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JOSÉ LUIS PASQUEL REÁTEGUI

Fracionamento e encapsulação de óleo-resina de copaíba (*Copaifera officinalis*) usando tecnologias supercríticas

Fractionation and encapsulation of copaiba oleoresin (*Copaifera officinalis*) using supercritical technologies

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Tese de doutorado apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Engenharia de Alimentos.

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RESUMO

Este trabalho foi dividido em três etapas. A etapa (I) consistiu na formação de emulsões contendo óleo-resina de copaíba (*Copaifera officinalis*), usando amidos modificados como agentes estabilizantes. Foram investigados os efeitos da concentração de amido modificado (g/L), concentração de óleo-resina (mg/mL) e proporção óleo-resina/água (%, v/v) no diâmetro médio das gotas formadas. O tempo de sonicação e a potência ultrassônica foram fixados em 6 min e 480 W, respectivamente. A formulação que proporcionou menor diâmetro de gota foi selecionada para um segundo planejamento, variando a potência ultrassônica e o tempo de sonicação. As emulsões que apresentaram maior estabilidade cinética e menor diâmetro da gota foram submetidas à secagem por *freeze-drying* e *spray-drying*. Ambas técnicas de secagem produziram partículas amorfas com diferentes tamanhos e baixa umidade. Além disso, conseguiu-se uma elevada eficiência de encapsulação e alta estabilidade contra degradação térmica. A análise morfológica indicou que a natureza dos amidos modificados não afetou sua microestrutura, mas as técnicas de secagem produziram partículas com diferentes microestruturas externas. A análise por CLSM (*Microscopia Confocal de Varredura a Laser*) confirmou a encapsulação do óleo-resina de copaíba dentro da matriz polimérica. A etapa II foi a encapsulação do óleo-resina de copaíba pela técnica de *Extração com Fluido Supercrítico de Emulsões* (SFEE), a partir da emulsão selecionada na etapa I (menor diâmetro da gota e maior estabilidade cinética). Inicialmente foram avaliadas a vazão de CO_2 e de emulsão, mantendo constantes temperatura, pressão e tempo de extração do solvente. O diâmetro do bocal coaxial utilizado para injetar a emulsão ao sistema SFEE foi de 177,8 μm. Após selecionar a condição da suspensão com menor concentração residual de acetato de etila, foi realizado outro estudo variando o tempo de extração do solvente, com o intuito de maximizar a taxa de remoção do solvente orgânico da suspensão na SFEE. A suspensão com a menor concentração de acetato de etila foi submetida à secagem. Os resultados obtidos evidenciaram uma redução na concentração de acetato de etila de 79588 (concentração de acetato de etila no início da emulsão) para 1484,5 ppm, atingindo desta forma o nível permitido pela FDA (*Food and Drug Administration*), que é de 5000 ppm por dia. Além disso, verificou-se que, quanto menor concentração de acetato de etila na suspensão, menor a recuperação de β-cariofileno e maior perda de óleo-resina no frasco de coleta. O tamanho das partículas suspensas mostrou pouca variação em relação ao tamanho das gotas da emulsão injetada no sistema SFEE. Verificou-se também que as partículas secas apresentaram características semelhantes às das partículas caracterizadas na etapa I. Finalmente, a etapa III consistiu no fracionamento do óleo-resina de

copaíba com CO_2 supercrítico, com o intuito de concentrar sesquiterpenos e ácidos diterpênicos. Primeiramente foram avaliados os efeitos da pressão e do material adsorvente na composição das frações recuperadas. Posteriormente, com o intuito de recuperar o óleo-resina retido nos materiais adsorventes utilizados no SFF (*Fracionamento com Fluido Supercrítico*), foi realizada uma extração de *Soxhlet* empregando acetato de etila seguido de etanol. Os resultados mostraram que a pressão e os materiais adsorventes tiveram efeito nas cinéticas de fracionamento e na composição química das frações. Também foi observada a produção de frações mais purificadas de β-cariofileno com a zeólita 13X a 9 MPa. Esta condição também permitiu concentrar e purificar o ácido copálico, que permaneceu adsorvido mesmo após a dessorção com CO_2 supercrítico, e foi recuperado por extração *Soxhlet* com acetato de etila.

Palavras-chave: Amido modificado, Nanoemulsão, Ultrassom, Micropartículas, Extração com Fluido Supercrítico de Emulsões, Fracionamento.

ABSTRACT

This work was divided in three stages. Stage (I) consisted in the formation of emulsions containing oleoresin from copaiba (*Copaifera officinalis*), using modified starches as stabilizers. The effects of modified starch concentration (g/L), oleoresin concentration (mg/mL) and oleoresin/water ratio (%, v/v) in the average diameter of the droplets were investigated. Sonication time and ultrasonic power were fixed at 6 min and 480 W, respectively. The formulation that provided the lowest droplet diameter was selected for a second planning, varying ultrasonic power and the sonication time. The emulsions with higher kinetic stability and lowest droplet diameter were subjected to drying by *freeze-drying* and *spray-drying*. Both drying techniques produced amorphous particles with different sizes and low humidity. Also, high encapsulation efficiency and high stability against thermal degradation were achieved. The morphological analysis indicated that the nature of the modified starches did not affect its microstructure, but drying techniques produced particles with different external microstructures. The CLSM (*Confocal Laser Scanning Microscopy*) analysis confirmed the encapsulation of copaiba oleoresin within the polymer matrix. Stage II of this work was the encapsulation of copaiba oleoresin by *Supercritical Fluid Extraction of Emulsions* (SFEE), from the emulsion selected in Stage I (lower droplet diameter and higher kinetic stability). Initially, CO_2 and emulsion flow rates were evaluated, maintaining temperature, pressure and solvent extraction time constant. The diameter of the coaxial nozzle used to inject the emulsion into SFEE system was 177.8 μm. After selecting the condition of suspension with the lowest residual ethyl acetate concentration, another study was carried out, varying the solvent extraction time, to maximize the rate of organic solvent removal from the suspension in SFEE. The suspension with the lowest concentration of ethyl acetate was subjected to drying. The results showed a reduction in the ethyl acetate concentration from 79588 (ethyl acetate concentration at the start of the emulsion) to 1484.5 ppm, thus reaching the level allowed by the FDA (*Food and Drug Administration*), which is 5000 ppm per day. Also, it was found that the lower the concentration of ethyl acetate in the suspension, the lower the recovery of β-caryophyllene and the greater the loss of oleoresin in the collection flask. The size of the suspended particles showed little variation from the droplet size of the emulsion injected into the SFEE system. It was also verified that the dried particles showed similar characteristics to the particles characterized in Stage (I). Finally, Stage III of this thesis consisted in the fractionation of copaiba oleoresin with supercritical CO_2, to concentrate sesquiterpenes and

diterpenic acids. First, the effects of pressure and adsorbent material on the composition of the recovered fractions were evaluated. Next, to recover the oleoresin retained in the adsorbent materials used in SFF (*Supercritical Fluid Fractionation*), a Soxhlet extraction was performed using ethyl acetate followed by ethanol. The results showed that pressure and adsorbent materials influenced the fractionation kinetics and the chemical composition of the fractions. The production of purified fractions of β-caryophyllene with zeolite 13X at 9 MPa was verified. This condition also allowed concentrating and purifing copalic acid, which remained adsorbed even after desorption with supercritical CO_2 and was recovered by Soxhlet extraction with ethyl acetate.

Keywords: Modified starch, Nanoemulsion, Ultrasound, Microparticles, Supercritical Fluid Extraction of Emulsions, Fractionation.

SUMÁRIO

CAPÍTULO 1 - INTRODUÇÃO, OBJETIVOS E ESTRUTURA DA TESE

1.1 INTRODUÇÃO

Poucos povos primitivos tiveram conhecimento tão vasto e complexo sobre as propriedades medicinais de seu ambiente botânico quanto os indígenas sul-americanos. A floresta brasileira é conhecida pela exuberância e variedades de suas plantas tropicais, que fornecem uma fonte alternativa de agentes terapêuticos para o tratamento de doenças. Atualmente, a obtenção de compostos bioativos de alto valor agregado justifica o crescente aumento no número de pesquisas realizadas, principalmente na área farmacológica.

As copaibeiras (*Copaifera* sp.) exsudam um líquido transparente de cheiro forte, cujas propriedades biológicas são amplamente descritas na literatura (VEIGA JUNIOR et al., 2001; VEIGA JUNIOR; PINTO, 2002). Os óleos de copaíba são, basicamente, misturas de sesquiterpenos e diterpenos, cuja composição pode variar entre espécies (CASCON; GILBERT, 2000; VEIGA JUNIOR et al., 2007). O óleo-resina de copaíba é um dos medicamentos mais utilizados na medicina popular, devido às suas diversas propriedades farmacêuticas, pois a população rural tem pouco acesso a produtos farmacêuticos comerciais (CASCON; GILBERT, 2000), sendo assim um dos mais importantes produtos amazônicos naturais comercializados. Alguns estudos recentes têm demostrado que a atividade anti-inflamatória do óleo-resina de copaíba está relacionada ao seu elevado teor de β-cariofileno, o sesquiterpeno de maior importância encontrado na fração volátil deste óleo (DIAS et al., 2012; VEIGA JUNIOR et al., 2007). Estudos realizados demostraram que o óleo-resina de copaíba, pela sua natureza insolúvel em água, tem seu uso tópico como medicamento anti-inflamatório prejudicado (DIAS et al., 2012). Neste contexto, o desenvolvimento de uma nova forma de dosagem tópica a partir de uma nanoemulsão traria benefícios relacionados à estabilização do óleo-resina de copaíba. Segundo BOUCHEMAL et al. (2004), nanoemulsões podem ser definidas como gotículas de óleo nano-dispersas em uma fase aquosa externa. Nesse sentido, as nanoemulsões apresentam várias vantagens, como o aumento da penetração na pele e capacidade de estabilizar algumas drogas, em comparação com as formulações convencionais. Uns dos métodos mais amplamente utilizados para obter nanoemulsões é a emulsificação espontânea. No entanto, a principal desvantagem desta técnica é uso de solventes orgânicos, que para sua posterior eliminação necessita elevadas temperaturas (BOUCHEMAL et al., 2004; KELMANN et al., 2007).

Novas técnicas de produção de nanopartículas utilizando fluidos supercríticos vêm sendo estudadas como alternativa a técnicas consolidadas, como spray-drying, freeze-drying,

coacervação, polimerização interfacial, emulsão/evaporação (COCERO et al., 2009). A aplicação de CO_2 supercrítico como anti-solvente foi investigada por diversos pesquisadores para obter nano- e micropartículas em meios aquosos (MARTÍN; COCERO, 2008; MATTEA et al., 2009; SOSA et al., 2011), encapsulando os princípios ativos de um composto em polímeros. Nos últimos anos foi desenvolvida uma nova tecnologia denominada extração com fluido supercrítico de emulsões (SFEE – *Supercritical Fluid Extraction of Emulsions*). Esta técnica consiste em extrair o solvente orgânico a partir de gotículas da fase oleosa de uma emulsão óleo em água, usando CO_2 supercrítico. A extração provoca a precipitação do soluto, formando partículas sólidas em uma suspensão (MATTEA et al., 2010). A aplicação deste processo é a combinação de técnicas de emulsão com o processo de precipitação por anti-solvente supercrítico (SAS – *Supercritical Antisolvent Precipitation*). Segundo DELLA PORTA; CAMPARDELLI; REVERCHON (2013), com este método combinado o tamanho das partículas formadas pode ser controlado, estando relacionado ao tamanho inicial das gotículas nas emulsões. Neste sentido, a obtenção de microesferas poliméricas utilizadas na encapsulação representa uma alternativa para proteger princípios ativos de alguns compostos que são altamente degradáveis em condições adversas (calor, luz e oxigênio), mantendo assim suas propriedades físico-químicas.

