Lavandin essential oil biocide formulations:

New products and processes

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Formulaciones biocidas de aceite de lavandin: Nuevos productos y procesos

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Certifican que:

Que la ingeniera SALIMA VARONA IGLESIAS ha realizado en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Universidad de Valladolid, bajo nuestra dirección el trabajo que, para optar al Grado de Doctor Europeo presenta con el título "*Lavandin Essential Oil Biocide Formulations: New Products and Processes*", cuyo título en castellano es "*Formulaciones biocidas de aceite esencial de lavandín: nuevos productos y procesos*". Siendo el Prof. Eckhard Weidner su tutor durante la estancia realizada en Ruhr Universität Bochum (Alemania).

Valladolid, a _____ de_____ de 2010

Fdo. María José Cocero Alonso Fdo. Ángel Martín Martínez

Reunido el tribunal que ha de juzgar la tesis doctoral titulada "*Lavandin Essential Oil Biocide Formulations: New Products and Processes*" presentada por la ingeniera Salima Varona Iglesias y en cumplimiento con lo establecido por el Real Decreto 1393/2007 de 29 de Octubre ha acordado conceder por_____ la calificación de _____.

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A MI FAMILIA Y AMIGOS...

Resumen

Formulaciones biocidas de aceite esencial de lavandín:
Nuevos productos y procesos.

Salima Varona Iglesias

1. Introducción

La preocupación pública por el uso de productos químicos en agricultura y ganadería ha aumentado y hay una mayor presión social para la utilización de productos naturales. En el caso de la agricultura esto se debe al uso extensivo de pesticidas y herbicidas (carbamatos y órganofosfatos) que generan contaminación ambiental y residuos que pueden pasar al organismo. Otro problema es el aumento del uso de antibióticos en ganadería y su posible contribución en la aparición de bacterias resistentes y su trasmisión a los humanos. En este contexto, una posible solución sería el uso aceites esenciales extraídos de plantas aromáticas con propiedades biocidas (bacterias, hongos, insectos y nematópodos). Este trabajo se ha centrado en el aceite de lavandín (*Lavandula hybrida*), debido al interés de su cultivo en la región de Castilla y León.

Los aceites esenciales presentan una serie de limitaciones para poder ser utilizados como biocidas, siendo las principales su alta volatilidad, baja solubilidad en agua, sensibilidad al calor, a la oxidación y a la luz ultravioleta. La actividad biocida de los aceites esenciales se puede mejorar mediante una formulación adecuada. Esta formulación debe ser físicamente estable y permitir la protección y liberación controlada de los compuestos activos de los aceites esenciales.

En este trabajo se han desarrollado varias formulaciones de aceites esenciales para su uso como biocidas, como emulsiones y microcápsulas. Tanto para la emulsión como para la encapsulación se han empleado polímeros biodegradables; almidones modificados con el grupo n-(octenil) anhídrido succínico (OSA), polietilenglicol, policaprolactonas y lecitina de soja.

El uso del aceite de lavandín como antibiótico por vía oral requiere una formulación especial. Diversos autores emplearon con éxito liposomas (vesículas formadas por fosfolípidos), para la administración oral de medicamentos como insulina y ciclosporina. Los liposomas tienen la capacidad de proteger el aceite encapsulado de la digestión, permitiendo su adsorción en el intestino y su liberación a través de la

membrana citoplasmática de las células, debido a la similitud de la estructura de los liposomas con la membrana celular. En este trabajo se han obtenido liposomas mediante el método convencional de Bangham y a partir de partículas de lecitina de soja formuladas mediante PGSS (partículas a partir de disoluciones saturadas de gas).

La encapsulación de aceites esenciales ha sido objeto de investigación durante años. Realizando una revisión en la literatura, se puede concluir que la técnica más empleada es el secado en espray de emulsiones de aceites esenciales. Otras técnicas como coacervación, liofilización, co-cristalización e inclusión en liposomas han sido también empleadas pero en menor proporción. Los procesos de formulación desarrollados en este trabajo fueron, a parte del secado en espray, otros que emplean CO_2 en estado supercrítico ($scCO_2$); PGSS (partículas a partir de disoluciones saturadas de gas), PGSS-secado e impregnación supercrítica. Los procesos de precipitación que usan CO_2 supercrítico presentan varias ventajas con respecto a los convencionales; permiten operar con condiciones suaves, se aumenta la eficacia de encapsulación y no utilizan disolvente orgánicos. El PGSS es el proceso con el que se han obtenido los mejores resultados con respecto a la eficiencia de encapsulación y cantidad de aceite encapsulado.

La actividad antibacteriana del aceite esencial de lavandín y de las formulaciones desarrolladas se evaluó para tres tipos de bacterias implicadas en enfermedades atribuidas a la comida. La concentración mínima de inhibición del aceite de lavandin fue de 7.1 mg/mL para *E.coli*, 7.1 mg/mL para *S.aureus* y 3.6 mg/mL para *B.cereus*. La encapsulación en todos los casos mejoro el efecto del aceite como inhibidor del crecimiento de las bacterias. La eficacia de esta acción fue función principalmente de la concentración de aceite y del agente encapsulante. La lecitina de soja resultó ser el encapsulante más eficaz debido a su capacidad de formar liposomas. El proceso empleado para la formación de las partículas también resultó ser un parámetro importante, siendo las partículas obtenidas mediante PGSS secado las que mostraron mayor actividad antimicrobiana para la misma cantidad de aceite encapsulado. *E.coli* (Gram-) resultó ser la cepa más sensible, mientras que *B.cereus* y *S.aureus* (Gram+) fueron las más resistentes.

2. Objetivos

El objetivo de esta tesis es realizar los estudios que permitan obtener diferentes formulaciones del aceite esencial de lavandín, con el fin de poder utilizarlas como biocidas para su uso en agricultura y ganadería. Para cumplir este objetivo se ha planteado los siguientes objetivos

- Desarrollo de formulaciones del aceite de lavandín empleando biopolímeros (almidón modificado y lecitina de soja), como emulsiones y microcápsulas.
    - Caracterización de las emulsiones y determinación de la influencia de las condiciones de operación (concentración de surfactante, volumen de aceite, energía suministrada a la emulsión) en sus características (estabilidad, diámetro de gota). Las emulsiones deben cumplir unos requisitos de estabilidad y tamaño de gota para poder ser procesadas con fluidos supecríticos.
    - Caracterización de las microcápsulas de aceite de lavandín (tamaño de partícula, distribución de tamaño de partícula, aceite encapsulado y aceite superficial, morfología y humedad).
    - El principal objetivo de la tesis es la aplicación de aceite de lavandín y de los formulados obtenidos como biocidas. Para ello se determinará su actividad antimicrobiana frente a bacterias patógenas causantes de enfermedades relacionadas con la comida.
- Estudio de varios procesos de encapsulación para la obtención de microcapsulas de aceite de lavandín.
    - Secado en espray de emulsiones. Estudio de la influencia de las condiciones de operación (temperatura entrada del aire, flujo de emulsión, velocidad del atomizador rotativo) en el producto final.

- PGSS empleado polímeros biodegradables (PEG, Policapolactona) como agentes encapsulantes. Estudio de la influencia de las variables de operación (temperatura y presión antes de la expansión, tamaño de la boquilla y relación de gas/disolución).

- Secado de emulsiones mediante el proceso PGSS. Estudio de la influencia de las variables de operación (temperatura y presión antes de la expansión, tamaño de la boquilla y relación de gas/disolución).

- Impregnación mediante CO_2 supercrítico del aceite de lavandín en almidón modificado. Estudio de la influencia de las variables de operación (presión, temperatura y relación polímero/aceite lavandín).

3. Resultados y discusión

Capítulo I. *Formulación de emulsiones de aceite de lavandín (Lavandula hybrida) empleando almidón modificado como emulgente.*

El objetivo de este capítulo es el desarrollo de emulsiones del aceite de lavandín para su uso como biocida. Las emulsiones se han obtenido empleado un equipo rotor estator y varios tipos de almidones modificados con el grupo OSA como surfactantes para su estabilización. Estos almidones están aprobados para su uso en alimentación por la Unión Europea y Estados Unidos (FDA).

La evaluación de la capacidad de los almidones modificados como surfactantes para el aceite de lavandín se realiza mediante la determinación de la concentración crítica de micela (mínima cantidad de surfactante para que se formen micelas). Esta concentración se encuentra entre 4,5 - 6,6 g/l para los cuatro tipos de almidones.

Las emulsiones se caracterizaron a través del diámetro de gota inicial, variación del diámetro de gota con el tiempo y separación de fases. El diámetro de gota de estas emulsiones se encuentra en el rango de 500 - 2800 nm y aproximadamente un 80% del aceite permanece emulsionado después de 50 días almacenado a 5ºC. La adsorción de los almidones en la interfase líquido-liquido de la emulsión también se ha estudiado. La cantidad de almidón adsorbido en la interfase resultó ser muy grande (hasta 370 mg/m^2) dando lugar a multicapas muy gruesas. En la figura 1 se presenta un ejemplo de isoterma de adsorción.

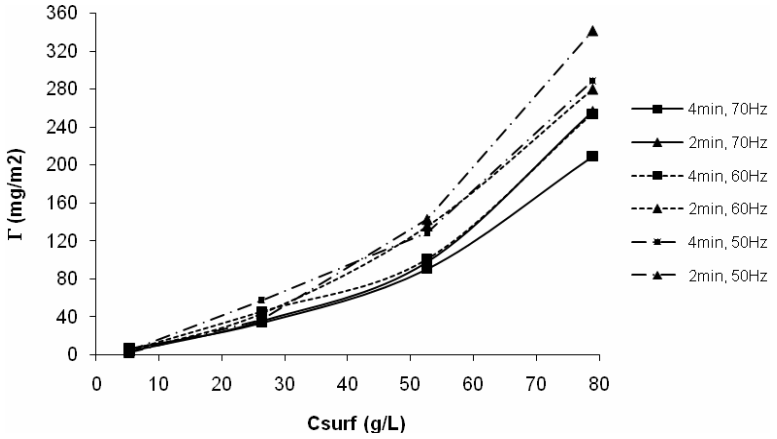


Figura 1. Isoterma de adsorción para el almidón modificado OSA2.

Se ha comparado la eficacia de los almidones modificados como surfactantes con dos surfactantes comerciales (tween20 y span20). Estos surfactantes comerciales son iónicos y hay que determinar el balance hidrofílico-lipofílico (HLB) del aceite de lavandín para saber el porcentaje de cada uno que se debe emplear para obtener una emulsión estable. Para evaluar un rango amplio de HLB se mezclaron tween 20 (HLB 16.7) y span20 (HLB 8.6), realizándose emulsiones con las mezclas y con los surfactantes puros. A partir de estos ensayos, se determinó que el HLB del aceite de lavandín está en el rango de 13.1-13.8. El diámetro de gota de las emulsiones de aceite de lavandín obtenidas, empleando una mezcla de tween20 y span20 de HLB 13.4, está en el rango de 620- 810 μm, dependiendo de la concentración de surfactante y la energía suministrada para la formación de la emulsión.

La distribución del tamaño de gota de las emulsiones se determino mediante DLS (Dispersión dinámica de la luz) y microscopia óptica. Como se puede observar en la figura 2 las emulsiones estabilizadas con surfactantes no-iónicos presentan una distribución estrecha y unimodal, mientras que las emulsiones con almidón modificado presentan una distribución de tamaños más ancha y bimodal. Los resultados demuestran la validez de los cuatro almidones modificados probados como agentes surfactantes para el aceite de lavandín. Las características de las emulsiones producidas con los anteriores

almidones son similares a las producidas con surfactantes comerciales tween20 y span20.

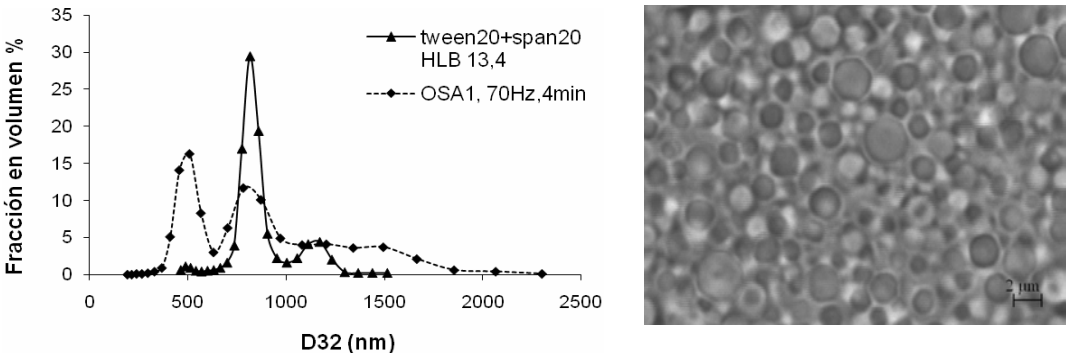


Figura 2. (a) Distribución de tamaño de gota (b) Foto microscópica de emulsiones tween20+span20.

Capítulo II. *Formulación de aceite de lavandín (Lavandula hybrida) en liposomas mediante el método de Bangham y secado-PGSS.*

En este capítulo se estudió la formulación del aceite de lavandín para la aplicación de sus propiedades biocidas y antivirales en ganadería, como sustitutos de los medicamentos sintéticos. La formulación propuesta para esta aplicación son los liposomas, comúnmente empleados como encapsulantes en la industria farmacéutica y cosmética. En concreto, se investigó la incorporación del aceite esencial de lavandín en liposomas formados por lecitina de soja comercial y colesterol.

Para la formación de los liposomas se emplearon el método convencional de Bangham y el proceso de formación de partículas a partir de disoluciones saturadas de gas (secado-PGSS). Los liposomas obtenidos por el método de Bahgham presentaron morfología multivesicular o uni/multilamelar y un diámetro medio de 0.4- 1.3 μm, como se puede observar en la figura 3. La eficiencia de encapsulación del aceite esencial de lavandín para este método fue del 66%.

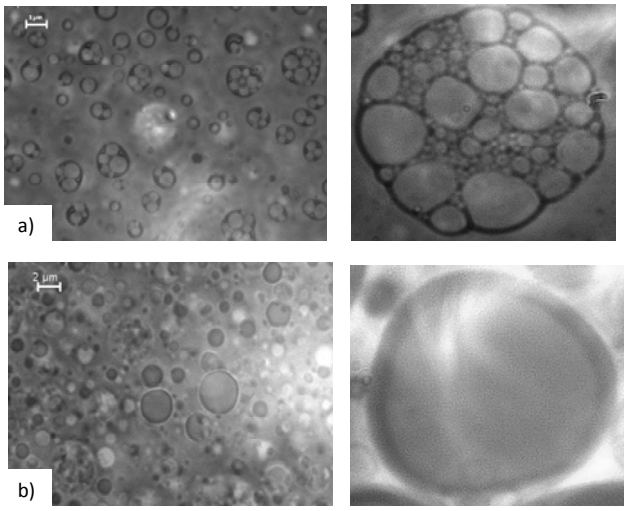


Figura 3. Fotos con microscopio óptico (100X) de lasformulaciones vesiculares obtenidas: Multivesiculares (a) and uni/multilamelares (b).

El diámetro de los liposomas dependió especialmente de la incorporación de aceite esencial, aumentando con la relación aceite de lavadin/lípidos (lecitina y colesterol). El colesterol fue añadido para mejorar la estabilidad y disminuir la permeabilidad de los liposomas, traduciéndose en una disminución de su diámetro, como se muestra en la figura 4. El agua de hidratación también resulto ser una variable importante, disminuyendo el diámetro de los liposomas con la cantidad de agua añadida para hidratarles. La estabilidad física de los liposomas se evaluó siguiendo la variación de su diámetro durante un mes. El diámetro de los liposomas aumento considerablemente durante los primeros 10 días permaneciendo después estable.

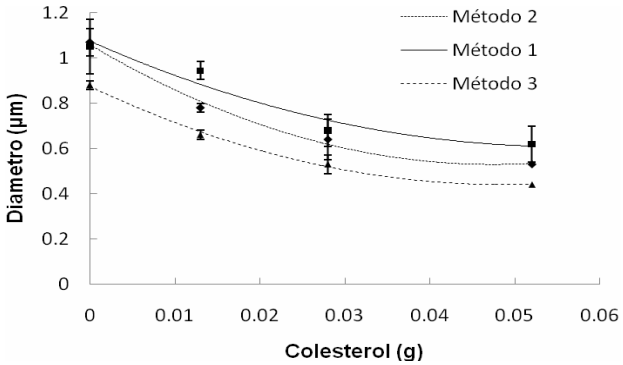


Figura 4. Influencia de la cantidad de colesterol en el diámetro de los liposomas.

En el proceso de secado-PGSS las partículas se obtuvieron tras el secado con dióxido de carbono supercrítico ($scCO_2$) de emulsiones de aceite de lavandín/agua estabilizadas con lecitina de soja y colesterol. Previamente se comprobó la estabilidad de dichas emulsiones en CO_2 a la presión y temperatura de operación (0.1-12 MPa, 20-120ºC) para evitar su desestabilización durante el proceso. Las partículas de lecitina de soja obtenidas presentaron un tamaño entre 1.4 μm y 24.8 μm y tendencia a aglomerase. La eficacia de encapsulación fue baja, encapsulándose entre el 3- 15% del aceite inicial y 3-39% de linalool inicial. La variable que más influyó en la eficiencia de encapsulación fue la relación de flujos de CO_2 /emulsión (GPR), disminuyendo al aumentar el GPR debido a la alta solubilidad del aceite de lavandín en CO_2. La eficiencia de encapsulación del proceso aumento en las condiciones en las que es se producen partículas más pequeñas (alta T y P pre-expansión), por lo tanto está relacionada con la eficiencia de atomización y el proceso de formación de partículas. La eficacia de encapsulación disminuyo con la concentración de lecitina en la emulsión, puesto que la lecitina forma disoluciones muy viscosas dificultándose su atomización.

La ventaja principal del secado-PGSS frente al método convencional, es que se evita el uso de disolventes y las partículas obtenidas pueden ser almacenadas y posteriormente dispersadas en agua para reconstruir los liposomas (figura 5). Los liposomas reconstruidos fueron uni/miltivesiculares y su tamaño se encontró entre 0.5 μm and 1.5 μm. Se determinó que hay una relación entre el tamaño de las partículas y el tamaño de los liposomas formado al hidratar las partículas.

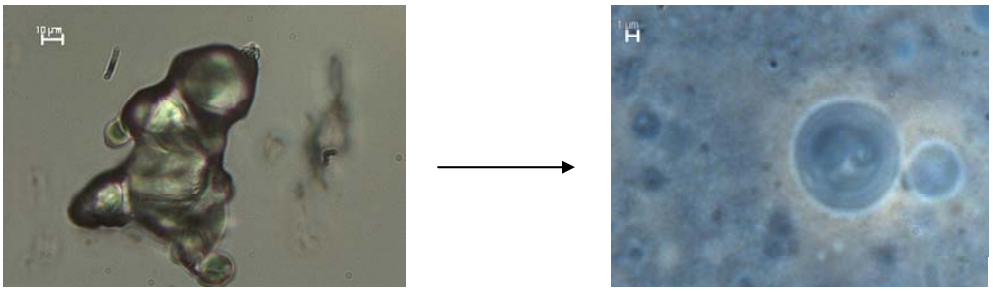


Figura 5. Fotos con microscopio óptico (100x) dde la recostrucción de los liposomas mediante hidratación de las partículas obtenidas por secado-PGSS.

Capítulo III. *Formulación del aceite esencial de lavandín (Lavandula hybrida) en microcápsulas para su aplicación como biocida en la agricultura. Desarrollo del los procesos PGSS y secado PGSS.*

En este capítulo se ha estudiado la encapsulación del aceite de lavandín en polímeros biodegradables como formulación para su uso como biocida. En concreto se han estudiado dos técnicas de precipitación que emplean dióxido de carbono en estado supercrítico ($scCO_2$); formación de partículas a partir de disoluciones saturadas de gas (PGSS) y formación de partículas a partir del secado de disoluciones saturadas de gas (secado-PGSS). En el proceso PGSS se ha empleado como agente encapsulante el polietilenglicol (PEG). En el caso del secado-PGSS, el aceite se ha encapsulado en almidón modificado con el grupo OSA (n-(octenil) anhídrido succínico) mediante el secado de emulsiones aceite de lavandín-agua estabilizadas con dicho almidón. Previamente se comprobó la estabilidad de la emulsión ($250g_{almidón}/L$, lavandín/almidón: 0.2, 0.3 y 0.4) en CO_2 a las condiciones de presión y temperatura de proceso (tabla 1). La emulsión permaneció estable en todo el rango de condiciones probadas durante varios minutos (7-60min), lo cual la hace apta para el proceso puesto el tiempo de residencia en el mezclador es inferior a un segundo

	Pre-exp P(MPa)	Pre-exp T(ºC)	GLR	T_{tower} (ºC)	Lav/almidón-OSA
PGSS	76-84	5.4-8.5	0.56-1.26	30	0.25-0.37
Secado-PGSS	9-12.5	108-131	24-40	75	0.2-1

Tabla 1. Condiciones de operación.

El aceite de lavandín se encapsulo más eficientemente en PEG, obteniéndose una eficacia de encapsulación entre 14% y 66%. La eficacia de encapsulación de linalool (componente con mayor actividad biocida del aceite de lavandín) también fue mayor en las partículas de PEG, obteniéndose valores de hasta el 80%.

Las partículas de PEG obtenidas presentaron una morfología esférica (figura 6a) y una distribución de tamaños estrecha y unimodal, características favorables para una liberación controlada del aceite. Las partículas de almidón modificado obtenidas mediante secado-PGSS presentaron dos tipos de morfologías: partículas esféricas y agujas (figura 6b, 6c) y una distribución de tamaño de partícula ancha y bimodal. Este resultado junto con la mayor eficacia de encapsulación obtenida, hacen que el PGSS sea el proceso más adecuado para la encapsulación del aceite de lavandín.

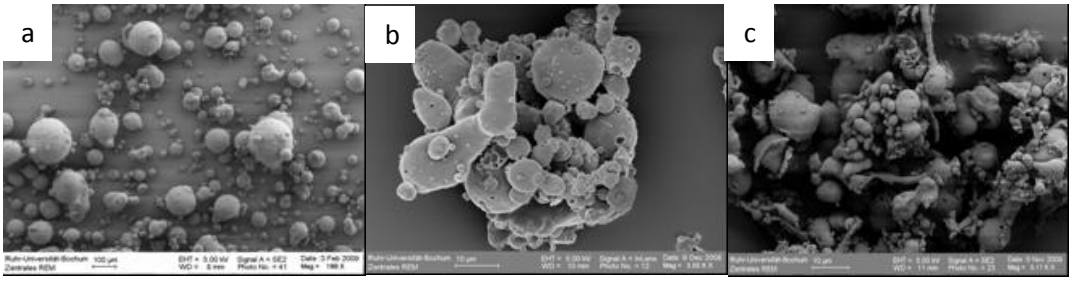


Figura 6. Fotos SEM: Partículas PEG (a) y partículas de almidón modificado (b,c).

Capítulo IV. *Impregnación con CO_2 supercrítico del aceite de lavandín en almidón modificado.*

En los últimos años ha aumentado el uso de procesos que emplean fluidos supercríticos (RESS, SAS, GAS y PGSS) para formulación de sistemas de liberación controlada de medicamentos. Procesos como RESS (Expansión rápida de disoluciones supercríticas), SAS (disoluciones supercríticas antisolventes) y PGSS (partículas a partir de disoluciones saturadas de gas) son los más empleados debido a los buenos resultados obtenidos. Sin embargo, estos procesos presentan la desventaja del difícil control de las características del producto final.

La impregnación en medio supercrítico ($scCO_2$) es una alternativa a los procesos anteriores. En la literatura se encuentran varios trabajos sobre impregnación de fármacos y fragancias obteniéndose formulaciones que permiten su liberación controlada. Las ventajas de este método están relacionadas con el efecto plastificante

(disminución del a temperatura de transición vítrea) y la capacidad para hinchar los polímeros del $scCO_2$., mejorando de la difusión de los solutos a través del polímero. Estas propiedades permiten el control de la carga y profundidad de penetración del principio activo mediante la variación de las condiciones de impregnación.

Este trabajo está enfocado a la impregnación de aceite de lavandín en almidón modificado empleando $scCO_2$ como disolvente. Los aceites esenciales son muy solubles en $scCO_2$, lo cual facilita su impregnación. La carga de aceite de lavandín obtenida en las partículas (25-144 mglavandín/g producto) fue similar a la obtenida con otros procesos como el PGSS o sacado-PGSS. Sin embargo, los rendimientos de impregnación fueron bajos, impregnándose entre el 4 y el 22% del aceite inicial.

El proceso global de impregnación es el resultado de varias interacciones; lavandín-$scCO_2$, lavandín-polímero y polímero-$scCO_2$. En este caso, el coeficiente de reparto del aceite entre el polímero y el disolvente ($scCO_2$) es desfavorable, lo que indica el aceite tiene poca afinidad por el polímero. En la figura 7 se puede observar que tanto el coeficiente de reparto como la cantidad de aceite impregnada disminuyen con la densidad del $scCO_2$. De este resultado se puede concluir que aunque el efecto plastificante y el poder de hinchar el polímero del CO_2 aumenté con la densidad, la cantidad de aceite impregnado disminuye debido al aumento las interacciones del aceite de lavadín-CO_2 y disminución de las interaciónes lavandín-polímero con la densidad del $scCO_2$.

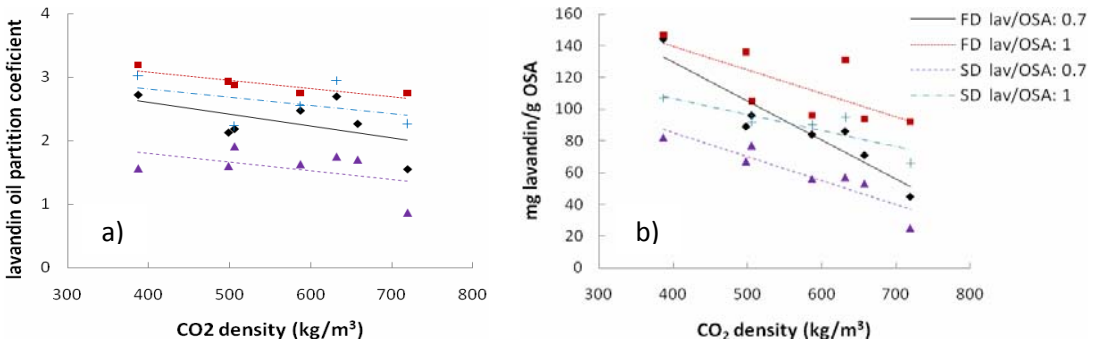


Figure 7. a) Coeficiente de reparto del aceite de lavandin en función del la densidad del CO_2. b) Cantidad de aceite de lavandin impregnado en funcíon de la densidad del CO_2.

Los resultados demuestran que la temperatura en una variable favorable para el proceso de impregnación, lo cual se justifica por un aumento de la solubilidad del aceite con la temperatura. Sin embargo, la presión es una variable desfavorable, puesto que la cantidad de aceite impregnado disminuye con la presión.

Capítulo V. *Actividad antimicrobiana de formulados de aceite esencial de lavandin (Lavandula hybrida).*

Este capítulo se ha centrado en el estudio de la actividad antimicrobiana del aceite de lavandín (*Lavandula Hybrida*) libre y encapsulado frente a tres tipos de bacterias causantes de enfermedades asociadas a la comida (gram-negativa: *Escherichia coli; gram-positivas: Staphylococcus aureus y Bacillus cereus*). Los procesos de encapsulación empleados fueron el secado en espray de emulsiones, la formación de partículas a partir de disoluciones saturadas de gas (PGSS) y formación de partículas a partir de disoluciones (emulsión lavandín/agua) saturadas de gas (secado-PGSS). Como agentes encapsulantes se emplearon lecitina de soja, almidón modificado OSA y poli-caprolactona. Las características de las partículas estudiadas y la concentración de aceite de lavandin alcanzada para el ensayo de actividad antimicrobiana, se muestran en la tabla 2.

Los resultados desmuestran que tanto el aceite de lavandín libre como encapsulado inhiben el crecimiento de los tres tipos de bacterias. La concentración mínima de inhibición de aceite de lavandín (concentración que inhibe el crecimiento de, por lo menos, el 90% de las bacterias) fue de 7.1 ml/mg para *E.coli* y *S.aureus* y de 3.6 ml/mg para *B.cereus*. El porcentaje de inhibición del crecimiento de las bacterias causado por las partículas se recoge en la figura 8. Como se puede observar, la *E.coli* (Gram-negativa) fue la bacteria que mayor sensibilidad presentó. También se observo que la actividad antimicrobiana de las partículas aumenta con la concentración de lavandín encapsulado.

Sample	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11
Process	SD	SD	SD	SD	SD	SD	SD	PGSS D	PGSS D	PGSS D	PGSS
Carrier	OSA	OSA	OSA	OSA	OSA	Lec.	Lec.	OSA	OSA	OSA	CAPA
T (ºC) $_{process}$	170	170	170	170	170	170	170	118	136	112	70
Lavandin/Carrier	0.4	1	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.2	0.5
$mg_{lav}/g_{product}$	165	361	231	96	102	24	63	33	57	43	153
$mg_{lin}/g_{product}$	84	205	59	29	49	8	27	15	40	17	82
$D_{0,5}$ part (µm)	14	14	19	14	17	25	9	32	53	65	85
C_{lav}(mg/mL)	0.008	0.079	0.044	0.018	0.028	0.005	0.014	0.007	0.013	0.009	0.03
$C_{linalool}$(mg/mL)	0.006	0.045	0.011	0.005	0.01	0.002	0.006	0.003	0.006	0.004	0.02
$C_{linalyl}$(mg/ml)	0.0007	0.0117	0.0098	0.0047	0.0050	0.0012	0.0031	0.0005	0.0011	0.0023	0.02

Tabla 2. Características de las partículas analizadas y concentración de lavadin alcanzada en el ensayo antimicrobiano.

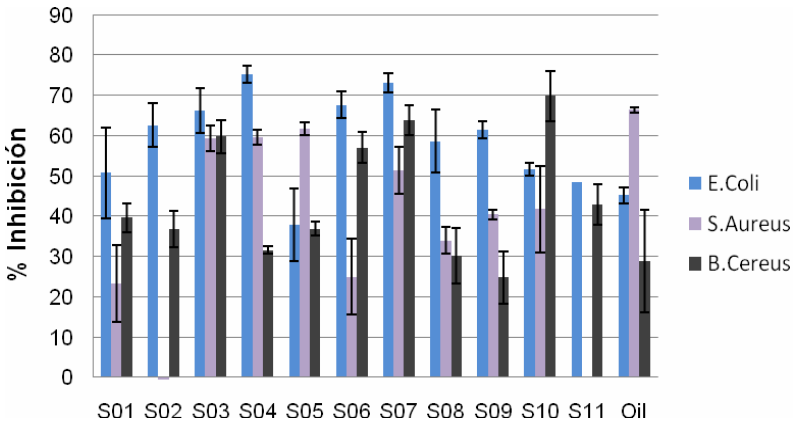


Figura 8. Inhibición causada por diferentes partículas (0.2mg/ml).

La lecitina resulto ser el encapsulante más eficaz para el aceite de lavandín para todas las bacterias evaluadas. En la tabla 4 se puede observar que la inhibición causada por partículas de lecitina (S07) resulto mayor que la causada por partículas de almidón modificado (S09) o policaprolactona (S11), todas con la misma carga de aceite de lavandín. Nuestra hipótesis es que, los liposomas debido a su semejanza con la membrana de fosfolípidos de las bacterias Gram-negativas pueden atravesarla con

mayor facilidad. Posteriormente, los liposomas tendrían que atravesar la capa fina de peptidoglicanos y la membrana citoplasmática (también fosfolipídica) para liberar el aceite en citoplasma. Por otro lado, las bacterias Gram-positivas presentan una capa gruesa de peptidoglicanos, lo cual dificulta la penetración de las partículas y liberación del aceite de lavandín.

4. Conclusiones

A continuación se presentan de las conclusiones más relevantes de este trabajo.

Formulación de emulsiones de aceite de lavandín (Lavandula hybrida) empleando almidón modificado como emulgente.

- Las emulsiones de aceite de lavandín preparadas con los cuatro tipos de almidones-OSA son estables y sus características (estabilidad y tamaño de gota) son similares a las preparadas con los surfactantes comerciales (mezcla de Tween 20 y Span20 con un HLB óptimo de 13).

- Los parámetros más influyentes en las propiedades de la emulsión son la concentración de surfactante, el volumen de aceite y la velocidad de homogenización (energía suministrada a la emulsión). Con las mejores condiciones de operación, es posible la obtención de emulsiones con un tamaño de gota medio de 700nm y estables (20% de aceite desemulsionado tras 50 días).

