AMANDA MARIA TEIXEIRA LAGO

ULTRASOUND-ASSISTED OIL-IN-WATER NANOEMULSION PRODUCED USING *Pereskia aculeata* Miller MUCILAGE

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, área de concentração em Ciência dos Alimentos, para a obtenção do título de Doutor.

Prof. Dr. Jaime Vilela de Resende
Orientador

Prof. Dr. Diego Alvarenga Botrel
Coorientador

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PRODUÇÃO DE NANOEMULSÃO ÓLEO-EM-ÁGUA USANDO MUCILAGEM DE *Pereskia aculeata* Miller ASSISTIDA POR ULTRASSOM

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, área de concentração em Ciência dos Alimentos, para a obtenção do título de Doutor.

APROVADA em 18 de abril de 2018.

Dr. Diego Alvarenga Botrel	UFLA
Dr. Fabiano Freire Costa	UFJF
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LAVRAS – MG
2018

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RESUMO

Cada vez mais há um considerável interesse por parte da indústria alimentícia, farmacêutica, agroquímica e outros segmentos pela utilização de dispersões coloidais para encapsular, proteger e distribuir componentes lipofílicos bioativos. Em certas aplicações, é desejável utilizar dispersões coloidais contendo gotículas bem menores, uma vez que apresentam vantagens potenciais em relação aos sistemas convencionais. Para o preparo de nanoemulsões, a escolha correta de emulsificantes, em conjunto com os métodos de emulsificação, influencia diretamente a qualidade do produto final. O presente estudo relata a preparação de nanoemulsões óleo-em-água empregando mucilagem de ora-pro-nóbis (OPN), emulsificante de origem vegetal, por meio da aplicação da técnica de ultrassom. Para tanto foi necessário otimizar a faixa de concentração da mucilagem (%) e óleo de soja (%), bem como os parâmetros operacionais do processo, amplitude (%) e tempo (min) da agitação mecânica do equipamento, com base no diâmetro médio das gotículas (d_{32}). O efeito da concentração da mucilagem e do óleo também foi verificado por meio das variáveis respostas polidispersão, densidade, turbidez, viscosidade, potencial zeta e tensão interfacial. O aumento da concentração de OPN (%), juntamente com menores quantidades de óleo de soja (%), favoreceu a formação das nanoemulsões ($116 \leq d_{32} \leq 171$ nm) e aumentou a polidispersão, a densidade e o potencial zeta. Já a turbidez dos tratamentos aumentou à medida que maiores porcentagens de mucilagem de OPN e de óleo de soja foram utilizadas. Todos os sistemas coloidais apresentaram comportamento Newtoniano e a viscosidade do meio aumentou em função do acréscimo na concentração da mucilagem de OPN na fase aquosa, em uma determinada concentração de óleo. Em adição, foram encontrados menores valores de tensão interfacial de equilíbrio com o aumento da concentração da mucilagem de OPN. Avaliações de marcação de compostos utilizando fluorocromos também foram realizadas, o que favoreceu a análise qualitativa das microestruturas dos sistemas e evidenciou a adsorção das moléculas de polissacarídeos e de proteínas na interface óleo-água. Por fim, a partir do teste de estabilidade, pode ser apontado que a concentração da mucilagem de OPN deve estar entre 1.0 a 1.5% e a concentração de óleo deve ser menor que 5%, para que sejam mantidos menores valores de d_{32} ao longo do tempo. Portanto, emulsões podem ser produzidas em escala nanométrica, pela utilização de mucilagem de OPN e por meio da aplicação da técnica de ultrassonicação, para o potencial uso no setor alimentício.

Palavras-chave: Emulsificante natural. Sistemas coloidais. Ultrassonicação.

ABSTRACT

There is considerable interest from the food industry, pharmaceutical, agrochemical and other segments for the use of colloidal dispersions to encapsulate, protect, and deliver lipophilic bioactive components. In certain applications, it is desirable to use colloidal dispersions containing much smaller droplets, because they have many potential advantages over conventional systems. For the preparation of nanoemulsions, the correct choice of emulsifiers, together with the emulsification methods, directly influences the final product quality. The present study reports the ultrasound-assisted preparation of oil-in-water nanoemulsions produced with mucilage extracted from leaves of *Pereskia aculeata* Miller (*ora-pro-nobis*; OPN). The OPN mucilage (%) and soybean oil (%) concentration range, and the process operating parameters, ultrasonic power amplitude (%) and sonication time (min), were optimized based on the mean droplet diameter (d_{32}). The effect of the mucilage and oil concentrations was also investigated by the response variables such as polydispersity, density, turbidity, viscosity, zeta-potential, and interfacial tension. The higher OPN mucilage concentrations (%) with lower amounts of soybean oil (%) favored nanoemulsion formations ($116 \leq d_{32} \leq 171$ nm) and increased polydispersity, density, and zeta-potential. On increasing OPN mucilage and soybean oil the turbidity of the dispersions increased. All colloidal systems showed Newtonian behavior, and the viscosity in the systems increased due to the greater OPN mucilage concentration in the aqueous phase at a certain oil concentration. In addition, lower values of equilibrium interfacial tension were found with increasing OPN mucilage concentrations. Qualitative analysis of system microstructures was also performed via compound labeling using fluorochromes, which evidenced the adsorption of polysaccharide and protein molecules at the oil-water interface. Finally, from the stability test, it can be pointed out that the OPN mucilage concentration should be between 1.0 and 1.5% and the oil concentration should be less than 5%, so that lower d_{32} values are maintained over time. Therefore, mucilage extracted from OPN and the ultrasound technique can be used in the preparation of nanoemulsions.

Keywords: Natural emulsifier. Colloidal systems. Ultrasonication.

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PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

Em um cenário de constante competitividade do setor alimentício, onde a concorrência é intensa e a inovação é vital, a área de nanotecnologia tem surgido como um auxílio potencial na produção de melhorias da qualidade de alimentos com propriedades funcionalizadas. Em consequência, existe um interesse acentuado por parte da indústria de alimentos e da comunidade científica na avaliação das possibilidades da sua aplicação em produtos e/ou processos. Os avanços no conhecimento sobre obtenção e estabilidade dos sistemas coloidais viabilizam o desenvolvimento de materiais diferenciados como, por exemplo, nanoemulsões, que além da inerente estabilidade cinética, apresentam facilidade de preparo e alto poder de dispersão, devido ao tamanho reduzido das gotículas.

A crescente exigência do consumidor por alimentos e bebidas inócuos requer sistemas de distribuições baseados em emulsão estabilizados, por emulsificantes naturais em substituição aos sintéticos. A grande maioria dos emulsificantes naturais empregados na indústria alimentícia brasileira é oriunda de produtos importados, apesar de o país possuir plantas com alto potencial de produção de hidrocoloides. Inúmeras espécies de cactos, com destaque para a *Pereskia aculeata* Miller, conhecida popularmente como ora-pro-nóbis, produzem grandes quantidades de mucilagem, com alta capacidade de absorção de água, o que lhes conferem excelentes perspectivas para uso como hidrocoloides em alimentos processados, devido às suas propriedades estabilizantes e emulsificantes.

As nanoemulsões, por serem sistemas termodinamicamente instáveis, necessitam de energia externa em quantidade suficiente para romper a interface óleo-água e, assim, reduzir o tamanho das gotículas do meio disperso durante o processo de emulsificação. Atualmente, métodos de alta energia, por serem considerados mais eficientes em operações industriais de alimentos, têm sido mais utilizados para a obtenção de nanoemulsões. Dentre estas abordagens, o uso de homogeneizadores ultrassônicos, que se baseiam na técnica de cavitação intensa, demonstram ser adequados para a formação de nanoemulsão óleo-em-água com tamanhos de gotículas inferiores e com melhor estabilidade.

Com base neste contexto, é razoável lançar a hipótese de que o uso de energia ultrassônica possa ser uma técnica eficiente para o desenvolvimento de nanoemulsões óleo-em-água preparadas com mucilagem de ora-pro-nóbis na fase aquosa e óleo de soja na fase dispersa. Com este propósito, objetivou-se com o presente estudo preparar nanoemulsões

óleo-em-água (O/A) empregando mucilagem de ora-pro-nóbis, emulsificante de origem vegetal, por meio da aplicação da técnica de ultrassom. Estudar a influência da concentração da mucilagem (%) e do óleo de soja (%) sobre o diâmetro médio das gotículas, assim como otimizar os parâmetros operacionais do processo, tempo (min) e amplitude (%) da agitação mecânica do equipamento na preparação das nanoemulsões. Em adição, potencializar o processo de obtenção das nanoemulsões fundamentado nos melhores desempenhos encontrados a partir das análises de determinação do diâmetro médio e distribuição do tamanho das gotículas, densidade, turbidez, comportamento reológico, potencial zeta, tensão interfacial e morfologia. Por fim, avaliar a estabilidade das nanoemulsões ao longo do tempo de armazenamento por meio do acompanhamento do diâmetro médio das gotículas e índice de cremeação (formação de creme no sistema coloidal).

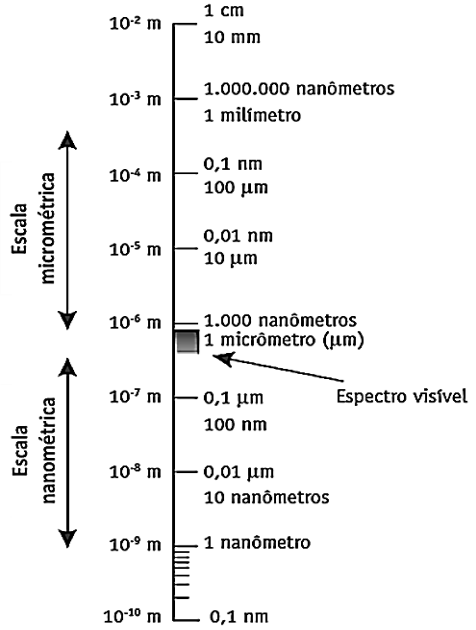
2 REFERENCIAL TEÓRICO

2.1 Nanociência e Nanotecnologia

Pesquisas na área de nanociência e nanotecnologia estão em crescimento acelerado, rápida evolução e inovação industrial com muitas potencialidades, sendo estes termos, atualmente, os mais amplamente empregados na literatura científica e na tecnologia moderna (DUDEFOI et al., 2017; DUMITRIU et al., 2018). Apesar de não existir distinção nítida entre eles, o termo "nanociência" é definido como "o estudo de fenômenos e manipulação de materiais em escalas atômicas, moleculares e macromoleculares, em que as propriedades diferem significativamente daquelas em escala maior". Já o termo "nanotecnologia" abrange uma vasta gama de ferramentas, técnicas e aplicações potenciais, e é referido como "o *design*, a caracterização, a produção e a aplicação de estruturas, dispositivos e sistemas, controlando a forma e o tamanho em escala nanométrica" (DOWLING et al., 2004).

O efeito impulsionador dos estudos em nanociência e nanotecnologia vem da reinvestigação dos campos científicos para melhorar o conhecimento e desenvolver novas ferramentas, que consideram a nanoescala como um nível relevante e aplicável (DUDEFOI et al., 2017). Em termos de dimensões, um nanômetro é um bilionésimo do metro ($1\ nm = 10^{-9}\ m$) e equivale a um milésimo do micrômetro (também conhecido como mícron), que, por sua vez, é um milésimo do milímetro (FIGURA 1).

Figura 1 – Representação gráfica da ordem de grandeza das estruturas em nanociência.



Fonte: Adaptada de Moore (2010).

A nanotecnologia é de relevância global e tem atraído mais financiamentos públicos que qualquer outro segmento de tecnologia, por ser uma área de pesquisa e desenvolvimento verdadeiramente multidisciplinar. É considerada uma disciplina emergente que aplica princípios da nanociência à criação de produtos e proporciona um enfoque novo ao processo produtivo, de tal forma que, a omissão em responder a esse desafio ameaçará a competitividade futura de muitas organizações e empresas (MOORE, 2010). Os nanomateriais ganharam importância em vários campos da ciência, tecnologia, medicina, tecnologias coloidais, diagnósticos, distribuição e liberação de medicamentos, com aplicações em cuidados pessoais, engenharia de tecidos, engenharia biomédica, entre outros, devido ao seu pequeno tamanho e características físico-químicas únicas (RANJAN et al., 2014).

Adicionalmente, apresenta uso crescente em produtos alimentícios e bebidas com propósito de alterar as suas características, o perfil de sabor, a aparência, as propriedades reológicas, a estabilidade e a processabilidade (JOYE; MCCLEMENTS, 2014), em razão de ser uma tecnologia chave que promove a inovação de produtos (KABRI et al., 2011). Esta ciência apresenta impacto sobre todas as fases de produção de alimentos, iniciando na agricultura, depois, no processamento (onde a criação de emulsões e encapsulamento evoluíram para a nanoescala) e no produto final (onde a área de embalagem aprimorou por meio da incorporação de nanopartículas) (CUSHEN et al., 2012). O campo da nanotecnologia é projetado para impactar a indústria agroalimentar e os consumidores, no que se refere aos métodos mais eficientes de produção, ao desenvolvimento de alimentos funcionais que ofereçam alegações de saúde, ao processamento de alimentos mais higiênicos e sua segurança, ao maior prazo de validade e à melhor rastreabilidade dos produtos alimentícios (HANDFORD et al., 2015; RANJAN et al., 2014).

Com a crescente demanda por produtos alimentares mais saudáveis e seguros, mais atenção tem sido dada à utilização da nanotecnologia para encapsular, proteger e fornecer compostos funcionais. As abordagens atuais estão considerando estritamente essas preocupações ao projetar soluções nanotecnológicas para os setores alimentícios. Em particular, as nanoemulsões apresentam uma grande capacidade de encapsular, proteger e liberar agentes bioativos, modificar as propriedades reológicas, ópticas e de estabilidade do material e alterar o destino gastrointestinal das substâncias encapsuladas, em comparação com as emulsões convencionais (MCCLEMENTS; JAFARI, 2018; MCCLEMENTS; XIAO, 2012; PATHAKOTI; MANUBOLU; HWANG, 2017).

2.2 Nanoemulsão

Nanoemulsões podem ser definidas como sistemas heterogêneos em que um líquido, na presença de um agente emulsificante, é disperso em outro na forma de pequenas gotículas (MCCLEMENTS, 2011; MCCLEMENTS; RAO, 2011). Comercialmente, as nanoemulsões são comumente utilizadas para encapsular componentes lipofílicos dispersos em meio aquoso e, portanto, sua abordagem limita-se, geralmente, em sistemas óleo-em-água (O/A), ainda que seja possível a preparação desses sistemas do tipo água-em-óleo (A/O) (JIN et al., 2016). Em comparação com as emulsões convencionais, as nanoemulsões possuem tamanhos relativamente pequenos de gotículas, no intervalo de 20 a 200 nm de diâmetro (HUANG; YU; RU, 2010). Usualmente têm melhor estabilidade à agregação de partículas e à separação gravitacional, floculação e coalescência, apresentam elevada área superficial (TADROS et al., 2004), aparência opticamente translúcida, parâmetros reológicos ajustáveis (GUPTA et al., 2016), elevada eficiência de encapsulação, alta biodisponibilidade e em sua preparação requer menor quantidade de emulsificantes (AHMED et al., 2018; QIAN; MCCLEMENTS, 2011).

