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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **10.132.086**

Solicitud de patente a nombre de **LABORATOIRE M2 INC.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **LABORATOIRE M2 INC.**, solicitante de la patente de la referencia, en respuesta al oficio No. **20096** notificado por fijación en lista el 6 de octubre de 2012, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial un nuevo capítulo reivindicatorio, el cual consta de 23 reivindicaciones y reemplaza completamente el capítulo presentado anteriormente.

En el nuevo pliego reivindicatorio no se han incluido las anteriores reivindicaciones 13-15 y 27-29.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones planteadas por el examinador en su concepto técnico.

2. Requisitos de Patentabilidad

El examinador ha rechazado las reivindicaciones 1-26 porque considera que se hubieran derivado de manera obvia en vista de los documentos D1, D2 y D3, por lo que respetuosamente nos permitimos decir que no nos encontramos de acuerdo con dichas premisas teniendo en cuenta las siguientes razones:

Es deseo del solicitante poner de presente inicialmente que las composiciones de D1, D2 y D3 todas comprenden un aceite esencial como agente antimicrobiano. Adicionalmente, D1 reconoce que los aceites esenciales comprenden un gran número de compuestos diferentes. De otra parte, el agente antimicrobiano de la composición de la presente invención no es un aceite esencial, sino por el contrario es un compuesto sintético o aislado puro. Las Reivindicaciones 1 y 6 se han modificado para mencionar

“compuestos fenólicos antimicrobianos sintéticos o aislados”, y enfatiza esta importante diferencia. El soporte para este cambio se puede encontrar en el párrafo [0049] en las páginas 8 – 9 de la descripción como se presentó. Por lo que no se ha agregado materia nueva.

En segundo lugar, el solicitante indica respetuosamente al Examinador que las nuevas reivindicaciones 1-7 y 13-23 no reclaman específicamente un aceite esencial. Por lo tanto, el Solicitante sostiene respetuosamente que para hacer no inventiva la presente invención como se reivindica actualmente en las reivindicaciones 1-7 y 13-23 en vista de las anteriores combinaciones, las referencias D1, D2 y D3 se tendrían que modificar todas en una forma que harían inoperables las referencias. Específicamente, con el fin de que las anteriores combinaciones lleven a una persona versada en la materia a obtener la presente invención, **el aceite esencial utilizado en D1, D2 y D3 se tendría que retirar de la composición.** El retiro de este componente es una modificación que dejaría inoperable la composición para su propósito puesto que perdería la actividad antimicrobiana del aceite esencial. Esta modificación no es una acción que el experto en la técnica contemplaría hacer en vista de la citada técnica anterior.

En tercer lugar, D1 utiliza un tensoactivo que forma una emulsión gelatinosa entre el solvente orgánico y la cantidad de agua. El tensoactivo utilizado en la presente invención, es un tensoactivo suficiente para formar una solución o dispersión del compuesto fenólico en el portador acuoso. Por lo tanto, los tensoactivos de D1 y aquellos de la presente invención son diferentes y tienen diferentes propiedades químicas. Adicionalmente, el surfactante de D1 corresponden a una sustancia similar a gel (como un sólido), aunque la composición de la presente invención es un líquido, y por lo tanto, son composiciones clara y completamente diferentes.

El solicitante presenta respetuosamente que D1 describe una formulación que forma una emulsión gelatinosa con un tensoactivo, un solvente y una cantidad de agua que contiene un aceite esencial natural (por ejemplo pág. 15 línea 4 de D1). Es bien sabido por el experto en la técnica que formular una emulsión gelatinosa que contiene un aceite esencial es mucho más fácil que formular un desinfectante basado en agua que contiene un aceite esencial o sus fenólicos aislados como se reivindicó actualmente en la presente invención. Una emulsión gelatinosa contendrá niveles de solventes y tensoactivos mucho mayores, que tienen propiedades lipófilas que les permite emulsificar los aceites, en razón a que no se alcanzará un estado gelatinoso sin dichas altas concentraciones. Las altas cantidades de tensoactivos y solvente solubilizarán una pequeña cantidad de aceite esencial.

La composición de la presente invención se basa en cambio en una cantidad mucho menor de estos ingredientes que se deben seleccionar y dosificar cuidadosamente el

compuesto fenólico aislado en una composición que contiene 98% de agua. El agua no es un compuesto fenólico, y solubilizar un compuesto fenólico (tal como el timol utilizado en una composición de la presente invención) en agua no es obvio, incluso para el experto en la técnica, cuando se utilizan pequeñas cantidades de solvente y tensoactivos.

El Solicitante también afirma respetuosamente que la composición descrita en D1 no sería muy efectiva como desinfectante. Un desinfectante debe ser capaz de cubrir la superficie sobre la que se aplica y penetrar las pequeñas imperfecciones en la superficie. La composición de la presente invención como se reivindicó actualmente tiene en cambio una muy baja tensión superficial y fluiría fácilmente sobre las superficies. Esto se ejemplifica en la Declaración Jurada del Sr. Ulysse Desranleau (Adjunta a este memorial), un gerente de desarrollo de producto para el Solicitante (véase Anexo 1 adjunto). En la Declaración Jurada, se explica que 300 g de la composición de la presente invención son capaces de cubrir aproximadamente 1 m² y que la naturaleza gelatinosa de la composición de D1 significa que la mezcla tiene una tensión superficial muy alta (es pegajosa y las mantiene juntas) y cubriría solo aproximadamente 0,03m², o aproximadamente 33 veces menos área superficial. No penetraría pequeñas imperfecciones de la superficie, proporcionando una completa desinfección.

La formulación de la presente invención también se puede aplicar utilizando un pulverizador, una lata de aerosol (ver párrafo [0059] de la descripción como se presentó) o un paño. Estos métodos de aplicaciones son los métodos preferidos para desinfectantes. La naturaleza gelatinosa de D1 significa que no se puede utilizar en estos métodos de aplicación, que adicionalmente limitan su uso.

La naturaleza gelatinosa y el relativamente bajo contenido de agua (pág. 13 describe cantidades de agua de aproximadamente 5-70%) de la composición de D1, también evita que sea formulado en un concentrado. Una forma diluida de la composición de la presente invención que contiene 0.23% de timol se puede concentrar hasta 33 veces, aunque la otra composición también que contiene 0.23% de timol también se puede concentrar hasta 100 veces. Esto ahorra costos de transporte y almacenaje, lo que es una mejora significativa sobre la composición de D1. También, la composición de D1 se formula **como una formulación sin agua**, lo que significa que se debe utilizar sin agua adicional (véase página 1, líneas 22-24 de D1). Por lo tanto, D1 no enseña este aspecto conveniente de la presente invención. Adicionalmente, uno puede presumir que la adición de agua a la composición de D1 puede cambiar la textura gelatinosa que es esencial para su uso. Por lo tanto, la combinación de las enseñanzas de D1 con aquellas de D2 y D3 **no** produciría una composición líquida tal como aquella de la presente invención como se reivindica actualmente, sino que daría una composición gelatinosa que no tiene las propiedades de desinfección deseadas.

La referencia D2 enseña una composición antimicrobiana acuosa que comprende un aceite esencial que exhibe propiedades antimicrobianas, un tensoactivo, un solvente orgánico, y agua. D2 no enseña ni sugiere un compuesto fenólico o un agente secuestrante, como se reivindica en la reivindicación 1. Adicionalmente, el Solicitante considera respetuosamente que el experto en la técnica en posesión de las enseñanzas de D1 en vista de D2 podría de nuevo buscar reproducir una composición gelatinosa, incluso cuando se utiliza uno cualquiera de los diferentes tensoactivos enseñados por D2.

La referencia D3 describe una formulación para limpieza a diferencia de desinfección, como la presente invención. D3 no describe ninguna eficacia antimicrobiana de su composición. Aunque D3 no indica que los aceites esenciales tienen alguna eficacia antimicrobiana, el Solicitante afirma respetuosamente que no esta no se formula para que tenga una gran eficacia antimicrobiana.

Específicamente, en el Anexo 1, el Sr. Desranleau Dandurand explica la pobre eficacia antimicrobiana de las composiciones preferidas de D3 (véase pág. 22 párrafo [0055] de D3). D3 describe una formulación concentrada que contiene 6.5% de aceite de pino que se diluye para producir una solución de pino-aceite de 0.10%. También compara esta composición diluida con el producto original Pine-Sol™ de Clorox™, que contiene 0.24% de aceite de pino una vez diluido. Sin embargo es bien sabido por aquellos expertos en la técnica que el Pine-Sol™ es un pobre agente antimicrobiano, y no cumple los estándares de desinfección más básicos establecidos presentados por muchas agencias reguladoras (regulaciones Europeas, Health Canada, U.S. EPA). El fabricante del producto original Pine-Sol™ de Clorox™, quien también es el solicitante de D3, recomienda aplicar el producto Pine-Sol™ original en toda su potencia (no diluido) durante 10 minutos para desinfectar. La formulación que utiliza 6.5% de aceite de pino sería por lo tanto aún menos efectiva que la formulación Pine-Sol™ original. En comparación, el Sr. Desranleau Dandurand explica que la formulación de la presentación es capaz de desinfectar bacterias en solo 2 minutos (5 veces menos que la formulación de aceite de pino de D3) con solo 0.23% de un compuesto fenólico (25 veces menos que la formulación de aceite de pino de 6.5% de D3).

Adicionalmente, el Solicitante declara respetuosamente que D3 describe formulaciones preferidas utilizando por lo menos 1.0% de tensoactivos para 0.2% de aceites esenciales, una relación de 5:1 (véase pág. 17 de D3), y que la relación de tensoactivo a aceite debe ser de por lo menos 2:1. La formulación de la presente invención es en cambio capaz de emulsificar un compuesto fenólico aislado utilizando solo 0.35% de tensoactivo para más de 0.2% de dicho compuesto, un índice de menos de 2:1 (más cerca a 1.5:1). Sin embargo la formulación de la presente invención se puede preparar como un concentrado a niveles mayores y requiere menos tensoactivo. Si bien se reducen costos para la producción de la formulación, también contribuye a mejorar la

eficacia antimicrobiana de la composición de la presente invención. De hecho, es bien sabido por los expertos en la técnica que el tensoactivo puede impedir la acción antimicrobiana de los aceites esenciales o compuestos fenólicos (tal como timol), como se muestra en el Anexo 3 – Ziani et al..

