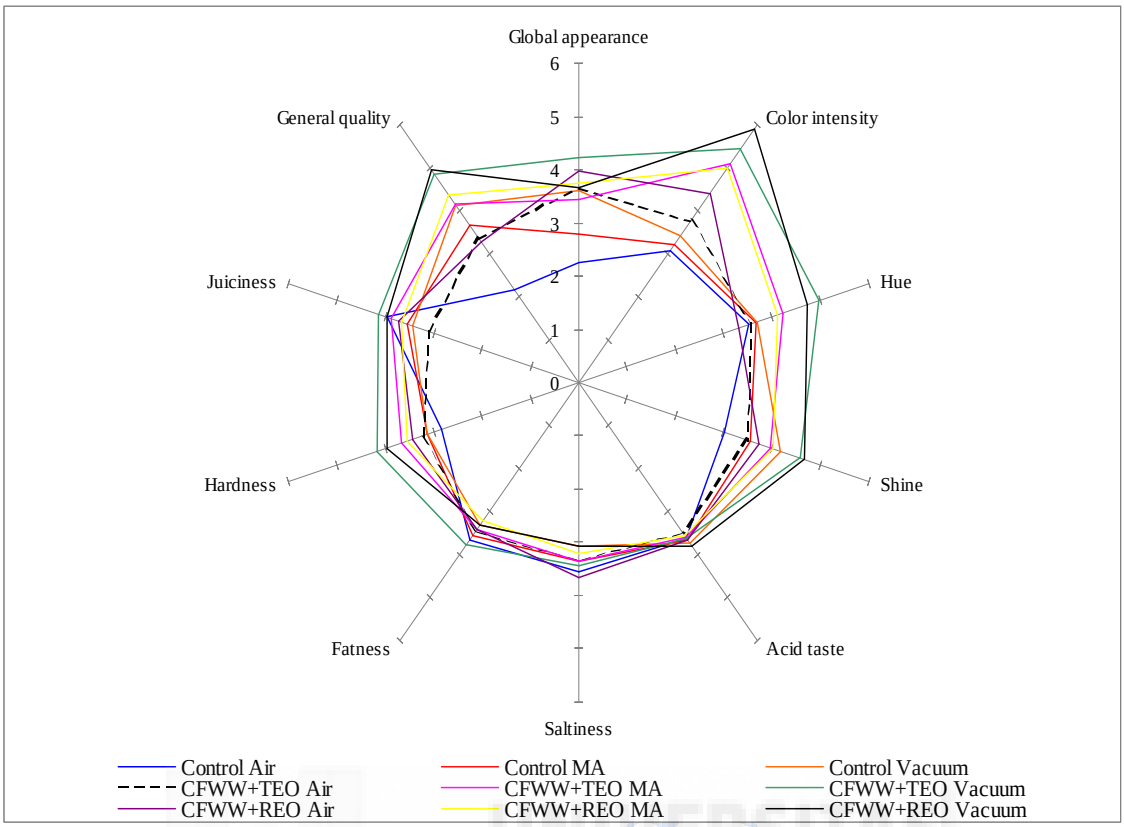

Figure 1. Results of the sensory evaluation (quantitative descriptive analysis) carried out in the samples formulated with different citrus fibre washing water and thyme or rosemary essential oil. Global appearance: 0 (from conventional *mortadella* appearance) to 10 (unexpected appearance); color intensity: 0 (extremely light) to 10 (extremely dark), hue: 0 (pale pink) to 10 (brown); shine: 0 (dull) to 10 (bright); acid taste, saltiness and fattiness: 0 (imperceptible) to 10 (extremely intense); hardness: 0 (extremely soft) to 10 (extremely tough); juiciness: 0 (extremely dry) to 10 (extremely moist); general quality: 0 (poor quality) to 10 high quality.

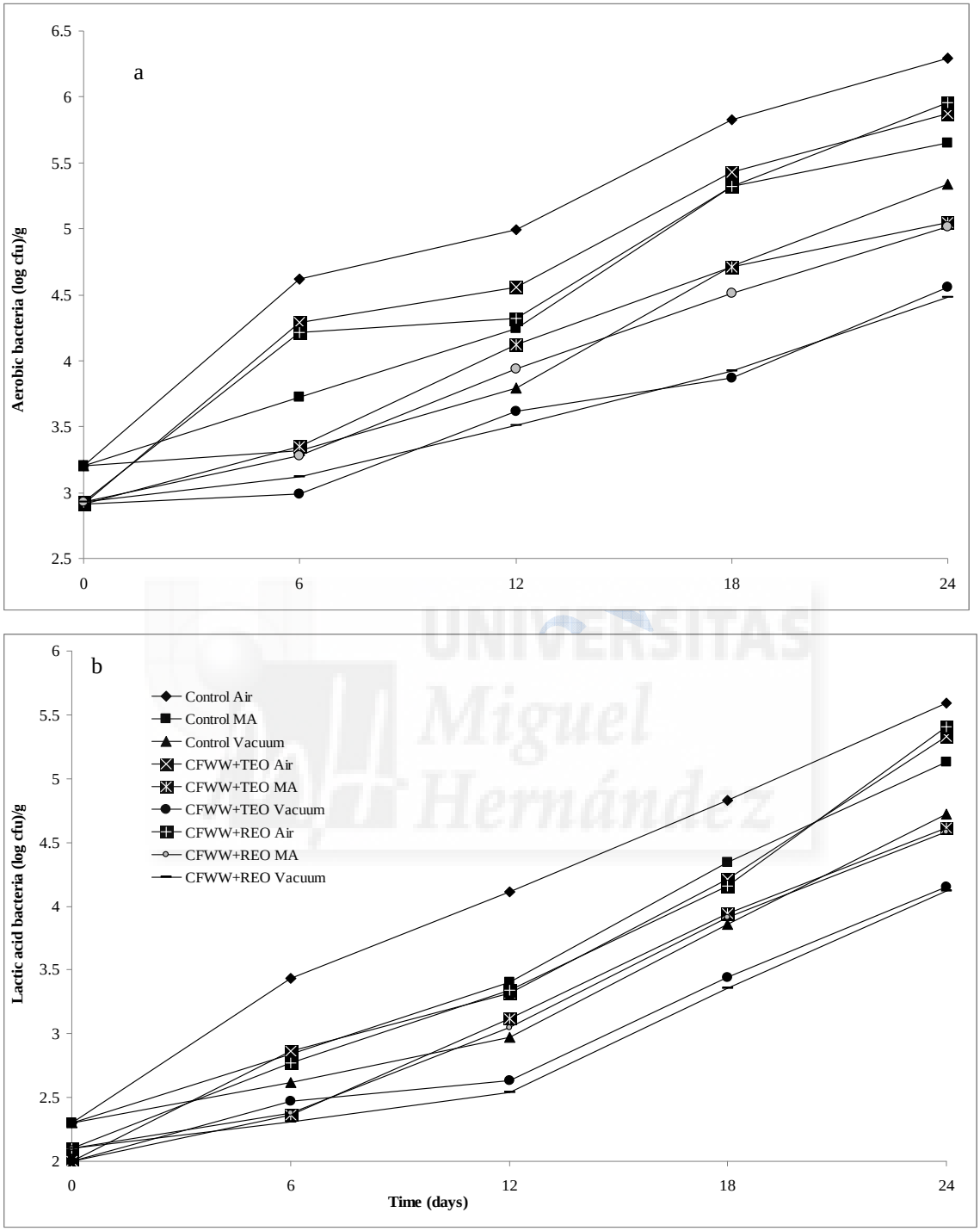

Figure 2. Evolution of counts of aerobic bacteria (a) and lactic acid bacteria (b) in *mortadellas* formulated with citrus fibre washing water and thyme or rosemary essential oil, with different storage conditions, during 24 days of storage