Uma nanoemulsão é um sistema de não-equilíbrio (ou seja, termodinamicamente instável) e, portanto, não pode ser formada espontaneamente. Como consequência, as nanoemulsões requerem certa quantidade de energia para auxiliar o processo de emulsificação, quebrando assim a interface e reduzindo o tamanho da gota de emulsão (SHANMUGAM; ASHOKKUMAR, 2014). Interface é a região do espaço compreendida entre as duas fases imiscíveis, onde pode ser observada variação das propriedades termodinâmicas intensivas verticalmente. Tal comportamento pode ser explicado a partir do excesso de energia livre de *Gibbs* (ΔG) por unidade de área existente nesta região, visto que surge uma tensão interfacial. Para as nanoemulsões, a energia livre da dispersão coloidal (gotículas em água) é mais elevada do que a energia livre das fases separadas (óleo e água), o que proporciona sua instabilidade termodinâmica. Deste modo, as nanoemulsões dependem da estabilidade cinética para se manterem estáveis por um longo período, quanto maior a barreira de energia para a separação de fase, maior a vida útil deste sistema coloidal. A taxa, cuja nanoemulsão reverte para as fases separadas, é determinada pela frequência com que as gotículas de óleo entram em contato umas com as outras, o que depende dos mecanismos, principais responsáveis pelos contatos entre gotículas, tais como movimento Browniano, tensão aplicada ou forças gravitacionais (MCCLEMENTS, 2012, 2015).

Em síntese, o processo de formação de uma nanoemulsão, requer, em geral, quatro componentes: (a) fase oleosa (b) fase aquosa (c) emulsificante e (d) energia (SIVAKUMAR;

TANG; TAN, 2014). A fase oleosa utilizada para formular uma nanoemulsão pode ser composta de vários componentes hidrofóbicos, incluindo triglicerídeos, óleos essenciais, óleos aromatizantes, vitaminas solúveis em óleo, nutracêuticos e fármacos. As fases oleosas podem, portanto, diferir em suas propriedades físico-químicas, tais como viscosidades, polaridade, índice de refração, ponto de fusão, tensão interfacial, que alteram sua capacidade de formar e estabilizar nanoemulsões (MCCLEMENTS; XIAO, 2012; SHAH; BHALODIA; SHELAT, 2010). Na indústria alimentar, os triglicerídeos são frequentemente desejáveis como fase oleosa devido à sua fonte de matéria-prima de baixo custo, não tóxica e abundante, incluindo óleo de soja, de girassol, de milho e de canola. Como exemplo de complexidade, a polaridade do óleo vegetal é geralmente muito baixa, mas a viscosidade é relativamente alta devido à presença de triacilglicerol de cadeia longa ou média. Portanto, geralmente é difícil preparar nanoemulsões com esses óleos usando métodos de temperatura de inversão de fase e homogeneização de alta pressão (JIN et al., 2016).

A fase aquosa é tipicamente composta pela água, contudo, pode também conter outros componentes que desempenham um papel importante, tais como tampões, sais, cossolventes, conservantes, proteínas e polissacarídeos. Assim, as fases aquosas diferem consideravelmente nas suas propriedades físico-químicas e fisiológicas, visto que alguns solutos em água alteram a viscosidade, o pH e a força iônica ou a polaridade da água, afetando a tensão interfacial e o comportamento de fase dos sistemas de emulsão. As nanoemulsões, geralmente, não podem ser formadas simplesmente homogeneizando um óleo e uma fase aquosa, em razão do sistema se separar rapidamente em suas fases componentes (LEE; MCCLEMENTS, 2010; MCCLEMENTS, 2015). Em vez disso, um número de ingredientes adicionais, muitas vezes referidos como emulsificantes, é tipicamente necessário para facilitar a formação e assegurar a estabilidade em longo prazo da nanoemulsão. Os emulsionantes variam consideravelmente em sua capacidade de formar e estabilizar a nanoemulsão, e é extremamente importante identificar o mais adequado para uma determinada aplicação (MCCLEMENTS; JAFARI, 2018). A adição de um emulsificante é crítica para a criação de gotículas de pequeno tamanho ao diminuir a tensão interfacial (GUPTA et al., 2016).

Consideráveis avanços foram alcançados na compreensão sobre os métodos que podem ser utilizados para elaborar as nanoemulsões, que podem ser fabricadas por meio de várias abordagens, mas geralmente são categorizadas como abordagens de alta energia ou baixa energia (ANTON; BENOIT; SAULNIER, 2008; LEONG et al., 2009; MCCLEMENTS, 2015). Abordagens de alta energia utilizam dispositivos mecânicos (homogeneizadores) que geram forças disruptivas intensas capazes de romper e misturar as

fases oleosa e aquosa em minúsculas gotículas de óleo, como exemplo, microfluidificadores, homogeneizadores de válvulas de alta pressão e homogeneizadores ultrassônicos (KENTISH et al., 2008; MCCLEMENTS; RAO, 2011; WOOSTER; GOLDING; SANGUANSRI, 2008). Atualmente, esta abordagem é o método mais eficiente para preparar nanoemulsões em operações industriais de alimentos (HAKANSSON; RAYNER, 2018). Já as abordagens de baixa energia baseiam-se na formação espontânea de minúsculas gotículas de óleo dentro de um sistema misturador de óleo-água-emulsificante, quando a solução ou as condições ambientais são alteradas, como exemplo, métodos de inversão de fase e emulsificação espontânea (OSTERTAG; WEISS; MCCLEMENTS, 2012; TADROS et al., 2004).

O projeto, a fabricação e o teste de nanoemulsões requerem conhecimento de sua composição, estrutura e propriedades e, portanto, ferramentas analíticas apropriadas são necessárias para fornecer informações sobre as características de gotículas (como concentração, estado de agregação de tamanho, carga e estado físico) e as propriedades físicas (tais como aparência, propriedades reológicas e estabilidade). Uma ampla variedade de ferramentas analíticas foi desenvolvida para caracterizar as propriedades das nanoemulsões: (I) a distribuição de tamanho e o diâmetro médio da gotícula são tipicamente determinados usando métodos dinâmicos ou de espalhamento de luz estática; (II) as características da carga de partículas são comumente medidas usando métodos de microeletroforese; (III) a estatística de agregação e a morfologia das nanoemulsões são frequentemente determinadas usando microscopia óptica ou de força atômica; (IV) o estado físico das gotículas em nanoemulsões pode ser convenientemente determinado utilizando calorimetria diferencial de varredura ou modos de espalhamento por raio-X; e (V) e as propriedades físicas, como turbidez (grau de transparência do meio) e comportamento reológico (viscosidade do meio) utilizando espectrofotômetro e reômetro, respectivamente (MCCLEMENTS; MCCLEMENTS, 2016).

O pequeno tamanho e a alta área superficial das gotículas das nanoemulsões O/A conferem-lhes uma série de caracterizações de propriedades físico-químicas e fisiológicas que as tornam altamente adequadas para determinadas aplicações. A aparência do produto pode ser controlada alterando o tamanho da gota de óleo. Para aplicação em que o produto final é turvo ou opaco, nanoemulsões com gotículas com diâmetro entre 100 e 200 nm podem ser utilizadas. Por outro lado, para aplicações nas quais o produto final deve ser opticamente transparente, nanoemulsões com gotículas com diâmetro menor que 50 nm devem ser usadas. Outra vantagem do uso de nanoemulsões, para a aplicação em alimentos, é que elas tendem a ser altamente estáveis à separação e agregação gravitacional, devido ao pequeno tamanho das gotículas, aumentando consideravelmente a estabilidade do produto. As nanoemulsões são,

também, particularmente adequadas para aumentar a biodisponibilidade oral de agentes bioativos hidrofóbicos (MCCLEMENTS; JAFARI, 2018). As características físico-químicas e as eventuais aplicações das nanoemulsões dependem da seleção dos ingredientes, principalmente no que se refere ao agente emulsificante, para a sua formulação, bem como dos parâmetros operacionais do processo de emulsificação (MCCLEMENTS, 2015).

2.3 Hidrocoloides

A seleção de um emulsionante (ou a combinação de emulsionantes) é um dos fatores mais importantes a ser considerado no preparo adequado de uma nanoemulsão. O agente emulsificante é uma molécula de superfície ativa capaz de se adsorver na interface óleo-água, o que facilita a ruptura das gotículas, devido à redução da tensão interfacial, e evita a desestabilização do sistema coloidal, em razão das forças repulsivas estéricas ou eletrostáticas geradas entre as gotículas (BAI et al., 2016; MCCLEMENTS; JAFARI, 2018; WOOSTER et al., 2016). Um grande número de emulsionantes está disponível para a formulação de emulsões e nanoemulsões, como polissacarídeos, proteínas, fosfolipídios, extratos naturais e surfactantes sintéticos (GUERRA-ROSAS et al., 2016; MIRHOSSEINI et al., 2008).

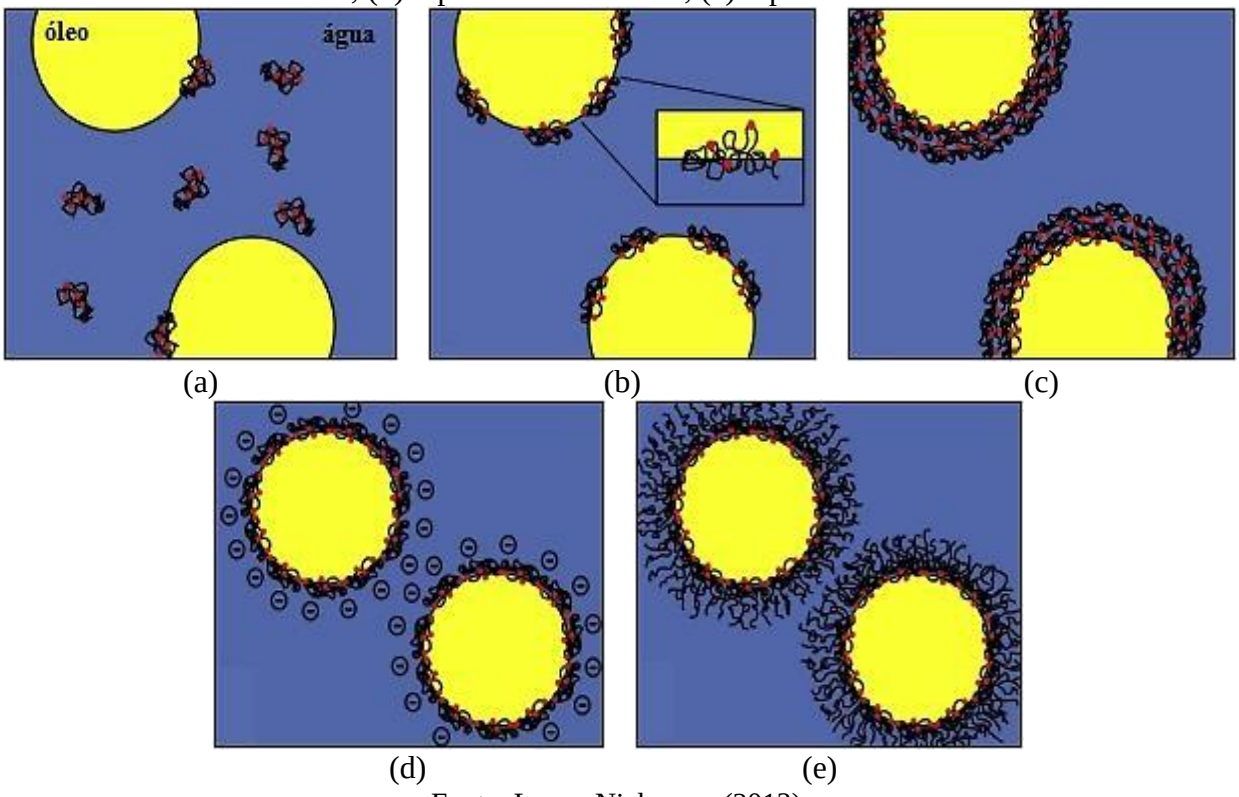
A maioria dos estudos realizados se concentra na utilização dos agentes tensoativos sintéticos e de baixa massa molecular, como os *Tweens* e *Spans*, para o preparo de nanoemulsões, devido à sua excelente difusividade interfacial, em comparação com grandes biopolímeros, como proteínas e polissacarídeos (GHOSH et al., 2013; QIAN; MCCLEMENTS, 2011). No entanto, preocupações sobre a segurança, toxicidade e metabolismo destes emulsionantes sintéticos no corpo humano limitam sua aplicação aos sistemas alimentares (ADJONU et al., 2014). Recentemente, os consumidores vêm exigindo produtos que contenham ingredientes "*label-friendly*", isto é, substâncias naturais de alta qualidade, seguras e inócuas à saúde. Em consequência, identificar, desenvolver e utilizar emulsificadores naturais, que podem ser empregados com sucesso em alimentos e bebidas a base de emulsão, para substituir alternativas sintéticas, tem atraído atenção considerável (OZTURK; MCCLEMENTS, 2016; YANG; MCCLEMENTS, 2013).

Os emulsionantes à base de biopolímeros naturais, como os hidrocoloides (proteínas e polissacarídeos), têm se mostrado eficientes agentes de superfície ativa de alto peso molecular para formar e estabilizar nanoemulsões óleo-em-água (TEO et al., 2016). O termo hidrocoloides refere-se a um grupo heterogêneo de polímeros de cadeia longa, caracterizado pela sua propriedade de formar dispersões viscosas e/ou géis quando dispersos em água

(ABDUL KHALIL et al., 2018; SAHA; BHATTACHARYA, 2010). Os hidrocoloides de origem vegetal são encontrados nas plantas superiores, obtidos de exsudados, sementes, folhas, frutos e tubérculos. Essas biomoléculas encontradas em espécies vegetais também são conhecidas como gomas ou mucilagens (CUNHA; PAULA; FEITOSA, 2009).

Os mecanismos de estabilização interfacial dos hidrocoloides são explicados pela presença de segmentos proteicos combinados ou fisicamente misturados aos polissacarídeos Estes agrupamentos proteicos são de particular interesse em termos de suas propriedades emulsificantes, devido à presença de grupos hidrofóbicos e hidrofílicos e à habilidade de formação de um filme ao redor das gotículas dos sistemas coloidais (FIGURA 2) (LAM; NICKERSON, 2013).

Figura 2 – Representação do processo de estabilização interfacial dos hidrocoloides em nanoemulsão óleo-em-água: (a) migração das proteínas para a interface óleo/água; (b) reorientação das proteínas na interface; (c) formação de um filme viscoelástico; (d) repulsão eletrostática; (e) repulsão estérica.



Fonte: Lam e Nickerson (2013).

Ao contrário dos emulsificadores de pequena massa molecular, como os surfactantes sintéticos, que se difundem rapidamente na interface para dar excelentes capacidades de formação de emulsão, as proteínas tendem a ser mais volumosas e difusas a uma taxa muito mais lenta (FIGURA 2a). Quando na interface, as proteínas se realinham para posicionar seus

aminoácidos hidrofóbicos superficiais dentro da fase oleosa e os aminoácidos hidrofílicos dentro da fase aquosa (FIGURA 2b). Desta forma, podem ser desenvolvidos filmes viscoelásticos fortes e coesos (FIGURA 2c) que resistem a tensões mecânicas e fornecem estabilização eletrostática (FIGURA 2d), dependendo das condições do solvente, e estérica, dependendo da proteína (FIGURA 2e) (MCCLEMENTS, 2015).