Finalmente, el Solicitante afirma respetuosamente que las propiedades antimicrobianas excepcionales de la composición de la presente invención se pueden apreciar en los resultados presentados en el Anexo 1. En esta Declaración Jurada, el Sr. Desranleau Dandurand presenta nuevos resultados comparativos entre una composición de la presente invención y las composiciones de la técnica anterior de D1, D2 y D3. Los resultados muestran claramente que la composición (que es una dilución de una formulación concentrada) es capaz de alcanzar “muerte total” de microorganismos más rápido que las composiciones de la técnica anterior, fue capaz de alcanzar una mayor reducción Log, que representa aproximadamente una mejora de 330 veces en provocación de muertes de microorganismos, si bien utiliza significativamente menos ingredientes activos. Este aumento de la eficacia muestra que la presente invención como reivindicó actualmente reduce los conteos microbianos en más de 6 Log. En comparación, D1 reivindica tener solo “eficacia antimicrobiana tópica”, que se define al tener una zona de inhibición de 2 milímetros (página 6 línea 24 de D1). Durante ensayos similares, la composición de la presente invención obtuvo zonas de inhibición de por lo menos 20 milímetros, lo que indica que la zona de inhibición cubre 100 veces más superficie que la composición de D1. Una composición de la presente invención como se reivindicó actualmente alcanza estos resultados con solo 0.23% de un compuesto fenólico aislado (timol), aunque la composición presentada en D1 utiliza más de dos veces esta cantidad de aceite esencial (0.6%). Utilizando timol como un compuesto puro permite a la presente invención alcanzar una mejor desinfección que la de los aceites esenciales. El balance de tensoactivo y solvente cuidadosamente seleccionado de la presente invención permite a un ingrediente lipófilo (como el compuesto fenólico aislado) ser suministrado más eficientemente en el microorganismo, y por lo tanto tener un aumento de eficacia. El Sr. Desranleau Dandurand da fe que estos resultados fueron sorprendentes y demuestran claramente la superioridad de la composición de la presente invención sobre la técnica anterior, y que éstos resultados son más de lo que el experto en la técnica habría esperado de la combinación de las composiciones de la técnica anterior citada.

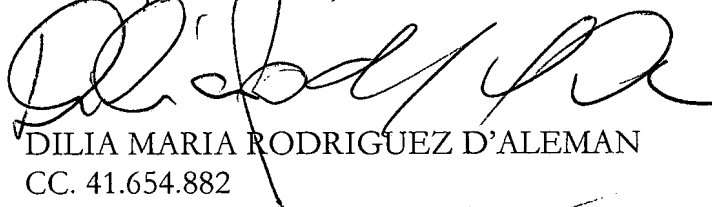
Por lo tanto, en vista de los anteriores argumentos, el Solicitante afirma respetuosamente que la presente invención como se reivindicó actualmente no se considera obvia por el documento D1 solo, o en combinación con el conocimiento general común. Respetuosamente se solicita la reconsideración y retiro de este rechazo por parte del Examinador.

Por consiguiente, teniendo en cuenta los argumentos presentados, se concluye que las enseñanzas de la anterioridad citada por su Despacho no lleva a un técnico versado en la materia a obtener la presente invención ya que no revelan o enseñan las características técnicas esenciales relacionadas con la misma, de manera tal como es reivindicada. Lo anterior si se tiene en cuenta que para el estudio de nivel inventivo el Examinador no puede adoptar la posición de un inventor y que además no puede partir de la solución dada sino simplemente de lo que expresamente revelen el o los documentos más cercanos del estado del arte.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el examinador considera que subsisten razones para objetar la presente invención rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *“habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares”* y *“(.) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso”*, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

Señor Superintendente,



DILIA MARIA RODRIGUEZ D'ALEMAN

CC. 41.654.882

TP 20824 CSJ

notificaciones@clarkemodet.com.co

REIVINDICACIONES

1.- Una formulación desinfectante acuosa, que comprende:

- a) por lo menos un compuesto fenólico antimicrobiano aislado o sintético de origen natural;
- b) un agente tensoactivo suficiente para formar una solución o dispersión de dicho compuesto fenólico en un vehículo acuoso;
- c) un solvente;
- d) un agente secuestrante; y
- e) agua suficiente para obtener un 100% en peso.

2.- La formulación desinfectante acuosa de conformidad con la reivindicación 1, caracterizada además porque dicha solución comprende:

- a) de aproximadamente 0.05% a aproximadamente 25% en peso dicho compuesto fenólico;
- b) de aproximadamente 0.1% a aproximadamente 15% en peso de dicho agente tensoactivo;
- c) de aproximadamente 0.1% a aproximadamente 40% en peso de dicho solvente; y
- d) de aproximadamente 0.01% a aproximadamente 10% en peso de dicho agente secuestrante.

3.- La formulación desinfectante acuosa de conformidad con la reivindicación 1, caracterizada además porque dicha solución comprende:

- a) de aproximadamente 5% a aproximadamente 25% en peso de dicho compuesto fenólico;
- b) de aproximadamente 5% a aproximadamente 15% en peso de dicho agente tensoactivo;
- c) de aproximadamente 5% a aproximadamente 35% en peso de dicho solvente; y
- d) de aproximadamente 1% a aproximadamente 5% en peso de dicho agente secuestrante.

4.- La formulación desinfectante acuosa de conformidad con la reivindicación 1, caracterizada además porque dicha solución comprende:

- a) de aproximadamente 15% a aproximadamente 25% en un peso de dicho compuesto fenólico;
- b) de aproximadamente 10% a aproximadamente 15% en peso de dicho agente tensoactivo;
- c) de aproximadamente 15% a aproximadamente 30% en peso de dicho solvente; y
- d) de aproximadamente 1% a aproximadamente 3% en peso de dicho agente secuestrante.

5.- La formulación desinfectante acuosa de conformidad con la reivindicación 1, caracterizada además porque dicho compuesto fenólico se selecciona del grupo que consiste de timol, carvacrol, citral y eugenol.

6.- La formulación desinfectante acuosa de conformidad con la reivindicación 1, caracterizada además porque dicho agente tensoactivo se selecciona del grupo que consiste de lauril sulfato de sodio, estearato de sorbitán, laureth sulfato de sodio, sarkosyl, cocoamidopropil betaína (CAPB), lauril éter sulfonato de sodio, alquilbencensulfatos, nonilfenol etoxilato, ésteres de sorbitán y éter etoxilato.

7.- La formulación desinfectante acuosa de conformidad con la reivindicación 1, caracterizada además porque dicho agente secuestrante se selecciona del grupo que consiste de sal de sodio de ácido etilendiaminotetraacético (EDTA), gluconato de sodio, citrato de sodio, ácido cítrico, NTA trisódico, disuccinato trisódico de etileno, fosfato de sodio y colato de sodio.

8.- La formulación desinfectante acuosa de conformidad con cualquiera de las reivindicaciones 1 a 7, caracterizada además porque comprende un aceite esencial.

9.- La formulación desinfectante acuosa de conformidad con la reivindicación 8, caracterizada además porque dicho aceite esencial se selecciona del grupo que consiste de aceite de orégano, aceite de timo, aceite de eucalipto y aceite de limón.

10.- La formulación desinfectante acuosa de conformidad con cualquiera de las reivindicaciones 1 a 9, caracterizada además porque comprende además fragancia.

11.- La formulación desinfectante acuosa de conformidad con la reivindicación 10, caracterizada además porque dicha fragancia es citral.

12.- La formulación desinfectante acuosa de conformidad con cualquiera de las reivindicaciones 1 a 11, caracterizada además porque comprende un PH que varía de aproximadamente 6 a aproximadamente 9.

13.- Una formulación desinfectante acuosa, que comprende:

- a) por lo menos un compuesto fenólico antimicrobiano aislado o sintético de origen natural;
- b) un agente tensoactivo suficiente para formar una solución o dispersión de dicho compuesto fenólico en un vehículo acuoso;
- c) un solvente; y
- d) agua suficiente para obtener 100% en peso.

14.- La formulación acuosa de conformidad con la reivindicación 13, caracterizada además porque dicha solución comprende:

- a) de aproximadamente 0.05% a aproximadamente 25% en peso de dicho compuesto fenólico;
- b) de aproximadamente 0.1% a aproximadamente 15% en peso de dicho agente tensoactivo; y
- c) de aproximadamente 0.1% a aproximadamente 40% en peso de dicho solvente.

15.- La formulación desinfectante acuosa de conformidad con la reivindicación 13, caracterizada además porque dicha solución comprende:

- a) de aproximadamente 5% a aproximadamente 25% en peso de dicho compuesto fenólico;
- b) de aproximadamente 5% a aproximadamente 15% en peso de dicho agente tensoactivo; y
- c) de aproximadamente 5% a aproximadamente 35% en peso de disco solvente.

16.- La formulación desinfectante acuosa de conformidad con la reivindicación 13, caracterizada además porque dicha solución comprende:

- a) de aproximadamente 15% a aproximadamente 25% en peso de dicho compuesto fenólico;
- b) de aproximadamente 10% a aproximadamente 15% en peso de dicho agente tensoactivo; y
- c) de aproximadamente 15% a aproximadamente 30% en peso de dicho solvente.

17.- La formulación desinfectante acuosa de conformidad con la reivindicación 13, caracterizada además porque dicho compuesto fenólico se selecciona del grupo que consiste de timol, carvacrol, citral y eugenol.

18.- La formulación desinfectante acuosa de conformidad con la reivindicación 13, caracterizada además porque dicho agente tensoactivo se selecciona del grupo que consiste de lauril sulfato de sodio, estearato de sorbitán, laureth sulfato de sodio, sarkosyl, cocoamidopropil betaína (CAPB), lauril éter sulfonato de sodio, alquilbencensulfonatos, nonifenol etoxilato, ésteres de sorbitán y éter etoxilato.

19.- La formulación desinfectante acuosa de conformidad con cualquiera de las reivindicaciones 13 a 18, caracterizada además porque comprende adicionalmente un aceite esencial.

20.- La formulación desinfectante acuosa de conformidad con la reivindicación 19, caracterizada además porque dicho aceite esencial se selecciona del grupo que consiste de aceite de orégano, aceite de timo, aceite de eucalipto y aceite de limón.

21.- La formulación desinfectante acuosa de conformidad con cualquiera de las reivindicaciones 13 a 20 caracterizada además porque comprende adicionalmente fragancia.

22.- La formulación desinfectante acuosa de conformidad con la reivindicación 21, caracterizada además porque dicha fragancia es citral.

23.- La formulación desinfectante acuosa de conformidad con cualquiera de las reivindicaciones 13 a 22, caracterizada además porque comprende un PH que varía de aproximadamente 6 a aproximadamente 9.

ANEXO 1

EN LA OFICINA DE PATENTES DE COLOMBIA

Daigle et al.

Solicitud No.: 10.132.086

Fecha de presentación: abril 08, 2009

Para: FORMULACIÓN DESINFECTANTE ACUOSA QUE COMPRENDE UN COMPUESTO FENÓLICO, UN TENSOACTIVO, Y UN SOLVENTE

DECLARACIÓN JURADA DE ULYSSE DESRANLEAU

ULYSSE DESRANLEAU DANDURAND declara y afirma:

- 1 - Que soy ciudadano del Canadá, que reside actualmente en QUEBEC, CANADÁ.
- 2 - Que soy microbiólogo, que participan en los productos basados en la presente invención.
- 3 - Que soy graduado de la Universidad de Montreal, Quebec, Canadá.
- 4 - Que mi formación académica y experiencia en el campo de la presente invención se enumeran en el currículum vitae adjunto.
- 5 - Que he leído y entiendo la especificación.
- 6 - Que una composición de la presente invención se comparó con las composiciones encontradas en los documentos de la técnica anterior citados por el examinador.

