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UTILIZAÇÃO DE QUITOSANA NO REVESTIMENTO DE FILÉS DE TILÁPIA DO NILO (*Oreochromis niloticus*) E NA PREPARAÇÃO DE FILMES INCORPORADOS COM ÓLEOS ESSENCIAIS

FÁBIO MARCEL DA SILVA SANTOS

Recife
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Orientador: Prof. Dr. Ranilson de Souza Bezerra
Coorientadora: Profa. Dra. Maria das Graças Carneiro da Cunha

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Data da aprovação: 29 / 08 / 2014.

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Aos meus pais, Audecides Santos e Juvenice Leopoldina;

a minha irmã Fabíola Leopoldina e a minha sobrinha Maria Clara,

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“A aparente dificuldade é um degrau para o sucesso.

Não devemos desanimar diante de uma situação difícil. Pelo contrário, devemos aproveitá-la de modo a aprender lições valiosas, pois só assim podemos evoluir. Em vez de esmorecer diante das dificuldades, vamos superá-las resolutamente e levemos avante a nossa vida com ânimo e atitude positiva.”

Seicho Taniguchi

RESUMO

Tilápia do Nilo (*Oreochromis niloticus*), uma das espécies de peixe mais cultivadas no mundo, é uma importante fonte de proteínas de alta qualidade para os seres humanos. No entanto, assim como o pescado de um modo geral, é altamente susceptível à deterioração microbiológica e química. Dentre os processos de conservação de alimentos desenvolvidos, a defumação é um dos mais antigos meios utilizados para conservação de pescado ou produtos de origem animal. A defumação líquida é um método que utiliza os componentes da fumaça sob a forma de extrato líquido concentrado; fornecendo vários benefícios, tais como a eliminação de compostos cancerígenos e controle uniforme da cor e sabor do produto. A quitosana é um polissacarídeo, capaz de formar filmes e revestimentos comestíveis, que é obtido a partir da hidrólise alcalina do grupo N-acetil da quitina, o componente principal das carapaças de crustáceos. Óleos essenciais de plantas (EOs) são agentes antimicrobianos naturais interessantes para serem incorporados em filmes comestíveis, devido a estes extratos vegetais apresentarem características adicionais, tais como efeitos antimicrobianos e antioxidantes. O objetivo geral deste estudo foi avaliar a eficácia do revestimento de quitosana e quitosana contendo glicerol sobre a qualidade de tilápia do Nilo e preparar filmes de quitosana incorporados com óleos essenciais. O primeiro estudo avaliou os filés de tilápia revestidos com quitosana e filés com revestimento de quitosana com glicerol. As amostras foram armazenadas a 4°C por 12 dias para avaliação físico-química e microbiológica. Estudos posteriores avaliaram também a qualidade dos filés de tilápia submetidos a defumação líquida e utilizando revestimento de quitosana com glicerol. A defumação do pescado foi realizado com a imersão dos filés em solução de 20% NaCl (p/v) e posteriormente em 20 % fumaça líquida (v/v). Em

seguida, os filés foram colocados em estufa a 50°C por 30 minutos. O revestimento com quitosana foi realizado antes das amostras irem para a estufa de secagem. O pescado defumado foi armazenado por 30 dias a 4°C. Posteriormente, a quitosana foi utilizada na preparação de filmes com incorporação de óleos essenciais (OEs), como o de citronela, copaíba e eucalipto, onde foram avaliadas as propriedades físicas e mecânicas desse filmes. A partir das análises pôde-se observar uma redução em alguns parâmetros físico-químicos, como pH e perda de massa em filés de tilápia revestidos com quitosana. O teor de umidade foi maior nos filés com o revestimento de quitosana e glicerol. A concentração de compostos reativos ao ácido tiobarbitúrico (TBARS) foi menor em filés revestidos, e melhores resultados foram observados com a adição de glicerol. A contagem total de micro-organismos mesófilos e psicrotróficos foi menor nos filés revestidos. Alguns parâmetros físico-químicos, como a dosagem de bases voláteis totais nitrogenadas (BVT-N) e TBARS, foram reduzidos nos filés de tilápia com a defumação líquida. Houve mudança nas características de cor, textura e atividade de água, onde o revestimento de quitosana em filés defumados aumentou o efeito de conservação. Os filmes de quitosana demonstraram mudanças nas suas características físicas e mecânicas quando foram incorporados os OEs. A adição de alguns tipos de óleos proporcionou uma característica mais hidrofóbica para os filmes, além de alterar a cor, opacidade, espessura, permeabilidade ao vapor de água (WVP), teor de umidade e solubilidade dos filmes. Como conclusão, o revestimento de quitosana pode levar a uma melhor qualidade do pescado e prolongar a vida de prateleira de filés de tilápia do Nilo; além disso, a adição de OEs (citronela, eucalipto e copaíba) mudou algumas propriedades dos filmes de quitosana, o que pode trazer novas aplicações biotecnológicas para estes filmes.

Palavras chaves: *Oreochromis niloticus*, quitosana, revestimento comestível, defumação líquida, filmes, óleos essenciais.

ABSTRACT

Nile tilapia *(Oreochromis niloticus)*, one of the most cultivated fish species in the world, is an important source of high-quality protein for humans. However, as the fish in general, is highly susceptible to microbiological and chemical deterioration. Among the food preservation processes developed, smoking is one of the oldest methods used for conservation of fish or animal products. The liquid smoking is a method that utilizes the smoke components in the form of concentrated liquid extract; providing several benefits, such as the elimination of carcinogenic compounds and uniform control of the color and flavor of the product. Chitosan is a polysaccharide capable of forming edible films and coatings, which is obtained from the alkaline hydrolysis of the N-acetyl group of chitin, the main component of the crustacean shells. Plant essential oils (EOs) are interesting natural antimicrobial agents to be incorporated into the edible films, due to these plant extracts exhibit additional characteristics such as antimicrobial and antioxidant effects. The aim of this study was to evaluate the efficacy of chitosan and chitosan containing glycerol coating on quality of Nile tilapia and prepare chitosan films incorporated with essential oils. The first study evaluated the tilapia fillets coated with chitosan and fillets with chitosan with glycerol coating. The samples were stored at 4°C for 12 days to physicochemical and microbiological evaluation. Later studies also evaluated the quality of tilapia fillets subjected to liquid smoking and using chitosan with glycerol coating. The smoked fish was performed by immersing the fillets in 20% NaCl solution (w/v) and 20% liquid smoke (v/v). Then the fillets were placed in a drying oven at 50°C for 30 minutes. The coating of chitosan was performed before samples going to the drying oven. The smoked fish was stored for 30 days at 4°C. Thereafter, the chitosan was used to prepare films incorporating essential oil (EOs) such

as citronella, eucalyptus and copaiba, where the physical and mechanical properties of films were evaluated. From the analysis it was observed a reduction in some physicochemical parameters such as pH and weight loss in tilapia fillets coated with chitosan. The moisture content was higher in the fillets with the coating of chitosan and glycerol. The concentration of reactive thiobarbituric acid (TBARS) was lower in the coated fillets, and best results were observed with the addition of glycerol. The total count of mesophilic and psychrotrophic microorganisms was lower in the coated fillets. Some physicochemical parameters such as dosage volatile nitrogenous bases (TVB-N) and TBARS were reduced in the liquid-smoked fillets of tilapia. There was a change in the color, texture and water activity, where the chitosan coating on liquid-smoked fillets increased the effect of conservation. The chitosan films showed changes in their physical and mechanical characteristics when EOs were added. The addition of certain types of oils provided a more hydrophobic character to the film, and change color, opacity, thickness, water vapor permeability (WVP), moisture content and solubility of the films. In conclusion, the chitosan coating can lead to a better quality of the fish and extend the shelf life of Nile tilapia fillets; moreover, the addition of EOs (citronella, eucalyptus and copaiba) changed some properties of the chitosan films, which can bring new biotechnological applications for these films.

Keywords: *Oreochromis niloticus*, Chitosan, edible coating, liquid smoking, essential oils, chitosan films.

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LISTA DE ABREVIATURAS

Aw: Atividade de água

Ch: Quitosana

ChG: Quitosana com glicerol

ChCi: Quitosana e óleo de citronela

ChCo: Quitosana e óleo de copaíba

ChE: Quitosana e óleo de eucalipto

EB: Alongamento

EOs: Óleos essenciais

LS: Defumação líquida

LSCh: Defumação líquida e quitosana

Mi: Massa inicial

Mf: Massa final

MM: Marcadores de peso molecular

SDS-PAGE: Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio

TBARS: Substâncias reativas ao ácido tiobarbitúrico

TEP: 1,1,3,3-tetraetoxiproprano

TS: Resistência à tração

TVB-N: Bases voláteis totais nitrogenadas

WVP: Permeabilidade ao vapor de água

SUMÁRIO

1. INTRODUÇÃO

Os resíduos provenientes do beneficiamento industrial de camarão são representados principalmente por cabeças, cascas e cauda, o que significa aproximadamente 30% a 50% do peso total produzido, este material representa uma fonte em potencial de biomoléculas como quitina e astaxantina. A extração de quitina e obtenção de quitosana torna-se uma atividade viável, não só ambientalmente, como também, economicamente.

A quitosana é um polissacarídeo catiônico presente na carapaça de crustáceos, conhecido como biopolímero formador de película, com uma ampla atividade antimicrobriana contra bactérias e fungos (Rabea et al., 2003). Filmes e revestimentos feitos de quitosana têm sido utilizados como uma barreira microbiana na indústria alimentícia para conservação de frutas e vegetais (DEVLIEGHERE et al., 2004), queijos (DUAN et al., 2007), e carne (OUATTARA et al., 2000), melhorando a qualidade desses alimentos, favorecendo seu armazenamento e aumentando o tempo de prateleira.

A tilápia (*Oreochromis niloticus*), espécie exótica introduzida no Brasil na década de 70, é um dos principais peixes de água doce cultivado no Brasil, representando cerca de 8% do total de pescado produzido no país, sendo 37% deste contingente proveniente da Região Nordeste (IBAMA, 2008).

O pescado é uma importante fonte de proteínas de alta qualidade para humanos. Entretanto, é altamente susceptível a deterioração química e microbiológica, devido a sua alta concentração de água, pH neutro, quantidades relativamente grandes de aminoácidos livres e presença de enzimas autolíticas (JEYASEKARAN et al., 2006). Os métodos mais utilizados para preservação do pescado são refrigeração ou

congelamento que não inibem completamente sua deterioração. Diversos estudos evidenciam a utilização de novas abordagens biotecnológicas para preservação avançada do pescado, diminuindo as perdas por deterioração.

A defumação é um processo que proporciona ao alimento um maior tempo de conservação. A defumação-líquida é um tipo de defumação que utiliza os componentes da fumaça na forma líquida que é aplicada no alimento, podendo ser utilizado como método de conservação de pescado e produtos de origem animal.

A aplicação de um revestimento comestível de quitosana em pescado pode melhorar a conservação do alimento e manter suas características nutricionais. Além disso, a utilização de quitosana na preparação de filmes com a adição de óleos essenciais traz uma nova abordagem biotecnológica para o aproveitamento de resíduos da indústria pesqueira.

2. REVISÃO DA LITERATURA

2.1. QUITOSANA

A quitosana é um polissacarídeo obtido a partir da hidrólise alcalina do grupamento N-acetil da quitina, principal componente do exoesqueleto de artrópodes e da parede celular de alguns fungos, e ainda o segundo polissacarídeo mais abundante na natureza depois da celulose. Atualmente a produção de quitina e quitosana é realizada através do tratamento de resíduos do processamento de crustáceos, matéria prima abundante em países como China, Japão, Índia e Brasil. São utilizadas carapaças de caranguejo, camarão, penas de lula e cabeças de camarão, nas quais o conteúdo de quitina chega a 11% (SYNOWIECKI & AL-KHATEEB, 2000). A produção de quitosana de cascas de crustáceos é viável economicamente, principalmente quando associada à recuperação de carotenóides, uma vez que elas contêm uma considerável quantidade de astaxantina (KUMAR, 2000).

A quitosana é um heteropolímero composto por ligação β (1→4) de N-acetil-D-glucosamina e D-glucosamina (Figura 1) e formada através da desacetilação parcial da quitina, numa faixa de 80 a 85%, ou superior.

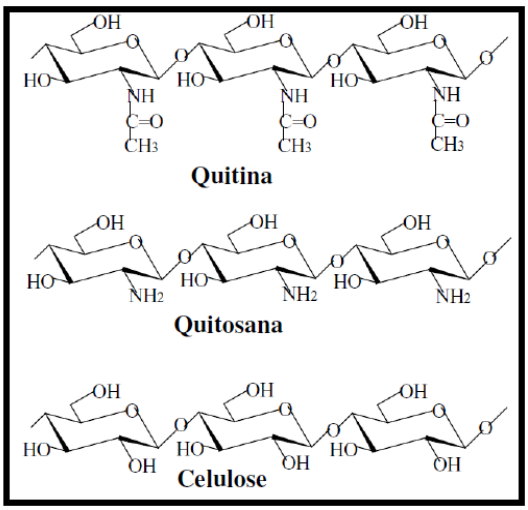

Figura 1. Estrutura da quitina, quitosana e celulose (Fonte: KRAJEWSKA, 2004)

Dependendo da fonte, a quitina pode ocorrer em duas formas, α e β. A forma α é a mais abundante, estando presente na parede celular de fungos, krill, lagostas, tendões e carapaça de caranguejos, cascas de camarão e cutículas de insetos. Por sua vez, a forma β-quitina é mais rara, encontrada, por exemplo, em penas de lula. A forma β mais reativa que a α , sendo esta uma importante propriedade relacionada às transformações enzimáticas e químicas da quitina (RINAUDO, 2006). A quitina pode ser encontrada parcialmente desacetilada em vários níveis de forma natural. As formas α e β da quitina têm microestrutura predominantemente cristalina, o que inviabiliza sua dissolução, na maioria dos solventes, sendo este um dos maiores problemas relacionado ao seu uso. Já a quitosana possui estrutura menos cristalina que a quitina, pela perda do grupo acetil, apresentando assim maior solubilidade em meio aquoso, embora seja virtualmente insolúvel em valores de pH neutros e alcalinos.

O grau de desacetilação determina características como, as propriedades ácido básicas e solubilidade. A protonação do grupamento amina da glucosamina cujo o pKa adquire valores entre 6,3 e 6,7, permite a dissolução em soluções ácidas diluídas. O peso molecular do polímero também influencia a viscosidade e solubilidade, afetando a acessibilidade e disponibilidade de sítios ativos. Uma característica peculiar adicional é a sua alta hidrofilicidade, devido a um grande número de grupos hidroxila no polímero.

A quitosana é largamente utilizada para tratamento de efluentes, pois tem a capacidade de absorver íons metálicos e espécies orgânicas por causa dos grupamentos amina e hidroxila na cadeia que formas ligações de coordenação e sítios reativos (JUANG et al., 2001).

Quitina e quitosana são polímeros com características interessantes para aplicações biotecnológicas e biomédicas. A natureza catiônica, rara entre polissacarídeos

(geralmente ácidos ou neutros), permite a interação eletrostática com moléculas carregadas negativamente, principalmente a quitosana que assume a carga positiva em valores de pH menores que 6,7. Dentre as possíveis aplicações estão: cultura de células, carreamento de fármacos, cobertura anti-corrosão, filmes comestíveis antimicrobianos, engenharia de tecidos, imobilização de proteínas, adsorção de íons metálicos e corantes. A reconhecida biocompatibilidade e não-toxicidade da quitosana tornam possível seu uso em diversas aplicações biomédicas como preparação de filmes e membranas (SAHOO, 2009).

2.2. REVESTIMENTOS E FILMES EDÍVEIS

As embalagens sintéticas plásticas têm originado sérios problemas ecológicos devido ao fato de não serem biodegradadáveis. Neste contexto, acredita-se que os biopolímeros constituem uma fonte alternativa para o desenvolvimento de embalagens devido à sua biodegradabilidade.

Atualmente, os biopolímeros não têm conseguido alcançar maturidade comercial, devido ao seu elevado custo e ao fato de os polímeros sintéticos apresentarem geralmente melhores propriedades. Além disso, não tem havido incentivos suficientes para que os materiais biodegradáveis sejam utilizados. Cerca de 150 milhões de toneladas de plásticos são produzidas anualmente em todo o mundo, sendo que o seu consumo continua a aumentar (PARRA et al., 2004). O impacto ecológico dos recursos de matéria-prima usados na produção e a sua eliminação final são considerações relevantes no seu projeto. Produtos designados como “eco-eficientes” são a nova geração de produtos com base biológica produzidos a partir de materiais sustentáveis

que estão em conformidade com os requisitos ecológicos e econômicos (NARAYAN, 1994).