Existe, ainda, grande interesse na produção de nanoemulsões com hidrocoloides, uma vez que os já existentes no mercado não são suficientemente eficazes na formação de nanoemulsões finas (JIN et al., 2016). Portanto, a busca por novas fontes de hidrocoloides alimentares com funcionalidade específica é uma área de pesquisa ativa (ZAMENI et al., 2015). No Brasil, apesar de existirem plantas nativas que apresentam alto potencial de produção, grande parte dos hidrocoloides utilizados em aplicações alimentares são provenientes de produtos importados, o que demanda grande aporte financeiro. Propostas de pesquisas com cactáceas, como possíveis fontes de mucilagens, têm sido requeridas devido à presença de grandes quantidades de polissacarídeos e de proteínas em sua estrutura (LIMA JUNIOR et al., 2013; MARTIN et al., 2017).

2.3.1 Mucilagem de ora-pro-nóbis

Ora-pro-nóbis (OPN), cientificaamente denominada de *Pereskia aculeata* Miller, é uma planta nativa, com característica de trepadeira, pertencente à família das Cactáceas (TAKEITI et al., 2009). Possuem flores pequenas e brancas, pequenos frutos amarelos e caules finos que podem alcançar até dez metros de altura, lenhosos ou sublenhosos, nos quais se inserem acúleos e folhas verdes escuras, largas, lisas e suculentas (FIGURA 3) (BRASIL, 2010; DUARTE; HAYASHI, 2005). Estas folhas não apresentam propriedades tóxicas e, ainda, possuem rico conteúdo nutricional, com elevados teores de ferro, zinco, manganês, cálcio, fósforo, magnésio, vitaminas A e C, ácido fólico e, principalmente, elevados teores de proteínas – 25% na matéria seca com 85% de digestabilidade – e de aminoácidos essenciais (TAKEITI et al., 2009). A OPN, devido a sua alta rusticidade, propaga-se em ambientes com baixo teor de água e nutrientes, possuindo ampla distribuição pelo Brasil e alto potencial de utilização (MARTINEVSKI et al., 2013).

Figura 3 – Ora-pro-nóbis (*Pereskia aculeata* Miller).

Fonte: Duarte e Hayashi (2005).

As plantas da família das Cactáceas produzem grandes quantidades de mucilagem, um carboidrato complexo com alta capacidade de absorção em água, que lhe confere excelentes perspectivas para uso como hidrocoloides em alimentos e bebidas (PETERA et al., 2015). As mucilagens de várias espécies de cactos, como a OPN, apresentam em suas folhas um arranjo de polissacarídeos rico em arabinogalactana com ramificações constituídas por galactose, arabinose, ramnose e ácido galacturônico, associado com proteínas (LIMA JUNIOR et al., 2013; MARTIN et al., 2017). Tal arranjo exerce um papel crucial na determinação das propriedades emulsificantes de hidrocoloides com alto valor comercial, como a goma arábica (LEE; MCCLEMENTS, 2010; YADAV et al., 2007).

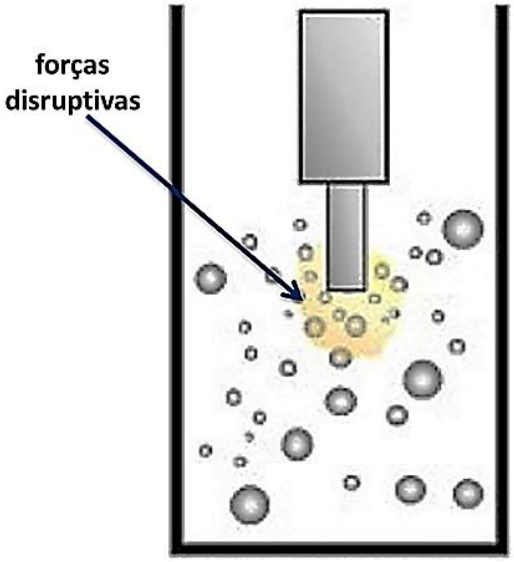
A goma arábica é um exsudato de ocorrência natural coletado de árvores de *Acacia senegal* e, em menor escala, de árvores de *Acacia seyal*. Considerada um dos aditivos industriais mais antigos e amplamente empregados nas indústrias alimentícia e farmacêutica, possui uma combinação apontada como única, de excelentes propriedades emulsificantes, estabilizantes e espessantes, que contribuiu para uma baixa viscosidade de soluções. Em adição, é classificada como a melhor goma em uso em sistemas diluídos de emulsão óleo-em-água, em que o componente proteico da goma arábica se incorpora no óleo, enquanto o componente carboidrato se estende para dentro da água (SAHA; BHATTACHARYA, 2010; YADAV et al., 2007). Poucas substâncias proteico-polissacarídicas apresentam mecanismos de emulsificação comparável, portanto, a busca por substitutos totais ou parciais para a goma arábica na indústria de alimentos tem sido cada vez mais incentivada e explorada (PORTO; CRISTIANINI, 2014).

Em estudos recentes, a mucilagem de OPN é apontada como uma fonte alternativa viável para aplicação em produtos alimentícios. Esta mucilagem apresenta um perfil macromolecular heterogêneo, com comportamento de polieletrólito, e potencial de uso como agente de emulsificação e de estabilização, em consequência das suas propriedades de adsorção interfacial (MARTIN et al., 2017). Revela alta capacidade da mucilagem em formar géis e emulsão, em virtude de um aumento linear da viscosidade do meio em função da concentração da mucilagem e sua alta higroscopicidade, e da estabilidade térmica da emulsão formada em diferentes temperaturas (CONCEIÇÃO et al., 2014; LIMA JUNIOR et al., 2013). Emulsões preparadas com maiores concentrações desta mucilagem apresentaram aumento no número e maior uniformidade no tamanho das gotas, além de estrutura morfológica estável (JUNQUEIRA et al., 2018). Em adição, a aplicação de misturas de hidrocoloides contendo a mucilagem de OPN, como componente majoritário, em bebidas lácteas fermentadas provou ser uma abordagem factível, visto que a mistura aumentou o teor proteico e a viscosidade do produto, bem como reduziu a sinérese (AMARAL et al., 2018a). A mucilagem assim obtida pode ser um material funcional alternativo natural e econômico para utilização em muitos processos industriais, em especial, no desenvolvimento de produtos alimentícios à base de emulsão (MONRROY et al., 2017).

2.4 Técnica de ultrassom

Para a obtenção de tamanhos ótimos das gotículas em nanoemulsões existe uma dependência não somente dos materiais utilizados na formulação, mas também da técnica de emulsificação ou dos métodos empregados para a sua obtenção (ANTON; BENOIT; SAULNIER, 2008; JIN et al., 2016). Dentre os diferentes equipamentos existentes, os homogeneizadores ultrassônicos, denominados também de sonicador, são os mais populares para a produção de nanoemulsões. Estes equipamentos utilizam ondas ultrassônicas, de alta intensidade, para criar as intensas forças disruptivas necessárias no rompimento das fases de óleo e água em gotículas muito pequenas. A entrada de energia é fornecida por meio de sondas sonicadoras, constituídas de cristais de quartzo piezoelétricos que se expandem e se contraem em resposta a uma tensão elétrica alternada. A ponta da sonda é inserida dentro do sistema coloidal, a ser homogeneizado, onde gera vibrações mecânicas intensas que levam a efeitos de cavitação, responsáveis pela formação, crescimento e colapso de pequenas bolhas no líquido e, consequentemente, ruptura das gotículas (FIGURA 4) (LEONG et al., 2009; MCCLEMENTS; RAO, 2011).

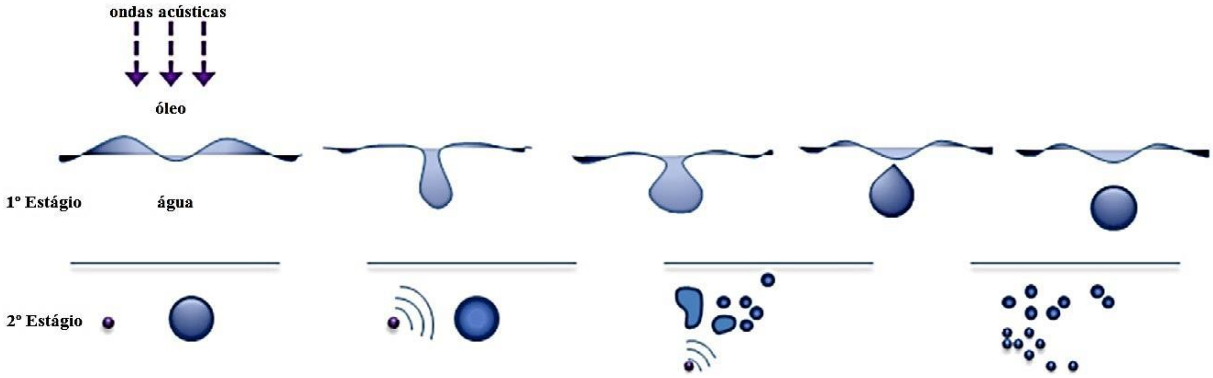
Figura 4 – Representação esquemática da técnica de ultrassom.



Fonte: McClements e Rao (2011).

O mecanismo de emulsificação por ultrassom pode ser dividido em dois estágios: (1) geração de gotículas primárias, onde o campo acústico produz as ondas interfaciais, cuja instabilidade causa a erupção da fase oleosa na fase aquosa na forma de gotículas; e (2) desmembramento de gotículas primárias, onde a cavitação acústica causa intensa turbulência localizada e forças de cisalhamento, responsáveis pela produção de bolhas que implodem, de forma violenta e assimétrica, e quebram ainda mais as gotículas primárias de óleo disperso, geradas no primeiro estágio, em gotículas de tamanhos nanométricos (FIGURA 5) (HAKANSSON; RAYNER, 2018).

Figura 5 – Emulsificação por ultrassom em dois estágios: formação e quebra das gotículas.



Fonte: Sivakumar, Tang e Tan (2014).

O primeiro estágio é controlado, principalmente, pelo tipo e quantidade de cisalhamento aplicado ao meio, e a resistência das gotículas à deformação é determinada pelo

tipo de emulsificante presente na fase aquosa. Já no segundo estágio, a taxa na qual o emulsificante é adsorvido na interface das gotículas recém-formadas é controlada pela atividade superficial do emulsificante e sua concentração. Assim, a seleção de emulsificante apropriado é de suma importância na produção de nanoemulsões com alta estabilidade (BORTHAKUR et al., 2016). A eficiência da homogeneização e o tamanho das gotículas produzidas também dependem da viscosidade das fases oleosa e aquosa, bem como da intensidade das ondas ultrassônicas e do tempo de permanência do sistema coloidal na zona de ruptura. Em contrapartida, há uma preocupação de que as altas intensidades locais envolvidas nos métodos de sonicação possam levar à desnaturação da proteína, despolimerização do polissacarídeo ou oxidação lipídica durante a homogeneização. Todos esses parâmetros devem, portanto, ser otimizados para produzir nanoemulsões contendo pequenas gotículas (SILVA et al., 2015; SIVAKUMAR; TANG; TAN, 2014).

Muitos estudos relataram que a técnica de ultrassom emergiu como uma excelente e poderosa ferramenta no processo de emulsificação em comparação com outros processos mecânicos, em termos de obtenção de um tamanho menor de gota e alta eficiência energética na geração de nanoemulsões. Adicionalmente, sob as mesmas condições, no caso de se produzir uma nanoemulsão com o diâmetro desejado, a quantidade necessária de agente emulsificante e o consumo de energia foram reduzidos e, em consequência, os custos operacionais foram consideravelmente baixos (HAKANSSON; RAYNER, 2018; HASHTJIN; ABBASI, 2015; MCCLEMENTS, 2015; PANIWNYK, 2017; SIVAKUMAR; TANG; TAN, 2014). No entanto, na área alimentícia ainda há muito a ser explorado em relação à aplicação prática desta técnica (CARPENTER; SAHARAN, 2017; LI; CHIANG, 2012).

3 CONCLUSÃO GERAL

As nanoemulsões óleo-em-água estão sendo cada vez mais investigadas para suas aplicações potenciais na área alimentícia, a fim de encapsular, proteger e liberar compostos bioativos e funcionais. Estes sistemas coloidais apresentam inúmeras vantagens em relação à emulsão convencional, devido à facilidade de preparo, tamanho reduzido de gotícula, alta estabilidade cinética e favorável relação superfície-volume. Emulsionantes desempenham um papel fundamental no desenvolvimento de nanoemulsões bem sucedidas, visto que facilitam a formação de pequenas gotículas durante a homogeneização, adsorvendo-se na interface óleo-água e reduzindo a tensão interfacial. Há um apelo crescente por produtos que contenham apenas ingredientes saudáveis, portanto, o uso de emulsificantes naturais em emulsões alimentares tem atraído considerável atenção. A mucilagem extraída de folhas de ora-pro-nóbis tem se mostrado uma fonte potencial para a aplicação industrial, por apresentar alta capacidade de formação de emulsões devido às suas propriedades como agente espessante, gelificante e/ou emulsificante. Além da escolha correta de emulsificantes para o preparo de nanoemulsões, os métodos de emulsificação influenciam diretamente a qualidade do produto final. O método de ultrassom tem sido apontado como uma técnica eficiente para a produção de nanoemulsões, pela obtenção de menores tamanhos de gotículas e pelo maior controle dos parâmetros operacionais do processo de produção. Em geral, a tecnologia de nanoemulsão tem aplicações potenciais nas indústrias de alimentos, no entanto, há muito a ser explorado quanto ao emprego em produtos alimentícios e ao uso de emulsificantes naturais e de técnicas de ultrassom para a sua obtenção.

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SEGUNDA PARTE – ARTIGO

ULTRASOUND-ASSISTED OIL-IN-WATER NANOEMULSION PRODUCED FROM *Pereskia aculeata* Miller MUCILAGE

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Abstract

For the preparation of nanoemulsions, the correct choice of emulsifiers, together with the emulsification methods, directly influences the final product quality. The present study reports the ultrasound-assisted preparation of oil-in-water nanoemulsions produced with mucilage extracted from leaves of *Pereskia aculeata* Miller (*ora-pro-nobis*; OPN). The OPN mucilage (%) and soybean oil (%) concentration range, and the process operating parameters, ultrasonic power amplitude (%) and sonication time (min), were optimized based on the mean droplet diameter (d_{32}). The effect of the mucilage and oil concentrations was also investigated by the response variables such as polydispersity, density, turbidity, viscosity, zeta-potential, and interfacial tension. The higher OPN mucilage concentrations (%) with lower amounts of soybean oil (%) favored nanoemulsion formations ($116 \leq d_{32} \leq 171$ nm) and increased polydispersity, density, and zeta-potential. On increasing OPN mucilage and soybean oil the turbidity of the dispersions increased. All colloidal systems showed Newtonian behavior, and the viscosity in the systems increased due to the greater OPN mucilage concentration in the aqueous phase at a certain oil concentration. In addition, lower values of equilibrium interfacial tension were found with increasing OPN mucilage concentrations. Finally, from the stability test, it can be pointed out that the OPN mucilage concentration should be between 1.0 and 1.5% and the oil concentration should be less than 5%, so that lower d_{32} values are maintained over time. Therefore, mucilage extracted from OPN and the ultrasound technique can be used in the preparation of nanoemulsions.

Keywords: Colloidal systems. Natural emulsifier. Ora-pro-nobis. Sonicator. Ultrasonication. Kinetic stability.