000011

7 - Que una composición de la presente invención es un líquido mientras que la composición de D1 es una composición gelatinosa, que tiene una cobertura de superficie pobre como se indica en la tabla 1.

Tabla 1 - cobertura de 300 g de composición

	Presente invención	D1
Área cubierta	1 m2	0,03 m2
Área relativa cubierta	33x	1x

8 - Que una composición de la presente invención tiene una mayor zona de inhibición que la composición de D1 como se indica en la tabla 2.

Tabla 2 - Log de reducción en carga bacteriana

	Presente invención	D1
Zona de inhibición	20 mm	2 mm
Cobertura de superficie	100x	1x

9 - Que la composición de la presente invención obtuvo zonas de inhibición de al menos 20 milímetros, lo que indica que la zona de inhibición cubre 100 veces más superficie que la composición de D1 con sólo 0,23% de un compuesto fenólico aislado (timol), mientras que la composición presentada en D1 utiliza más de dos veces la cantidad de aceite esencial (0,6%).

000012

10 - Que las composiciones de D3 muestran pobre eficacia antimicrobiana en comparación con la composición de la presente invención, como se indica en la tabla 3.

Tabla 3 - actividad antimicrobiana de la composición de D3

	Present e invenci ón	Composició n de D3 sin diluir	Composició n de D3 1:65	Pine-Sol TM Original 1:64	Pino- Sol TM Origin al sin diluir
aceite de pino	-	6.5%	0.10%	0.24%	15%
timol Aislado	0.23%	-	-	-	-
Tiempo para desinfección	2 minutos (muerte total)	Sin desinfecci ón	Sin desinfecci ón	Sin desinfecci ón	10 minuto s
cantidad antimicrobia na activa Relativa	1x	28x	2.3 x	2.4 x	65x
Cumplimiento de estándares de desinfección	Sí	No	No	No	No

000013

11 - Que los resultados de la tabla 3 muestran que las composiciones preferidas de D3, y los productos de limpieza Original Pine-Sol™ son muy pobres composiciones antimicrobianas.

12 - Que el tiempo de contacto requerido para lograr una "muerte total" según el estándar de la "Asociación de Comunidades Analíticas" (AOAC) es de 2 minutos.

13 - Que una composición antimicrobiana de la presente invención (0.21% timol, 0.35% de tensoactivo, 0.90% de disolvente y 0.09% de agente secuestrante; véase páginas 9 a 23 de la solicitud presentada) ofrece una "muerte total" por el método de Prueba de Dilución por Uso (UDT)/Prueba de Pulverización de Germicida (GST) de estándares AOAC (DIS/TSS-1) por el tiempo indicado en la Tabla 4.

Tabla 4 - La hora de la destrucción total

	Presente invención	D2
tiempo de contacto requerido AOAC UDT y GST	2 minutos	10 minutos

14 - que las pruebas se llevaron a cabo sin fallas de la composición de la presente invención, lo que significa que ninguno de los 180 cultivos mostraron crecimiento.

000014

15 - Que la composición de la presente invención era capaz de efectuar una "muerte total" mucho más rápido que las composiciones de D2.

16 - que las composiciones de la técnica anterior sólo requieren una reducción de 4 Log (Conteo de portador), mientras que la composición de la presente invención es capaz de matar más de 6.5 Log bajo la misma prueba (ver Tabla 5).

Tabla 5 - log de reducción de la carga bacteriana

	Presente invención	D2
Pruebas AOAC UDT y GST para Reducción Log	6.5 Log	4 Log

17 - Que un grupo completo de resultados de ensayo para la composición de la presente invención está a disposición del Examinador en el Documento 2, adjunto a la presente.

18 - Que esta diferencia de 2.5 log representa una enorme brecha en el rendimiento lo que significa que se matan 330 veces más bacterias por la composición de la presente invención.

19 - Esta es una mejora crítica, ya que una diferencia de esta magnitud proporciona en realidad una protección adicional a las personas expuestas a las superficies desinfectadas.

000015

20 - Que esto es especialmente notable ya que la teoría de desinfección enseña, a través de la ley de Chick y la teoría del "valor D", que la desinfección sigue a una progresión lineal. Esto significa que, aunque la composición de la presente invención alcanza una "muerte total" de 6.5 Log en 2 minutos, esa composición de la citada técnica sólo ha conseguido matar aproximadamente 1 o 1,5 Log en 2 minutos (ver Tabla 4).

21 - Esta es también una mejora crítica ya que es bien conocido por los expertos en la técnica que el cumplimiento del usuario en la desinfección disminuye con la duración del tiempo de contacto requerido. Cuanto menor sea el tiempo de contacto, más probable es que el usuario deba cumplir con este. Por ello es fundamental que la desinfección sea alcanzada tan rápido como sea posible.

22 - Que para el alcohol etílico, es bien ejemplificado en D2 columna 9 composición "D" y "E" en la Tabla IV y V que el alcohol etílico 15% solo es capaz de lograr un buen nivel de desinfección, a pesar de que no es suficiente lograr una desinfección completa.

23 - Que también es un hecho bien conocido que agregar más ingredientes activos resulta en aumento de la eficacia.

24 - Que esto hace a la composición preferida de la presente invención aún más única, ya que logra un nivel de desinfección mucho más alto, y una desinfección más rápida, utilizando ingredientes significativamente menos activos (al menos nueve

veces menos; véanse las Tablas 6 y 7 adelante). Esto no se podría haber predicho, incluso por aquellos expertos en la técnica.

Tabla 3 - Comparación de ingrediente activo

	Presente invención	D1	D2	D2
Cantidades de Ingredientes Activos	timol 0.23%	Alcoholes minerales 32%	Aceite de tomillo 0.20%	Aceite de tomillo 0.15%
	-	aceite de orégano 0.6%	Alcohol de etilo 85%	Alcohol de etilo 15%
Total	0.23%	32.6%	85.20%	15.15%

Tabla 4 - Comparación de ingrediente activo

	Presente invención	D3*	D3*	D3
Cantidades de Ingredientes Activos	timol 0.23%	aceite de pino 6.5%	aceite de pino 0.1%	aceite de pino 15%
	-	Isopropanol 1.5%	Isopropanol 0.02%	Isopropanol desconocido

Total	0.23%	8%	0.12%	por lo menos 15%
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* No hay actividad antibacteriana medida

25 - Que considero estos resultados comparativos superiores sorprendentes y no obvios, y que en vista de las composiciones de la técnica anterior, las composiciones de la presente invención no son composiciones obvias que resultan de la simple combinación de la técnica anterior que no producen más de lo que cabe esperar de dicha combinación.

26 - Que en vista de los resultados y argumentos anteriores, la invención reivindicada no se hace evidente por los documentos de la citada técnica anterior, solos o en combinación, según los rechazos del examinador.

(Aparece firma ilegible)

Ulysse Desranleau Dandurand

Firmado ante mí en Sherbrooke el día 22 de enero de 2013.

(Aparece firma ilegible)

Testigo

Nombre; GAÉTAN LEMIRE

000018

Exhibit 1

IN THE COLOMBIAN PATENT OFFICE

Daigle et al.

Application. No.: 10.132.086

Filed: April 8, 2009

For: AQUEOUS DISINFECTANT FORMULATION COMPRISING A PHENOLIC
COMPOUND, A SURFACTANT, AND A SOLVENT

* * * * *

AFFIDAVIT OF ULYSSE DESRANLEAU DANDURAND

ULYSSE DESRANLEAU DANDURAND hereby declares and says:

- 1- That I am a citizen of the Canada, presently residing at QUEBEC, CANADA.
- 2- That I am a microbiologist, involved in the products based on the present invention.
- 3- That I am a graduate of the University of Montréal, Québec, Canada.
- 4- That my academic background and experiences in the field of the present invention are listed on the enclosed *curriculum vitae*.
- 5- That I have read and understand the specification.
- 6- That a composition of the present invention was compared to the compositions found in the prior art documents cited by the Examiner.
- 7- That a composition of the present invention is a liquid while the composition of D1 is a gelatinous composition, having poor surface coverage as indicated in table 1.

Table 1 – coverage of 300 g of composition

	Present invention	D1
Area covered	1 m ²	0.03 m ²
Relative area covered	33x	1x

- 8- That a composition of the present invention has a larger inhibition zone than the composition of D1 as indicated in table 2.

Table 2 – Log reduction in bacterial load

	Present invention	D1
Inhibition zone	20 mm	2 mm
Surface coverage	100x	1x

- 9- That the composition of the present invention obtained inhibition zones of at least 20 millimeters, which indicates the inhibition zone covers 100 times more surface than the composition of D1 with only 0.23% of an isolated phenolic compound (thymol), while the composition presented in D1 use more than twice that amount of essential oil (0.6%).
- 10- That the compositions of D3 show poor antimicrobial efficacy compared to the composition of the present invention, as indicated in table 3.

Table 3 – antimicrobial activity of the composition of D3

	Present invention	Composition of D3 undiluted	Composition of D3 1:65	Original Pine-Sol™ 1:64	Original Pine-Sol™ undiluted
Pine oil	-	6.5%	0.10%	0.24%	15%
Isolated thymol	0.23%	-	-	-	-
Time for disinfection	2 minutes (total kill)	No disinfection	No disinfection	No disinfection	10 minutes
Relative active antimicrobial amount	1x	28x	2.3x	2.4x	65x
Meeting disinfection standards	Yes	No	No	No	No

- 11- That the results of table 3 show that the preferred compositions of D3, and the Original Pine-Sol™ cleaning products are very poor antimicrobial composition.
- 12- That the contact time required to achieve a “total kill” as per the “Association of Analytical Communities” (AOAC) standard is 2 minutes.
- 13- That an antimicrobial composition of the present invention (0.21% thymol, 0.35% surfactant, 0.90% solvent and 0.09% sequestering agent; see pages 9 to 23 of the application as filed) delivers a “total kill” by AOAC standards Use Dilution Test (UDT)/Germicidal Spray Test (GST) method (DIS/TSS-1) by the time indicated in Table 4.

Table 4 – Time for total kill

	Present invention	D2
Required contact time AOAC UDT and GST	2 minutes	10 minutes

- 14- That the tests were conducted with NO failures from the composition of the present invention, meaning that none of the 180 cultures showed growth.
- 15- That the composition of the present invention was capable of effecting a "total kill" much faster than the compositions of D2.
- 16- That the compositions of the prior art required only a 4 Log reduction (carrier count), while the composition of the present invention is capable to kill over 6.5 Log under the same test (see Table 5).

Table 5 – Log reduction in bacterial load

	Present invention	D2
Log reduction AOAC UDT and GST Testing	6.5 Log	4 Log

- 17- That a full set of test results for the composition of the present invention is available to the Examiner in Exhibit 2, enclosed herewith.
- 18- That this 2.5 Log difference represents a huge gap in performance which means that 330 times more bacteria are killed by the composition of the present invention.
- 19- That this is a critical improvement, since a difference of this magnitude actually provides additional protection to the persons exposed to the disinfected surfaces.
- 20- That this is especially impressive since disinfection theory teaches, through Chick's law and "D value" theory, that disinfection follows a linear progression. This means that, while the composition of the present invention reaches a "full kill" of 6.5 Log in 2 minutes, that the composition of the cited prior art have only achieved to kill about 1 or 1.5 Log in 2 minutes (see Table 4).