Dentre os métodos usados com pouca frequência para a separação de compostos, o fracionamento com CO_2 supercrítico vem ganhando cada vez mais espaço na indústria farmacêutica (DAVARNEJAD et al., 2008). O SFF (*Fracionamento com Fluido Supercrítico*) pode ser realizado de duas formas: a) empregando uma coluna recheada com material adsorvente, quando o material a separar é um liquido e, b) pela diferença de solubilidade dos diferentes compostos na matriz a extrair. A adsorção/dessorção com fluido supercrítico é realizada seguindo os mesmos princípios e procedimentos operacionais das extrações supercríticas convencionais a partir de partículas sólidas (DANIELSKI et al., 2008). O processo supercrítico de adsorção/dessorção geralmente emprega óxido de silício como adsorvente, mas outros adsorventes como óxido de alumínio, zeólitas e carvão ativado podem ser usados.

É importante ressaltar que, até o momento, não foram encontrados na literatura trabalhos sobre encapsulação e fracionamento (adsorção/dessorção) do óleo-resina de copaíba empregando tecnologia supercrítica, o que faz desta tese um trabalho inédito.

Diante do exposto, a proposta principal deste trabalho consiste em: i) produzir partículas poliméricas contendo óleo-resina de copaíba (*Copaifera officinalis*), pela técnica de Extração com Fluido Supercrítico de Emulsões (SFEE), visando à proteção do composto ativo

β-cariofileno; e ii) aplicar adsorção/dessorção com fluido supercrítico ao óleo-resina de copaíba, com o intuito de concentrar sesquiterpenos e ácidos diterpênicos.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Produzir partículas poliméricas a partir do óleo-resina de copaíba (*Copaifera officinalis*) usando a técnica de extração supercrítica de emulsões (SFEE) e fracionar o óleo-resina de copaíba utilizando CO_2 supercrítico.

1.2.2 Objetivo específicos

- Avaliar a composição química do óleo-resina de copaíba (*Copaifera officinalis)*, através da técnica de cromatografia gasosa de alta resolução acoplada à espectrometria de massas (CG/EM);
- Produzir emulsões a partir de óleo-resina de copaíba, visando avaliar a influência de parâmetros como: concentração de biopolímero (g/L), concentração de óleo-resina de copaíba (mg/mL) e proporção entre acetato de etila e água (%, v/v);
- Caracterizar as emulsões em relação ao diâmetro médio das gotas, estabilidade cinética e microscopia óptica;
- Avaliar o efeito da potência ultrassónica (160, 320, 480 e 640 W) e do tempo de sonicação (2, 4, 6 e 8 min) sobre o diâmetro médio das gotículas;
- Estudar o efeito dos parâmetros do processo SFEE, tais como vazão de CO_2 (g/min), vazão de emulsão (mL/min) e tempo de extração do solvente (min), sobre o teor residual de acetato de etila na suspensão produzida;
- Caracterizar as suspensões produzidas por SFEE em relação ao diâmetro médio das partículas, recuperação de β-cariofileno e perda de óleo-resina ao final do processo;
- Caracterizar as partículas secas quanto ao teor de umidade, atividade de água, óleo superficial, eficiência de encapsulação, distribuição e tamanho de partículas, estabilidade oxidativa pelo método Rancimat, difração de raio-X e morfologia por FESEM e CLSM;
- Avaliar a influência da pressão e dos materiais adsorventes nas frações de óleo-resina de copaíba recuperadas após o processo de adsorção/dessorção com CO_2 supercrítico.

1.3 ESTRUTURA DA TESE

Esta tese se encontra dividida em capítulos, os quais apresentam o desenvolvimento deste trabalho. O **Capitulo 1** é composto pela introdução, o objetivo geral e os objetivos específicos desenvolvidos na realização da tese, além da sua estrutura. No **Capitulo 2** está apresentada uma breve revisão bibliográfica, abordando a matéria-prima utilizada, emulsões, formação de partículas por extração supercrítica de emulsões e fracionamento com fluido supercrítico (SFF). Os **Capítulos 3, 4 e 5** consistem em artigos publicados ou submetidos para publicação em periódicos internacionais.

O **Capitulo 3 – Production of Copaiba oleoresin particles from emulsions stabilized with modified starches** mostrou o efeito da concentração de biopolímero, da concentração de óleo-resina de copaíba e da proporção acetato de etila/água sobre o diâmetro médio da gota de emulsões óleo em água contendo óleo-resina de copaíba. Além disso, é apresentado o efeito da potência ultrassônica e do tempo de sonicação sobre o diâmetro médio das gotículas. As emulsões com menor diâmetro de gotículas foram submetidas a secagem por *spray-drying* e *freeze-drying*. Finalmente, as partículas secas foram caraterizadas em termos de umidade, distribuição de tamanho das partículas, eficiência de encapsulação, óleo superficial, estabilidade oxidativa, difração de raios-x e análise morfológica pelas técnicas FESEM (Field Emission Scanning Electron Microscopy) e CLSM (Confocal Laser Scanning Microscopy).

A técnica de extração com fluido supercrítico de emulsões (SFEE) foi utilizada como alternativa aos processos convencionais de remoção de solvente e formação de partículas. Desta forma, o **Capitulo 4 – Production of copaiba (*Copaifera officinalis)* oleoresin particles by supercritical fluid extraction of emulsions** apresenta um estudo detalhado da influência das condições operacionais (vazão de CO_2, vazão de emulsão e tempo de extração do solvente) sobre a remoção de acetato de etila no processo SFEE. Além disso, neste artigo foram avaliados o diâmetro médio das partículas suspensas, a recuperação de β-cariofileno e a perda de óleo-resina no final do processo. Finalmente, nas suspensões produzidas nas melhores condições para remoção de acetato etila, foi realizada a secagem por *spray-drying* e *freeze-drying*.

Como alternativa às desvantagens dos processos convencionais de fracionamento, o processo de fracionamento através de adsorção/dessorção com CO_2 supercrítico foi utilizado, explorando a capacidade de alguns materiais para induzir a concentração de compostos específicos. Desta forma, o **Capitulo 5 – Supercritical carbon dioxide fractionation of sesquiterpenes and diterpenic acids from copaiba (*Copaifera officinalis*) oleoresin**,

apresenta as condições ótimas para concentrar o β-cariofileno e ácido copálico, avaliando a influência da pressão e de três materiais adsorventes. Além disso, para separar o óleo-resina de copaíba retido no material adsorvente após o processo de fracionamento, foi sugerida uma extração a baixa pressão.

No **Capitulo 6** são realizadas discussões gerais a respeito dos resultados obtidos nos capítulos 3, 4 e 5. Finalmente, no **Capitulo 7** são apresentadas as conclusões gerais obtidas e sugestões para trabalhos futuros. Além disso, é apresentada a memória do período de doutorando com todos os trabalhos acadêmicos realizados paralelamente ao desenvolvimento desta tese. Por fim, o **Capitulo 8** apresenta as referências bibliográficas utilizadas e no **Apêndice** são apresentadas informações suplementares dos artigos desenvolvidos nesta pesquisa.

O trabalho foi realizado, em sua maioria, no Laboratório de Alta Pressão em Engenharia de Alimentos (LAPEA) - DEA/FEA/UNICAMP e no CPQBA (Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas), Campinas-SP. A Figura 1 mostra o diagrama com as atividades realizadas durante este doutorado.

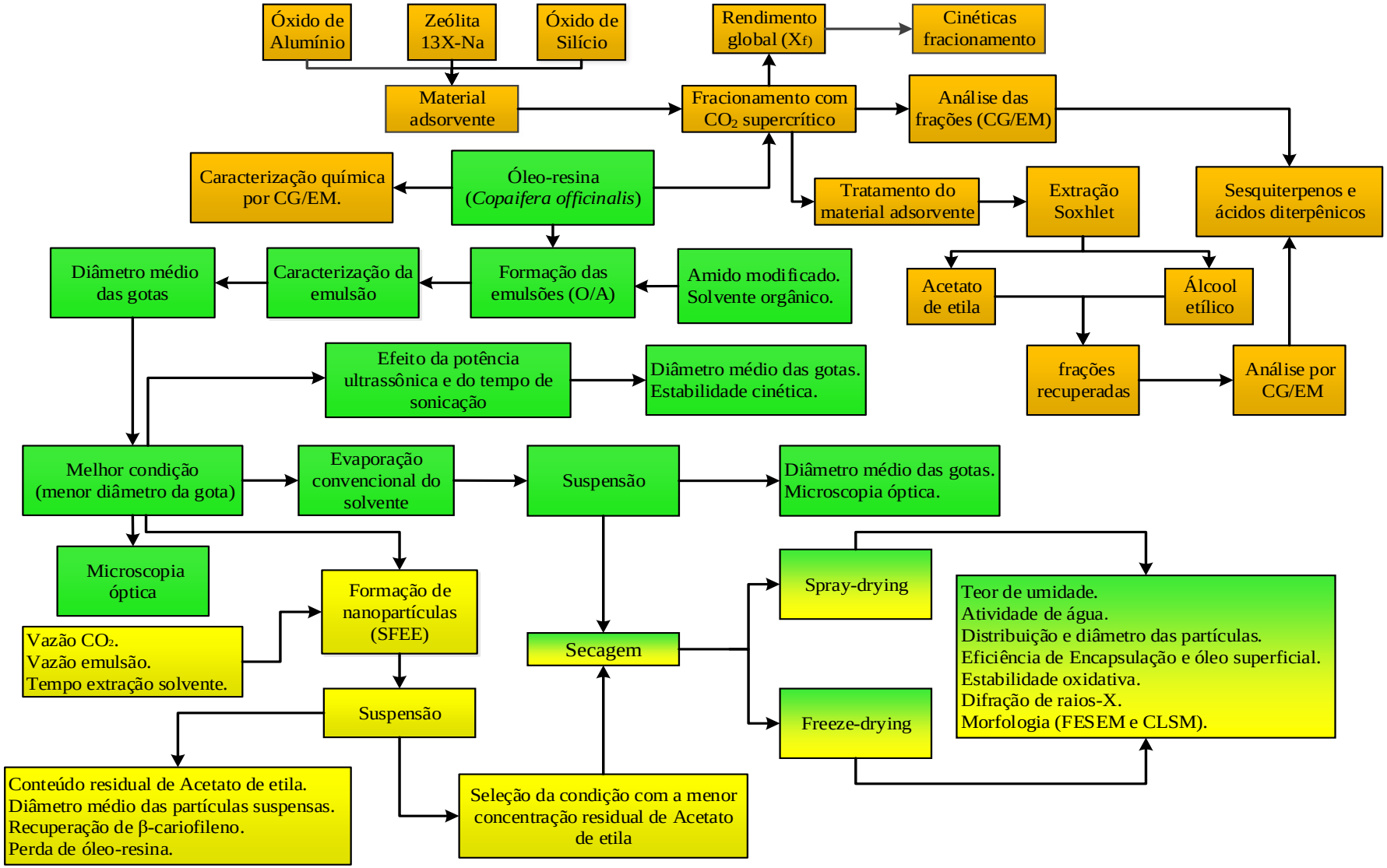

Figura 1. Diagrama com as atividades desenvolvidas durante a realização do trabalho.

CAPÍTULO 2 - REVISÃO BIBLIOGRÁFICA

2.1 REVISÃO BIBLIOGRÁFICA

2.1.1 Óleo-resina de copaíba (*Copaifera* sp.)