Table 1. Redness (a*) and yellowness (b*) colour parameters of *mortadellas* formulated with citrus fibre washing water and spice essential oils, with different storage conditions, during 24 days of storage.

Coordinate	Sample	Storage conditions	Time (d)				
			0	6	12	18	24
a*	Control	Air	9.33±0.66^{aA}	8.31±0.29^{bA}	7.84±0.74^{bcA}	7.01±0.17^{cA}	6.06±0.28^{dA}
	Control	MA	9.33±0.66^{aA}	8.43±0.66^{abA}	7.99±0.17^{bA}	7.09±0.66^{cA}	6.17±0.18^{dA}
	Control	Vacuum	9.33±0.66^{aA}	8.71±0.52^{abA}	8.06±0.25^{bA}	7.19±0.25^{cA}	6.21±0.38^{dA}
	CFWW+TEO	Air	9.33±0.66^{aA}	8.31±0.29^{bA}	7.84±0.74^{cA}	7.01±0.17^{cA}	6.06±0.28^{dA}
	CFWW+TEO	MA	9.33±0.66^{aA}	8.43±0.66^{aA}	7.99±0.17^{aA}	7.09±0.66^{bA}	6.17±0.18^{cA}
	CFWW+TEO	Vacuum	9.33±0.66^{aA}	8.71±0.52^{abA}	8.06±0.25^{bA}	7.19±0.25^{cA}	6.21±0.38^{dA}
	CFWW+REO	Air	9.20±0.20^{aA}	8.82±0.28^{abA}	8.39±0.45^{bA}	7.35±0.31^{cA}	6.22±0.23^{dA}
	CFWW+REO	MA	9.20±0.20^{aA}	8.79±0.52^{abA}	8.30±0.27^{bA}	7.43±0.27^{cA}	6.55±0.21^{dA}
	CFWW+REO	Vacuum	9.20±0.20^{aA}	8.73±0.56^{abA}	8.28±0.48^{bA}	7.50±0.33^{cA}	6.69±0.37^{dA}
b*	Control	Air	7.27±0.49^{aA}	7.52±0.44^{aA}	7.67±0.22^{abA}	8.20±0.46^{bcA}	8.65±0.25^{cA}
	Control	MA	7.27±0.49^{aA}	7.63±0.13^{aA}	8.03±0.12^{bA}	8.61±0.20^{cA}	8.68±0.20^{cA}
	Control	Vacuum	7.27±0.49^{aA}	7.71±0.25^{aA}	8.07±0.09^{bA}	8.65±0.22^{cA}	8.74±0.23^{cA}
	CFWW+TEO	Air	7.27±0.49^{aA}	7.52±0.44^{aA}	7.67±0.22^{aA}	8.20±0.46^{abA}	8.65±0.25^{bA}
	CFWW+TEO	MA	7.27±0.49^{aA}	7.63±0.13^{aA}	8.03±0.12^{bA}	8.61±0.20^{cA}	8.68±0.20^{cA}
	CFWW+TEO	Vacuum	7.27±0.49^{aA}	7.71±0.25^{aA}	8.07±0.09^{bA}	8.65±0.22^{cA}	8.74±0.23^{cA}
	CFWW+REO	Air	7.31±0.20^{aA}	7.42±0.25^{aA}	7.84±0.70^{aA}	8.31±0.30^{abA}	8.61±0.32^{abA}
	CFWW+REO	MA	7.31±0.20^{aA}	7.49±0.33^{aA}	7.89±0.36^{aA}	8.29±0.37^{abA}	8.55±0.19^{abA}
	CFWW+REO	Vacuum	7.31±0.20^{aA}	7.51±0.28^{abA}	7.92±0.36^{bcA}	8.39±0.24^{cA}	8.48±0.27^{cA}

For a same coordinate, values followed by the same small letter within the same line are not significantly different (p>0.05) according to Tukey's Multiple Range Test

For a same coordinate, values followed by the same capital letter within the same column are not significantly different (p>0.05) according to Tukey's Multiple Range Test

Table 2. TBA values (mg malonaldehyde/kg sample) of *mortadellas* formulated with citrus fibre washing water and spice essential oils, with different storage conditions, during 24 days of storage.

Sample	Storage conditions	TBA values (mg MAD/kg sample)				
		Time (d)				
		0	6	12	18	24
Control	Air	4.15±0.03^{aA}	5.41±0.00^{bA}	6.01±0.00^{cA}	6.52±0.06^{dA}	7.38±0.05^{eA}
	MA	4.15±0.03^{aA}	5.36±0.01^{bB}	5.89±0.00^{cB}	6.31±0.00^{dB}	6.42±0.00^{eB}
	Vacuum	4.15±0.03^{aA}	5.34±0.00^{cC}	5.76±0.02^{cC}	6.18±0.04^{dC}	6.26±0.00^{eC}
CFWW+TEO	Air	4.11±0.01^{aA}	5.40±0.01^{bA}	6.00±0.01^{cA}	6.44±0.01^{dD}	7.08±0.01^{eD}
	MA	4.11±0.01^{aA}	5.35±0.00^{bB}	5.88±0.01^{cB}	6.28±0.01^{dE}	6.34±0.00^{eE}
	Vacuum	4.11±0.01^{aA}	5.32±0.00^{bD}	5.65±0.00^{cD}	6.02±0.00^{dF}	6.22±0.00^{eF}
CFWW+REO	Air	4.11±0.00^{aA}	5.40±0.01^{bA}	6.00±0.01^{cA}	6.41±0.01^{dG}	7.00±0.01^{eG}
	MA	4.11±0.00^{aA}	5.34±0.01^{bBC}	5.85±0.00^{cE}	6.27±0.01^{dE}	6.32±0.01^{eH}
	Vacuum	4.11±0.00^{aA}	5.30±0.01^{bE}	5.63±0.00^{cF}	6.01±0.01^{dF}	6.22±0.00^{eF}

Values followed by the same small letter within the same line are not significantly different (p>0.05) according to Tukey's Multiple Range Test.