- 21- That this is also a critical improvement as it is well known to those skilled in the art that user's compliance in disinfection diminishes with the duration of the contact time required. The lower the contact time, the more likely the user is to comply with it. It is therefore critical that disinfection be achieved as fast as possible.
- 22- That for ethyl alcohol, it is well exemplified in D2 column 9 composition "D" and "E" in Table IV and V that ethyl alcohol 15% alone is able to achieve a good level of disinfection, even though it is not enough to achieve full disinfection.
- 23- That it is also a well-known fact that adding more active ingredients results in heightened efficacy.
- 24- That this renders the preferred composition of the present invention even more unique, as it achieves a much higher disinfection level, and a faster disinfection, using **significantly less active ingredients** (at least nine times less; see Tables 6 and 7 below). This could not have been predicted, even by those skilled in the art.

Table 3 – Active ingredient comparison

	Present invention	D1	D2	D2
Active Ingredients Amounts	Thymol 0,23%	Mineral spirits 32%	Thyme Oil 0,20%	Thyme Oil 0,15%
	-	Oil of oregano 0.6%	Ethyl alcohol 85%	Ethyl alcohol 15%
TOTAL	0,23%	32.6%	85,20%	15,15%

Table 4 – Active ingredient comparison

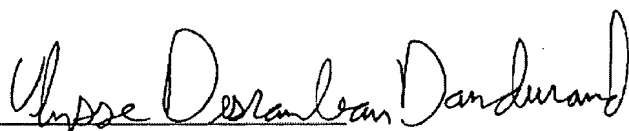
	Present invention	D3*	D3*	D3
Active Ingredients Amounts	Thymol 0,23%	Pine oil 6.5%	Pine oil 0.1%	Pine oil 15%
	-	Isopropanol 1.5%	Isopropanol 0.02%	Isopropanol unknown
TOTAL	0,23%	8%%	0.12%	at least 15%

* No antibacterial activity measured

25- That I consider these superior comparative results surprising and not obvious, and that in view of the compositions of the prior art, the compositions of the present invention are not obvious compositions resulting from the simple combination of the prior art that yield no more than one would expect from such combination.

26- That in view of the above results and arguments, the claimed invention is not rendered obvious by the cited prior art documents alone or in combination, as per the Examiner's rejections.

Application No. 10.132.086
Affidavit of Mr. Desranleau Dandurand


Ulysse Desranleau Dandurand

Signed before me in Sherbrooke this 22 day of January 2013.


Witness
Name : GAËTAN LEMIRE

ANEXO 2

**Tabla 1: Resultados de Eficacia Bactericida para Pulverizador Desinfectante
Thymox®**

Microorganismos de Prueba	Carga de tierra Orgánica	Tiempo de Exposición	Resultados		
			Lote #	Números de portadores que muestran crecimiento*	Resultados de Control de Población Portadora (CFU/portador)
<i>Pseudomonas aeruginosa</i> ATCC 15442	5% de suero fetal de bovino	1 min		0/60	3.4×10^6
		2 min		0/60 0/60	4.1×10^6
<i>Salmonella enterica</i> ATCC 10708	5% de suero fetal de bovino	1 min		0/60 0/60 0/60	1.8×10^4
<i>Staphylococcus aureus</i> ATCC 6538	5% de suero fetal de bovino	2 min		0/60	1.55×10^6
				0/60 0/60	3.21×10^6
<i>Escherichia coli</i> ATCC 11229	5% de suero fetal de bovino	2 min		0/10 0/10	9.1×10^4
<i>Escherichia coli</i> O157:H7 ATCC 35150	5% de suero fetal de bovino	2 min		0/10 0/10	4.0×10^5
Methicillin-Resistant <i>Staphylococcus aureus</i> MRSA ATCC 33592	5% de suero fetal de bovino	2 min		0/10 0/10	1.76×10^6
Vancomycin-Resistant <i>Enterococcus faecalis</i> VRE ATCC 51575	5% de suero fetal de bovino	2 min		0/10 0/10	1.20×10^5

<i>Listeria monocytogenes</i> ATCC 18117	5% de suero fetal de bovino	2 min		0/10 0/10	1.02×10^5
<i>Klebsiella pneumonia</i> – NDM-1 positive CDC 1000527	5% de suero fetal de bovino	2 min		0/10 0/10	1.66×10^5
<i>Streptococcus suis</i> ATCC 43765	5% de suero fetal de bovino	2 min		0/10 0/10	3.24×10^5

Tabla 2: Resultados de Eficacia Fungicida para Pulverizador Desinfectante Thymox®

Microorganismos de Prueba	Carga de tierra Orgánica	Tiempo de Exposición	Resultados		
			Lote #	Números de portadores que muestran crecimiento*	Resultados de Control de Población Portadora (CFU/portador)
<i>Trichophyton mentagrophytes</i> ATCC 9533	5% de suero fetal de bovino	3 min		0/10 0/10 0/10	9.77×10^4
<i>Candida albicans</i> – ATCC 10231	5% de suero fetal de bovino	3 min		0/10 0/10	1.15×10^6

*Los resultados mostrados para cada lote ensayado. No se observa crecimiento en los subcultivos primarios y secundarios para cada portador

Tabla 3: Resultados de Eficacia Virucida para Pulverizador Desinfectante Thymos®

Microorganismos de Prueba	Carga de tierra Orgánica	Tiempo de Exposición	Resultados	
			Lote #	Reducción de título vírico
Influenza A de porcino H1N1 ATCC VR-333, Cepa /Porcino/Iowa/15/30	5% de suero fetal de bovino	1 min		$\geq 5.75 \log_{10}$
Virus de inmunodeficiencia humano tipo 1, Cepa HTLV-IIIb	5% de suero fetal de bovino	1 min		$\geq 3.75 \log_{10}$
Coronavirus Humano, ATCC VR-740, Cepa 229E	5% de suero fetal de bovino	1 min		$\geq 3.25 \log_{10}$

Tabla 4: Resultados de Eficacia Sanitaria para Pulverizador Desinfectante Thymos®

Microorganismos de Prueba	Carga de tierra Orgánica	Tiempo de Exposición	Resultados		
			Lote #	Reducción de título vírico	
<i>Staphylococcus aureus</i> ATCC 6538	5% de suero fetal de bovino	30 seg		>99.999%	2.29 x 10 ⁶
<i>Enterobacter aerogenes</i> ATCC 13048	5% de suero fetal de bovino	30 seg		>99.999%	1.17 x 10 ⁷

Las pruebas se realizaron utilizando 5 portadores de pruebas y 3 portadores de control

Table 1: Bactericidal Efficacy Results for Thymox® Disinfectant Spray

Test Microorganisms	Organic Soil Load	Exposure time	Results		
			Lot #	Numbers of carriers showing growth*	Carrier Population Control Results (CFU/carrier)
<i>Pseudomonas aeruginosa</i> ATCC 15442	5% fetal bovine serum	1 min		0/60	3.4 x 10 ⁶
		2 min		0/60 0/60	4.1 x 10 ⁶
<i>Salmonella enterica</i> ATCC 10708	5% fetal bovine serum	1 min		0/60 0/60 0/60	1.8 x 10 ⁴
<i>Staphylococcus aureus</i> ATCC 6538	5% fetal bovine serum	2 min		0/60	1.55 x 10 ⁶
				0/60 0/60	3.21 x 10 ⁶
<i>Escherichia coli</i> ATCC 11229	5% fetal bovine serum	2 min		0/10 0/10	9.1 x 10 ⁴
<i>Escherichia coli</i> O157:H7 ATCC 35150	5% fetal bovine serum	2 min		0/10 0/10	4.0 x 10 ⁵
Methicillin-Resistant <i>Staphylococcus aureus</i> MRSA ATCC 33592	5% fetal bovine serum	2 min		0/10 0/10	1.76 x 10 ⁶
Vancomycin-Resistant <i>Enterococcus faecalis</i> VRE ATCC 51575	5% fetal bovine serum	2 min		0/10 0/10	1.20 x 10 ⁵
<i>Listeria monocytogenes</i> ATCC 18117	5% fetal bovine serum	2 min		0/10 0/10	1.02 x 10 ⁵
<i>Klebsiella pneumonia</i> – NDM-1 positive CDC 1000527	5% fetal bovine serum	2 min		0/10 0/10	1.66 x 10 ⁵
<i>Streptococcus suis</i> ATCC 43765	5% fetal bovine serum	2 min		0/10 0/10	3.24 x 10 ⁵

* Results shown for each lot tested.

Table 2: Fungicidal Efficacy Results for Thymox® Disinfectant Spray

Test Microorganisms	Organic Soil Load	Exposure time	Results		
			Lot #	Numbers of carriers showing growth*	Carrier Population Control Results (CFU/carrier)
<i>Trichophyton mentagrophytes</i> ATCC 9533	5% fetal bovine serum	3 min		0/10 0/10 0/10	9.77x 10 ⁴
<i>Candida albicans</i> – ATCC 10231	5% fetal bovine serum	3 min		0/10 0/10	1.15 x 10 ⁶

* Results shown for each lot tested. Growth was not observed in primary and secondary subcultures for each carrier

Table 3: Virucidal Efficacy Results for Thymox® Disinfectant Spray

Test Microorganisms	Organic Soil Load	Exposure time	Results	
			Lot #	Reduction in viral titer
Swine Influenza A H1N1 ATCC VR-333, Strain A/Swine/Iowa/15/30	5% fetal bovine serum	1 min		≥ 5.75 log ₁₀
Human immunodeficiency Virus type 1, Strain HTLV-III _B	5% fetal bovine serum	1 min		≥ 3.75 log ₁₀
Human Coronavirus, ATCC VR-740, Strain 229E	5% fetal bovine serum	1 min		≥ 3.25 log ₁₀

Table 4: Sanitizer Efficacy Results for Thymox® Disinfectant Spray

Test Microorganisms	Organic Soil Load	Exposure time	Results		
			Lot #	Percent reduction	Carrier Population Control Results (CFU/carrier)
<i>Staphylococcus aureus</i> ATCC 6538	5% fetal bovine serum	30 seconds		>99.999%	2.29 x 10 ⁶
<i>Enterobacter aerogenes</i> ATCC 13048	5% fetal bovine serum	30 seconds		>99.999%	1.17 x 10 ⁷

Tests were performed using 5 test carriers and 3 control carriers

Influence of Surfactant Charge on Antimicrobial Efficacy of Surfactant-Stabilized Thyme Oil Nanoemulsions

Khalid Ziani, Yuhua Chang, Lynne McLandsborough, and David Julian McClements*

Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Amherst, Massachusetts 01003, United States

ABSTRACT: Thyme oil-in-water nanoemulsions stabilized by a nonionic surfactant (Tween 80, T80) were prepared as potential antimicrobial delivery systems (pH 4). The nanoemulsions were highly unstable to droplet growth and phase separation, which was attributed to Ostwald ripening due to the relatively high water solubility of thyme oil. Ostwald ripening could be inhibited by incorporating $\geq 75\%$ of corn oil (a hydrophobic material with a low water solubility) into the nanoemulsion droplets. The electrical characteristics of the droplets in the nanoemulsions were varied by incorporating ionic surfactants with different charges after homogenization: a cationic surfactant (lauric arginate, LAE) or an anionic surfactant (sodium dodecyl sulfate, SDS). The antifungal activity of nanoemulsions containing positive, negative, or neutral thymol droplets was then conducted against four strains of acid-resistant spoilage yeasts: *Zygosaccharomyces bailli*, *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Brettanomyces naardensis*. The antifungal properties of the three surfactants (T80, LAE, SDS) were also tested in the absence of thymol droplets. Both ionic surfactants showed strong antifungal activity in the absence of thymol droplets, but no antimicrobial activity in their presence. This effect was attributed to partitioning of the antimicrobial surfactant molecules between the oil droplet and microbial surfaces, thereby reducing the effective concentration of active surfactants available to act as antimicrobials. This study shows oil droplets may decrease the efficacy of surfactant-based antimicrobials, which has important consequences for formulating effective antimicrobial agents for utilization in emulsion-based food and beverage products.