1. Introduction

Nanoemulsions have aroused great interest in the scientific community, as this technology has more potential advantages over conventional emulsions for specific applications in food and beverages [1]. Compared with emulsions, nanoemulsions have relatively small droplet sizes in the range from 20 to 200 nm in diameter [2]. Usually they have improved stability for particle aggregation and gravitational separation, flocculation and coalescence, present high surface area [3], an optically translucent appearance, adjustable rheological behavior [4], high encapsulation efficiency, high bioavailability, and in their preparation require a lower concentration of emulsifiers [5,6].

The basic nanoemulsion formulation can be divided into oil phase and aqueous phase containing emulsifier, which helps in the system modulation [1]. Thus, nanoemulsions are heterogeneous systems in which a liquid, in the presence of an emulsifying agent, is dispersed in another as small droplets [7]. The emulsifying agents are surfactant materials that adsorb at the contact interface of the immiscible phases and facilitate the production of small droplets, lowering the interfacial tension during homogenization [8]. A wide variety of emulsifiers is legally acceptable for use in the food industry, with a growing trend towards replacing synthetic emulsifiers with natural. As a result, there is greater focus on the identification of natural surfactants that can be used successfully in emulsified food products [9].

Several types of emulsifiers are accessible to form nanoemulsions, including small molecules, surfactants, proteins and hydrocolloids [5]. The term ‘hydrocolloid’ refers to a heterogeneous group of long chain polymers, characterized by their viscous dispersion or gel forming properties when diffused in water [10,11]. Hydrocolloids of vegetable origin are found in the upper plants obtained from exudates, seeds, leaves, fruits and tubers. These biomolecules found in vegetable species are also known as gums or mucilages [12]. The search for new sources of food hydrocolloids with specific functionality is an active area of research [13]. In Brazil, although there are native plants that present high production potential, most of the hydrocolloids used in food applications come from imported products, which demands great financial support [14].

Research proposals with cacti, as possible hydrocolloid source, have been required due to the presence of large amounts of polysaccharides and proteins in their structure. Among the Cactacea family, the species *Pereskia aculeata* Miller, popularly known in Portuguese as *ora-pro-nobis* (OPN), presents, in its leaves, arabinogalactan-rich polysaccharide arrangements made up of galactose, arabinose, rhamnose and galacturonic acid, associated with proteins

[14,15]. This arrangement plays a crucial role in determination of the emulsifying properties of hydrocolloids with high commercial value such as gum Arabic [16,17], which has an unique behavior. Few protein-polysaccharide substances have comparable emulsifying mechanisms, therefore the search for total or partial replacement of gum Arabic in the food industry is increasingly encouraged [18].

In recent studies, OPN mucilage has shown to be a viable alternative source for application in food products. It presents a heterogeneous macromolecular profile, with polyelectrolyte behavior, and potential use as an emulsifying and stabilizing agent, due to its interfacial adsorption properties [15]. It also reveals the high ability of the mucilage to form gels and emulsions, because of a linear increase of the system viscosity as a function of the mucilage concentration and its high hygroscopicity, and thermal stability of the emulsion formed at different temperatures [14,19]. Emulsions prepared with higher concentrations of this mucilage present an increase in the number of drops and greater droplet size uniformity, besides a stable morphological structure [20]. In addition, the application of hydrocolloid blends containing OPN mucilage, as the major component, in fermented milk beverages proved to be a feasible approach, since the mixture increased the protein content and viscosity of the product, as well as reduced syneresis [21]. In this context, it is possible to infer that the use of this mucilage to prepare nanoemulsions is a promising path to be investigated, in order to provide improvements in the stability of these colloidal systems, due to droplet and production cost reduction, by lower emulsifier usage and replacement of imported emulsifiers.

The optimum size of the nanoemulsion droplets depends not only on the materials used in the formulation, but also on the emulsification technique or the methods employed to obtain them [1,22]. Nanoemulsions can be considered as kinetically stable systems due to small droplets and long-term stability and as such, require input energy for their preparation. Nanoemulsions are prepared using several methods, which can be generally classified as low and high energy approaches. Low-energy methods, such as membrane emulsification, spontaneous emulsification and phase inversion, are commonly employed and rely on the natural formation of fine droplets when changing environmental conditions of the systems (oil/water-containing emulsifiers). High-energy approaches rely on different mechanical devices such as high-speed and high-pressure homogenizers, ultrasonics and microfluidizers, which can provide intensive energy to the system, resulting in the formation of small dispersed phase droplets [1,23].

High-energy approaches are pointed out as the most used methods because of their greater efficiency in the preparation of nanoemulsions in industrial food operations [22]. In

recent years, the interest in using the ultrasound technique to process liquid foods has been increasing [24], because the ultrasonic homogenizer (sonicator) uses ultrasound waves of high intensity to generate disruptive forces that transform immiscible solutions into small and stable droplets [25,26]. Compared to other devices the ultrasound prepares monodisperse and more stable nanoemulsions without the need for high amounts of emulsifiers, presents low operational cost and is also efficient in reducing droplet size. In the food area there is still much to be explored regarding the practical application of this device [27–29].

The formation and droplet size are mainly dependent on the ultrasonic power amplitude and sonication time applied. The resistance to droplets deformation is also dependent on the emulsifier surface activity and its concentration in the continuous phase [30,31]. For this purpose, the aim of this study was to prepare oil-in-water (O/W) nanoemulsions using OPN mucilage (natural emulsifier) by the ultrasound technique; as well as to verify the influence of the OPN mucilage (%) and soybean oil (%) concentrations on the mean droplet diameter, and to optimize the process operating parameters, ultrasonic power amplitude (%) and sonication time (min).

2. Material and methods

2.1. Material

Nanoemulsions were prepared using commercial soybean oil (Liza, Cargill Agrícola S/A, Uberlândia, Brazil), freshly-prepared ultra-pure water (OR Prolab, WS Proaqua, Brazil) and OPN mucilage obtained by a process adapted from Lima Júnior et al. [14]. The chemical reagents used in the experiments presented analytical grade: sodium phosphate dibasic (99%), sodium phosphate monobasic (98%) and ethanol (99.8%) (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil); dimethyl sulfoxide (99.9%, DMSO ACS), Nile Red (N3013-100MG), and fluorescein isothiocyanate isomer I (90%, F2502-250MG) (Sigma-Aldrich Co. LLC, Saint Louis, USA); and rhodamine B (90%, CAS [81-88-9]) (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil).

2.2. Extraction of OPN mucilage

Fresh OPN leaves were collected (Lavras, MG, Brazil), packed in polyethylene bags and stored under freezing (−18 °C). The production of the OPN extract was made from the

homogenization of 1 kg of leaves with 2.5 L of boiling water in an industrial blender (Metvisa, LG10, São Paulo, SP, Brazil) for 10 min. The resulting extract was transferred to beakers and kept at 75 °C in a thermostatic water bath (Quimis, 215-2 q, São Paulo, SP, Brazil) for 6 h. Subsequently, this extract was filtered in organza fabric and the obtained liquid was subjected to vacuum filtration using a Buchner funnel (Primar, MC 1284, Itu, SP, Brazil). The filter element was a layer of three organza cloths. Finally, the filtrate was centrifuged in a refrigerated centrifuge (SP Labor, SP-701, Presidente Prudente, SP, Brazil) (7 min, 6000 rpm, 4677 × g) for maximal fiber removal, and ethanol was added to the supernatant at a ratio of 1:3 (extract:alcohol). The precipitate was subjected to freezing in an ultra-freezer with a static air system (Coldlab, CL120-86V, Piracicaba, SP, Brazil) at −75 °C, and freeze-dried (Edwards High Vacuum, L4KR, São Paulo, SP, Brazil) at −40 °C and a vacuum pressure of 0.998 mbar. The powdered products were hermetically packed and stored in vacuum desiccators for use in subsequent steps as a natural emulsifier derived from OPN leaves for the production of nanoemulsions.

2.3. Experimental design

Firstly, for the optimization of the parameters used in the process of obtaining the O/W emulsions, soybean oil percentage (dispersed phase) was set at 10%, and three independent variables were studied: natural emulsifier concentration (0.5%, 1.0%, 2.0%, and 3.0%), sonication time (5, 10, and 15 min) and ultrasonic power amplitude (50, 70, and 90%). Thus, a full factorial design ($3^2 \times 4$) was used totaling 36 treatments, studied in 3 replicates at each point to calculate the error and standard deviation (SD) [32].

The minimum and maximum limits for the concentration of the natural emulsifier (%) were established because of the results presented by Junqueira et al. [20] for emulsions prepared with OPN mucilage. The limits for homogenizing equipment, time (min) and power amplitude (%), were defined from the evaluation of the critical parameters for the preparation of nanoemulsions described by Shahavi et al. [33]; and the fixed value of the oil concentration was determined from research carried out with nanoemulsions containing natural emulsifiers, as shown by Bai and McClements [34]. The emulsions produced according to each treatment within the experimental design were evaluated for mean droplet diameter (d_{32}), and from these results the optimum power amplitude and time were selected to be used in the sonicator, as well as the of OPN mucilage concentration range to obtain smaller droplet sizes.

After optimization of the operating parameters, sonication time (5 min) and ultrasonic power amplitude (90%), a new full factorial design (3 × 5) was tested totaling 15 treatments studied in 3 replicates. At this stage, different OPN mucilage concentrations (0.5%, 1.0% and 1.5%) and soybean oil (1.0%, 2.5%, 5.0%, 7.5% and 10.0%) were evaluated in the preparation of the nanoemulsions. The treatments were subjected to analysis of d_{32}, polydispersity (Span), density, turbidity, flow curve, zeta-potential, morphological and interfacial tension. Samples that showed oil droplet sizes at the nano scale (d_{32} < 200 nm) were evaluated for their stability over time.

2.4. Preparation of emulsions and nanoemulsions

The continuous phase of the emulsions and nanoemulsions was prepared by dissolving OPN mucilage in sodium phosphate buffer (pH 7.0) under heating (80 °C) and constant magnetic stirring (Diagtech, DT3120H, São Paulo, SP, Brazil) for 1 h. After this period, the mixture was allowed to stand overnight to ensure complete solubilization and, in sequence, the pre-homogenization of the continuous and dispersed phases was performed in a Turratec homogenizer (Tecnal, TE-102, Piracicaba, SP, Brazil) operating at 15000 rpm for 5 min. To obtain the emulsified systems an ultrasonic processor (Sonifier Cell Disruptor Branson, 450D, Manchester, UK) was used, operating at 20 kHz with a maximum power output of 400 W, according to the treatments defined in the experimental design.

2.5. Mean droplet diameter and size distribution

The polydispersity and mean droplet diameter of emulsions and nanoemulsions were measured using a laser diffraction technique (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK) at 25 °C. The refractive indices of the soybean oil and aqueous phase used were 1.481 and 1.33, respectively. The droplet diameter of each sample was represented as the surface-weighted mean diameter (d_{32}), and the polydispersity (also known as the Span) was calculated from Eq. (1).

$$Span = \frac{(d_{90} - d_{10})}{d_{50}} \quad (1)$$

Where d_{10}, d_{50}, and d_{90} are droplet diameters (μm) at 10, 50, and 90% cumulative volume, respectively [34,35]. Five measurements were considered per sample.

2.6. Density

Density measurement of the emulsions and nanoemulsions, of the aqueous phase containing OPN mucilage, and of the oil phase were carried out by a digital densitometer (Schmidt Haensch, EDM 4000, Berlin, Germany) with an accuracy of 10^{-4} g/cm³ at room temperature (25 ± 0.5 °C) [36].

2.7. Turbidity

Turbidity (τ) of treatments was measured using a spectrophotometer (Nova Instruments, 2000UV, Piracicaba, SP, Brazil) at 600 nm, according to the oil droplet concentrations. Samples were diluted to a ratio of 1:500 (v/v) in sodium phosphate buffer pH 7.0 [9,37].

2.8. Rheological

Rheological behavior of the samples was determined at 25 °C using a rheometer (HAAKE ReoStress 6000, Thermo Scientific, Karlsruhe, Germany), equipped with a thermostatic bath (HAAKE A10, Thermo Scientific) and a universal temperature control system (HAAKE UTM Controller, Thermo Scientific). The measurements were carried out using a double-gap concentric cylindrical sensor (DG41, inner gap of 0.25 mm and outer gap of 0.30 mm). To eliminate thixotropy (the influence of time on the solution flow behavior), each sample was subjected to three continuous shear rate ramps (rising, downward, and rising) ranging from 0 to 100 s^{-1} for 2 min for each curve [38,39]. The Newton's law (Eq. 2), Power Law (Eq. 3), and Herschel-Buckley (Eq. 4) models were fitted to experimental data of the second rising curve (flow curve).

$$\sigma = \mu\dot{\gamma} \tag{2}$$

$$\sigma = k\dot{\gamma}^n \tag{3}$$

$$\sigma = \sigma_0 + k\dot{\gamma}^n \tag{4}$$

Where σ is the shear stress (Pa), μ is the Newtonian viscosity (Pa·s), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency index ($Pa \cdot s^n$), n is the flow behavior index (dimensionless), and σ_0 is the yield stress (Pa).

2.9. Zeta-potential

The zeta-potential (ζ) was determined by measuring the electrophoretic mobility of droplets using dynamic light scattering (Zetasizer Nano ZS, Malvern Instrument Ltd., Worcestershire, UK). Samples were diluted with a sodium phosphate buffer pH 7.0 (1:100 v/v) to avoid multiple scattering effects in the measurements done at room temperature (25 °C). The electrophoretic mobility was evaluated by measuring the direction and velocity of droplet movements when subjected to the applied electric field [40], and converted into ζ-potential values (mV) through Henry's function (Eq. 5), adopting the Smoluchowski approximation ($f(ka) = 1.5$) [41,42].

$$\zeta = \frac{3\,\eta\,\mu}{2\,\varepsilon\,f(ka)} \tag{5}$$

Where η is the solvent viscosity (cP), μ is the electrophoretic mobility ($\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$), ε is the medium dielectric constant (dimensionless), and $f(ka)$ is the Henry's function (dimensionless). Five measurements were considered per sample.

2.10. Interfacial tension

The interfacial tension between oil phase and aqueous phase was measured at 25 °C using a pendant drop tensiometer (Sinterface Technologies, PAT-1M, Berlin, Germany) [43]. A pendant liquid drop of continuous phase solution containing OPN mucilage (0.5%, 1.0%, and 1.5%) was formed at the capillary tip within the oil phase, with an interfacial area of 12 mm^2. The equilibrium interfacial tension (γ_{eq}, $mN \cdot m^{-1}$) values were estimated using a long-time extrapolation of an empirical exponential decay fitted model (Eq. 6) after an experimental time of 1 h [44].

$$\gamma = \gamma_{eq} + A \cdot e^{-b\sqrt{t}} \quad (6)$$

Where γ is the interfacial tension ($mN \cdot m^{-1}$) at time t (s), A is a fitting constant ($mN \cdot m^{-1}$) and b is a constant related to decay rate ($s^{-0.5}$) of γ until reaching the equilibrium value γ_{eq}.