Revestimentos e filmes comestíveis são termos usados na área alimentar e, muitas vezes sem distinção. Contudo, é importante fazer a distinção destes dois termos: o filme é uma película formada pela secagem (*casting*) da solução do biopolímero preparada separadamente do alimento, que é posteriormente aplicado; enquanto que o revestimento pode ser uma suspensão ou uma emulsão aplicada diretamente na superfície do alimento que após secagem leva à formação de um filme.

O uso de revestimentos/filmes comestíveis baseados em polímeros naturais e em aditivos reconhecidos como seguros tem aumentado na indústria alimentar. Os revestimentos/filmes podem ser produzidos utilizando uma grande variedade de produtos, tais como polissacarídeos, proteínas, lípideos, resinas, com a adição de plasticizantes e surfactantes.

A funcionalidade e o comportamento dos filmes e revestimentos comestíveis dependem principalmente das suas propriedades mecânicas e de transporte, que por sua vez dependem da composição do filme, do seu processo de formação e do método de aplicação no produto.

O método de imersão é o método geralmente usado para revestir frutos, queijos, vegetais, peixes e carnes. Neste método, o produto é diretamente imerso na formulação do revestimento (em meio aquoso), o excesso é removido e o revestimento é seco, formando-se um filme sobre a superfície do produto.

Os revestimentos comestíveis estão ganhando cada vez mais importância, uma vez que dão resposta a vários desafios relacionados com o armazenamento e marketing dos

produtos alimentares e surgem como uma alternativa para reduzir os efeitos prejudiciais impostos pelo processamento dos alimentos.

A barreira semipermeável criada pelos revestimentos comestíveis tem como objectivo aumentar o tempo de prateleira através da redução da humidade, da migração dos solutos, das trocas de gases, das taxas de respiração e das reacções oxidativas, assim como pela diminuição de desordens fisiológicas em frutos frescos e cortados (WONG et al., 1994).

Estes revestimentos/filmes comestíveis e biodegradáveis têm sido utilizados com sucesso em várias aplicações comerciais: gelatina para cápsulas, suplementos, fármacos e encapsulação de aromas; zeína de milho para revestimentos, suplementos e compridos; colagéno para envolver produtos de carne; revestimentos de amido para comprimidos e frutos secos; revestimentos de celulose para suplementos e comprimidos; ésteres de sacarose de ácidos graxos como revestimento de produtos frescos; revestimentos de cera e óleo para produtos frescos, suplementos e comprimidos (KROCHTA, 2002).

2.2.1. Utilização de quitosana como revestimento

Os polissacarídeos são polímeros naturais que dependendo da sua fonte podem ser neutros ou carregados. Estão envolvidos no metabolismo energético de plantas (amido) e animais (glicogênio), agindo também na função estrutural de células vegetais (celulose, pectina) ou no esqueleto de insetos e outros animais (quitina) [9].

Os polissacarídeos avaliados e/ou usados para formar revestimentos/filmes edíveis incluem; amido, alginatos, carragenatos, quitosana e gomas. Como fonte de gomas naturais tem-se, por exemplo, extratos de algas marinhas (alginatos, agar), gomas de sementes (galactomananos) ou raízes.

Os revestimentos/filmes de polissacarídeos caracterizam-se por ser uma boa barreira ao CO_2 e O_2 e uma fraca barreira ao vapor de água (NELSON & COX, 2010).

Devido a características como biodegradabilidade, biocompatibilidade e perfil atóxico, a quitosana e os seus derivados têm sido objeto de estudo para aplicação em diferentes áreas como: produção de cosméticos, formulação de medicamentos, aditivos alimentares, adsorção de metais pesados, tratamento de efluentes industriais das indústrias fotográfica, têxtil, de corantes e de papel. Os materiais à base de quitosana podem ser também utilizados para produzir filmes e revestimentos edíveis devido às suas características viscoelásticas, dando origem a filmes resistentes, duradouros e flexíveis. A maioria das propriedades mecânicas de filmes de quitosana são comparáveis aos de muitos polímeros comerciais (BUTLER et al., 1996).

A formação de filmes e revestimentos edíveis com base em polissacarídeos exige na maioria dos casos, a presença de um plasticizante. Os filmes sem plasticizante apresentam uma estrutura frágil e dura, devido às interações entre as moléculas do polímero. A água é um dos plasticizantes mais eficazes na composição de filmes e revestimentos, sendo a humidade relativa de armazenagem dos filmes um dos parâ-metros mais analisados devido à sua influência na estrutura do filme. Os plastificantes são agentes de baixo peso molecular que uma vez incorporados no filme polimérico são capazes de se posicionar entre as moléculas. Eles interferem com as interações polímero-polímero e originam um aumento da flexibilidade e da capacidade de processamento. A maioria dos plasticizantes são muito hidrofílicos e higroscópicos e podem atrair moléculas de água. Em filmes e revestimentos edíveis à base de polissacarídeos, os plasticizantes podem romper pontes de hidrogênio, aumentando a distância entre as moléculas do polímero e reduzindo desta forma a proporção de

regiões cristalinas em relação às amorfas (KROCHTA, 2002). Em resumo, a adição de plasticizantes pode modificar o módulo de elasticidade e outras propriedades mecânicas, permitindo uma melhor resistência dos filmes e revestimentos à penetração de vapores e gases.

Os surfactantes são substâncias anfipáticas devido às suas propriedades simultâneas de hidrofilicidade e hidrofobicidade e são geralmente adicionados para aumentar a estabilidade da emulsão na formulação de filmes. Os surfactantes podem ser incorporados no revestimento para reduzir a tensão superficial da solução, melhorando a capacidade molhante dos revestimentos (KROCHTA, 2002).

Ao escolher uma composição de revestimento adequado para um determinado tipo de produto alimentar, há uma série de critérios que devem ser considerados. A eficácia dos revestimentos edíveis para conservação de alimentos depende, numa primeira fase, do controle da capacidade molhante do revestimento de modo a garantir uma superfície uniformemente revestida (CASARIEGO et al., 2008). Outros fatores que afetam a eficácia do revestimento são as propriedades mecânicas e de transporte, cor e solubilidade. Estes parâmetros também devem ser considerados a fim de:

- Diminuir a perda de água (ou seja, menor valor de permeabilidade ao vapor da água);
- Diminuir a permeabilidade ao O_2 (ou seja, valores inferiores de permeabilidade ao O_2), uma vez que uma menor concentração de O_2 prolonga o tempo de prateleira de alguns alimentos, retardando a decomposição oxidativa de substratos complexos (FARBER et al., 2003) e reduz a produção de etileno, um elemento chave no processo de maturação de frutos (LEE et al., 1996). Além disso, em contato com o queijo, o O_2 contribui para a oxidação de gorduras e para o crescimento de microrganismos indesejáveis;

- Aumentar a fase lag e o tempo de formação durante a fase de crescimento logarítmico dos microrganismos indesejáveis, leveduras e bolores (ROBERTSON et al., 1996), que é alcançado mantendo os valores de permeabilidade ao CO_2 elevados;
- Melhorar a resistência mecânica dos revestimentos/filmes, com o objetivo de preservar a sua integridade;
- Diminuir a incidência de luz (a luz promove a oxidação de gorduras) (ROBERTSON et al., 2006) ou seja, elevados valores de opacidade.

Os filmes e revestimentos constituídos por polissacarídeos devem ser compatíveis com os atuais processos de produção dos filmes sintéticos e com os processos de revestimento dos alimentos, sem investimento significativo. Os filmes e revestimentos comestíveis são sistemas promissores para a melhoria da qualidade dos alimentos, tempo de prateleira, segurança e funcionalidade.

2.3. TILÁPIA DO NILO

O aumento na demanda por produtos pesqueiros tem resultado em um constante crescimento da produção aquícola mundial. Em 2011, foram produzidos 154 milhões de toneladas de pescado, das quais 90,4 milhões foram oriundos da pesca e 63,6 milhões, da aquicultura. Aproximadamente 130 milhões foram destinados ao consumo humano e 23,2 milhões a produção de farinha e óleo de peixe (FAO, 2012).

Embora em termos percentuais a captura de organismos aquáticos ainda seja responsável por quase 59% do total de pescado fornecido, essa atividade vem apresentando estabilidade de produção desde a década de 80, onde no período de 2002 a 2010, houve uma diminuição de 93 para 88,6 milhões de toneladas. Entretanto, nas últimas três décadas (1980-2010), a produção mundial da aquicultura se expandiu por quase 12 vezes mais, com uma taxa média de crescimento anual de 8,8 % (FAO, 2012).

Atualmente, a aquicultura é um dos sistemas de produção de alimentos com maior taxa de crescimento no mundo, o que coloca esta atividade em foco pela grande oportunidade de produção de alimentos, geração de postos de trabalho e desenvolvimento de negócios (HOWARTH, 1996). Com destaque para produção de peixes de água doce, que em 2010 representaram 56,4% (33,7 milhões de toneladas), dos quais a tilápia é o segundo maior gênero mais cultivado do mundo (FAO, 2012).

No século 20 o cultivo de tilápias foi estabelecido. Este desenvolvimento se deu principalmente pelo cultivo na África, China e América do Sul, o melhoramento genético, a expansão do cultivo em águas salinas nas Filipinas e pela demanda do filé pelos Estados Unidos, onde a China se tornou o maior produtor e grande fornecedor dos EUA (GUERRERO, 2008). A produção mundial de tilápia passou de aproximadamente 400 mil de toneladas em 1991 para cerca de 3,0 milhões de toneladas em 2010, sendo o continente asiático o maior produtor (72%), especialmente a China e o Sudeste da Ásia, seguidos pelos continentes africano (19%) e americano (9%) (FAO, 2012).

De acordo com Wing-Keong e Hanin (2007), *Oreochromis niloticus* (Figura 1) representa 80% das espécies de tilápia cultivadas no mundo, sendo considerada a mais importante.

Figura 2. Tilápia do Nilo (*Oreochromis niloticus*)

Fonte: http://portuguese.alibaba.com/product-gs/supply-best-fresh-water-nile-tilapia-fish-493832368.html

A tilápia é um peixe de água doce pertencente à família Cichlidae. É nativa da África, mas foi introduzida em muitas regiões tropicais, subtropicais e temperadas do mundo durante a segunda metade do século 20 (EL-SAYED, 2006).

Devido as suas características biológicas e mercadológicas como rápido crescimento, rusticidade, alimentação em baixos níveis tróficos, tolerância a variações ambientais, resistência a doenças, ausência de espinhos intra-musculares, dentre outros, a tilápia do Nilo é considerada uma das principais espécies da piscicultura mundial e a principal espécie brasileira.

2.4. MICROBIOLOGIA DO PESCADO

Enquanto o peixe está vivo, sua pele atua como uma barreira mecânica à penetração de bactérias, razão pela qual seu músculo é considerado estéril. Logo após a morte, o peixe perde suas defesas tornando-se vulnerável ao ataque microbiano. O tipo de deterioração observado pode ser, em grande parte, atribuído à alteração dos tecidos dos peixes, causadas pelo ataque de tipos específicos de bactérias e produtos gerados por elas. A extensão da deterioração é determinada pela carga microbiana inicial, pela temperatura do músculo do peixe, pelo tempo decorrido depois de sua morte e pelas práticas sanitárias adotadas (LEITÃO, 1977).

A decomposição do pescado é principalmente causada por bactérias e uma das maneiras de retardar essa decomposição é diminuir a temperatura até um nível em que as bactérias não cresçam ou o faça muito lentamente. A entrada de microrganismos na carne de pescado e a decomposição gradual das substâncias nitrogenadas começam quase que simultaneamente à autólise. Se o pescado é mantido sob gelo, ocorre inibição da atividade bacteriana e o processo de autólise é mais intenso que a decomposição

bacteriana. Quando a temperatura é maior, a decomposição bacteriana predomina (BEIRÃO et al. 2000).

O habitat da *Salmonella* é o trato intestinal, e a sua presença indica provável contaminação fecal de fontes humanas ou animais. Peixes capturados em águas não poluídas estão isentos de *Salmonella* pelo fato desta não fazer parte da microbiota natural do pescado, sendo que sua presença neste alimento origina-se normalmente do manuseio ou contato com superfícies higienizadas inadequadamente. A presença de *Salmonella* é razão suficiente para que o mesmo seja condenado (LEITÃO, 1977). No Brasil, sua ocorrência foi observada em todos os tipos de alimentos, principalmente os de origem animal (BONILHA e FALCÃO, 1994).

O gênero *Staphyloccocus* é o agente responsável por aproximadamente 45% das toxinfecções do mundo. O *Staphyloccocus aureus* é um dos agentes patogênicos mais comuns, responsáveis por surtos de contaminação de origem alimentar, sendo normalmente transmitido aos alimentos por manipulação (CUNHA NETO, SILVA e STAMFORD, 2002). No Brasil pesquisas realizadas em diferentes regiões do país, mostraram a ocorrência de *S. aureus* em pescado (DAMS, BEIRÃO e TEIXEIRA 1996; HYLUY *et al*. 1996). Tilápias (*Oreochromis niloticus*) recém-capturadas foram analisadas quanto à presença de *S. aureus* por Vieira et al. (2000) e todas as amostras apresentaram valores que variaram de <10 a 10,6 x 10^2 UFC/g.

A presença do *Clostridium botulinum* nos alimentos tem muita importância sanitária, devido à alta periculosidade da toxina produzida por estes microorganismos, provavelmente a mais potente de todas as toxinas produzidas pelas bactérias. A atividade de água (Aw) mínima para o crescimento do *Clostridium botulinum* tipos A,

B, e E seria 0,95, 0,94 e 0,97 respectivamente, o que corresponde a concentrações salinas que variam entre 6,5% e 11% (TROLLER, 1989).

O indicador microbiológico de contaminação fecal mais empregado é o grupo coliforme. Os coliformes são bactérias Gram-negativas, não esporuladas, na forma de bastonetes, e que fermentam a lactose com formação de gás a 35ºC. *Escherichia coli* é o indicador clássico da possível presença de patógenos entéricos na água, nos moluscos, em produtos lácteos e outros alimentos. *Escherichia coli* é um microrganismo cujo habitat natural é o trato entérico do homem e do animal. Por isso, a sua presença em um alimento, sugere uma falta geral de higiene no manuseio do mesmo e um armazenamento inadequado (OGAWA e MAIA, 1999).

2.3. DEFUMAÇÃO

Muito antigamente, o cozimento de alimentos era feito utilizando o fogo da madeira. Essa foi à base para a proteção da carne por cozimento parcial e defumação contra os problemas de desperdícios ocasionados pela putrefação do alimento. A partir de 1915 foram realizados estudos sobre a utilização de fumaça para conservação de produtos alimentícios. Nesta data, pela primeira vez, relataram-se as propriedades bacteriostáticas da fumaça da madeira quando testadas com *Proteus* e *Staphylococcus sp*. Em 1944 foi demonstrado o efeito bacteriostático da fumaça quando se avaliou a vida de prateleira de bacon Wilshire defumado e não defumado. Em 1954 demonstraram o efeito antibacteriano da fumaça em peixes obtendo bons resultados sobre culturas de *Staphylococcus aureus*, *Bacillus subtillis* e *Proteus vulgaris* (LOHMEYER, 1999).

Mendes et al. (2002) estudaram os aspectos microbiológicos e a vida de prateleira de camarões defumados. Os autores observaram que, após o processo de

defumação, os camarões marinhos não apresentaram coliformes totais, presentes inicialmente na matériaprima. A vida de prateleira do camarão foi maximizada quando o produto foi defumado. O produto estocado sob refrigeração apresentou validade de 12 dias.

2.3.1. Composição da Fumaça

O conhecimento da composição da fumaça é um pré-requisito para o estudo do desenvolvimento do sabor e cor, assim como para o entendimento das propriedades bacteriostáticas e antioxidantes dos alimentos defumados.

As possíveis reações que acontecem durante a combustão dos três principais componentes da madeira (celulose, hemicelulose e lignina) resultam em mais de 200 compostos. Estes podem ser divididos em quatro grupos principais: compostos ácidos, fenólicos, carbonílicos e os hidrocarbonetos (SCHINDLER, 1996).

a) *ácidos*: Os componentes ácidos proporcionam sabor de defumado;

b) fenólicos: Além do sabor defumado, conferem brilho ao produto ao reagirem com compostos carbonílicos. A quantidade e natureza dos fenóis presentes na fumaça estão diretamente relacionadas com a temperatura de pirólise da madeira. A presença de fenóis e ácidos confere à fumaça propriedades bacteriostáticas e bactericidas (YAMADA e GALVÃO, 1991). Compostos fenólicos possuem ação antioxidante, o que permite atuar na conservação do produto tratado. Sérot e Lafficher (2003) identificaram os 10 compostos fenólicos mais importantes presentes no peixe defumado, como sendo, fenol, p-cresol, o-cresol, guaicol, 4-metil guaicol, 4-etil guaiacol, siringol, eugenol, 4 propil- guaicol e isoeugenol.

c) Carbonílicos: Os compostos carbonílicos são responsáveis pela cor característica do produto (marrom dourado). Atuam de forma mais efetiva sobre a coloração do que no sabor dos produtos defumados (ADICON, 1998)

d) Hidrocarbonetos: Os hidrocarbonetos aromáticos policíclicos, (3-4 benzopireno) não são desejáveis por serem carcinogênicos. O 3,4 benzopireno tem sido considerado um indicador contaminante nos produtos alimentares. Sua quantidade pode variar desde várias centenas de ppb (mg/kg) a traços não quantificados. As quantidades de 3,4 benzopireno dependem, entre outros, da tecnologia da defumação (ADICON, 1998).