- La velocidad de adsorción de los almidones modificados en la interfase aceite-agua está gobernada por el área interfacial creado durante la emulsificación y la cantidad de almidón disponible, dando lugar a la formación de multicapas gruesas (alcanzando valores de hasta 370 mg/m^2). Este dato explica la alta estabilidad de la emulsiones de aceite de lavandín en agua con almidones modificados como surfactantes.

Formulación del aceite de lavandín en liposomas mediante el método de Bangham y mediante PGSS (formación de partículas a partir de disoluciones saturadas con gas).

- El aceite de lavandín fue encapsulado en liposomas empleando como emulgentes lecitina de soja y colesterol.

- Los liposomas obtenidos por el método convencional de Bangham son uni/multilamelares y multivesiculares y con un diámetro entre 0.6 - 1.3 μm. El tamaño de estos liposomas depende sobre todo de la composición de la

membrana (relación lecitina /colesterol), de la carga de aceite y del método usado para reducir el diámetro tras la hidratación de los liposomas. La eficiencia de encapsulación del aceite de lavandín se encuentra entre el 6 y 60%.

- Se obtuvieron liposomas uni/multivesiculares, con diámetro entre 0.5 μm y 1.5 μm, a partir de la hidratación de partículas de lecitina de soja con aceite de lavandín encapsulado. Las partículas de lecitina producidas mediante el secado de emulsiones por PGSS presentan un diámetro en el rango de 1.4-25 μm. La eficacia de encapsulación del aceite de lavandín es baja (6-14%), debido a las pobres cualidades de la lecitina de soja como emulgente. Esta eficacia se podría mejorar empleando condiciones en las que el CO_2 es más soluble en la emulsión, puesto que la expansión sería más rápida y efectiva, permitiendo la formación de partículas pequeñas. La concentración de lecitina en la emulsión también es un parámetro importante, puesto que altas concentraciones dan lugar a emulsiones muy viscosas dificultando la difusión del CO_2 en el mezclador y en la boquilla.

Formulación del aceite esencial de lavandín (Lavandula hybrida) en microcápsulas para su aplicación como biocida en la agricultura. Desarrollo del los procesos PGSS y secado PGSS.

- El aceite de lavandín se encapsuló en polímeros biodegradables (almidón OSA y PEG) mediante los procesos PGSS y PGSS-secado. Las características de las partículas obtenidas (diámetro, morfología, densidad aparente y aceite encapsulado) dependen de las condiciones de operación (temperatura y presión antes de la expansión y relación de CO_2/alimentación).

- El proceso más eficaz para la encapsulación del aceite de lavandín es el PGSS, debido a su eficacia y a las características de las partículas obtenidas. Las partículas producidas presentan una morfología esférica, distribución de tamaños estrecha y un tamaño medio entre 31 -91μm. La eficacia de encapsulación de aceite del lavandín se encuentra en el rango 14% al 66%. Este resultado se

explica puesto que este proceso permite operar con condiciones más suaves (menor temperatura y presión), resultando en una reducción de la pérdida de aceite por evaporación o solubilización en CO_2. Por el contrario, mediante PGSS-secado la eficacia de encapsulación es menor (6-55%) y las partículas de almidón-OSA obtenidas presentan dos tipos de morfología: esferas y agujas irregulares.

- La liberación del aceite de lavandín de las cápsulas de almidón-OSA depende principalmente de la concentración de lavandín encapsulado. Relaciones de lavandín/almidón OSA inferiores a 0.2 dan lugar a una liberación del 20% del aceite después de 20 días de almacenamiento, mientras que relaciones superiores dan lugar a una liberación del aceite de hasta el 60%.

Impregnación con CO_2 supercrítico del aceite de lavandín en almidón modificado.

- Se ha llevado a cabo la impregnación con $scCO_2$ del aceite de lavandín en almidón modificado. La carga de lavandín impregnada es alta, sin embargo, la eficacia del proceso es baja comparada con las eficacias obtenidas en otros procesos.

- Se ha determinado que la densidad del $scCO_2$ es el parámetro más influyente en la impregnación. En concreto, la impregnación se ve favorecida por la temperatura, puesto que al aumentar aumenta también la solubilidad del lavandín. Por otro lado, la presión es un parámetro desfavorable, puesto que tanto el coeficiente de reparto como la cantidad de aceite impregnado disminuyen con la presión. Esto se puede explicar por un debilitamiento de las interacciones lavandín-polímero y un aumento de las interacciones lavandín-$scCO_2$.

Actividad antimicrobiana de formulaciones con aceite de lavandín.

- En esta investigación se ha demostrado que el aceite de lavadín tiene propiedades antimicrobianas, las cuales mejoran mediante su encapsulación.

Esta actividad antimicrobiana depende sobre todo de la concentración de aceite esencial y del agente encapsulante.

- La bacteria *E.coli* (Gram-) es la cepa más sensible, mientras que el *B.cereus* (Gram+) y *S.aureus* (Gram+) presentan una mayor resistencia al aceite de lavandín y a sus formulados. Estos resultados se deben a las diferencias en la membrana celular de estas cepas (gram+ y gram-).

- El material portador de aceite es un factor importante, puesto que determina el modo de acción del agente antimicrobiano en la membrana. La lecitina de soja es el agente encapsulante más eficaz, debido a su capacidad de formar espontáneamente liposomas en contacto con agua.

- El proceso de formación de las partículas también es un parámetro importante, puesto que determina las propiedades de estas (material, cantidad y composición del aceite lavandín encapsulado, tamaño y morfología). Las partículas formadas por PGSS-secado, muestran una mayor actividad antibacteriana que las partículas formadas por secado en espray con la misma cantidad de aceite de lavandín encapsulado.

Summary

Lavandin essential oil biocide formulations:
New products and processes

Salima Varona

In recent years, there has been an increase of public concern about the use of chemical products in agriculture and livestock. Also, there is an increasing social pressure for the replacement of these compounds by natural products. In the case of agriculture, an excessive use of pesticides and herbicides generates environmental pollution and toxic wastes which are harmful for human health. In this context, one solution could be the use of essential oils, extracted from aromatic plants, with biocide properties (bacteria, fungus, insects and nematodes). Far this study, lavandin essential oil has been selected, partly due to the interest of its cultivation in the geographic area of Castilla y León (Spain).

Essential oils have several limitations to be used as biocide, being the most important their high volatility, low solubility in water and heat, oxygen and uv-light sensitivity. These limitations can be overcome by means of a suitable formulation, with the consequent improvement of the biocide activity of the essential oils. This formulation has to be physically stable, protect the oil and allow the control release of essential oils.

During this work several essential oil formulations (emulsions, liposomes and microcapsules) have been developed. Biodegradable polymers have been used with all formulations; starches modified with the group n-octenyl succinic (OSA), poly-ethyleneglycol (PEG), poly-caprolactones and soybean lecithin.

In **chapter I**, the emulsification of lavandin oil in water, using OSA-starches as emulgents was studied. Results showed that OSA-starches can be used as effective surfactant agents, obtaining stable emulsions. After the determination of the critical micelle concentration (cmc), the influence of the main process variables on the physical properties and stability of the emulsion (drop diameter and creaming velocity) was studied. The process variables studied were surfactant concentrations above the cmc (5 - 79 g/L), starch/oil ratio (1:8, 1:3, 1:1 and 3:1), homogenisation velocity of the rotor-stator equipment (50 - 70 Hz) and operation time (2 - 4 min). Obtained oil droplet sizes were in the range 0.5 - 1.3 μm, whereas approximately 80% of the oil remained stabilized in the emulsion after 50 days of storage at 5ºC. The surface loads of the starch were very high, in some cases of up to 370 mg/m^2, which corresponded to a very thick

adsorbed multilayer. It has been shown that for this application OSA-modified starches presented similar surfactant capabilities as synthetic non-ionic surfactants.

The application of lavandín oil as antibiotic for oral administration requires a special formulation. The most suitable formulation for this application could be in the form of liposomes. In fact, several authors applied successfully liposomes for oral administration of drugs like insulin and cyclosporine. Liposomes have the ability to protect the essential oil from digestion, enhancing its absorption in the intestinal epithelium and its release through the cytoplasmic cell membrane, due to its similarity to the cell membrane. In **chapter II**, two different liposome production methods have been tested: a modification of the Bangham thin-film method, and the Particles from Gas Saturated Solutions (PGSS) – drying process. Liposomes obtained with the thin-film method were multivesicular or unilamellar/multilamellar with a mean diameter of 0.4 – 1.3 μm and the incorporation efficiency of essential oil up to 66%. The obtained liposomes were stable at least for one month. PGSS drying of lavandin oil emulsion stabilized with soybean lecithin allowed to obtain dry but aggregated particles, which size range between 1.4 - 24.8 μm. Encapsulation efficiency of essential oil was low, ranging between 3 - 14.5%. These particles could be dispersed in water, producing liposomes whose size ranged between 0.5 - 1.5 μm.

Essential oil encapsulation has been investigated over years. Based on a literature review, it can be concluded that spray-drying of essential oils emulsions is the most widely used technique. Other techniques like coacervation, freeze drying, co-crystallization and liposomal have been used in a smaller proportion. Formulation processes developed in this work were, as well as spray-drying, PGSS (particles form gas saturated solutions), PGSS-drying and impregnation. These precipitation processes which use supercritical CO_2 have several advantages with regard to conventional processes; mild operating conditions, improvement of encapsulation efficiency and avoid the use of organic solvents.

In **chapter III**, two high pressure precipitation techniques have been applied to perform the lavandin oil encapsulation. PGSS process has been applied to encapsulate the oil in PEG and PGSS-drying has been applied to encapsulated the oil in OSA starches by

removing the water form an oil-in-water emulsion stabilized using the OSA-starches as surfactants. Operating conditions were selected in order to reduce oil losses due to its dissolution in supercritical CO_2 or due to emulsion destabilization. Results revealed that encapsulation efficiencies of lavandin oil were higher for PEG microcapsules obtained by PGSS (14-66%). The particles obtained showed spherical morphology and narrow particle size distribution, which is favourable for a controlled release of lavandin.

Supercritical impregnation of lavandin oil in OSA-starch was developed in **chapter IV**. The influence of operational pressure (10 - 12 MPa), temperature (313-323 K) and lavandin oil to starch ratio (0.2 - 1) in the essential oil impregnation load and efficiency, was studied. Impregnation time was kept constant and equal to 2 hours. Essential oil loads obtained (25 - 147 mg lavadin /g starch) depended on CO_2 density, decreasing with this parameter. Specifically impregnation load increased with temperature and decreases with pressure. The distribution coefficient of essential oil between the starch and the supercritical phase, as well decreased with the CO_2 density. In general, the solubility of the active compound in CO_2 is expected to increase when pressure is increased. Indeed, results show that higher impregnation loads were obtained in conditions that are in general unfavourable for the solubility of the active compound in CO_2, which demostrated that this parameter had no influence in this study. It can be concluded that impregnation is mainly influenced by other specific interactions like lavandin-scCO_2, which increased with pressure leading to lower impregnation loads or lavandin-polymer interactions which become weaker with CO_2 density, due to swelling effect of CO_2.

Antibacterial activity against three borne bacteria of lavandin essential oil and the formulations developed has been determined in **chapter V**. Minimum inhibition concentration of lavandin oil was 7.1 mg/mL for *E.coli* , 7.1 mg/mL for *S.aureus* and 3.6 mg/mL for *B.cereus*. Encapsulation of lavandin oil led to an enhancement in its antimicrobial activity, which mainly depended on the essential oil concentration and encapsulating agent. Soybean lecithin results to be the most efficient carrier material due to its capacity to spontaneously form liposomes. Particles produced by PGSS-drying showed more antibacterial activity than particles formed by spray-drying with a

similar essential oil load. *E.coli* (Gram-) resulted to be the most sensitive strain, while *B.cereus* and *S.aureus* (Gram+) were more resistant.

Objectives

The main aim of this thesis is the development of different lavandin essential oil formulations, in order to use them as biocide in agriculture and livestock.

With the general aim already presented the thesis was organized with the following partial objectives.

- Development of lavandin oil formulations using biopolymers (n-octenyl succinic anhydride (OSA)-modified starches and soybean lecithin), being the proposed formulations emulsions and microcapsules.
    - Characterization of emulsions and determination of the effect of the process variables (surfactant concentration, oil volume percentage and energy supplied to the emulsion) over their characteristics (stability, drop size and amount of starch adsorbed in the interface). Emulsions have to fulfill specifications of stability and drop size to be able to be processed with supercritical fluids.
    - Characterization of the microcapsules (particle size, particle size distribution, encapsulated and superficial essential oil morphology and humidity).
    - The main goal of the thesis is the application of lavandin oil and the obtained lavandin oil microcapsules as biocides. With this objective, their antimicrobial activity against food borne pathogenic bacteria has to be determinate.
- Development of several encapsulation processes to obtain lavandin oil microcapsules.
    - Spray drying of emulsions. Effect of the operating variables (air inlet temperature, emulsion flow, speed of the rotary spray) on the final product.
    - PGSS using biodegradable polymers (PEG, Policaprolactone) as encapsulating agent. Effect of the operating variables (pre-expansion

temperature and pressure, nozzle size, static mixer and gas to solution ratio) over the final product.

- PGSS drying of emulsions stabilized with OSA modified starches and soybean lecithin. Effect of the operating variables (pre-expansion temperature and pressure, nozzle size, static mixer and gas to solution ratio) over the final product.

- Supercritical CO_2 impregnation of lavandin oil in OSA modified starch. Effect of the operating variables (temperature, pressure and lavandin oil to starch ratio) over the final product.

Chapter I

Formulation of a natural biocide based on lavandin essential oil (*Lavandula hybrida*) by emulsification using modified starches

The formulation of lavandin essential oil as an emulsion for agrochemical applications has been studied. Four different biodegradable and non-toxic n-octenyl succinic anhydride (OSA)-modified starches have been tested as surfactants for this purpose. The results obtained with these surfactants have been compared with emulsions prepared with synthetic non-ionic surfactants (span20 and tween20). Furthermore the adsorption equilibrium of the OSA-modified starches on lavandin oil droplets has been studied because is of crucial importance to the formation and stabilization of emulsions. Results demonstrate that stable emulsions could be prepared with all types of OSA-modified starches tested in this work. Obtained oil droplet sizes were in the range 0.5 – 1.3 μm, whereas approximately 80% of the oil remained stabilized in the emulsion after 50 days of storage at 5ºC. The surface loads of the starch were very high, in some cases of up to 370 mg/m^2, which correspond to a very thick adsorbed multilayer. It has been shown that for this application OSA-modified starches present similar surfactant capabilities as synthetic non-ionic surfactants. The results of this study show that OSA-modified starches can be used as effective surfactant agents for the formulation of natural biocides.

1. Introduction

Nowadays, synthetic chemical pesticides and herbicides (e.g. carbamates and organophosphates) are extensively used to prevent crop loss. The use of these substances is associated with environmental pollution and presence of toxic residues in the products. An alternative is the use of reduced-risk pesticides and herbicides based on plant essential oils, which are also biodegradable [1]. Essential oils extracted from certain aromatic plants and their derivates have long been reputed to present antimicrobial properties and repellent activity against insects. Recent investigations confirm the biocide action of some essential oils. These essential oils show contact and fumigant insecticidal actions against a number of economically important insect and mite pests, as well as against plant pathogens and fungi [1].

Lavandin (*lavandula hybrida*) essential oil is readily available in many areas of the world and particularly in the region of Castilla-León (Valladolid), where the results of this investigation are intended to be applied. Although the biocide activity of lavandin essential oil has not been studied in detail yet, it can be inferred by an analysis of the activity of its individual components. A detailed composition analysis of the lavandin essential oil used in this work is presented in Table 1 [2]. Monoterpenes (α-terpineol, terpinen-4-ol, linalool and β-pinene) have lipophilic nature that can alter cell permeability and also change membrane properties and functions by increasing membrane fluidity and changing cell permeability [3]. The inhibitory effect of several terpenoids on microbial oxygen uptake and oxidative phosphorylation has also been demonstrated. Linalool, whose concentration in lavandin oil is around 30%, induces a reduction in cell size and abnormal germination and increases membrane permeability. 1-8 cineole (8% of lavandin oil composition) also inhibit citoquines production, which are proteins produced by lymphocytes and macrophages responsible of intercellular communication and anti-inflammatory mechanism [4].

Component	CAS No.	wt. %
Linalool	000078-70-6	33.2
Linalyl acetate	000115-95-7	29.7
Camphor	000464-49-3	7.1
1,8-Cineole	000470-82-6	7.6
terpinen-4-ol	000562-74-3	3.3
Lavandulyl acetate	025905-14-0	2.6
endo-Borneol	000507-70-0	2.7
β-Farnesene	028973-97-9	1.9
β-Caryophyllene	000087-44-5	1.4
α-Terpineol	000562-74-3	1.5
Limonene	000138-86-3	0.9
trans-β-Ocimene	003779-61-1	0.4
cis-β-Ocimene	003338-55-4	0.4
Germacrene-d	023986-74-5	0.7
β-Myrcene	000123-35-3	0.5
α-Bisabolol	072691-24-8	0.4

Table1. Chemical composition of the essential oil of Lavandin (*Lavandula hybrida)*

Essential oils typically are volatile and they rapidly evaporate from surfaces. It is thus desirable to formulate them in a way that allows protecting the oil from high temperature, oxidation and UV light, minimizes the evaporation, allows a selective release and increases the shelf life of the oil. For this purpose, the formulation of the essential oils in an oil-in-water (O/W) emulsion has been studied in this work. Such formulations can be produced without using toxic or contaminant products and therefore they can be suitable for agricultural and food industry applications. Moreover, by controlling the droplet size distribution of the oil, it is possible to enhance deposition and spreading of the oil over the crop, which may enhance biological activity. One of the main challenges in the development of these O/W emulsions is the control of their physical stability in order to achieve an adequate shelf life [5].

As far as the authors know, there are no previous studies in the open literature on the emulsification of lavandin oil. However, studies on the emulsification of other essentials oils are available. To mention only a few of the most representative examples, Orafidiya et al. [6] studied the antibacterial potential of *Ocimum gratissimum* essential oil designing liquid and semisolid formulations including emulsions with tween80. Orafidiya et al. [7] also emulsified eucalyptus, peppermint and lippia essential oils with

span80 and tween80. Baranauskiené et al. [8] emulsified and then encapsulated peppermint oil with modified starches. Three essential oils, oregano, red thyme and cassia were emulsified and encapsulated by phase separation into zein nanospheres by Parris et al. [9]. Beristain et al. [10] produced cardamom oil emulsions with mesquite gum with mean volume surface droplet size between 3-2.5 μm, in order to produce microcapsules by spray –drying. One innovative process was the microencapsulation of peppermint oil and orange peel oil in bakers' yeast cells developed by Bishop et al. [11].

The choice of the surfactant used to stabilize the emulsion is crucial since it must fulfil several requirements: besides being able to stabilize an emulsion with adequate physical properties, for agricultural and food industry applications the surfactant must be biodegradable and non toxic. For this reason, it has been decided to use biopolymers as surfactants, and in particular n-octenyl succinic anhydride (OSA)-modified starches. These starches have been approved for its use in food products by the US Food and Drug Administration (FDA) and by the European Union. The surfactant activity of these starches is obtained through chemical modification that introduces hydrophobic groups into the polymeric structure of the starch. Due to its high molecular weight and its branched polymeric structure, the modified starch that is adsorbed at the interface is able to stabilize the emulsion by steric hindrance, and it is possible to produce stable emulsions at low OSA-starch concentrations [12]. Moreover, when these starches are used as surfactants, the emulsification process can be coupled with a precipitation process performed by spray-drying, freeze-drying or supercritical precipitation technologies, in which the OSA-modified starch that was used as surfactant in the emulsification step can perform the function of carrier material, thus allowing to obtain a more functional product which can prevent oxidative deterioration and can provide controlled release characteristics.

In this work, four different OSA-modified starches have been tested in order to select the most suitable starch for the formulation of lavandin essential oil in O/W emulsions, considering the droplet size distribution as well as the stability of the emulsions against creaming obtained with each surfactant. Emulsions formed using synthetic surfactants (combinations of tween20 and span20) have also been produced and characterized as a

benchmark for comparison with the emulsions formed with OSA-modified starches. Furthermore, the adsorption equilibrium of the starches at the liquid-liquid interface has been studied at different operating conditions.

2. Materials and Methods

2.1 Materials

Lavandin oil used in this project was purchased to Silvestris & Szilas (Kerepes, Hungary), which is a certified manufacturer of fragrances, essential oils and hydrosols in Hungary. This lavandin oil was produced by steam distillation. The composition of this essential oil is presented in Table 1. Surfactant Span®20 was supplied by SIGMA (Madrid, Spain) and Tween®20 was supplied by PANREAC (Barcelona, Spain). The following n-octenyl succinic anhydre (OSA)-modified starches were used as emulsifier agents: OSA-starch derived from waxy maize (OSA1), OSA-starch derived from waxy maize blend with dried glucose syrup (OSA2), OSA-Dextrin derived from waxy maize (OSA3) and OSA- Dextrin derived from tapioca (OSA4), which were kindly provided by National Starch Group (New Jersey, USA).

2.2 Emulsion preparation

Water in oil emulsions were prepared by a two step process. A surfactant suspension was initially prepared by dispersing the surfactant in deionised water (Milli-Q, Millipore) at 50ºC with the aid of a Magnetic stirrer (IKA, Staufen, Germany)). If the surfactant is non-ionic (span20 or tween20) it is not necessary to heat the water because its solubility in water is high enough. Afterwards the oil in the specified ratio was gradually added to the suspension under continuous agitation for 5 min and a crude emulsion was obtained. The resulting coarsely-dispersed raw emulsion was then fed into the rotor-stator machine (IKA® LABOR PILOT 2000/4) whose capacity is 200 mL and processed during 2 or 4 min for fine emulsification. The rotor-stator machine was cooled by ethylene glycol that circulates through a jacket, which allows to remove the heat generated by the equipment and to operate at temperatures between 5ºC and 25ºC.

2.3 Determination of droplet size

Lavandin oil droplet size distribution of the emulsion was determined by Dynamic Light Scattering (Autosizer Lo-C (Manern instruments). Sampling was carried out after a gentle rotation of the emulsion container in order to obtain an ever dispersion of the droplets and further dilution with deionized water to less than 0.4% w/w to prevent multiple scattering effects. Particle size measurements are reported as volume distribution as defined as the average emulsion diameter (d_{32}). Each diameter value is the average of three freshly prepared emulsion samples measured ten times each. The following optical parameters were applied: lavandin oil refractive index: 1.460 and water refractive index: 1.331.

The method described before can be used only if drop diameter is less than 2 μm. For higher diameter the mean drop diameter had been determined using a Microscope Leica DM4000B (Wetzlar, Germany).

2.4 Determination of the surface load of OSA-modified starch

The amount of OSA-starch adsorbed at the emulsion oil droplet surface (Γ, $mg \cdot m^{-2}$) was inferred from measurements of the concentration of starch remaining in the serum phase after centrifugation. Thus, following equilibration for 24 hours, the emulsion was centrifuged at 50 Hz for 60 min using a KUBOTA 5100 centrifuge and then separated continuous phase was recovered. It was observed that the emulsions were very resistant to coalescence during centrifugation. The amount of modified starch in the continuous phase was determinate by measure of the optical rotation in a Digital Automatic Saccharimeter (Sucromat®). In order to minimize the calibration error, lines for each modified starch were developed. The absorbed amount of modified starch was calculated from the different between the initial amount of starch in the continuous phase and the amount in the subnatant after separation of the emulsion.

$$c_{adsorbed} = c_{initial} - c_{sub} \quad (1)$$

The most representative measure of the adsorption is the adsorbed amount or surface load (Γ), This parameter was obtained by relating the adsorbed amount to the specific surface area of the emulsion.

$$\Gamma = \frac{c_{adsorbed} d_{32}}{6\varphi} = \left[\frac{mg}{m^2}\right] \quad (2)$$

Where d_{32} is the area-weighted droplet diameter and φ is the dispersed phase (oil phase) volume fraction.

2.5 Determination of the stability of emulsions

To evaluate the kinetics of destabilization a portion of 10 mL of emulsion was transferred to a polyestyrene screw cap tube (internal diameter: 15 mm, height: 100mm), stored at 5ºC and periodically visually examined. The height of visible supernatant oil layer, H_t, was recorded with storage time over a period of several months. The volume of supernatant oil, V_t, was calculated from the height of supernatant oil and oil density. The phase separation was expressed as in Eq. (3), where V_o is the total volume of lavandin oil in the emulsion.

$$\%V = \frac{V_t}{V_o} 100 \quad (3)$$

2.6 Determination of surface tension

To determinate critical micelle concentration of the surfactants tested in this project is crucial to know the surface tension. The equipment employed to determine the surface tension is a tensiometer EasyDyne from Krüss (Hamburg, Germany) and the method that had been used is Wilhelmy plate.

3. Results and Discussion

3.1 Polymeric surfactants

Four different OSA-modified starches have been tested in order to determine the one which gives the most stable emulsion and therefore is the most suitable for the application.

As a first step, the critical micelle concentration (cmc), which is the minimum concentration of surfactant above which micelles are spontaneously formed, has been determined. This has been done by determination of the surface tension of solutions with different concentrations of starch. Below the cmc, the surface tension strongly decreases with the concentration of surfactant, while above the cmc the surface tension stays more constant. Therefore the cmc can be determined as the concentration at which the surface tension reaches a nearly constant lowest value. Figure 1 presents the surface tensions obtained with different surfactants as a function of its concentration. The critical micelle concentrations obtained from this Figure are reported in Table 2. It can be seen that the critical micelle concentrations obtained with all the OSA-modified starches are of about 5 g/L. On the other hand, lower surface tensions were obtained with OSA4 and OSA3, while OSA2 and OSA1 gave higher surface tensions, as it is presented in figure 1.

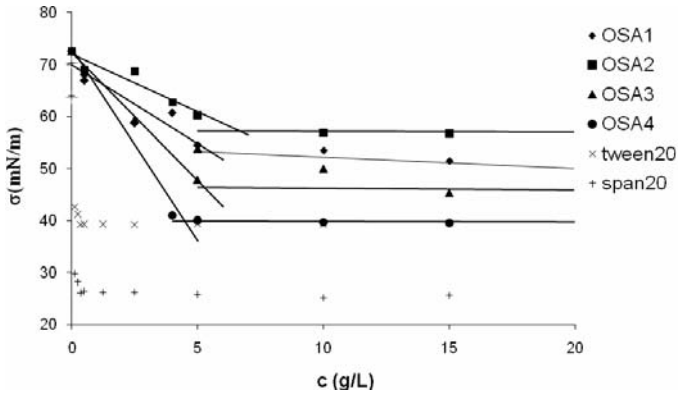


Figure 1. Surface tension vs. surfactant concentration for OSA-modified starches, tween20 and span20.

	CMC (g/L)	S (error)
OSA1	5,3	± 0,4
OSA2	6,6	± 0,6
OSA3	5,2	± 0,4
OSA4	4,5	± 0,3
Tween20	0,26	± 0,06
Span20	0,36	± 0,02

Table 2. Critical micelle concentrations of OSA-modified starches, tween20 and span20.

After the determination of the cmc, the influence of the main process variables (operation time, homogenization velocity and surfactant concentration) on the physical properties and stability of the emulsion (drop diameter and creaming velocity) has been studied. The process variables considered are the surfactant concentrations (5 g/L, 25 g/L, 53 g/L and 79 g/L, all above the cmc), starch/oil ratio (1/8, 1/3, 1/1 and 3/1 respectively), homogenisation velocity of the rotor-stator machine (50 Hz, 60 Hz and 70 Hz) and operation time (2 min and 4 min). The results obtained are reported in Table 3 and Table 4.

Table 3 presents the variation of the mean droplet diameter of the emulsion with the process variables. Obtained emulsions had droplet diameters in the range 500 – 1300 nm. It can be seen that there is a certain variation of the mean droplet size with the time of operation, and in most cases smaller droplet diameters are obtained when the operation time is increased. On the other hand, a considerable reduction of the droplet size is obtained when the surfactant concentration is increased or when the velocity of the rotor-stator machine is increased. Of these parameters, the homogenisation velocity has the greatest impact on droplet size. In general, the influence of this parameter, which is related to the energy input provided for the formation of the emulsion, depends on the coalescence rate. If surfactant is adsorbed slowly at the newly formed interface, the coalescence rate increases with the energy input, because the collision rate increased due to an increase in the droplets concentration [13]. In the case of emulsion stabilized with OSA-modified starches, an increase of the energy input has a bigger impact on the reduction of droplet size than on the increase of coalescence, and the mean diameter decreases with increasing energy input. This can be explained because adsorption of surfactant in the interface is relative fast (lower than 1min). This has been determined by measuring variation with time of the interfacial tension between lavandin oil and the dissolution of OSA-starches in water. It is noticeable that similar droplet diameters are obtained when the emulsions are prepared with different surfactants but with the same process conditions.

Surfactant	v (Hz)	t (min)	d_{32} (nm)			
			c = 5 g/L	c = 25 g/L	c = 53 g/L	c = 79 g/L
OSA1	70	2	1020 ± 46	916 ± 20	730 ± 10	503 ± 4
		4	1020 ± 10	806 ± 2	770 ± 6	580 ± 30
	60	2	1220 ± 30	1044 ± 37	970 ± 13	860 ± 4
		4	1050 ± 7	1063 ± 61	930 ± 16	815 ± 30
	50	2	1440 ± 5	1382 ± 36	1400 ± 60	850 ± 35
		4	1260 ± 51	1210 ± 14	1370 ± 14	780 ± 35
OSA2	70	2	900 ± 40	790 ± 40	673 ± 16	590 ± 1
		4	950 ± 40	772 ± 14	648 ± 14	480 ± 5
	60	2	1136 ± 50	1010 ± 24	960 ± 14	650 ± 2
		4	1120 ± 50	960 ± 24	717 ± 4	590 ± 10
	50	2	1290 ± 40	1410 ± 20	1030 ± 33	790 ± 30
		4	1260 ± 20	1165 ± 14	930 ± 51	660 ± 30
OSA3	70	2	850 ± 15	813 ± 50	660 ± 30	600 ± 14
		4	860 ± 65	732 ± 35	620 ± 12	480 ± 16
	60	2	980 ± 60	950 ± 95	1000 ± 35	660 ± 30
		4	950 ± 50	860 ± 20	802 ± 50	670 ± 20
	50	2	1390 ± 5	1340 ± 42	1004 ± 10	790 ± 30
		4	1200 ± 70	1140 ± 44.3	940 ± 30	810 ± 40
OSA4	70	2	850 ± 20	792.6 ± 40	800 ± 14	620 ± 14
		4	790 ± 19	777 ± 37	720 ± 12	540 ± 20
	60	2	1005 ± 22	1026 ± 43	852 ± 26	730 ± 30
		4	904 ± 20	942 ± 74	773 ± 40	630 ± 20
	50	2	1320 ± 18	1296 ± 55	1030 ± 17	880 ± 32
		4	1160 ± 20	1205 ± 28	1005 ± 12	820 ± 40

Table 3. Initial diameter for different OSA-modified starches at different experimental conditions.

Table 4 presents the variation of the height of the separated supernatant oil with process variables after storage at 5ºC for 15 days and for 50 days. It can be seen that the fraction of supernatant oil after 50 days varies between 12% and 48%. With respect to the trends of variation of the fraction of supernatant oil with process variables, it can be seen that they are the same observed for the initial diameter in Table 3. Therefore the conditions that allow to produce an emulsion with a smaller droplet size also allow to produce a more stable emulsion against creaming.