Values followed by the same capital letter within the same column are not significantly different (p>0.05) according to Tukey's Multiple Range Test.

Table 3. Flavonoid content of *mortadellas* formulated with citrus fibre washing water and spice essential oils, with different storage conditions, during 24 days of storage.

Compound	Sample	Storage conditions	Time (d)				
			0	6	12	18	24
Hesperidin (µg/g sample)	CFWW+TEO	Air	10.44±0.04^{aA}	9.69±0.01^{bA}	9.01±0.00^{cA}	8.78±0.01^{dA}	8.54±0.03^{eA}
	CFWW+TEO	MA	10.44±0.04^{aA}	9.82±0.05^{bB}	9.58±0.02^{cB}	9.02±0.03^{dB}	8.75±0.03^{eB}
	CFWW+TEO	Vacuum	10.44±0.04^{aA}	9.95±0.04^{bC}	9.67±0.03^{cC}	9.15±0.04^{dC}	9.01±0.04^{eC}
	CFWW+REO	Air	10.36±0.05^{aA}	9.65±0.02^{bD}	9.11±0.04^{cD}	8.81±0.02^{dA}	8.49±0.02^{eA}
	CFWW+REO	MA	10.36±0.05^{aA}	9.75±0.05^{bB}	9.63±0.02^{cE}	8.97±0.04^{dB}	8.78±0.01^{eB}
	CFWW+REO	Vacuum	10.36±0.05^{aA}	9.91±0.03^{bC}	9.72±0.02^{cC}	9.12±0.02^{dC}	8.98±0.02^{eC}
Narirutin (µg/g sample)	CFWW+TEO	Air	5.77±0.02^{aA}	4.32±0.03^{bA}	4.18±0.04^{cA}	4.05±0.03^{dA}	3.86±0.01^{eA}
	CFWW+TEO	MA	5.77±0.02^{aA}	4.68±0.02^{bB}	4.35±0.03^{cB}	4.15±0.02^{dB}	4.06±0.02^{eB}
	CFWW+TEO	Vacuum	5.77±0.02^{aA}	4.86±0.04^{bC}	4.59±0.03^{cC}	4.29±0.02^{dC}	4.12±0.03^{eC}
	CFWW+REO	Air	5.81±0.03^{aA}	4.31±0.01^{bA}	4.22±0.01^{cA}	4.09±0.04^{dA}	3.91±0.04^{eA}
	CFWW+REO	MA	5.81±0.03^{aA}	4.71±0.02^{bB}	4.40±0.02^{cB}	4.19±0.02^{dB}	4.03±0.02^{eB}
	CFWW+REO	Vacuum	5.81±0.03^{aA}	4.85±0.05^{bC}	4.68±0.01^{cD}	4.22±0.02^{dD}	4.16±0.02^{eC}

For a same compound, values followed by the same small letter within the same line are not significantly different (p>0.05) according to Tukey's Multiple Range Test

For a same compound, values followed by the same capital letter within the same column are not significantly different (p>0.05) according to Tukey's Multiple Range Test

TITULO:	Effect of packaging conditions on shelf-life of bologna sausages made with orange juice wastewater and oregano essential oil.
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EFFECT OF PACKAGING CONDITIONS ON SHELF-LIFE OF BOLOGNA SAUSAGES MADE WITH ORANGE JUICE WASTEWATER AND OREGANO ESSENTIAL OIL.

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1 INTRODUCTION

Fruit juices have experienced growing popularity within the last years. In tropical and subtropical areas the predominant fruit juices production and consumption is from citrus fruits and specifically orange. Citrus juice production provides many by-products which constitute about 45–60% of the fruit. Due to the large amount being processing into juice, citrus by-products represent a growing problem for the citrus juice industry.[1] However, their composition has the potential to be used to obtain dietary fibre. The process of obtaining the fibre from citrus fruits also generates a series of by-products, being of special interest the large washing water wastes which, besides having economic impact, are important from an environmental point of view.

One way of avoiding this problem would be to re-cycle the water to take advantage of the large quantity of potentially beneficial substances and to re-use the water, once depurated, which would represent a saving both in economic and environmental terms.

Research is currently being undertaken to improve techniques for extracting the bioactive compounds found in such co-products for use in the production of functional foods.[2] Many of these bioactive compounds are of a polyphenolic nature and are eliminated during the elaboration of foodstuffs. It is therefore important to know exactly which polyphenolic compounds are present in the co-products generated by the food industry in general, and the citrus juice extraction industry in particular. The essential oils of spices can also be used as functional ingredients. Its contain phenolic diterpenes which act as primary antioxidants, showing a high synergistic effect with other antioxidants.[3]

The aim of this work was to study the effect of (i) the addition of orange juice wastewater (OJWW) and oregano essential oil (OEO) and (ii) storage conditions on the chemical, sensory and microbiological properties of bologna sausages.

2 MATERIAL AND METHODS

2.1 Sausage manufacture

Bolognas were manufactured according to a traditional formula (only meat percentages add up to 100% while the percentages of others ingredients are related to meat): 50% lean pork meat, and 50% pork backfat; 15% water (in the form of ice, w/w), 3% potato starch (w/w), 2.5% sodium chloride (w/w), 300 mg/kg sodium tripolyphosphate, 500 mg/kg sodium ascorbate, 150 mg/kg sodium nitrite, spices (0.01% black pepper, 0.005% nutmeg and 0.2% garlic powder). This original mixture was used as control sample.

To assess the influence of the concentration of OJWW, and OEO, water content was replaced in the control formula by OJWW (5%) and OEO were added (0.02%). The orange juice wastewater was obtained by the method described by Fernández-Ginés, et al.[4] and oregano and thyme essential oils were supplied by Ravetllat Aromatics (Barcelona, Spain).

The products were prepared in a pilot plant and followed industrial processing techniques. Frozen raw material of animal origin, except pork backfat, was transferred to the cutter (Tecator 1094 Homogeneizer, Tekator, Höganäs, Sweden) with the sodium chloride to extract salt-soluble proteins; after comminute, the other ingredients and additives were added. Then, pork backfat, previously divided into cubes 10 x 10 x 10 cm, was added. After homogenization, the mixture was stuffed into artificial casing Fibran-Pack (Fibran, Girona,Spain) and cooked in a water bath. The sausages were kept in the bath until the coldest point reached 72 °C. When the endpoint temperature was achieved, the sausages were immediately chilled in ice. After reaching room temperature, the product was transferred to the lab in insulated boxes containing ice.