2.3.2. Técnicas de Defumação

2.3.2.1 Defumação a quente

Na defumação a quente o produto é exposto a uma temperatura acima de 80ºC, ocorrendo a desnaturação enzimática e uma breve esterilização, resultando em um grau maior de preservação, podendo ser consumido sem cozimento prévio algum (SUBASINHE,1993). O produto obtido pela defumação a quente destina-se ao consumo imediato, sendo necessário somente um reaquecimento antes da ingestão. Neste processo, o pescado pode ser total ou parcialmente cozido, sendo o teor de sal baixo, de modo que não seja necessária uma operação de dessalga (BERAQUET, 1984).

Souza et a*l.* (2004) estudaram o efeito da defumação a quente em tilápias do Nilo (*Oreochromis niloticus*) inteiras evisceradas e filés, nas características sensoriais (aparência, aroma, sabor, textura, teor de sal e aceitação global). Antes da defumação a quente, que foi realizada de 50 a 90ºC por cerca de 5 horas para o peixe inteiro e 4 horas para o filé, os peixes foram salgados a uma concentração de 30% por 45 minutos, lavados, drenados por 60 minutos e pré-secados a 40ºC por 50 minutos. O filé defumado

teve maior aceitação geral, principalmente quanto à aparência, e o peixe inteiro defumado teve maior aceitação quanto ao sabor e teor de sal quando comparado aos atributos cor, textura, aparência e aroma.

Santos et al. (2007) utilizando uma câmara de defumação com aquecimento a gás, avaliaram os efeitos das formas de processamento e do alecrim na defumação dos troncos e filés sem pele de tilápia do Nilo sobre o rendimento e as características sensoriais. Independente da forma de processamento aplicada, os filés defumados na presença do alecrim apresentaram menor rendimento. Foi também observado que os filés obtidos a partir dos troncos defumados proporcionaram maiores rendimentos. Analisando a forma de processamento dos filés defumados, os provadores apresentaram maior aceitação para filés defumados em relação aos filés obtidos a partir dos troncos defumados. A presença de alecrim nos filés, independente da forma de obtenção do produto final, não foi significativo para aparência, cor, aroma e aceitação geral. Mesmo sendo um peixe considerado magro pelo baixo teor de lipídios, apenas 5,57% no filé *in natura*, não sendo este indicado para defumação, os filés defumados tiveram boa aceitação pelos provadores.

2.3.2.2. Defumação a frio

A defumação a frio ocorre em temperaturas moderadas, em torno de 40ºC, a fim de se evitar o cozimento do produto.

É um processo bastante comum na Europa, especialmente para defumação de arenque e salmão. Pode ser dividido em duas fases distintas. Na primeira, a temperatura do fumeiro eleva-se a 32ºC, facilitando a secagem do peixe. É nesta fase que o fogo queima sem a serragem. Na segunda, a temperatura baixa até 27ºC ou 24ºC, em

conseqüência do abafamento do fogo com a serragem; é a defumação propriamente dita. Nesta etapa, a circulação do ar no fumeiro tem que ser regulada, assim como a propagação da fumaça. Os produtos resultantes da defumação a frio têm longa duração, pois são expostos à fumaça por tempo prolongado, mas exigem cocção antes de serem consumidos (SANCHEZ, 1989).

O salmão é normalmente preservado por dois tratamentos: o primeiro com sal, por algumas horas, e então defumação à baixa temperatura (15 a 30ºC), por cerca de 1 a 3 semanas (RAMACHANDRAN e TERUSHIGUE, 1994).

2.3.2.3. Defumação líquida

A indústria de aditivos e ingredientes iniciou na década de 60 nos Estados Unidos a produção de extratos líquidos empregados no processo de defumação, conhecido como fumaça líquida. A produção desses extratos é realizada pela absorção em água dos componentes gerados na pirólise da serragem da madeira, onde a temperatura do processo, a concentração de oxigênio e a umidade da matéria prima são variáveis controladas. O produto de fundo da coluna de absorção é decantado (processo de envelhecimento), ocorrendo à formação de produtos de condensação ou polimerização, que fornecem uma cor escura ao extrato. O alcatrão e os compostos policíclicos são removidos por filtração (SCHINDLER, 1996).

Os benefícios da fumaça líquida são:

- Minimização da poluição do ar (como medida primária) e minimização da carga de serragem lançada no esgoto;
- Processo de defumação realizado sem riscos de fogo e /ou explosão;
- Controle uniforme da cor e sabor do defumado;
- Simplificação da limpeza e manutenção das condições de defumação;

- Fim da coleta de alcatrão, cinza e outros resíduos;

- Eliminação da presença de elementos carcinogênicos nos produtos defumados;

- Aumento da produtividade com redução dos custos do processo;

- Possui propriedades antioxidantes e bacteriostáticas;

Gonçalves e Prentice-hernández (1998) utilizaram a fumaça líquida em filés de anchova em uma concentração de 20% a qual apresentou grande aceitação sensorial. A utilização de salmoura a 20% por 15 minutos assegurou a estabilidade microbiológica e a utilização de uma pré-secagem de 45 minutos e 49ºC antes da aplicação da fumaça líquida favoreceu uma maior aplicação da mesma no músculo de anchova. Obteve-se baixa contagem microbiana e ausência de coliformes fecais e de salmonela, tanto na matéria-prima como no produto final.

Ribeito (2000) utilizou fumaça líquida (extrato vegetal da nogueira) para a defumação de filé de matrinchã (*Brycon cephalus*), através da técnica de imersão. Neste trabalho foram variadas as concentrações de fumaça líquida de 20, 25 e 30% v/v, temperatura de 40, 50 e 60ºC e tempo de imersão de 20, 25 e 30 segundos. Através da análise sensorial, verificou-se que, a condição de melhor aceitação por parte dos consumidores foi a de maior temperatura, menor concentração da fumaça e maior tempo de imersão.

Hattula et al. (2001) estudaram a aplicação de fumaça líquida na defumação de truta de arco–íris em substituição ao método comumente utilizado, a defumação a frio, e verificaram que o processo de defumação líquida diminuiu a emissão de hidrocarbonetos poliaromáticos (PAH).

3. OBJETIVOS

3.1. Objetivo geral

Avaliar a eficácia do revestimento de quitosana na qualidade dos filés de tilápia do Nilo (*Oreochromis niloticus*) e preparar filmes de quitosana com óleos essenciais incorporados.

3.2.Objetivos específicos

- Realizar análises físico-químicas dos filés de tilápia do Nilo submetidos à defumação e ao revestimento com quitosana durante um período de armazenamento em baixa temperatura;
- Realizar análises microbiológicas para micro-organismos mesófilos e psicrotróficos no pescado.
- Obter o perfil eletroforético dos filés de tilápia do Nilo que foram submetidos ao revestimento e a defumação líquida;
- Avaliar as propriedades físicas e mecânicas dos filmes de quitosana e com incorporação de óleos essenciais (Citronela, Copaíba e Eucalipto).

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5. CAPÍTULO 1

ARTIGO CIENTÍFICO:

Edible chitosan coatings increase the shelf life of Nile tilapia fillets (*Oreochromis niloticus*)

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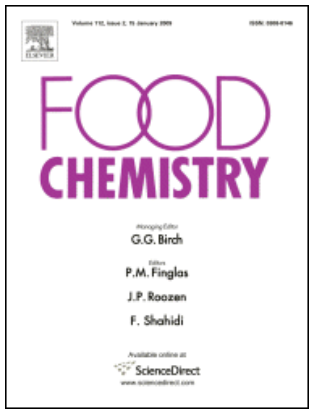

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Edible chitosan coatings effects in shelf life of Nile tilapia fillets (*Oreochromis niloticus*)

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Abstract

Nile tilapia (*Oreochromis niloticus*) is one of the most important species of farmed fish in Brazil. Fish fillet rapid deterioration is one of the main challangers of fishery industry due to the need of high costs of preservation during transport and storage. The aim of this study was to evaluate the effectiveness of the chitosan coating in preservation of tilapia fillets during cold storage for 12 days. Physicochemical and microbiological analysis of tilapia fillets was performed. There was a reduction in some physicochemical parameters such as pH and mass loss in chitosan coated fillets. The moisture content was higher in fillets coated with chitosan and glycerol (ChG). The color values were present mainly in the samples of white to yellowish color. The concentration of thiobarbituric acid reactive substances (TBARS) was lower in fillets coated. The addition of glycerol (ChG) made the fillets present lower lipid oxidation. The total plate count and psychrotrophic count was higher in the control treatment, and lower in both chitosan (Ch) and ChG coated samples. This type of coating can lead to a better quality of food and extend the shelf-life of fillets.

Keywords: Edible coating; Chitosan; *Oreochromis niloticus*

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important species of farmed fish in Brazil, with a production of 253,824 tons in brazilian aquaculture in 2013 (MPA, 2013). Fish is a important source of nutrients in the human diet, as certain molecules such as proteins and lipids can be found in large concentrations in its composition. Seafood is an extremely perishable compared with other fresh commodities (Sathivel, 2005; Vásconez, Flores, Campos, Alvarado & Gerschenson, 2009). Cold storage and freezing are normally employed methods for fish preservation, but they do not completely inhibit the quality deterioration of fish (Jeon, Kamil &Shahidi, 2002). During storage, fish quality is reduced quickly due to chemical and enzymatic reactions and microbial spoilage.

The application of an edible film or coating is a method to protect its quality (Vásconez et al., 2009). Edible coating is a thin layer of material formed as a coating on a food product, while an edible film is a preformed thin layer, made of edible material, which once formed can be placed on or between food components (Falguera, Quintero, Jiménez, Munoz, & Ibarz, 2011). The most commonly used materials for edible film production are biopolymers such as polysaccharides and proteins (Pereda, Ponce, Marcovich, Ruseckaite, & Martucci, 2011; Souza et al., 2010).

Chitosan, a cationic polysaccharide obtained from crustacean shells, is a well-known film-forming biopolymer with a broad antimicrobial activity against bacteria and fungi (Cagri, Ustunol & Ryser, 2004; Rabea, Badawy, Stevens, Smagghe & Steurbaut, 2003). Chitosan based films and coatings have been applied as a microbial hurdle in a variety of food, including fruits and vegetables (Devlieghere, Vermeulen & Debevere, 2004), eggs (Kim, Daeschel & Zhao, 2008), cheeses (Duan, Park, Daeschel & Zhao, 2007) and

meat (Ouattara, Simard, Piette, Bégin & Holley, 2000), for improving overall food quality and prolonging storage life. This biopolymer has also been applied as an edible invisible film for preserving fresh fillets of Atlantic cod, lingcod and herring, and its preservative efficacy has been exhibited by the reduced moisture loss, lipid oxidation, and growth of microorganisms in the tested fishes (Duan, Cherian & Zhao, 2010; Jeon et al., 2002).

The incorporation of other compounds, such as plasticizers and lipids, is common in order to improve properties of edible coating. Glycerol is a major by-product of biodiesel production which has significantly increased and is often regarded as a waste stream with an associated cost (Cerqueira, Souza, Teixeira & Vicente, 2012, Fountoulakis & Manios, 2009). The use of glycerol as the plasticizer may aid in reducing the hydrophobicity of the coating, resulting in better interaction with the lipids of the food.

The increasing demand for fresh refrigerated seafood with an extended shelf life has intensified the search for technologies that support fresh fish utilization, numerous studies being currently focused on using natural ingredients to enhance fish quality and shelf life (Abbas, Mohamed, Jamilah & Ebrahimian, 2008). The aim of this study was to evaluate the effectiveness of the coating of chitosan in preservation of tilapia fillets during cold storage.

2. Materials and methods

2.1. *Preparation of solutions and coating application*

Chitosan was obtained from shrimp heads of the species *Litopenaeus vanammei* according methodology described by Cahú et al. (2012) .The coating solutions were prepared by dissolving chitosan in 1% lactic acid (v/v) using a magnetic stirrer for 2

hours. Two solutions were prepared: a solution of 0.5% chitosan (w/v) only and a solution with the addition of 0.1% glycerol (v/v). The solutions will be previously sterilized using UV light for 15 minutes before being used in the coating of the fillets.

Nile tilapia fillets were obtained from a local fish industry (Noronha Pescados).The fillets were immersed in the solution of chitosan for 10 seconds and dried for 1 minute in a sterile stainless steel screen at 25 °C. The fillets were separated into three groups (n = 3) according to the treatment used: fillets without chitosan coating (Control), fillets coated with chitosan solution (Ch) and chitosan with glycerol (ChG).

The coated and uncoated fillets were placed in sterile plastic storage bags and stored at 4±1 °C for 12 days. The physicochemical and microbiological analyzes of samples were carried out during the experimental period (0, 4, 8, 12 days).

2.4. Physicochemical analyses

For pH measurement, approximately 10 g of minced fillets samples were placed in a 100-mL beaker and homogenized with 100 mL distilled water. The pH of homogenized sample was measured using a pH meter (PM 608, ANALION, São Paulo, Brazil).

Moisture content was measured by drying about 10 g of minced fish samples in a conventional oven (TE-394/1, TECNAL, São Paulo, Brasil) at 105°C for 12h. The weight of the initial and dried samples was recorded. The moisture content (%) was calculated as weight change after drying divided by the initial weight of samples x 100% (IAL, 2008). The mass loss was performed by weighing the samples at the beginning and end of the storage time, where the results were expressed as weight change after the storage time divided by the initial weight of samples x 100%.

The colour of fish samples was determined using a colorimeter (Model Chroma Meter CR-400, Konica Minolta, Ltda., Japan) and reported in the CIELAB colour profile system, where L^* denotes the lightness of colour (0–100, black to white), a^* corresponds to red to green and b^* presents yellow to blue.

2.5. Lipid oxidation

The lipid oxidation of samples was performed by measuring thiobarbituric acid-reactive substances (TBARS) as described by Buege and Aust (1978). A portion (3g) of minced fish samples was homogenised with 25 mL of solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 M HCl. The mixture was heated in a boiling water bath (100°C) for 10 min for developing a pink colour, then cooled and centrifuged at 3600g at 25°C for 20 min. The absorbance of the supernatant was measured by a spectrophotometer (SmartSpec^{TM} 3000 Spectrophotometer, Bio-Rad, USA) at 532 nm, and 1,1,3,3-tetraethoxypropane (TEP) (Sigma–Aldrich) was used as the standards. TBARS was expressed as mg malonaldehyde equivalents/kg muscle (mg MA eq/kg muscle).

2.6. Microbiological analysis

A 25g sample of each treatment (n=3) was removed aseptically and homogenized with 225 mL of 0.85% NaCl sterile solution. Serial dilutions were performed for each sample and 1 mL of each dilution was taken and placed in petri dishes. Pour-plate method using plate count agar was used to determine the total plate and psychrotrophic counts in the fish samples. The inoculated agar plates were incubated at 35°C for 48 h for determining total plate counts, and at 4°C for 7 d for psychrotrophic counts. Microbiological data were transformed into logarithms of the number of colony-forming units (CFU/g).

2.7. Electrophoresis

Proteins from fillets of *O. niloticus* coated and uncoated were submitted analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% (w/v) stacking gel and a 12.5% (w/v) separating gel (Laemmli, 1970). The gel was stained with 0.1% (w/v) Coomasie Blue for 120 min and destained in 10% (v/v) acetic acid and 25% (v/v) methanol. Molecular weight markers were used.

2.8. Statistical analysis

Statistical difference within treatment groups were established by ANOVA test. Differences between treatments were established by Tukey test at $p < 0.05$. The software used was Origin 6.0 Professional.

2. Results and Discussion

3.1. Physicochemical analyses

In Figure 1, the pH of samples is illustrated. The pH of the samples increased during the 12 days of storage at 4 °C with a significant difference between the coated and uncoated samples ($p < 0.05$). During the course of storage time, samples coated showed values lower than uncoated. The coating treatments had pH values between 7.33 and 7.76, while the uncoated samples showed pH values between 7.48 and 8.26. On day 0, the pH values were similar for all samples. After 4 days of storage can be observed pH values of 7.68 for uncoated samples. On the same day found lower values of pH for the treatments, 7.50 and 7.40 for Ch and ChG, respectively.