A espécie *Copaifera* sp. é uma planta decídua a semidecídua, heliófita e xerófita, popularmente conhecida como “copaíba”, “bálsamo” ou “pau-de-óleo”. No Brasil, esta espécie cresce principalmente nos estados do Amazonas, Pará, Ceará. As copaibeiras são árvores de crescimento lento, alcançam uma altura de 25 a 40 metros, podendo viver até 400 anos. Seu tronco é cilíndrico, tortuoso e geralmente curto. A casca é aromática e tem coloração avermelhada (jovem) e marrom (adulta), sendo que a casca interna exala uma resina de sabor amargo. Segundo VEIGA JUNIOR; PINTO (2002), as copaibeiras são árvores nativas de regiões tropicais da América Latina e também da África Ocidental. Na América Latina são encontradas espécies na região que se estende do México ao norte da Argentina, conforme mostra a Figura 2.

Figura 2. Regiões onde é encontrado o gênero *Copaifera* sp. (destacadas em verde)
Fonte: adaptado de VEIGA JUNIOR, V. F., PINTO, A. C. (2002).

No Brasil, a espécie *Copaifera langsdorffii* Desf. é particularmente importante por estar distribuída em todo o território (da Amazônia a Santa Catarina, no nordeste e centro-oeste) e por possuir quatro diferentes variedades: *Copaifera langsdorffii* var., *grandifolia*, *laxa* e *glabra*. Segundo PIERI; MUSSI; MOREIRA (2009), entre as espécies mais abundantes destacam-se: *Copaifera officinalis* L. (Norte do Amazonas, Roraima, Colômbia, Peru, Venezuela e El Salvador), *Copaifera guianensis* Desf. (Guianas), *Copaifera reticulata* Ducke, *Copaifera multijuga* Hayne (Amazônia), *Copaifera confertiflora* Bth (Piauí), *Copaifera langsdorffii* Desf. (Brasil, Argentina e Paraguai), *Copaifera cariacea* Mart (Bahia) e *Copaifera cearensis* Huber ex Ducke (Ceará). Na África Ocidental são descritas 19 espécies nas regiões do Congo, Camarões, Guiné e Angola. Destas, as espécies *Copaifera convertifolia*, *Copaifera demeusii* (Copal do Congo), *Copaifera coleosperma* (Copal da Rodésia), *Copaifera conjugata*, *Copaifera hymenaefolia*, *Copaifera chodatiana* e *Copaifera fissicuspis* são descritas como pertencentes ao gênero *Copaifera*.

A Figura 3 ilustra como a coleta do óleo-resina é realizada de forma sustentável com a utilização de um trado (Figura 3A), com o qual se faz um pequeno orifício na casca do caule da árvore (Figura 3B) (PAIVA et al., 2004). Após a extração, o orifício é vedado com uma rosca para facilitar futuras extrações (Figura 3C).

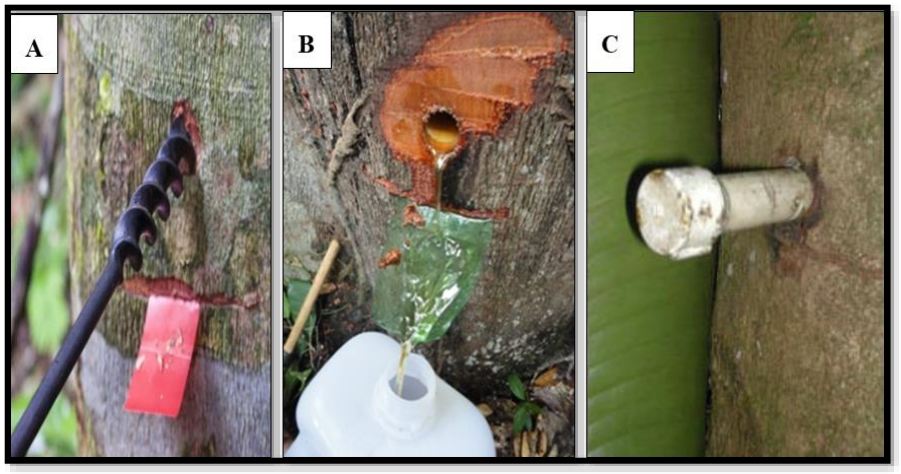

Figura 3. Extração de óleo-resina de copaíba.
Fonte: adaptado de ROMERO (2007).

Segundo PINTO et al. (2000) e VEIGA JUNIOR et al. (2001), a época mais indicada para extração de óleo-resina de copaíba é a das chuvas. As extrações feitas no período seco resultam na produção de um óleo bem mais denso. O óleo-resina de copaíba é um líquido

de viscosidade variável, cuja coloração varia do amarelo ouro ao marrom claro, como pode ser observado na Figura 4.

Figura 4. Diferentes cores e tonalidades do óleo-resina de copaíba.
Fonte: adaptado de ROMERO (2007).

Para a utilização farmacológica, os óleos mais escuros e viscosos são os preferidos, e somente na espécie *Copaifera langsdorffii* o óleo de copaíba apresenta-se semelhante ao sangue de dragão (*Croton* sp.), recebendo denominação popular de copaíba vermelha (VEIGA JUNIOR; PINTO, 2002). Os óleos comerciais de copaíba não têm identificação de espécies botânicas (VEIGA JUNIOR et al., 2001), e apenas suas características físicas, como densidade e cor, são comumente usadas para diferenciar os óleos de copaíba.

A resina de copaíba tem sido amplamente utilizada na medicina popular como: anti-inflamatório (PAIVA et al., 2002), anticâncer (LIMA et al., 2003), antioxidante (PAIVA et al., 2002), antitetânico, antisséptico e é indicada para tratar a gonorreia, sífilis, bronquite e feridas (PAIVA et al., 2004). Além disso, verificou-se que os óleos de diferentes espécies de *Copaifera* coletados no Brasil são ativos no tratamento contra promastigotas e amastigotas, formas de *Leishmaniose amazonensis* (SANTOS et al., 2008), que é um parasita protozoário responsável por diversos problemas de saúde pública na América Latina (SERENO et al., 2007). Não existem vacinas contra a leishmaniose, e o seu tratamento é dependente de um número limitado de drogas (DESJEUX, 2004; SERENO et al., 2007) como o antimônio pentavalente, anfotericina B, formulações lipídicas de anfotericina B no caso da leishmaniose visceral e miltefosine, medicamente administrado apenas por via oral (ALNAIM et al., 2007; PARIS et al., 2004).

O óleo de copaíba é extensivamente comercializado como óleo bruto e em vários produtos farmacêuticos, tais como cápsulas (GOMES et al., 2007). Na indústria, é utilizado como fixador para perfumes, em cosméticos (xampus, cremes, sabões, loções e espumas de banho), em vernizes e como solvente para tintas em pó (VEIGA JUNIOR; PINTO, 2002).

2.1.1.1 Constituintes químicos do óleo-resina de copaíba (*Copaifera* sp.)

Estudos fitoquímicos do óleo-resina de copaíba revelaram a presença de grandes quantidades de compostos voláteis (8%; β-cariofileno, óxido cariofileno, β-elemeno, α-cis-bergamoteno, ar-curcumeno e α-trans-bergamoteno), resinas não voláteis e pequenas quantidades de ácidos orgânicos (GRAMOSA; BRIGIDO; SILVEIRA, 1996). VEIGA JUNIOR; PINTO (2002) e RIGAMONTE-AZEVEDO; WADT; WADT (2006) reportam 72 sesquiterpenos e 27 diterpenos, conforme apresentado na Tabela 1, destacando-se o α-copaeno, β-cariofileno, α-humuleno, bergamoteno e δ-cadineno entre os principais sesquiterpenos. Segundo SILVA (2004), os 27 diterpenos descritos na literatura pertencem aos esqueletos caurano, labdano e clerodano, como mostrado na Figura 5, e entre os ácidos diterpênicos mais representativos estão o ácido copálico, ácido hardwíckiico, ácido caurenóico e ácido colavênico, conforme ilustrado na Figura 6.

O ácido copálico é usado como referência do óleo-resina de copaíba, pois foi o único encontrado em todos os óleos analisados por cromatografia gasosa para identificação da composição dos mesmos (VEIGA JUNIOR; PINTO, 2002).

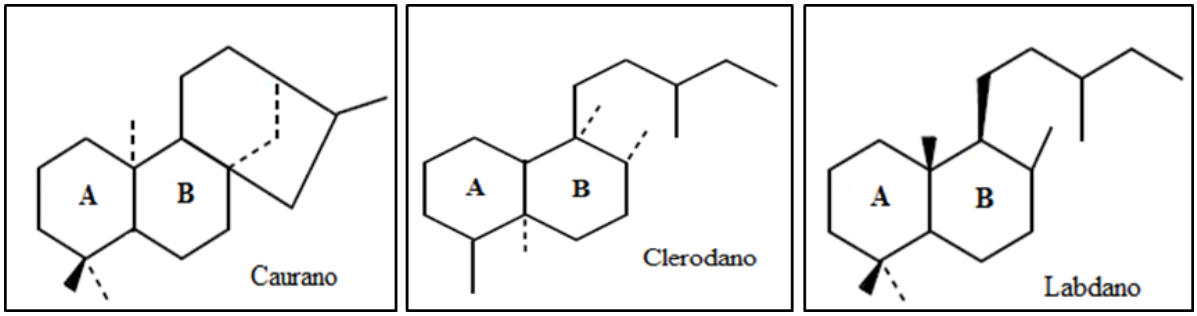

Figura 5. Estereoquímica dos esqueletos diterpênicos: caurano, clerodano e labdano. Sistema decalínico representado pelos anéis A e B.

Fonte: adaptado de VEIGA JUNIOR; PINTO (2002).

De acordo com VEIGA JUNIOR; PATITUCCI; PINTO (1997), muitos trabalhos foram publicados relatando que o óleo-resina de copaíba obtido a partir da *Copaifera langsdorffii* L. é constituído por 50% de hidrocarbonetos sesquiterpênicos e 25% de ácidos diterpênicos com esqueletos (-)-labdano e (-)-caurano.

Tabela 1. Sesquiterpenos e diterpenos encontrados no óleo-resina de copaíba.