KEYWORDS: emulsions, nanoemulsions, thyme oil, stability, charge, antifungal, antimicrobial, fungi, yeast

■ INTRODUCTION

Emulsion-based systems are being increasingly used as delivery systems to encapsulate lipophilic bioactive components, such as antitumor agents,^{1,2} anti-inflammatory agents,² vitamins,^{3,4} and antimicrobials.^{5–9} Emulsion-based systems may be divided into either conventional emulsions (radius > 100 nm) or nanoemulsions (radius < 100 nm) depending on the dimensions of the droplets they contain.^{10,11} In this study, we investigated the potential of using nanoemulsions as antimicrobial delivery systems suitable for utilization within the food and other industries. Oil-in-water nanoemulsions consist of fine oil droplets dispersed within an aqueous liquid.^{12,13} Like conventional emulsions, nanoemulsions are thermodynamically unstable systems that tend to break down due to a variety of physicochemical mechanisms, such as gravitational separation, flocculation, coalescence, and Ostwald ripening.¹⁴ However, the small size of the droplets in nanoemulsions means they have physicochemical and biological properties different from those of conventional emulsions, which provides advantages for certain industrial applications.¹⁵ The relatively small droplet size in nanoemulsions means that (i) they are more stable to gravitational separation, flocculation, and coalescence than conventional emulsions; (ii) they scatter light less efficiently than conventional emulsions and so may be transparent or only slightly turbid; (iii) they interact with biological surfaces differently from conventional emulsions, which may affect the bioactivity of any encapsulated components.¹⁶ In general, emulsion-based systems also offer the potential of incorporating multiple active components into a single delivery system, which may increase their efficacy.

This study focuses on the development of antimicrobial nanoemulsions containing two different antimicrobial agents: essential oils and ionic surfactants. Essential oils have been shown to have antibacterial, antiviral, and antifungal activities.^{17–19} Commercial essential oils contain a complex mixture of different constituents that vary in their molecular and physicochemical properties, for example, molecular weight, polarity, solubility, partitioning, and biological activity. Some of the most potent antimicrobial components in essential oils are hydrophobic compounds, such as cinnamic aldehyde, carvacrol, thymol, and eugenol.²⁰ Consequently, they must usually be encapsulated within colloidal delivery systems if they are going to be incorporated into aqueous-based foods and beverages.^{21–24} An essential oil extracted from thyme was utilized in this study to formulate antimicrobial nanoemulsions. Thyme oil is obtained commercially by hydrodistillation of the flowering plants of common thyme (*Thymus vulgaris*).²⁵ It is commonly used as a flavoring agent in food products,²⁶ but it also has applications in cosmetics, personal care products, and pharmaceuticals.^{27,28} Thyme oil has been shown to have inhibitory activities against various bacteria and yeasts.²⁰ Thymol, the major component of thyme oil,²⁹ has also been shown to exhibit antimicrobial activity against several bacteria and fungi.^{30,31} The antimicrobial activity of thymol has been attributed to its ability to penetrate into and disturb the structure of microbial cell membranes, thereby leading to leakage of ions and other cell contents.¹⁷

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A number of ionic surfactants have also been shown to exhibit strong antimicrobial activity, such as lauric arginate (a cationic surfactant) and sodium dodecyl sulfate (an anionic surfactant).^{32–36} The antimicrobial activity of ionic surfactants has been attributed to their ability to be incorporated into the lipid membrane of microbial cells, thereby disrupting normal cellular function.³⁷ Ionic surfactants may also influence the antimicrobial activity of nanoemulsions by altering the electrical characteristics of the oil droplets. The surfaces of microorganisms are typically negatively charged,^{38,39} and therefore positively charged droplets might be expected to be electrostatically attracted to their surfaces, whereas negatively charged droplets would be expected to be repelled.³⁸ Consequently, there should be a higher concentration of any active component encapsulated by lipid droplets within the immediate vicinity of the microbial cell surface for positive droplets than for negative droplets. Thus, the antimicrobial activity of any encapsulated substances might be expected to increase when they are incorporated into positively charged droplets. The electrical charge on the droplets in nanoemulsions can be manipulated in a number of different ways, for example, by using one or more emulsifiers with different charge characteristics or by adsorbing charged substances onto the droplet surfaces.¹⁵ Consequently, controlling the electrical characteristics of nanoemulsion delivery systems may be an effective way of increasing their antimicrobial activity. Conversely, antimicrobial surfactants may remain adsorbed to the surfaces of oil droplets in nanoemulsions, which could reduce the amount present to interact with the microorganisms, thereby decreasing their efficacy. It is therefore important to establish whether nanoemulsions containing a combination of different antimicrobials are more or less effective than the individual components themselves.

The main objectives of the current research were to prepare physically stable nanoemulsions containing both thyme oil and ionic surfactants and then to test their antimicrobial activity against a number of acid-resistant yeasts. In addition, we aimed to determine whether using a combination of antimicrobial agents (essential oils and ionic surfactants) within a single delivery system had synergistic or antagonistic effects on the overall antimicrobial efficacy. The results of this study have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries.

MATERIALS AND METHODS

Materials. Thyme oil was purchased from Optimal Health Solutions (La Pine, OR). Corn oil was obtained from a local supermarket. Both oils were used without further purification. A nonionic surfactant (Tween 80, T80) and an anionic surfactant (sodium dodecyl sulfate, SDS) were purchased from Sigma-Aldrich Co. (St. Louis, MO), and a cationic surfactant (Mirenat-N) was provided by Vedeqsa (Grupo Lamirsa Terrassa, Spain). For confocal microscopy work, technical grade Nile Red dye (CAS Registry No. 7385-67-3) was purchased from Sigma-Aldrich.

Nanoemulsion Preparation. The aqueous phase used to prepare the nanoemulsions consisted of 0.5% (w/w) T80 dispersed in an aqueous buffer solution (10 mM acetate, pH 4). Lipid phases were prepared by mixing different mass ratios of thyme oil and corn oil (from 0 to 100 wt % corn oil) prior to homogenization. The lipid phase (5% w/w) was mixed with the aqueous phase (95% w/w) using a high-speed blender for 60 s. The resulting emulsion premix was then homogenized by passing it five times through a high-pressure homogenizer at 9 kPa (Microfluidics 110 L, Microfluidics Corp., Newton, MA) to further reduce the particle size. After preparation, the nanoemulsions formed were stored at 20 °C

prior to analysis. To change the interfacial charge of the previously formed droplets, different amounts of either SDS solution or lauric arginate (LAE) solution were added.

Nanoemulsion Characterization. *Particle Size Measurements.* The mean particle diameters (Z-averages) of the nanoemulsions were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, U.K.). This instrument determines the particle size from intensity–time fluctuations of a laser beam (633 nm) scattered from a sample at an angle of 173°. Each individual measurement was an average of 13 runs. The nanoemulsions were diluted using buffer solution (10 mM acetate buffer, pH 4) prior to analysis to avoid the effects of multiple scattering.

Particle Charge Measurements. The electrical charge (ζ -potential) of the droplets in the nanoemulsions was measured using a particle electrophoresis instrument (Zetasizer Nanoseries ZS, Malvern Instruments, Worcestershire, U.K.). Initially, the nanoemulsions were diluted 100-fold using buffer solution (10 mM acetate, pH 4) to avoid multiple scattering effects. Diluted samples were then placed in a disposable cuvette that acted as a measurement chamber, and the ζ -potential was determined by measuring the direction and velocity that the particles moved in the applied electric field. The Smoluchowsky model was used by the instrument's software program to convert the electrophoretic mobility measurements into ζ -potential values.

Determination of Antifungal Activity. *Minimum Inhibitory Concentration (MIC) Assay.* Four strains of acid-resistant spoilage yeasts were provided by the Pepsico R&D Culture Collection (Valhalla, NY): *Zygosaccharomyces bailii* (ZB), *Saccharomyces cerevisiae* (SC), *Brettanomyces bruxellensis* (BB), and *Brettanomyces naardenensis* (BN). Yeast cultures were kept frozen at –70 °C in 25% glycerol. The yeast strains were refreshed on malt extract agar plates (Becton Dickinson, Sparks, MD) before treatments. A single yeast colony from the plate was inoculated into 10 mL of malt extract broth (MEB) medium (Becton Dickinson, Sparks, MD), which was adjusted to pH 4.0 by citrate buffer with the final strength of 10 mM. The culture was then incubated at 25 °C under mild agitation (150 rpm in a rotary shaker) for about 2 days until the optical density at 600 nm (OD_{600}) was around 1.0. As a guideline, at an OD_{600} of 1.0, cultures of yeast strains contain approximately 5×10^6 CFU/mL. The cultures were then diluted 100-fold using fresh pH 4.0 MEB medium to determine the MIC.

The MIC was determined for each of the four yeast strains mentioned above using 96-well microtiter plates (Falcon 3075) by a 2-fold serial dilution method. The microtiter plate wells of the 1st and 12th columns were filled with 150 μ L of blank MEB medium to establish potential contamination, and the rest of the wells were filled with 150 μ L of 1:100 inoculated pH 4.0 MEB medium as stated above (about 5×10^4 CFU/mL inoculation level). One hundred and fifty microliters of inoculated MEB medium (pH 4.0) containing 10% of the treatment solution (Table 1) was added to each of the eight wells of the second column and mixed thoroughly; 150 μ L of the mixed medium from the second column were then transferred to the wells of the third column, and this procedure was continued so as to make successive 2-fold dilutions up to the 11th column. The final concentrations of each treatment solution from the 2nd column to the 11th columns were therefore 5, 2.5, 1.25, 0.6, 0.3, 0.16, 0.8, 0.4, 0.2, and 0.1%, respectively. Plates were incubated at 25 °C for 5 days, and the MICs were assessed visually as the lowest concentration showing complete growth inhibition.