			% supernatant oil after 15 days				% supernatant oil after 50 days			
	V (Hz)	t (min)	5 g/L	25 g/L	53 g/L	79 g/L	5 g/L	25 g/L	53 g/L	79 g/L
	70	2	29.30	21.11	10.30	7.08	31.2	29.9	24.2	18.45
		4	26.45	25.20	10.55	9.83	30.3	29.1	23.6	18.90
OSA1	60	2	30.54	24.50	15.00	13.47	33.4	31.2	22.5	20.45
		4	29.50	28.90	12.70	11.18	32.2	30	24.1	21.04
	50	2	32.18	31.40	15.35	14.45	35.7	34.6	25.5	21.9
		4	31.56	30.66	14.90	12.72	33.1	30.7	24.4	22.54
	70	2	29.07	21.58	24.26	13.03	36.3	35.1	24.8	24.84
		4	34.53	33.41	22.17	11.53	35.8	34.7	23.9	23.91
OSA2	60	2	30.45	26.12	25.58	12.31	38.9	37.9	35.6	20.72
		4	34.04	26.00	29.21	15.55	36.7	27.1	34.4	23.66
	50	2	40.06	39.90	11.26	6.71	47.9	46.8	44	12.63
		4	35.65	16.10	10.49	12.16	46.1	46.2	41.6	17.55
	70	2	29.97	23.60	19.26	14.32	37.2	35	26.2	21.34
		4	32.67	31.49	22.73	13.58	35.6	33.5	23.2	20.45
OSA3	60	2	30.20	23.47	17.74	14.45	38.4	34.7	27.8	22.76
		4	29.32	28.07	21.91	6.28	36.4	32.9	21.9	22.13
	50	2	36.78	34.74	20.98	12.59	39.6	35.2	29.1	23.55
		4	34.56	18.82	16.62	11.45	38.3	29.9	25.7	21.95
	70	2	27.25	23.68	22.33	13.46	35.9	35	24.2	21.53
		4	24.21	23.14	19.39	14.19	34.5	33.3	20.4	20.03
OSA4	60	2	28.45	14.83	17.79	12.16	37	35.2	23.8	21.98
		4	27.65	25.62	22.23	13.86	35.7	33.7	23.3	23.54
	50	2	30.45	22.41	10.64	5.92	37.9	29	28.8	22.33
		4	28.56	20.79	11.31	7.84	36.2	25.1	24.1	23.68

Table 4. Percentage of desemulsionated oil for different OSA-starches at different experimental conditions.

Furthermore the effect of the percentage of disperse phase (lavandin oil) on emulsion stability has been studied using the OSA-starch OSA1 as surfactant. The results are reported in Table 5. It can be seen that the mean droplet diameter and the amount of supernatant oil after 50 days of storage increases as the disperse phase volume increases. The reason for this is that when the disperse phase volume is increased at a constant surfactant concentration, the relationship surfactant-disperse phase decreases causing an increase in the mean droplet size and a reduction of emulsion stability. Moreover, an increase in the number of oil droplets results in an increase in coalescence and therefore a faster destabilization of the emulsion [12]. Apart from that, the effect of

other process parameters is the same as in the results presented in Tables 3 and 4: smaller droplets are produced when the operation time is increased or when the surfactant concentration is increased.

OSA 1 (%wt)	Oil (% wt)	Experimental conditions	d_{32} (nm)	%supernatant oil after 50 days
5	45		922	27.0
5	5	70Hz 4min	774	23.6
2,5	47,5		834	32.9
2,5	7,5		806	29.1
5	45		1021	33.9
5	5	70Hz 2min	732	24.2
2,5	47,5		1432	37.3
2,5	7,5		917	29.9

Table 5. Influence of disperse phase volume in drop diameter.

Finally the results of the surface load of the OSA-modified starches are presented in Figure 2 as surface adsorption isotherms, which give the relationship between total bulk concentration of surfactant and surface load adsorption. It can be seen that very high loads up to 370 mg/m^2 are obtained. High loads were also observed by Nilsson and Bergenstahl [14] during the preparation of oil emulsions with OSA-modified starches. Furthermore it can be seen that the adsorption isothems obtained for all OSA-modified starches appear not to reach a maximum plateau level within the range of bulk concentrations of surfactant investigated. These results suggest that the adsorption of the different OSA-starch is a non-equilibrium process and probably the surface load may depend on polymer availability versus surface area, as were suggested by Walstra [14]. The generation of high surface loads can be due several reasons as the formation of multilayer at the interface and configurational changes or reorientation of polymers at interface, as was study by Nilsson et al [15]. The formation of multilayer at interface in case of hydrophobically modified polymers, could be due to the hydrophobic interaction between nonadsorbed substituents on the polymer backbone leading to some form of aggregation at surface. In case of OSA-starch, hydrophobic interactions could also give

rise to the formation of inclusion complexes between adsorbed and nonadsorbed OSA-starch. A possibility is that adsorption rate controls the adsorbed amount and a thick adsorbed multilayer of surfactant is formed in this system if polymers adsorb rapidly due to the lack of time for rearrangement.

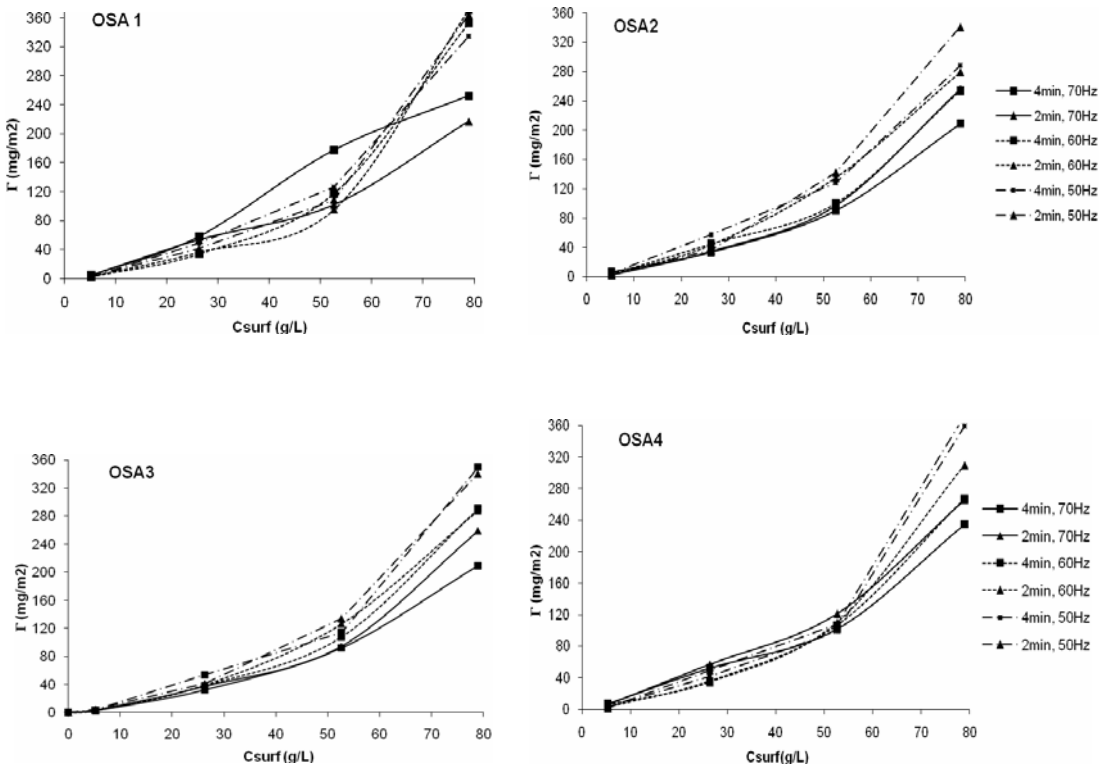


Figure 2. Adsorption isotherms of each OSA-modified starch.

In the present study it could be interesting relate the surface load versus the dynamic surface load, that is the amount of OSA-starch available per specific emulsion surface area, is shown in Figure 3. From this figure is clear that the surface load increase linearly with dynamic surface load, thus more surface is created rather than high surface load.

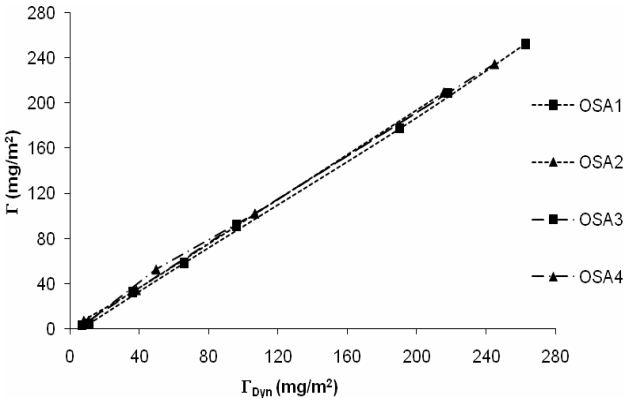


Figure 3. Emulsion surface load (Γ) in emulsion with different levels of dynamic surface load (Γ_{dyn}). Experimental conditions: 70Hz and 4min.

3.2 Non-ionic surfactants

Emulsions stabilized with two non-ionic surfactants, span20 and tween20, have been produced as a benchmark for evaluating the performance of the OSA-modified starches. The selection of different non-ionic surfactants for the preparation of the emulsion was made on an empirical basis. A semi-empirical scale for selecting surfactants is the hydrophilic – lipophilic balance, or HLB number, developed by Griffin [16]. The required HLB for essential oils is around 13 as was investigated by Orafidiya et al. [7]. Thus the emulsion was prepared using a combination of two surfactants, span20 whose HLB is 8.6 and tween20 whose HLB is 16.7. The proportion between these two surfactants was changed in order to obtain HLB values between 12 and 15, calculating the HLB of the mixture according to Eq. (4).

$$HLB = x_A HLB_A + x_B HLB_B \quad (4)$$

The mean droplet diameters obtained with surfactant mixtures with different HLB are presented in Figure 4, and the fractions of supernatant oil after 15 – 50 days are presented in Figure 5. Both curves pass through a minimum in the HLB range 13.1 – 13.8. The corresponding minimum diameter varies between 620 nm and 810 nm depending on the total surfactant concentration.

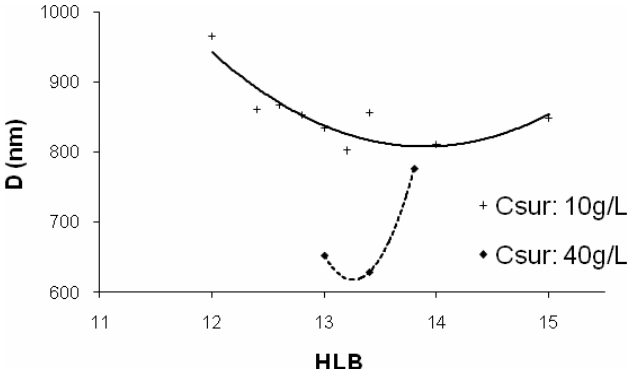


Figure 4. Droplet diameter as a function of HLB of mixtures of span20 and tween20.

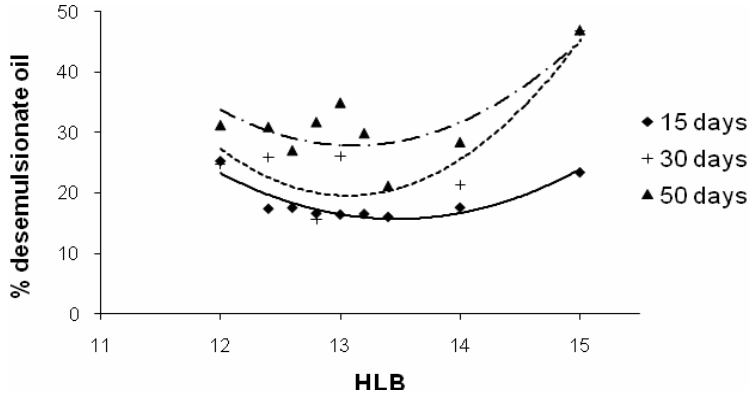


Figure 5. Fraction of supernatant oil after 15 – 50 days as a function of HLB of mixtures of span20 and tween20.

Furthermore it has been studied the effect of the fraction of disperse phase (lavandin oil) on emulsion stability using tween20 and span20 as emulsifiers. The proportion between both non-ionic surfactants has been selected in order to have HLB = 13.4, which is near the optimum. As it can be seen in the results reported in Table 6, the mean diameter and the supernatant oil fraction increases as the disperse phase volume increases, so the behaviour is the same as that of OSA-modified starch emulsions. Also in this case the droplet diameter decreases when the rotor-stator homogenization speed and operation time is increased or when the surfactant concentration is increased.

	Oil (%wt)	Surfactant (%wt)	d_{32} (nm)	%supernatant oil after 50
70 Hz, 4min	50	1	1495	29.6
	50	2	1540	27.2
	45	5	608	19.3
70 Hz 2min	50	1	1701	30.2
	50	2	1414	28.0
	45	5	687	20.3
70 Hz, 4min	21	1	856	26.3

Table 6. Influence of disperse phase volume in droplet diameter of emulsions prepared with span20 + tween20 mixtures with HLB = 13.4.

3.3 Comparative of results obtained with OSA-modified starches and with commonly used non-ionic surfactants

As previously discussed, there were no big differences in the properties of the emulsions prepared with the four OSA-modified starches considered in this work. The smallest droplet diameter was observed in an emulsion prepared with OSA3 and the lowest fraction of supernatant oil after 50 days was observed in an emulsion prepared with OSA4. Table 7 presents a comparative of the properties of the emulsions produced with these starches and with a mixture of span20 and tween20 with HLB = 13.4. It can be seen that the properties of the emulsions prepared with OSA-modified starches are very similar to the properties obtained with non-ionic surfactants.

Surfactant	OSA3	OSA4	Span20 + tween20
Experimental conditions	70 Hz, 4min, 5%surf	50 Hz, 2min, 5%surf	70 Hz, 4min, 4%surf
$d_{32,min}$ (nm)	623.6 ± 12.4	720.0 ± 12.4	622
%supernatant oil (50 days)	23.2	20.4	21

Table 7. Comparison of properties of emulsions obtained with OSA-modified starches and non-ionic surfactants.

Microscopic observations of the emulsions showed the presence of spherical drops, as it can be seen in Figure 6. The size distribution of the droplets of an emulsion produced with an OSA-modified starch and with the non-ionic surfactants is presented in Figure

6. It can be seen that the emulsion obtained with the non-ionic surfactants present a narrower droplet size distribution with only two peaks, compared with the three peaks obtained with the OSA-modified starch. This observation can explain the slightly higher stability of the emulsions prepared with non-ionic surfactants.

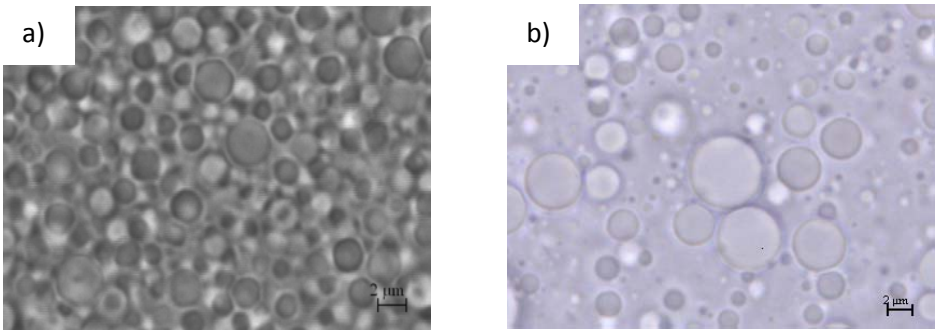


Figure 6. Microscopic pictures of emulsion drops obtained using a) tween20+span20 and b) OSA1. Scale bar 2µm.

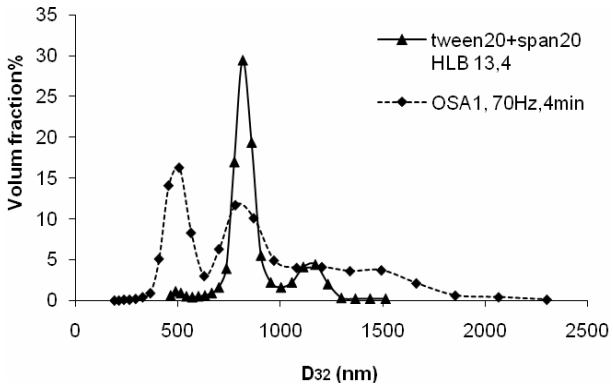


Figure 7: Drop diameter distribution of emulsions produced with HI-CAP100 or with non-ionic surfactants.

4. Conclusions

Due to the potential applications of emulsions of essential oils in agrochemical formulations, a detailed study has been performed in order to obtain a stable emulsion of lavandin essential oil using biopolymers (OSA-modified starches) as surfactants. The results obtained with these surfactants have been compared to results obtained with commonly used commercial non-ionic surfactants (span20 and tween20).

It has been found that stable emulsions could be prepared with four types of OSA-modified starches. Similar results were obtained with all these four surfactants. The surfactant concentration, oil volume, and homogenisation velocity represent important parameters that affect the properties of the emulsion and particularly the droplet size and the stability: emulsions with smaller droplet size and a better stability were produced when the surfactant concentration and the energy input was increased, and an increase in the fraction of oil reduced the stability of the emulsion. With the best process conditions, it was possible to produce emulsions with a mean droplet size of about 700 nm and in which only 20% of the oil was demulsified after 50 days of storage. These results were compared with the properties of an emulsion prepared with a mixture of two commercial non-ionic surfactants, span20 and tween20, with an optimum hydrophilic-lipophilic balance (HLB). The results obtained with the OSA-modified starches were very similar to the results achieved with the non-ionic surfactants.

Adsorption isotherms of OSA-modified starches on the droplets of lavandin oil have been determined. The results obtained suggest that the adsorption rate controls the adsorbed amounts and a very thick multilayer of surfactant have been formed, reaching values of up to 370 mg/m^2. Adsorption is likely to be governed by the amount of surface area created during emulsification and amount of OSA-starch available.

5. Acknowledgements

The authors thank National Starch Group for supplying the OSA-modified starches and giving permission to publish the results obtained with them. This project has been financed by Diputación de Valladolid.

6. Symbols

C : Concentration [$g.L^{-1}$]

d : diameter [nm]

d_{32} : Area-weighted droplet diameter [nm]

φ : dispersed phase volume fraction.

V_t : Volume of supernatant oil [mL]

V_o : Initial emulsion volume [mL]

σ : Surface tension [$mN.s^{-1}$]

Γ : surface load [$mg.m-^{2}$]

7. References

[1] M. B. Isman. Plant essential oils for pest and disease management. Crop Protection. 19 (2000) 603-608.

[2] M. Cerpa, M. J. Cocero. Hydroextraction of essential oil from Lavandin Super, in: Proceedings of the 36th International Symposium on Essential Oils, Budapest, Hungary, 2005.

[3] F. F. Boyom, V. Ngouana, A. P. H. Zollo, C. Menut, J. M. Bessiere, J. Gut, P. Rosenthal. Composition and anti-plasmodial activities of essential oils from some Cameroonian medicinal plants. Phytochemistry. 64 (2003)1269-1275.

[4] S. G. Griffin, G. Wyllie, J. L. Markham, D. N. Leach. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour and Fragrance Journal. 14 (1999) 322-332.

[5] T. F. Tadros, Applied Surfactants, Ed. Wiley-VCH (2005)

[6] L. O. Orafidiya, A. O. Oyedele, A. O. Shittu, A. A. Elujoba. The formulation of an effective topical product containing *Ocimum gratissimum* leaf essential oil. Int. J. Pharm. 224 (2001) 117-183.

[7] L. O. Orafidiya, F. A. Oladimeji. Determination of the required HLB values of some essential oils. J. Pharmaceutics. 237 (2002) 241-249.

[8] R. Baranauskiené, E. Bylaitè, J. Zukauskaite, R. Venskutonis. Flavor Retention of Peppermint (Mentha piperita L.) Essential Oil Spray-Dried in Modified Straches during Encapsultaion and Storage. J. Agric. Food Chem, 55 (2007) 3027-3036.

[9] N. Parris, P. H. Cooke, K. B. Hicks. Encapsulation of Essential oils in Zein Nanospherical Particles. J. Agric. Food Chem, 53 (2005) 4788-4792.

[10] C. I. Beristain, H. S. Garcm, E. J. Vernon-Carter. Spray-dried Encapsulation of Cardamom (Elettaria cardamomum) Essential Oil with Mesquite (Prosopis juli-ora) Gum. Lebensm.-Wiss. u.-Technol. 34 (2001) 398-401.

[11] J. R. Bishop, G. Nelson, J. Lamb. Microencapsulation in yeast cells. J. Microencapsulation. 15, 6 (1998) 761-773.

[12] S. Tsech, Ch. Gerhards, H. Schubert. Stabilization of emulsions by OSA starches. J. Food Eng. 54 (2002) 167-174.

[13] M. Stang, H. Karbstein, H. Schubert. Adsorption kinetics of emulsifiers at oil-water interfaces and their effect on mechanical emulsification. Chem. Eng. Process. Process. 33 (1994) 307-311.

[14] P. Walstra. In Encyclopedia of emulsion technology volume 1: Basic Theory; P. Becher, Ed.; Marcel Dekker Inc.: New York, (1983) 57-127.

[15] L. Nilsson, B. Bersgenstahl. Adsorption of hydrophobically modified anionic starch at oppositely charged oil/water interfaces. J. Colloid Interface Sci. 308 (2007) 508-513.

[16] W. C. Griffin. Classification of surface active agents by HLB. J. Soc. Cosmet. Chem. 1 (1949) 311-326.

Chapter II

Formulation of lavandin essential oil (*Lavandula hybrida*) in liposomes by thin-film hydration method and by PGSS

Lavandin (Lavandula Hybrida) essential oil contains components with biocide and antiviral properties that can be used as substitutes of synthetic drugs in livestock. This application requires an appropriate formulation of the essential oil. Liposomes are promising drug carriers because they can enhance the skin penetration of drugs, deliver the entrapped drugs across cell membranes and improve essential oil stability and bioavailability. In this work, the incorporation of lavandin essential oil in liposomes produced with commercially available lecithin and cholesterol has been studied. Two different liposome production methods have been tested: a modification of the Bangham thin-film method, and the Particles from Gas Saturated Solutions (PGSS) – drying process. Liposomes obtained with the thin-film method were multivesicular or unilamellar/multilamellar with a mean diameter between 0.4 – 1.3 μm. These liposomes were stable during at least one month and the encapsulation efficiency of essential oil of up to 66%. On the other hand, by application of the PGSS process, dry and fine but aggregated soy lecithin particles were produced, which size ranged between 1.4 – 24.8 μm. The encapsulation efficiency of essential oil was poor ranging between 3- 14.5%. These particles could be dispersed in water, producing liposomes whose size range between 0.5 - 1.5 μm.

1. Introduction

In recent years there has been a growing social demand for environmentally-friendly technologies and for natural products, particularly in sensitive fields such as food applications. These demands are reflected in changes in legislation, which has become more restrictive with respect to the application of synthetic chemicals in agriculture, livestock farming and food. Therefore, there is a considerable interest in the development of new livestock health and growth promoters as substitutes of the conventional synthetic chemicals.

Nowadays, public concern about the use of antibiotics in livestock feed has increased due to their possible contribution to emergence of antibiotic resistant bacteria and their transmission from livestock to humans. In the European Union, these concerns drove to the prohibition in year 1999 of most antibiotics as additives in livestock feed, and a total ban of the remaining synthetic health and growth promoters since 2006. Similar initiatives have been taken in other countries, as for example the Preservation of Antibiotics for Medical Treatment Act in the United States. Livestock farming is thus under social, political and economical pressure towards the development of alternative additives. The solution to this problem can be found in nature itself, as some plants contain active components with demonstrated biocide, antiseptic, insecticide, acaricidal and nematicidal activity, and they can be a source of new effective additives for the livestock farming industry. Some of these plants have been sources of active components for the pharmaceutical industry for decades.

Aromatic plants such as lavender, rosemary or thyme are natural sources of different compounds, including essential oils and antioxidants. Besides their well-known applications as cosmetics and food additives, some components of essential oils present antimicrobial activity [1,2,3]. Due to this, it has been proven that essential oils and their active components can improve the health of non-ruminant species [4, 5]. Essential oils can also improve the efficiency of use of energy and proteins by rumen, optimizing the production of livestock and acting as growth promoters [6]. However, dosing of these compounds is still problematic and excessive doses can cause negative effects. Moreover, essential oils are very volatile, and they are sensitive compounds that can be

degraded easily by the action of heat, oxygen and light. Therefore, a suitable formulation for the dosing and protection of the essential oils must be developed.

Liposomes are formed spontaneously by self-assembly of phospholipids in aqueous solutions, producing vesicles consisting of an aqueous medium surrounded by a lipid membrane. These structures can retain water-soluble substances in the inner aqueous phase and oil-soluble substances in the lipid bilayer membrane. Essential oils are hydrophobic and therefore they are accumulated in the lipid bilayer. Liposomes can enhance the delivery of essential oils to cells because the lipid bilayer of liposomes can protect the encapsulated substances from gastrointestinal digestion [7]. Intact liposomes can bind to intestinal mucosa [8] and deliver biocide and antiviral agents through the cytoplasmic barrier of cells [9].

In recent years liposomes have been extensively studied as carrier systems but only a few works dealing with essential oils have been published. For example, *Aremisia arborescens* essential oil was incorporated in soya phosphatidyl choline and hydrogenated phosphatidyl choline producing multilamellar liposomes and greatly improving its antiviral activity [10]. *Santolina insularis* essential oil was incorporated in hydrogenated soya phosphatydil choline and cholesterol liposomes enhancing its antiviral efficiency [11]. Carvacrol and thymol isolated from *Origanum dictamnus* essential oil were successfully encapsulated in phosphatidyl choline-based liposomes [12].

The method used to produce liposomes should achieve a high entrapment efficiency, narrow size distribution, long-term stability and protective properties. In this work, the suitability of three different techniques for liposome generation, based on the Bangham method, has been studied. The Bangham method or thin-film hydration method is one of the most widely used and simplest techniques for the formulation of liposomes [13], and because of this it has been used in this work as a reference method. However this method has limited applications in commercial scale due to a reduced production capacity, presence of organic solvent traces in the final product and homogenisation limitations. As an alternative method, dry liposomes were also produced by Particles from Gas Saturated Solutions (PGSS) – drying of lavandin oil emulsions using lecithin as surfactant. The PGSS – drying process is a novel technique based on supercritical

fluids which has the advantages of producing particles of a controlled size operating at mild temperatures (typically 40 – 60ºC) in an inert environment and using only carbon dioxide and water as solvents [14,15]. These features can contribute to avoid essential oil losses due to evaporation, as well as contamination and degradation of the final product. Several methods for producing liposomes with supercritical fluids were described by Meure et al. [9]. Some authors have also used supercritical fluid precipitation methods to produce pure lecithin particles, without incorporation of essential oils. According to previous literature, the RESS (Rapid Expansion of Supercritical Solutions) process was not effective in micronizing soy lecithin [16], but the SAS (Supecritical Anti Solvent) method yielded microspheres of lecithin in a wide range of operating conditions whose diameters ranged between 1 - 40 μm [17,18]. Precipitation of a puerarin complex rich in phospholipids was achieved by GAS (Gas Anti Solvent) and SEDS (Supercritical Enhanced Dispersion of Solutions) processes, obtaining aggregated particles with a diameter around 5.5 μm [18]. In previous works of the authors, the formulation of emulsions of essential oil with modified starches was studied [19], and the PGSS-drying process was used to encapsulate essential oils in biopolymers such as Poly Ethylene Glycol and starches, with the aim of obtaining a powder that could be used to reconstitute an emulsion by hydration for agricultural applications [20]. As far as the authors know, the application of the PGSS-drying method to produce liposomes has not been studied yet.

The objective of this research is to formulate lavandin essential oil (approximate composition: 45 %wt. linalool and 25 %wt. linalyl acetate) in soybean lecithin-based liposomes by conventional method (Bangham method) and PGSS drying, for applications as antimicrobial and antiviral agent in livestock. Soybean lecithin (complex mixture composed of phospholipids like phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol [21]) has been used as carrier material, because is product generally accepted by consumers and legislators as natural and safe.

2. Materials and Methods

2.1 Materials

Lavandin essential oil "super" used in this project was purchased from COCOPE (Valladolid, Spain). This oil was produced by steam distillation. Soybean lecithin (97% phospholipids) was obtained from Glama-Sot (SOTYA, Madrid, Spain). CO_2 was provided by Carburos Metálicos (Barcelona, Spain). Cholesterol (grade ≥ 99%) was purchased from Sigma-Aldrich (Spain). Chloroform (PA Grade) was supplied by Panreac (Barcelona, Spain). Trans-2-Hexen-1-al 98% was provided by Sigma-Aldrich (Madrid, Spain).

2.2 Preparation of liposomes by thin-film method

Liposomes were prepared with the thin-film hydration method. Lavandin essential oil, soybean lecithin and cholesterol were dissolved together with 10 mL of chloroform in a 50 mL round-bottom flask. Liposomes were obtained using lavandin oil (0.1-0.4 mg), soybean lecithin (0.1-0.4 mg), cholesterol (0.013-0.052 mg) and hydration water (1-2 mL). Afterwards, the organic solvent was evaporated with a rotary evaporator working at 40ºC until a thin film was formed on the walls of the flask. The lipid film was suspended in 1-2 mL of water. Liposomes were obtained by three different procedures and each experiment was performed three fold in order to check the reproducibility. In the first method, the lipid film was heated at 60ºC (above the lipid transition temperature, because below this temperature lipids are in gel state and usually they can not form continuous closed bilayered structures [22]) for 20 min. and then sonicated in an ultrasound bath (J.P Selecta, 50Hz, 100W, Barcelona, Spain) during 30 min. to reduce size and homogenize the liposomes. With the second method, the lipid film suspended in water was shaken in a vortex mixer (Vortex V-3, 50-3400 rpm, Spain) at 1700 rpm for 15 min and this suspension was allowed to hydrate for 2 h in the dark at room temperature, in order to avoid any structural defects. In a third method, the lipid film was heated at 60ºC (above lipid transition temperature) during 20 min and then shaken in a vortex mixer at 1700 rpm during15 min.

2.3 Preparation of essential oil-in-water emulsions

Essential oil in water emulsions were prepared by a two step process. A suspension of lecithin (38 g/L, 62 g/L ,88 g/L) in deionized water (Milli-Q, Millipore) was prepared at 50ºC with the aid of a magnetic stirrer (IKA). Afterwards 20g lavandin oil was gradually added to 200 mL of these the suspension under continuous agitation with the magnetic stirrer and a crude emulsion was obtained. The resulting coarsely-dispersed raw emulsion was then fed into a rotor-stator machine (IKA® LABOR PILOT 2000/4, Staufen, Germany) whose capacity is 200 mL and processed during 4 min. for fine emulsification.

2.4 Determination of the stability of emulsions in supercritical CO_2

The stability of lavandin oil emulsions in a CO_2 environment at different pressure (0.1 -12 MPa) and temperature (20 – 120ºC) conditions was studied using a high-pressure view cell with an inner volume of 25mL. The stability of the emulsions was check by visual observation, considering that an emulsion was not stable when two phases appeared or when creaming occurred. A schematic diagram of the equipment is presented in Figure 1. This cell is equipped with two sapphire windows and it can withstand pressures of up to 50 MPa. A heating jacked allows operating at constant temperature, which is measured by a thermocouple fitted inside of the cell. There is also a magnetic stirrer which allows an optimal mixing of the phases.

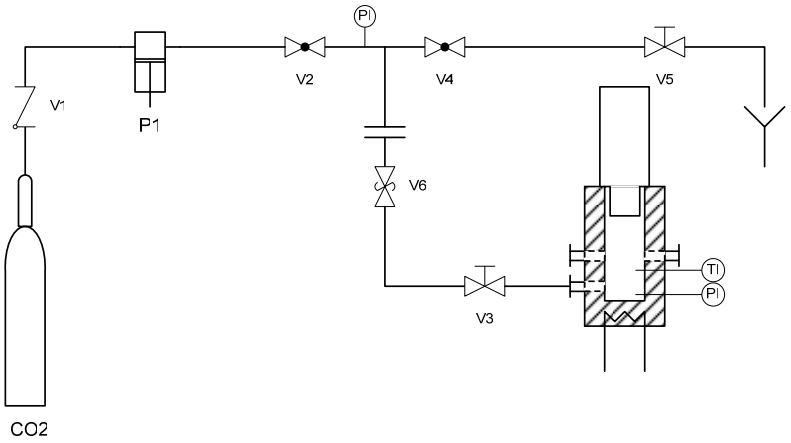


Figure 1. Schematic diagram of the high-pressure view cell.

2.5 Preparation of liposomes by PGSS-drying of emulsions

Once a stable oil-in-water emulsion had been obtained, PGSS drying was applied to remove water and to produce dry liposomes. Figure 2 presents a flow diagram of the installation used for PGSS-drying in this work, which allows operating with a maximum CO_2 flow rate of 15 kg/h.