2.2 Storage conditions

Immediately after manufacture, some slices (1.5 cm thick) were aseptically removed from each bologna sausage. The trays containing the slices were packed either in vacuum, and air pouches made of polyethylene and polyamide laminate of water vapour permeability 1.1 g/m^2/24 h at 23 °C, nitrogen permeability 10 cm^3/m^2/24 h at 23 °C, carbon dioxide permeability 140 cm^3/m^2/24 h at 23 °C, and oxygen permeability 30 cm^3/m^2/24 h at 23 °C (Fibran, Girona, Spain). The pouches were heat-sealed and stored at 4 ± 1 °C in a cabinet simulating supermarket retail conditions. The cabinet was illuminated by a standard supermarket fluorescent lamp (OSRAM, Germany). All the samples were exposed to continuous lighting at 1000 lux at the surface. The positions of the samples in the cabinet were rotated every 24 h to minimize light intensity differences and possible temperature variations on the meat surface. The packs were stored for 24 d. Samples from each treatment and storage conditions were taken at 0, 6, 12, 18, and 24 d (storage time) and analyzed on the same day.

2.3 Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) assays following the recommendations of Buege and Aust.[5] TBARS values were calculated from a standard curve of malonaldehyde (MAD) and expressed as mg MAD/kg sample.

2.4 Sensory evaluation

Non-trained panellists (30) were recruited from the staff and students of the Miguel Hernández University, Alicante, Spain. Panellists were chosen on the basis of previous experience in consuming traditional bolognas. Furthermore, a preparatory session was held prior to testing, so that each panel could thoroughly discuss and clarify each attribute to be evaluated in bolognas. Testing was initiated after the panellists agreed on the specifications. A Quantitative Descriptive Analysis was carried out.[6] During evaluation, the panellists set in private booths under incandescent/fluorescent light, with an intensity of approximately 350 lux. Rectangular pieces approximately 1.5 cm × 2 cm were cut from the centre of bologna slices and served at room temperature. Each panellist evaluated three replicates of all the treatment groups; the sample presentation order was randomized for each panellist. Tap water was provided between samples to cleanse the palate. The sensory attributes were measured in unstructured scales with descriptors at both ends, no standards were provided. The attributes measured and their descriptors were as follows: for "external evaluation": global appearance (from conventional bologna appearance to unexpected appearance), colour (from extremely light to extremely dark), shine (from dull to bright), hue (from pale pink to brown) and homogeneity perception (from particulate to no particles observed); for odour (from imperceptible to extremely intense); for "taste": acid taste, saltiness, and fatness (from imperceptible to extremely intense); and for "texture": hardness (from extremely soft to extremely tough), juiciness (from extremely dry to extremely moist). At the end of the test, panellists were asked to give a score for product quality from 0 to 10.

2.5 Microbiological analysis

Samples (25 g) were excised from the interior of the sausages with a sterile scalpel and forceps and then homogeneized with sterile 1.5% peptone water in a Stomacher 400 (Colworth, London, U.K.) for 1.5 min. Total viable counts were determined on Plate Count Agar, *Enterobacteriaceae* using Violet Red Bile Glucose Agar (VRBGA) and Lactic acid bacteria (LAB) were counted on double layer MRS Agar at pH 5.6. In all cases, plates were incubated at 35 °C for 48 h. Psychrotrophic microbiota was determined on Plate Count Agar, and the plates were incubated at 7 °C for 10 d. Culture media were from Oxoid (Oxoid Basingtoke, Hampshire, U.K.).

2.6 Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations. Statistical analysis (ANOVA) was applied to the data to determine differences ($P < 0.05$). To discover whether there were significant differences between the levels of the main factor, contrasts (Tukey test) between means were made.[7] For the bologna self-life determination, ANOVAs with 3 factors (storage time: 0, 6, 12, 18 and 24; packaging conditions: vacuum and air; treatments: control and OJWW+OEO) were applied for each parameter. The Statistical analyses were made using Statgraphics 5.1 for Windows.

3 RESULTS AND DISCUSION

3.1 Lipid oxidation

Chopping is one of the most important steps in meat emulsions manufacturing. Chopping and heating can catalyze the lipid oxidation because they disrupt cellular protective

compounds contained in cell membranes such as vitamin E and hydrogen donors.[8] Lipid oxidation was evaluated by determining the levels of TBARS (Table 1).

Table 1 TBA values (mg MAD/kg sample) of bologna control and bologna formulate with OJWW+OEO, stored on vacuum package and air package, during 24 days of storage.

Sample	Storage conditions	Time (d)				
		0	6	12	18	24
Control	Air	4.15 ± 0.03^{aw}	5.41 ± 0.01^{bw}	6.01 ± 0.01^{cw}	6.52 ± 0.06^{dw}	7.14 ± 0.00^{ew}
	Vacuum	4.15 ± 0.03^{aw}	5.34 ± 0.00^{bx}	5.76 ± 0.02^{cx}	6.18 ± 0.04^{dx}	6.26 ± 0.00^{ex}
OJWW+OEO	Air	4.10 ± 0.00^{az}	5.39 ± 0.01^{bw}	5.98 ± 0.01^{cw}	6.40 ± 0.00^{dy}	6.94 ± 0.01^{ey}
	Vacuum	4.10 ± 0.00^{az}	5.28 ± 0.01^{by}	5.60 ± 0.02^{cy}	5.97 ± 0.03^{dz}	6.15 ± 0.00^{ez}

$^{a-e}$ Means within a row with different letters are significantly different ($p< 0.05$)
$^{w-z}$ Means within a column with different letters are significantly different ($p< 0.05$)

At day 0, the OJWW+OEO samples showed lower oxidation values ($P<0.05$) than the control. At day 6, the lowest oxidation values recorded were in the OJWW+OEO samples packed in vacuum while no statistically significant differences were observed between the air packed control sample and the OJWW+OEO sample air packed. At days 12 and 18, the vacuum-packed OJWW+OEO samples continued to show the lowest oxidation values, while the air-packed OJWW+OEO sample showed higher ($P<0.05$) values than the vacuum-packed control (6.40 and 6.18 mgMAD/kg sample, respectively). At the end of the experiment (24 days), the vacuum-packed OJWW+OEO treated sample showed the lowest oxidation degree ($P<0.05$) of all the samples, regardless of packaging type, while both the air-packed control and air-packed OJWW+OEO samples showed the highest values for this parameter.

The antioxidant activity of co-products from the industrial manipulation of oranges has been widely demonstrated.[4,9] Such activity is basically due to their composition: mainly due to phenolic compounds and flavonoids. However, the action mechanism responsible for the antioxidant activity is not clear, although several mechanisms have been proposed such as the scavengers of free radicals, hydrogen donation, metallic ion chelation or even acting as substrate of radicals such as superoxide or hydroxyl.[10] The activities of flavonoids such as antioxidants depend not only on their structural features but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists.[11] Furthermore, components of essential oils, including thyme and oregano EOs, show their own antioxidant activity.[12,13] These bioactive compounds with antioxidant activities may also interfere with the propagation reactions[14] besides inhibiting the enzymatic systems involved in initiation reactions.[15]

3.2 Sensory analysis

Figure 1 shows the results obtained for the sensory evaluation carried out at the end (day 24) of the assay. The panellists clearly detected the loss of colour produced in the samples. The fat character, acid taste, salty taste and odour values were very similar. It should be noted that despite the marked aroma of oregano, this was not found unpleasant by the panellists, who valued the samples containing this essential oil almost equally with the controls. In general, greatest differences for all parameters were detected between control and OJWW+OEO samples, control samples obtained the poorest scores.