2.11. Morphology of the emulsion and nanoemulsion

Fluorescent labeling of macromolecules of the samples were observed using a Laser Confocal Microscope (Zeiss Observer Z.1, LSM780, Gottingen, Germany) in 100x EC-Plan Neofluar/1.3 Oil M27 objective, equipped with Zen 2010 software (Image Program). For the location of the different macromolecules the following procedures were established: (I) Nile red (NR) labeling (lipid fluorochrome) in Ch2 detector, 543 nm Beam splitter MBS, 103 μm pinhole, 30% laser, 410 Master Gain, 1.0 Digital Gain, 616-656 nm emission filter; (II) Rhodamine B (RB) labeling (polysaccharide fluorochrome) in Ch2 detector, 543 nm Beam splitter MBS, 103 μm pinhole, 50% laser, 370 Master Gain, 1.0 Digital Gain, 607-631 nm emission filter; (III) Fluorescein isothiocyanate isomer I (FITC) labeling (proteins fluorochrome) in ChS1 detector, 488 nm Beam splitter MBS, 87 μm pinhole, 26% laser, 500 Master Gain, 1.0 Digital Gain, and 518-553 nm emission filter. The fluorochromes (1 mM NR, 4% RB and 4% FITC) were dispersed in DMSO and 2 μL used in 4 μL of sample. The acquired images were edited and diagrammed by the software Corel Photo-Paint 12 and Corel Draw 12 (Corel Corporation, Ottawa, Canada, 2003).

2.12. Stability test

The stability of the nanoemulsions (d_{32} < 200 nm) was evaluated over 30 days (0, 1, 2, 3, 7, 14, 21, and 30 days) by analysis of d_{32}. In addition, immediately after preparation of the nanoemulsions, aliquots of each nanoemulsion sample were packaged in glass test bottles, tightly sealed and stored at room temperature (25 ± 2 °C). Thus, creaming stability (creaming index, CI %) was measured from the volume of the cream layer (v_c, mL) formed after 30 days of storage, and the total volume of the nanoemulsion (v_t, mL) [45].

2.13. Statistical analysis

Response surface methodology (RSM) was used to evaluate response patterns and to determine the best combination of variables (OPN mucilage concentration, sonication time and ultrasonic power amplitude). A polynomial regression equation (Eq. 7) was adjusted, in a generalized manner, to data in order to assess each parameter tested.

$$\hat{Y} = a + bx + cz + dw + exz + fxw + gzw + hxzw + ix^2 + jz^2 + kw^2 \quad (7)$$

Where, $\hat{Y}$ is the predicted analysis response, x is the OPN mucilage concentration (%) in the aqueous phase, z is the sonication time (min), w is the ultrasonic power amplitude (%), and a, b, c, d, e, f, g, h, i, j, and k are estimated model coefficients. Subsequently, the variables of OPN mucilage (x) and soybean oil (z) concentrations were evaluated by Eq. (8).

$$\hat{Y} = a + bx + cz + dxz + ex^2 + fz^2 \quad (8)$$

The analysis of variance (ANOVA) was used to examine the statistical significance of the terms in the regression equation using the statistical package Statistical Analysis System (SAS University Edition, Cary, USA, 2016). The models were fitted to the experimental data of the flow curves using SAS. All graphs were plotted using SigmaPlot 11.0 software (Systat Software Inc., California, USA, 2008).

3. Results and discussion

3.1. Effects of emulsifier concentration and process operating parameters on emulsion formation

The initial analysis of the independent variables (OPN mucilage concentration, sonication time and ultrasonic power amplitude) was evaluated by the d_{32} response variable. The factorial design of the experiment with real levels of each independent variable, and the mean values and standard deviation found for d_{32} of each treatment, are shown in Table 1.

Table 1
Full factorial design with the independent variables (real levels) and the experimental response for the process optimization.

Treatments	Independent variables			Response variable
	OPN (%)	Time (min)	Amplitude (%)	d_{32} (nm)
1	0.5	5	50	643 (±4.6)
2	0.5	5	70	368 (±1.9)
3	0.5	5	90	304 (±1.0)
4	0.5	10	50	469 (±4.0)
5	0.5	10	70	424 (±1.5)
6	0.5	10	90	375 (±1.5)
7	0.5	15	50	715 (±6.4)
8	0.5	15	70	454 (±2.3)
9	0.5	15	90	414 (±0.6)
10	1.0	5	50	708 (±3.2)
11	1.0	5	70	445 (±3.5)
12	1.0	5	90	308 (±1.5)
13	1.0	10	50	773 (±6.1)
14	1.0	10	70	456 (±2.1)
15	1.0	10	90	318 (±3.0)
16	1.0	15	50	844 (±2.1)
17	1.0	15	70	455 (±1.1)
18	1.0	15	90	321 (±1.0)
19	2.0	5	50	863 (±3.5)
20	2.0	5	70	684 (±2.2)
21	2.0	5	90	467 (±1.7)
22	2.0	10	50	1110 (±10.8)
23	2.0	10	70	765 (±6.9)
24	2.0	10	90	478 (±0.6)
25	2.0	15	50	1407 (±15.3)
26	2.0	15	70	959 (±1.7)
27	2.0	15	90	495 (±1.5)
28	3.0	5	50	1203 (±15.7)
29	3.0	5	70	678 (±8.1)
30	3.0	5	90	451 (±1.4)
31	3.0	10	50	1760 (±30.1)
32	3.0	10	70	1233 (±20.5)
33	3.0	10	90	869 (±5.2)
34	3.0	15	50	1530 (±10.1)
35	3.0	15	70	1103 (±19.8)
36	3.0	15	90	870 (±7.4)

OPN = OPN mucilage concentration, d_{32} = mean droplet diameter.

According to Table 1, the experimental range obtained varied from 304 to 1760 nm, therefore, there was no nanoemulsion formation (d_{32} < 200 nm) at this first moment of the study. The regression model adjusted equation for this response variable is presented at the

top of Fig. 1, which demonstrates the behavior of the experimental values in comparison to the predicted values. The suggested optimization model was a quadratic polynomial ($P < 0.0001$), which presented a good coefficient of determination ($R^2 = 0.9257$).

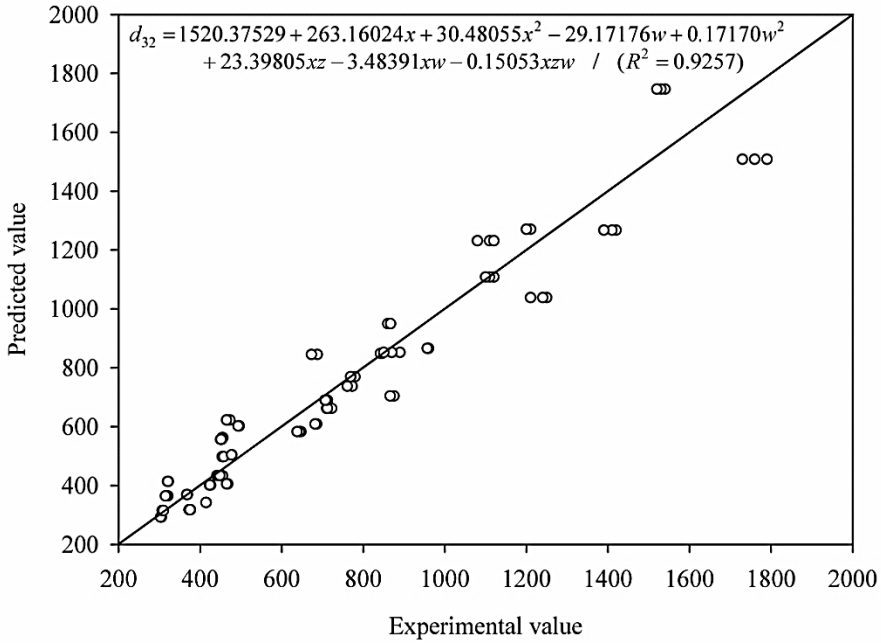

Fig. 1. Comparison of the predicted and experimental for the d_{32} values of the emulsions prepared with OPN mucilage. d_{32} = mean droplet diameter, x = OPN mucilage (%) in the aqueous phase, z = sonication time (min), and w = ultrasonic power amplitude, R^2 = coefficient of determination.

The model equation reveals positive individual effect of the OPN mucilage (x) percentage in the aqueous phase and the negative effect of the ultrasonic power amplitude (w) on the d_{32}, in this sense, individually the sonication time (z) did not significantly influence ($P > 0.05$) the d_{32} values. The interaction between the variables of OPN mucilage and sonication time (xz) concentrations had a positive effect, while the interaction of the OPN mucilage with the power amplitude (xw) had a negative effect. Regarding the interaction among the three independent variables (xzw), a negative magnitude was determined. In summary, based on the numerical values of the equation coefficients and the findings presented on the separate effects of each variable and the interactions among them, it can be inferred that the decrease in the addition range of the OPN mucilage concentration, along with a high power amplitude and shorter emulsion sonication time result in acceptable values of d_{32} (< 200 nm). Such behavior can be visually understood by the response surfaces for d_{32}, presented in Fig. 2.

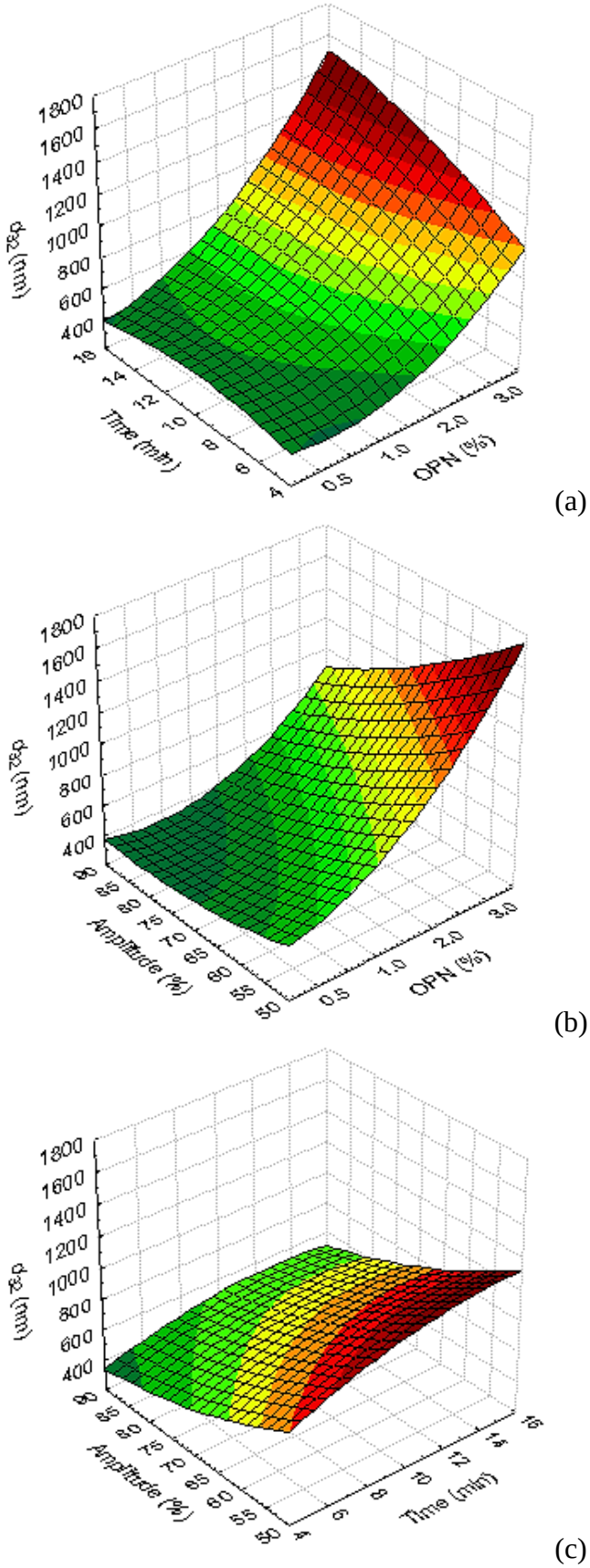

Fig. 2. Response surfaces for the mean droplet diameter (d_{32}) of the emulsions as a function of the interaction between the independent variables (a) OPN mucilage × tempo, (b) OPN mucilage × amplitude, and (c) time × amplitude.

The curves of Figs. 2a and 2b show that the OPN mucilage concentration must be less than 2%, under the conditions tested, to obtain lower values of d_{32}. In practice, it was observed that the higher the OPN mucilage concentration used the higher the viscosity of the continuous phase. Martin et al. [15] also observed that in a solution when higher mucilage concentrations of OPN were used, there was a trend towards molecule migration to the solution surface with consequent formation of agglomerates due to the association among the polymer chains, which made its dissolution in the system difficult. As described by Sivakumar et al. [30], in ultrasonic devices, the generated shear is predominantly supplied by cavitation, defined as a combined phenomenon of formation, growth and implosive collapse of the bubbles in liquid medium. Piorkowski and McClements [46] report that the amount of emulsifier present in the aqueous phase and the oil phase viscosity directly affect the homogenization efficiency because the sonicator is particularly suitable for less viscous systems. In addition, McClements [47] explains that the higher viscosity of the systems, promoted by the greater addition of emulsifier or oil, hinders the generation of impact force necessary to break the oil droplets, resulting in the formation of larger droplets after the emulsification system.

Regarding the ultrasonic power amplitude, it was observed (Fig. 2b) that the best results were found at higher amplitudes. The introduction of a large amount of mechanical energy is essential for the production of smaller droplets. This shear energy input is the product between the power amplitude and the sonication time [30,31]. However, in this study the sonication time did not present an isolated effect on the homogenization process and its interaction with the amplitude was not significant ($P > 0.05$) for the result. In practice, the increase in time, together with higher amplitude values, provided excessive energy to the system which caused heating of the equipment and, consequently, increased the formation temperature of the emulsion, contributing to the generation of larger droplets. According to Cavazos-Garduño et al. [48] and McClements [47], a greater turbulence in the emulsification, together with the heating of the system, promotes a higher frequency of collision between the oil globules and destabilizes the emulsion, a phenomenon known as over-processing. As a result, higher d_{32} values can be observed due to the coalescence of oil droplets, before these droplets have been completely coated by emulsifying molecules. In addition, these same authors reported that the effect of ultrasonic power amplitude is influenced by the type and concentration of emulsifier and oil added for the emulsion formation.

Thus, for the continuation of the study the amplitude was fixed at 90% and the sonication time at 5 min. In addition, the above mentioned optimization showed the need to evaluate another independent variable, soybean oil concentration (%) and the need to reduce the study range of the OPN mucilage concentration (%).

3.2. Effects of emulsifier and oil concentrations on nanoemulsion formation

3.2.1. Mean droplet diameter and size distribution

The values of the mean droplets diameter and Span as a function of the independent variables (OPN mucilage and soybean oil) are shown in Table 2, and the model adjustment equation for the response variable d_{32} (nm) is also shown at the bottom.

Table 2
Full factorial design with independent variables (real levels) and the dependent variable for the preparation of nanoemulsions.