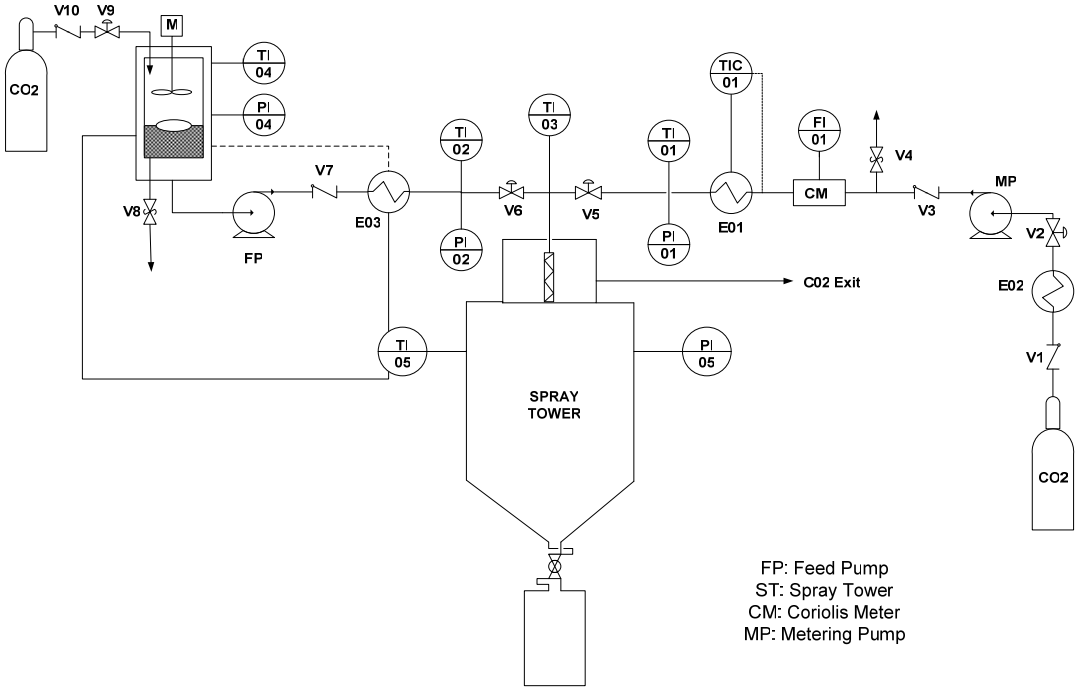


Figure 2. Schematic diagram of the PGSS-drying system used in this work.

In this processes the emulsion is first saturated with CO_2 in a vessel equipped with a mechanical stirrer, operating at 10 MPa and 30ºC. This allows for a decrease of the emulsion viscosity and facilitates the pumping of this viscous fluid. Then the emulsion saturated with CO_2 is pumped and put into contact with supercritical CO_2 in a static mixer operating at high pressure and temperature (60 bar < P < 100 bar and 100ºC < T < 130ºC). The residence time in the static mixer is of the order of a few seconds, and its purpose is to achieve an intimate mixing of the emulsion and the supercritical fluid.

Immediately afterwards, this biphasic mixture is depressurized down to atmospheric pressure using a capillary nozzle. This causes a sudden vaporization and expansion of CO_2, producing a very intense atomization of the emulsion which facilitates the formation of extremely fine droplets which dry very fast resulting in fine powders. In order to obtain dry powder, temperature conditions in the spray tower must be above the dew line of the temperature-composition phase equilibrium diagram of CO_2 and water [15, 16]. Different conditions of pre-expansion pressure (6-10 MPa), pre-expansion temperature (104 -130ºC) and gas to product ratio (GPR) (5-35) were assayed in order to study their influence in powder characteristics. Each experiment was performed three fold in order to test the reproducibility. Particle size of all replies was analyzed, but encapsulation efficiency was only analyzed for two triplicates.

2.6 Determination of the efficiency of incorporation of essential oil in liposomes produced by thin-film methods

The efficiency of incorporation of lavandin essential oil in soybean lecithin based liposomes was determined with the following procedure: Liposomes were subjected to centrifugation (Microcentrifuge 24D Labnet, Woodbridge NJ, USA) at 13 000 rpm during 30 min. Then the subnadant (water with non-encapsulated oil) was separated with the aid of a syringe and 1 mL acetone was added to the liposomal solution to disrupt the vesicles. The obtained sample was filtered to eliminate the lecithin and then heated at 60ºC for one hour in an oven to evaporate the acetone. The obtained essential oil was analysed with a gas chromatograph coupled with a mass spectrometer (GC-MS) Agilent 6890/5973 (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5ms Capillary GC column. For this, the sample was diluted in Hexane including n-hexenal as internal standard, in order to be able to determinate the amount of essential oil through a previous calibration. The operating conditions were the following: Helium was the carrier gas at 0.7 mL/min, split mode injection (200:1), injection temperature 250ºC and injection volume 1µL. The oven temperature was programmed as follows: 5 min at 65ºC and 4ºC/min to 220ºC. Identification of compounds was based on relative retention times, matching with NIST MS library or by comparison of their relative

retention times with those of authentic samples. More details were provided in a previous work [20].

2.7 Determination of the efficiency of encapsulation of essential oil in soybean lecithin microparticles produced by PGSS-drying

The encapsulation efficiency of soybean lecithin microparticles was determined by dissolving 500 mg of the power in 2 mL of acetone. The mixture was vigorously vortexed during 1 min. and filtered to eliminate lecithin particles. The extracted lavandin oil was analyzed by gas chromatography as described in section 2.6. Superficial, non-encapsulated oil was determined by washing 500 mg of powder with 2 mL of a dissolution of 0.5 % wt/wt of n-hexenal in hexane. The suspension was filtered and samples were analyzed by gas chromatography.

2.8 Determination of particle size

Particle size of liposomes was measured by Dynamic Light Scattering (Autosizer Lo-C, Malvern instruments). Sampling was carried out after a gentle rotation of the container of liposomes in order to obtain an adequate dispersion of the liposomes and further dilution with deionized water to less than 0.4% wt/wt to prevent multiple scattering effects. In this work, particle size measurements are reported as the average diameter in the volume distribution (d_{32}). Reported values are the average of ten measurements made on at least three freshly prepared samples. The following optical parameters were applied: lavandin oil refractive index: 1.46 and water refractive index: 1.331.

2.9 Reconstitution of liposomes from lavandin oil load lecithin particles

Re-hydration of particles produced by PGSS-drying was performed by dissolving 0.5 g of particles in 2 mL of water and vortex mixed for 10 min.

2.10 Microscopy

An automated Upright Microscope system for Life Science Research Leica DM4000 B (Wetzlar, Germany) was used to obtain microscopic images of liposomes.

3. Results and Discussion

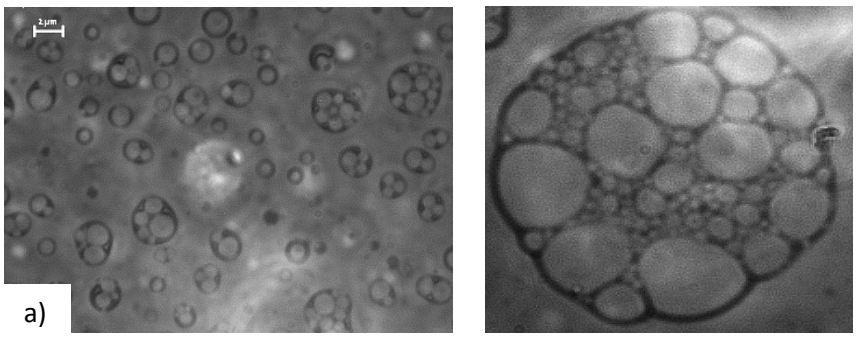
3.1 Thin-film methods

Formulations were prepared by the three different thin-film methods described in Section 2.2. First, the influence of lavandin oil/ lecithin mass ratio was evaluated using a fixed amount of lecithin (0.1 g) and cholesterol (0.013 g) and different amounts of lavandin oil (0.1-0.4 g). The diameter of liposomes was determined by Dynamic Light Scattering and their morphology was evaluated using optical microscopy. Table 1 reports the results obtained. Liposome size was strongly dependent on the incorporation of essential oil, increasing with the lavandin oil/lipids ratio. On the other hand, empty vesicles (d = 1.32-2.47μm) were larger than essential oil loaded vesicles (d = 0.42-1.29 μm), which can be explained by the higher cohesion packing among the apolar chains in the membrane of vesicles [23]. The hydration water also plays an important role, since liposome diameter increased as the amount of water was increased. In fact, for entrapping a higher amount of water with the same quantity of lipid vesicles must be larger. The concentration of lecithin also influenced the size of liposomes, and it was found that liposome diameter decreased when the concentration of lecithin increased with all preparation methods, as shown in Table 1.

		Method 1		Method 2		Method 3	
Lav/Lipids	Water/Lipids	Diameter	Structure	Diameter	Structure	Diameter	Structure
1.0	10	0.68±0.04	MVV	0.42±0.01	MVV	0.75±0.09	MVV
1.8		0.781±0.04	SUV/SM	0.65±0.07	SUV/SM	0.77+0.02	SUV/SM
2.4		0.77±0.17	SUV/SM	0.75±0.02	SUV/SM	0.81±0.03	SUV/SM
3.5		0.94±0.09	SUV/SM	1.26±0.08	SUV/SM	0.94±0.03	SUV/SM
1.0	15	0.75±0.05	MVV	0.84±0.02	MVV	0.60±0.09	MVV
1.8		0.75±0.04	SUV/SM	0.92±0.02	SUV/SM	0.73±0.03	SUV/SM
2.4		0.99±0.02	SUV/SM	1.05±0.09	SUV/SM	0.80±0.02	SUV/SM
3.5		1.03±0.04	SUV/SM	1.09±0.02	SUV/SM	0.89±0.02	SUV/SM
0	20	2.47±0.24	SUV/SM	1.32±0.07	SUV/SM		
1.0		0.94±0.01	MVV	0.78±0.12	MVV	0.66±0.02	MVV
1.8		1.07±0.03	SUV/SM	0.87±0.02	MVV	0.81±0.04	MVV
2.4		1.22±0.05	SUV/SM	0.89±0.05	SUV/SM	0.88±0.04	SUV/SM
3.5		1.29±0.42	SUV/SM	0.93±0.02	SUV/SM	0.87±0.06	SUV/SM

Table 1. Influence of the lavandin/lipids mass ratio on the size and morphology of liposomes produced with thin-film methods. Formulations were prepared 0.1g of lecithin and 0.013g of cholesterol, and variable amounts of lavandin oil ranging from 0.1 g to 0.4 g. Structures observed were small unilamellar vesicles (SUV), small multilamellar vesicles (SMV) and multivesicular vesicles (MVV).

Different vesicular formulations like small unilamellar/multilamellar vesicles (SUV/SMV) and multivesicular vesicles (MVV) were obtained, as it is shown in Figure 3. In most experiments, SUV or SMV were obtained. However, when the ratio lavandin oil/lipids was smaller than 1.8, MVV were obtained. When the amount of hydration water was increased, SUV/SMV were produced at higher lavandin oil/lipids ratio. For example, when 2 mL of water were added, MVV were obtained with lavandin oil to lipids ratio of 1.8, as shown in Table 1.



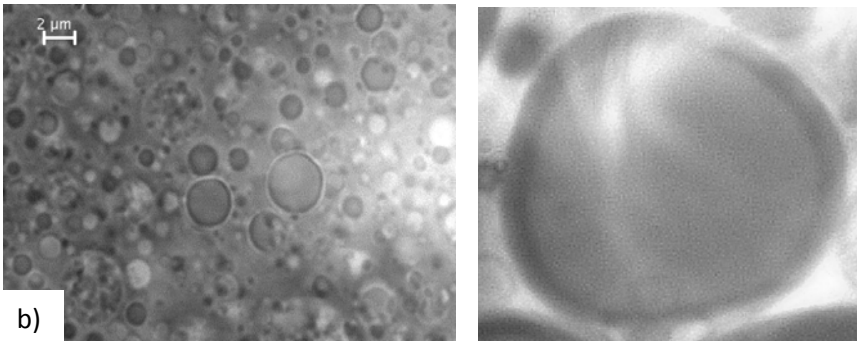


Figure 3. Microscopy images (100x) of different vesicular formulations obtained: Multivesicular (a) and multilamellar (b) vesicles. Scale bar 2µm.

The influence of the amount of cholesterol on liposome size had been also determined. Results shown in figure 4, demonstrated that liposome size decreases with the amount of cholesterol. The addition of cholesterol can cause changes in the degree of head group dissociation and interaction with lipophilic compounds. Usually cholesterol molecules will be oriented with its steroid nucleus among the fatty acid chains of phospholipids molecules and its hydroxyl group facing towards water. Therefore cholesterol is often added to improve liposomes in vitro and in vivo stability and to decrease membrane permeability [23].

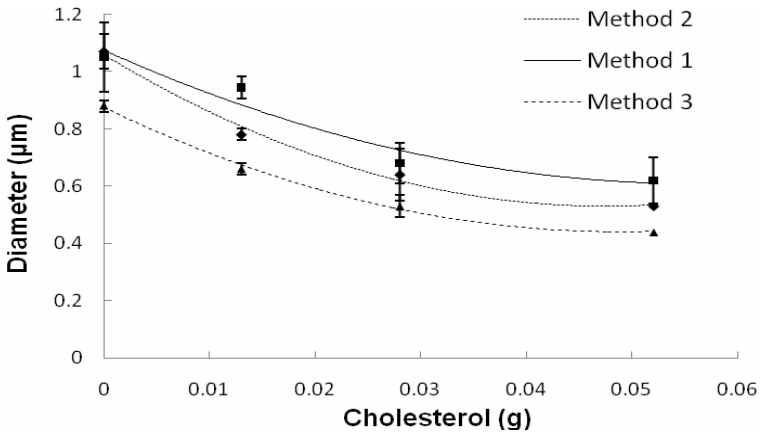


Figure 4. Influence of cholesterol on vesicle size for different methods. Vesicles were prepared with 0.1g lecithin, 0.1g lavandin oil and 2mL of water.

Figure 5 presents the efficiency of the incorporation of lavandin oil in liposomes, expressed as the fraction of the total oil in final product that was effectively incorporated in the liposomes, as a function of the lavandin oil/lipid ratio and of the method of preparation. It can be observed that, for methods 1 and 2 the incorporation efficiency increased when the lavandin oil/lipids ratio was increased, while method 3 followed an opposite trend. It can be concluded that method 2, involving vortex mixing followed by a two hours hydration period, results to be the most efficient in terms of essential oil load and vesicles stability. It fact, vortex mixing gave small uni/multilamelar vesicles whose size ranged from 0.6 μm to 0.94 μm, while sonicated oil-loaded vesicles presented sizes ranging from 0.68 μm to 1.29 μm. Hydration of liposomes in darkness for two hours improved their stability and the efficiency of incorporation of essential oil. That could be explained because the longer time allowed for layer formation with this method permitted layer defects to be repaired.

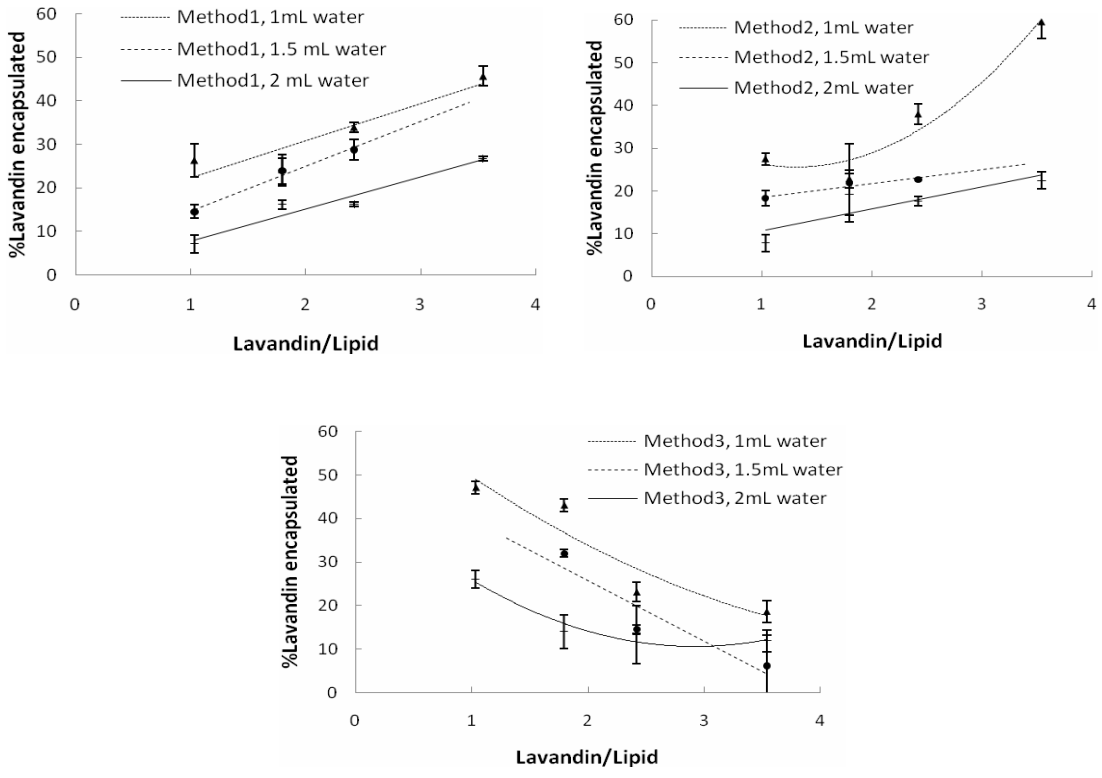


Figure 5. Efficiency of incorporation of lavandin oil in liposomes as a function of lavandin oil/lipids ratio with different preparation methods and amounts of hydration water (1, 1.5 and 2mL).

The physical stability of liposomes was estimated by following their variations in size over one month. Liposomes were stored in 3mL clear glass snap cap vial at 5ºC. Results presented in Figure 6, shown in all case a considerable increase of liposome size during the first 10 days. Afterwards the size remained stable.

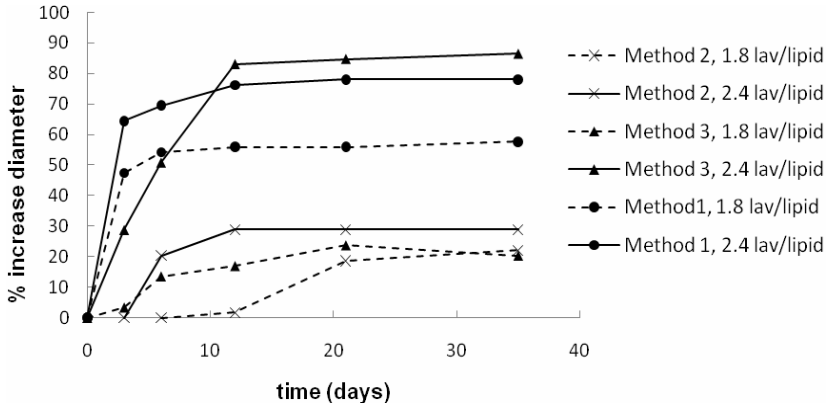


Figure 6. Evolution of the diameter of liposomes with time.

In order to estimate the stability of the incorporation of essential oil in liposomes, the amount of essential oil released after 50 days was analyzed. Liposomes were stored in 3mL clear glass snap cap vial at 5ºC. Results are presented in Table 2. Except in the case of liposomes with the highest lavandin oil/lipids ratio prepared with method 1, lavandin oil was released slowly, as only 7- 33% of the oil was released after 50 days. The main parameters influencing the release of essential oil from liposomes were the lavandin oil/lipids ratio, with a faster release of oil when this ratio was increased, and the method of preparation. Method 3 yielded the slowest release of essential oil, although with this method the release of linalyl acetate was unusually fast compared to that of other components of the essential oil.

	Method 1		Method 2		Method 3	
Lavandin oil/lipids ratio	1.8	3.5	1.8	3.5	1.8	3.5
Released oil after 50 days (%)	33	94	21	25	7	19
Released linalool after 50 days (%)	15	100	18	43	3	7
Released linalyl acetate after 50 days (%)	4	100	11	14	33	36

Table 2. Fraction of lavandin oil, linalool and linalyl acetate released from liposomes after 50 days.

3.2 PGSS-drying

PGSS-drying experiments were performed on lavandin oil emulsion prepared with lecithin, in which lavandin oil concentration was 5 wt%. and soybean lecithin concentrations ranged between 38 g/L and 85 g/L. In preliminary experiments performed with a high pressure view-cell, it was established that these emulsions were stable in a CO_2 environment at different pressures and temperatures, as it is required for application of the PGSS-drying process. From the results shown in Table 3 it can be observed that dried particles could only be obtained in a narrow range of conditions. The efficiency of encapsulation of lavandin oil was low in general, ranging from 6% to 14.5%, probably due to the poor packing ability of phospholipids bilayers. In comparison, in a previous work higher encapsulation efficiencies (up to 50%) were achieved by PGSS-drying of starch particles (*19*). On the other hand, lavandin oil was not detected on particles surface, implying that all lavandin oil present in the final product was encapsulated inside the particles.

$C_{Lecithin}$ (g/l)	T_{Mixer} (K)	T_{Tower} (K)	$P_{before\ exp}$ (MPa)	GPR	Particles d (μm)	%Encapsulated			Liposome d (μm)
						Lavandin	Linalool	Linalyl acetate	
38	389	312	7	17	6.22±0.38	14.5	11.6	0	0.93
	387	324	7.5	31	1.46±0.10	14.1	1.67	0.96	0.75
	387	311	7	35	1.39±0.21	13.5	9.4	10.96	0.71
62	379	318	6.4	14	6.89±0.76	7.5	4.9	4.8	0.86
	382	323	6	25	2.69±0.11	6.8	2.4	6.2	0.52
	381	318	6.6	29	2.61±0.27	6	1.4	3.2	0.54
	393	315	6.4	30	18.02±2.36	8	3.45	1.66	0.82
	393	318	6.9	34	24.84±2.18	7.2	0	7.9	1.19
	396	315	6.9	34	8.85±0.86	7.6	1.3	35.4	0.95
	400	314	7	20	19.10±2.0	8	8.71	7.46	0.85
	377	323	7.4	34	2.38±0.20	5.6±0.2	2.9±0.5	2.8±0.1	0.63
	383	325	7.4	33	1.98±0.23	9.1	5.2	3.5	0.47
	385	323	7.5	18	4.12±1.1	2.7	4.3	8.9	0.49
	391	314	7.1	34	14.43±5.4	9.1	5.9	4.1	1.19
	402	316	7.2	19	20.31±3.9	4.6±1.1	8.5±0.3	5.0±1.8	1.42
	394	314	8.1	34	17.34±1.2	10.7	4.4	1.4	1.02
	378	323	10.3	5	6.30±0.30	10.5	8,9	7.5	
85	386	323	7.3	16	2.99±0.65	9.6	39.1	36	1.05
	383	320	7.3	35	6.63±0.55	7.2	3.2	5.7	1.47
	390	314	7	34	2.52±0.19	9.8	4.67	0	0.75

Table 3. Results of PGSS-drying of lavandin oil emulsions using soybean lecithin as surfactant.

According to the results, represented in Figure 7, GPR seems to be the variable which more influences the encapsulation efficiency. In fact, dry particles were only obtained with GPR higher than 5 and as the GPR was increased, lavandin and linalool encapsulation efficiency decreased, probably due to loses of essential oil by evaporation in the static mixer and spray tower. Like GPR ratio, pre-expansion temperature and pressure greatly influenced lavandin oil and linalool encapsulation efficiency. This efficiency increased when pre-expansion temperature or pre-expansion pressure were increased. These conditions allowed producing smaller particles, so in this case the effect of these parameters seems to be related to the efficiency of atomization and particle formation. Finally, the encapsulation efficiency decreased when the concentration of lecithin increased. This behaviour is also associated with an increase in particle size as discussed later.

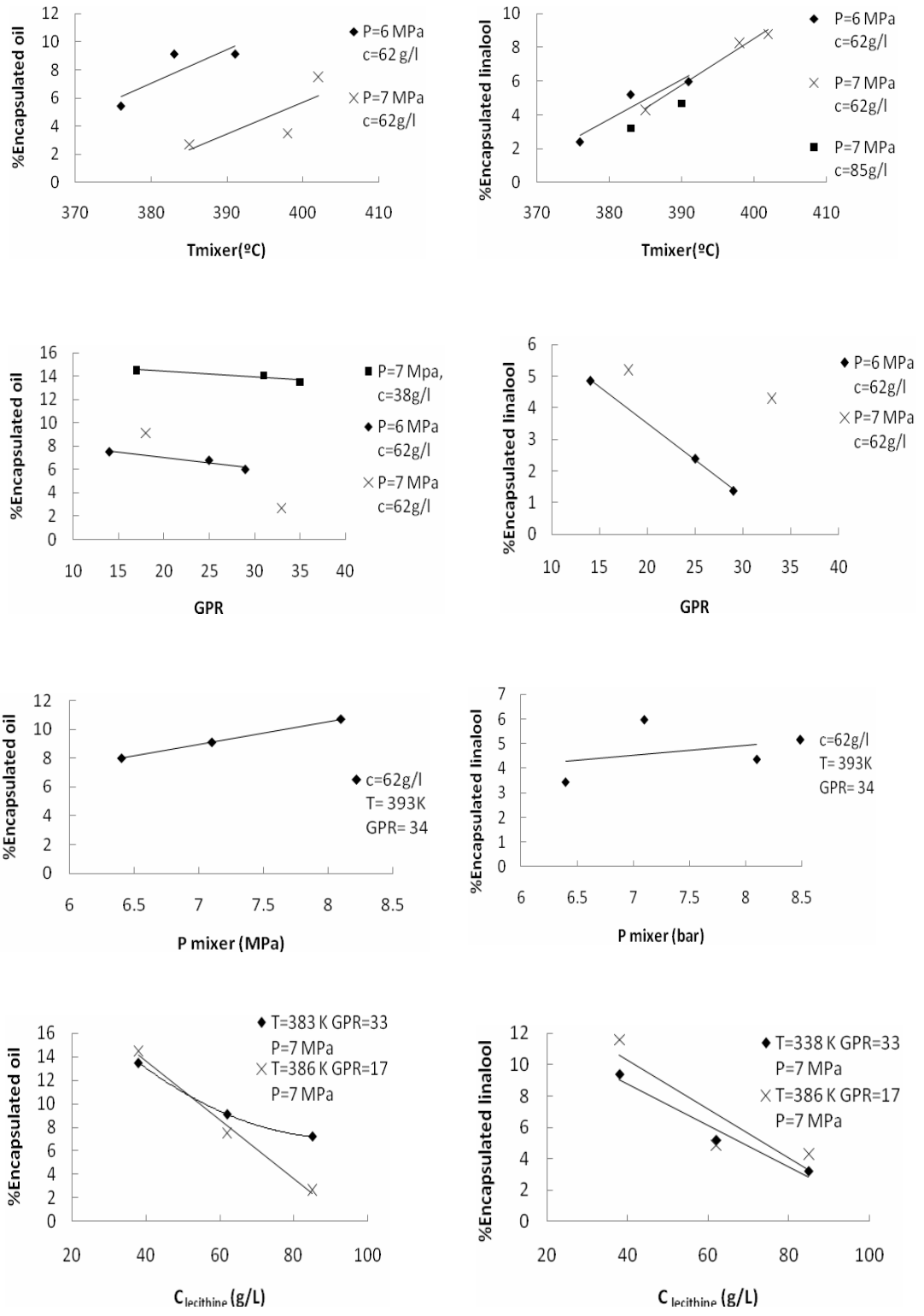


Figure 7. Effect of different variables on the efficiency of encapsulation of lavandin oil and linalool by PGSS-drying.

The influence of different parameters on particle size was also analyzed. As it can be seen in Figure 8, GPR also had a strong effect on particle size. As GPR was increased, particle size decreased. This result is consistent with previous experience with the PGSS-drying process [15], and it could be due to an increase in the concentration of CO_2 in the emulsion after the static mixer, improving the atomization during the expansion and promoting the formation of smaller particles [24]. Particle size also decreased when pre-expansion temperature and pressure decreased, because with these variations of parameters the amount of CO_2 dissolved in the emulsion also increased [24]. In the case of the concentration of lecithin in emulsion, as it increases the emulsion becomes more viscous making the atomization more difficult and producing bigger particles.

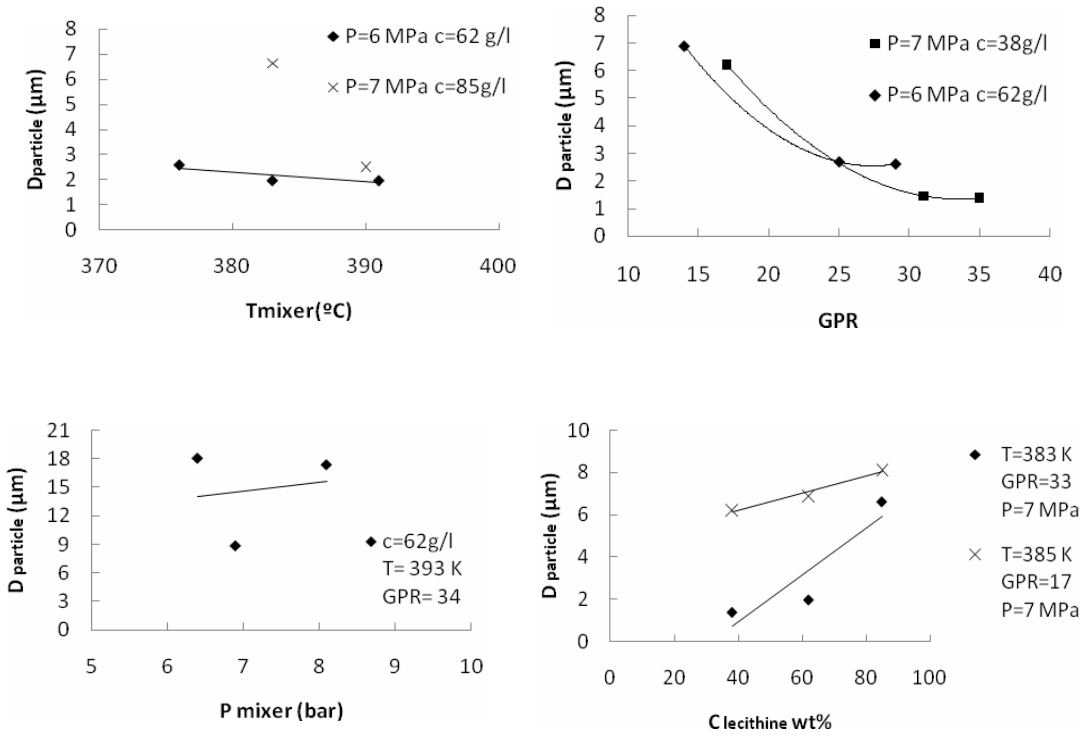


Figure 8. Effect of different variables on particle size obtained by PGSS-drying.

Finally it was verified that liposomes could be reconstituted by re-hydration of particles produced by PGSS-drying. Liposomes obtained were multilamellar and it size ranged

between 0.47±0.02μm and 1.47±0.12 μm. Figure 9 shows microscopic pictures of the particles obtained by PGSS-drying and of liposomes produced by re-hydration of these particles. As presented in Figure 10, the size of reconstituted liposomes depended on particle size and therefore on the operating parameters of the PGSS-drying process, particularly pre-expansion temperature and lecithin concentration.

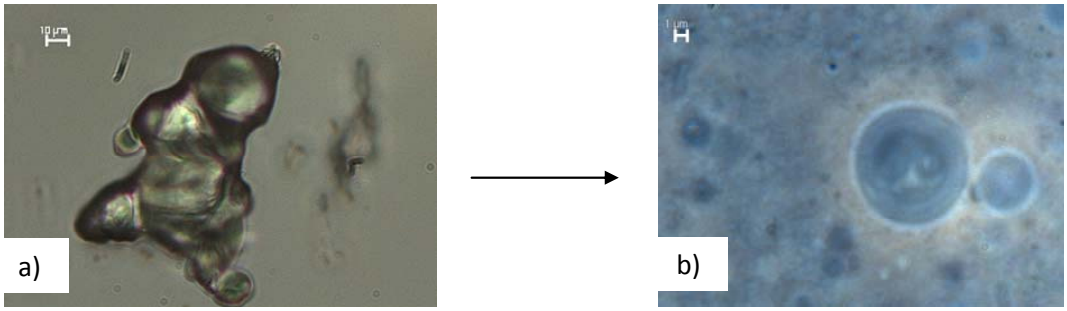


Figure 9. Microscopy images (100x) of lecithine particles obtained by PGSS drying (a) and liposomes reconstuted by hydration of lecithin particles (b). Scale bar 10 μm and 1 μm respectively.