The best scored sample was the OJWW+OEO sample packaged in vacuum, and the least acceptable the control sample exposed to air.

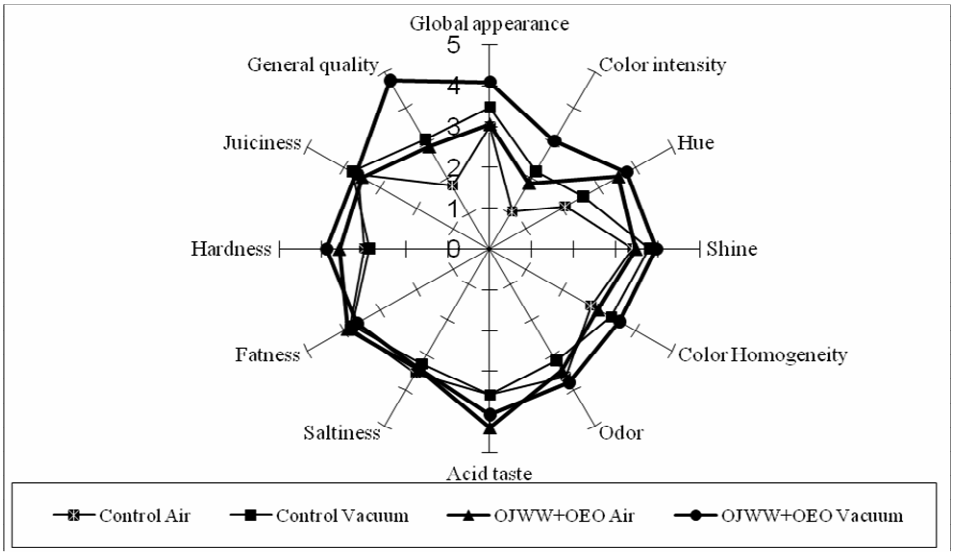

Figure 1 Quantitative descriptive analysis carried out at day 24 of control bolognas and bolognas formulated with Orange juice waste water and oregano essential oil

3.3 Microbiological analysis

The growth of aerobic and lactic bacteria was greater ($P<0.05$) in the control samples than in those with OJWW+OEO (Figure 2 a and b) at all times and in all packaging conditions,

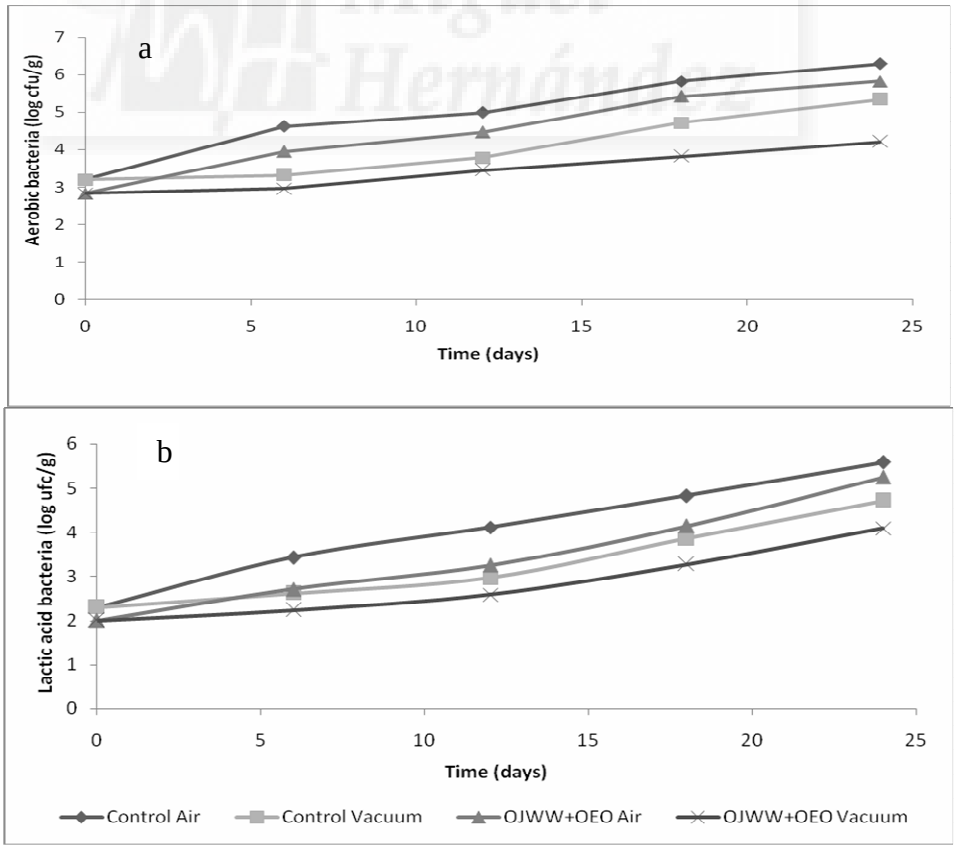

Figure 2 Evolution of counts of aerobic bacteria (a) and counts of lactic acid bacteria (b) of bolognas control and formulated with orange juice waste water and oregano essential oil

except in the OJWW +OEO sample packed in air at six days, which showed higher values than the controls packed in vacuum, a difference that was maintained until the end of the experiment (24 days). No *enterobacteria* or psycotrophic bacteria were found in any of the treatments (control and OJWW+OEO), regardless packaging and storage time of storage, probably due to the effectiveness of the cooking, aseptic slicing process together with the presence of the sodium chloride contained in the products.[16]

In all the samples, the total aerobic bacteria and lactic acid counts at the end of the experiment were below those considered as representing a degraded product (higher viscosity, colour changes, off-flavours).

4 CONCLUSIONS

The addition of orange juice wastewater and oregano essential oil seems to be a viable alternative for elaborating cooked meat products because of the "natural" image of the products is improved. Their addition increase the acceptance rates of the products their oxidative stability and the shelf-life of the products and reduce microbial growth.

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Antioxidant activity of orange fibre added with spices essential oils

J.A. Pérez-Alvarez*, J. Fernández-López, M. Viuda-Martos, Y. Ruiz-Navajas
Miguel Hernandez University, Spain

Citrus fruits are processed, mainly to obtain juice. Worldwide industrial citrus wastes may be estimated at more than 15 x 10^6 tons. Citrus co-product has many applications for the food industry as antioxidant fibre, pectin and flavonoids source. Isolation of functional compounds from citrus co-products can be of interest to the food industry as they can retard oxidative changes in food and thereby improve the quality and nutritional value of food. The aim of this work was to determine the antioxidant activity of the orange fibre and the orange fibre added with oregano, thyme and rosemary essential oil at several concentrations (15 and 30 μL/g of fibre) for eighteen days at different temperatures (20, 40 and 60ºC).