The increase of pH values may be related to the fast spoilage of the product, which may indicate bacterial growth in fish (Souza et al., 2010; Duan et al., 2010). The colonization by bacteria leads to increased production of nitrogenous compounds such as ammonia, which results in an increase in pH and changes in quality of the fish.

Similar results were found in experiments using other species such as sardines and hake (Ababouch, Souibri, Rhaliby, Ouahdi, Battal & Busta, 1996; Nunes, Batista & Morâo de Campos, 1992; Ruiz-Capillas & Moral, 2001). The coating was more effective in maintaining pH, where their pH values were lower than the uncoated fillets at the end of the storage period.

The values of moisture content of the uncoated fillets were determined and the results are shown in Table 2. The moisture content was reduced in uncoated fillets during the period of storage. There was no significant difference in the results of the coated fillets during storage. On the 12th day, treatment ChG had a lower moisture loss (78.31 ± 0.61%) compared with the chitosan coating only (77.11 ± 0.32%). These results showed that the coating with chitosan can delay water loss. Sathivel (2005) reported a 4.1% moisture loss in pink salmon fillets after 3 months of frozen storage.

The weight loss during storage of *O. niloticus* fillets for 12 days at 4 ° C is shown in Table 1. During the storage period (12 days at 4 °C) there was a weight loss of both coated and uncoated samples. The control showed the greatest weight loss (3.70 ± 0.03%) while the coated samples showed lower values. Treatment of fillets with chitosan and glycerol was more effective (3.13 ± 0.02%) than only the coating with chitosan (3.57 ± 0.02%).

Color characteristics of the treatments in the fillets can be shown in Figure 2. The values of *L** increased during the storage period. On day 12 , the Control sample showed values of *L** higher (54.5 ± 0.85) than in Ch (52,45 ± 1,02) and ChG (52.54 ± 0,65). Duan et al. (2010) reported a decrease in *L** values in frozen fillets of Ophiodon elongates during three of storage. In *a** and *b** values of all treatments were similar and

showed changes from 0 to -2 for *a** and -3 to 3 for *b**. These values show that in general the samples were in white to yellowish color.

3.2. Lipid oxidation

TBARS values in the samples were very similar at inicial day (Figure 3). Over the days the Control group showed higher values than in Ch and ChG. This increase in TBARS in fillets Control started from day 4 (0.21±0.01 mg MA eq/kg), resulting in a value of 0.25 ± 0.01 mg MA eq/kg on day 12. TBARS is a parameter that indicates the degree of lipid oxidation in a food. Oxidation of lipids may be due to the contact with oxygen and the presence of microbial growth in fish.

TBARS values were stable for Ch and ChG. On the last day of storage, Ch treatment had higher value than ChG, demonstrating that glycerol aided the antioxidant effect of chitosan in the coating. On the last day of storage, treatment Ch showed a value of 0.19 ± 0.01 mg MA eq/kg while ChG was 0.17 ± 0.01 mg MA eq/kg. The results showed that glycerol aided the antioxidant effect of chitosan in the coating. Jeon et al.(2002) found lower TBARS values in chitosan-coated herring and Atlantic cod samples throughout a 12 d cold storage. Sathivel (2005) reported that chitosan coatings reduced the lipid oxidation in pink salmon fillets during the frozen storage.

Chitosan coatings reduced the lipid oxidation in fish fillets (Fan, Sun, Chen, Qiu, Zhang & Chi, 2009). The ability of chitosan to combine with lipid also plays a role in its antioxidative activity (Xue, Yu, Hirata, Terao, & Lin, 1998).

2.3. Microbiological analyses

Microbiological analysis of coated and uncoated fillets was performed and is shown in Figure 6a and 6b. There growth of microorganisms at the initial day which may be due a contamination in the preparation of fillets. The total count plate of uncoated

samples showed initial values of 2.10 log CFU/g and after eight days the values increased to 5.96 log CFU/g. The Ch and ChG treatments demonstrated a growth inhibition of microorganisms when compared with the Control. After 12 days of storage, the total plate count in Ch (5.50 log CFU/g) and ChG (5.70 log CFU/g) had lower values than the Control (7.44 log CFU/g).

The values of psychrotrophic counts were lower on the first day of analysis in fillets coated, probably due to antimicrobial action of chitosan. The microorganisms count was higher in samples without coating. The control group had 3.10 CFU/g on day 0 and had the highest value after 12 days (6.04 CFU/g). ChG had the lowest psychrotrophic count on the last day of storage (5.10 CFU/g), demonstrating higher efficacy of this treatment.

Only the Control after 12 days of storage exceeded the international limit (7 log CFU/g) the amount of aerobic microorganisms in fish (ICMSF, 1986). Souza et al. (2010) found a low microbiological growth in salmon fillets coated with chitosan. Chitosan coatings significantly inhibited the total plate count in lingcod, with 0.60–1.19 log CFU/g reductions being obtained in the coated samples (Duan et al., 2010).

Chitosan is well-known for its excellent film-forming property and broad antimicrobial activity against bacteria and fungi (Rabea et al., 2003). The antimicrobial action of chitosan appears to be mediated by the interactions between the positively charged chitosan and negatively charged microbial cell membranes, which induces the leakage of cellular proteins and other intracellular constituents (Duan et al., 2010).

2.4. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of tilapia fillets minced samples is shown in Figure 6. The electrophoresis revealed the presence of a larger number of bands below the 53 kDa on the last day of storage in all groups.

This may be due to degradation of the protein chains of high molecular weight by microbial action. The electrophoretic profile of Ch and ChG were similar, but the treatment ChG showed less intense bands of lower molecular weight. This may suggest that there was a better protection from the degradation of proteins by ChG.

Conclusions

This study demonstrated the protective effects of chitosan coating on tilapia fillets. The antimicrobial coating gave better control of microorganisms in fish. There was a decreased lipid oxidation in the food promoting the reduction of off-flavor. The addition of glycerol as plasticizer increased the protective effect of the coating, this may be due to the formation of a more uniform coating on the fish. This type of coating can lead to a better quality of food and extend the shelf-life of fillets.

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Tables

Table 1. Weight loss of the *O. niloticus* fillets during cold storage at 4°C. Different lowercase letters represent significant difference (Tukey test $p<0.05$).

Time storage (days)	**Weight Loss (%)**		
	Control	Ch	ChG
12	3.70 ± 0.03^{a}	3.57 ± 0.02^{b}	$3,13 \pm 0.02^{c}$

Table 2. Moisture content of *O. niloticus* fillets during cold storage at 4°C.

Time storage (days)	**Moisture content (%)**		
	Control	Ch	ChG
0	76.30 ± 0.71^{Aa}	77.02 ± 1.26^{Aa}	77.32 ± 0.95^{Aa}
4	75.63 ± 0.68^{ABa}	76.34 ± 0.89^{Aab}	77.63 ± 1.09^{Ab}
8	74.49 ± 0.19^{BCa}	77.19 ± 0.34^{Ab}	78.26 ± 1.22^{Ab}
12	74.69 ± 0.12^{Ca}	77.11 ± 0.32^{Ab}	78.31 ± 0.61^{Ac}

Means followed by the lowercase letters in the same row within each treatment and each measurement are significantly different ($p < 0.05$).

Figures

Figure 1. pH of *O. niloticus* fillets during cold storage at 4°C.

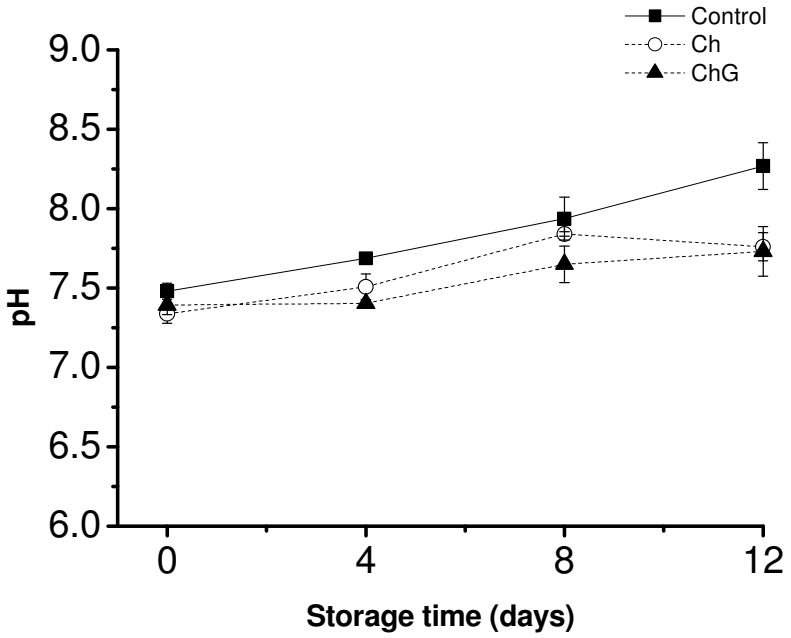


Figure 2. Colour of *O. niloticus* fillets during cold storage at 4°C.

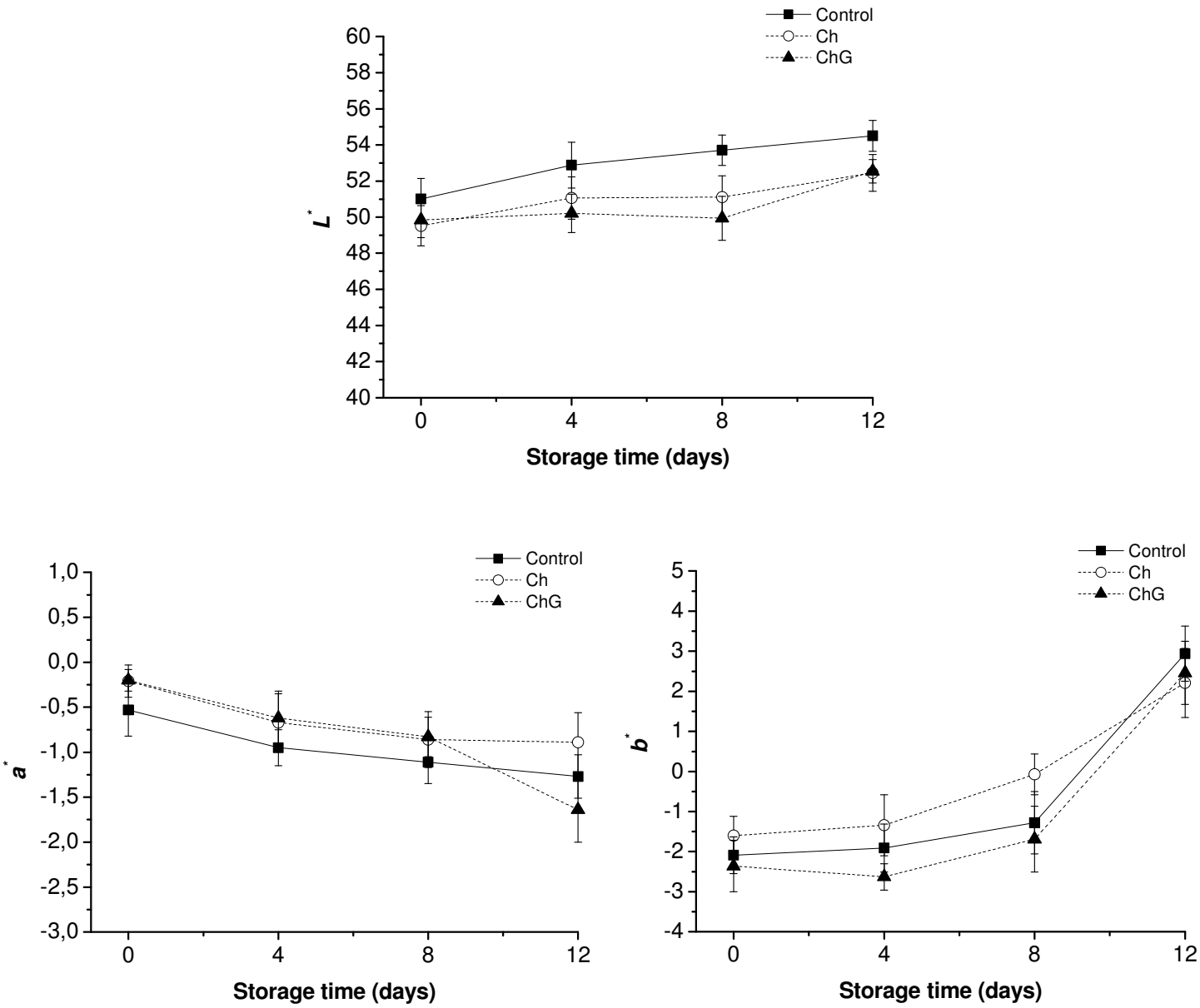


Figure 3. Thiobarbituric acid-reactive substances (TBARS) of *O. niloticus* fillets during cold storage at 4°C. Different letters in the same day indicate a statistically significant difference (Tukey test, $p < 0.05$).

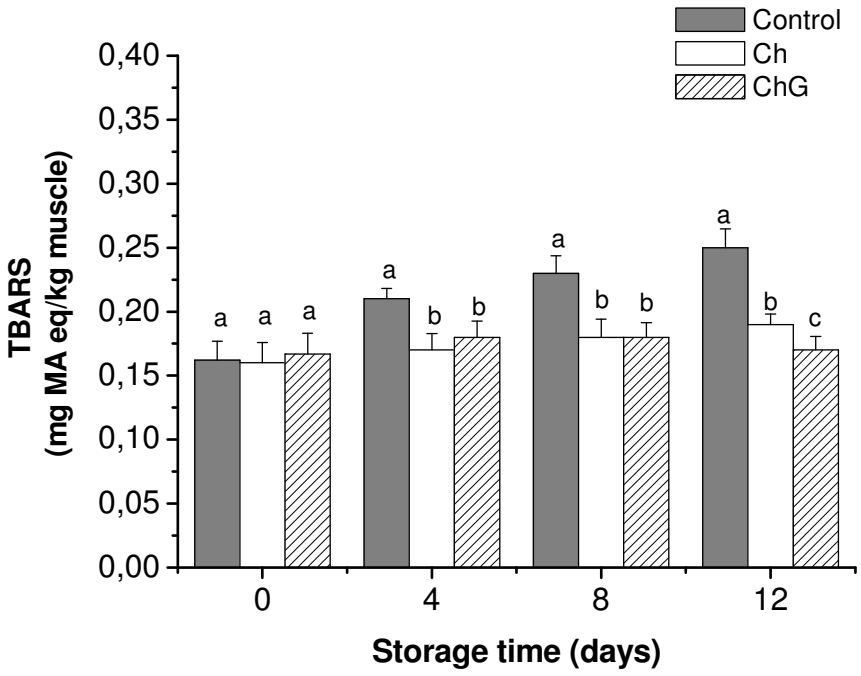


Figure 4. Microbiological analysis of *O. niloticus* fillets. (A) Total plate count during cold storage at 4°C; (B) psychrotrophic count during cold storage at 4°C.