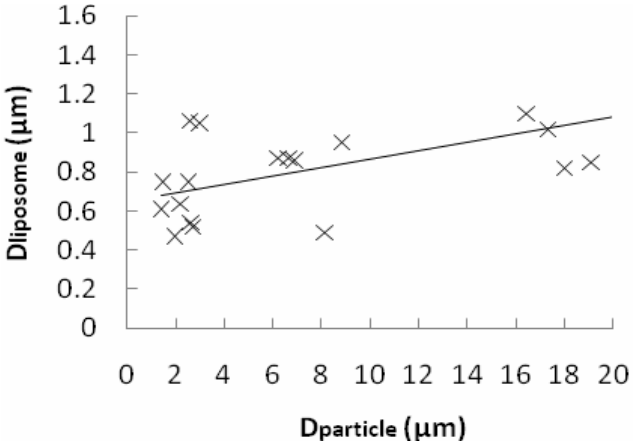


Figure 10. Diameter of liposomes as a function of particle diameter.

4. Conclusions

Uni/multilamellar and multivesicle vesicles with a diameter between 0.42-1.29 μm and encapsulation efficiency between 6-60% were obtained. The size of liposomes produced by thin-film methods was strongly dependent on bilayer composition, lavandin oil load and the experimental method used. The liposomes size increased with the lavandin oil/lipids ratio and decreased with the amount of cholesterol. The structure of liposomes depended on the lavandin oil/lipids ratio and the amount of hydration water. With lavandin oil/lipids ratio smaller than 1, MVV were formed, while higher ratios yielded SUV/SMV structures, regardless of the method used.

Method 2, involving vortex mixing followed by a two hours hydration period, was the most efficient in terms of essential oil load and vesicles stability. Hydration of liposomes in darkness for two hours improved their stability and the efficiency of incorporation of essential oil. Entrapping efficiency for method 1 or 2 (consisting in sonication or vortex mixing followed by a long hydration period) increased with lavandin oil/lipids ratio, while with method 3 (consisting in vortex mixing of the thin film without subsequent hydration period) entrapping efficiency decreased with this ratio. Encapsulation efficiency decreased with the amount of hydration water.

Liposomes showed a good stability for at least one month. The mean size of liposomes changed during the first ten days and remained stable after this period, confirming that fusion and breakage of vesicles did not occur after the initial period of 10 days. The release rate of lavandin oil was slow, with only a 7% of oil released after 50 days in the best case

PGSS was effective in micronizing soy lecithin, forming spherical aggregated particles (d = 1.4 – 25 μm). The efficiency of encapsulation of essential oil in these particles was in general low (6 - 14.5%), and it could be improved by modifying process conditions in order to increase the solubility of carbon dioxide in the emulsion in the static mixer (higher gas/product ratio, pre-expansion temperature and pre-expansion pressure). These conditions enhanced an effective and fast expansion which reduced the contact time between lavandin oil and carbon dioxide and reduced losses by evaporation. These conditions also allowed producing smaller particles. The

concentration of soy lecithin in the emulsion played an important role because with high concentrations the emulsion becomes highly viscous and diffusional effects might take place during the micronization process. As a consequence entrapping efficiency decreased and particle size increased as soybean concentration was increased.

5. Acknowledgments

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6. References

[1] S. Burt. Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol. 94 (2004) 223-253.

[2] L.S. Nerio, J. Olivero-Verbel, E.Stashenko. Repellent activity of essential oils: A review. Biores. Tech. 101 (2010) 372-378.

[3] R. Di Pasqua, G. Betts, N. Hoskins, M. Edwards, D. Ercolini, G. Mauriello. Membrane Toxicity of Antimicrobial Compounds from Essential Oils. J. Agr. Food Chem. 55 (2007) 4863.

[4] J.P. Lalles, P. Bosi, P. Janczyk, S.J. Koopmans, D. Torrallardona. Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. Animal. 3 (2009) 1625-1643.

[5] R. Krieg, W. Vahjen, W. Awad, M. Sysel, S. Kroeger, E. Zocher, H.W. Hulan, G. Arndt, J. Zentek. Performance, digestive disorders and the intestinal microbiota in weaning rabbits are affected by a herbal feed additive. World Rabbit Sci. 17 (2009) 1-9.

[6] C. Benchaar, S. Calsamiglia, A.V. Chaves, G.R. Fraser, D. Colombatto, T.A. McAllister, K.A. Beauchemin. A review of plant-derived essential oils in ruminant nutrition and production. Anim. Feed Sci. Technol. 145 (2008) 209-228

[7] M. Takahashi, K. Inafuku, T.Miyagi, H. Oku, K. Wada, T. Imura, D. Kitamoto. Preparation of Liposomes Encapsulating Food Materials Using Lecithins by a Mechanochemical Method. J. Oleo. Sci. 56 (2007) 35-42.

[8] N. Zang, Q.N. Ping, G.H. Huang, W.F. Xu. Investigation of lectin-modified insulin liposomes as carriers for oral administration. Int. J. Parm. 294 (2005) 247-259.

[9] L.A. Meure, N.R. Foster, F. Dehghani. Conventional and dense gas techniques for the production of liposomes. AAPS Pharm. Sci. Tech. 9 (2008) 798-809.

[10] C. Sinico, A. De Logu, O. Lai, F. D. Valenti, M. Manconi, G. Loy, L. Bonsignore, A.M. Fadda. Liposomal incorporation of Artemisia arborescens L. essential oil and in vitro antiviral activity. Eur J. Pharm. Biopharm. 59 (2005) 161–168.

[11] D. Valenti, A. De Logu, G. Loy, C. Sinico, L. Bonsignore, F. Cottiglia, D. Garau, A.M. Fadda. Liposome-incorporated santolina insularis essential oil: preparation, characterization and in vitro antiviral activity. J. Liposome Res. 11 (2001) 73-90.

[12] C.C. Liolios, O. Gortzi, S. Lalas, J. Tsaknis, I. Chinou. Liposomal incorporation of carvacrol and thymol isolated from the essential oil Origanum dictamnus L. And in vitro antimicrobial activity. Food Chem. 112 (2009) 77–83.

[13] E. Dickinson. Towards more natural emulsifiers. Trends Food Sci. Tech. 4 (1993) 330-334.

[14] A.D. Bangham, M.M. Standish, J.C. Watkins. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13 (1965) 238-252.

[15] E. Weidner. High pressure micronization for food applications, J. Supercrit. Fluids. 47 (2009) 556-565.

[16] A. Martín, H.M. Pham, A. Kilzer, S. Kareth, E. Weidner. Micronization of polyethylene glycol by PGSS (Particles from Gas Saturated Solutions) – Drying of aqueous solutions. Chem. Eng. Proc. 49 (2010) 1259-1266.

[17] E. Badens, C. Magnan, G. Charbit. Microparticles of soy lecithin formed by supercritical processes. Biotechnol. Bioeng. 72 (2001) 194-204.

[18] C. Magnan, E. Badens, N. Commenges, G. Charbit. Soy lecithin micronization by precipitation with a compressed fluid antisolvent — influence of process parameters. J. Supercrit. Fluids. 19 (2000) 69-77.

[19] Y. Li, D. Yang, S. Chen, S Chen, A.S. Chan. Comparative Physicochemical Characterization of Phospholipids Complex of Puerarin Formulated by Conventional and Supercritical Methods. Pharm Res. 25 (2008) 563-577.

[20] S. Varona, Á. Martín, M.J. Cocero. Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches. Chem. Eng. Proc. 48 (2009) 1121-1128.

[21] S. Varona, S. Kareth, Á. Martín, M.J. Cocero. Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture. J. Supercrit. Fluids. 54 (2010) 369-377.

[22] M.R. Mozafari. Liposomes: an overview of manufacturing techniques. Cell Mol. Biol. Lett. 10 (2005) 711-719.

[23] Y.H: Chan, B.H. Chen, C.P. Chiu, Y. Lu. The influence of phytosterols on the encapsulation efficiency of cholesterol liposomes. Int. J. Food. Sci. Tech. 39 (2004) 985-995.

[24] Á. Martín, E. Weidner. PGSS-drying: mechanisms and modelling. J. Supercrit. Fluids. 5 (2010) 271-281.

Chapter III

Formulation of lavandin essential oil (*Lavandula hybrida*) in microcapsules for its application as biocide in ecological: Development of the processes PGSS and PGSS-drying

Essential oils, and in particular lavandin (lavandula hybrida) essential oil, can be used as natural biocides as an alternative to synthetic chemical biocides. For this purpose, agrochemical formulations of the essential oils should be physically stable in the long term and should enhance the biological performance of the agrochemical. In this work, such a formulation of lavandin essential oil obtained by encapsulation of the oil in a biodegradable polymer has been studied. Two high pressure precipitation techniques, Particles from Gas Saturated Solutions (PGSS) and PGSS-drying, have been applied to perform the encapsulation. The PGSS process has been used to encapsulate the oil in polyethylene glycol (PEG). With PGSS-drying the oil has been encapsulated in n-octenyl succinic (OSA)-modified starches, by removing the water from an oil-in-water emulsion stabilized using the OSA-starches as surfactants. Operating conditions were selected in order to reduce oil losses due to its dissolution in supercritical CO_2 or due to emulsion destabilization. A comparison between the characteristics of the particles obtained by encapsulation in PEG with PGSS and by encapsulation in OSA-starches with PGSS-drying was done. Results revealed that encapsulation efficiencies of lavandin oil were higher in PEG microcapsules obtained by PGSS (14-66% of initial oil encapsulated). This particles show a spherical morphology and a narrow size distribution, which is favourable for a controlled release of lavandin essential oil.

1. Introduction

Essential oils obtained by steam distillation of certain aromatic plants and their derivates can be used as antimicrobial and insect repellents, and they are considered as an alternative to conventional synthetic chemical pesticides, due to their reduced health risk and their biodegradability [1]. Recent investigations confirm the biocide action of some essential oils (fennel, peppermint, caraway, eucalyptus, geranium and lemon). These essential oils show control and fumigant insecticidal actions against a number of economically important insect, mite pests and plant pathogen fungi. [1,2].

In this work, lavandin (*lavandula hybrida*) essential oil has been selected as active compound because of its availability in the region of Castilla y León (Northwest Spain), where this project has been carried out, and for its biocide properties. The main constituents of this oil are linalool (33 wt%), linalyl acetate (29 wt%), camphor (7.1 wt%), 1,8-cineole (7.6 wt%) and terpinen-4-ol (3.3 wt%) [3]. Among these components, it has been demonstrated that several monoterpenes (α-terpineol, terpinen-4-ol and α-pinene) [4], linalool [5] and 1-8-cineole [6] show the strongest biocide activity.

A formulation that allows protecting the essential oil from high temperatures, oxidation and UV light must be found. There are different agrochemical formulations and in this case microencapsulation has been considered as the most appropriate. The main advantages of the encapsulation are minimized evaporation, increased shelf live and controlled release, which may increase the biological efficiency [7]. Conventional encapsulation techniques (solvent evaporation, phase separation and spray drying) require relatively high temperatures, which can be inadequate for preserving the stability of essential oils. Moreover, they are not suited for producing microspheres with controlled particle size. High pressure technology allows producing powders with properties difficult to achieve by classical methods [8, 9]. One promising possibility of some of these processes, like PGSS (Particles form Gas Saturated Solutions) or PGSS-drying, is to produce microparticles filled with solids or liquids [10, 11, 12].

For this application the carrier or shell material must be biodegradable and non toxic. In this work, OSA-modified starches and polyethylene glycol 9000 (PEG 9000) have been

used, because they have been approved for their use in food and medicine. Lavandin oil can be encapsulated in PEG with a PGSS process and it can be encapsulated in starch by removing the water from an oil-in-water emulsion with a PGSS-drying process. Moreover, PEG precipitation by PGSS has been thoroughly studied [10], and oil-in-water emulsions of lavandin essential oil using OSA-starches as surfactants, which are the starting point for a PGSS-drying process, have been prepared and characterized in a previous work [3]. The most suitable process and polymer have been selected based on the amount of encapsulated oil, particle size and particle morphology.

2. Materials and methods

2.1 Materials

Lavandin oil used in this project was purchased to Silvestris & Szilas (Kerepes, Hungary).This lavandin oil was produced by steam distillation. As shell material modified OSA starch derived from waxy maize kindly provided from National Starch Group and polyethylene glycol 9000 (PEG 9000) were used.

2.2. Preparation of emulsions

Water in oil emulsions were prepared by a two step process. First a suspension of the coating material was prepared by dispersing the surfactant (modified OSA-starch) in distilled water with the aid of a Ultra-Turrax® UTC (Staufen, Germany) homogenizer set at 3000 rpm during 15 min. Afterwards the oil was gradually dispersed in the aqueous phase to the suspension and the emulsion was agitated for 5 min at 6400 rpm.

2.3 Determination of the stability of emulsions in supercritical CO_2

The stability of lavandin oil emulsions in a CO_2 environment at different pressure and temperature conditions was studied using a high-pressure view cell. A schematic diagram of the equipment is presented in Figure 1. This cell is equipped with two sapphire windows and it can withstand pressures of up to 20 MPa. A heating jacked allows operating at constant temperature, which is measured by a thermocouple fitted on the cell. There is also a stirrer which allows an optimal mixing of the phases.

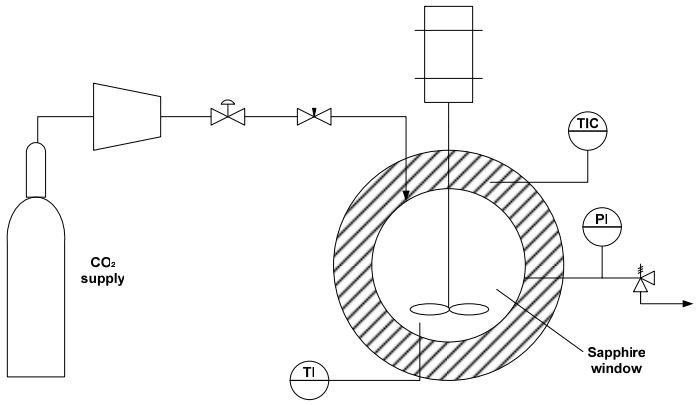


Figure 1. View cell used for emulsion stability determinations

2.4 Precipitation by Particles from Gas Saturated Solutions (PGSS)

PEG particles loaded with lavandin essential oil were produced using the PGSS process. With this technique, melted PEG and lavandin oil are dosed into a static mixing system were the two substances are intensively mixed in presence of heated CO_2 under high pressure. In this mixer CO_2 is dissolved into the melted polymer, and microdroplets of the essential oil are dispersed in the melted polymer. Upon a rapid expansion through a nozzle to ambient pressure very fine particles are produced as the gas come out of the solution. The driving force for particle formation is the strong cooling due to the Joule Thomson effect during expansion. Due to this sudden reduction in temperature the shell material solidifies and forms a covering layer around the essential oil droplets. The particles are collected in a spray tower and a cyclone.

A flow diagram of the pilot plant used for PGSS experiments is presented in Figure 2. Maximum operating pressure and temperature of this plant are 35 MPa and 200ºC. Maximum mass flow rates are 50 kg/h for shell material (PEG), 10 kg/h for core material (lavandin essential oil) and 150 kg/h for carbon dioxide. Operating conditions of PGSS in this project are reported in table 1.

Experiment	E1	E2	E3	E4	E5
Lavandin/PEG mass ratio	0,25	0,32	0,29	0,37	0,34
$T_{before\ exp}$ (ºC)	80	84	77	82	76
$P_{before\ exp}$ (MPa)	5.6	5.7	5.4	5.6	8.5
GPR ($m_{CO2}/m_{PEG+Lav}$)	0.68	0.66	0.58	0.92	1.26
T_{tower} (ºC)	33	34	34	31	30

Table 1. Experimental conditions for PGSS process

In this plant, PEG and lavandin oil were loaded inside of the vessels V1 and V2, respectively. The vessel of the PEG was heated and pressurized so CO_2 was dissolved in PEG decreasing the melting temperature and viscosity of the polymer [12]. Lavandin oil was keep at ambient temperature to avoid the degradation of its components. Both fluids were pumped through thermally insulated pipes to a static mixer (SM), where a flow of CO_2 is added and an intensive mixing between the fluids occurs. After the mixing zone the mixture is expanded in a nozzle and particles are formed in the expanding gas. Particles are collected in a spray tower (ST) and a cyclone. More details about the experimental set-up and procedure can be found in previous works [14].

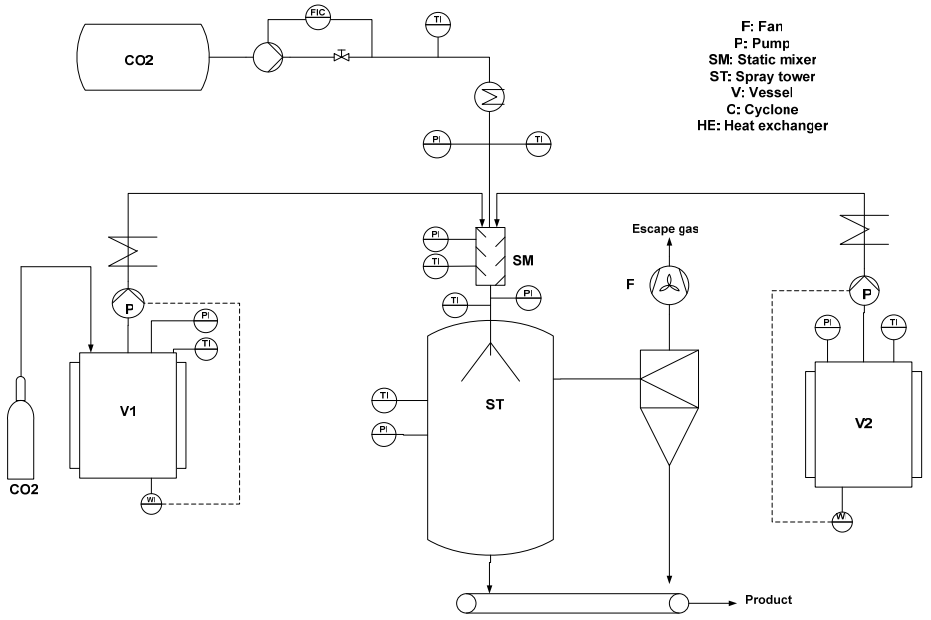


Figure 2. Flowsheet of the PGSS pilot plant.

2.5 Precipitation by PGSS-drying

The flowsheet of the pilot plant used for PGSS-drying experiments is presented in Figure 3. The first step of the operating procedure is to condition it at the desired operating conditions, which are reported in table 2.

Experiment	E1	E2	E3	E7	E 8	E 4	E 5	E 6	E 9	E 10	E 11
Oil/starch mass ratio	0.2	0.2	0.2	0.2	0,3	0.4	0.4	0.4	0.4	1.0	1.0
Pre-exp P (MPa)	12.4	12.0	10.0	10.3	10.4	12.0	12.1	11.7	10.5	10.6	9.0
Pre-exp T (ºC)	127	117	129	100	116	112	131	127	113	114	108
GPR ($m_{CO2}/m_{emulsion}$)	22.5	27.3	22.4	33.2	35.8	25.1	24.1	33.3	41.2	28.0	35.5
T (°C)	64	66	72	68	60	71	70	72	75	73	74
m_{CO2} (kg/h)	77	75	75	79	79	72	73	90	91	88	77
Nozzle diameter (mm)	1.2	1.4	1.4	1.4	1.4	1	1	1.4	1.4	1.4	1.4
$m_{emulsion}$ (kg/h)	3.7	3.6	3.9	2.4	2.9	4.1	3.6	2.7	2.6	3.6	2.6
Static mixer	Yes	Yes	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes

Table 2. Experimental conditions for PGSS-drying process.

In this process, an oil-in-water emulsion is intensively mixed with supercritical carbon dioxide using a static mixer (Sulzer SMX with an internal diameter of 10 mm and 10 mixing elements). In this mixer, a certain amount of water is extracted from the emulsion by carbon dioxide, and CO_2 is dissolved into the liquid phase. This biphasic mixture is sprayed and expanded down to atmospheric conditions through a nozzle. The release and expansion of the carbon dioxide dissolved into the liquid promotes the atomization of the emulsion into small droplets, and conditions inside the spray tower are chosen so all the remaining water from the emulsion is evaporated. Finally the produced powder is collected inside the spray tower and with a cyclone. More details about the experimental set-up can be found in previous works [15, 16]

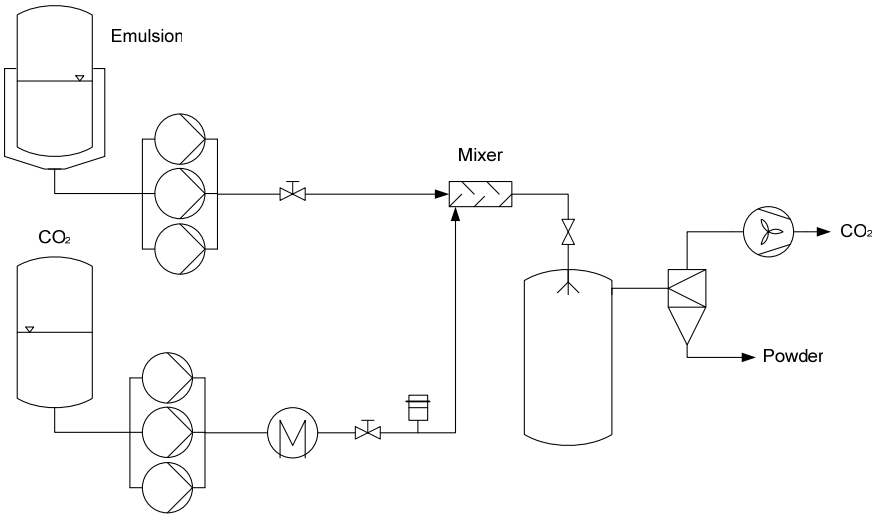


Figure 3. Flowsheet of the PGSS-drying pilot plant

2.6 Powder characterization

Particle size and particle size distribution of the samples obtained by PGSS and PGSS-drying were measured with a laser diffraction method using a Mastersizer 2000 particle analyzer (Worcestershire, U. K). The morphology of the particles was examined with a scanning electron microscope (SEM). The moisture content of the samples was measured by Karl Fischer volumetric titration using an 870 KF Titrino plus from Metrohm (Zofingen, Switzerland).

Bulk density was determined by the tapping method. A 25 mL graduated cylinder was filled with the powder. The cylinder containing the powder was tapped on a flat surface until a constant volume of 25 mL was reached. The final weight of the powder was recorded and bulk density was calculated by dividing the sample weight by its volume.

2.7 Determination of the amount of encapsulated oil

About 10 g of microcapsules were washed with 500 ml of absolute ethanol to remove the surface essential oil, because the polymer is not soluble in ethanol, in order to assure that only oil encapsulated oil is measured. The amount of oil encapsulated in the powder was determined gravimetrically. For this, 10 g of powder were placed in an

extraction thimble. Essential oil in the powder was extracted with hexane for 4 hours. Afterwards, the extract was evaporated to dryness at 40ºC and 335 mbar on a rotary evaporator. The total encapsulated oil was determined by weighing the solid residue.

Composition analyses of the essential oil were carried out with a gas-chromatograph coupled with a mass spectrometer (GC-MS) Agilent 6890/5973 (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5ms Capillary GC column. The operating conditions were as follows: Helium was the carrier gas at 0.7 mL/min, the sample was diluted in Hexane including n-hexenal as internal standard and injected in the split mode (200:1), injection temperature 250ºC and injection volume 1 µl. The oven temperature was programmed as follows: 5 min at 65ºC followed by a temperature ramp of 4ºC/min until 220ºC. Identification of compounds was based on their relative retention times.

3. Results and Discussion

3.1 Stability of lavandin oil emulsions in CO_2

The stability of lavandin oil emulsions was determined at different pressures, temperatures and lavandin oil concentrations in the emulsion. Conditions used in experiments are shown in Table 3. Since the OSA-modified starch performs the double function of surfactant and carrier material, the maximum possible concentration of starch was used in the experiments to improve the encapsulation efficiency. This concentration was 250 g/L, which is close to the solubility of the starch in water. Then different amounts of lavandin essential oil were added to prepare the emulsion with lavandin oil/starch mass ratios of 0.2, 0.3 and 0.4, according to the procedure described in Section 2.2. The emulsion was placed inside the view cell described in Section 2.3 and the desired pressure and temperature were set. Afterwards, the time that the emulsion remained stable was registered, considering that the emulsion was not stable when two phases appeared or when creaming occurred. Two examples of emulsion destabilization by creaming are presented in Figure 4.

Results shown in Table 3 demonstrate that temperature variations have a minor effect, but emulsion stability is drastically reduced when pressure is increased. This is in

agreement with previous studies of the mass transfer of CO_2 to emulsion droplets [17], which demonstrated that the diffusion of CO_2 to the emulsion droplets causes an increase of the oil-water interfacial tension, therefore destabilizing the emulsion, and that the concentration of CO_2 in the emulsion droplets and therefore its destabilizing effect increases when pressure is increased. Nevertheless, the results presented in Table 3 show that for P = 10 MPa, which is a typical operating pressure of PGSS-drying processes, the emulsion is stable during a period of several minutes, which is enough for the experiments because the time of contact between emulsion and CO_2 before spraying it into the tower is much shorter [16].

	Lavandin/Hi-CAP = 0.2					Lavandin/Hi-CAP = 0.3					Lavandin/Hi-CAP = 0.4				
T (ºC) /P (MPa)	0.1	7.5	8	9	10	0.1	7.5	8	9	10	0.1	7.5	8	9	10
40	60	45	45	30	10	60	45	30	30	15	60	45	15	15	15
60	60	45	35	12	8	60	45	-	-	-	60	30	35	15	12
70	60	-	-	-	-	60	45	-	-	-	60	-	-	-	-
80	60	40	-	12	7	60	45	-	-	-	60	-	-	15	12

Table 3. Stability of lavandin oil emulsions in CO_2 at different pressures, temperatures and concentrations. Time is expressed in minutes.

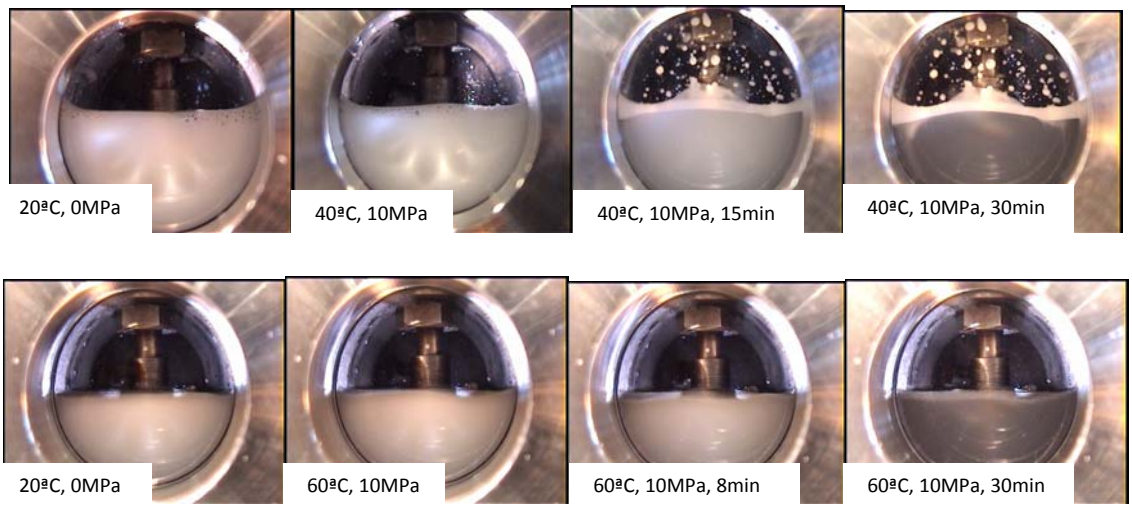


Figure 4. Pictures of the evolution of lavadin oil emulsions in CO_2 at different pressures and temperatures. Creaming can be observed after15 min at 40°C and after 8 min at 60°C.

3.2 Encapsulation of lavandin oil in PEG by PGSS

Lavandin oil was encapsulated in PEG by PGSS precipitation. Experiments were carried out varying the lavandin oil/PEG ratio, gas to product ratio (GPR) and pre-expansion pressure, as shown in Table 1. Temperature before expansion and temperature in the collection vessel of the final product were kept constant. Experimental conditions used were; pressure (5 - 9 MPa), GPR (0.50- 1.3) and lavandin oil/PEG (0.25 - 0.40). Experiments E1 and E2 are duplicates and the obtained error for lavandin and linalool encapsulation yield and mean particle size are ±1.4, ±1.2 and ±7.3 respectively. Results of the experiments are also reported in Table 4.

	E1	E2	E3	E4	E5
%Lavandin encapulated	63	66	44	19	14
%Linalool encapsulated	80	77	37	18	0
$mg_{lavandin}/g_{product}$	216	283	126	69	48
$mg_{linalool}/g_{product}$	63	76	33	10	0
$d_{0,5}$ (μm)	80±2	95±1	31±1	88±2	44±1
Specific area (m^2/g)	0.11	0.09	0.10	0.13	0.08
Bulk density (kg/m^3)	512	540	461	367	213

Table 4. PGSS results.

The efficiency of encapsulation of the essential oil varied between 14-66% in weight. Figure 5 shows the variation of encapsulation efficiency with the main process parameters. Efficiency decreases when pressure is increased, reaching a minimum value of 14% at a pressure of 8.5 MPa. This can be explained considering that the solubility of lavandin oil in CO_2 increases when pressure is increased, becoming completely miscible with CO_2 at pressures above the mixture critical point [18]. Encapsulation efficiency increases when pre-expansion temperature is increased, and it decreases when GPR is increased. The latter can be explained considering that an increase in the relationship between gas and liquid flowrates facilitates that more essential oil is extracted to the gas phase, thus reducing the encapsulation efficiency.

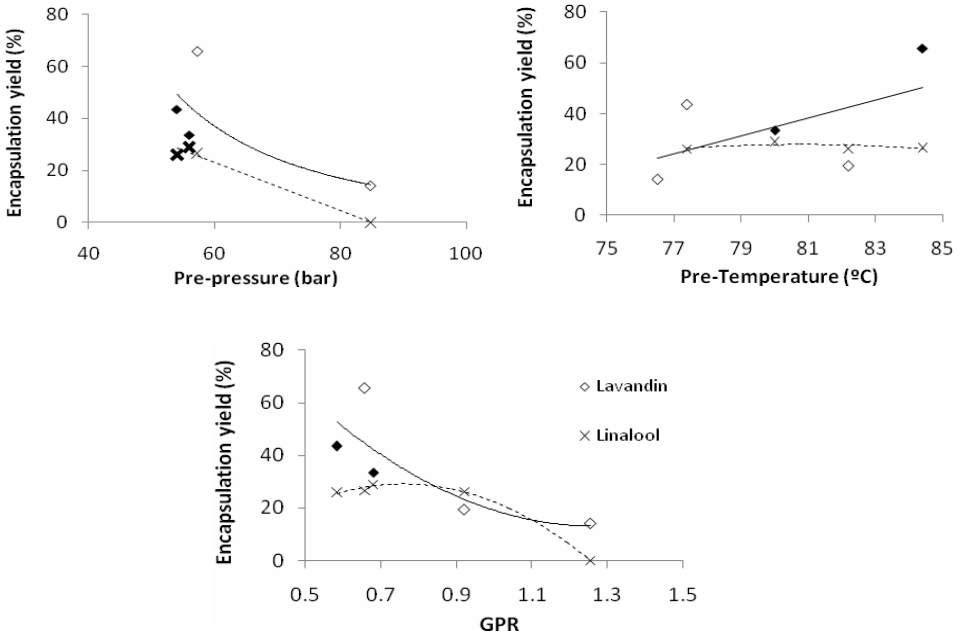


Figure 5. Influence of different process parameters on encapsulation efficiency for linalool in PGSS experiments (solid line) and lavandin oil (dashed line). Bold data are experiment developed exactly at same conditions.

As shown in Table 4, particles produced have sizes ranging from 30 μm to 100 μm. The influence of process parameters in particle size is presented in Figure 6. It can be seen that particle size decreases when pre-expansion pressure is increased, when GLR is increased or when pre-expansion temperature is decreased. In the three cases, the trend of variation of particle size can be explained considering that higher pre-expansion pressures, higher amounts of gas with respect to the liquid and lower pre-expansion temperatures cause a more intense cooling due to Joule-Thomson effect, and therefore a faster solidification of the PEG leading to the formation of smaller particles. Particle size distributions are shown in Figure 7. It can be seen that the distribution is relatively narrow, with $d_{0.1}$ = 10 μm and $d_{0.9}$ = 500 μm, approximately. SEM micrographs of particles obtained in all experiments reported in Table 1 are presented in Figure 8. It can be seen that spherical particles were obtained. Some agglomeration occurs when pre-expansion temperature is increased, and especially when the oil/PEG mass ratio is

increased, as in experiments E4 and E5, suggesting that some fraction of oil that is not encapsulated makes particles sticky.