Sesquiterpenos		Diterpenos
S1 Alo-Aromadendreno	***S40*** β-Guaieno	***Cauranos***
S2 Ar-Curcumeno	***S50*** γ-Guaieno	***D1*** Ácido *ent*-16-β-caurano-19-óico
S3 α-Bergamoteno	***S51*** Guaiol	***D2*** Ácido *ent*-caura-16-eno-19-óico
S4 β-Bergamoteno	***S52*** α-Gurjuneno	***Clerodanos***
S5 Biciclogermacreno	***S53*** Himacheleno	***D3*** Ácido 3,13-clerodadieno-15,16-olídeo-18-óico (Ácido patagônico)
S6 β-Bisaboleno	***S54*** Humuleno	***D4*** Ácido 3-clerodeno-15,18-dióico
S7 β-Bisabolol	***S55*** α-Humuleno	***D5*** Ácido 13-clerodeno-15,16-olídeo-18-óico
S8 α -Bourbouneno	***S56*** β-Humuleno	***D6*** Ácido clerodano-15,18-dióico
S9 Cadaleno	***S57*** γ-Humuleno	***D7*** Ácido *ent*-15,16-epóxi-13(16),14-clerodadieno-18-óico (Ácido clorechínico)
S10 Cadineno	***S58*** Ledol	***D8*** Ácido *ent*-15,16-epóxi-3,13(16),14-clerodatrieno-18-óico (Ácido hardwíckiico)
S11 α-Cadineno	***S59*** Longicicleno	***D9*** Ácido 15,16-epóxi-7β-acetóxi-3,13(16),14-clerodatrieno-18-óico (Ácido 7-acetóxi-hardwíckiico; 7a-acetoxibacchotriconeatina D)
S12 δ-Cadineno	***S60*** Longifoleno	***D10*** Ácido 3,13-clerodadieno-15-óico (Ácido colavênico)
S13 γ-Cadineno	***S61*** Longipineno	***D11*** 3,13-clerodadieno-15-ol (Colavenol)
S14 α-Cadinol	***S62*** α-Multijugenol	***D12*** Ácido *ent*-15,16-epóxi-7β-hidróxi-3,13(16),14-clerodatrieno-18-óico (Ácido 7-hidróxi-hardwíckiico)
S15 Calameneno	***S63*** t-Muurolol	***D13*** *ent*-(19a)-3,13-clerodadieno-15-ol (cis-colavenol)
S16 Calareno	***S64*** α-Muuroleno	***D14*** *ent-neo*-4(18),13-clerodadien-15-ol
S17 Cariofileno	***S65*** γ-Muuroleno	***Labdanos***
S18 β-Cariofileno	***S66*** Óxido de cariofileno	***D15*** Ácido 18-hidróxi-8(17), 13-labdadieno-15-óico (Ácido copaiferólico)
S19 α-Cariofilenol	***S67*** α-Selineno	***D16*** Ácido 8(17), 13E-labdadieno-15-óico (Ácido copaiférico)
S20 Cedrol	***S68*** β-Selineno	***D17*** Ácido (13S)-7-labdeno-15-óico (Ácido catívico)
S21 α-Cedreno	***S69*** β-Sesquifelandreno	***D18*** 3β-hidróxi-15,16-dinorlabda-8(17)-eno-13-ona
S22 Cipereno	***S70*** Veridiflorol	***D19*** 8(17), 13-labdadieno-15-ol
S23 Copaeno	***S71*** β-Vetiveneno	***D20*** Ácido *ent*-11-hidróxi-labda-8(17), 13-dieno-15-óico (Ácido 11-hidróxi-copálico)
S24 α-Copaeno	***S72*** α-Ylangene	***D21*** Ácido *ent*-3-hidróxi-labda-8(17),13-dieno-15-óico
S25 β-Copaeno		***D22*** Ácido *ent*-8(17),13-labdadieno-15,19-dióico (Ácido *ent*-agático)
S26 Cubebeno		***D23*** Ácido *ent*-8(17)-labdeno-15-óico (Ácido ePeruico)
S27 α-Cubebeno		***D24*** Ácido *ent*-8(17)-labdeno-15,18-dióico (Ácido eperu-8 (20)-15, 18-dióico)
S28 β-Cubebeno		***D25*** Ácido *ent*-15,16-epóxi-8(17), 13(16),14-labdatrieno-18-óico (Ácido poliáltico)
S29 1,5-Dimetil-8- isopropilciclodeca-1,4-dien-8-ol		***D26*** Ácido *ent*-8(17)-13E-labdadieno-15-óico (Ácido copálico)
S30 α-Elemeno		***D27*** Ácido *ent*-11-acetóxi-8(17)-13E-labdadieno-15-óico (Ácido 11-acetóxi-copálico)
S31 β-Elemeno		
S32 δ-Elemeno		
S33 γ-Elemeno		
S34 β-Farneseno		
S35 *trans*-β-Farneseno		
S36 Fonenol		
S37 Germacreno B		
S38 Germacreno D		
S39 α-Guaieno		

Fonte: adaptado de VEIGA JUNIOR; PINTO (2002).

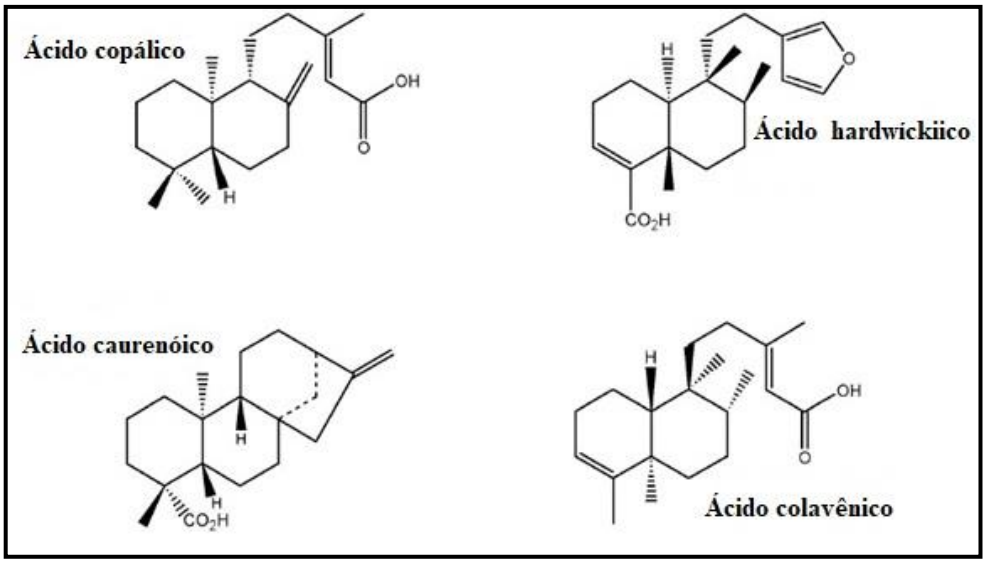

Figura 6. Principais ácidos diterpênicos presentes no óleo de copaíba.
Fonte: adaptado de VEIGA JUNIOR et al. (1997).

É importante salientar que α-copaeno, β-cariofileno, e α-humuleno, mostrados na Figura 7, são os marcadores químicos dos óleos voláteis da copaíba (PINTO et al., 2000). Assim, uma grande diversidade de estudos envolvendo esses três compostos tem sido realizada, demonstrando o seu elevado potencial como anti-inflamatório (FERNANDES et al., 2007), antialérgico (PASSOS et al., 2007), antiespasmódico (BOYOM et al., 2003), antimicrobiano e inseticida (KOTAN et al., 2008). A composição química do óleo extraído varia em função da concentração e da natureza dos diterpenos e sesquiterpenos presentes, dependendo principalmente da espécie, da estação e das características geográficas e climáticas da região onde a árvore cresce (RAMOS, 2006).

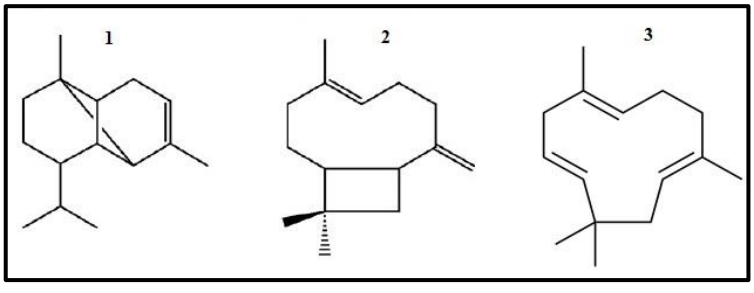

Figura 7. Estruturas Químicas de 1: α-copaeno, 2: β-cariofileno, e 3: α-humuleno.
Fonte: adaptado de SOUSA et al. (2011).

Uma vez que a natureza insolúvel em água do óleo-resina de copaíba possa lhe conferir uma sensação desagradável quando usado topicamente, o desenvolvimento de uma

forma de dosagem tópica pode conferir certos benefícios (DIAS et al., 2014). Neste contexto, as emulsões do tipo óleo em água são amplamente utilizadas na indústria farmacêutica.

2.1.2 Emulsões

Emulsões são dispersões bifásicas de dois líquidos imiscíveis: gotas de água em óleo (A/O) ou óleo em água (O/A) estabilizadas por um surfactante. As emulsões do tipo óleo em água (O/A) são amplamente utilizadas nas indústrias alimentícia e farmacêutica, já que atuam como veículos, permitindo o aumento da retenção e estabilidade dos compostos ativos. Segundo GHARSALLAOUI et al. (2007) e GOUIN (2004), as emulsões O/A podem estar presentes no produto final ou criar produtos intermediários, tais como partículas contendo um composto ativo de interesse. O principal desafio para a aplicação de emulsões na indústria é sua instabilidade termodinâmica, já que a estabilidade de uma emulsão pode se manifestar através de diferentes mecanismos físico-químicos, incluindo separação gravitacional, coalescência, floculação, etc. (MCCLEMENTS, 2004). A instabilidade termodinâmica é o resultado de duas características físico-químicas diferentes da emulsão. Em primeiro lugar, o contato entre as moléculas de água e óleo é energeticamente desfavorável, e assim o sistema reduz a área de contato entre o óleo e água. Em segundo lugar, os óleos normalmente possuem densidade mais baixa que as soluções aquosas, e assim o óleo tende a se deslocar para cima (MCCLEMENTS, 2000). De acordo com SILVA et al. (2015); YANG et al. (2012); HENRY et al. (2010) e ANTON; VANDAMME (2009), existem muitas técnicas de adaptação para nanoemulsões que podem ser categorizados em alta energia (ultrassom, microfluidificadores e homogeneizadores de alta pressão) ou baixa energia (emulsificação espontânea e pelo método de inversão) ou uma combinação de ambas. O critério mais importante na elaboração de nanoemulsões é a obtenção do tamanho da gota desejado com distribuição monomodal. Biopolímeros, tais como proteínas e polissacarídeos, têm sido utilizados como agentes emulsionantes e estabilizadores para a produção de emulsões altamente estáveis (BOUYER et al., 2012). No entanto, a estabilização das emulsões utilizando biopolímeros ainda não é completamente compreendida, devido às estruturas complexas das micromoléculas (AMINE et al., 2014).

Dependendo do tamanho da gota, as emulsões podem ser classificadas em macroemulsão (Instável (0,1 - 100 μm), nanoemulsão (Instável (20 - 200 nm) e microemulão (Estável (5 - 50 nm) (JAFARI et al., 2008; MCCLEMENTS, 2010). Em geral, as emulsões tradicionais são formadas por gotículas com raio variando de 100 nm a 100 mm, que

opticamente são opacas (MCCLEMENTS; RAO, 2011). No entanto, as nanoemulsões são formadas por gotículas muitas pequenas, com raios que variam de 10 a 300 nm, o que significa que são transparentes ou ligeiramente turvas (GRUMEZESCU, 2016). As propriedades termodinâmicas da nanoemulsões são semelhantes às das emulsões convencionais. Segundo DEY et al. (2012), a principal diferença entre elas é que as nanoemulsões apresentam desestabilização cinética mais lenta, fazendo com que sejam mais estáveis, devido ao tamanho das gotículas e ao alto movimento browniano. Segundo ANTON; BENOIT; SAULNIER (2008), a maior particularidade das nanoemulsões é a elevada estabilidade das gotículas pequenas contra fenômenos de coalescência e separação de fases, conhecida como "cremeação".

No campo farmacêutico as emulsões são amplamente utilizadas como sistemas de entrega de determinados compostos por via oral ou tópica (cutânea e ocular). O principal interesse na produção das emulsões é encapsular uma molécula ativa hidrofílica ou lipofílica no interior da fase dispersa, garantindo assim a sua proteção contra o stress ambiental e degradação (oxigênio, luz, enzimas, acidez, etc.) e permitindo, desta forma, uma liberação controlada (BOUYER et al., 2012). A formação de emulsões também é utilizada para mascarar um odor (desagradável ou agradável) ou sabor (LEY, 2008), reduzir a toxicidade de fármacos (BRIME et al., 2002) e aumentar a sua penetração através da pele (KOGAN; GARTI, 2006).