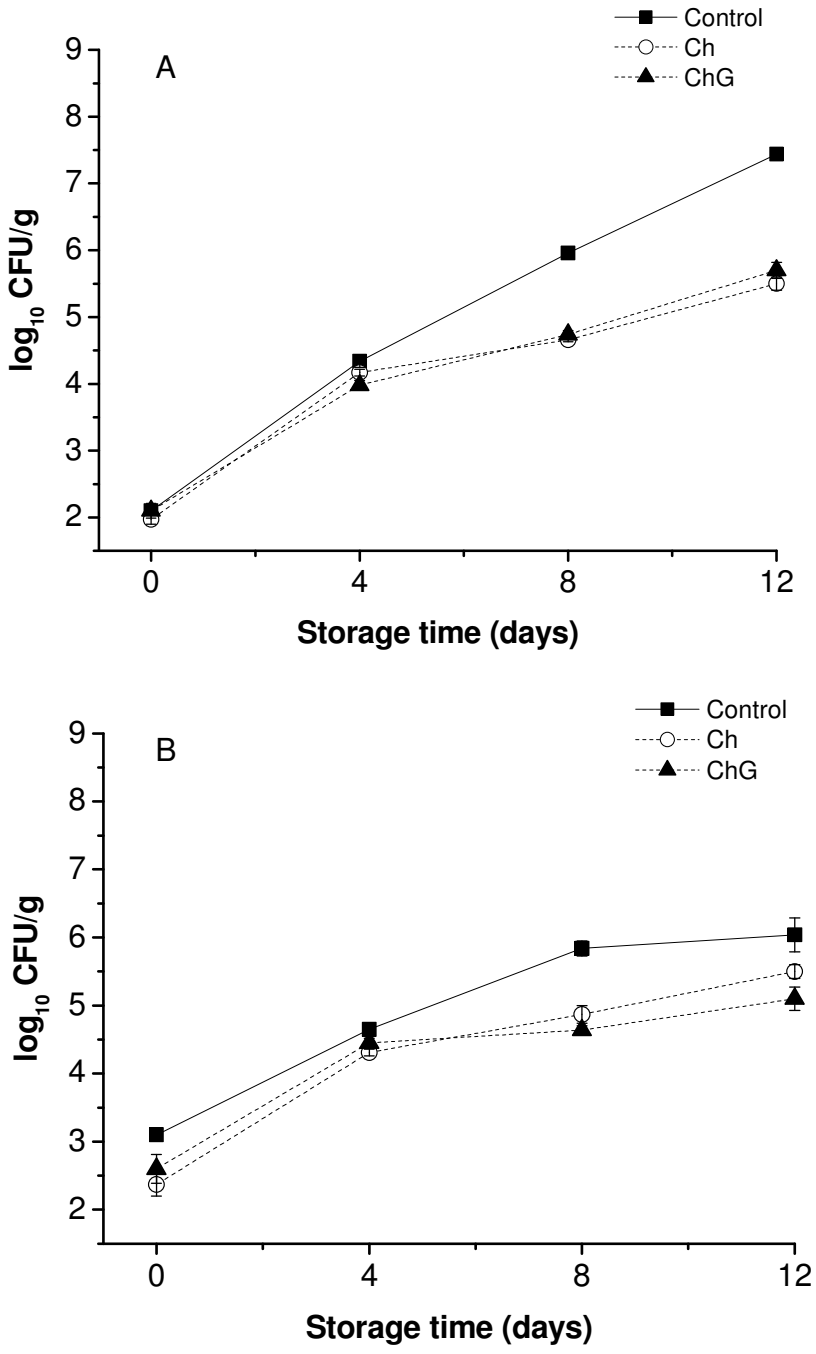
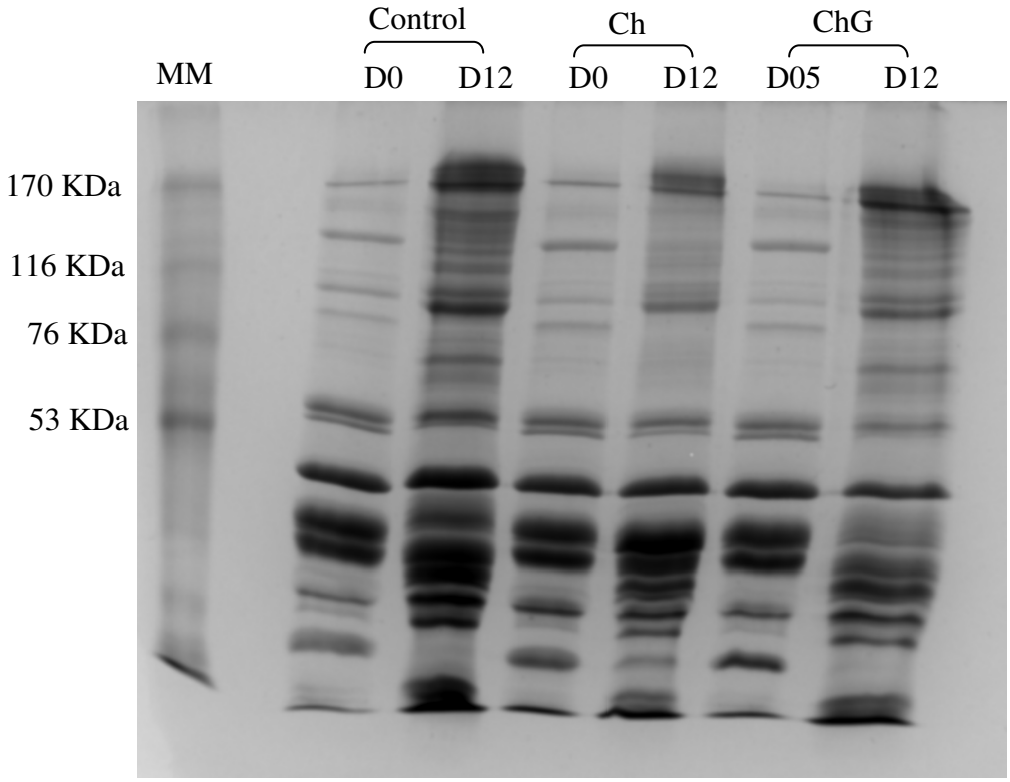


Figure 5. SDS-PAGE of tilapia fillets during cold storage at 4°C. Lanes: MM – molecular mass markers – α2-Macroglobulin (170 kDa), β-galactosidase (116 kDa), Transferrin (76 kDa), Glutamic dehydrogenase (53 kDa); D0 - day 0, D12 – day 12.

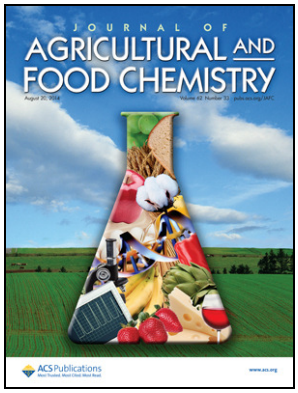


6. CAPÍTULO 2

ARTIGO CIENTÍFICO:

Use of chitosan coating in increasing the shelf life of liquid-smoked Nile tilapia fillets (*Oreochromis niloticus*)

Este artigo será submetido à revista "Journal of Agricultural and Food Chemistry"



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Use of chitosan coating in increasing the shelf life of liquid-smoked Nile tilapia fillets (*Oreochromis niloticus*)

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Abstract

The aim of this research was to evaluate the efficiency of liquid-smoking and coating of chitosan in shelf life of Nile tilapia (*Oreochromis niloticus*) fillets. Fillets without liquid-smoked and chitosan coating (Control), fillets with liquid-smoked (LS) and fillets with liquid-smoked and coated with chitosan (LSCh) were stored at 4±1 °C for 30 days. The physicochemical (pH, moisture content, water activity - A_w, color, texture, total volatile bases nitrogen - TVB-N and thiobarbituric acid reactive substances - TBARS), microbiological analysis (mesophilic and psychrotrophic counts) and electrophoretic profile of samples were carried out during the experimental period. Some physicochemical parameters, such as TVB-N and TBARS, were reduced in the tilapia fillets with liquid smoking.The chitosan coating on smoked fillets increased the effect of maintenance. The number of microorganisms was better controlled in the presence of the coating of chitosan.This work showed that addition of a chitosan coating in liquid-smoked fillets can further enhance the effect of preservation.

Keywords: Chitosan, Liquid-smoked, *Oreochromis niloticus*.

INTRODUCTION

Smoking is one of the oldest methods of preserving fish and meat. The preservative effect is due to the presence of some antioxidant and antimicrobial compounds in smoke such as phenols and formaldehyde (Tülsner, 1994). The preservation of the food is guaranteed by the antioxidant and antimicrobial properties of certain molecules (Cornu et al., 2006) such as phenolic compounds generated by the combustion combined with the temperature and the conditions of smoking can reduce the microbiological development and the oxidation (Kja¨llstrand & Petersson, 2001).

The smoking process is nowadays very looked after for the flavouring of the food and the typical organoleptic qualities that this process confers to the smoked food (Varlet et al., 2007). The liquid smoking is a method that utilizes the components of the smoke in the form of liquid extract; it provides several benefits such as the elimination of carcinogenic compounds and uniform control of the color and flavor of the product.

Nile tilapia *(Oreochromis niloticus)* is one of the most widely cultivated species of fish in the world and is an important source of high-quality proteins for humans. However, it is highly susceptible to both microbiological and chemical deterioration, due to its high water activity, neutral pH, relatively large quantities of free amino acids, and presence of autolytic enzymes (Jeyasekaran, Ganesan, Anandaraj, Shakila, & Sukumar, 2006).

Chitosan is a polysaccharide obtained from the alkaline hydrolysis of N-acetyl group of chitin, the main component of the crustacean shells. Chitosan has been reported to have a number of functional properties that make it technically and physiologically useful in nutrition (Gallaher et al., 2002; Shahidi, Arachchi, & Jeon, 1999). Technically, these include its antimicrobial activity and its ability to form protective films (Cuero, 1999; Jeon, Kamil, & Shahidi, 2002), its texturizing (Benjakul, Visessanguan,

Phatchrat, & Tanaka, 2003), and binding action (No, Lee, & Meyers, 2000); and its antioxidant activity (Kamil, Jeon, & Shahidi, 2002).

The aim of this research was to evaluate the efficiency of liquid-smoked and chitosan coating in enhancing the shelf life of Nile tilapia fillets. The physicochemical and microbiological characterization of the samples was performed. The ability of liquid smoking and chitosan in inhibiting lipid oxidation was also studied.

MATERIALS AND METHODS

Liquid smoking of fillets

Nile tilapia fillets were obtained from a local fish industry and taken in ice boxes to the Fish Technology Laboratory of the Department of Fisheries Engineering, situated at the Federal University of Ceará.

The liquid smoking was perfomed by immersing the fillets in a solution of 20% NaCl (w/v) for 10 minutes, then placed in a stainless steel to drain for 1 min. After this step the fillets were immersed in a solution of 20% liquid smoke (v/v) (TRIPOBET) for 10 minutes and then removing the excess solution on a stainless steel. The fillets were placed in a drying oven at 50 ° C for 30 minutes, then placed in sterile plastic bags and stored at 4 ± 1 °C. The temperature of the muscle tissue of the fillets during storage experiment was 5.49 ± 0.36 °C.

Preparation of solutions and coating application

Chitosan was obtained from shrimp heads of the species *Litopenaeus vanammei* according methodology described by Cahú et al. (2012). The coating solutions were prepared by dissolving 1% chitosan and 0.1% glycerol in 1% lactic acid (v/v) using a magnetic stirrer for 2 hours. The solutions will be previously sterilized using UV light for 15 minutes before being used in the coating of the fillets.

The application of the coating was done by aspersion the solution of chitosan in the liquid-smoked tilapia fillets. The fillets were dried for 1 minute in a stainless steel at 25°C and placed in a drying oven at 50°C for 30 minutes. The fillets were separated into three groups (n = 3) according to the treatment used: fillets without smoking and chitosan coating (Control), liquid-smoked fillets (LS) and liquid-smoked fillets coated with chitosan solution (LSCh).

The samples were placed in sterile plastic storage bags and stored at 4 ± 1 °C for 30 days. The physicochemical and microbiological analysis of samples was carried out with intervals of 5 in 5 days.

Physicochemical analyses

For pH measurement, approximately 10 g of minced fish samples were placed in a 100-mL beaker and homogenized with 100 mL distilled water. The pH of homogenized sample was measured using a pH meter (PM 608, ANALION, São Paulo, Brazil). Moisture content was measured by drying about 10 g of minced fish samples in a conventional oven (TE-394/1, TECNAL, São Paulo, Brasil) at 105°C for 12h. The weight of the initial and dried samples was recorded. The moisture content (%) was calculated as weight change after drying divided by the initial weight of samples x 100% (IAL, 2008).

The determination of the Water Activity (A_w) of the samples was performed in Water Activity Analyzer CX-2 (AquaLab). The color of the fish samples was determined with the help of a colorimeter (Model Chroma Meter CR-400, Konica Minolta, Ltda., Japan). L* (brightness), a* (+a, red;–a, green) and b* (+b, yellow; –b, blue) values were measured.

Texture

The texture of the samples of tilapia fillets was evaluated using the Texture Analyzer TA.XT Plus (Stable Micro Systems). The shear force needed to cut the samples is given in Kg.

Total Volatile Base Nitrogen (TVB-N)

The analysis of TVB-N was performed homogenizing 100 g of fish sample with 200 mL of 7.5% (v/v) aqueous trichloroacetic acid (TCA) solution. The homogenate was filtered through Whatman no. 1 filter paper. TVB-N was measured by steam distillation of the TCA-fish extract, using the modified method of Malle and Tao (*26*). The amounts of TVB-N were calculated from the volume of sulfuric acid used for titration, and the results were expressed in milligrams of nitrogen per 100 g of sample.

Determination of the TBA Reactive Substances (TBARS)

The lipid oxidation of samples was performed by measuring thiobarbituric acid-reactive substances (TBARS) as described by Buege and Aust (1978). A portion (3g) of minced fish samples was homogenised with 25 mL of solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 M HCl. The mixture was heated in a boiling water bath (100°C) for 10 min for developing a pink colour, then cooled and centrifuged at 3600g at 25°C for 20 min. The absorbance of the supernatant was measured by a spectrophotometer at 532 nm, and 1,1,3,3-tetraethoxypropane (TEP) was used as the standards. TBARS was expressed as mg malonaldehyde equivalents/kg muscle (mg MA eq/kg muscle).

Microbiological Analysis

A 25g sample of each treatment was removed aseptically and homogenized with 225 mL of 0.85% NaCl solution. Serial dilutions were performed for each sample and 1 mL

of each dilution was taken and placed in petri dishes. Pour-plate method using plate count agar was used to determine the total plate and psychrotrophic counts in the fish samples. The inoculated agar plates were incubated at 37°C for 48 h for determining total plate counts, and at 4°C for 10 d for psychrotrophic counts. Microbiological data were transformed into logarithms of the number of colony-forming units (CFU/g).

Electrophoresis

Proteins from fillets of *O. niloticus* were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The gel was stained with 0.1% (w/v) Coomasie Blue for 120 min and destained in 10% (v/v) acetic acid and 25% (v/v) methanol. Molecular weight markers were used.

Statistical analysis

Statistical difference within treatment groups were established by ANOVA test. Differences between treatments were established by Tukey test at $p < 0.05$. The software utilized was Origin 6.0 Professional.

RESULTS AND DISCUSSION

Physicochemical analyses

The pH of tilapia fillets during storage at 4 ° C is shown in Figure 1. The smoking process caused a reduction in pH tilapia fillets on initial day of storage when compared with the control group. LS and LSCh showed values of 6.14 ± 0.10 and 6.18 ± 0.08 respectively, while the control was 6.57 ± 0.06 on day 0. From day 20 the pH of the Control significantly increased to 7:39 ± 0.06, with the value of 7.47 ± 0.08) on last day. The pH values were similar for the smoked fillets on the last day of storage (LS, 6.56 ± 0.04; LSCh, 6.60 ± 0.06). The increase in pH can also be found in some studies with other fish species such as sardine and hake. (Ababouch et al., 1996; Nunes, Batista, &

Morâo de Campos, 1992; Ruiz-Capillas & Moral, 2001). This pH increase has a pronounced effect on the quality of the product during storage, especially in terms of sensorial characteristics such as odor, color, and texture, which are negatively affected (Shenderyuk, 1989). Spoilage bacteria utilize low molecular weight compounds such as amino acids present in fish muscle and induce the accumulation of alkaline ammonia components, resulting in the rise of pH (Campos, Rodríguez, Losada, Aubourg, & Barros-Velázquez, 2005).

The moisture content is shown in Table 1. The tratament LS and LSCh showed a moisture content initial lower than the group Control. This may be due to the smoking process pass through a drying step in an oven. The control group had a decrease of approximately 1.5% during the 30 days of storage.Sathivel (2005) reported a 4.1% moisture loss in pink salmon fillets after 3 months of frozen storage. There was no moisture content variation in liquid-smoked fillets during storage at 4 ° C. The moisture content of the Control (74.79 ± 0.38%) was same as for smoked fillets (LS, 75.52 ± 0.84%; LSCh 74.27 ± 0.44%) on the last day.

The fish has a high water activity (A_w) in tissue, favoring the proliferation of microorganisms. A reduction of water is one way to increase the shelf life of the fish. The control samples showed initial values of A_w of 0.963 (Table 1). The process of liquid smoking reduced the initial values of A_w fillets of tilapia (LS, 0.955; LSCh, 0.951). The coating of chitosan in the liquid-smoked fillets maintained A_w during 30 days. The Aw of the controls was reduced to 0.939, probably due to loss of water during storage.

Some variations in color between treatment and during storage in the cold (Table 2) were observed. On day 0 there were no differences in *L** value between treatments.

Only LS suffered significant variation in *L** after 30 days of storage (47.99 - 54.55). The *b** values of the LS treatment (3.19) and LSCH (2.83) were higher than the control (0.62). This characteristic is due to aggregation of the compounds of liquid smoke in fillets, giving a yellowish color to the fish. The smoked fillets did not cause variations in the values of *b** (2.83-3.56) after storage. The coating of chitosan increased the *a** after 30 days (-0.27), while the Control decreased the value to -1.45. LS remained constant value *a** on the last day (-0.43).

Texture

The texture of fish fillets were evaluated by shear force (kg) in the initial and final day (Table1). The lowest value was found in the texture of fresh fillets (0.277 ± 0.063 Kg). The liquid-smoked fillets had a firmer texture of muscle fibers and did not signficantes differences ($p < 0.05$) in initial day (LS, 0.407 ± 0.034 Kg; LSCh, 0.358 ± 0.029). On the last day of the analysis of chitosan coating made with the texture of muscle in fillets stay more firm (0.656 ± 0.053), while the tratament LS and Control had no change in texture.