Treatments	Independent variables		Response variable	
	OPN (%)	Soybean oil (%)	d_{32} (nm)	Span
1	0.5	1.0	147 (±1.48)	2.577 (±0.021)
2	0.5	2.5	337 (±6.02)	2.885 (±0.028)
3	0.5	5.0	348 (±5.64)	3.926 (±0.036)
4	0.5	7.5	376 (±2.70)	6.817 (±0.289)
5	0.5	10.0	385 (±1.52)	6.496 (±0.350)
6	1.0	1.0	116 (±0.84)	2.440 (±0.016)
7	1.0	2.5	156 (±1.10)	3.393 (±0.019)
8	1.0	5.0	285 (±1.48)	3.208 (±0.035)
9	1.0	7.5	296 (±0.55)	4.157 (±0.024)
10	1.0	10.0	320 (±1.82)	5.052 (±0.048)
11	1.5	1.0	125 (±1.30)	5.693 (±0.088)
12	1.5	2.5	134 (±0.45)	3.795 (±0.030)
13	1.5	5.0	171 (±2.30)	2.852 (±0.024)
14	1.5	7.5	383 (±8.32)	3.171 (±0.036)
15	1.5	10.0	423 (±2.70)	3.625 (±0.066)
Regression model (d_{32})	$d_{32} = 404.75285 - 542.79220x + 193.44000x^2 + 32.39425z - 1.95445z^2 + 16.22927xz$		R^2_{REG}	0.8553
			$P > F$	< 0.0001

OPN = OPN mucilage, d_{32} = mean droplet diameter, x = OPN mucilage (%) in the aqueous phase, z = soybean oil (%), R^2 = coefficient of determination.

Table 2 shows that the response variable (d_{32}) presented values between 116-423 nm and, among these results, 6 treatments were considered nanoemulsions (d_{32} < 200 nm). The

mean droplet diameter for the treatments considered nanoemulsions ranged from $116 \leq d_{32} \leq 171$ nm. The nanoemulsions formed were the treatments defined as: (1) 0.5% OPN mucilage with 1.0% soybean oil; (6) and (7) 1.0% OPN mucilage with 1.0% and 2.5% soybean oil, respectively; and (11), (12), and (13) 1.5% OPN mucilage with 1.0%, 2.5% and 5.0% soybean oil, in this order. The model equation shows the dependence of d_{32} relative to the single effects of OPN mucilage and soybean oil concentrations, as well as the interaction between these independent variables. Increased OPN mucilage concentration (%), together with lower soybean oil concentrations (%), favored the nanoemulsion formation. For better understanding, the experimental data can be seen through the 3D plot (response surface) for d_{32} (Fig. 3).

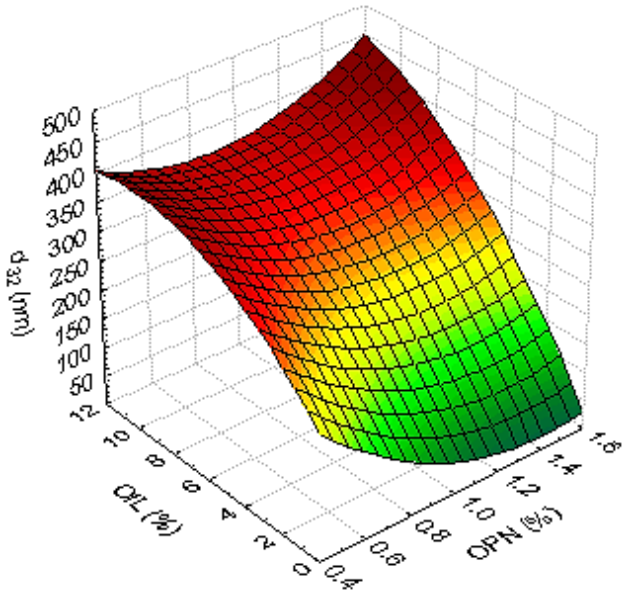

Fig. 3. Response surface for the mean droplet diameter (d_{32}) of the emulsions and nanoemulsions as a function of the interaction between the independent variables, OPN mucilage × soybean oil.

The curves of Fig. 3 indicate that the OPN mucilage concentration should be between 1.0 and 1.5% and the oil concentration less than 5% in order to reach lower d_{32} values. Ahmed et al. [6], in studies with nanoemulsions prepared with different oil and surfactant percentages, determined that a small increase in the percentage of oil addition leads to a significant increase in the mean droplet diameter. Likewise, according to McClements [47] with the excessive increase of the oil concentration there is not enough emulsifier present in the medium to coat all surfaces of the droplets formed during the homogenization process,

favoring coalescence. Furthermore, higher emulsifier concentrations combined with high oil concentrations increase the viscosity of the colloidal system, which makes sonication difficult.

In general, Span range of treatments was from 2.440 to 6.817, as shown in Table 2. According to Chan et al. [49], Span values less than or equal to 2 indicate low polydispersity with a narrower droplet size distribution. The highest Span values correspond to the treatments with the highest concentration of mucilage together with a lower concentration of the oil or vice versa. This may be justified by the fact that the excessive OPN mucilage in the system eased the agglomeration of its molecules and the lower OPN mucilage concentration caused insufficient coating on the oil droplets. Events that contributed to the coalescence of a part of the droplets of the dispersed phase, causing droplets of different sizes within the same sample, which favored the increase of the Span. The evaluation of the droplet size distribution of the formed nanoemulsions (d_{32} < 200 nm) is shown in Fig. 4.

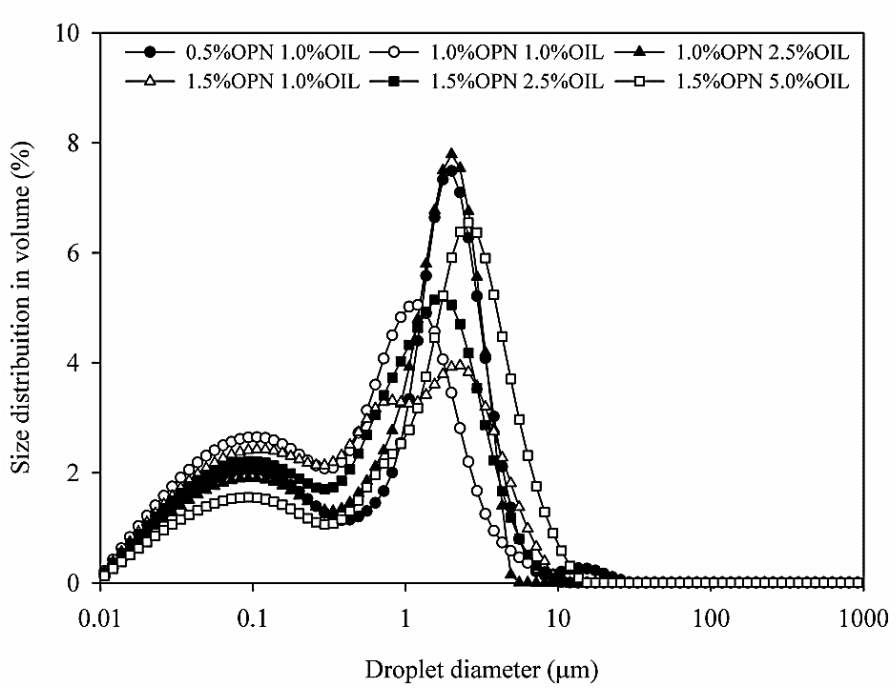

Fig. 4. Droplet size distribution of the nanoemulsions prepared with OPN mucilage in the aqueous phase and soybean oil.

In general, the nanoemulsions showed a very similar behavior relative to the droplet size distribution, where a high peak around 0.1 μm (d_{32}), equivalent to 100 nm, and an additional narrow peak can be observed. According to Rao and McClements [37], this bimodal behavior may be associated with a destabilization phenomenon (droplet coalescence). However, visually there was no phase separation or creaming for these treatments.

3.2.2. Density

Prior to the preparation of the nanoemulsions were measured the densities of the dispersed phase (soybean oil) and the continuous phases for each OPN mucilage concentration. The determined values were 1000.0, 1001.9, and 1004.0 $kg \cdot m^{-3}$ for the continuous phases containing 0.5, 1.0, and 1.5% OPN mucilage, respectively, and 891 $kg \cdot m^{-3}$ for the oil phase. These data, besides helping to understand the densities found in the different treatments (Table 3), were used as input data in determining the interfacial tension between the aqueous phase and the oil phase.

Table 3
Density of emulsions and nanoemulsions containing 0.5%, 1.0%, and 1.5% OPN mucilage in the aqueous phase at different concentrations of soybean oil (25 ± 0.5 °C).

Soybean oil (%)	Density ($kg \cdot m^{-3}$)		
	0.5% OPN	1.0% OPN	1.5% OPN
1.0	999.5 (±0.1)	1001.1 (±0.1)	1003.1 (±0.0)
2.5	998.8 (±0.1)	999.9 (±0.1)	1002.1 (±0.1)
5.0	998.7 (±0.1)	999.4 (±0.2)	1000.6 (±0.1)
7.5	996.6 (±0.2)	996.7 (±0.1)	999.2 (±0.2)
10.0	995.8 (±0.2)	995.6 (±0.0)	997.4 (±0.4)
Regression model (density)	$Density = 999.60214 + 1.89224x^2 - 0.33633z - 0.21682xz$		
R^2_{REG}	0.9696	$P > F$	< 0.0001

OPN = OPN mucilage, x = OPN mucilage (%) in the aqueous phase, z = soybean oil (%), R^2 = coefficient of determination.

Through the model equation that predicts the effect of the independent variables on the variation of the experimental values of density, it was possible to verify that the isolated effects of each variable and the interaction between them had a significant influence ($P < 0.05$) on this response variable. A positive magnitude was observed for the coefficient referring to the OPN mucilage concentration in the aqueous phase, and negative magnitude for those corresponding to the soybean oil concentration and the interaction of the independent variables.

The density of the phases employed directly influenced the final density of emulsions and nanoemulsions, ranging from 995.6 to 1003.1 $kg \cdot m^{-3}$, with the highest values corresponding to nanoemulsions (999.5 to 1003.1 $kg \cdot m^{-3}$). For the same OPN mucilage concentration it was possible to observe a decrease in the density of the treatments with increase of the oil concentration, because the oil has a lower density than water. On the other

hand, for the same oil concentration, an increase in the density value was observed for higher emulsifier concentrations, which may be justified by the increase in the density value found for the continuous phases when more mucilage was added to the colloidal system.

Homayoonfal et al. [36], determined density values between 994.3 to 1002.8 $kg \cdot m^{-3}$ in nanoemulsions. The difference was attributed to the direct effect of the aqueous phase containing the surfactants, and to the reverse effect of the oil phase on the system density. Gharibzahedi et al. [50] and Mirhosseini et al. [51] in studies of O/W emulsions, also observed an increase in the system density with the addition of higher ratios of gum Arabic in the aqueous phase and a rapid decrease with increasing nut oil concentration. The authors ended that increased gum Arabic eased the formation of relatively smaller droplets, which absorbed more gum at the interface by increasing its density and, consequently, decreasing the creaming index.

3.2.3. Turbidity

Table 4 presents the turbidity values of the treatments and the regression model adjusted to the experimental data.

Table 4
Turbidity of emulsions and nanoemulsions containing 0.5%, 1.0% and 1.5% OPN mucilage in the aqueous phase at different concentrations of soybean oil.

Soybean oil (%)	Turbidity (τ)		
	0.5% OPN	1.0% OPN	1.5% OPN
1.0	0.147 (±0.001)	0.175 (±0.004)	0.203 (±0.006)
2.5	0.165 (±0.004)	0.234 (±0.003)	0.284 (±0.001)
5.0	0.229 (±0.001)	0.289 (±0.011)	0.296 (±0.001)
7.5	0.265 (±0.003)	0.314 (±0.006)	0.320 (±0.001)
10.0	0.293 (±0.002)	0.352 (±0.003)	0.452 (±0.009)
Regression model (turbidity)	$Turbidity = 0.10570 + 0.06041x + 0.01356z + 0.00596xz$		
R^2_{REG}	0.9028	$P > F$	< 0.0001

OPN = OPN mucilage, x = OPN mucilage (%) in the aqueous phase, z = soybean oil (%), R^2 = coefficient of determination.

The turbidity of the treatments increased linearly as higher OPN mucilage and soybean oil concentrations were used for the nanoemulsion formation, and the interaction between these two variables also influenced the result, as verified in the adjusted regression model. Piorkowski and McClements [46], point out that the main factors that determine the optical

properties of nanoemulsions refer to the ratio of refractive index, oil concentration and droplet size in the colloidal distribution. According to the same authors for nanoemulsions to be translucent, they must have a d_{32} less than 50 nm, above that value the system appears to be cloudy. In this sense, a possible justification for increasing turbidity with increasing oil concentration would be the increase in the number of droplets in the dispersed phase present in the system and the mean values found for nanoemulsions between 116-171 nm (d_{32} > 50 nm). Rao and McClements [37], in nanoemulsion studies, observed a sharp increase in turbidity, characterized by a transparency decrease with consequent sample opacity, with the increasing the oil concentration and d_{32} of droplets formed.

Furthermore, there was also an effect of increasing emulsifier concentration on turbidity, which contributed to the opacity of the treatments. OPN mucilage used in this experiment shows a dark color, unlike the gums already available on the market, which affected the color of the formed nanoemulsion. For many practical applications, according to Yang and McClements [9], knowledge of the different optical properties of the emulsion-based systems is important. For example, in clear beverages it is necessary to use transparent systems, whereas in cloudy beverages it may be desirable to employ opaque systems to confer optimal coloration.

3.2.4. Rheological behavior

Among the models used to describe the rheological behavior of the treatments, Newton's Law model presented the best adjustments to the experimental data, with high coefficient of determination ($0.9842 \leq R^2 \leq 0.9999$) values and low root mean square error ($0.00131 \leq RMSE \leq 0.05570$) values. Regarding the Newtonian viscosity (μ) determined by this model, there was no interaction correlation (P = 0.2956) between the independent variables (OPN mucilage concentration in the aqueous phase and soybean oil concentration), only the isolated effect of each variable (Table 5).

Table 5
Rheological parameters of Newton's Law model for emulsions and nanoemulsions containing 0.5%, 1.0% and 1.5% OPN mucilage in the aqueous phase at different soybean oil concentrations.

Oil (%)	Newton's Law (μ)			R^2_{REG}	$P > F$
	0.5% OPN	1.0% OPN	1.5% OPN		
1.0	0.002072 (±0.000017)	0.003662 (±0.000054)	0.004474 (±0.000074)	0.9648	0.0005
2.5	0.002203 (±0.000036)	0.004318 (±0.000117)	0.006464 (±0.000069)	0.9796	0.0002
5.0	0.002498 (±0.000127)	0.004477 (±0.000088)	0.007196 (±0.000172)	0.9947	< 0.0001
7.5	0.003768 (±0.000147)	0.005710 (±0.000004)	0.008714 (±0.000004)	0.9987	< 0.0001
10.0	0.004252 (±0.000545)	0.011988 (±0.000762)	0.023579 (±0.001288)	0.9805	0.0001
R^2_{REG}	0.9099	0.8775	0.8588		
$P > F$	< 0.0001	< 0.0001	< 0.0001		

OPN = OPN mucilage, μ = Newtonian viscosity (Pa·s), RMSE = root mean square error, R^2 = coefficient of determination.

Based on the presented results it was observed that there was an increased in the viscosity of the colloidal system due to the increase in the OPN mucilage concentration in the aqueous phase, in a fixed oil concentration. The same behavior was observed for a fixed OPN mucilage value at different oil concentrations. According to Campelo et al. [52] the viscosity increases due to emulsion solids and adding oil to the system. In the literature, the most frequently reported effect on the polysaccharide in the presence of solutions is its ability to modify the rheological properties of the medium, in solutions or colloidal systems such as emulsions [53]. OPN mucilage has a branched macromolecular structure with long chains, so the increase in its concentration in the medium provides greater intermolecular interactions and changes in chain entanglement, which explains the increase in viscosity of the system [14,15,19]. But the oil, when dispersed in the continuous phase, changes the rheological properties of the system because it has a higher viscosity than water [54].