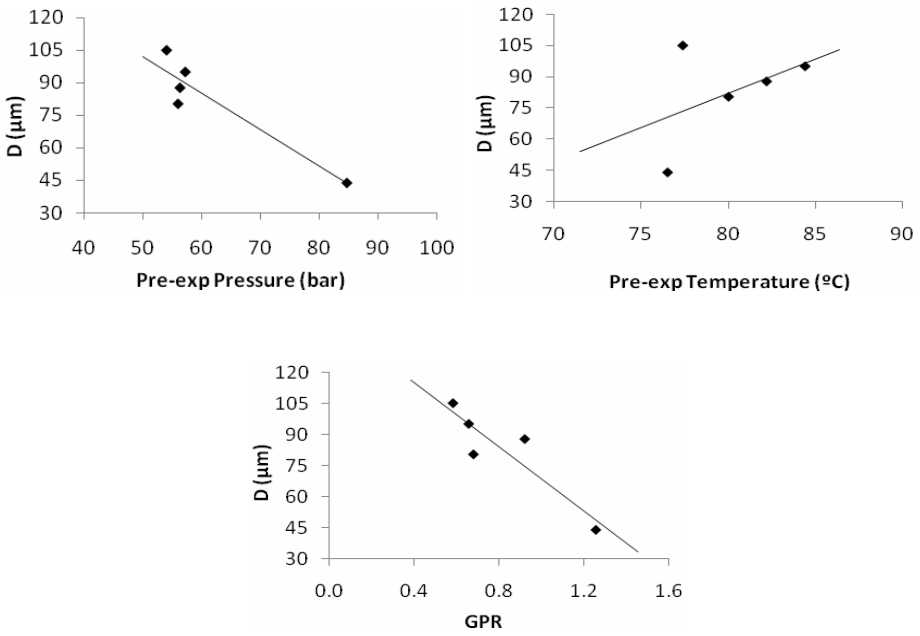


Figure 6: Variation of particle size with operating conditions in PGSS experiments.

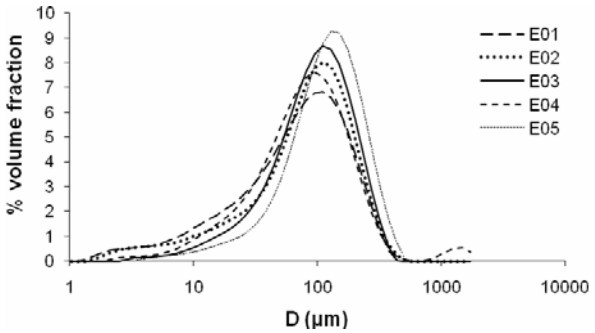
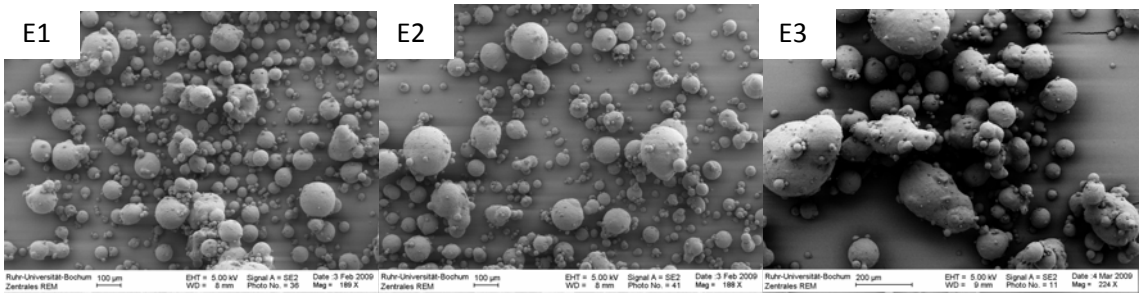


Figure 7. Particle size distribution of particles obtained by PGSS.



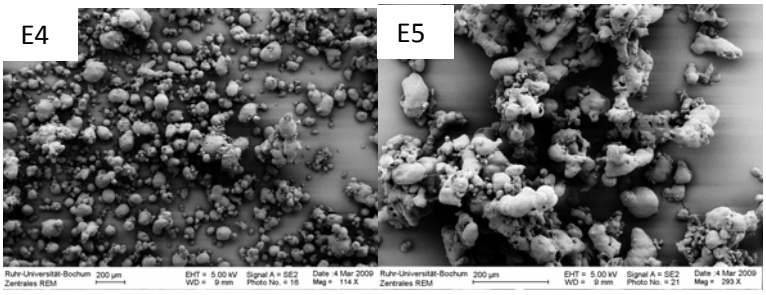


Figure 8. SEM micrographs of lavandin oil-loaded PEG particles produced by PGSS.

3.3 Encapsulation of lavandin oil in starch by PGSS-drying

Lavandin oil was encapsulated in OSA-modified starches by PGSS-drying. For doing so, an oil-in water emulsion was prepared in which the essential oil constitutes the dispersed phase and the OSA-starch acts as a surfactant, and water was removed from this emulsion by PGSS-drying so the OSA-starch became solid encapsulating the essential oil. To study the morphology, particle size distribution, bulk density and efficiency of encapsulation of lavandin oil, experiments were carried out varying the lavandin oil concentration in the emulsion, gas to product ratio (GPR) pre-expansion temperature, pre-expansion pressure. Experiments with and without static mixer were also performed. Experimental conditions and results are shown in Table 5.

	E1	E2	E3	E7	E 8	E 4	E 5	E 6	E 9	E 10	E 11
Encapsulated oil %	30	52	18	34	32	18	6	13	43	46	45
Encapsulated linalool%	32	86	15	46	24	35	9	27	60	51	78
mg $_{lavandin}$/g $_{product}$	46	82	40	92	70	48	18	35	117	211	199
mg $_{linalool}$/g $_{product}$	15	42	11	39	17	29	8	22	87	64	108
mg oil deliver/g product in	0.2	6.1	2.8	3.5	8.7	17.	16.2	9.0	55.6	145.5	160.7
d(0,5) µm	21±1	25±0	194±	49±	25±0	29±	15±0	40±0	33±0	46±0	48±1
Specific surface (m^2/g)	1,26	0,30	0,64	0,5	0,92	1,7	1,12	0,48	-	-	-
Bulk density (kg/m^3)	167	337	260	175	150	-	-	-	156	114	181
Residual moisture %	6.71	5.88	4.78	4.1	5.75	5.0	4.72	5.15	5.49	4.57	5.26

Table 5. PGSS-drying results.

As presented in table 5, the efficiency of encapsulation of lavandin oil and linalool in OSA-starch microcapsules varies in the ranges 6-52% and 9-85%, respectively. It can be seen that both the encapsulation efficiency and the amount of oil encapsulated per unit mass of product ($m_{lavandin}/m_{product}$) decrease when the initial oil/starch ratio is increased, because the formed emulsion is less stable as the amount of lavandin oil increases. It can also be seen that the use of the static mixer is another important parameter, because when the static mixer is not used the encapsulation efficiency decreases to 6-18%. As shown in Figure 9, encapsulation efficiency strongly increases when pre-expansion temperature is decreased, because lavandin cooling rate increases allowing particles to form early. Pre-expansion pressure and GPR have a smaller effect on encapsulation efficiency. Nevertheless it can be appreciated that encapsulation efficiency slightly increases with pre-expansion pressure because Joule-Thomson effect is stronger. The residual moisture content, measured in samples obtained, was in the range 6.7- 4.1 wt%, which is similar to the water content in unprocessed OSA-starch (5 wt%).

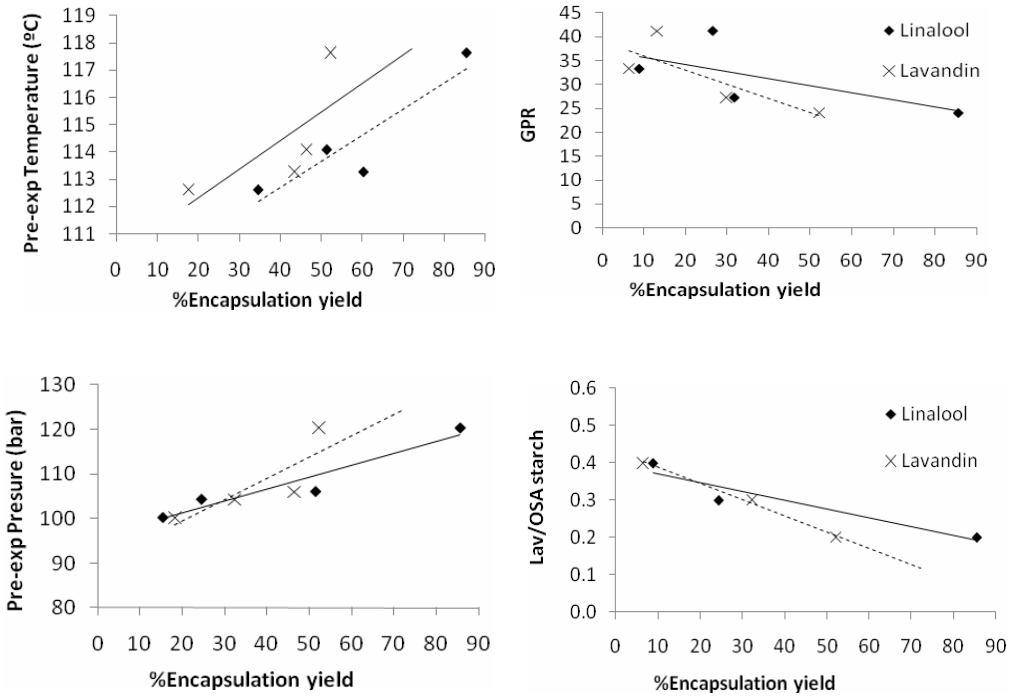


Figure 9. Influence of process parameters on encapsulation efficiency of linalool (solid line) and lavandin oil (dashed line) by PGSS-drying.

Figure 10 shows the influence of different process parameters on particle size. Particle size decreases when pre-expansion temperature is decreased, when pre-expansion pressure is increased and when the gas/liquid ratio is increased. All these variations in process parameters promote a higher concentration of CO_2 in the liquid phase after the static mixer, which enhances the atomization during the depressurization, leading to the formation of smaller particles [16]. As shown in Figure 11, in some experiments bimodal particle size distributions were obtained, which can be due to agglomeration caused by non-encapsulated essential oil, or to formation of mixtures of microspheres and needles, as it will be discussed in the next paragraphs.

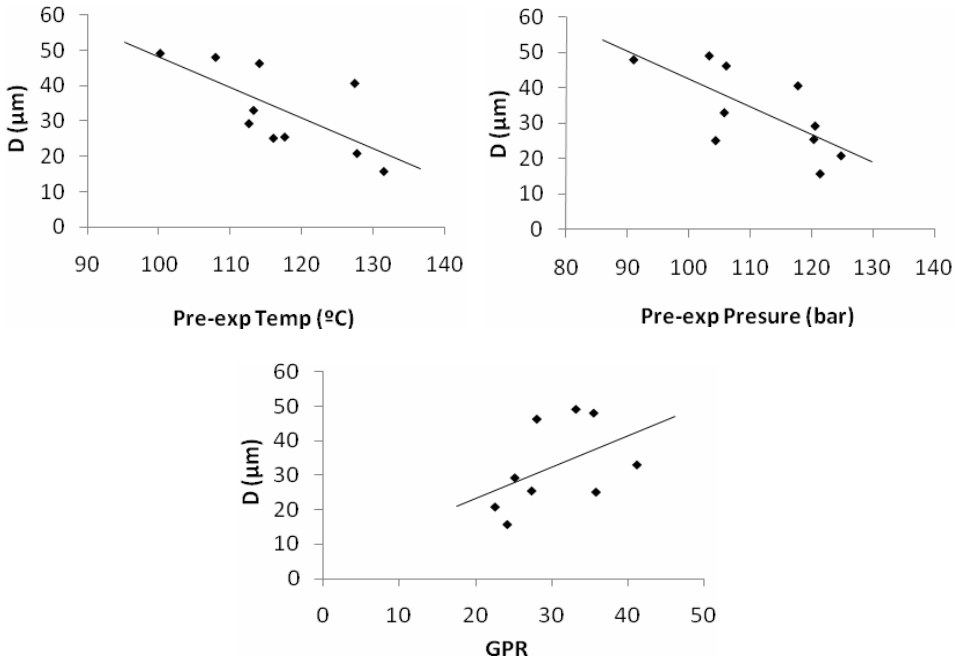


Figure 10. Influence of different parameters on particle size of particles obtained by PGSS-drying.

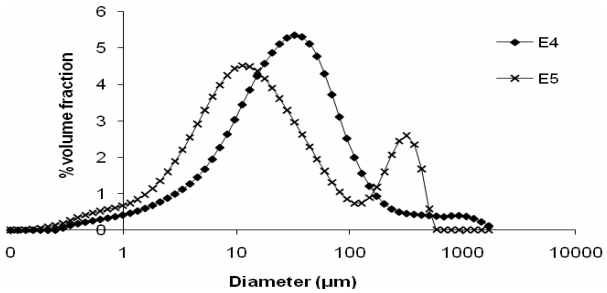


Figure 11. Particle size distributions of particles obtained by PGSS drying.

Figure 12 shows SEM micrographs of particles obtained by PGSS-drying. Two main different particle morphologies were obtained by PGSS-drying: spheres and needles. Needles are probably constituted by OSA-starch without encapsulated oil, while spheres may be loaded with essential oil. It can be seen that at low pre-expansion temperatures many needles and few spheres are produced (Figure 12b, 12d, 12h, 12k, 12i and 12j), while at high pre-expansion temperatures mostly small spheres are produced (Figure 12a, 12c, 12e and 12f). Similarly, when pre-expansion pressure is high the sphere morphology is predominant (Figure 12a, 12e, 12f), but at lower pre-expansion pressure more needles are produced. Therefore, it can be concluded that the generation of spheres is favoured by high pre-expansion temperatures and pressures. These results are coherent with the observations of Wendt et al. [12]. It can be observed that in many cases some agglomerates are formed, because the oil is binding together spheres via capillary forces.

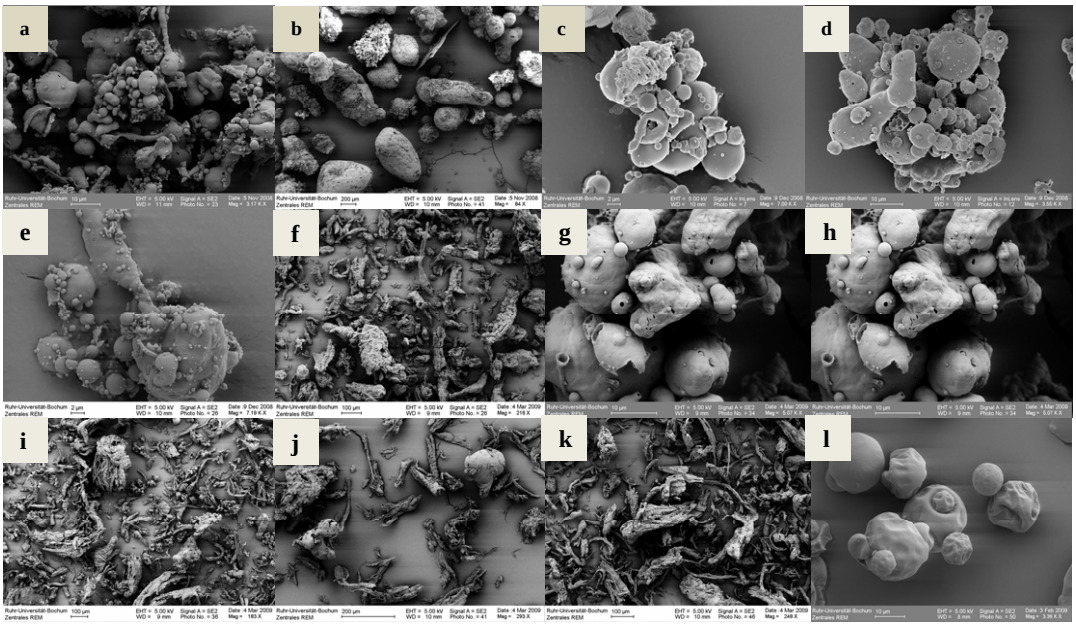


Figure 12. lavandin oil-loaded OSA starch particles produced by PGSS-drying a)experiment E1, b) experiment E2, c) experiment E3, d) experiment E4, e) experiment E5, f) experiment E6, g) experiment E7, h) experiment E8, i) experiment E9, j) experiment E10, k) experiment E11, l) Particles produced by PGSS-drying of OSA-starch without lavandin essential oil.

In the experiments with the highest encapsulation efficiency, spherical particles with a smooth and closed surface were obtained (e.g. Figure 12a and 12b), without dark areas in the surface, which would indicate the presence of non-encapsulated essential oil. In other cases, the spherical particles are broken or show pores (e.g. Figure 12e). In these cases smaller encapsulation efficiencies were also observed. The main factor determining whether open or closed composites are generated is the different between the velocities of solidification and phase separation of the emulsion. If the amount of oil in the emulsion is increased, the oil droplets in the emulsion are bigger, and the emulsion becomes less stable. As a results phase separation occurs faster than solidification and open composited are formed when the amount of lavandin oil in the emulsions increases.

In order to study the stability of the encapsulation of oil, the amount of oil released after 20 days was determined for all samples, following the method indicated in section 2.6. The obtained results, shown in Table 4 suggest that the proportion of oil released is mainly related to the amount of oil in the initial emulsion and the amount of oil in the final product, as shown in Figure 13. In the experiments with low oil concentrations, the fraction of oil released after 20 days was lower than 20%, but when the concentration of oil was higher, as much as 60% of the encapsulated oil was released after 20 days.

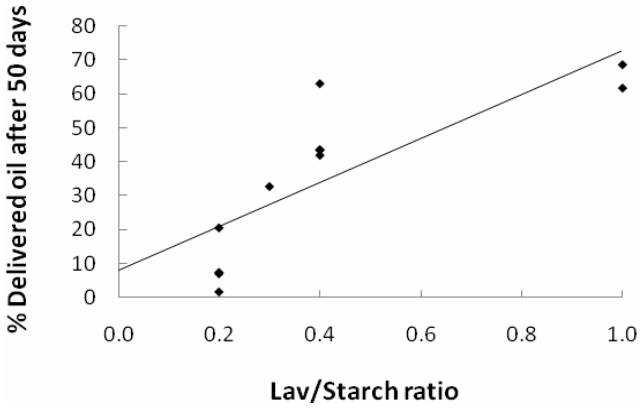


Figure 13: Fraction of oil released after 20 days of storage.

4. Conclusions

Due to their potential applications as agrochemical formulations, an exhaustive study was performed in order to obtain stable capsules of lavandin oil using biodegradable polymers (OSA-starch and polyethylene glycol) as carrier materials. High pressure technology (PGSS-drying and PGSS) was successfully applied for the production of OSA starch and PEG microcapsules filled with lavandin oil. Results show whether and how particle size, morphology, bulk density and percentage of encapsulated oil are influenced by operating conditions.

The effectiveness of microencapsulation is the most important property of the product. Encapsulation efficiency obtained by PGSS-drying varied between 6-55%. Higher encapsulation efficiency varying between 14 - 66 % was obtained in PEG microspheres produced by PGSS. This result can be expected, because for PGSS no water has to be removed and operating conditions are milder comparing with PGSS-drying. In both processes a fraction of the oil has been lost by evaporation, solubilised in CO_2.

Lavandin oil release from OSA microcapsules was studied in order to establish that the formulation suits its purpose. The fraction of oil released after 20 days was found to be mainly dependent on the concentration of lavandin oil in the initial emulsion and the final product.

Particles produced obtained by PGSS drying usually present two types of morphology: spherical particles and small irregular shaped crystals and needles. Results suggest that, starting from an emulsion, particle morphology, particle size distribution and encapsulation efficiency are nor only related to the precipitation process but also related to solvent elimination process. It can be concluded that PGSS-drying has some limitations, because the needle morphology is not appropriate if a controlled release of the oil is required. In case of PGSS spherical particles with some agglomeration had been obtained. This result together with the higher encapsulation efficiency achieved in the encapsulation in PEG by PGSS allow concluding that of the two processes studied, PGSS is more adequate for the encapsulation of lavandin essential oil.

5. Acknowledgements

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6. References

[1] Isman. MB. Plant essential oils for pest and disease management. Crop. Prot. 19 (2000) 603-608.

[2] MA Abo-El Seoud et al. Biocides formulation of Essentials oils having antimicrobial activity. Archives of phytopathology and plant protection. 38 (2005) 175-184.

[3] S. Varona, Á. Martín, M. J. Cocero. Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches. Chem. Eng. Proc. 48 (2009) 1121-1128.

[4] Ruberto G And Baratta M. Antioxidant activity of selected essential oil compoents in two lipid model systems. Food Chem. 69 (2000) 167-174.

[5] Boyom F F, Ngouana V, Zollo APH, Menut C, Bessiere JM, Gut J, Rosenthal P, 2003. Composition and anti-plasmodial activities of essential oils from some Cameroonian medicinal plants. Phytochemistry. 64 (2003) 1269-1275.

[6] Griffin SG, Wyllie G, Markham JL, Leach DN. (1999). The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour Frag. J. 14 (1999) 322-332.

[87] Á. Martín, S. Varona, A. Navarrete, M. J. Cocero. Encapsulation and co-precipitation processes with supercritical fluids: applications with essential oils. The Open Chem. Eng. J. 4 (2010) 31-41.

[8] F. Miguel, Á. Martín, F. Mattea, M. J. Cocero. Precipitation of lutein and co precipitation of lutein and poly lactic acid with the supercritical anti solvent process. Chem. Eng. Proc. 47 (2008) 1594-1602.

[9] M. J. Cocero, A. Martín, F. Mattea, S. Varona. Encapsulation and co precipitation processes with supercritical fluids: fundamentals and applications. J. Supercrit. Fluids. (2009) 546-555.

[10] E. Weidner. High pressure micronization for food applications. J. Supercrit. Fluids. 47 (2009) 556-565.

[11] G. Brandin. Micro particles filled with liquid using the particles from gas saturated solutions technology, in AIChE Annual Meeting, conference proceedings (2006).

[12] T. Wendt, G. Brandin, A. Kilzer, E. Weidner, M. Peterman. Generation of fluid-filled micro particles using PGSS technology. Proceedings of European Congress of Chemical Engineering (ECCE-6) Copenhagen (2007).

[13] E. Weidner, V. Wiesmet, Z. Knez, M. Skerget. Phase equilibrium (solid-liquid-gas) in polyethyleneglycol – carbon dioxide systems. J. Supercrit. Fluids. 10 (1997) 139-147.

[14] E. Weidner, M. Petermann, K. Blatter, V. Rekowski. Manufacture of powder coatings by spraying of gas-enriched melts. Chem. Eng. Technol. 24 (2001) 529-533.

[15] D. Meterc, M. Petermann, E. Weidner. Drying of aqueous green tea extracts using a supercritical fluid spray process. J. Supercrit. Fluids. 45 (2008) 253-259.

[16] Á. Martín, H. M. Pham, A. Kilzer, E. Weidner. Particles from gas saturated solutions (PGSS)-drying process: fundamentals and application to Micronization of polyethyleme glycol by PGSS (Particles from Gas Saturated Soluitons)-drying of aqueous solutions. Chem. Eng. J. 49 (2010) 1259-1266.

[17] F. Mattea, Á. Martín, C. Schulz, P. Jaeger, R. Eggers, M. J. Cocero. Behavior of an organic solvent drop during the supercritical extraction of emulsions. AIChE J. 56 (2009) 1184-1194.

[18] Á. Martín, V. Silva, L. Pérez, J. García-Serna, M.J. Cocero. Direct synthesis of linalyl acetate from linalool in supercritical carbon dioxide. Chem. Eng. Technol 30, 2 (2007) 726-731.

Chapter IV

Supercritical impregnation of lavandin essential oil (*Lavandula hybrida*) in modified starch

Lavandin (Lavandula Hybrida) essential oil contains components with biocide and antiviral properties that can be used as substitutes of synthetic drugs in livestock. This application requires an appropriate formulation of the essential oil. Supercritical impregnation of lavandin oil has been proposed as a possible formulation process, due to the high solubility of lavandin essential oil in supercritical carbon dioxide. The polymer used in this work as a matrix for impregnation was modified starch with the group n-octenil succinate (OSA) in form of powder of 27μm. The effects of operational pressure (10-12 MPa), temperature (313-323 K) and lavandin oil to starch ratio (0.2-1) were studied on the essential oil yield. Impregnation time was kept constant and equal to 2 hours. Impregnation loads ranging between 25 and 147 mg lavandin oil/g OSA-starch were obtained. The distribution coefficient of essential oil between the starch and the supercritical phase as well as the impregnation load depended on the density of CO_2.

1. Introduction

Nowadays there is a growing social demand for environmentally-friendly technologies and for natural products, particularly in sensitive fields such as food applications. These demands are reflected in changes in legislation, which has become more restrictive with respect to the application of chemicals in agriculture, livestock farming and food. Therefore, there is a considerable interest in the development of new biocides and growth promoters as substitutes of the conventional chemical compounds used in these applications. This work is part of a global research whose main aim is to achieve an optimal formulation for the use of lavandin essential oil as biocide in agriculture and livestock.

In recent years, the relevance of supercritical fluid processes in the production of drug delivery system has sharply increased, especially due to their environmentally friendly characteristics. Processes like RESS (Rapid Expansion of Supercritical Solutions), GAS (Gas Anti-Solvent), SAS (Supercritical Anti-Solvent) and PGSS (Particles from Gas Saturated Solutions) have been widely studied with promising results. However, the control of the characteristics of particles produced with these processes still remains a difficulty, particularly in the case of co-precipitation. Impregnation in supercritical fluid media ($scCO_2$) can be an alternative to obtain drug delivery systems, allowing to incorporate the active compound into previously formed particles of carrier material. This method presents advantages related to the plasticizing effect (glass transition temperature depression) and swelling effect caused by supercritical fluids on polymers [1,2]. CO_2 at high pressure can be dissolved in polymers reaching a concentration up to 10-30 %wt, resulting in polymers swelling due to intermolecular forces (Van der Waals, hydrogen bond, acid-base and donor-acceptor). The increase in the free volume of CO_2-swollen polymers (transition from glassy state into a rubbery state) improves the diffusion rate and penetration of solute molecules into the polymeric matrix [3]. The impregnation of drug molecules can occur as a consequence of the balance of interactions between drug, polymer and supercritical fluids, resulting in an adsorption or a physic-chemical attachment of drug molecules to the polymeric matrix. Alternatively, after a quick decompression the solvent power of the $scCO_2$ decreases, causing the

precipitation of the active compound entrapped in the nets of the polymer, as well as a contraction of the carrier matrix [4].

There are several studies on supercritical impregnation of active pharmaceutical ingredients (APIs) toward producing controlled release drugs. Some of the supports used in these works are chitosan derivatives [5], activated carbon [6], poly(methyl methacrylate) [7], poly(vinylpyrrolidone) [8], poly(DL-lactide-co-glycolide) [9], poly(ε-caprolactone) [10], silica matrices [11], wood [12], commercial soft contact lenses [13] or cotton fibres [14]. All these authors have explored quite similar impregnation conditions of pressure (9-25 MPa) and temperature (303-328 K).

This work is focused on the impregnation of lavandin essential oil on a n-octenyl succinate (OSA) modified starch. Essential oils are highly soluble in $scCO_2$, and therefore the supercritical fluid can be an effective fluid medium for impregnating these compounds into a polymer. In fact, one of the first applications of this process with CO_2 was impregnation of synthetic polymers with fragrances [15, 16]. The influence of the main process conditions (pressure, temperature, composition) on the impregnation process (essential oil loading and distribution coefficient) has been analyzed.

2. Materials and Methods

2.1 Materials

Lavandin essential oil "super" used in this project was purchased from COCOPE (Valladolid, Spain). This oil was produced by steam distillation. CO_2 was provided by Praxair (Portugal). Modified OSA starch derived from waxy maize was provided by National Starch Group (Hamburg, Germany).

2.2 Supercritical impregnation experiments

The supercritical impregnation apparatus used in this work is schematically represented in Figure 1. This discontinuous apparatus is comprised of a high pressure stainless steel impregnation vessel/cell (22 cm^3 of internal volume), a high pressure CO_2 liquid pump, a temperature controlled bath, a magnetic stirring plate and a pressure transducer. The cell contains the lavandin oil (0.43 g) at the bottom and the modified starch to be

impregnated (0.44-0.64 g) in a stainless mesh elevated 3 cm from the bottom by a support.

An assay consists of immersing the impregnation cell in the water bath until the desired temperature is achieved and then introducing CO_2 in the cell until the desired pressure. In these conditions, lavandin oil is solubilised in $scCO_2$ and homogenization is achieved by magnetic stirring. Impregnation time was kept constant at 2 hours in all the experiments. Afterwards, the system was depressurized. Two different depressurization methods were tested: fast depressurization (total depressurization time: approximately 1 minute) and slow depressurization (depressurization rate of 0.007-0.015 MPa/min) after decreasing the temperature down to 10°C in order to decrease the solubility of lavandin oil in CO_2 before depressurization. Several operational conditions (pressure: 10-12 MPa, temperature: 313-323 K) and lavandin oil/modified starch ratio (0.6-1) were tested in the impregnation experiments, and the effect of these parameters on the impregnation yield was evaluated considering the total amount of impregnated essential oil.

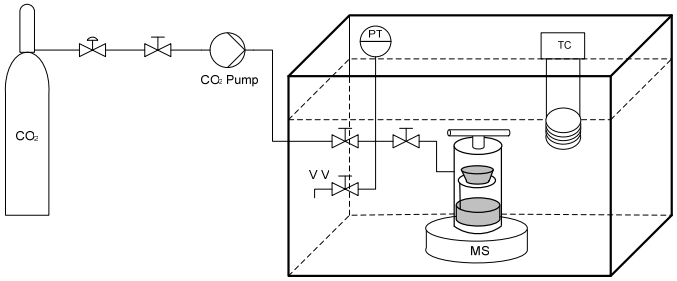

Figure 1. Diagram of Supercritical Impregnation Plant. MS: Magnetic stirrer, VV: vent valve, PT: High pressure transducer, TC: Temperature controler.

2.3 Determination of the amount of lavandin oil impregnated in modified starch.

The amount of essential oil impregnated in OSA-starch microparticles was determined by two independent methods: gravimetrically, determining the mass of the sample

before and after the impregnation, and by chromatographic analysis. In the second case, the impregnated lavandin oil was extracted with hexane. This extraction was performed dissolving 0.2 g of the powder in 2 mL of a dissolution 0.5 % wt/wt n-hexenal (internal standard) in hexane. The mixture was vigorously vortexed during 1 minute and filtered to eliminate starch particles. The extracted essential oil was analysed with a gas chromatograph coupled with a mass spectrometer (GC-MS) Agilent 6890/5973 (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5ms Capillary GC column. The operating conditions were the following: Helium was the carrier gas at 0.7 mL/min, split mode injection (200:1), injection temperature 250ºC and injection volume 1µL. The oven temperature was programmed as follows: 5 min at 65ºC and 4ºC/min to 220ºC. Identification of compounds was based on relative retention times, matching with NIST MS library or by comparison of their relative retention times with those of authentic samples.

2.4 Particle size and morphology analysis

The morphology of the particles was examined with environmental scanning electron microscope (ESEM-Quanta 200-F).

3. Results

Impregnation experiments were carried out following the experimental protocol described in section 2.2. The employed experimental conditions and results (lavandin oil loading of particles and impregnation efficiency) are presented in Table 1. Several experiments were performed in triplicate in order to test the reproducibility. The corresponding standard deviation evaluated from these repetitions ranged between 1 and 14%.

Process conditions are represented in Figure 2 with respect to the phase boundaries. Experimental conditions (pressure and temperature) were maintained above the critical point of the mixture lavandin key compounds (linalool and linalyl acetate) and CO_2, and therefore a practically complete miscibility of the essential oil in the supercritical fluid is expected.