Total Volatile Base Nitrogen (TVB-N)

TVB-N, a parameter that quantifies the compounds composed of ammonia and primary, secondary, and tertiary amines, is widely used as an indicator of deterioration of muscle tissues (Fan et al., 2009). TVB-N of all samples showed low values at initial day (1.79, 1.77 and 1.75mg TVB-N/100g for Control, LS and LSCH respectively) (Figure 2). During storage the control group significantly increased the concentration of TVB-N. The control showed the value of 13.92 mg / 100g on day 15. The values of TVB-N after 25 days (31.58 mg/100g) exceeded the recommended limit of 30mg TVB-N/100g.

The smoked fillets showed lower values of TVB-N. The coating of chitosan showed the lowest concentrations in the TVB-N samples, demonstrating a better contribution to maintaining the quality of the fish. On the last day, the values for TVB-N were from 8.12 mg/100g for LS and 4.17 mg/100g to LSCh. Using whole cod fillets and different types of soluble chitosan coatings, Jeon et al. (2002) reported reduction of 33–50% in the formation TVBN at the end of a 12-day storage period.

Determination of the TBA reactive substances (TBARS)

The lipids present in the food can deteriorate in the course of time, this can be due to the action of hydrolytic enzymes or by oxidation of the sample by contact with oxygen. The presence of microorganisms can also degrade the lipids. The TBA value is a widely used index of lipid oxidation (Souza et al., 2010).

The initial TBARS values were higher in the control than in LS and LSCH. The liquid smoking may have removed some compounds of lipid oxidation during the process. TBARS values continued to increase and showed maximum value at day 30 (0.30mg MA eq/kg). The smoked fillets had similar values until day 15. On day 30, the value LS (0.19mg MA eq/kg) was greater than LSCh (0.15mg MA eq/kg). Chitosan showed an antioxidant effect because decreased the oxidation of lipids in tilapia fillets. Jeon et al. (2002) found lower TBARS values in chitosan-coated herring and Atlantic cod samples throughout a 12 d cold storage. Salmon coated with chitosan showed TBARS values (1.08 mg MA/kg) less than the uncoated fillets (1.76 mg MA/kg) after 18 days (Souza, 2010).

Lipid oxidation causes undesirable rancid off-flavours and potentially toxic products, which lead to the qualitative deterioration of fish (Eymard et al., 2005). With the good oxygen barrier properties, chitosan coatings applied on the surface of fish may act as a

barrier between the fillet and its surroundings, thus slowing down the diffusion of oxygen from the surrounding to the surface of fillet and retarding the lipid oxidation (Sathivel, 2005).

Microbiological Analysis

The initial mesophilic counts were lower in LSCh than in LS and Control groups. After 30 days of storage, the LS and LSCh groups showed the lowest values (4.54 and 3.61 log CFU/g respectively). Psychrotrophic counts of the Control increased from 6.42 log CFU/g on day 0 to 8.05 log CFU/g on day 30. LSCh had the lowest psychrotrophic counts on day 30 (7.36 log CFU/g). The number of microorganisms was better controlled in the presence of the coating of chitosan.

Electrophoresis

The electrophoretic profile of tilapia fillets on day 0 and 30 are shown in Figure 4. A higher number of bands after 30 days of storage was observed between 30 kDa and 45kDa for all treatments. Among this range of molecular weight also observed a lower intensity of these bands in LS and LSCh. The deterioration by microorganisms of the fish may result in the formation of polypeptide chains of low molecular weight.

In conclusion, this work demonstrated the beneficial effects of smoking and chitosan coating fillets of O. niloticus. A reduction in the levels of TVB-N and TBARS was observed in liquid smoked fillets. Antimicrobial and antioxidant activity of chitosan made the coated fillets had better quality. The liquid smoking associated with the coating of chitosan may promote greater shelf life of the fish.

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Tables

Table 1. Moisture (%), Water activity (A_w) and Texture of tilapia fillets during storage at 4℃.

	Moisture (%)		A_w		Texture (kg)	
	D0	D30	D0	D30	D0	D30
Control	76.24 ± 0.47^{Aa}	74.79 ± 0.38^{Ab}	0.963 ± 0.002^{Aa}	0.939 ± 0.004^{Ab}	0.277 ± 0.063^{Aa}	0.253 ± 0.045^{Aa}
LS	74.82 ± 0.53^{Ba}	75.52 ± 0.84^{Aa}	0.955 ± 0.002^{Ba}	0.943 ± 0.001^{Ab}	0.407 ± 0.034^{Ba}	0.400 ± 0.060^{Ba}
LSCh	73.45 ± 0.17^{Ca}	74.27 ± 0.44^{Aa}	0.951 ± 0.003^{Ba}	0.953 ± 0.001^{Ba}	0.358 ± 0.029^{Ba}	0.656 ± 0.053^{Cb}

Values followed by the different capital letters in the same column are significantly different ($P < 0.05$).
Values followed by the lowercase letters in the same row within each coating method are significantly different ($P < 0.05$).
D0 – Values of proximate composition on day 0.
D30 – Values of proximate composition on day 30.

Table 2. Colour of tilapia fillets during cold storage at 4°C.

	*L**		*a**		*b**	
	D0	D30	D0	D30	D0	D30
Control	49.94 ± 1.83^{Aa}	51.70 ± 2.15^{ABa}	-0.11 ± 0.26^{Aa}	-1.45 ± 0.58^{Ab}	0.62 ± 0.24^{Aa}	2.14 ± 0.48^{Ab}
LS	47.99 ± 2.27^{Aa}	54.55 ± 1.72^{Ab}	-0.76 ± 0.53^{ABa}	-0.43 ± 0.71^{ABa}	3.19 ± 0.72^{Ba}	3.56 ± 0.79^{Ba}
LSCh	51.88 ± 1.94^{Aa}	49.76 ± 0.98^{Ba}	-1.12 ± 0.29^{Ba}	-0.27 ± 0.52^{Bb}	2.83 ± 0.55^{Ba}	3.38 ± 0.76^{ABa}

Values followed by the different capital letters in the same column are significantly different ($P < 0.05$).
Values followed by the lowercase letters in the same row within each coating method are significantly different ($P < 0.05$).
D0 – Values of proximate composition on day 0.
D30 – Values of proximate composition on day 30.

Figures

Figure 1. pH of tilapia fillets stored at 4℃.

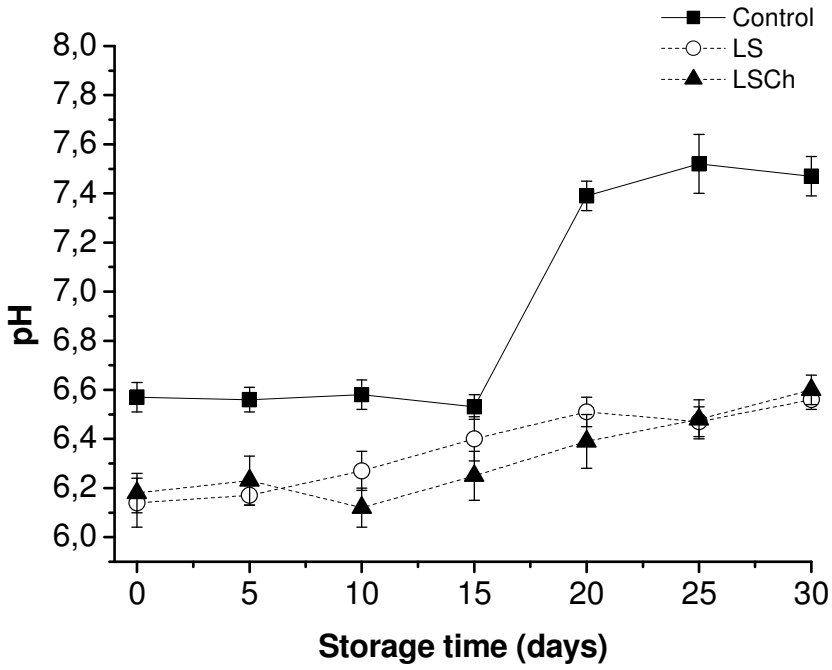


Figure 2. Total volatile base nitrogen (TVB-N) of tilapia fillets stored at 4°C. The horizontal line represents the rejection limit in fishflesh, which is 30 mg of TVB-N/100 g. Different letters in the same day indicate a statistically significant difference (Tukey test, $p < 0.05$).

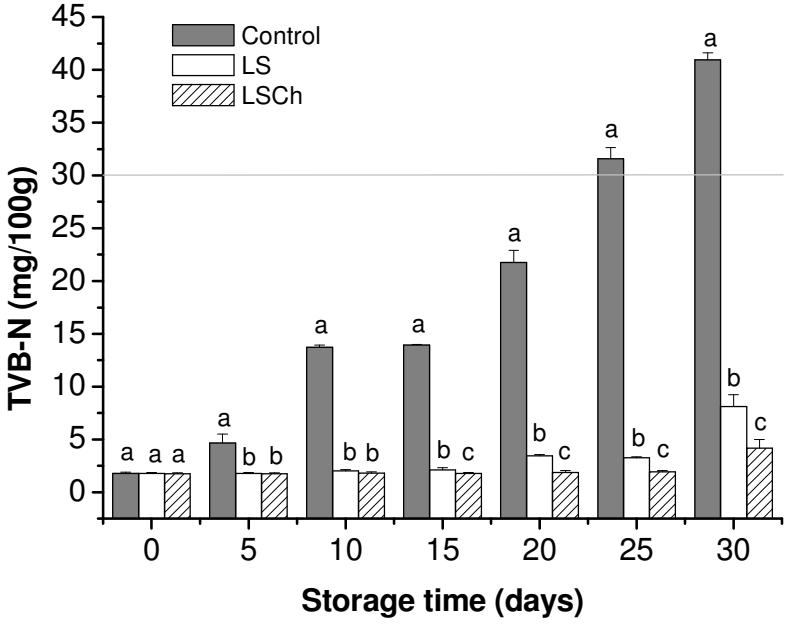


Figure 3. Thiobarbituric acid-reactive substances (TBARS) of tilapia fillets during cold storage at 4°C. Different letters in the same day indicate a statistically significant difference (Tukey test, $p < 0.05$).

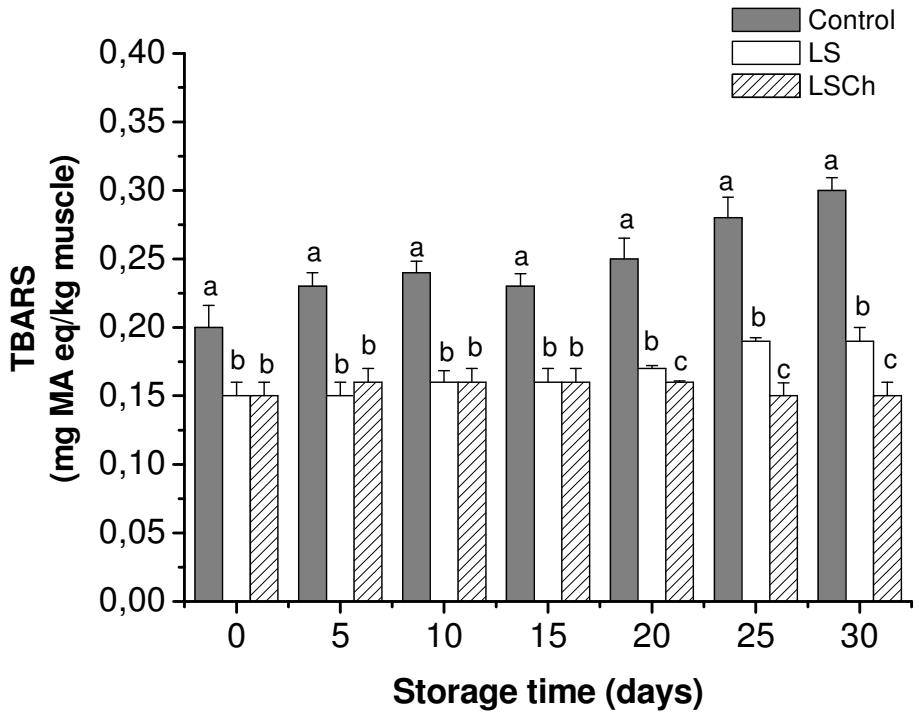


Figure 4. Microbiological analysis of tilapia fillets. (A) Total plate count during cold storage at 4°C; (B) psychrotrophic count during cold storage at 4°C.

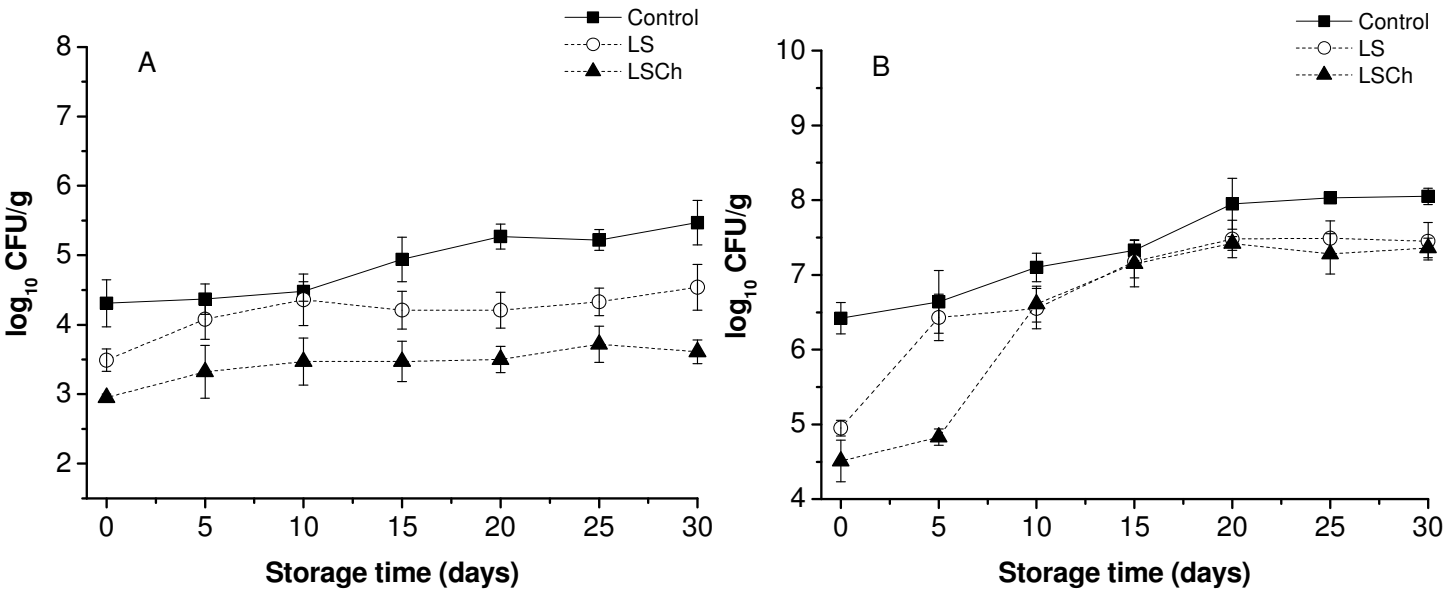
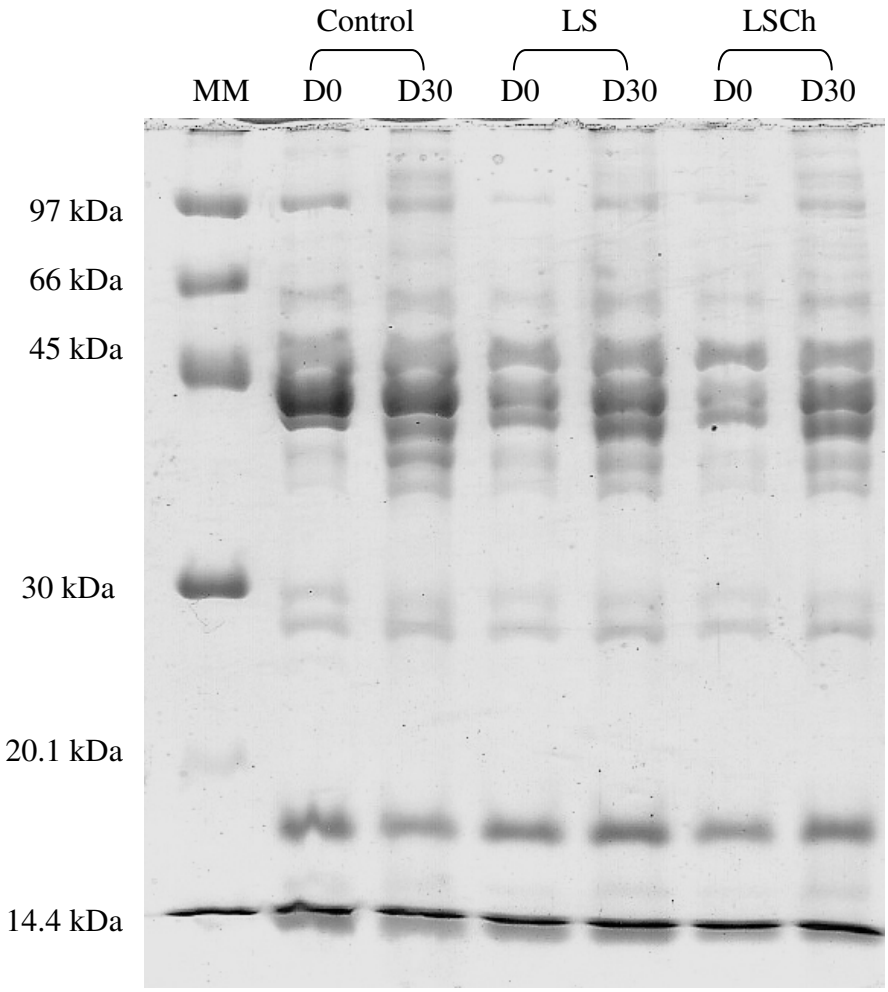


Figure 5. SDS-PAGE of tilapia fillets during cold storage at 4°C. Lanes: MM – molecular mass markers – Phophorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 KDa), Trypsin inhibitor (20.1 kDa), α-Lactoalbumin (14.4); D0 - day 0, D30 - day 30.