The droplets size, produced in a sonicator, depends on the viscosity ratio between the two phases being homogenized. It is only possible to obtain droplets with smaller sizes (d_{32}) if there is sufficient emulsifier to coat the new surfaces of droplets formed, provided there is no system saturation and an excessive increase in the viscosity of the medium, making it difficult to adsorb these molecules on the surfaces to avoid coalescence during the emulsion process [31]. Fig. 5 shows the shear stress variation as a function of the applied shear rate (0 to 100 s^{-1}) for the different emulsifier concentrations used.

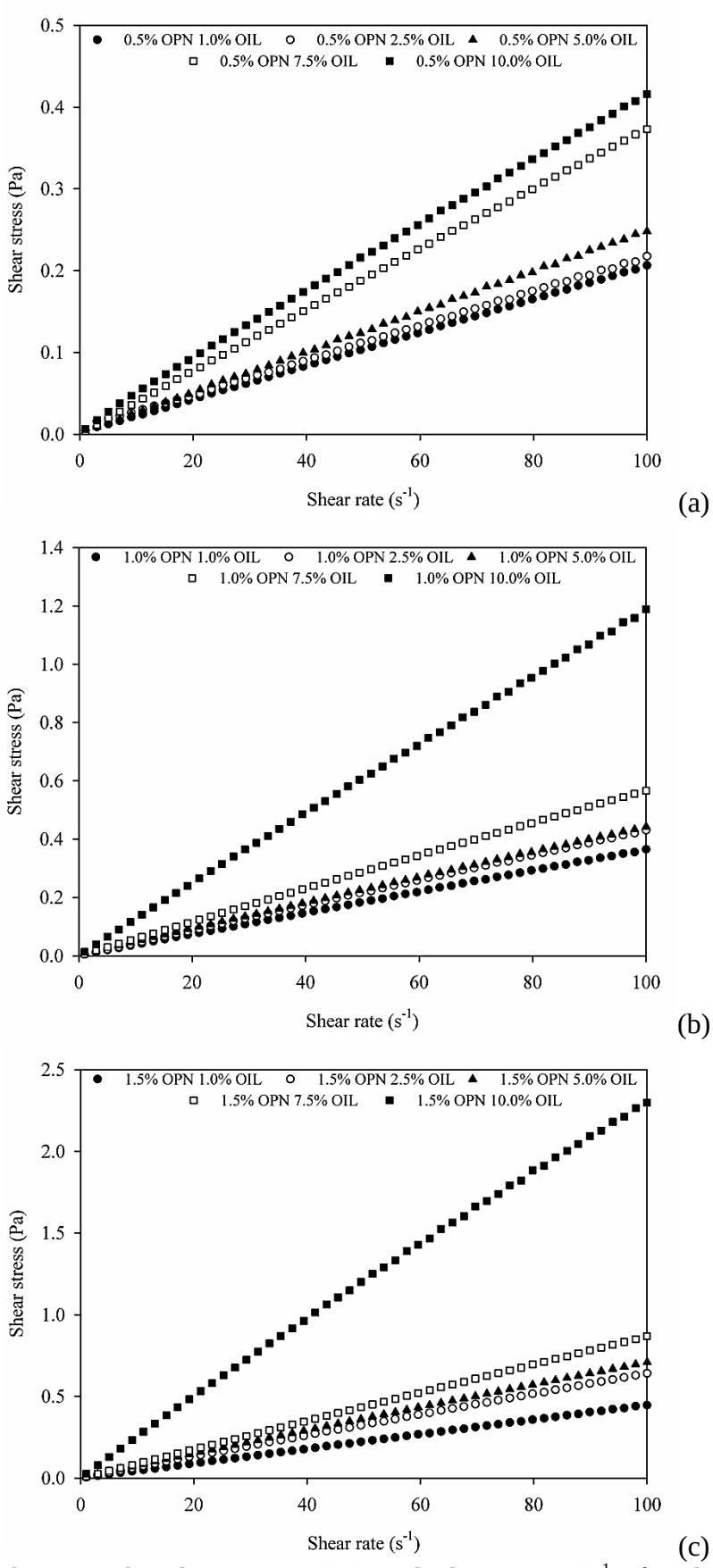

Fig. 5. Relation between the shear stress (Pa) and shear rate (s^{-1}) for the emulsions and nanoemulsions prepared with 0.5% (a), 1.0% (b), and 1.5% (c) OPN mucilage in the aqueous phase at different soybean oil concentrations.

In the rheograms, there is an evident linear trend between shear stress (Pa) and shear rate (s^{-1}), typical behavior of Newtonian fluids (Figs. 5a, 5b, and 5c). Such behavior suggests that, for a given temperature, the viscosity remains constant, regardless of the application time of shear fluid and the applied shear rate [55,56]. Nanoemulsions may have different rheological properties when compared to conventional emulsions, with the same oil content, because of the relatively small droplet size [23]. These are generally characterized as low viscosity fluids, often exhibiting Newtonian type rheological behavior, as observed in the studies by Dias et al. [40], Jin et al. [1], and Sivakumar et al. [30]. Emulsions are generally characterized as fluids with non-Newtonian behavior (pseudoplastic), described by the Power Law model, which show viscosity variation as a function of shear rate as verified by Campelo et al. [52]; Lima Junior et al. [14], and Silva et al. [54].

Junqueira et al. [20], when preparing emulsions with OPN mucilage, observed that with increasing emulsifier concentration in the continuous phase, i.e., increasing the concentration of proteins and polysaccharides in the medium, the pseudoplastic behavior of the emulsion was more evident, resulting in a higher apparent viscosity of the system. With a shear rate of 100 s^{-1} at 0.5, 1.0 and 1.5% OPN mucilage concentrations, the values of the apparent viscosity of the emulsions were 0.011, 0.035, and 0.073 Pa·s, and the d_{32} ranged from 10.89 to 29.30 μm. In this present study, the nanoemulsions presented Newtonian viscosity between 0.0020 and 0.0072 Pa·s (Table 5), and d_{32} between 0.116 and 0.171 μm (Table 2). Based on these results, the lower viscosity of the nanoemulsions compared to the emulsions at the same OPN mucilage concentrations is evident, which confirms the direct relationship between viscosity and mean droplet diameter.

3.2.5. Zeta-potential

The emulsion and nanoemulsion droplets typically have an electrical surface potential due to the adsorption of charged molecules. These electrical properties of the droplets are characterized in terms of their ζ-potential, which depends on the type of emulsifier and the conditions of the medium, and plays an important role in the system stability, because it is related to the generation of repulsive electric forces among the dispersed phase droplets, thus making coalescence difficult [41,57]. The ζ-potential values for the treatments, at pH 7.0, and regression model adjusted are shown in Table 6.

Table 6
Electric charge of droplet surfaces (ζ-potential) of emulsions and nanoemulsions containing 0.5%, 1.0%, and 1.5% OPN mucilage in the aqueous phase at different soybean oil concentrations.

Soybean oil (%)	ζ-potential (mV)		
	0.5% OPN	1.0% OPN	1.5% OPN
1.0	−26.3 (±0.1)	−27.8 (±0.1)	−27.1 (±0.1)
2.5	−25.1 (±0.1)	−26.3 (±0.1)	−26.5 (±0.1)
5.0	−25.0 (±0.0)	−25.0 (±0.1)	−25.6 (±0.1)
7.5	−24.7 (±0.2)	−24.1 (±0.1)	−24.1 (±0.1)
10.0	−23.8 (±0.1)	−23.6 (±0.1)	−23.5 (±0.1)
Regression model (ζ-potential)	$\zeta\text{-}potencial = -25.72561 - 1.38537x + 0.17480z + 0.18949xz$		
R^2_{REG}	0.9125	$P > F$	< 0.0001

OPN = OPN mucilage, x = OPN mucilage (%) in the aqueous phase, z = soybean oil (%), R^2 = coefficient of determination.

Considering the absolute values for the ζ-potential ($|\zeta|$), the increase in the emulsifier concentration positively influenced the ζ-potential, since the soybean oil concentration and the interaction between these independent variables contributed negatively, as can be observed in the equation adjustment model. Regarding the values presented for nanoemulsions (−25.6 a −27.8 mV), it was possible that the highest ζ-potential values (Table 6) in modulus refer to the treatments with the lowest d_{32} values (Table 2). Ozturk et al. [58] in studies with nanoemulsions prepared using gum Arabic, determined a ζ-potential value around −30 mV (at pH 7.0). Droplets with ζ-potential values between ± 25 mV and ± 30 mV usually have a considerable degree of stability, which agrees with the greater visual stability observed for the nanoemulsions. However, under these conditions, the systems present a dispersion threshold [59].

Martin et al. [15], in a study with solutions prepared using OPN mucilage determined a ζ-potential of the solution in water at −25 mV (at pH 5.5). Pathak et al. [60] describe that this surface charge of the droplets can be attributed to the carboxylic groups of the galacturonic acid structure and to the side chains of the amino acids present in the macromolecular structure of the OPN mucilage, responsible for the excess of negative electrical charges at the O/W interface. According to O'Brien [61], an increase in ζ-potential can be explained by the rupturing of emulsifier chains by ultrasonic homogenization during the cavitation process. In addition, Campelo et al. [62] point out that emulsions with higher

ζ -potential tend to produce less viscous systems results, a result found in this study, because the high surface load avoids droplet agglomeration and sedimentation.

3.2.6. Interfacial tension

Interfacial tension curves (γ) between the aqueous phase containing OPN mucilage (0.5, 1.0, and 1.5%) and the dispersed phase (soybean oil) showed a very similar temporal decreasing profile (Fig. 6).

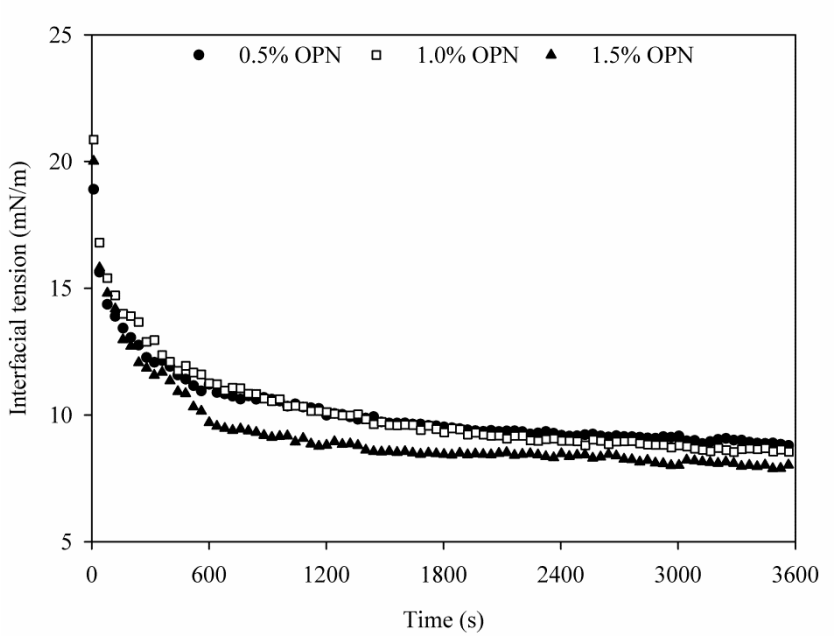

Fig. 6. Decay profiles of interfacial tension between soybean oil in contact with aqueous phases containing different OPN mucilage concentrations (0.5%, 1.0%, and 1.5%).

In the first moments, the interfacial tension increased rapidly due to protein diffusion, present in the continuous phase containing OPN mucilage, to the O/W interface for consequent adsorption. Then, a less pronounced decay was observed, represented by conformational changes in the three-dimensional structure of the adsorbed protein and a continuous adsorption process, as reported by Neves et al. [63]. As described by Perez et al. [64] and Persson et al. [39], molecular structural rearrangements expose the hydrophobic groups to the oil phase and the hydrophilic amino acid residues to the aqueous phase, which ensures the reduction of interfacial tension and, consequently, improves the stability of the system by preventing the coalescing ability of the droplets. An empirical exponential model

was adjusted to the experimental decay data of γ and the corresponding parameters are given in Table 7, in which its good fit ($R^2 \geq 0.9863$ and $RMSE \leq 0.2591$) can also be observed.

Table 7
Parameters of the exponential empirical model adjusted to data of interfacial tension decay between soybean oil contact with aqueous phases containing different OPN mucilage (0.5%, 1.0%, and 1.5%).

Aqueous phase	Adjusted parameters: $\gamma = \gamma_{eq} + A \cdot e^{-b\sqrt{t}}$			RMSE	R^2
	γ_{eq} (mN·m^{-1})	A (mN·m^{-1})	b (s$^{-0.5}$)		
0.5% OPN	8.867060 (±0.111920)	11.77840 (±0.127113)	0.069568 (±0.007495)	0.2182	0.9863
1.0% OPN	8.474476 (±0.100624)	14.35135 (±0.510151)	0.066134 (±0.006826)	0.2591	0.9875
1.5% OPN	8.050978 (±0.087765)	15.41184 (±0.446057)	0.085586 (±0.001107)	0.1734	0.9939
Regression model (γ_{eq})	$\gamma_{eq} = 9.28025 - 0.81608x$			R^2_{REG}	0.9161
				$P > F$	0.0027

OPN = OPN mucilage, γ (mN·m^{-1}) = interfacial tension at time t (s), γ_{eq} (mN·m^{-1}) = equilibrium interfacial tension, A (mN·m^{-1}) and b (s$^{-0.5}$) = fitting constants, x = OPN mucilage (%) in the aqueous phase, RMSE = root mean square error, R^2 = coefficient of determination.

The systems showed lower of equilibrium interfacial tension values as a function of the increase in the OPN mucilage concentration, indicating that the greater presence of this hydrocolloid positively affected the γ_{eq}, as can be seen by the negative coefficient found in the regression model. This behavior can be explained by the higher protein content present in the medium when higher emulsifier concentrations are used, favoring the formation of a denser and cohesive film of these molecules, which decreases Gibbs free energy excess at the interface due to the unfavorable interactions between the immiscible phases. As described by McClements [65] for nanoemulsions, the free energy of the colloidal dispersion (droplets in water) is higher than the free energy of the separated phases (oil and water), which provides their thermodynamic instability. Martin et al. [15] describe that this OPN mucilage performance is explained by the presence of O-acetyl and O-methyl groups in the polysaccharides and by the amino acids side chain (R group), which induce polarity changes on the less polar surface, and involves the ability of the hydrocolloid to act as an emulsifier.

According to Bai et al. [35] and De Souza Soares et al. [44], the decay of interfacial tension indicates that the emulsifier was able to adsorb at the O/W interface and therefore exerted an effect of interfacial/emulsifiers and, when added in suspensions, may provide improvements in the kinetic stability of emulsions and nanoemulsions. Bai et al. [35] when

preparing solutions with 2% natural emulsifiers, determined interfacial tension values of 9 $mN \cdot m^{-1}$ and 16 $mN \cdot m^{-1}$ for treatments with added whey protein isolate and gum Arabic, respectively. In addition, Nazarzadeh et al. [66] found that high energy methods of obtaining nanoemulsions, such as ultrasonic generators and high pressure homogenizers, also cause reduction of interfacial tension of the system, mainly if the system γ_{eq} is in the range of 1 to 10 $mN \cdot m^{-1}$. Thus, the results ($8 < \gamma_{eq} < 9$ $mN \cdot m^{-1}$) strengthen the premise that OPN mucilage can be considered a good substitute, partial or total, for emulsifiers traditionally used in the food industry, such as gum Arabic.