Microbial Adhesion to Hydrocarbon (MATH) Assay. The surface hydrophobicity of yeast cells after treatments with nanoemulsions was measured by a MATH assay described by Rosenberg⁴⁰ with some modifications. In these experiments, only *S. cerevisiae* (SC) cells were used as representative yeast cells. One and a half milliliters of freshly grown SC cells in MEB medium (pH 4.0) was collected by centrifugation and then resuspended in 1 mL of either 5% nonionic emulsion (oil + 0.5% T80), 5% cationic emulsion (oil + 0.5% T80 + 0.32% LAE),

Table 1. Antifungal Properties of Different Treatments against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, and *Brettanomyces naardenensis*^a

initial delivery system composition (%)				fungus name	final amount of delivery system added to MEB (wt %)				
oil ^c	T80	SDS	LAE		5	2.5	1.25	0.6	0.3
0	0.5	0	0	<i>Zygosaccharomyces bailii</i>	+	+	+	+	+
0	0.5	0.8	0		–	–	+	+	+
0	0.5	0	0.32		–	–	–	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+
0	0.5	0	0	<i>Brettanomyces naardenensis</i>	+	+	+	+	+
0	0.5	0.8	0		–	+	+	+	+
0	0.5	0	0.32		–	–	+	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+
0	0.5	0	0	<i>Brettanomyces bruxellensis</i>	+	+	+	+	+
0	0.5	0.8	0		–	+	+	+	+
0	0.5	0	0.32		–	–	+	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+
0	0.5	0	0	<i>Saccharomyces cerevisiae</i>	+	+	+	+	+
0	0.5	0.8	0		–	–	+	+	+
0	0.5	0	0.32		–	–	–	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+

^aInitial delivery systems consisted of either surfactant only or surfactant-stabilized thymol oil droplets. ^bCalculated final compositions of delivery systems in microbial broth:

concn of delivery system in MBE broth (wt %)	thyme oil (wt %)	T80 (wt %)	LAE (wt %)	SDS (wt %)
5	0.0625	0.025	0.016	0.04
2.5	0.03125	0.0125	0.008	0.02
1.2	0.016	0.00625	0.004	0.01
0.6	0.0078	0.0031	0.002	0.005
0.3	0.0039	0.0015	0.001	0.0025

^c25% thyme oil/75% corn oil.

5% anionic emulsion (oil + 0.5% T80 + 0.8% SDS), or acetate buffer (10 mM, pH 4.0) as a control. After 2 h of treatment at room temperature, the yeast cells were centrifuged, the treatment solutions were poured off, and the yeast cells were then washed twice with 1.2 mL of acetate buffer to remove the remaining treatment solutions. The yeast cells were then resuspended in appropriate amounts of acetate buffer to achieve an initial absorbance (A_1) of 1.0 cm^{−1} at 600 nm, using a UV–visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech). In a clean borosilicate round-bottom glass tube (12 × 75 mm), 1 mL of hexadecane (Sigma, purity ≥ 99%) was added to 2 mL of yeast suspension. The tube was vortexed for 2 min and set aside to rest for

15 min to allow the hexadecane phase to fully rise and separate due to gravity. Next, the yeast suspension was collected with a clean Pasteur pipet; great care was taken to avoid collection of the hexadecane layer. The yeast suspension was then transferred to a cuvette for the final absorbance measurement (A_F) at 600 nm. Adhesion of yeasts to the hydrocarbons was evaluated as the fraction partitioned to the hydrocarbon phase, FPC, which was calculated as $FPC = 1 - A_F/A_1$. The value of FPC could be used to evaluate the surface hydrophobicity of yeast cells. The MATH assays were performed in duplicate.

Surface Charge (ξ-Potential) Measurements of Yeast Cells. Additional information about the interactions between nanoemulsion

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droplets and yeast cells was determined by measuring the surface charge of mixed systems. The ζ -potential of yeast cells (SC) after treatment with different nanoemulsions (nonionic, cationic, anionic) was measured using a particle microelectrophoresis instrument (Zetasizer Nano-ZS). The yeast cells were treated exactly the same way as described above, and then they were resuspended in appropriate amounts of acetate buffer (10 mM, pH 4.0) to achieve an OD₆₀₀ of around 1.0 cm⁻¹. The ζ -potential of the resulting yeast cell suspensions was then measured at room temperature. Each measurement was conducted in duplicate.

Confocal Fluorescence Microscopy. Interactions between nanoemulsion droplets and freshly grown yeast cells (SC) were observed using confocal fluorescence microscopy. The yeast cells (Syto 9) and droplets (Nile Red) were stained using two different fluorescent dyes so that we could distinguish them. Nile Red is a lipid soluble stain that gives a red-fluorescent color. A Nile Red solution was prepared by dissolving 0.1 mg of Nile Red in 10 mL of methanol. Syto 9 is a nucleic acid stain that dyes both live and dead cells a green-fluorescent color. A Syto 9 solution was prepared by dispersing the dye in an aqueous solution according to the manufacturer instructions (L7012; Molecular Probes, Carlsbad, CA) and then incubated with the yeast cells. The yeast cells were then washed twice with acetate buffer (10 mM, pH 4.0) to remove the residual Syto 9, and the stained yeast cells were incubated for 2 h with nanoemulsions. For the control, the yeast cells were incubated with buffer solution. Two drops of Nile Red dye solution were then added to the samples prior to transfer onto glass microscope slides. Syto 9 fluorescence was detected by excitation at 488 nm, and emission was collected with a 500–530 nm band-pass filter. Nile Red fluorescence was detected by excitation at 605 nm, and emission was collected with a 565–615 nm band-pass filter. All images were acquired and processed using the instrument's software program (EZ-CS1 version 3.8, Nikon, Melville, NY).

RESULTS

Impact of Lipid Phase Composition on Nanoemulsion Formation and Stability. If nanoemulsions are going to be used as delivery systems for antimicrobial agents, then it is important that they have good long-term stability during storage. We therefore carried out a series of preliminary experiments to establish the physical stability of the nanoemulsions to droplet growth and phase separation.

Initially, we attempted to prepare a nanoemulsion by homogenizing pure thyme oil and an aqueous nonionic surfactant solution: 5% thyme oil, 0.5% T80, pH 4.0. However, the resulting emulsions were highly unstable to droplet growth and phase separation; after 3 days of storage, the mean particle diameter was >7000 nm, and there was visible evidence of creaming and oiling off (Figure 1). The high instability of this system can be attributed to the fact that thyme oil has an appreciable water solubility ($\approx 1 \text{ g L}^{-1}$ for thymol at 25 °C, MSDS sheet), which means that it is highly susceptible to Ostwald ripening (OR). OR is the growth of large oil droplets at the expense of smaller oil droplets due to diffusion of oil molecules through the intervening aqueous phase.^{41,42} The increase in mean droplet diameter (d) with time (t) due to Ostwald ripening of a one-component emulsified lipid in the steady state regime is given by the following equation:⁴³

$$d^3 - d_0^3 = \omega t \quad \frac{32}{9} \alpha c D t \quad (1)$$

Here, ω represents the Ostwald ripening rate, $\alpha (= 2\gamma V_m/RT)$, γ is the interfacial tension, V_m is the molar volume of the lipid, R is the gas constant, T is the absolute temperature, c is the solubility of the lipid in the aqueous phase, and D is the translational

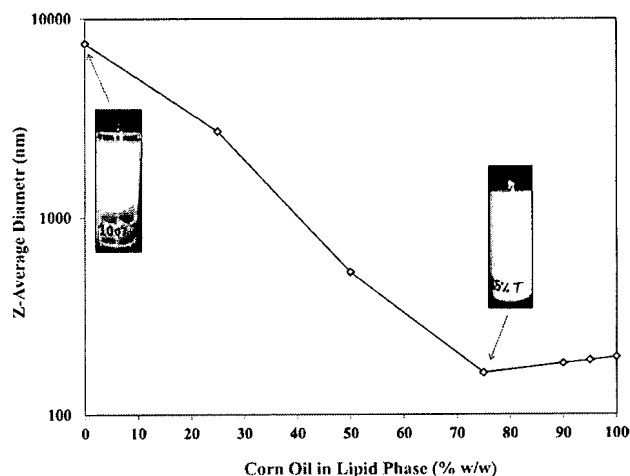


Figure 1. Dependence of mean droplet diameter after 2 days of storage at ambient temperature on oil phase composition for 5% oil-in-water emulsions containing different amounts of thyme oil and corn oil in the lipid phase (0.5% Tween 80, 10 mM acetate buffer, pH 4).

diffusion coefficient of the lipid in the aqueous phase. This equation highlights that the rate of OR is directly proportional to the solubility of the oil in the aqueous phase, which accounts for the observed increase in droplet size during storage observed in the thyme oil nanoemulsions (Figure 1).

Previous studies have shown that OR can be retarded or prevented in emulsions by mixing an oil that has a relatively high water solubility with another oil that has a very low water solubility prior to homogenization.^{42,44,45} This second oil can be referred to as a “ripening inhibitor”, and it is usually a highly nonpolar substance with a relatively high molecular weight. The amount of ripening inhibitor required to prevent OR depends on the molecular weights and solubilities of the low and high water-soluble oils. In the current study, we examined the possibility of improving the OR stability of our emulsions by mixing thyme oil with corn oil, because this is a food-grade oil that could be used as part of an edible delivery system. Corn oil is primarily composed of highly nonpolar triacylglycerol molecules with relatively high molecular weights and low water solubilities and is therefore a highly effective ripening inhibitor.⁴⁴

After homogenization, samples with different droplet compositions (thyme oil/corn oil) were stored for 2 days at ambient temperature, mixed to ensure they were homogeneous, and then their particle size was measured (Figure 1). As mentioned earlier, emulsions prepared using a lipid phase consisting of only thyme oil (0% corn oil) were highly unstable, being susceptible to droplet growth, creaming, and oiling off. We postulate that the droplet size increased due to OR, which led to rapid creaming because of the relatively large droplet size ($d > 5000 \text{ nm}$) and relatively low density of thyme oil ($\rho = 923 \text{ kg m}^{-3}$) compared to water ($\rho = 1000 \text{ kg m}^{-3}$). As the concentration of corn oil in the lipid phase was increased, the extent of droplet growth observed after 2 days of storage decreased (Figure 1). From 0 to 75% corn oil, there was a steep decrease in mean particle diameter with increasing corn oil concentration, which can be attributed to the ability of the triacylglycerol oil to inhibit OR.^{39,46} The smallest droplet diameter (163 nm) that could be produced occurred when about 75% of corn oil was present in the lipid phase prior to