De acordo com DE PAZ et al. (2013), formulações baseadas em emulsões têm sido particularmente bem-sucedidas, uma vez que a emulsão pode proporcionar um modelo que permite controlar e reduzir o tamanho de partícula, facilitando a encapsulação de partículas em um material polimérico

2.1.3 Formação de Partículas

Diferentes métodos têm sido relatados para a formação de micro (1,0 - 5000 μm) e nanopartículas (< 1 μm). Tais técnicas podem ser a polimerização em dispersão (ZHANG; SHEN; FAN, 2008), nanoprecipitação (BARICHELLO et al., 1999), secagem por pulverização (VEHRING, 2008), emulsão/evaporação e a coacervação. Estas técnicas, são amplamente utilizadas para a secagem de alimentos sensíveis ao calor (GEORGETTI et al., 2008), produtos farmacêuticos (WEERAKODY; FAGAN; KOSARAJU, 2008) e óleos essenciais (BARANAUSKIENĖ et al., 2007). Estas técnicas apresentam algumas desvantagens, tais como a remoção incompleta de solventes tóxicos (FREITAS; MERKLE; GANDER, 2005), baixa

eficiência de encapsulação (LI; ROUAUD; PONCELET, 2008) e os problemas de poluição ambiental associada ao uso de grandes quantidades de solventes orgânicos.

Técnicas de microencapsulação utilizando fluidos supercríticos têm sido desenvolvidas para enfrentar algumas desvantagens das técnicas convencionais (JUNG; PERRUT, 2001; MARR; GAMSE, 2000; REVERCHON, 1999; TOM; DEBENEDETTI, 1991). O principal motivador destas pesquisas é a possibilidade de explorar as propriedades físico-químicas dos fluidos supercríticos. Em particular, a solubilidade do CO_2 supercrítico é muito importante para o sucesso de processos como a extração com fluido supercrítico de emulsões (SFEE – *Supercritical Fluid Extraction of Emulsions*) (CAMPARDELLI et al., 2013; DE PAZ et al., 2013; DELLA PORTA et al., 2013; FALCO; REVERCHON; DELLA PORTA, 2013; KLUGE et al., 2012; LUTHER; BRAEUER, 2012; PORTA; FALCO; REVERCHON, 2011; SANTOS et al., 2012); expansão rápida de soluções supercríticas ou precipitação de soluções supercríticas (RESS) (SANTOS et al., 2013; HUANG et al., 2005; KAYRAK; AKMAN; HORTAÇSU, 2003); precipitação por anti-solvente supercrítico (SAS) (ADELI, 2014; DE MARCO; REVERCHON, 2011; CHANG; LEE; LIN, 2007; REVERCHON; DE MARCO; TORINO, 2007); dispersão da solução expandida pelo fluido supercrítico (SEDS) (PRIAMO et al., 2011; JUN et al., 2005; TOROPAINEN et al., 2006) e processos relacionados, como o sistema de extração do solvente por aerosol (ASES) (LEE et al., 2005; STECKEL; PICHERT; MÜLLER, 2004). Muitas destas técnicas foram desenvolvidas originalmente para produzir compostos sólidos. Porém, com algumas modificações é possível obter compostos sólido-líquidos. Segundo YEO; KIRAN (2005), uma ampla variedade de materiais orgânicos e inorgânicos foram processados sob a forma de partículas, fibras e filmes, empregando fluidos supercríticos como solventes ou como anti-solvente. Dentro deste contexto, o emprego de fluidos supercríticos como anti-solventes na encapsulação e precipitação de compostos ativos em biopolímeros mostra-se uma tecnologia atraente, uma vez que é possível obter produtos praticamente livres de solvente orgânico, evitando desta forma etapas pós-processamento. O composto ativo, quando encapsulado no interior de matrizes poliméricas, é liberado após o início do processo de degradação do polímero. Por isso, o biopolímero utilizado na preparação das microesferas deve ser compatível e hidroliticamente degradável quando em contato com o organismo (SCHAFFAZICK; GUTERREZ, 2003). De acordo com BAHRAMI; RANJBARIAN (2007), a encapsulação de compostos ativos pode ser realizada basicamente por duas maneiras: os compostos podem ser encapsulados, situação em que ocorre a formação

de uma fina camada de polímero recobrindo o composto de interesse, e também podes ser co-precipitados, onde várias partículas do composto encontram-se dentro da partícula de polímero.

MATTEA; MARTIN; COCERO (2008) realizaram a co-precipitação de carotenoides e β-caroteno, respectivamente, empregando biopolímeros. Nesse sentido, YEO; KIRAN (2005) e COCERO et al. (2009) relataram que biopolímeros naturais ou sintéticos podem ser empregados como agentes encapsulantes de compostos bioativos, por apresentar biocompatibilidade e biodegradabilidade.

A produção de nano- e micropartículas empregando técnicas de precipitação que utilizam fluidos supercríticos é atraente por oferecer soluções alternativas para vários problemas encontrados nas técnicas tradicionais. Uma dessas soluções é a plasticidade inerente dos polímeros na presença de CO_2 supercrítico. A alta viscosidade do sistema polímero-CO_2 durante o processo de extração do solvente conduz à aglomeração das partículas, inconsistência do tamanho das mesmas e, em alguns casos, à encapsulação incompleta. Assim, alguns polímeros amorfos, como poli (ácido láctico-co-glicólico) (PLGA), polimetilmetacrilato (PMMA) e policaprolactona (PCL), não são adequados para processamento utilizando CO_2 supercrítico (CHATTOPADHYAY; HUFF; SHEKUNOV, 2006).

2.1.3.1 Extração com Fluido Supercrítico de Emulsões (SFEE)

A SFEE é um processo que combina técnicas de emulsão convencionais com o processo de precipitação por anti-solvente supercrítico (SAS). Esta técnica surge como solução para evitar os principais problemas de cada uma destas tecnologias, quando são empregadas separadamente. Técnicas de emulsão como a emulsificação espontânea (FERNANDEZ et al., 2004; FÜREDI-MILHOFER; GARTI; KAMYSHNY, 1999; LANDFESTER; EISENBLÄTTER; ROTHE, 2004; TROTTA et al., 2003; TROTTA et al., 2001) geralmente envolvem grande quantidade de solventes orgânicos, cuja remoção requer etapas de separação adicionais que geralmente ocorrem em temperaturas elevadas. Por outro lado, a técnica SAS geralmente não é capaz de produzir partículas dentro da escala nanométrica, e os produtos obtidos apresentam problemas de aglomeração que resultam em partículas grandes. A combinação destas duas técnicas foi apresentada pela primeira vez por PERRUT; JUNG; LEBOEUF (2004), e em seguida patenteou-se um processo para a produção de micropartículas e nanopartículas, chamado de extração supercrítica de emulsões (SFEE).

A técnica SFEE consiste em extrair o solvente orgânico a partir das gotas da fase oleosa de uma emulsão óleo em água (O/A), usando CO_2 supercrítico. Isto é, se um soluto está dissolvido no solvente orgânico, a extração deste solvente provoca a precipitação do soluto, formando partículas sólidas em uma suspensão (MATTEA et al., 2010), como é mostrado na Figura 8. Dessa forma, o tamanho das partículas é controlado através do tamanho das gotas da emulsão. A aglomeração é reduzida graças aos surfactantes, que formam um filme nas gotículas, ajudando a impedir a floculação e a coalescência, aumentando assim a estabilidade (TADROS et al., 2004). A emulsão, segundo LUTHER; BRAEUER (2012), é formada em condições de pressão ambiente, considerando alguns parâmetros adicionais como preparação, estabilidade da emulsão, material surfactante, concentração de surfactante, etc.

A principal limitação da SFEE é o controle da emulsão. Outro inconveniente da técnica SFEE é que, em vez de se obterem partículas secas, o produto final normalmente consiste em uma micro ou nano-suspensão de partículas insolúveis ou pouco solúveis em água, como óleo-resina de pimenta (AGUIAR et al., 2016), carotenoides (MATTEA; MARTÍN; COCERO, 2009; SANTOS et al., 2012), β-caroteno (DE PAZ et al., 2013; MATTEA et al., 2009) e outras substâncias com aplicações farmacêuticas (KLUGE; FUSARO; CASAS et al., 2009; KLUGE; FUSARO; MAZZOTTI et al., 2009; KLUGE et al., 2012).

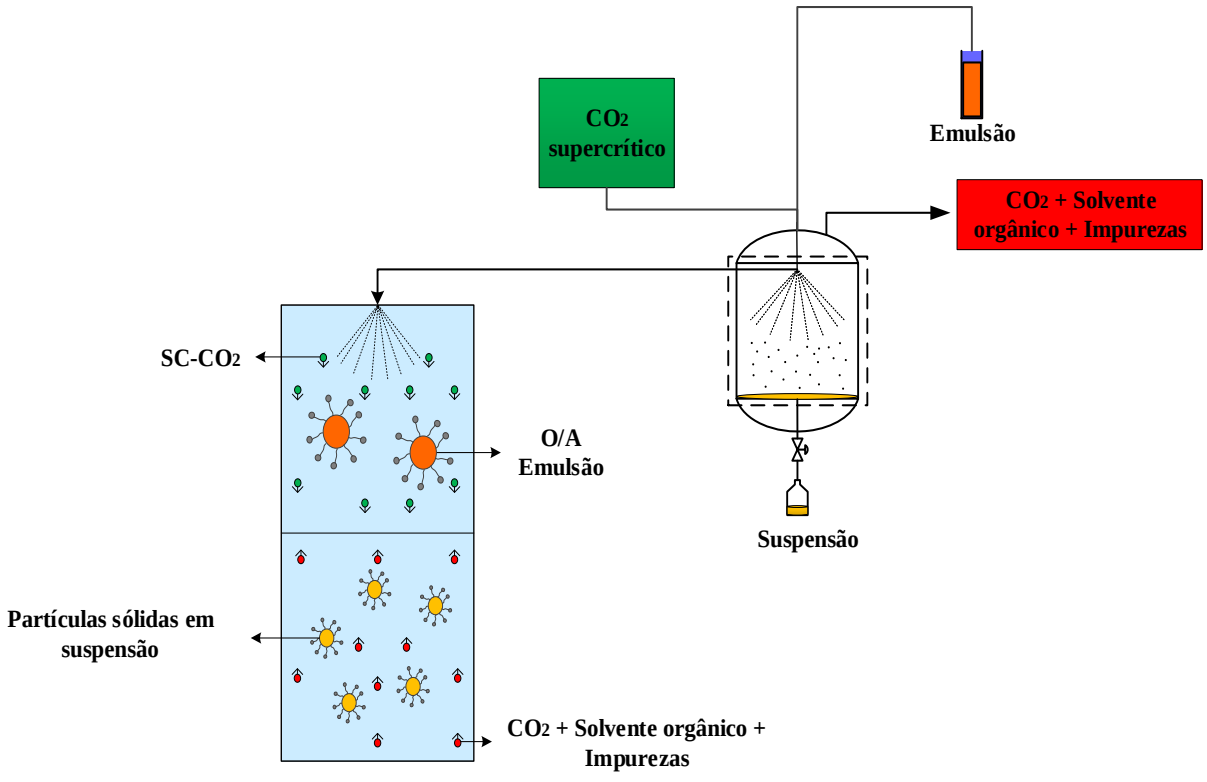

Figura 8. Ilustração esquemática da formação de partículas no processo SFEE.

Em quase todos os artigos publicados com aplicação de SFEE é observada uma relação direta entre o tamanho da partícula e o tamanho inicial das gotas da emulsão. Assim, variáveis como temperatura, pressão e tempo de processamento apresentam pouca influência no tamanho final das partículas suspensão após processo SFEE (COCERO et al., 2009). Dois pré-requisitos para o processo de SFEE devem ser cumpridos: i) o solvente orgânico deve ser miscível com o CO_2 supercrítico; ii) o soluto e a água não devem ser solúveis no CO_2 supercrítico (LUTHER; BRAEUER, 2012). A eficiência de extração utilizando CO_2 supercrítico é muito mais elevada em relação com à remoção de solvente pelas técnicas convencionais e, portanto, proporciona uma remoção rápida e completa do solvente, além de extrair outras impurezas de baixa massa molecular (CHATTOPADHYAY et al., 2007).