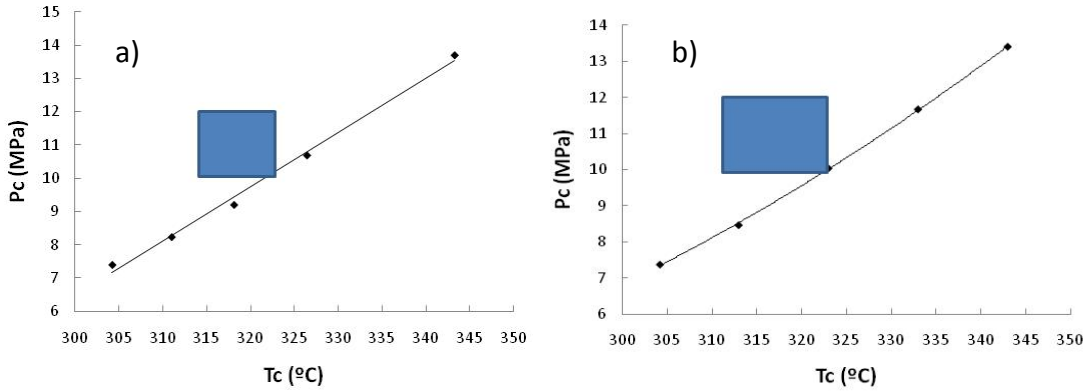

Figure 2. Critical points for a) linalool-CO_2 mixtures [17,18] and b) linalyl acetate-CO_2 mixtures [19].

Results revealed that the impregnation loads achieved (25-147 $mg_{lavandin}/g_{product}$) were in the range of loads obtained by other processes like PGSS (particles from gas saturated solutions) or PGSS-drying [20]. With respect to the fraction of the essential oil introduced in the system which is impregnated in the starch, low efficiencies were obtained, ranging from 4% to 23%. However, it must be noted that this efficiency depends on the mass balance: lavandin oil will be distributed between the starch and the supercritical fluid according to the equilibrium conditions, and if the amount of supercritical phase with respect to the amount of starch is increased, the fraction of essential oil impregnated will decrease. Therefore the efficiency of impregnation of essential oil could be optimized with a careful design of the cell and an optimization of the amounts of CO_2, essential oil and starch used.

Operational conditions				Product characteristics			
Depressurization	P(MPa)	T (K)	Lav/OSA	$mg_{lav}/g_{product}$	$mg_{lin}/g_{product}$	%Lavandin	%Linalool
Fast	10	323	0.21	31		15	
	10	323	0.68	144±1	62±2	22±1	18±1
	11		0.68	96	56	14	16
	12		0.64	84	0	13	14
	10	318	0.69	89	0	13	0
	12		0.68	71	41	10	12
	10	313	0.66	86	44	13	13
	12		0.68	45	25	7	7
	10	323	1	147	65	15	13
	11		1	105	5	11	1
	12		1	96	58	10	11
	10	318	1	136		14	13
	12		1	94	24	9	8
	10	313	1	131±2	78±4	13±2	15±3
	12		1	92	30	9	6
Slow	10	323	0.21	27		13	
	10	323-283	0.67	82	44	12	13
	11		0.68	77	77	11	22
	12		0.65	56	56	9	0
	10	318-283	0.69	67	0	10	0
	12		0.68	53	30	8	8
	10	313-283	0.67	57	32	8	9
	12		0.68	25	14	4	4
	10	323-283	1	107	77	11	15
	11		1	92	0	9	0
	12		1	90	47	9	9
	10	313-283	1	95	51	10	10
	12		1	66	0	7	0

Table 1. Operational conditions and results (particles lavandín oil load and encapsulation efficiency) of lavandin oil supercritical impregnation of modified starch.

Figure 3 shows the amount of lavandin oil impregnated in OSA-starch as a function of temperature, pressure and the ratio between the amounts of lavandin oil and OSA-starch used in the experiments. It can be observed that the amount of essential oil impregnated per unit mass of OSA-starch increases when temperature is increased and when pressure is decreased. In general, the solubility of the active compound in CO_2 is expected to

increase when pressure is increased (and, in this range of conditions, when temperature is decreased, although this depends on the specific phase behaviour of each system and the corresponding crossover temperature). However, this factor is not relevant for the present study, because as previously discussed and shown in Figure 2, process conditions were selected in order to operate above the critical mixture point and to have complete miscibility between CO_2 and essential oil. Indeed, results show that higher impregnation loads were obtained in conditions that are in general unfavourable for the solubility of the active compound in CO_2, demonstrating that this parameter had no influence in this study.

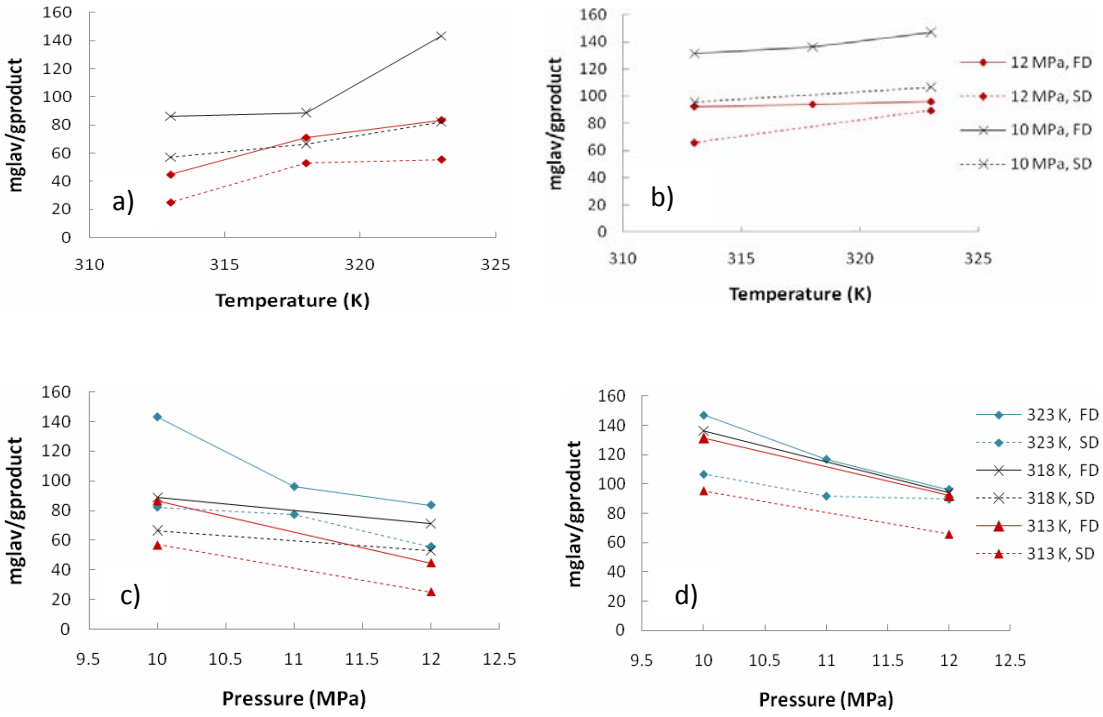

Figure 3. Influence of impregnation parameters on impregnation load. **SD**: slow depressurization **FD**: fast depressurization.a) 0.6 lav/OSA, b) 1 lav/OSA, c) 0.6 lav/OSA, d) 1 lav/OSA.

Having eliminated the solubility of the active compound in CO_2 as an influencing factor over the process, other aspects to be considered are the degree of polymer swelling caused by CO_2, and the balance of interactions between CO_2, OSA-starch and essential oil. As far as authors know, there is no experimental information available related to the

phase behaviour and the swelling of CO_2 + OSA-starch systems. However, in general higher CO_2 solubility and a stronger swelling effect are expected when pressure is increases [20], leading to a better diffusion of $scCO_2$ + drug phase into the polymer. On the other hand, as pressure increases $scCO_2$-lavandin oil interactions are stronger [6, 15] and the swelling of the polymer matrix causes the weakening of chemical interactions between lavandin oil and the basic sites of polymer matrix (OSA starch) [22]. This produces a higher affinity between lavandin oil and $scCO_2$, leading to lower impregnation loads. Figure 4 presents the impregnation loads of essential oil as a function of CO_2 density. It can be observed that the load of essential oil is drastically reduced when CO_2 density is increased, as a consequence of the variations in the interactions between compounds.

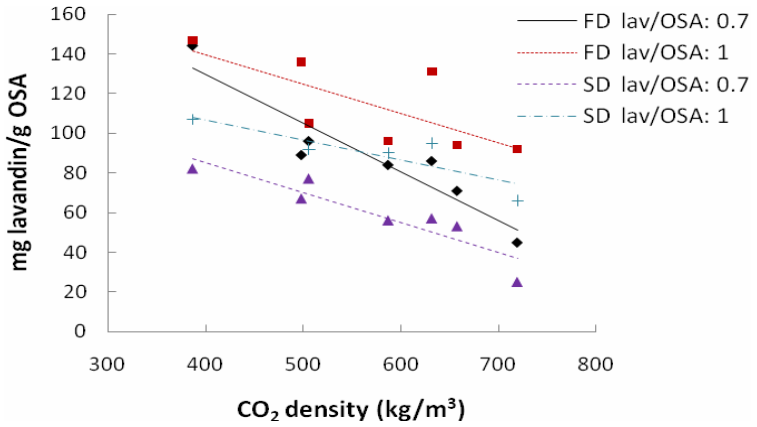

Figure 4. Lavandin oil impregnation load as a funtion of CO_2 density, the ratio between lavandin oil and OSA-starch and the method of depressurization (**SD**: slow depressurization **FD**: fast depressurization).

As shown in Figure 3 and Figure 5, the values of lavandin oil load are significantly different according to the method of depressurization. A fast depressurization gives higher loads than a slow depressurization after a decreasing of the temperature at 10ºC. Comparing the results obtained at the same pressure and temperature and different lavandin oil/OSA-starch ratios, it can be observed that, as it may have been expected, higher impregnation loads were obtained when this ratio was increased.

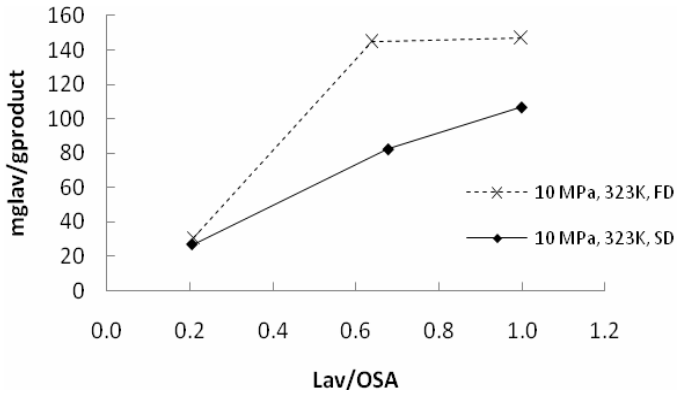

Figure 5. Lavandin oil impregnation load as a funtion of the method of depressurization and the ratio lavandin/OSA-starch

The influence of process conditions on the impregnation load can also be characterized studying the variation of the partition coefficient of lavandin oil between phases. As shown in Eq. (1), the partition coefficient is defined as the concentration of essential oil in the polymer over its concentration in the fluid phase and determines the amount of active drug that can be placed in the matrix.

$$Lavandin\ Partition\ Coeficient\ g/g = \frac{M_{lavadin\ impregnated} / M_{OSA}}{M_{lavandin\ \ initial} / M_{CO2}} \qquad (1)$$

As shown in Figure 6, lavandin oil partition coefficient of lavandin oil (g/g) slightly decreases as CO_2 density increases in all cases. Lavandin oil impregnated load follows the same trend, decreasing as the CO_2 density increases. This could be explained because although swelling effect contributes to the ease of the impregnation, but the partition between the fluid and the polymer phase is a major factor governing the amount of additive absorbed. This results sustain the previously hypothesis, confirming that lavandin oil-modified starch interactions increase with density. In Figure 6 it can be seen that favourable partition coefficients for the impregnation, higher than 1, have been obtained.

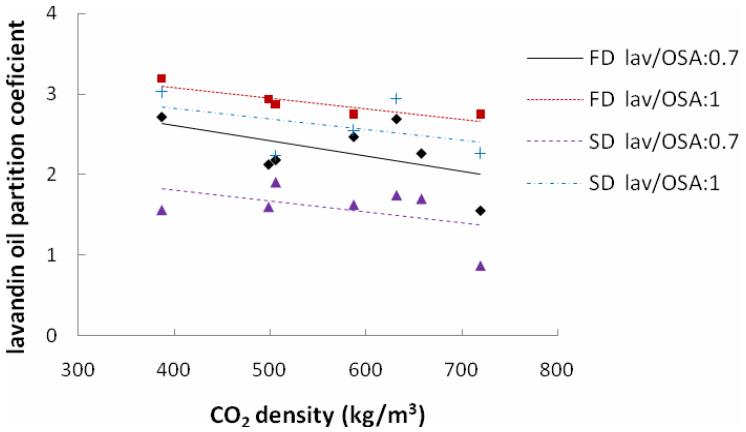

Figure 6. (A) Lavandin oil partition coeficient as a funtion of CO_2 density. (B) Lavandin oil impregnation load as a funtion of CO_2 density.

Figure 7 shows SEM micrographs of the initial modified starch (mean particle size of 27 μm) and the modified starch after impregnation. It can be appreciated that particles did not suffer any significant change on their morphology, but seem to become more agglomerated due to lavandin oil impregnation.

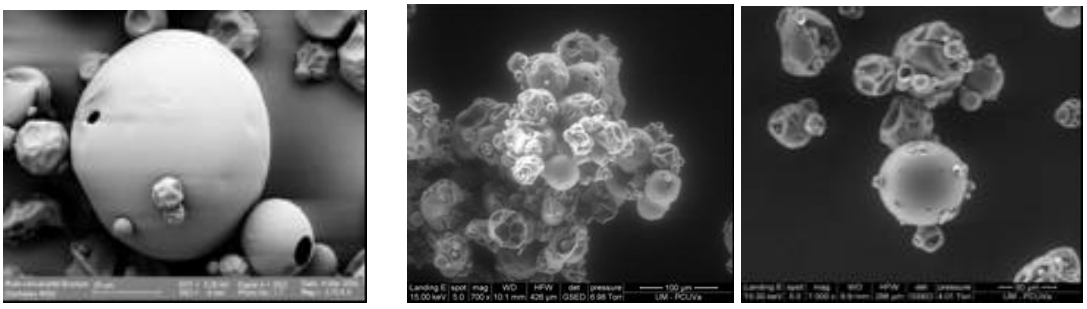

Figure 75. SEM micrographs of modified starch particles before (1) and after impregnation (2,3).

4. Conclusions

In this work, lavandin oil was impregnated in modified starch using $scCO_2$ as impregnation solvent. Lavandin oil loadings obtained in supercritical impregnation were similar to the ones obtained using other processes like PGSS (particles from gas

saturated solutions) or PGSS-drying [21]. On the other hand, the efficiency of impregnation, defined as the fraction of lavandin oil fed to the system that is impregnated in particles, is lower for the same load that the efficiency obtained by PGSS and PGSS-drying, as presented in Table 2.

Process	$mg_{lav}/g_{product}$	$mg_{lin}/g_{product}$	%lavandín encap	%linalool encap
PGSS-drying	117	87	45	78
	70	17	32	24
PGSS	126	33	44	37
	69	10	19	18
Impregnation	107	77	11	15
	71	41	10	12

Table 2. Lavandin oil particles load ($mg_{lav}/g_{product}$) and efficiency for different supercritical fluid processes.

Results indicated that not only essential oil solubility, but also other phenomena, are involved in the impregnation process, as it is referred in the literature [22]. In fact the global process will be the result of several specific interactions as essential oil-scCO_2, essential oil-polymer and polymer-scCO_2 (sweeling and plasticizing effect). The best results both in terms of essential oil load and partition coefficient were obtained with conditions that weakened the interactions between CO_2 and essential oil (i.e., with lower CO_2 densities).

Acknowledgments

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References

[1] S.G. Kazarian, N.H. Brantley, B.L. West, B.L., M.F. Vincent, C.A. Eckert. In situ spectroscopy of polymers subjected to supercritical CO_2: plasticization and dye impregnation. Appl.Spectrosc. 51 (1997) 491-494.

[2] A.R. Berens, G.S. Huvard, R.W. Korsmeyer, F.W. Kunig. Application of compressed carbon dioxide in the incorporation of additives into polymers. Journal of Applied Polymer Science. 46 (1992) 231.

[3] I. Kikic, F. Vecchione. Supercritical impregnation of polymers. Current Opinion in Solid State and Materials Science. 7 (2003) 399–405

[4] A. Cortesi, P. Alessi, I. Kikic, S. Kirchmayer, F. Vecchione, Supercritical fluids chromatography for impregnation optimization. J. Supercrit. Fluids. 19 (2000) 61–68.

[5] M.E.M. Braga, M.T. V. Pato, H. S.R. Costa Silva, E. I. Ferreira, M.H. Gil, C.M.M. Duarte, H. de Sousa. Supercritical solvent impregnation of ophthalmic drugs on chitosan derivatives. J. Supercrit. Fluids. 44 (2008) 245–257.

[6] I. Kikic, P. Alessi, A. Cortesi, S.J. Macnaughton, N. Foster, B. Spicka, An experimental study of supercritical adsorption equilibria of salicylic acid on activated carbon. Fluid Phase Equilibria. 117 (1996) 304–311.

[7] M. Temtem, D. Pompeu, G. Jaraquemada, E.J. Cabrita, T. Casimiro, A. Aguiar-Ricardo. Development of PMMA membranes functionalized with hydroxypropyl-β-cyclodextrins for controlled drug delivery using a supercritical CO_2-assisted technology. Int. J. Pharm. 376 (2009) 110-115.

[8] J.R. Bush, A. Akgerman, K.R. Hall. Synthesis of controlled release device with supercritical CO_2 and co-solvent. J. Supercrit. Fluids. 41 (2007) 311–316.

[9. O. Guney, A. Akgerman. Synthesis of controlled-release products in supercritical medium. AIChE Journal. 48 (2002) 856–866.

[10] M.V. Natu, M.H. Gil, H.C. de Sousa. Supercritical solvent impregnation of poly(e-caprolactone)/ poly(oxyethylene-b-oxypropylene-b-oxyethylene) and poly(e-caprolactone)/poly(ethylene-vinyl acetate) blends for controlled release applications. J. Supercrit. Fluids. 47 (2008) 93-102.

[11] V.P. Costa, M.E.M. Braga, J.P. Guerra, A.R.C. Duarte, C. M.M. Duarte, E. O.B., Leite, M.H. Gil, H.C. de Sousa. Development of therapeutic contact lenses using a supercritical solvent impregnation method. J. Supercrit. Fluids. 52 (2010) 306–316.

[12] F. Belhadj-Ahmed, E. Badens, P. Llewellyn, R. Denoyel, G. Charbit. Impregnation of vitamin E acetate on silica mesoporous phases using supercritical carbon dioxide. J. Supercrit. Fluids. 51 (2009) 278-286.

[13] S. Lucas, E. Gonzales, M.P. Calvo, C. Palencia, E. Alonso, M.J. Cocero, Supercritical CO_2 impregnation of Radiata pine with organic fungicides: effect of operating conditions and two-parameters modeling, J. Supercrit. Fluids. 40 (2007) 462–469.

[14] M. Van der Kraan, M.V. Fernandez Cid, G.F. Woerlee, W.J.T. Veugelers, G.J. Witkamp. Dyeing of natural and synthetic textiles in supercritical carbon dioxide with disperse reactive dyes. J. Supercrit. Fluids. 40 (2007) 470–476

[15] A.R. Berens, G.S. Huvard, R.W., Korsmeyer. Process for Incorporating an Additive into a Polymer and Product Produced Thereby. US Patent 4 820 752 (1986).

[16] M.L Sand. Method for Impregnating a Thermoplastic Polymer, US Patent 4 598 006 (1986).

[17] A. Martín, V. Silva, L. Pérez, J. Garcia Serna, M.J. Cocero. Direct Synthesis of Linalyl Acetate from Linalool in Supercritical Carbon Dioxide: A Thermodynamic Study. Chem.l Eng. Technol. 30 (6) (2007) 726-731.

[18] S. Raeissi, C.J. Peters. Bubble-point pressures of the binary system carbon dioxide+linalool. J. Supercrit. Fluids. 20 (2001) 221–228.

[19] E. Franceschi, M.B. Grings, C.D. Frizzo, J.V. Oliveira, C. Dariva. Phase behavior of lemon and bergamot peel oils in supercritical CO_2. Fluid Phase Equilibria. 226 (2004) 1-8.

[20] Á. Martín, H. M. Pham, A. Kilzer, S. Kareth, E. Weidner. Phase equilibria of carbon dioxide + poly ethylene glycol + water mixtures at high pressure: Measurements and modeling. Fluid Phase Equilibria. 286 (2009) 162–169.

[21] S. Varona, S. Kareth, A. Martín, M.J. Cocero. Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture. J. Supercrit. Fluids. 54 (3) (2010) 369-377.

[22] S. G. Kazarian, M. F. Vincent, B. L. West, C. A. Eckert. Partitioning of solutes and cosolvents between supercritical CO_2 and polymer phases. J. Supercrit. Fluids. (1998) 107–112.

Chapter V

Antimicrobial activity of lavandin essential oil formulation

Lavandin (Lavandula Hybrida) essential oil contains components with biocide and antiviral properties that can be used as substitutes of antibiotics. This application requires an appropriate formulation of the essential oil. In the present work, the antimicrobial activity of lavandin essential oil and encapsulated lavandin oil in soybean lecithin, modified starch and poly-caprolactone against three pathogenic bacteria gram-negative: Escherichia coli; gram-positive: Staphylococcus aureus and Bacillus cereus) was determined. The formulations were prepared using innovative high-pressure techniques (PGSS and PGSS-drying) as well as spray-drying. Minimum inhibition concentration of lavandin oil was 7.1 mg/mL for E.coli and S.aureus and 3.6 mg/mL for B.cereus. Results demonstrate that lavandin oil and encapsulated lavandin oil are efficient antimicrobial agents. Enhancement of antibacterial activity by encapsulation was achieved in all the cases.

1. Introduction

Nowadays, public concern about the use of antibiotics in livestock feed has increased, because of their contribution to the emergence of antibiotic resistant bacteria, and their possible transmission from livestock to humans. In the European Union, these concerns drove to the prohibition in year 1999 of most antibiotics as additives in livestock feed, and a total prohibition of the remaining synthetic health and growth promoters since 2006. Similarly, the use of chemical pesticides and herbicides in agriculture is associated with the pollution of the environment and the presence of toxic residues in the plants [1]. Agriculture and livestock farming is thus under social, political and economical pressure towards the development of alternative additives. The solution to this problem can be found in nature itself, as some plants contain active components with demonstrated biocide, antiseptic, insecticide, acaricidal and nematicidal activity, and they can be a source of new effective additives for the farming industry. Some of these plants have been sources of active components for the pharmaceutical industry for decades.

Some essential oils extracted from plants contain compounds which can be used as antibacterial additives [2] or bioactive phytochemicals. The antibacterial activity of essentials oils is mainly associated with their lipophilicity, leading them to cross the plasma membrane causing the disruption of the permeability barrier of the cell membrane structures and the accompanying loss of chemiosmotic control [3]. Several studies have identified mitochondria as one of the possible targets of essential oils, causing enzyme inhibition, mitochondrial respiratory chain inhibition and cell death [4]. The antibacterial properties of essential oils and their components are exploited in diverse commercial products as dental root canal sealers [5], antiseptics [6, 7, 8] and feed supplements for lactating sows and weaned piglets [9, 10]. Some essential oils also have antifungal and insecticide activity [1, 11]. Essential oils are recognized as safe substances (ESO, GRAS - 182.20) and therefore, if correctly dosed, they can be used in food products, for example to prevent post-harvest growth of native and contaminant bacteria [12]. However, the required concentration of the essential oil for an effective biocide action can be about 100 times higher than that of a standard antibiotic (e.g.

streptomycin and nystatin) [13]. Due to this, their use can be limited by organoleptic criteria. Moreover, to provide a good antimicrobial activity, essential oils must be adequately formulated, in order to protect them from degradation by ambient conditions, facilitate the handling and dosage, and provide a controlled release of the active compounds. Some studies focused on improving antibacterial activity of several antibiotics (aminoglycosides, quinolones, polypeptides and betalactames) by encapsulation. These studies reveal that the minimal inhibition concentration for several bacteria generally was reduced by encapsulation. However, if bacteria had a strong resistance against the drugs, even the transport of encapsulated drugs into the cytoplasm did not improve bacteria killing [14].

In order to evaluate the potential applications of essential oil formulations as antimicrobial compounds, it is necessary to evaluate the antimicrobial activity of these formulations and to determine the minimum concentration of the formulation required to inhibit the growth of pathogenic bacteria. In this work, the antimocrobial activity of lavandin (*Lavandula Hybrida*) essential oil formulations (main active compounds of the essential oil: linalool, 44 %wt and linalyl acetate, 30 %wt) against three food-borne bacteria strains (gram-positive and gram-negative) has been evaluated. The bacteria strains chosen for this work were *E. coli*, *S. aureus* and *B. cereus*. *E. coli* is responsible for intestinal tract infections and diarrhoea and is usually found in products derived from cattle. It has been chosen as a model of a bacterial infection in livestock treated by ingestion of the essential oil formulations. *S. aureus* is responsible for sepsis and skin infections, and in this work it has been studied as a model infection that could be treated by a topical treatment with the essential oil formulations. *B. cereus* is responsible for diarrheal and emetic (vomiting) syndrome and it can contaminate rice and vegetables (as well as dairy products and meat), and therefore it is a possible target for essential oil formulations added as biocides to the irrigation of such cultivations.

The formulations tested in this work were prepared using three types of carriers: lecithin, n-octenyl succinic anhydride (OSA) - modified starch and polycaprolactone. The formulations prepared with the two first carrier materials can be easily dispersed in water, stabilizing the essential oil in the form of liposomes, if lecithin is used, or in the micelles of an emulsion, in the case of OSA-starch. On the contrary, polycaprolactone is

insoluble in water and shows a slow degradation in the digestive tract. Therefore, this carrier material can be used to prepare a formulation providing a slow release of the active compounds when ingested.

As encapsulation processes PGSS (particles from gas saturated solutions), PGSS-drying (drying of gas saturated solutions) and spray-drying (SD) of lavandin oil emulsions have been proposed. Spray-drying is one of the best-known conventional technologies for the precipitation and co-precipitation of particles for food and pharmaceutical application, but its main drawback is the high temperature needed. PGSS and PGSS drying are new technologies which use carbon dioxide as solvent. This solvent is non toxic, environmental friendly and will completely leave the product upon depressurization in the last step of the process, thus avoiding the contamination of the product with residual solvent. In comparison with spray-drying, PGSS and PGSS-drying processes allow to work at lower temperatures and therefore reduce lavandina oil losses by evaporation increasing process efficiency. The PGSS process takes advantage to the fact that polymers can be saturated with carbon dioxide decreasing their melting temperature [15]. In the case of spray-drying and PGSS-drying, lavandin oil has to be emulsioned with the carrier material (OSA-starch or soybean lecithin) and these emulsions have to been stable at the corresponding operational conditions. The application of these processes and the characterization of the formulations prepared with them have been described in detail in previous works [16,17].

2. Materials and Methods

2.1 Materials

Lavandin essential oil “super” used in this project was purchased from COCOPE (Valladolid, Spain). This oil was produced by steam distillation. As shell material modified OSA starch derived from waxy maize was provided from National Starch Group (New Jersey, USA), Poly-(ε-caprolactone) 2403D (mean molecular weight: 4000 g/mol; melting temperature: 55ºC - 60ºC) was supplied by Solvay Caprolactones (Solvay Interox Ltd., United Kingdom). Soybean lecithin (97% phospholipids) was

obtained from Glama-Sot (SOTYA, Madrid, Spain). Sodium Chloride was provided by Sigma-Aldrich (Madrid, Spain).

2.2 Preparation of formulations

2.2.1 Spray-drying

Oil-in-water emulsions were dried by spray drying. The spray drier used was Mobile Minor model MM-Basic PSR from GEA Niro. This equipment has a maximum treatment capacity of 4 l/h, maximum inlet air temperature of 330ºC and rotary spray maximum capacity of 15 Nm^3/h.

2.2.2 Particles from Gas Saturated Solutions (PGSS)-drying

Oil-in-water emulsions were drying applying the PGSS-drying process. In this processes the emulsion is saturated with CO_2 causing a decrease of the emulsion viscosity. The emulsion saturated with CO_2 is contacted with the supercritical CO_2 in a static mixer and then expanded in a nozzle, which facilitates the formation of extremely fine droplets which dry very fast resulting in fine powders. By the combination of co-extraction in the static mixer and evaporation of residual water in the spray tower the demand of CO_2 is reduced [18]. A flow diagram of the plant used for PGSS-drying experiments is presented in Figure 1.

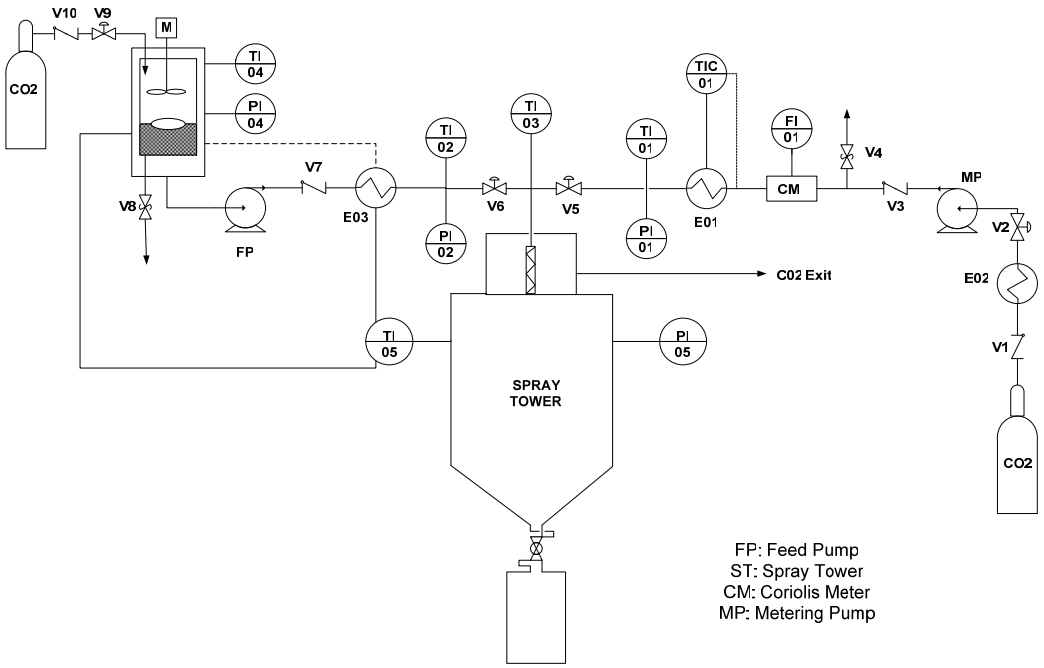

Figure 1. Flow diagram of the PGSS-drying plant

2.2.3 Particles from Gas Saturated Solutions (PGSS)

Poly-caprolactone particles loaded with lavandin essential oil were produced using the PGSS process. Poly-caprolactone and lavandin oil were filled together in a pressure cell where they were intensively mixed in presence of heated CO_2 under high pressure and by magnetic stirring. After a period of 2 h, long enough to reach phase equilibrium, the mixture is depressurized. Upon a rapid expansion through a nozzle to ambient pressure very fine particles, which are collected in a vessel, are produced as the gas comes out of the solution. The driving force for particle formation is the strong cooling as a consequence of Joule Thomson effect during expansion. Due to this sudden reduction in temperature the shell material solidifies and forms a covering layer around the essential oil droplets. A flow diagram of the PGSS plant is presented in Figure 2. Maximum operating pressure and temperature of this plant are 35 MPa and 80ºC respectively. The volumen of the cell was 50 ml and the nozzle diameter was 300 μm (Spraying System Co., Illinois, USA).

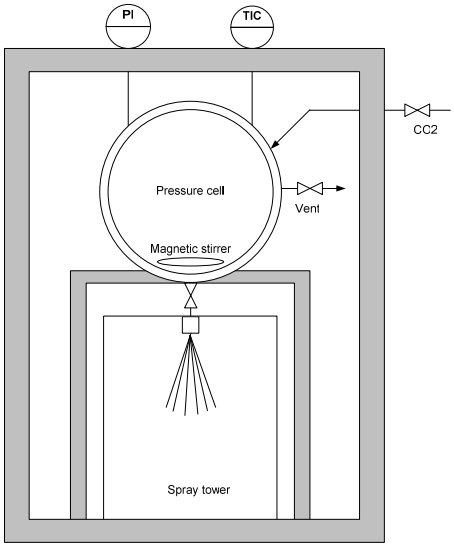

Figure 2. Flow diagram of PGSS plant.