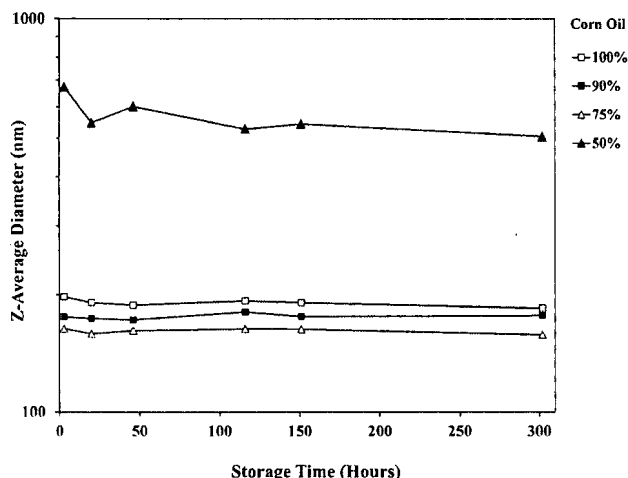


Figure 2. Evolution of mean droplet diameter with time at ambient temperature for diluted 5 wt % oil-in-water emulsions containing different ratios of thyme and corn oil in the lipid phase (0.5% Tween 80, 10 mM acetate buffer, pH 4).

homogenization. A further increase in corn oil concentration caused a slight increase in mean droplet diameter (196 nm for pure corn oil), which can be attributed to the increase in lipid phase viscosity and interfacial tension, both of which oppose the formation of small droplets in a high-pressure homogenizer.^{47,48}

The ability of low water solubility oils to retard OR in emulsions containing high water solubility oils can be attributed to an entropy of mixing effect.^{42,44,45} Consider an oil-in-water emulsion that contains droplets composed of two different lipid components: a water-insoluble component (such as corn oil) and a water-soluble component (such as thyme oil). The water-soluble oil molecules will diffuse from the small to the large droplets due to OR. Consequently, there will be a greater percentage of water-soluble oil molecules in the larger droplets than in the smaller droplets after OR occurs. Differences in the composition of emulsion droplets are thermodynamically unfavorable because of entropy of mixing; it is more favorable to have the two oils distributed evenly throughout all of the lipid droplets rather than concentrated in particular droplets. Consequently, there is a thermodynamic driving force that operates in opposition to the OR effect. The change in droplet size distribution with time then depends on the concentration and solubility of the two components within the oil droplets.

Practically, one would like to maximize the amount of an active ingredient (in this case thyme oil) present in a delivery system. Our study suggests that nanoemulsions with relatively small droplets ($r < 85$ nm) can be produced using a lipid phase of 25% corn oil and 75% thyme oil. We also measured the long-term stability of these systems to droplet growth by measuring the change in mean particle diameter with time (Figure 2) and the particle size distribution (Figure 3) before and after storage. There was no appreciable change in the mean droplet diameter or particle size distribution of the samples containing 75% or more corn oil up to 300 h (12.5 days) of storage. Samples containing 50% corn oil and 50% thyme oil had larger mean diameters, but they also appeared to remain relatively stable to droplet growth during storage, indicating that OR had been largely inhibited.

Impact of Surfactant Type and Concentration on Nanoemulsion Droplet Charge. The functional performance of

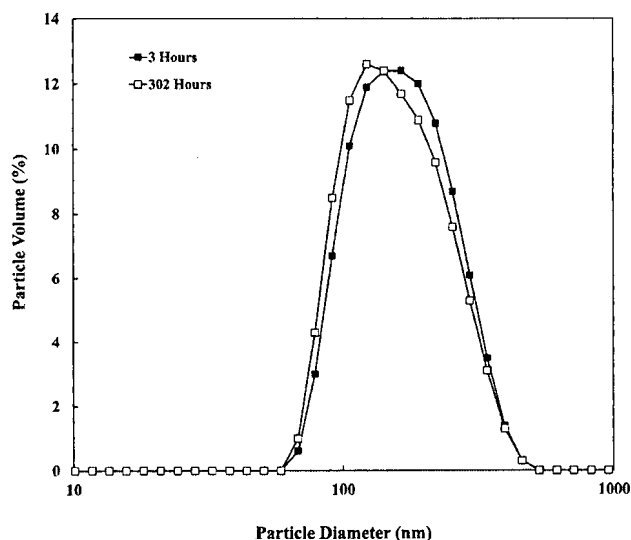


Figure 3. Dependence of particle size distributions before and after storage of diluted 5% oil-in-water emulsions containing 25% thyme oil and 75% corn oil in the lipid phase (0.5% Tween 80, 10 mM acetate buffer, pH 4).

nanoemulsions containing antimicrobial essential oils is likely to depend on the electrical characteristics of the oil droplets, because this would be expected to affect their interactions with the anionic surfaces of microorganisms. We therefore examined the possibility of preparing nanoemulsion droplets with different electrical characteristics by mixing the nonionic surfactant stabilized nanoemulsions with different types and concentrations of ionic surfactants after homogenization. The initial nanoemulsions prepared in these experiments consisted of 5% lipid phase (25% thyme oil/75% corn oil) stabilized by 0.5% T80. A cationic surfactant (LAE) was used to create positively charged droplets, whereas an anionic surfactant (SDS) was used to create negatively charged droplets.

The impact of adding different types and concentrations of ionic surfactants on the ζ -potentials of the nanoemulsion droplets was determined (Figure 4). The ζ -potential of the initial nanoemulsion (containing only T80) was slightly negative (≈ -4 mV), which can be attributed to the presence of some anionic impurities in the surfactant (such as free fatty acids) or adsorption of anionic species from the water (such as hydroxyl ions) to the droplet surfaces.⁴⁹ When SDS was added to the initial nanoemulsion, the droplet charge became much more strongly anionic. There was a steep increase in the negative charge on the droplets from -4 to -44 mV when the SDS concentration was increased from 0 to 0.1%, after which the charge remained relatively constant upon further addition of SDS. This suggested that the anionic SDS molecules adsorbed to the oil–water interface and displaced some or all of the nonionic T80 molecules. Indeed, the ζ -potential of the droplets in a nanoemulsion prepared using only SDS was measured to be ≈ -67 mV, which suggested that the droplets were covered predominately by SDS rather than T80 at high SDS concentrations. Conversely, when LAE was added to the initial nanoemulsion, the droplet charge became increasingly cationic. There was an appreciable increase in the positive charge on the droplets from -4 to $+39$ mV when the LAE concentration was increased from 0 to 0.32%. The change in droplet charge with increasing

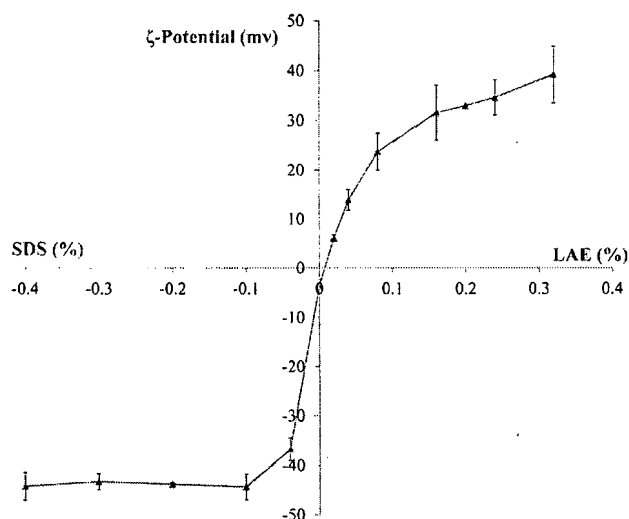


Figure 4. Effect of addition of different levels of anionic surfactant (SDS) or cationic surfactant (LAE) to T80-stabilized 5% oil-in-water nanoemulsions on the droplet ζ -potential. The lipid phase consisted of 25% thyme oil and 75% corn oil. For comparison, the ζ -potential of nanoemulsions prepared with only SDS was -67 mV, and that of those prepared with only LAE was $+73$ mV.

LAE concentration was steepest from 0 to 0.08%, but still increased gradually from 0.08 to 0.32%, and did not appear to reach a plateau region. This suggested that the cationic LAE molecules had adsorbed to the oil–water interface and displaced at least some of the nonionic T80 molecules. The ζ -potential on a nanoemulsion prepared using only LAE was measured to be $\approx +73$ mV, which suggested that the droplet surfaces were not completely saturated with LAE in the presence of T80 (Figure 4).

The initial change in particle charge with increasing ionic surfactant concentration was higher for SDS than for LAE (Figure 4), which may be attributed to differences in their molecular characteristics. The molecular weight of LAE (421.0 g mol^{-1}) is considerably higher than that of SDS (288.4 g mol^{-1}). When the results were expressed on a molar basis, rather than a mass basis, then the change in droplet charge with increasing surfactant concentration is more similar for LAE and SDS (data not shown). However, the SDS molecules still appeared to have a somewhat higher affinity for the T80-coated droplets than the LAE molecules. SDS and LAE both have similar nonpolar tails consisting of a lauric acid chain (12 carbon atoms), but they do have different head groups. The cationic headgroup (arginate) on LAE is considerably larger than the anionic headgroup (sulfate) on SDS, which suggests that the SDS molecules may be able to pack more effectively at the oil–water interface.

These results indicate that nanoemulsions with a range of different charge characteristics can be prepared by mixing nonionic coated droplets with different types and concentrations of ionic surfactant solutions. In the following sections, the antimicrobial activities of nanoemulsions with different charge characteristic were tested. For these studies we prepared three nanoemulsions with different electrical characteristics: anionic (0.5% T80 + 0.8% SDS), nonionic (0.5% T80, 0% SDS or LAE), and cationic (0.5% T80 + 0.32% LAE).

Antifungal Activity of Surfactant Solutions. Many surfactants are known to have strong antimicrobial activity,³⁶ and therefore we initially measured the ability of SDS, LAE, and

Table 2. Physicochemical Characteristics of the Surfactant Micelles Used in This Study

	T80	T80/SDS	T80/LAE
ζ -potential (mV)	-0.1	-18	$+16.3$
Z-average diameter (nm)	12.6	7.4	6.9

Tween 80 solutions to inhibit microbial growth using similar conditions as were used to test the nanoemulsions (Table 1). The antifungal activities of the three surfactants against four yeast strains, *Z. bailli* (ZB), *S. cerevisiae* (SC), *B. bruxellensis* (BB), and *B. naardenensis* (BN), were tested by measuring the MIC (Table 1). The MIC was determined by calculating the final amount of surfactant in the broth that was required to completely inhibit observable microbial growth. The nonionic surfactant (T80) did not show any antifungal activity at the levels used in this study; that is, fungal growth occurred at all concentrations used. On the other hand, both ionic surfactants were able to inhibit microbial growth above a certain concentration. For convenience, we express the surfactant concentrations as micrograms per milliliter for the microbiology experiments, which is equivalent to 0.0001%. For the anionic surfactant, the presence of $200 \mu\text{g/mL}$ (0.02%) of SDS inhibited the growth of ZB and SC, whereas the presence of $400 \mu\text{g/mL}$ (0.04%) of SDS inhibited the growth of BB and BN. For the cationic surfactant, the presence of $40 \mu\text{g/mL}$ (0.004%) of LAE inhibited the growth of ZB and SC, whereas the presence of $80 \mu\text{g/mL}$ (0.008%) of LAE inhibited the growth of BB and BN. These results show that LAE was more effective at inhibiting microbial growth than SDS and that T80 did not show any antifungal effects against all four tested yeast strains.