Antioxidant activity was assessed by the 2-thiobarbituric acid (TBA) method. Thiobarbituric acid reactive substances (TBARS) values were expressed as mg malonaldehyde/kg sample. Polyphenols composition, quantified by HPLC was also determined.

The orange fibre and the orange fibre added with spices essential oils showed a protective effect upon the oxidation, being this effect greater in terms of concentration. During the study, the oxidation degree, independently of essential oil concentration used, was increased by the effect of the time and temperature studied. The thyme essential oil at concentration of 30 μL/g of fibre showed the highest protective effect from oxidation. All the fibre samples analyzed retain hesperidin and naringenin as main flavonoids presents in the orange fibre The hesperidin is the phenolic compound that presents highest concentration in all the fibre samples analyzed. The temperature and the time provoke a slight reduction in the concentration of these flavonoids.

Fibre added with essential oils used in foods (meat, dairy and bakery products) could help to retard lipid oxidation and avoid the use of synthetic antioxidants as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

Keywords: Citrus fibre, Antioxidant, Flavonoids, TBA Method

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[171]

Characterization of "tomato fibre" a new functional food ingredient

M. Cámara*, MªC. Sánchez-Mata, A. González, P. García
University Complutense Madrid, Spain

Introduction:
Advances in Food Science and Technology are centered on developing "Novel Foods and New Food Ingredients" with special reference to healthy foods. In addition, in the last years there has been a considerable emphasis on the recovery, recycling and utilization of the residues obtained in the vegetables products industry, for legal, environmental and economic reasons. According to the previous experience of this research group, tomato byproducts are a good source of bioactive compounds, potentially useful for the food industry.

This research work is aimed to evaluate the utilization of "tomato fibre", as a useful food ingredient.

Methods:
"Tomato fibre" is produced from tomato peels by AGROGAL (Spanish industry located in Mengabril, Badajoz), patent (p200001264. 19/05/2000). Samples for analysis were collected on 2 tomato seasons 2005 and 2006.

To evaluate the "nutritional quality", different parameters has been analyzed as proteins, fibre, carbohydrates, minerals and antioxidants as carotenoids.

Results:
Tomato fibre is a dry product (3% moisture) with a particle size lower than 1.19 mm, with the main fraction between 0.3 y 0.5 mm, it is rich in insoluble fibre (72%), soluble fibre (6%), with a carbohydrate content of 11%, 6% of proteins and 2% of minerals with a potential antioxidant capacity (due to its licopene content).

Discussion:
The development of new ingredients from tomato by-products will affect agricultural economics and provide a new source of healthful nutrients. Our results can justify the consideration of "tomato fibre" as new functional ingredient for the food industry.

Acknowledgments: This work is part of a research proposal funded by UCM-PR1-06-14423-A. Samples were provided by AGROGAL, S.A.T. Badajoz, Spain.

Keywords: tomato fibre, functional ingredients

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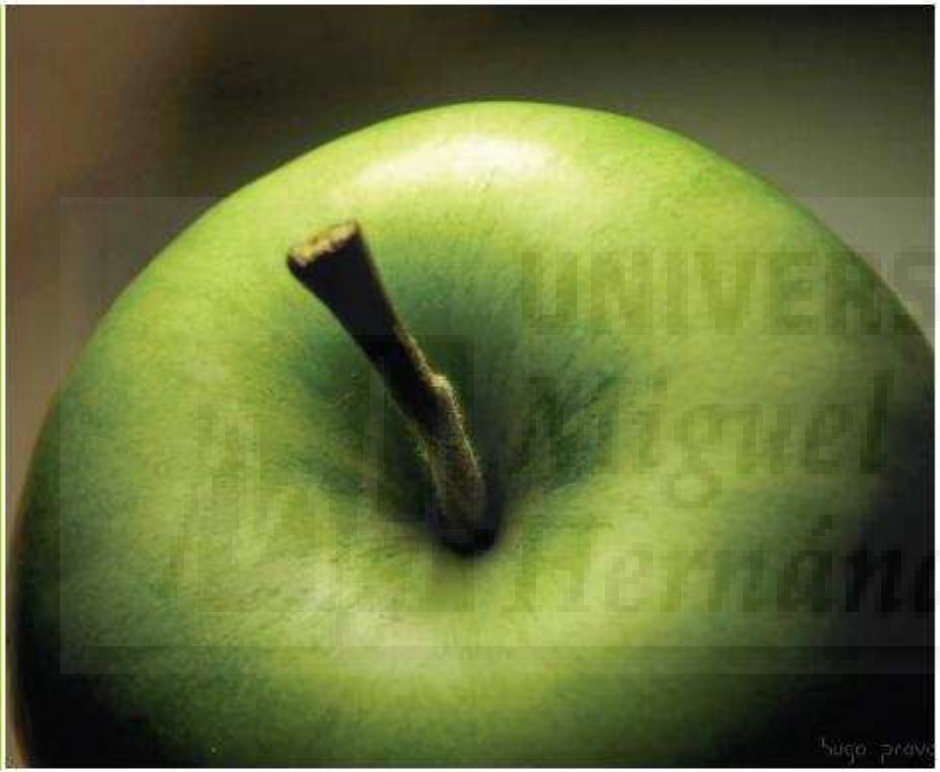

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The Association BioEurolatina (BEL), the European Federation of Biotechnology (EFB) and the Escola Superior de Biotecnologia of the Portuguese Catholic University (ESB-UCP) have joined efforts to organize this Debate, within the framework of the EULAFF Project "Europe-Latin America Action on Functional Foods", sponsored by the European Commission. This project is focused on the promotion of innovation partnership links between the S&T and industry communities of functional foods in the European Union and Latin America.

SCOPE

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TARGET

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Physical and chemical properties of cooked meat products added with orange juice wastewater and thyme essential oil

Viuda-Martos, M., Fernández-López, J., Sendra, E., Sayas, M.E., Navarro, C., Sánchez-Zapata, E. and **Pérez-Alvarez, J.A.**

Industrialización de Productos de Origen Animal, Grupo 1 UMH y Grupo REVIV Generalitat Valenciana, Universidad Miguel Hernández, Escuela Politécnica Superior de Orihuela, Orihuela, Alicante, Spain.

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There is a growing demand of natural products in human diet, both due to the possible negative effects of synthetic food additives on human health and to the increased consumer perception of this problem in the recent years. The important role which diet plays in preventing and treating illnesses is widely accepted.
The classical concept of "adequate nutrition" is tending to be replaced by the concept of "optimal nutrition", which includes the potential of food to promote health, improve general well-being and reduce the risk of developing certain illnesses.