The interfacial tension of the O/W, when in low values, favors the rupture of the drops with the reduction of their d_{32} during the homogenization process [65]. In addition, Neumann et al. [43] state that the viscosity parameters of the emulsion and the interfacial tension of the solutions influence the droplet breaking behavior during the emulsification processes. The increase of proteins at the interface of the droplets reduces the interfacial tension and slightly increases the viscosity of the emulsion. In the present study, as the viscosity of the treatments was affected by the greater addition of OPN mucilage (Table 5) in the aqueous phase as well as the interfacial tension, it is possible to infer that droplet rupture was directly influenced by these parameters. This result can be confirmed by the values found for d_{32} (nm) (Table 2), where the highest number of treatments considered nanoemulsions ($d_{32} < 200$ nm) were those with 1.5% OPN mucilage and lower concentrations of soybean oil (0.5, 2.5, and 5.0%).

3.2.7. Morphology of the nanoemulsion

Compounds labeling (lipid, polysaccharide and protein) using fluorochromes provided a qualitative evaluation of the microstructures of emulsions and nanoemulsions (Fig. 7).

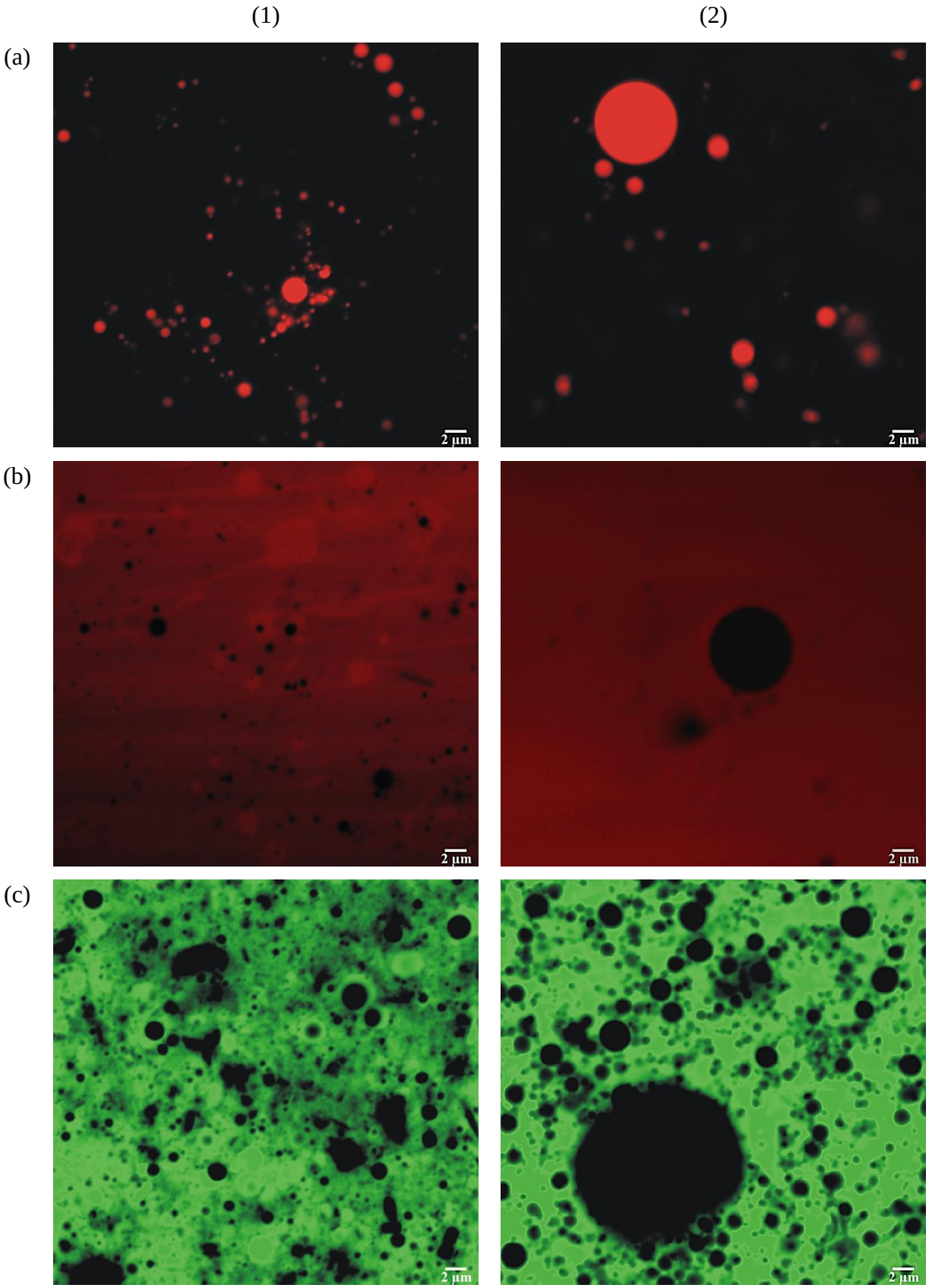

Fig. 7. Fluorescence photomicrographs of (1) nanoemulsion with 1.5% OPN mucilage and 1.0% soybean oil; (2) emulsion with 1.5% OPN mucilage and 10% soybean oil; (a) Nile red labeling (lipid fluorochrome), (b) Rhodamine B labeling (polysaccharide fluorochrome); and (c) Fluorescein labeling (proteins fluorochrome).

Column (1) of Fig. 7 represents the nanoemulsion prepared with 1.5% OPN mucilage in the aqueous phase and 1.0% soybean oil (d_{32} = 125 nm); Column (2) shows the emulsion prepared with 1.5% OPN mucilage in the aqueous phase and 10% soybean oil (d_{32} = 423 nm). The photomicrographs clearly show the difference between the d_{32} of the nanoemulsion (Fig. 7.1a) compared to the emulsion (Fig. 7.2a), through Nile red (NR) labeling (lipid fluorochrome). In general, the photomicrographs confirm the polydispersity detected in all samples, since different droplet sizes can be found in the same system.

Rhodamine B and fluorescein labeling showed fluorescence around the oil droplets of the nanoemulsion (Figs. 7.1b and 7.2b) and to a lesser degree in the emulsion (Figs. 7.1c and 7.2c), which shows that the polysaccharide and protein molecules, respectively, were adsorbed to the O/W interface. The photomicrographs of the emulsion indicate that due to the high concentration of oil droplets there was not enough emulsifier to coat all surfaces of the droplets and consequently there was droplets coalescence. This fact justifies the higher d_{32} determined for this system.

The adsorption of molecules around the droplets in the nanoemulsion shows that the OPN mucilage acted as emulsifier and stabilizer. According to Junqueira et al. [20] the protein molecules present in the OPN mucilage can be oriented in such a way that their hydrophobic and hydrophilic extremes interact at the interface, preventing oil droplet aggregation.

3.2.8. Stability test of nanoemulsions

The d_{32} measurements are considered a good indicator of stability. A nanoemulsion is considered stable if it has similar d_{32} during the stability test, while instability can be confirmed if there is a large variation in the d_{32} value along with any increase in the creaming index value (%CI) [29,67]. Fig. 8 shows the d_{32} behavior of the nanoemulsions subjected to the stability test for 30 days.

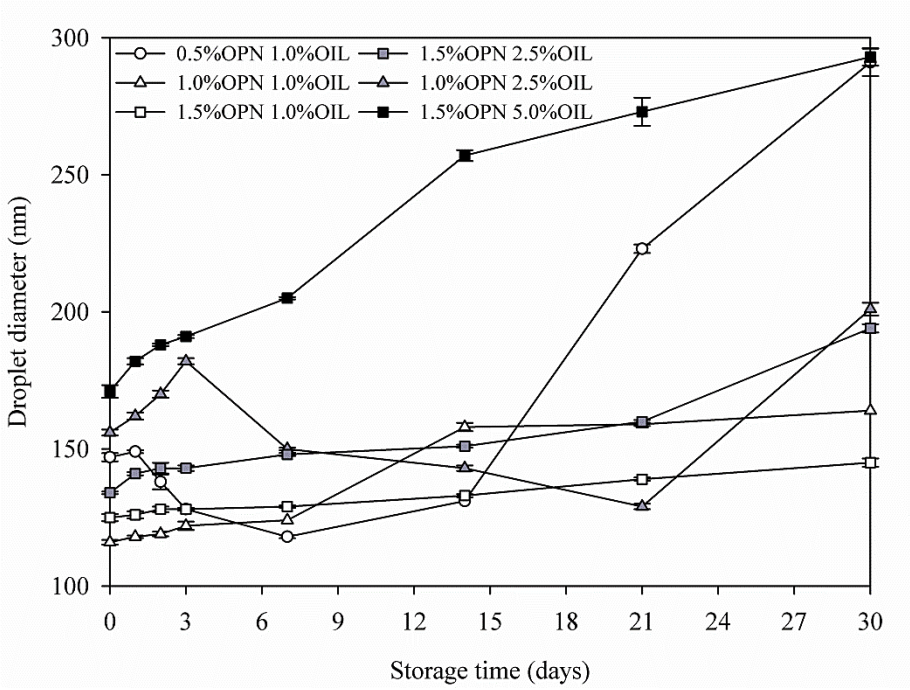

Fig. 8. Stability of the nanoemulsions d_{32} (with standard deviation bars) prepared with OPN mucilage in the aqueous phase and soybean oil during the storage time (0, 1, 2, 3, 7, 14, 21, and 30 days).

Nanoemulsions containing 1.5% OPN mucilage in the aqueous phase and 1.0% soybean oil showed relatively low d_{32} variation, with values between 125 and 145 nm with over the storage days. The treatments with 1.0% OPN mucilage and 1.0% soybean oil (116 to 164 nm) and 1.5% OPN mucilage and 2.5% soybean oil (134 to 194 nm) showed relatively higher values, mainly from the 14th day. Despite the higher d_{32} increase observed for some treatments, these samples were maintained within the range defined for the d_{32} characteristic of nanoemulsion (d_{32} < 200 nm). Regarding the treatment containing 1.5% OPN mucilage and 5.0% soybean oil, d_{32} values found ranged from 171 to 293 nm, and from the 7th storage day the values were no longer within acceptable ranges for nanoemulsions (d_{32} > 200 nm). According to Henry et al. [68], the increase in droplet size over time is due to the movement of the dispersed phase through the continuous phase, thus increasing the chance of collisions between droplets, which favors the coalescence process.

For the treatments with 0.5% OPN mucilage and 1.0% soybean oil, and 1.0% OPN mucilage and 2.5% soybean oil, a different destabilization process is observed relative to those previously mentioned. These samples presented similar behavior, in which an increase of d_{32} values was observed first, followed by a decrease and, subsequently, a new addition.

As described by Chung and McClements [69] over time, nanoemulsions can destabilize by forming a cream layer at the top (upward movement of the droplets that occurs when the density of the dispersed phase is less than that of the surrounding liquid), or a serum layer at the bottom (downward movement of the droplets that occurs when the density of the dispersed phase is less than that of the surrounding liquid). As shown in the density topic, the oil has a lower density than the continuous phase aqueous solution and, therefore, the droplets of these O/W nanoemulsions tend to form cream during the destabilization process. However, these same authors point out that sedimentation can be observed in O/W nanoemulsions when the oil droplets are coated by a relatively thick and dense layer emulsifier. In these treatments, both cream formation and sedimentation were observed, which justifies the decrease in d_{32}, since the remaining liquid of the colloidal system contained only a few droplets which had not yet undergone coalescence.

Overall, after 30 days of storage time, cream formation was verified in all treatments, with creaming index of: (I) 10.0% for treatments 1.0% OPN mucilage/2.5% soybean oil and 1.5% OPN mucilage/1.0% soybean oil; (II) 13:33% for treatment 1.0% OPN mucilage/1.0% soybean oil; (III) 15.0% for treatment 1.5% OPN mucilage/2.5% soybean oil; (IV) 16.67% for treatment 0.5% OPN mucilage/1.0% soybean oil; and (V) 20.0% for treatment 1.5% OPN mucilage/5.0% soybean oil. Creaming can be observed visually in Fig. 9.

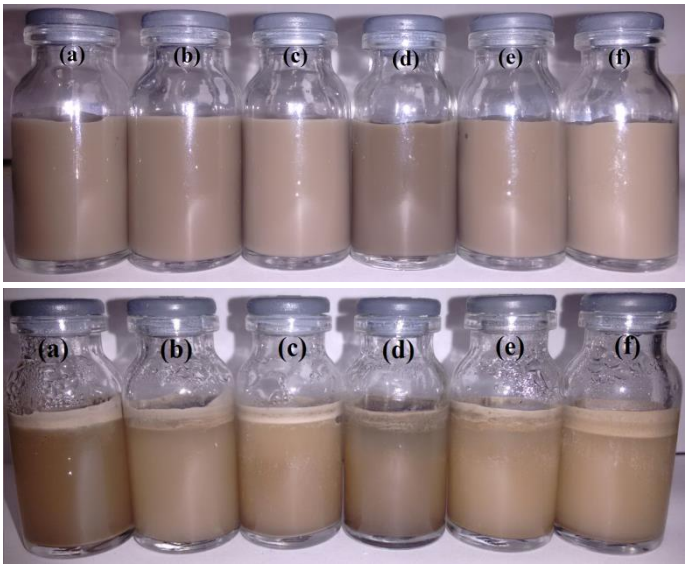

Fig. 9. Photographs of the stability test of nanoemulsions prepared with OPN mucilage in the aqueous phase and soybean oil: (1) 0 Day; (2) 30 Days; (a) 0.5% OPN 1.0% oil; (b) 1.0% OPN 1.0% oil; (c) 1.0% OPN 2.5% oil; (d) 1.5% OPN 1.0% oil; (e) 1.5% OPN 2.5% oil; (f) 1.5% OPN 5.0% oil.

4. Conclusion

The study shows that it is possible to obtain nanoemulsions containing OPN mucilage in the aqueous phase and soybean oil as the dispersed phase by applying the ultrasound technique. The operational parameters can be established in a 5 min sonication time and 90% ultrasonic power amplitude for the preparation of the nanoemulsions. The OPN mucilage concentrations are established between 1.0% and 1.5% and soybean oil concentration should be less than 5.0%, to achieve lower mean droplet diameter values. The addition of OPN mucilage in the aqueous phase, together with soybean oil concentrations, influences the mean droplet diameter, density, turbidity, viscosity, zeta-potential and interfacial tension parameters. For the viscosity parameter we found an isolated effect of OPN mucilage and soybean oil. Among the nanoemulsions formed, considering the mean droplet diameter values during the stability test period, the treatments containing 1.0% OPN mucilage and 1.0% soybean oil concentrations, and 1.5% OPN mucilage for the concentrations of 1.0% and 2.5% soybean oil present values within the range established for nanoemulsions. Based on the results it is possible to infer that the treatment containing 1.5% OPN mucilage and 1.0% soybean oil presents better kinetic stability, since low mean droplet diameter values are maintained during the 30 days of storage time. Therefore, OPN mucilage can be used as a natural emulsifier in the preparation of nanoemulsions, being considered as a good alternative for partial or total replacement of emulsifiers already available in the market.

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