2.3 Particle characterization

2.3.1. Analytical Quantification

The total oil content in the OSA-starch particles was determined by distilling 10 g of powder for 3h in a Clevenger apparatus, weighting then the lavandin essential oil collected in the cold trap. The surface oil was determined by washing 10 g of powder with 500 mL of absolute ethanol. The encapsulation efficiency of soybean lecithin and poly-caprolactone microparticles was determined by dissolving 0.5 g of the power in 2 mL of acetone. The mixture was vigorously vortexed during 1 minute and filtered to eliminate lecithin particles. Superficial, non-encapsulated oil was determined by washing 0.5 g of powder with 2 mL of a solution of 0.5 % wt/wt of n-hexenal in hexane. The suspension was filtered and samples were analyzed by gas chromatography.

Composition analyses of the essential oil were carried out with a gas-chromatograph coupled with a mass spectrometer (GC-MS) Agilent 6890/5973 (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5ms Capillary GC column. The operating conditions were as follows: helium was the carrier gas at 0.7 mL/min, the sample was diluted in hexane including n-hexenal as internal standard and injected in the split mode (200:1), injection temperature 250ºC and injection volume 1 µl. The oven

temperature was programmed as follows: 5 min at 65ºC followed by a temperature ramp of 4ºC/min until 220ºC. Identification of compounds was based on their relative retention times.

2.3.2. Particle size and morphology analysis

Particle size and particle size distribution of the samples obtained by PGSS and PGSS-drying were measured with a laser diffraction method using a Mastersizer 2000 particle analyzer. The morphology of the particles was examined with an environmental scanning electron microscope (ESEM-Quanta 200-F).

2.4 Antibacterial assays

2.4.1. Bacterial strains and culture media

All microorganisms used in this study were obtained from American Type Culture Collection (ATCC), Manassas, VA. The strains studied were E. coli (ATCC 10798), S. aureus (ATCC 6538) and B. cereus (ATCC 14579. The culture media used for each strain was Tryptone Soya Broth (TSB) purchased from Oxoid Inc.

2.4.2 Microdilution assay

Microdilution assay was used to determine the antibacterial activity of the Lavandin essential oil. Briefly, the wells of 96-well microplate were filled with 100 µL of bacteria growing culture plus 100 µL of various essential oil concentrations diluted in TBS (final concentrations range between 0 and 8 mg/mL). For all bacteria, the inoculum tested was 10^6cfu/mL. Then, plates were aerobically incubated during 24 h at 37 °C for *E. coli* and *S. aureus* or at 30 °C for *B. cereus*. The microorganism growth was followed measured the absorbance before and after incubation at 620nm in a PowerWave Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The results obtained were expressed in terms of % of inhibition relative to the control (inoculum without lavandin oil) according to eq (1)

$$\%Inhibition = \frac{\left(A_{24h} - A_0\right)_{oil+inoculum} - \left(A_{24h} - A_0\right)_{control\ \ oil}}{\left(A_{24h} - A_0\right)_{inoculum\ \ free\ growth}} 100 \qquad (1)$$

2.4.3 Plate count assay

The plate count assay was performed as previously described [19]. Briefly, a determined quantity of essential oil (1.13 mg), particles (2 mg) or carrier material (2 mg) were dissolved in 10 ml bacteria suspension (10^6 CFU/mL) in TSB medium and incubated at 37ºC (except for *B.cereus* whose optimal growth temperature is 25ºC) during 24h with a gentle agitation (100 rpm). Control was performed with bacteria suspension only. After incubation, appropriate dilutions of each culture in physiologic solution were done and spread on the surface of solidified agar plates (Tryptone Soya agar medium). Microorganism colonies were counted after 24 h of incubation in order to calculate de percentage of inhibition of each system according with eq (2), where C is the bacterial colonies counted for the control or samples. All assays were performed in duplicate and experimental errors were estimated using standard statistical techniques.

$$\%Inhibition = \frac{C_{freegrowth} - C_{sample}}{C_{freegrowth}} 100 \qquad (2)$$

3. Results and Discussion

Before studying the activity of the formulations with encapsulated essential oil, the antibacterial activity of pure lavandin essential oil was assayed *in vitro* by a micro-dilution method against the three pathogenic bacteria. Figure 3 presents the variation of the inhibition capacity with the concentration of essential oil in the medium. It can be seen that the % of inhibition increases when the concentration is increased, reaching 80% - nearly 100% inhibition efficiencies with essential oil concentrations in the range 2 - 7 mg/mL. Results presented in Table 1 and Figure 3 show that of the three bacterial strains tested, *B. Cereus* has the stronger resistance to the essential oil. This result is in agreement with other experimental results [20] that could find no differences related to the gram-straining reaction. However, most studies reported that generally whole essential oils are slightly more active against Gram+ bacteria than Gram- bacteria [21, 22, 23, 24]. Table 1 summarizes the minimum inhibition concentration (MIC) of lavandin oil for each strain tested, which is the lowest concentration inhibiting the

growth of at least 90% of microbial strain. In this Table, MIC of lavandin oil obtained in this work are compared to the values obtained by others authors [25].

	E. Coli	*S. Aureus*	*B. Cereus*
MIC (10^6 CFU/mL)	7.1	7.1	3.6
MIC (10^3 CFU/mL)	-	-	1.8
MIC (Oussalah et al., 2007)	7.1	7.1	-

Table 1. Minimum Inhibition Concentration (mg/mL) of lavandin oil for two diferent inoculum (10^3 and10^6 CFU/mL) of the microorganim strains tested.

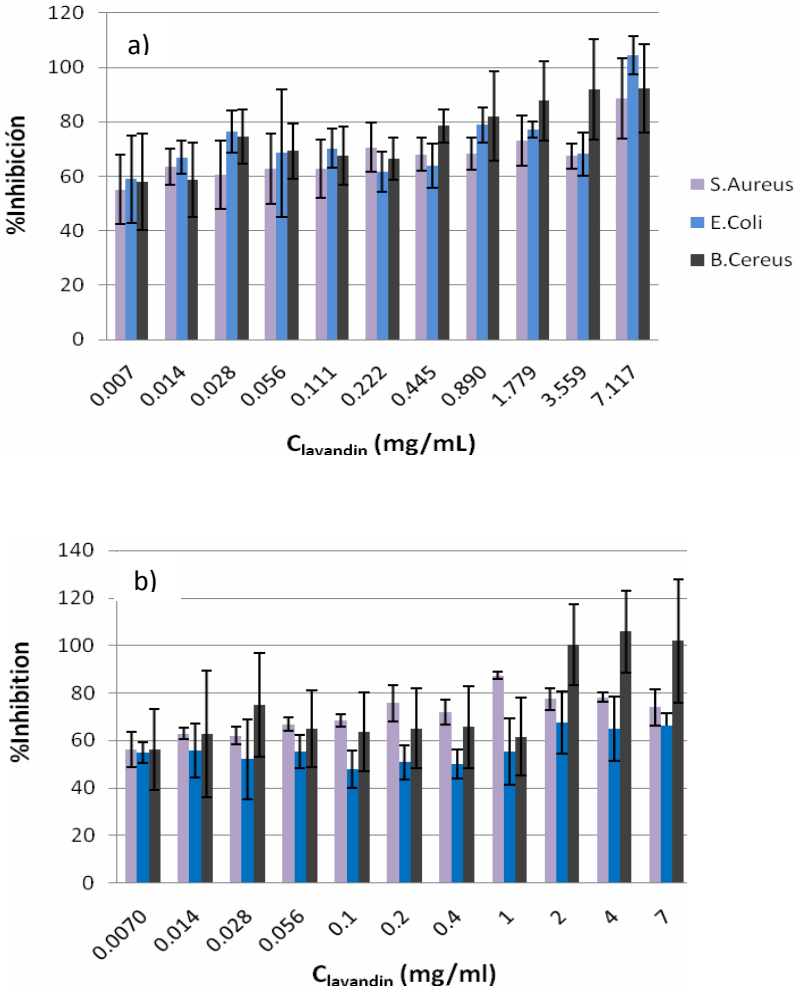

Figure 3. Inhibition caused by crude essential oil for different inoculum (a: 10^6 CFU/ml and b:10^3 CFU/mL) of different bacterial strains.

The antimicrobial activity of each carrier material against each bacteria strain was also tested in order to determine their contribution to the activity of the formulations prepared using these carrier materials. Tests have been performed using the same concentration of carrier material used in the assays with essential oil formulations. These results are presented in Table 2. It can be appreciated that *E.coli* is highly sensitive to lecithin and less sensitive to the modified starch OSA-starch. On the other hand, *S.aureus* is able to hydrolyze lecithin and OSA-starch using these compounds as energy source for their growth. *B.cereus* is also able to metabolize lecithin improving its growth, but it is sensitive to OSA-starch and polycaprolactone.

Strain	*E.coli*	*S.aureus*	*B.cereus*
Lecithin	68±3	-37±9	-56±9
OSA-starch	35±3	-40±6	27±3
CAPA 2403D	-	-	19±4

Table 2. % BacteriaGrowth Inhibition of 0.2 mg/mL of carrier material.

After these preliminary assays, the antibacterial activity of essential oil formulations prepared by encapsulation in OSA-starch, lecithin and polycaprolactone using spray-drying, PGSS and PGSS-drying techniques was analyzed. Table 3 presents the characteristics of the formulations analyzed (particle size, carrier material, lavandin and linalool load), as well as the process used for the precipitation of particles and the temperature, pressure and lavadin/carrier material ratio of process. Figure 4 presents SEM micrographs of some of the particles tested.

Sample	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11
Process	SD	SD	SD	SD	SD	SD	SD	PGSS D	PGSS D	PGSS D	PGSS
Carrier	OSA	OSA	OSA	OSA	OSA	Lec.	Lec.	OSA	OSA	OSA	CAPA
T (ºC) $_{process}$	170	170	170	170	170	170	170	118	136	112	70
Lavandin/Carrier	0.4	1	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.2	0.5
$mg_{lav}/g_{product}$	165	361	231	96	102	24	63	33	57	43	153
$mg_{lin}/g_{product}$	84	205	59	29	49	8	27	15	40	17	82
$D_{0,5}$ part (μm)	14	14	19	14	17	25	9	32	53	65	85
C_{lav}(mg/mL)	0.008	0.079	0.044	0.018	0.028	0.005	0.014	0.007	0.013	0.009	0.03
$C_{linalool}$(mg/mL)	0.006	0.045	0.011	0.005	0.01	0.002	0.006	0.003	0.006	0.004	0.02
$C_{linalyl}$(mg/ml)	0.0007	0.0117	0.0098	0.0047	0.0050	0.0012	0.0031	0.0005	0.0011	0.0023	0.02

Table 3. Characteristic of the particles tested and lavandin oil, linalool and linalyl acetate reached in the antimicrobial assay.

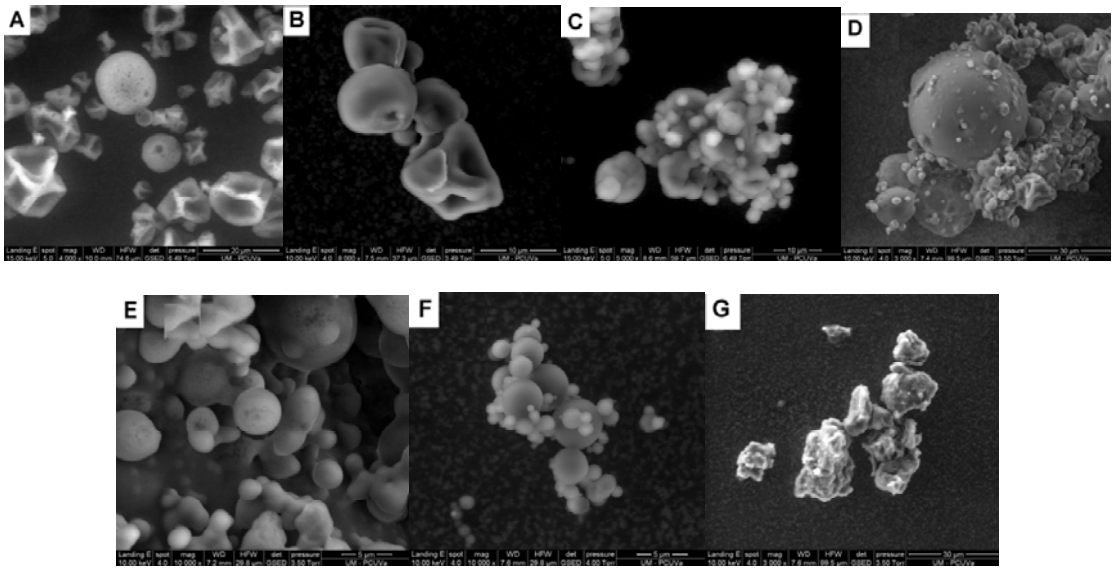

Figure 4. SEM micrographs of some particles tested. a.S01, b. S06, c. S07, d.S08, e. S09, f. S10, g. S11.

The antimicrobial activity of each system against tested strains is presented in figure 6 and table 4. The concentration of particles used was 0.2 mg/ml, because the concentration of lavandin oil reached was almost the minimum inhibition concentration. All bacterial strains demonstrate some degree of sensitivity to lavandin oil and lavandin oil particles. However, the strongest antibacterial activity was seen against *E.coli* (Gram-negative bacteria) and *S.aureus* (Gram-positive bacteria). *B. cereus* (Gram-

positive bacteria) was found to be the most resistant bacteria, in agreement with the results obtained with crude essential oil.

Sample	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	Free oil
S.Aureus	23	-13	59	60	62	25	51	34	41	42	-	66
E.Coli	51	63	66	75	38	68	73	59	61	52	49	45
B.Cereus	40	37	60	32	37	57	26	30	25	70	43	29

Table 4. Inhibition of diferents strains for diferent systems (0.2 mg particles/ml and 0.013mg oil/mL).

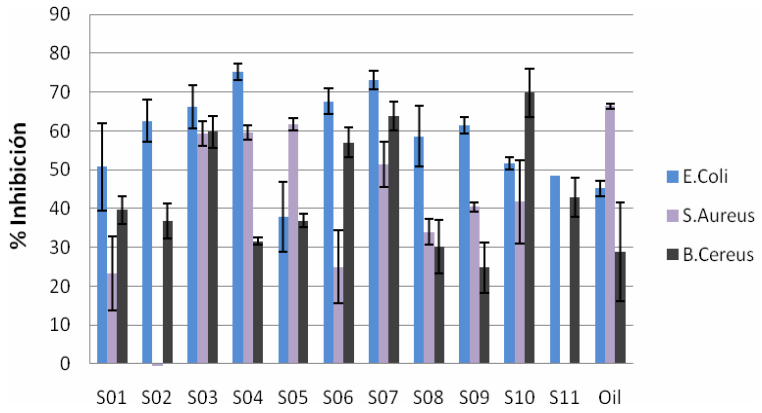

Figure 5. Inhibition of diferents strains for diferent systems (0.2mg particles/ml and 0.013mg oil/mL).

Results presented in figure 6 show that there is a clear relation between the lavandin oil concentration in the particles (table 3) and the inhibition concentration of each particle (figure 4). This relation could be related to the concentration of linalool [26], compound that is known to possess antibacterial activity. Anyway, minor constituents of essential oils may play an important role in the antibacterial activities via a synergistic effect.

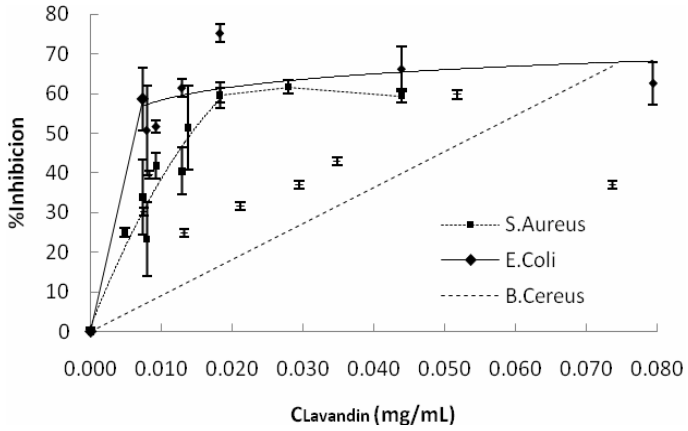

Figure 6. Inhibition of diferents strains (S.Aureus, E.Coli and B.Cereus) as a function of lavandin oil concentration of each system.

Soybean lecithin results to be the most efficient carrier material for lavandin essential oil. As it is shown in table 3, inhibition of soybean particle (S07) is higher than inhibition of OSA-starch particle (S09) or poly-caprolactone particle (S11), having all the particles a similar lavandin oil and linalool load. Soybean lecithin is a complex mixture of phospholipids which forms liposomes spontaneously by self-assembly in aqueous solutions. These structures can retain water-soluble substances in the inner aqueous phase and oil-soluble substances (lavandin essential oil) in the lipid bilayer membrane. Due to similarity to cell membranes, liposomes can transport biocide and antiviral agents across cells membranes and cytoplasmatic barrier of cells, enhancing intracellular drug delivery [27]. Gram-negative bacteria have an outer membrane structure formed by a phospholipids bilayer with anchored lipopolysaccharides (Lipid A, core polysaccharide and O-polysaccharide). Outside this membrane, gram-negative bacteria have a thin peptidoglycan layer and finally the inner cytoplasmatic layer. In contrast, Gram-positive bacteria only have a thick peptidoglycan layer and then the inner cytoplasmatic layer. A hypothesis is that liposomes formed by soybean lecithin could cross both phospholipids layers of gram-negative bacteria and deliver inside of the cell the essential oil. The thick peptidoglycan layer of gram-postive bacterias may difficult the action of the particles in gram-postive particles.

Encapsulation process is another important parameter determining the antimicrobial activity, because it determines the lavandin oil load and composition of particles. Particles obtained by PGSS-drying (experiment S08 and S09) show a higher inhibition capacity than particles produced by spray drying (S01) with an equivalent concentration of lavandin oil and linalool. This can be explained based on the fact that spray drying uses high temperatures which could compromise the biological activity of the essential oil antimicrobial compounds.

4. Conclusions

The present study shows that particles loaded with lavandin essential oil are effective antimicrobial agents. The effectiveness of this action mainly depends on the concentration of essential oil and the encapsulating agent. *E.coli* (Gram-) resulted to be the most sensitive strain, while *B.cereus* and S.aureus (Gram+) were more resistant. The influence of the carrier material is related to the differences in the mode of action of antimicrobial agents due to the differences in the cell membrane of these bacterial groups [28]. Soybean lecithin results to be the most efficient carrier material due to its capacity to spontaneously form liposomes. Particles produced by PGSS-drying showed more antibacterial activity than particles formed by spray-drying with a similar essential oil load.

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References

[1] L.S. Nerio, J. Olivero-Verbel, E. Stashenko. Repellent activity of essential oils: A review. Biores. Tech. 101 (2010) 372-378.

[2] D. Mišić, I. Zizovic, M. Stamenić, R. Ašanin, M. Ristić, S. D. Petrović, D. Skala. Antimicrobial activity of celery fruit isolates and SFE process modeling. Biochem. Eng. J. 42 (2) (2008) 148-152.

[3] S.D. Cox, C.M. Mann, J.L. Markham, J.E. Gustafson, J.R. Warmington, S.G. Wyllie. 2001. Determination of antimicrobial action of tea tree oil. Molecules. 6 (2001) 87-91.

[4] J. Usta, S. Kreydiyyeh, K. Khuzama, P. Barnabe, Y. Bou-Moughlabay. 2009. Linalool decreases HepG2 viability by inhibing mitochondrial complexes I and II, increasing reactive oxygen species and decreasing ATP and GSH levels. Chem. Bio. Interact. 180 (2009) 39-46.

[5] A. Manabe, S. Nakayama, K .Sakamoto. Effects of essential oils on erythrocytes and hepatocytes from rats and dipalitoylphosphatidylcholine-liposomes. Jpn. J. Pharmacol. 44 (1987) 77-84.

[6] S.D. Cox, C.M. Mann, J.L. Markham, H.C. Bell, J.E. Gustafson, J.R. Warmingtong, S.G. Wyllie. 2000. The mode of antimicrobial action of essential oil of Melaleuca alternifola. J. App. Microbiol. 88 (2000) 170-175.

[7] K. Bauer, D. Garbe, H. Surburg. Common Fragance and Flavor Materials. Preparation, Properties and Uses. Willey-VCH, Weinheim (2001) 293.

[8] N.Y.O Muyima, G. Zulu, T. Bhengu, D. Popplewell. The potencial application of some novel essential oils as natural cosmetic preservatives in an aqueous cream formulation. Flavor and Fragrance. 17(4) (2002) 258-266.

[9] M.M. Van Krimpen, G.P. Binnendijk. Ropadiar as alternative for microbial growth promoter in diets of weanling pigs. Lelystad, Praktijkonderzoek Veehouderij. ISSN 0169-3689 (2001)14.

[10] S. Ilsley, H. Miller, H. Greathead, C. Kamel. Herbal sow diets boost pre-weaning growth. Pig Process 18(4) (2002) 8-10.

[11] T.Yen , S.Chang**.** Synergistic effects of cinnamaldehyde in combination with eugenol against wood decay fungi. Bioresource Technology. 99 (2008) 232–236.

[12] R.J.W. Lambert, P.N. Skandamis, P. Coote, G. Nychas. A study of the minumum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 91 (2001) 453-462.

[13] M.S. Hanamanthagouda, S.B. Kakkalameli, P.M. Naik, P. Nagella, H.R. Seetharamareddy, H.N. Murthy. 2010. Essential oils of *Lavandula bippinata* and their antimicrobial activities. Food Chem. 118 (2010) 836-839.

[14] Z. Drulis-Kawa, A. Dorotkiewicz-Jach. 2010. Liposomes as delivery systems for antibiotics. Int. J. Pharm. 387 (2010) 187-198.

[15] De Paz E, Martín A, Rodríguez-Rojo S, Herreras J, Cocero MJ. 2010. Determination of Phase Equilibruim (Solid-Liquid-Gas) in Poly-(ε-caprolactone)-Carbon dioxide Systems. J. Chem. Eng. 55:2781–2785.

[16] Varona S, Martín A, Cocero MJ. 2009. Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches**.** Chem. Eng. Process. 48 (2009)1121-1128.

[17] S. Varona, S. Kareth, Á. Martín, M.J. Cocero. Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture. J. Supercrit. Fluids. 54 (2010) 369-377.

[18] E. Weidner. 2009. High pressure micronization fro food applications. J Supercrit. Fluids. 47 (2009) 556-565.

[19] J. Yu, J. Lei, H. Yu, X. Cai, G. Zou. Chemical composition and antimicrobial activity of the essential oil of Scutellaria barbata. Phytochem. 65 (2004) 881–884.

[20] S.G. Deans, G. Ritchie. 1987. Antibacterial properties of plant essential oils. Int. J. Food Microbiol. 5 (1987) 165-180.

[21] L.L Zaika. Spices and herbs. Their antimicrobial activity and its determination. J Food Nutrition. 9 (1988) 97-118.

[22] S. Burt. Essential oils: Their antibacterial properties and potential applications in foods- A review. Int. J. Food Microbiol. 94 (200) 223-253.

[23] P. Goñi, P. López, C. Sánchez, R. Gómez-Lus, R. Becerril, C. Nerín. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. Food Chem. 116 (2009) 982–989.

[24] L.A. Meure, N.R. Foster, F. Dehghani. 2008. Conventional and dense gas techniques for the production of liposomes. AAPS Pharm. Sci. Tech. 9 (2008) 798-809.

[25] M. Oussalah, M. Caillet, L. Saucier, M. Lacroix. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: E. coli O157:H7, Salmonella Typhimurium, Staphylococcus aureus and Listeria monocytogenes. Food Control. 18 (2007) 414–420.

[26] S.G. Griffin, S.G. Wyllie, J.L. Markham, D.N. Leach, The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour Frag. J. 14 (5) (1999) 322–332.

[27] O.O. Okon, A.P. Sadimenko, A.J. Afolaya. Comparative evaluation of the antibacterial activities of the essential oils of *Rosmarinus officinalis L.* obtained by hydrodistillation and solvent free microwave extraction methods. Food Chem. 120 (2010) 308-312.

[28] L. Kokoska, Z. Polesny, V. Rada, A. Nepovim, T. Vanek. Screening of some siberian medicinal plants for antimicrobial activity. J. Ethnopharmacol. 82 (2002) 51-53.

Conclusions

This work is a contribution for the development of new biodegradable and natural biocides for their use in agriculture and livestock, due to the promising results obtained. The final conclusions of this work, are presented bellow.

Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches

- Stable emulsions of lavandin oil were obtained with the four types of modified starches used as surfactants. Those emulsions have similar characteristics than emulsions prepared with commercial surfactants (mixer of tween 20 and span 20 with an optimal HLB of 13).

- The parameters which more influence have on the emulsion properties are the surfactant concentration, oil volumen and homogenization speed (energy gave to the emulsion). With the best process conditions, it is possible to produce emulsions with a mean droplet size of about 700 nm and very stable (20% of the oil was demulsified after 50 days).

- Adsorption of OSA-starch at the interfase is likely to be governed by the amount of surface area created during emulsification and amount of OSA-starch available. Very thick multilayer of surfactant is formed, reaching values of up to 370 mg/m^2. This fact can justify the high stability of lavandin oil emulsion stabilized with modified starches.

Liposomal incorporation of lavandin essential oil by thin-film hydration method and by PGSS (Particles from Gas Saturated Solutions)

- Lavandin essential oil was encapsulated in liposomes using commercial soybean lecithin and cholesterol as emulgents.

- Liposomes obtained by conventional Banghan method are uni/multilamellars and multivesicular vesicles which diameter range between 0.6-1.3 μm. Liposome size mainly depends on the bilayer composition (lecithin/cholesterol ratio), essential oil load and method used to reduce their diameter after

hydration. Lavanidn oil encapsulation efficiency of this process is between 6-60%.

- PGSS is an efficient process for soybean lecithin micronization, obtaining spherical aggregated particles (d = 1.4 – 25 μm). The encapsulation efficiency of essential oil for this process is quite low (6 - 15%). In any case, this efficiency can be improved by modifying process conditions in order to increase the solubility of carbon dioxide in the emulsion though the static mixer (higher gas/product ratio, pre-expansion temperature and pre-expansion pressure). Uni/multivesicular liposomes (0.5 - 1.5 μm) are obtained by hydration of the previous lecithin particles.

Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture

- Lavandin essential oil was encapsulated in biodegradable polymers (OSA-starches and PEG) by the supercritical process PGSS and PGSS-drying. The characteristic of the obtained particles (size, morphology, bulk density and essential oil load) mainly depend on the operation conditions (temperature and pressure before expansion and CO_2/feed ratio).

- The most efficient process for the encapsulation of lavandin oil is PGSS, due to its encapsulation efficiency and particles characteristics. Particles formed by this process show spherical morphology, narrow size distribution and mean diameter ranging between 31-91 μm. Lavandin oil encapsulation efficiency of this process range between 14-66%. This result could be explained by the fact that this process allows to operate at milder conditions, leading to a decrease of essential oil loss by evaporation or CO_2 solubilization. On the contrary, PGSS-drying show lower encapsulation efficiency and OSA-starch particles produced show two types of morphology; spheres and irregular needles.

- Lavandin oil released from OSA-starch microcapsules mainly depends on the lavandin oil load of the microcapsules. In fact, lavandin/OSA-starch ratios lower

than 0.2 lead to 20% of oil released after 20 days, while higher ratios lead to 60% of the encapsulated oil was released.

Supercritical impregnation of lavandin essential oil in modified starch

- Lavandin essential oil has been impregnated in OSA-starch using $scCO_2$ as dissolvent. Lavandin oil loads of impregnation (25-147 $mg_{lavandin}/g_{product}$) are in the range of values obtained with other processes (PGSS, PGSS-drying), however encapsulation efficiency (4-22%) is lower than the obtained with these processes.

- Supercritical $scCO_2$ density is the most important parameter affecting lavandin oil impregnation. In particular, impregnation is favored by the temperature, because as temperature increases lavandin oil solubility increases. On the other hand, pressure is an unfavorable parameter, due to the fact that both partition coefficient and impregnated lavandin oil decrease with pressure. This can be explained by a weakened of the specific interactions lavandin-polymer and an increase of lavandin-$scCO_2$ interactions.

Antimicrobial activity of lavandin oil formulations.

- In this study lavandin oil antimicrobial activity has been determinate. Antimicrobial activity of lavandin oil improves by its encapsulation and this improvement mainly depends on lavandin oil concentration and encapsulating agent.

- *E.coli* (gram-) results to be the most sensitive strain, while *B.cereus* (gram+) and *S.aureus* (gram+) are more resistant against lavandin oil and its formulates. These different behaviors are due to membrane differences between strains.

- Carrier material for essential oil encapsulation is very important, because it determines the mode of action of the antimicrobial agent at the membrane. Soybean lecithin is the most efficient encapsulating agent, due to its capacity to spontaneously form liposomes in contact with water.

- Particle formation process is an important parameter relating to antimicrobial activity, because it determines the properties of the particles (encapsulating agent, load and quality of encapsulated lavandin oil, size and morphology). Lavandin oil load OSA-starch particles formed by PGSS-drying show higher antimicrobial activity than particles obtained by spray-drying with the same lavandin oil load.

Future work

The use of natural products, like essentials oils, as natural biocides is a potential alternative to the use of synthetic chemicals in agriculture and livestock. Owing to the promising results obtained it is recommended to continue the research focusing on the following points.

- Use of new carrier materials for the encapsulation of essential oils, with the aim of improving the control released and protection (stability and preservation of volatile compounds) of the essential oil.
- It would be of considerable interest to determine the effect of the obtained essential oil formulations in the inhibition of other bacteria, fungus, insects and nematodes. As well as, to determine the effect of formulation composition, type and size on the antimicrobial activity. This study will help us to gain insight into lavandin oil antimicrobial mode of action and developed a targeted formulation.
- Determination of the biocide activity of lavandin oil and lavandin oil formulations in a real stage, that is, to carry out assays in the field and with animals.

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List of publications

Publications

- S.Varona, A.Martin, M.J.Cocero, T.Gamse. Supercritical carbon dioxide fractionation of Lavandin essential oil. Experiments and modeling. The Journal of Supercritical Fluids, 2008, 45, 181-188.
- M.J. Cocero, A.Martín, F.Mattea, S.Varona. Encapsulation and co-precipitation processes with supercritical fluids: Fundamentals and applications. The Journal of Supercritical Fluids, 2009, 546-555.
- S.Varona, A.Martín, M.J. Cocero. Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches. J. Chemical Engineering and Processing, 2009, 48, 1121-1128.
- A.Martín, S.Varona, A.Navarrete, M.J.Cocero. Encapsulation and Co-Precipitation Processes with Supercritical Fluids: Applications with Essential Oils. The Open Chemical Engineering Journal, 2009.
- S.Varona, S.Kareth, Á.Martín, M.J.Cocero. Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture. The Journal of Supercritical Fluids, 2010, 54, 369-377.
- S. Varona, Á. Martín, M.J. Cocero. Liposomal incorporation of Lavandin essential oil by a thin-film hydration method and by PGSS (Particles from Gas Saturated Solutions). Journal of Industrial & Engineering Chemistry Research. Submitted for publication (2010).
- S. Varona, S. Rodríguez-Rojo, Á. Martín, M. J. Cocero, A.T. Serra, T. Crespo, C.M.M. Duarte. Antimicrobial activity of lavandin essential oil formulations. Biochemical Engineering Journal. Submitted for publication (2010).

Participation y congress

- S.Varona, A. Martín, M.J. Cocero, T.Gamse. Supercritical carbon dioxide fractionation of Lavandin essential oil: Experiments and modeling. 5th International Syposium in Chemical Engineering and Pressure Proceses. Segovia, Spain. (July 2007).

- S. Varona, M.J. Cocero. Essential oils encapsulation for its use as biocides using green technologies. 18th International congress of Chemical and Process Engineering.Praga, Czech Republic. (August 2008).

- S. Varona, S. Kareth, M.J. Cocero.. Encapsulation of essentials oils using biopolymers for their use in ecological agriculture. 9th International Symposium on Supercritical Fluids. Arcachon (France), 18-20 Mayo 2009.

- S. Varona, S.Kareth, A. Martín, M.J. Cocero. Formulation of essential oils using biopolymers for application as biocides in ecological agriculture. II Iberoamerican Conference on Supercritical Fluids, Natal, Brazil (April 2010)

- S. Varona, A. Martín, M.J. Cocero. Liposomal incorporation of Lavandin (Lavandula hybrid) essential oil by conventional methods and PGSS. 18th International Congress of Chemical and Process Engineering. Graz, Austria. (May 2010)

- S. Varona, S. Rodríguez-Rojo, Á. Martín, M. J. Cocero, A.T. Serra, T. Crespo, C.M.M. Duarte. Antimicrobial activity of lavandin essential oil formulations. I International Conference on Antimicrobial Research. Valladolid, Spain (November, 2010).