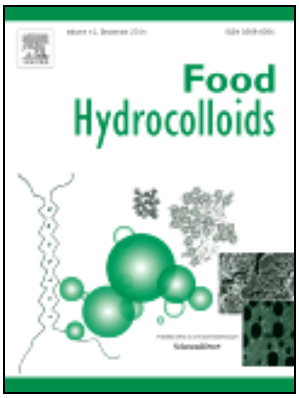


7. CAPÍTULO 3

ARTIGO CIENTÍFICO:

Physical and mechanical characteristics of chitosan films incorporated with essential oils

Este artigo será submetido à revista "Food Hydrocolloids"



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Physical and mechanical properties of chitosan films incorporated with essential oils

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Abstract

Chitosan films were performed with and without the incorporation of essential oils (EOs) (citronella, copaiba and eucalyptus). The physical and mechanical properties of these films were evaluated. The results showed that there was a change in the characteristics of chitosan film was when incorporated EOs. The interactions of oil with the polymer were varied, giving different results of tensile strenght - TS and elongation at break - EB between different EOs used. The values of moisture content confirmed the presence of water inside the films. There was a decrease in the solubility of some films in the presence of EOs. Addition of EOs alters the color and opacity property of films of chitosan. The color values of films were in white and yellowish color. The contact angle measurement was used to determine the hydrophobic or hydrophilic characteristics of the film surface. Addition of some kinds of oils made films to stay with a more hydrophobic character. This work showed that the addition of EOs changed some properties of the chitosan film, which can bring new biotechnological applications for these films.

Keywords: Chitosan films, essential oils, mechanical properties.

1. Introduction

The shelf life of food depends greatly on packaging materials and characteristics such as gas and water vapor barrier properties and atmosphere modification. Thus, packaging plays a vital role in food preservation. It also protects food from physical damage and conveysmarketing and statutory information about the product.

Edible packaging materials usually consist of proteins, lipids and polysaccharides. Chitosan is a natural polymer derived by deacetylation of chitin, the second most abundant biopolymer in nature after cellulose (Shahidi, Arachchi, & Jeon, 1999). When compared with other polysaccharides, chitosan has important properties such as biocompatibility, biodegradability and no toxicity; and several studies indicated chitosan as bacteriostatic and fungistatic (Yi et al., 2005).

Plant essential oils (EOs) are interesting natural antimicrobial agents to be incorporated into the edible films due to these plant extracts exhibit additional characteristics, such as antimicrobial and antioxidant effects (Atarés et al., 2010; Bagamboula, Uyttendaele, & Debevere, 2004; Fisher & Phillips, 2006; Pelissari, Grossmann, Yamashita, & Pineda, 2009). Moreover, in the application of EOs, it is also important to evaluate their effects on the physical, optical and structural properties of the resulting film (Altiok, Altiok, & Tihminlioglu, 2010; Ojagh et al., 2010; Sánchez-González et al., 2010). The purpose of this study was to investigate the effect of three types of essential oils: citronella (*Cymbopogon nardus G.*), copaiba (Copaifera multijuga) and eucalyptus (*Corymbia citriodora*) on the physical and mechanical properties of chitosan films.

2. Materials and methods

2.1. Materials

Chitosan was obtained from shrimp heads of the species *Litopenaeus vanammei* according methodology describe by Cahú et al. (2012). Essential oils used in this study were provided by União Vegetal Suplementos Nutricionais Ltda. (Ceará, Brazil).

2.2. Films preparation

A chitosan solution was prepared by diluting chitosan 2% (v / v) in 1% lactic acid (v / v) at 25 ° C for 4h acid. After being diluted chitosan were added 0.1% glycerol and 1% essential oils (EOs). The solutions Ch (chitosan), ChCi (chitosan and citronella), ChCo (chitosan and copaiba) and ChE (eucalyptus and chitosan) were homogenized for 30 min at 25°C.The films were prepared with a constant amount (28 mL) of solution which was cast onto a 9 cm diameter Petri plate. The films were dried in an oven at 35 °C for 20 h and maintained at 20 °C and 55% RH (relative humidity), until further use.

2.3. Film thickness

The film thickness was measured with a digital micrometer (No.293-561, Mitutoyo, Japan). Five thickness measurementswere taken on each testing sample in different points and the mean valueswere used to calculate permeability and mechanical properties.

2.4. Water vapor permeability (WVP) measurement

The measurement of water vapor permeability (WVP) was performed gravimetrically based on ASTM E96-92 method (Guillard, Broyart, Bonazzi, Guilbert, & Gontard, 2003; McHugh, Avena-Bustillos, & Krochta, 1993). The film was sealed on the top of a permeation cell containing distilled water (100% RH; 2337 Pa vapor pressure at 20°C), placed in a desiccator at 20°C and 0% RH (0 Pa water vapor pressure) containing silica. The cells were weighted at 2 h intervals during 10 h. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation

constant outside the test cell by using a miniature fan inside the desiccator (Guillard et al., 2003). The slope of weight loss versus time was obtained by linear regression. The measured (WVP) of the films was determined as follows:

$$WVP = \frac{WVTR \cdot L}{\Delta P}$$

where WVTR is the measured water vapor transmission rate through a film, L is the mean film thickness (m), and ΔP is the partial water vapor pressure difference (Pa) across the two sides of the film. Three replicates were obtained or each film.

2.5. Tensile strength (TS) and elongation-at-break (EB)

TS and EB were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation) following the guidelines of ASTM Standard Method D 882-91 (ASTM-D-882-91, 1991). The initial grip separation was set at 30 mm and the crosshead speed was set at 5 mm min^{-1}. TS was expressed in Pa and calculated by dividing the maximum load (N) by the initial crosssectional area (m^2) of the specimen. EB was calculated as the ratio of the final length at the point of sample rupture to the initial length of a specimen (30 mm) and expressed as a percentage. According to the ASTM standard, film strips with a length of 45 mm and a width of 20 mm were used. TS and EB tests were replicated at least three times for each type of film.

2.6. Moisture content

To determine the moisture content of films about 50 mg of film were dried at 105°C during 24 h (until the equilibrium weight was attained). The weight loss of the sample was determined, from which the moisture content was calculated using the following equation:

$$\text{Moisture content} = \frac{(Mi - Mf)}{Mi} \times 100$$

where *Mi* and *Mf* are the masses of initial and dried samples, respectively.

2.7. Film solubility and swelling degree

The solubility and swelling degree of the films were determined according to the methods described by Silva, Bierhalz, and Kieckbusch (2009) and Zhong et al. (2011) with some modifications. Film pieces (20 x 20 mm) were dried at 105°C for 24 h in a vacuum oven to get the initial dry mass (M1). Then the films were placed in 100 mL beakers containing 50 mL distilled water. The beakers were covered with plastic wraps and stored at 25°C for 24 h.Water remaining in the beakerswas discarded and the residual film pieces (M2) were dried superficially with filter paper. The residual film pieces were again dried at 70°C for 24 h in a vacuum oven to determine the final dry mass (M3). Three measurements were taken for each film sample. Film solubility and swelling degree were calculated by using the following equations, respectively:

$$\text{Film solubility} = \frac{(M1 - M3) \times 100}{M1}$$

$$\text{Swelling degree} = \frac{(M2 - M1)}{M1}$$

2.8. Film color and opacity

The colour of the fish samples was determined with the help of a colorimeter (Model Chroma Meter CR-400, Konica Minolta, Ltda., Japan). L* (brightness), a* (+a, red;−a, green) and b* (+b, yellow; −b, blue) values were measured.

The film opacity was determined according to the method of Park and Zhao (2004) by measuring the absorbance at 600 nm with a spectrophotometer. The opacity of the films was calculated by the following equation:

$$O = Abs_{600}/L$$

Where O was the opacity, Abs600 was the value of absorbance at 600 nm and L was the film thickness (mm). Four repetitions were performed for each sample.

2.9. Contact angle analysis

Contact angle (°) of the nanolayered film surface was measured in a face contact angle meter (OCA 20, Dataphysics, Germany) using the sessile drop method (Newman & Kwok, 1999). A 2mL droplet of ultra pure water was placed on the horizontal surface with a 500mL syringe (Hamilton, Switzerland), with a needle (0.75mmdiameter). Measurementsweremadeat 0s, 30s and 60s. Ten replicates of contact angle measurements were performed at 20.5±0.3 °C.

2.10. Statistical analysis

Statistical difference within treatment groups were established by ANOVA test. Differences between treatments were established by Tukey test at $p < 0.05$. The software utilized was Origin 6.0 Professional.

3. Results and discussion

3.1. Tchickness and Water vapor permeability (WVP) measurement of films

Thickness and WVP values of the films are shown in Table 1. Results showed that the addition of EOs significantly ($P < 0.05$) increased the thickness of ChCo and ChE films. The highest values of WVP were found in ChCi and ChCo films. The ChE treatment showed no significant difference from Ch. The water vapor permeability is the most extensively studied property of edible films mainly because of the importance of the water in deteriorative reactions (Cerqueira et al., 2009). Essential oils as lipid compounds were known to enhance the water barrier properties of polymer based films because of their hydrophobic nature (Sánchez-González et al., 2009).

3.2. Tensile strength (TS) and elongation-at-break (EB)

Tensile strength and elongation at break were usually related to the film network microstructure and the intermolecular force (Atarés et al., 2010).The interactions of oil

with the polymer were varied, giving different results of TS and EB between EOs. ChE showed the highest value of TS (19.4 MPa), while ChCi showed the lowest value (15.2 MPa) between the EOs (Table 1). The film of chitosan (Ch) had no significant differences with ChE. The increase of 12.31% TS with the incorporation of cinnamon oil on chitosan films was evidenced by Peng and Li (2014). Sánchez-González et al. (2010) reported that the tensile strength and elongation at break of chitosan film decreased with the incorporation of bergamot EO. Corroborating the values found for TS, ChE showed one of the lowest values of EB (64.3%). The addition of oils of citronella (ChCi) and copaiba (ChCo) increased by 8.7% and 5.5% values of EB, respectively.

3.3. *Moisture content*

The moisture content shows the presence of water within the film matrix. Values were found between 13:04 - 18.61% in the films analyzed (Table 2). ChCo showed the lowest value of moisture content (13.04%). The chitosan films without EOs had similar values when compared with ChCi and ChE. Some substances incorporated, such as glycerol, may decrease or increase the water concentration. Glycerol, due to its hydrophilic nature, retains water in the film matrix (Cerqueira et al., 2012). The essential oils of copaiba proved to be more hydrophobic than the other oils in the chitosan matrix.

3.4. *Water solubility and swelling degree*

Solubility and swelling degree are important characteristic for biodegradable films because they can affect the resistance of film to water, especially in a humid environment (Peng & Li, 2014). ChCo showed a decrease in solubility of 8.45% of the films of chitosan and the addition of eucalyptus oil decreased by only 1.3% solubility

(Table 2). For chitosan films, the hydrophobic character of oil changes the film structure leading to a less soluble film (i.e. decreases the number of O-H bonds and the presence of an aliphatic groups) (Cerqueira et al., 2012). The values of the degree of swelling are shown in Table 2. There was little variation in the degree of swelling of the films with and without EOs (2.20 to 2.55).

3.5. Film color and opacity

The differences in color could be ascribed to the natural yellow of EOs (Atarés et al., 2010). Addition of EOs alter the color and opacity property of films of chitosan. The color values of films were in white and yellowish color, wish was illustrated by *L** value > 36, *a** value ranging from 36.86 to 37.63, and *b** value ranging from -0.54 to -0.66 (Table 3). There were no differences between values of *a** films analyzed. ChE showed more yellowish (b * 2:52) and with more opacity (2.28). The copaiba oil also presented one of the greatest values in the opacity (2.32). Low opacity values were found in the films of chitosan (0.70) and citronella (0.78).

3.6. Contact angle analysis

The contact angle measurement is a useful tool to determine the hydrophobic or hydrophilic characteristics of a surface. The most wettable surfaces present low values (< 20◦) and the hydrophobic surfaces, on the contrary, show high values (> 70◦) of the contact angle (Carneiro-da-Cunha et al., 2010). The contact angle of the films is shown in Figure 1. Ch and CHCI presented more hydrophilic characteristics in the early 30s of contact with the drop of water on the surface of the films. The contact angle of Ch and 0s in ChCi was 38.65° and 36.20° respectively. ChCo and ChE were more hydrophobic in 0s than in the other films. These films showed a reduction in contact angle over time, reaching values of 39.68° (ChCo) and 37.10° (ChE) in 60s.

4. Conclusions

The analysis of the films of chitosan was important to evaluate the physical and mechanical characteristics when incorporated essential oils. The oils of citronella, eucalyptus and copaiba modified some of these properties. There was increased WVP of the films due to the presence of certain EOs. Analysis of TS, EB, color and opacity varied depending on the oil used. It was also shown that some EOs cause an increase in hydrophobicity of the films, this can facilitate the interaction with some hydrophobic substances. The incorporation of EOs in the chitosan matrix can provide property important features for the films.

Acknowledgments

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Tables

Table 1. Values of film thickness, water vapor permeability (WVP), tensile strength (TS) and elongation-at-break (EB) for Ch, ChCi, ChCo and ChE films.

Films	**Film Thickness (mm)**	**WVP x 10^{-10} (g m^{-1} s^{-1} Pa^{-1})**	**TS (MPa)**	**EB (%)**
Ch	0.089 ± 0.01a	3.58 ± 0.06a	17.0 ± 0.8a	67.0 ± 0.7a
ChCi	0.097 ± 0.01a	4.16 ± 0.44b	15.2 ± 0.4b	75.7 ± 1.6b
ChCo	0.138 ± 0.01b	4.36 ± 0.38b	16.4 ± 0.6a	72.5 ± 2.6b
ChE	0.111 ± 0.01c	3.25 ± 0.35a	19.4 ± 0.7c	64.3 ± 2.6a

Values reported are the means ± standard deviations.
Different letters in the same column indicate a statistically significant difference ($p<0.05$).

Table 2. Values of moisture content (%), solubility (%) and swelling degree for Ch, ChCi, ChCo and ChE films.

Films	Moisture content (%)	Solubility (%)	Swelling degree
Ch	18.61 ± 1.46a	24.92 ± 0.72a	2.20 ± 0.17a
ChCi	17.78 ± 1.38a	24.83 ± 1.20ac	2.55 ± 0.15bc
ChCo	13.04 ± 0.66b	16.47 ± 0.44b	2.35 ± 0.11ab
ChE	17.70 ± 0.93a	23.62 ± 0.17c	2.49 ± 0.02c

Values reported are the means ± standard deviations.
Different letters in the same column indicate a statistically significant difference ($p<0.05$).