2.1.4 Fracionamento com CO_2 Supercrítico (SFF)

Óleos vegetais em estado bruto são amplamente utilizados nas indústrias farmacêuticas e de alimentos. Normalmente, esses óleos devem ser refinados para remover compostos indesejáveis antes do consumo. Durante o processo de refino, compostos valiosos contidos nos óleos também podem ser perdidos. Nesse sentido, o uso de dióxido de carbono (CO_2) supercrítico é uma alternativa interessante para fracionar diferentes tipos de óleos e, assim, obter os compostos de interesse com alta pureza (PEREIRA; MEIRELES, 2010).

A aplicação de fluidos supercríticos para dissolver materiais sólidos foi descrita pela primeira vez em (1879) por Hannay e Hogarth, na reunião da Royal Society de Londres. Estes pesquisadores demostraram que vários sais inorgânicos, como o cloreto de cobalto, podiam ser dissolvidos em etanol supercrítico a pressões elevadas. Este comportamento peculiar incentivou muitos estudos posteriores sobre o poder de solvatação dos fluidos supercríticos, levando a algumas aplicações industriais na década de 1960. Desde então a técnica tem atraído grande interesse científico. Já no final dos anos 1970, os fluidos supercríticos eram utilizados para isolar produtos naturais (ERKEY, 2011).

O desenvolvimento de novos processos e equipamentos começou se expandir e as indústrias se interessaram cada vez mais em tecnologias supercríticas (BRUNNER, 2005). Este interesse também se reflete na grande quantidade de artigos científicos que tratam de SFF, publicados nos últimos anos. As propriedades críticas (T_c, P_c, ρ_c) de algumas substâncias que podem ser utilizadas como solventes supercríticos, são mostradas na Tabela 2.

Tabela 2. Propriedades críticas de alguns compostos usados como fluidos supercríticos.

Fluidos Supercríticos	T_c (°C)	P_c (MPa)	ρ_c (kg m^{-3})
Dióxido de carbono	31,2	7,38	468
Água	374,1	22,1	317
Acetona	235	4,76	273
Etileno	9,5	5,06	220
Etanol	243,1	6,39	280
Metanol	240	7,95	2,75

T_c = Temperatura crítica; P_c = Pressão crítica; ρ_c = Densidade crítica
Fonte: adaptado de ERKEY (2011).

Como pode ser observado na Tabela 2, o CO_2 apresenta pressão e temperatura críticas relativamente baixas em relação aos demais fluidos. O CO_2 é o fluido supercrítico mais usado como solvente em aplicações farmacêuticas e alimentares, já que em temperaturas amenas impede a degradação de compostos termicamente sensíveis (POURMORTAZAVI; HAJIMIRSADEGHI, 2007). Não é apenas barato, mas também seguro, facilmente disponível em alta pureza e não inflamável, ao contrário de outros fluidos como etileno, etano, propano e n-pentano. Além disso, pode ser facilmente separado do extrato por simples despressurização.

O uso de fluidos supercríticos, em especial o CO_2, para o fracionamento e purificação de misturas complexas tem sido cada vez mais estudado e aplicado. Segundo MARTÍN; COCERO (2007), o fracionamento com fluido supercrítico proporciona vantagens interessantes em relação aos métodos de fracionamento tradicionais (destilação sob vácuo ou extração liquido-liquido), como: (a) ausência de resíduos de solvente no produto final; (b) seletividade do CO_2 com pequenas alterações de temperatura e pressão; (c) o CO_2 é um solvente não tóxico e não inflamável; e (d) o processo de fracionamento geralmente acontece em temperaturas moderadas. Assim, o fracionamento com fluido supercrítico tem um grande potencial não apenas para a substituição de processos convencionais, mas também como uma ferramenta para a concepção de novos produtos enriquecidos com os compostos de interesse.

Uma das aplicações mais estudadas do CO_2 supercrítico é o fracionamento de óleos essenciais. Segundo GAÑÁN; BRIGNOLE (2013), óleos essenciais são misturas complexas de compostos voláteis de muitas espécies vegetais, que são responsáveis pelo seu odor característico. Além disso, os óleos essenciais são constituídos principalmente por

hidrocarbonetos monoterpênicos, monoterpenos oxigenados, sesquiterpenos e compostos de massa molecular mais elevada (di- e triterpenos, ceras, pigmentos, etc.). Nesse sentido, o princípio de separação do fracionamento com CO_2 supercrítico pode ser realizado pela diferença de solubilidade dos diferentes compostos na matriz, empregando uma coluna recheada com material adsorvente (quando o material a separar é um líquido).

Existe uma grande quantidade de trabalhos referentes ao fracionamento com fluido supercrítico aproveitando o princípio de diferença de solubilidade, tais como: fracionamento de ésteres a partir de óleo de peixe (RIHA; BRUNNER, 2000); remoção de terpenos a partir de óleos de cítricos (MARTÍN; COCERO, 2007; SATO; GOTO; HIROSE, 1995; 1996). BENVENUTI; GIRONI; LAMBERTI (2001) e GIRONI; MASCHIETTI (2008) estudaram o fracionamento do óleo essencial de limão com CO_2 supercrítico em diferentes condições de pressão e temperatura. Estes autores observaram que, em pressões baixas, o óleo essencial apresenta solubilidade baixa em CO_2 supercrítico e elevada seletividade para terpenoides não oxigenados. GIRONI; MASCHIETTI (2005) também apresentaram outro estudo sobre a deterpenação do óleo essencial de óleos essenciais de *Tagetes minuta*, *Salvia officinalis* e limão com CO_2 supercrítico, por meio de um processo descontinuo com refluxo externo. Além disso, GAÑÁN; BRIGNOLE (2013) e GAÑÁN et al. (2015) publicaram dois trabalhos referentes ao fracionamento do óleo essencial obtido a partir da hortelã de menta.

Vários autores têm investigado maneiras de melhorar a seletividade de novos produtos. Uma dessas linhas de investigação foi a adsorção/dessorção (BARTH et al., 1994; REVERCHON, 1997).

2.1.4.1 Adsorção/dessorção com fluido supercrítico

A adsorção é uma técnica utilizada para a separação de uma ou mais substâncias a partir de uma mistura na qual os componentes apresentam propriedades físicas semelhantes (volatilidade, solubilidade, etc.) ou concentrações muito baixas (MEIRELES, 2008). Segundo REVERCHON; DE MARCO (2006), é possível aplicar processos de adsorção/dessorção para obter diferentes frações a partir de um material líquido, existindo a possibilidade de tornar o processo continuo. Os fenômenos de adsorção/dessorção exploram a capacidade de alguns materiais sólidos em fazer com que substâncias específicas de uma solução se concentrem na sua superfície, permitindo desta forma concentrar ou purificar soluções. Existem dois tipos de adsorção: *fisissorção*, caracterizada pelas forças de van der Waals (fenômeno facilmente

reversível) e a *quimissorção*, que é baseada na interação química entre o sólido e a substância adsorvida, ou seja, as forças de adesão na *quimissorção* são maiores em relação à *fisissorção*, resultando assim em um processo reversível. Além disso, na dessorção o composto adsorvido sofre grandes alterações químicas. Nesse sentido, a Tabela 3 apresenta alguns exemplos referentes ao isolamento e obtenção de alguns compostos com elevada pureza.

Tabela 3. Trabalhos sobre fracionamento de misturas com CO_2 supercrítico, empregando uma coluna recheada com material adsorvente.

Objeto de estudo	Referência
Extratos de artemisina	(NEGI et al., 2018)
Óleo de Buriti (*Mauritia flexuosa Mart.*)	(CUNHA et al., 2012)
Ácido benzoico	(PÉREZ MOLINA; JOHANNSEN, 2010)
Óleo de casca de tangerina (*Citrus reticulta*)	(DANIELSKI et al., 2008)
Óleo de manteiga	(MOHAMED et al., 2000)
Óleo de casca de bergamota	(REVERCHON; IACUZIO, 1997)
Óleo de casca de citrinos	(REVERCHON, ERNESTO, 1997)
Óleo de casca de bergamota	(CHOUCHI et al., 1995)
Óleo essencial de laranja doce e limão	(DUGO et al., 1995)
Óleo de casca de limão	(BARTH et al., 1994)

Existe uma grande quantidade de matérias adsorventes utilizados para o fracionamento, sendo os mais utilizados as zeólitas, óxido de silício, óxido de alumínio, argila ativada, carvão ativado, entre outras.

2.1.5 Materiais adsorventes

O tamanho de partícula dos adsorventes varia de 50 μm a 1,2 cm. Os adsorventes também podem ser classificados, de acordo com o tamanho do poro, em microporoso (< 20 Å), mesoporoso (20–500 Å) e macroporoso (> 500 Å). Os adsorventes podem ter até 85% do volume de partículas micro e mesoporosas, e a distribuição do tamanho dos poros pode determinar a seletividade da adsorção (MEIRELES, 2008).

2.1.5.1 Zeólitas

Zeólitas são adsorventes que englobam uma grande quantidade de minerais naturais e sintéticos que possuem características estruturais comuns. A zeólita é composta de tetraedros de sílica e alumina dispostos de várias maneiras e unidos entre si através de átomos de oxigênio. A estrutura cristalina destes materiais adsorventes proporciona uma distribuição porosa uniforme de dimensões moleculares, que faz com que a sua superfície interna seja extremadamente grande em relação à sua superfície externa. O tamanho dos poros da zeólita pode variar entre 3 e 10 Å, tornando-se desta maneira importante para processos de purificação, adsorção, entre outros (PACE, 2000).

As operações de separação realizadas com zeólitas podem ser baseadas na exclusão de tamanho ou pela diferença de afinidade com os componentes a serem separados. Embora a segunda opção seja a mais utilizada, o intervalo estreito de tamanho dos poros da zeólita possibilita a separação de moléculas com pequena diferença de tamanho (MEIRELES, 2008). A grande maioria das aplicações das zeólitas é de processos de purificação nos quais a zeólita é usada para remover impurezas. Outros processos de separação incluem a separação de hidrocarbonetos lineares e ramificados e o isolamento de isômeros de xileno.

2.1.5.2 Óxido de Silício (SiO_2)

De acordo com COLLINS; BRAGA; BONATO (2006), o óxido de silício apresenta caráter ácido, sendo considerado um dos materiais adsorventes mais utilizados na adsorção e pode ser natural ou sintético, cristalino ou amorfo. Além disso, é altamente poroso. Em geral, o óxido de silício é empregado na separação de compostos lipofílicos como aldeídos, cetonas, fenóis, ácidos graxos, aminoácidos, alcaloides, terpenoides e esteroides.

2.1.5.3 Óxido de Alumínio (Al_2O_3)

Também chamado de alumina, o óxido de alumínio é, depois do óxido de silício, o adsorvente mais utilizado. Este óxido possui características alcalinas e pode ser preparada para apresentar característica ácida e neutra. A alumina geralmente é empregada na separação de compostos lipofílicos e, pelo fato de poder ser preparada com característica ácida, neutra e alcalina, é bastante útil na separação de substâncias que apresentam várias dessas

características, como os hidrocarbonetos policíclicos, alcaloides, aminas e vitaminas lipossolúveis (COLLINS; BRAGA; BONATO, 2006).