The objective of this work was to study the effect of the addition of different concentrations of orange juice wastewaters (5 %-10 %) and thyme essential oil (0.02 %) on the physical and chemical characteristics of bologna sausage.

Moisture, ash, water activity, and fat content were determined by AOAC methods. pH and color properties of CIELAB color space were studied. Residual nitrite level (mg $NaNO_2$/kg sample) was determined in agreement with standards ISO. Lipid oxidation was assessed by the 2-thiobarbituric acid (TBA) method. Polyphenols composition, quantified by HPLC was also determined.

TBA values of control samples were higher ($P<0.05$) than samples with orange juice wastewaters and samples with orange juice wastewaters and thyme essential oil. The orange juice wastewaters addition to this cooked meat product producesd a significant decrease ($P<0.05$) in residual nitrite level. Samples with orange juice wastewaters and thyme essential oil showed a higher decline in residual nitrite levels than the samples without thyme essential oil. The HPLC analysis of the different samples of bologna sausage detected two phenolic compounds narirutin and hesperidin. The highest peak corresponded to hesperidin.

The use of orange juice wastewaters and thyme essential oil as ingredients in cooked meat product has a protective effect from oxidation and can contribute to decrease the risk of nitrosamines and nitrosamides formation.

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Effect of packaging conditions on shelf-life of bologna sausages made with citrus fibre washing water and rosemary essential oil.

M. Viuda-Martos, J. Fernández-López, A.M. Martín-Sánchez, E. Sendra, E. Sayas, J.A. Pérez-Alvarez

Abstract— The aim of this work was to study the effect of (i) the addition of citrus fibre washing water (CFWW) obtained as ecoefficient process and rosemary essential oil (REO) and (ii) storage conditions on the chemical, microbiological and sensorial properties of bologna sausages. Traditional formula was used as control sample and experimental bolognas contained 5% CFWW and 0.02% REO. Samples were packed either in vacuum or air pouches and stored for 28 days Samples from each treatment and storage conditions were taken at 0, 6, 12, 18 and 24 days and analyzed on the same day. Lipid oxidation was assessed by TBA method, for sensory evaluation a quantitative descriptive analysis was carried out. Microbiological counts were also determined. Samples with CFWW+REO stored on vacuum packaging showed the lowest TBA values. *Enterobacteriaceae* and psychrotrophic bacteria were not detected in any sample. Samples with CFWW+REO stored on vacuum packaging showed the lowest counts of aerobic bacteria and lactic acid bacteria. Sensory evaluation results showed similar quality scores for samples added with CFWW+REO and stored either on air or vacuum packaging. The lowest scores were for control bolognas stored on air packaging.

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***Index Terms*—Bologna, Co-product, essential oil, shelf-life**

I. Introduction

Everyday, the scientific literature contains new references to the beneficial effects of different ingredients and/or bioactive compounds with functional properties, and it is interesting that many of these functional ingredients are obtained from the co-products of the agro-food industry itself.

The preparation of extracts rich in dietary fibre and natural antioxidants from the co-products of the citrus processing industry could be used as a functional ingredient in, among others, the meat [1] and dairy product [2] industries. The process of obtaining the fibre from citric fruits also generates other co-products, among them the washing water used since this process uses large quantities of water, which, besides having economic importance, is important from an environmental point of view. One way of avoiding this problem would be to re-cycle the water to take advantage of the large quantity of potentially beneficial substances it contains and to re-use the water, once depurated, which would represent a saving both in economic and environmental terms.

Research is currently being undertaken to improve techniques for extracting the bioactive compounds found in such co-products [3] for use in the production of functional foods.

The aim of this work was to study the effect of (i) the addition of citrus fibre washing water (CFWW) and rosemary essential oil (REO) and (ii) storage conditions on the chemical, microbiological and sensorial properties of bologna sausages. Traditional formula was used as control sample and experimental bolognas contained 5% CFWW and 0.02% REO.

II. Materials and Methods

Sausage manufacture

Bolognas were manufactured according to a traditional formula (only the meat percentages add up to 100% while the percentage of the other ingredients are related to meat): 50% lean pork meat and 50% pork backfat; 15% water (ice, w/w), 3% potato starch (w/w), 2.5% sodium chloride (w/w), 300 mg/kg sodium tripolyphosphate, 500 mg/kg sodium ascorbate, 150 mg/kg sodium nitrite, spices (0.01% black pepper, 0.005% nutmeg and 0.2% garlic powder). This original mixture was used as control sample while to assess the influence of the concentration of citrus fibre washing water and rosemary essential oil, water content were replaced in the control formula by citrus fibre washing water (5%) and rosemary essential oil (0.02%) was added. The citrus fibre washing water was obtained by the method described by Lario et al., [4] and rosemary essential oil (REO) was supplied by Ravetllat Aromatics (Barcelona, Spain).

The products were prepared in a pilot plant and followed industrial processing techniques. Frozen raw material of animal origin, except pork backfat, was transferred to the cutter (Tecator 1094 Homogeneizer, Tekator, Höganäs, Sweden) with the sodium chloride to extract salt-soluble proteins; after comminution, the other ingredients and additives were added. Then, pork backfat, previously divided into cubes 10 x 10 x 10 cm, was added. After homogenization, the mixture was stuffed into artificial casing Fibran-Pack (Fibran, Girona,Spain) 100 x150 mm long, clipped at both ends (Polyclip system/Niedecker, Germany) and cooked in a water bath. The sausages were kept in the bath until the coldest point reached 72 °C (geometric centre of bologna, which corresponds to the thickest part of the product). A thermocouple probe (Omega Engineering, Inc., Stamford, Conn., U.S.A.) positioned in the geometric centre of the bologna was used to monitor product temperature. When the end-point temperature was achieved, the sausages were immediately chilled in ice. After reaching room temperature, the product was transferred to the lab in insulated boxes containing ice.

Storage conditions

Immediately after manufacture, some slices (1.5 cm thick) were aseptically removed from each bologna sausage. The trays containing the slices were packed either in vacuum, or air pouches made of polyethylene and polyamide laminate of water vapour permeability 1.1 g/m^2/24 h at 23 °C, nitrogen permeability 10 cm^3/m^2/24 h at 23 °C, carbon dioxide permeability 140 cm^3/m^2/24 h at 23 °C, and oxygen permeability 30 cm^3/m^2/24 h at 23 °C (Fibran, Girona, Spain). The pouches were heat-sealed and stored at 4 ± 1 °C in a cabinet simulating supermarket retail conditions. The cabinet was illuminated by a standard supermarket fluorescent lamp (OSRAM, Germany). All the samples were exposed to continuous lighting at 1000 lux at the surface, measured using a luxometer Lutron LX-102 (Taiwan). The positions of the samples in the cabinet were rotated every 24 h to minimize light intensity differences and possible temperature variations on the meat surface. The packs were stored for 24 d. Samples from each treatment and storage conditions were taken at 0, 6, 12, 18, and 24 d (storage time) and analyzed on the same day.

Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) assays following the recommendations of Buege and Aust [5]. TBARS values were calculated from a standard curve of malonaldehyde (MA) and expressed as mg MA/kg sample.

Sensory evaluation

Non-trained panellists (30) were recruited from the staff and students of the Miguel Hernández University, Alicante, Spain. Panellists were chosen on the basis of previous experience in consuming traditional bolognas. Furthermore, a preparatory session was held prior to testing, so that each panel could thoroughly discuss and clarify each attribute to be evaluated in bolognas. Testing was initiated after the panellists agreed on the specifications. A Quantitative Descriptive Analysis was carried out [6]. All sensory work was carried out in the sensory laboratory at the University, which fulfils requirements according to the international standards [7, 8]. During evaluation, the panellists set in private booths under incandescent/fluorescent light, with an intensity of approximately 350 lux. Rectangular pieces approximately 1.5 cm × 2 cm were cut from the centre of bologna slices and served at room temperature [9]. Each panellist evaluated three replicates of all the treatment groups; the sample presentation order was randomized for each panellist. Tap water was provided between samples to cleanse the palate. The sensory attributes were measured in unstructured scales with descriptors at both ends, no standards were provided. The attributes measured and their descriptors were as follows: for “external evaluation”: global appearance (from conventional bologna appearance to unexpected appearance), colour (from extremely light to extremely dark), shine (from dull to bright), hue (from pale pink to brown) and homogeneity perception (from particulate to no particles observed); for odour (from imperceptible to extremely intense); for “taste”: acid taste, saltiness, and fatness (from imperceptible to extremely intense); and for “texture”: hardness (from extremely soft to extremely tough), juiciness (from extremely dry to extremely moist). At the end of the test, panellists were asked to give a score for product quality from 0 to 10.

Microbiological analysis

Samples (25 g) were excised from the interior of the sausages with a sterile scalpel and forceps and then homogeneized with sterile 1.5% peptone water in a Stomacher 400 (Colworth, London, U.K.) for 1.5 min.

Total viable counts were determined on Plate Count Agar, *Enterobacteriaceae* using Violet Red Bile Glucose Agar (VRBGA) and Lactic acid bacteria (LAB) were counted on double layer MRS Agar at pH 5.6. In all cases, plates were incubated at 35 °C for 48 h. Psychrotrophic microbiota was determined on Plate Count Agar, and the plates were incubated at 7 °C for 10 d. Culture media were from Oxoid (Oxoid Unipath Ltd. Basingtoke, Hampshire, U.K.).

III. RESULTS AND DISCUSSION

Lipid Oxidation

Figure 1 shows how lipid oxidation (TBARS) compared during the experiment in both treatments and in the different types of packaging.

At day 0, the CFWW+REO samples showed lower oxidation values ($P<0.05$) than the control in all two types of packaging. At day 6, the lowest oxidation values recorded were in the CFWW+REO samples packed in vacuum (5.30 mgMA/kg), while no statistically significant differences were observed between the air packed CFWW+REO sample and the control sample packed under vacuum. At days 12 and 18,

vacuum-packed CFWW+REO samples continued to show the lowest oxidation values, while the air-packed CFWW+REO sample showed higher ($P<0.05$) values than the Vacuum-packed control (6.41 and 6.18 mgMA/kg sample, respectively).

At the end of the experiment (24 days), the vacuum-packed CFWW+REO treated sample showed the lowest oxidation degree ($P<0.05$) of all the samples, regardless of packaging type, while both the air-packed control and air-packed CFWW+REO samples showed the highest values for this parameter.

Figure 1. Evolution of TBA in bolognas (control and formulated with CFWW+REO) exposed to light and stored for 24 d.

Chopping and heating can catalyze the lipid oxidation because of disrupts cellular protective compounds contained in cell membranes such as vitamin E, electron, and hydrogen donors [10].

The lower lipid oxidation values mentioned above obtained with the CFWW+REO treatments would be due to the protective effect of the same. The agents responsible for the antioxidant activity in both orange fibre and oregano essential oil are the bioactive compounds they contain and, mainly, polyphenols.

This antioxidant activity is related with the capacity of polyphenols to act as metal-chelators, free radical scavengers, hydrogen donators and inhibitors of the enzymatic systems responsible for initiating oxidation reaction. Furthermore, they can act as substrate for free radicals like superoxide or hydroxyl or intervene in propagation reactions [11].

Microbiological analysis

The microbiological stability of cooked meat products depends on intrinsic factors, such as their composition, and extrinsic factors, especially the packaging and storage temperature. In our experiment, no *enterobacteria* nor psycotrophic bacteria were found in either of the treatments (control and CFWW+REO), regardless of packaging method or time of storage, probably due to the sodium chloride and phosphates contained in the products [12].

The growth of aerobic and lactic bacteria was greater ($P<0.05$) in the control samples than in those with added CFWW+REO (Figures2 and 3) at all times and in all packaging conditions, except in the CFWW+REO sample packed in air at six days, which showed higher values than the controls packed in vacuum, a difference that was maintained until the end of the experiment (24 days).

Figure 2. Evolution of counts of aerobic bacteria in bolognas (control and formulated with CFWW+REO) exposed to light and stored for 24 d.

Figure 3. Evolution of counts of lactic acid bacteria in bolognas (control and formulated with CFWW+REO) exposed to light and stored for 24 d.

In all the samples, the total aerobic bacteria and lactic acid counts at the end of the experiment were below those considered as representing a degraded product

Sensorial analysis

Figure 4 shows the results of a sensorial evaluation of the different samples assayed.

For global appearance CFWW+REO vacuum packed showed the highest values while control air packed showed the lowest scores in this respect.

For shine and hue CFWW+REO vacuum packed showed the highest values follow by control sample vacuum packed.

In the case of odor, similar values were obtained in all samples, despite the marked odour of rosemary, this was not judged negatively by the panellists who scored the samples containing REO in a similar way or even higher than the rest of the samples. Colour homogeneity, acid taste, saltiness and fatness obtained similar values

The sample containing 5% CFWW and 0.02% REO was best regarded by the panellists.

Figure 4. Results of quantitative descriptive analysis carried out at day cero of bolognas (control and formulated with CFWW+REO) exposed to light.

IV. Conclusion

The addition of citrus fibre washing water and rosemary essential oil is a technologically viable alternative in fine paste meat products, since they improve their acceptance and have desirable effects as regards oxidative stability and reduced microbial growth, which contribute to prolonging their shelf-life. Also maintain polyphenolic compounds, such as flavonoids, which may have a beneficial effect on human health since they have been linked with the prevention of various illnesses.

Acknowledgement

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