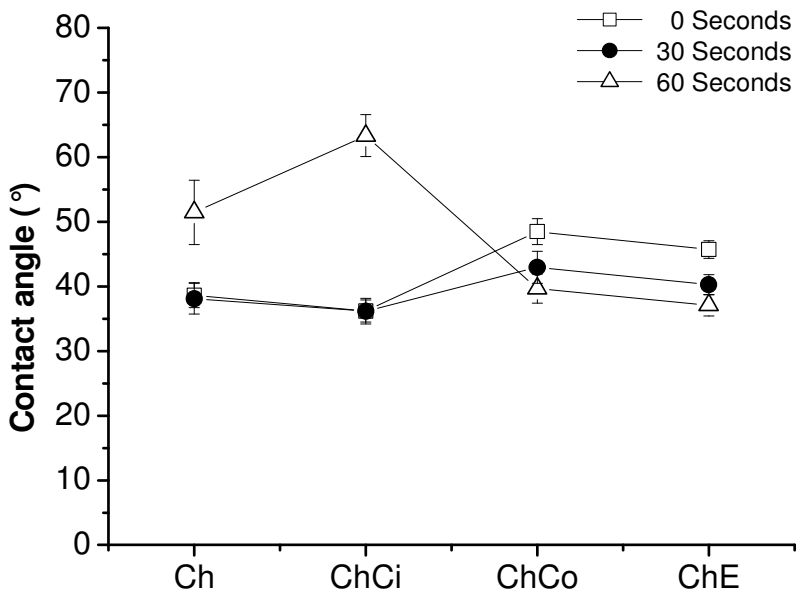
Table 3. Color and opacity of Ch, ChCi, ChCo and CE films.

Films	***L****	***a****	***b****	**Opacity**
Ch	36.86 ± 0.33a	-0.62 ± 0.06a	1.73 ± 0.24a	0.70 ± 0.09a
ChCi	37.17 ± 0.29a	-0.66 ± 0.05a	1.78 ± 0.14a	0.78 ± 0.18a
ChCo	37.63 ± 0.47ab	-0.54 ± 0.13a	1.56 ± 0.40a	2.32 ± 0.23b
ChE	38.23 ± 0.37b	-0.60 ± 0.08a	2.52 ± 0.36b	2.28 ± 0.48b

Values reported are the means ± standard deviations.
Different letters in the same column indicate a statistically significant difference ($p<0.05$).

Figures

Figure 1. The contact angle measured on Ch, ChCi, ChCo and ChE films (measured 0, 30 and 60 s after drop application). Each data point is an average of 10 determinations and the error bars represent the standard deviation.



8. CONCLUSÃO

O revestimento de quitosana demonstrou uma melhor conservação dos filés de tilápia do *Oreochromis niloticus* armazenados em baixa temperatura. A adição de grlicerol como plasticizante auxiliou o revestimento de quitosana melhorando suas propriedades.

A oxidação lipídica e número de microorganismo apresentou uma redução nas amostras com o revestimento de quitosana. A diminuição da deterioração lipídica favorece a redução do *off-flavor* no pescado.

A defumação utilizando fumaça líquida demonstrou uma melhor conservação do pescado quando se comparou com os filés frescos. A concentração de BVT-N e TBARS foram menores nos filés defumados durante o período de armazenamento. O revestimento de quitosana proporcionou uma melhor ação antioxidante e antimicrobiológica no filés de tilápia do Nilo.

A defumação líquida associada com o revestimento de quitosana pode promover uma melhor conservação do pescado e aumentar o tempo de prateleira.

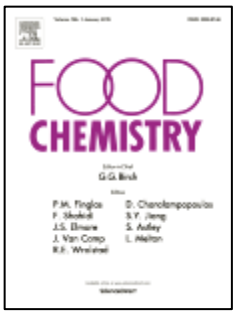
A adição de óleos essencias (citronela, copaíba e eucalipto) alterou algumas propriedades físicas e mecânicas dos filmes de quitosana. Alguns óleos proporcionaram mais hidrofobicidade a matriz de quitosana. Análises de tensão e deformação mostraram diferenças entre os filmes.

A incorporação de óleos essencias pode proporcionar características biotecnológicas importantes para os filmes elaborados com quitosana.

9. ANEXOS

9.1. ANEXO 1

Normas da revista Food Chemistry (ISSN: 0308-8146)



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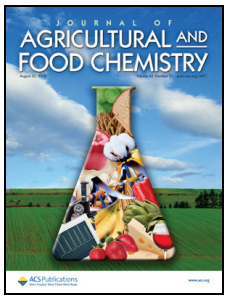

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Title and authorship (single page)
Abstract and keywords (single page)
Introduction
Materials and Methods (including Safety information)
Results/Discussion
Abbreviations Used
Acknowledgment
Supporting Information description
References
Figure captions
Tables
Figure graphics
Graphic for table of contents

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Discuss relationships of the study to previously published work, but do not reiterate or attempt to provide a complete literature survey. Use of *Chemical Abstracts/Scifinder* and other appropriate databases is encouraged to ensure that important prior publications or patents are cited and that the manuscript does not duplicate previously published work. **The purpose or reason for the research being reported, and its significance, originality, or contribution to new knowledge in the field, should be clearly and concisely stated.**

Do not include or summarize current findings in this section.

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Describe statistical design and methods in this section.

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ACKNOWLEDGMENT

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2. Smith, L; Caldwell, A. Chapter title. In *Book Title*, edition no.; Keys, F., Park, G., Eds.; Publisher: City, State (or Country if non-U.S.), Year; Vol. no., pp.
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Structural Formulas. Structural formulas should be included for all new chemicals and for existing chemicals for which chemical nomenclature and/or trivial names do not convey the structure adequately. Structural formulas are valuable in expressing concisely the precise nature of the compounds under discussion and revealing the essence of the subject to readers unfamiliar with the topic, without their necessary recourse to reference materials. The use of chemical names without accompanying structures may cause readers to overlook the significance of the paper.

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Gas Chromatographic Methods. For manuscripts in which gas chromatographic methods are used, see "Reporting of Gas Chromatographic Methods", by Morton Beroza and Irwin Hornstein [*J. Agric. Food Chem.* **1973**, *21*, 7A (located at the back of the January 1973 issue or as a link from the *Journal's* Author Information page)]. Consult recent issues for examples of GC, LC, and other instrument parameter descriptions.

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Mass Spectra. List the molecular ion and about 10 of the major ions with their intensities in parentheses, or more preferably use the method outlined by H. S. Hertz, R. A. Hites, and K. Biemann (*Anal. Chem.* **1971**, *43*, 681–691). This method involves dividing the spectrum into consecutive regions of 14 mass units starting at *m*/*z* 6 (i.e., 6–19, 20–33, 34–47, 48–61, etc.). The two most intense ions in each region are then listed. Intensities, relative to the most intense ion, the intensity of which is taken as 100, are shown in parentheses immediately following the *m*/*z* value; for example: hexanal, mass spectrum found (70 eV, two most intense ions each 14 mass units above *m*/*z* 34): 43 (86), 44 (100), 56 (86), 57 (65), 71 (28), 72 (33), 82 (18), 85 (5), 97 (2), 100 (2). If the molecular ion does not appear in this presentation, the author should indicate it separately.

Nuclear Magnetic Resonance (1H NMR or 13C NMR) Spectra. A document providing detailed information for the presentation of NMR data is now available through "Information for Authors and Reviewers" on the Journal's home page.

The frequency, the solvent, and also the temperature (if other than ambient) used are first specified. The type of unit used (δ or τ) is then stated, followed by the position of the center of gravity of the sharp line, broad line, or spin–spin multiplet in these units. This is then followed by information in parentheses which (1) describes the type of splitting, that is, singlet as s, doublet as d, triplet as t, quadruplet as qd, multiplet as m; (2) gives the value of the number of protons the area represents; (3) gives the coupling constant *J*; and (4) gives the part of the molecule connected with the particular absorption with the protons involved underlined.

An example would be 1H NMR for ethanol (60 MHz, CCl4): δ 1.22 (t, 3, J = 7 Hz, CH2CH3), 2.58 (s, 1, OH), 3.70 (qd, 2, J = 7 Hz, OCH2CH3).

Other Spectra. In general, list position and intensity of the maxima. In some cases it may be desirable to list points of inflection.
A brief explanation should be given for any abbreviations not in common use.
Examples:
• Reporting liquid chromatography (HPLC) and HPLC/MS: "Analysis of Polyphenolic Antioxidants from the Fruits of Three *Pouteria* Species by Selected Ion Monitoring Liquid Chromatography–Mass Spectrometry", by Jun Ma et al. *J. Agric. Food Chem.* **2004**, *52*, 5873–5878.

• Reporting data in detail, including UV shifts and IR spectra: "Characterization of Vegetable Oils: Detailed Compositional Fingerprints Derived from Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry", by Zhigang Wu et al. *J. Agric. Food Chem.* **2004**, *52*, 5322–5328.

Novel Compound Characterization. For a discussion of the *Journal's* expectations for compound characterization, please read "Compound Identification: A *Journal of Agricultural and Food Chemistry* Perspective" by R. J. Molyneux and P. Schieberle. *J. Agric. Food Chem.* 2007, *55*, 4625–4629 (DOI: 10.1021/jf070242j). It is essential that novel compounds, either synthetic or isolated from natural sources, be characterized rigorously and unequivocally. Supporting data normally include physical form, melting point (if solid), UV/IR spectra if appropriate, 1H and 13C NMR, mass spectrometric data, and optical rotation (when compounds have chiral centers).
Examples:
• Reporting X-ray data: "Racemic and Enantiopure Synthesis and Physicochemical Characterization of the Novel Taste Enhancer *N*-(1-Carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol Inner Salt", by Renaud Villard et al. *J. Agric. Food Chem.* **2004**, *51*, 4040–4045.

• Reporting data in detail, including UV shifts: "Novel Flavonol Glycoside, 7-*O*-Methyl Mearnsitrin, from *Sageretia theezans* and Its Antioxidant Effect", by Shin-Kyo Chung et al. *J. Agric. Food Chem.* **2004**, *52*, 4664–4668.
Flavor Constituents. Manuscripts reporting on flavor constituents should conform to the recommendations made by the International Organization of the Flavor Industry [for details, see the Editorial in the October 1996 issue of *J. Agric. Food Chem.* (*44*, 2941–2941)]. In brief, any identification of a flavoring substance must pass scrutiny of the latest forms of available analytical techniques. **In practice, this means that any particular substance must have its identity confirmed by at least two methods, for example, comparison of chromatographic and spectrometric data (which may include GC, MS, IR, and NMR) with those of an authentic sample.** If only one method has been applied (MS data alone or retention index or Kovats index alone), the identification shall be labeled "tentative". In addition, authors are encouraged to include at least semiquantitative data on the concentration of an identified component in the original source, for example, foodstuff or plant part. Ranges such as <1 μg/kg, 1–10 μg/kg, and 10–100 μg/kg are acceptable.
Flavor is evoked by smell (aroma) and taste. A good example showing the correct characterization of taste compounds is the study by Czepa and Hofmann (*J. Agric. Food Chem.* **2003**, *51*, 3865–3873). A good example for aroma compound identification is the study by Milo and Grosch (*J. Agric. Food Chem.* **1996**, *48*, 2366–2371).
The use of reference compounds is a must, if data on sensory properties of single compounds are reported. Odor, which is perceived during sniffing of a food extract at a

certain retention index, may be indicative of the presence of a given compound, but not conclusive unless substantiated by chromatographic and/or spectrometric data and comparison with an authentic reference compound.

Soil Classification. Soils used in research should be described down to the family level according to the soil classification scheme given in *Soil Taxonomy, A Basic System of Soil Classification for Making and Interpreting Soil Surveys*, 2nd ed. (Agricultural Handbook 436; U.S. Government Printing Office: Washington, DC, 1999) (available on-line at http://soils.usda.gov/technical/classification/taxonomy/). Also give series name if known.

This requirement is to allow comparison and extrapolation to other work giving similar soil classifications, as published in journals such as the *Journal of Soil Science*, *Soil Science Society of America Journal*, *Journal of Environmental Quality*, and *Geoderma*. If information is unavailable to classify the soils at the desired family level, classification should be described or estimated at least to the great group level in the same classification system.

Statistics. Manuscripts reporting analytical, biological activity, composition, and related data must include relevant statistical information to support discussion of differences or similarities in data sets. Refer to a standard statistics reference such as *Statistical Methods*, 8th ed.; Snedecor, G. W., Cochran, W. G., Eds.; University Press: Ames, IA, 1989.

Metabolomics. This category considers applications of metabolomics as related to research topics in agriculture, food, and nutrition, in particular metabolite-targeted analysis and progress in the development of analytical platforms for metabolomics approaches. A metabolome is the quantitative set of chemical compounds in a biological system, i.e., a food, at a given time. However, also metabonomics studies, focused on changes in a given metabolome, e.g., induced by environmental conditions or diseases, fall into this category.

Metabolic profiling and metabolomic fingerprinting correlated with multivariate or data-mining methods are acceptable, if presented in a targeted way. For additional information consult "Targeted Metabolomics: A New Section in the *Journal of Agricultural and Food Chemistry*" by J. N. Seiber, R. J. Molyneux, and P. Schieberle, *J. Agric. Food Chem.* **2013**, DOI: 10.1021/jf4046254.

Animal or Human Studies. Manuscripts describing studies in which the use of live animals or human subjects is involved must include under Materials and Methods a statement that such experiments were performed in compliance with the appropriate laws and institutional guidelines, and also name the institutional committee that approved the experiments. For experiments with human subjects, a statement that informed consent was obtained from each individual must be included and the consent forms made available to the *Journal* on request. Reviewers of manuscripts involving animal or human experiments will be asked to comment specifically on the appropriateness and conformity to regulations of such experiments. **Authors are encouraged to note the approval code or number or give the name of the approving office of official.**

Animal Subjects. The use of animals in a study should be employed only when there are no alternative methods for investigating the fundamental questions of the study. In such cases, **it is the ethical responsibility of all authors to ensure that the care of animals is of the highest possible order, that pain and/or distress is minimized, and that the numbers involved are strictly limited** to those essential to fulfill the experimental

design. In the United States the care and use of laboratory animals is regulated by the U.S. Department of Agriculture (USDA) under the Animal Welfare Act. Links to the regulations and other information are available at http://www.aphis.usda.gov/animal_welfare/links.shtml. It is recognized that researchers in other countries may be governed by different laws and regulations. In such cases, experiments should be designed to conform either to the above USDA regulations or to the International Guiding Principles for Biomedical Research Involving Animals (1985), available at http://www.cioms.ch/publications/guidelines/1985_texts_of_guidelines.htm.

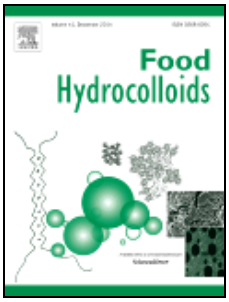
Human Subjects. **The use of human subjects in experimental studies requires informed consent.** Such consent requires that the subjects be informed completely not only about the procedures involved but also about the aims, design, and expected outcomes of the study. Consent must be obtained not only when subjects are involved directly in the study but also when samples (tissue, blood, plasma, etc.) are required for in vitro experiments. In the United States the protection of human research subjects is regulated by the U.S. Department of Health and Human Services (HHS). Regulations are available at http://www.hhs.gov/ohrp/. Laws and regulations governing researchers in other countries must be observed, but experiments should be designed to conform to the intent of the HHS regulations as far as possible.

In relation to the subject matter of the *Journal*, experiments involving taste and food quality evaluation and consumer acceptance are exempt from the above regulations [CFR 46.101 (b) (6)]. However, it should be noted that this would not exempt studies in which extracts, isolates, pure compounds, etc., obtained from conventional food sources are subjected to such evaluation.

The *Journal* will reject any manuscript for which there is reason to believe that animals have been subjected to unnecessary pain or distress or when informed consent of human subjects is absent or incomplete.

9.3. ANEXO 3

Normas da revista Food Hydrocolloids (ISSN: 0268-005X)



GUIDE FOR AUTHORS

INTRODUCTION

Food Hydrocolloids only publishes original and novel research that is of high scientific quality. Research areas include basic and applied aspects of the characteristics, properties, functionality and use of macromolecules in food systems. Hydrocolloids in this context include polysaccharides, modified polysaccharides and proteins acting alone, or in mixture with other food components, as thickening agents, gelling agents, film formers or surface-active agents. Included within the scope of the journal are studies of real and model food colloids - dispersions, emulsions and foams - and the associated physicochemical stability phenomena - creaming, sedimentation, flocculation and coalescence.

In particular, *Food Hydrocolloids* covers: the full scope of hydrocolloid behaviour, including isolation procedures, chemical and physicochemical characterization, through to end use and analysis in finished food products; structural characterization of established food hydrocolloids and new ones
ultimately seeking food approval; gelling mechanisms, syneresis and polymer synergism in the gelation process; rheological investigations where these can be correlated with hydrocolloids functionality, colloid stability or organoleptic properties; theoretical, computational or simulation approaches to the study of colloidal stability, provided that they have a clear relationship to food systems; surface properties of absorbed films, and their relationship to foaming and emulsifying behaviour; phase behaviour of low-molecular-weight surfactants or soluble polymers, and their relationship to food colloid stability; droplet and bubble growth, bubble nucleation, thin-film drainage and rupture processes; fat and water crystallization and the influence of hydrocolloids on these phenomena, with respect to stability and texture; direct applications of hydrocolloids in finished food products in all branches of the food industry, including their interactions with other food components;and toxicological, physiological and metabolic studies of hydrocolloids.

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