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CAPÍTULO 3 - PRODUÇÃO DE PARTÍCULAS DE ÓLEO-RESINA DE COPAÍBA A PARTIR DE EMULSÕES ESTABILIZADAS COM AMIDOS MODIFICADOS

Production of Copaiba oleoresin particles from emulsions stabilized with modified starches

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Production of Copaiba oleoresin particles from emulsions stabilized with modified starches

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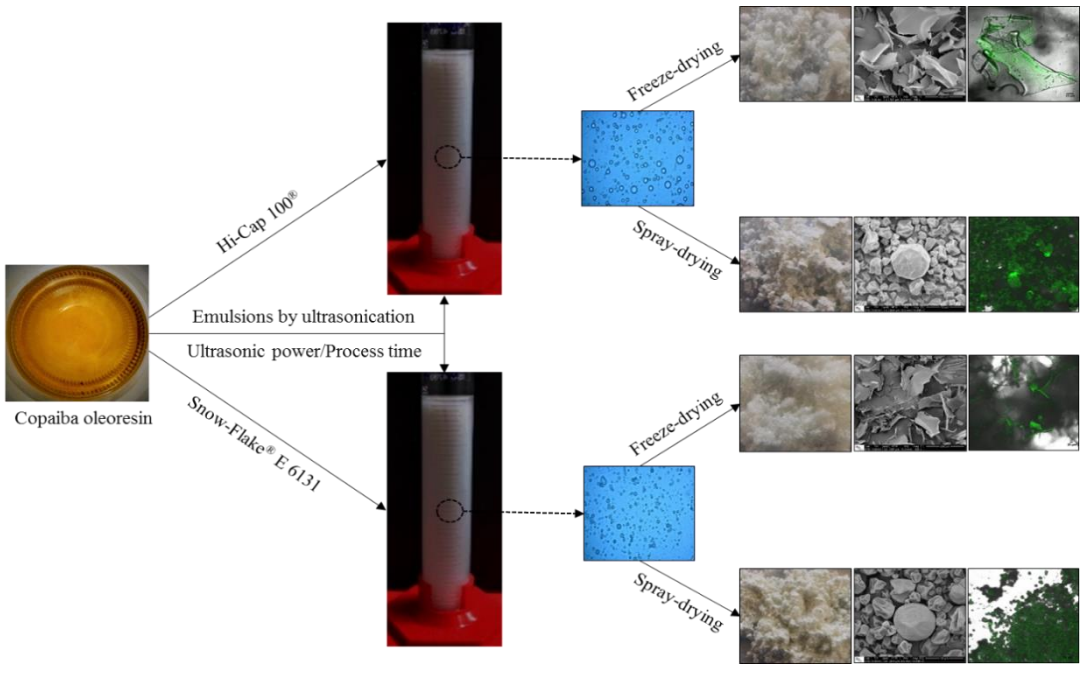
Highlights

1- The use of ultrasound yielded highly stable emulsions.
2- The obtained particles maintain the amorphous structure present in the biopolymers used.
3- The encapsulation process produced particles with high oxidative stability.
4- The surface of particles obtained by SD was smooth and without breaks.
5- Both polymers demonstrated good encapsulation efficiency.



ABSTRACT. The oleoresin of copaiba has many pharmacological properties, such as anti-inflammatory, antiseptic, and cicatrizing, besides helping to treat skin diseases, among other applications. In order to improve its activity, copaiba oleoresin was encapsulated within polymeric particles through emulsification assisted by ultrasound followed by a drying process. Ultrasound-assisted emulsification led to the formation of kinetically stable emulsions. The emulsions with increased stability and the lowest droplet diameters (80.95 ± 2.1 nm (Hi-Cap 100®) and 71.8 ± 2.9 nm (Snow-Flake® E 6131)) were subjected to freeze-drying and spray-drying to produce dry particles. The particles were characterized in terms of moisture content, particle size distribution, encapsulation efficiency, surface oil, oxidative stability, X-ray diffraction and morphological analysis by FESEM and CLSM. Both drying techniques produced amorphous particles with different sizes and low moisture content. Furthermore, high encapsulation efficiency and high stability against thermal degradation were achieved. The analysis of the particles' morphology indicated that the nature of the biopolymers used (Hi-Cap 100® and Snow-Flake® E 6131) did not affect their microstructure. However, as expected, the freeze-drying and spray-drying techniques produced particles with different external microstructures. SD particles were spherical, whereas FD particles presented irregular structures similar to sheets.

***Keywords*:** Biopolymer; Ultrasonic emulsification; Encapsulation; Copaiba; Scanning Electron Microscopy.

3.1 INTRODUCTION

The Brazilian rainforest is known for its exuberance and variety of tropical plants that provide alternative sources of therapeutic agents for the treatment of certain diseases. The copaiba tree (*Copaifera* sp.) exudes oleoresin, a clear smelly liquid with biological properties widely described in the literature (Veiga Junior et al., 2001). Copaiba oleoresins are mixtures of sesquiterpenes and diterpenes, which composition varied depending on the species (Cascon and Gilbert, 2000; Veiga Junior et al., 2007).

Due to the hydrophobic nature of copaiba oleoresin, its direct application on the skin may cause an unpleasant sensation. Therefore, the development of a topical dosage product may be beneficial. Oil-in-water emulsions (O/W) are widely used in the pharmaceutical industry for this purpose because they act as carriers, increasing the stability and retention of active compounds (Dias et al., 2014). According to McClements (2004), the main drawback for the industrial application of emulsions is their thermodynamic instability. Many physical-chemical mechanisms contribute to this instability, including density separation, coalescence, and flocculation. Depending on the droplet size, emulsions can be classified into micro- (10-100 nm), nano- (20-200 nm) and macro-emulsions (0.5-100 μm) (Henry et al., 2010). The terms micro-emulsion and nano-emulsion were first described by Schulman and Montagne (1961) and Calvo et al. (1996), respectively. According to McClements (2012), the difference among those terms is the stabilization principle of the emulsion. The kinetically stabilized emulsions are known as nano-emulsions, and those thermodynamically stabilized are called micro-emulsions. The main advantage of a nano-emulsion over a macro-emulsion is the high stability of its droplets against coalescence, phase separation or creaming (Anton et al., 2008).

According to Silva et al. (2015a), there are several methods to produce emulsions, such as high-energy emulsification methods including high-pressure, microfluidization and emulsification assisted by ultrasound. The use of ultrasound presents several advantages: i) it produces more stable emulsions with droplets of smaller size; ii) it requires minimal amounts of surfactant agents; iii) it is easy to operate, control and clean. The droplet size can be controlled through various process parameters, such as oil and emulsifier concentrations, oil/emulsifier ratio, viscosity of the continuous phase and emulsification time (Nakabayashi et al., 2011). The stability of emulsions can be improved by the addition of surfactants or biopolymers, which are often proteins or polysaccharides obtained from natural sources (Dickinson, 2009; Dokić et al., 2012).

Emulsions are widely used in pharmaceutical products for the delivery systems of certain ingredients by oral or topical (skin and eye) use. The main interest in the production of emulsions is to encapsulate an active hydrophilic or lipophilic component within the dispersed phase, thus ensuring its protection against environmental stress and degradation (oxygen, light, enzymes, acidity, etc.), and enabling a controlled release (Bouyer et al., 2012). According to de Paz et al. (2013), formulations based on emulsions have been particularly successful, since the emulsion allows controlling and reducing the particle size, therefore enhancing the encapsulation of particles in a polymeric material.

The drying of emulsions to obtain a particulate powder is frequently carried out through freeze-drying and spray-drying. The physical and chemical properties of the active compound are mandatory to select the drying technique to be applied (Silva et al., 2016). Furthermore, the selected technique will also have effect over the encapsulation efficiency, which is one of the most important quality parameters for encapsulation of bioactive compounds, since it indicates the relative amount of active compound that is protected within the polymeric matrix against adverse conditions that may cause its degradation. Finally, the presence of oil over the particle surface is undesirable, since it not only affects the powder wettability and dispersibility, but also makes the particles readily susceptible to oxidation and rancidity (Jafari et al., 2008).

Therefore, the objective of this work was to encapsulate the copaiba oleoresin within polymer matrices through emulsification assisted by ultrasound followed by freeze-drying (FD) or spray-drying (SD). Highly stable emulsions were obtained and the effects of ultrasonic power and sonication time on their mean droplet diameter were evaluated. Next, the emulsions with the smallest droplets were subjected to FD and SD to obtain dry particles.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and biopolymers used as emulsifiers

Copaiba (*Copaifera officinalis*) oleoresin was purchased from Ferquima Industria Trade Ltda, located in Vargem Grande – SP, Brazil. The oleoresin was stored in a hermetic dark-colored bottle at 5°C until the preparation of the emulsions.

The modified starches Hi-Cap 100® (HC) and Snow-Flake® E 6131 (SF), both derived from maize starch, were the biopolymers used to produce the emulsions. Both were

donated by Ingredion Brazil Industrial Ingredients Ltda. (Mogi Guaçu-SP, Brazil). HC (used in the encapsulation of oils and flavours), is a fine white powder that presents good resistance to oxidation and can form stable emulsions. SF (a light yellow powder with hydrophilic/lipophilic properties) is usually applied in the encapsulation of flavors. The polymers were characterized in terms of water content (3.45 ± 0.23% (HC) and 4.33 ± 0.05% (SF)) by the method 925.10 (AOAC, 1997), mineral residue (0.77 ± 0.05% (HC) and 0.24 ± 0.01% (SF)) by the method 972.15 (AOAC, 1997), protein content (0.28 ± 0.03% (HC) and 0.25 ± 0.02% (SF)) by the method 970.22 (AOAC, 1997), and total lipids (0,236 ± 0,004% (HC) and 0,482 ± 0.03% (SF)) using the method 963.15 (AOAC, 1997), with petroleum ether P.A - A.C.S (Synth, São Paulo, Brazil, Lot 185268) as solvent under reflux in Soxhlet apparatus.

3.2.2 Emulsions formation

3.2.2.1 Effect of formulation

Emulsions with several formulations were prepared following a Box-Behnken experimental design, in which the mean droplet diameter was the response variable. Experimental conditions were generated using the software Statistica (StatSoft - USA) and consisted of three central points and twelve different combinations of independent variables (Table 1), which were biopolymer concentration (g/L) (X_1), copaiba oleoresin concentration (mg/mL) (X_2) and the ethyl acetate/water proportion (%, v/v) (X_3), with levels based on the results of preliminary tests.

The emulsions were prepared according to the method described by Luther and Braeuer (2012), with some modifications. Table 1 presents the copaiba oleoresin and biopolymer concentrations for each experiment, as well as the ethyl acetate/water proportion. The total volume of each emulsion was fixed at 200 mL. The corresponding volumes of ethyl acetate and water solutions, as well as the masses of copaiba oleoresin and biopolymer, were calculated based on this volume. First, an aliquot of copaiba oleoresin was dissolved in the corresponding volume of ethyl acetate to obtain the concentration defined in the experimental design. In another flask, a mass of biopolymer (Hi-Cap 100® or Snow-Flake® E 6131) was dispersed in deionized water in an ultrasonic bath (Unique, model USC1450, Brazil) at room temperature, until complete dissolution to obtain the required biopolymer concentration. This biopolymer solution was let to rest for two hours at room temperature to ensure complete

saturation of the biopolymer molecules. Later, the corresponding proportions of these two solutions were mixed under magnetic stirring for 3 minutes to obtain a homogeneous dispersion. Finally, the dispersion was emulsified in ultrasound at 480 W during six minutes with an ultrasonic probe (Unique, Model DES500, Campinas, SP), as shown in (Fig. 1 (a)). The probe tip was submerged 1 cm below the liquid surface in all experiments.

The results were analyzed with a confidence level of 95% for the response variable (droplet mean diameter), generating a polynomial model (Equation (1)).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the response variable (mean droplet diameter); β_0 is an independent parameter; β_1, β_2 and β_3 are the regression coefficients of the linear effects; β_{11}, β_{22} and β_{33} are the terms of the quadratic effect and β_{12}, β_{13} and β_{23} are the terms of the interactions. The coefficients of the polynomial model without significant effect ($p > 0.05$) were eliminated to generate a reduced model. To assess whether the model properly explains the variation in the experimental data, the F significance test was performed.