We also measured the characteristics of the micelles formed by the three types of surfactant (Table 2). The T80 micelles were slightly larger in diameter than the T80/SDS or T80/LAE micelles. As would be expected, the T80 micelles had an electrical charge close to zero, the T80/SDS micelles were strongly anionic, and the T80/LAE micelles were strongly cationic. It has been proposed that the antimicrobial activity of ionic surfactants is at least partly due to their ability to penetrate into microbial cell membranes, leading to membrane disruption and loss of biological function.³³ One might expect that cationic surfactants, which have an opposite charge to bacterial membranes, would adsorb more strongly and be more effective at disrupting the membranes. In addition, previous studies have shown that both anionic and cationic surfactants promote denaturation of globular proteins above a critical level, whereas nonionic surfactants are not particularly effective.⁵⁰ These surfactants may therefore have been able to interfere with functional proteins associated with the cell membranes (e.g., transport or signaling proteins). Further research is clearly required to establish the physicochemical and/or biochemical mechanisms by which surfactants inhibit microbial growth.

Antifungal Activity of Nanoemulsions. Initially, oil-in-water nanoemulsions were prepared by homogenizing 5% lipid phase (25% thyme oil; 75% corn oil) with 95% aqueous phase (0.5% T80, pH 4.0). Nanoemulsions containing either anionic or cationic droplets were then prepared by mixing the original nanoemulsion with either 0.8% SDS or 0.32% LAE, respectively.

For all three surfactants, thyme oil nanoemulsions did not show any antifungal effects, even at the highest concentrations tested ($625 \mu\text{g/mL}$ (0.0625%) thyme oil in broth). This was

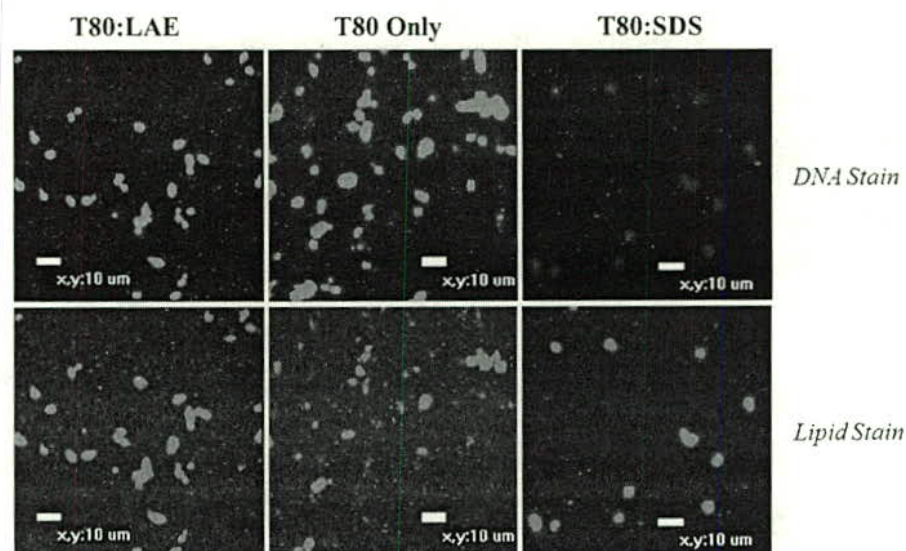


Figure 5. Confocal microscopy images of mixed systems containing nanoemulsions and yeast cell (SC) suspensions. The yeast cells were stained green with a DNA stain (Syto 9), whereas the oil droplets and yeast cell membranes were stained red with a nonpolar stain (Nile Red). The nanoemulsions consisted of 5% oil phase (25% thyme oil/75% corn oil), 0.5% nonionic surfactant (Tween 80), and additional LAE, buffer, or SDS (10 mM acetate buffer, pH 4).

probably because the highest concentration of thyme oil used was lower than the MIC. Paster et al. reported that thyme oil did not inhibit mycelial growth of *Aspergillus ochraceus* at 700 $\mu\text{g/mL}$.⁵¹ Higher thyme oil concentrations were not investigated in this study because the yeast's growth could not be accurately determined by the MIC measurement protocol due to the fact the samples are too opaque to measure optical densities. In addition, higher thyme oil concentrations would be unacceptable for most food applications due to the distinct flavor characteristics of this essential oil. Finally, the presence of the corn oil may have affected the ability of the thyme oil to become incorporated into the microbial membranes, for example, by acting as a hydrophobic sink.

Initially, we hypothesized that when thyme oil was incorporated within positively charged (LAE) nanoemulsion particles, it would be a more effective antimicrobial because it would be attracted to the surfaces of negatively charged yeast cells. Conversely, we hypothesized that when thyme oil was incorporated into negatively charged (SDS) nanoemulsion particles, it would be less effective as an antimicrobial because it would be repelled from negatively charged yeast cell surfaces. In practice, we found that thyme oil droplets actually decreased the antimicrobial activity of both LAE and SDS, which were found to be antimicrobial when used in isolation but ineffective when used in the presence of the oil droplets (Table 1). In other words, the presence of nanosized lipid droplets actually reduced the antifungal activity of the ionic surfactants. We hypothesize that this effect was due to partitioning of the surfactants between the lipid droplet surfaces and the yeast cell surfaces. The presence of the lipid droplets would reduce the effective concentration of surfactants in the aqueous phase, thereby reducing their ability to interact with the microbial membranes. The small size of the lipid droplets in nanoemulsions means that they have a high surface area per unit mass of oil, which would exacerbate this effect. Similar results have been reported for microemulsion systems, in which the presence of milk fat droplets was found to decrease the antimicrobial efficacy of surfactant microemulsions containing an essential oil (eugenol).⁵²

Nature of Interactions between Nanoemulsion Droplets and Yeast Cells. In this section, we used a number of methods to provide more information about the interaction of the various kinds of nanoemulsion droplets with the yeast cells. Because all strains of yeast behaved fairly similarly in the antimicrobial assays (Table 1), we used only SC cells as a representative strain in these studies.

Some insight into the nature of the interaction between nanoemulsion droplets and yeast cell surfaces was obtained using a MATH assay. Yeast surface hydrophobicity after nanoemulsion treatments was expressed by their FPC value: control = 0.06 ± 0.03 ; LAE/T80 = 0.63 ± 0.07 ; SDS/T80 = 0.07 ± 0.02 ; T80 = 0.05 ± 0.03 . These results show that the yeast cells became much more hydrophobic after treatment with cationic nanoemulsions, but showed little change in hydrophobicity when treated with either nonionic or anionic nanoemulsions. It is possible that the positively charged oil droplets in the cationic nanoemulsion attached to the negatively charged yeast cell surfaces, thereby increasing their hydrophobicity. Alternatively, the yeast cell surfaces may have become more hydrophobic because the cell membrane was disrupted by the cationic surfactant.

Additional information about the interaction of nanoemulsion droplets with yeast cells was obtained using ζ -potential measurements. The yeast cells were highly anionic before treatment with nanoemulsions: -18.1 ± 1.2 mV. After treatment, the ζ -potential of the yeast cells depended on the type of surfactant initially coating the nanoemulsion droplets: LAE/T80 = 2.0 ± 0.4 mV; SDS/T80 = -18.4 ± 0.5 mV; T80 = -18.7 ± 0.5 mV. These results support the MATH data, indicating that there was a strong interaction between the cationic droplets and the yeast cells, but little interaction between the nonionic or anionic droplets and the yeast cells. The fact that the yeast cells became positively charged after being in contact with the cationic nanoemulsion suggests that at least some of the cationic surfactant adsorbed to the yeast cell surfaces. Whether the LAE was adsorbed as individual molecules, micelles, or lipid droplets is currently unknown and would be a useful topic for future study.

Finally, we used confocal microscopy to observe differences in the microstructure of suspensions containing a mixture of yeast cells (SC) and nanoemulsions (Figure 5). The yeast cells were stained green with a DNA dye (Syto 9), whereas the oil droplets were stained red with a nonpolar dye (Nile Red). The separated fluorescent images of the two stains showed that Syto 9 stained only yeast cells but that Nile Red stained both nanoemulsion droplets and yeast cells (Figure 5). The intensity of the green dye in the yeast cells was appreciably lower for the SDS-containing systems than for the other systems, which may have been because this anionic surfactant interfered with the cationic Syto 9 stain. Presumably, the Nile Red was located in the lipophilic environments of both the nanoemulsion droplet interiors and the phospholipid-rich microbial membranes. Because the Nile Red stained both the yeast cells and the nanoemulsion droplets, it was not possible to ascertain whether droplets had preferentially accumulated around the yeast cells. Nevertheless, the microscopy images indicated that there were lipid droplets dispersed within the aqueous phase surrounding the yeast cells for all three nanoemulsion types (Figure 5). However, there appeared to be somewhat fewer droplets in the aqueous phase of the samples containing LAE (lower intensity of small red dots), which may indicate that more cationic LAE-coated droplets were absorbed to the yeast cell surfaces. Nevertheless, further work would be required to confirm this. It should be noted that it is not possible to observe the individual nanoemulsion droplets in the images because their dimensions were below the resolution of optical microscopy. The fact that many of the lipid droplets were present in the aqueous phase surrounding the yeast cells may account for the fact that the ionic surfactants were less effective as antimicrobials in the presence of nanoemulsions. As mentioned earlier, some of the ionic surfactants would be located at the surfaces of the lipid droplets and would therefore be unavailable for interacting with the microbial surfaces.

In future studies, it would be useful to carry out a detailed analysis of the physicochemical and biological mechanisms by which ionic surfactants act as antimicrobial agents and to establish the impact of lipid droplets on these mechanisms. Understanding the interactions of surfactants and lipid droplets with microbial surfaces may lead to the design of more effective antimicrobial delivery systems. In this study, we have shown that physically stable thyme oil-in-water nanoemulsions containing droplets with a range of different electrical characteristics, from highly positive to highly negative, can be produced by adding different types and amounts of ionic surfactants (cationic or anionic) after homogenization. In the absence of nanoemulsion droplets, the cationic and anionic surfactants both had appreciable antimicrobial activity, which was attributed to their ability to adsorb to the surfaces of the yeast cells and disrupt their lipid membranes. However, the antimicrobial activity of the ionic surfactants was lost when they were incorporated into nanoemulsions, which was attributed to the tendency for the surfactants to partition between the oil droplet surfaces and the cell membranes. In conclusion, the combination of an antimicrobial oil (thyme oil) and antimicrobial surfactant (SDS or LAE) had an antagonist, rather than a synergistic, impact on the overall antimicrobial efficacy. The results of this study have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries.

AUTHOR INFORMATION

Corresponding Author

*Phone: (413) 545-1019. Fax: (413) 545-1262. E-mail: mcdlements@foodsci.umass.edu.

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