3.2.2.2 Effect of ultrasonic power and sonication time

The emulsion formulations that provided the smallest droplet mean diameters for each biopolymer (experiments 04 (HC) and 12 (SF) – see Table 1) were selected for the second experimental design, which evaluated the effects of ultrasound power (160, 320, 480 and 640 W) and sonication time (2, 4, 6 and 8 minutes) on the mean droplet diameter. Each emulsion formulation was prepared totalizing 200 mL, as follows:

Experiment 04: 3.4 g of Hi-Cap 100® dissolved in 170 mL of deionized water, and 1.2 g of copaiba oleoresin dissolved in 30 mL of ethyl acetate.

Experiment 12: 2.4 g of Snow-Flake® 6131 dissolved in 160 mL of deionized water, and 1.6 g of copaiba oleoresin dissolved in 40 mL of ethyl acetate.

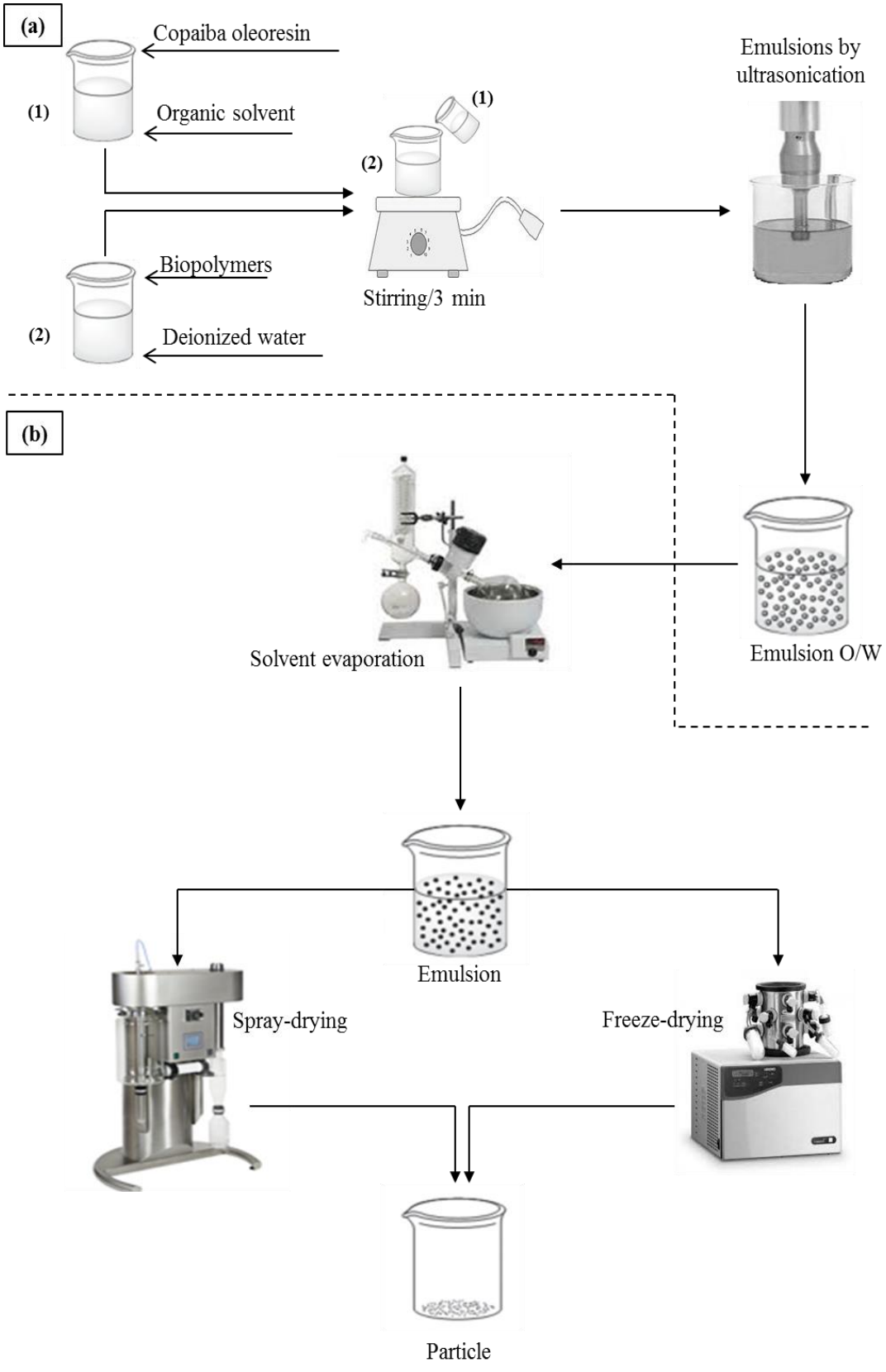


Fig. 1. Scheme diagrams: (a) emulsification assisted by ultrasound and (b) drying process by freeze-drying and spray-drying.

The results were statistically evaluated through analysis of variance (ANOVA), using the software Statistica (StatSoft - USA) in a confidence level of 95%.

3.2.3 Characterization of the emulsions

All the emulsions were characterized according to the mean droplet diameter, as described in Section 3.2.3.1. The emulsions with smallest mean droplet diameter were also evaluated in terms of physical stability and optical microscopy, as described in Sections 3.2.3.2 and 3.2.3.3, respectively.

3.2.3.1 Mean Droplet Diameter

The mean droplet diameters of the emulsions and suspensions were determined by light scattering (PCS) using a Zeta Potential Analyzer (Brookhaven Instruments Corporation, USA), which is a gauge of particle size by spreading of light, with a 15 mW power solid-state laser and wavelength of 675 nm. The software Bi-but Zeta Plus Particle Sizing version 2.27 was used for data collection. The droplet diameters were measured in triplicate.

3.2.3.2 Stability

Twenty-five milliliters of emulsion were transferred to tubes, which were sealed to avoid evaporation of ethyl acetate and subsequently stored in the dark at room temperature for 24 hours. The volume of the aqueous phase was measured every 30 minutes during the first two hours and, finally, 24 hours after the preparation of the emulsions. After the storage time, the emulsion stability was evaluated by calculating the creaming index (*CI*) (Ye and Singh, 2006), defined by the ratio between the height of the serum phase of the emulsion (h_s) and the initial height (h_e), as shown in Equation (2).

$$CI\ (\%) = \left(\frac{h_s}{h_e}\right) \times 100 \qquad (2)$$

The determination of *CI* was performed in triplicate, and the results were expressed as mean ± standard deviation.

3.2.3.3 Optical microscopy

The emulsion microstructure was evaluated by optical microscopy, using a Carl Zeiss microscope (model Axio Scope A1, Göttingen, Germany). Aliquots of the emulsions were placed on glass slides, covered with coverslips, and then visualized with 100x magnification lens, operating under oil immersion.

3.2.4 Drying of the emulsions

The emulsions with the smallest droplets (experiment 04 (HC) and experiment 12 (SF)) were dried to obtain solid particles. Drying was performed in two steps: 1) complete evaporation under vacuum of ethyl acetate in a rotary evaporator (Marconi, Model MA 120/E, Campinas, SP) at 45 °C; 2) water removal through freeze-drying and spray-drying (Fig. 1 (b)).

3.2.4.1 Freeze-drying (FD)

The emulsions were frozen in glass vials in a domestic freezer (Metalfrio DA420, Sao Paulo, SP, Brazil) at -18 °C for 24 hours and then submitted to FD at -40 °C for 72 hours in a lyophilizer (Liobras, L 101, São Carlos-SP, Brazil). Experimental tests were carried out in duplicate.

3.2.4.2 Spray-drying (SD)

A laboratory scale spray dryer was used (mini spray dryer) (Mark Labplant UK Ltd, model SD 06, United Kingdom), which has a drying chamber of 215 mm × 500 mm and a double fluid type nozzle atomizer (0.5 mm inner diameter). The emulsion was pumped into the drying chamber by a peristaltic pump, at 25 °C. The drying air flow rate was 300 m^3/h, the compressed air pressure was 4 bar and the compressed air flow rate was 1.7 m^3/h. The drying air temperature was set at 170 °C and outlet air temperature was 90 °C. The emulsion flow rate (11.6 mL/min) was defined as the maximum flow rate in which there was no dripping from the sample in the atomization chamber of atomization.

3.2.5 Particle characterization

3.2.5.1 Moisture content

The particle moisture content was determined according to the method 925.10 (AOAC, 1997), based on the removal of water by heating. The particles were placed on previously weighed glass plates, and oven dried under forced air circulation (model NT 395-1, Tecnal, São Paulo, SP) at 60°C for 4 hours or until constant mass was achieved.

3.2.5.2 Particle size distribution

The particle size distributions and their mean diameter were determined by laser diffraction on a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, United Kingdom). The calculation of the mean particle diameter was based on the mean diameter of a sphere of the same volume, named the diameter of De Brouckere $D_{[4.3]}$, as shown in Equation (3). The samples were analyzed in quintuplicate, by wet method, dispersed in ethanol (99.5%).

$$D_{[4,3]} = \frac{\sum n_i {d_i}^4}{\sum n_i {d_i}^3} \quad (3)$$

Where d_i is the particle diameter and n_i is the number of particles.

3.2.5.3 Encapsulation efficiency and surface oil

The oleoresin amount on the particle surface was quantified using the method described by Bae and Lee (2008), with some modifications. 20 mL of hexane were added to 0.1 g of particles in a glass jar with lid and stirred manually for two minutes at room temperature, to extract the superficial oil. The extract was filtered in a nylon membrane (Chromafil® Xtra PA-20/25 0.20 µm). The powder retained on the filter was washed three times with 20 mL hexane, and the solvent with extracted oleoresin was collected for further treatment. Since the biopolymers are insoluble in hexane, the particles keep unbroken and, therefore, the encapsulated oleoresin remains inside the particle and only the external oleoresin is driven away by hexane. Next, the solvent was removed in a rotary evaporator under vacuum (Marconi,

Model MA 120/E, Campinas, SP), to determine the non-encapsulated oleoresin (surface oil). The encapsulation efficiency (EE) was calculated according to Equation (4).

$$EE\ (\%) = \frac{(Oil_{Total} - Oil_{Surface})}{Oil_{Total}} x\ 100 \quad (4)$$

Where: Oil_{Total} is the oil total mass used to prepare the emulsion (g) and $Oil_{Surface}$ is the mass of oil extracted from the particles (g).

3.2.5.4 Oxidative Stability

The oxidative stabilities of the particles and copaiba oleoresin were evaluated through the accelerated Rancimat method (Läubli and Bruttel, 1986). Samples were subjected to an air flow of 20 L/h at 120 °C in a 873 Biodiesel Rancimat device (Metrohm, Herisau, Switzerland).

3.2.5.5 X-ray diffraction (XRD)

The X-ray diffraction patterns of the particles and polymers were obtained in a diffractometer Shimadzu XRD-7000 (Tokyo, Japan). The analyses were carried out with radiation source from Cu-K_α (λ = 1.5406 Å), operating at 40 kV/30 mA, and the angle (2θ) was scanned from 5 to 50° at a 2 °/min rate.

3.2.5.6 Field Emission Scanning Electron Microscopy (FESEM)

The morphology of the particles was analyzed in a scanning electron microscope equipped with a field emission gun (FESEM - FEI Quanta 650, USA). Prior to analysis, the samples were coated with gold in a SCD 050 sputter coater (Oerlikon-Balzers, Balzers, Liechtenstein). Both equipments were available at the National Laboratory of Nanotechnology (LNNano), located in Campinas-SP/Brazil. Analyses of the sample surfaces were carried out under vacuum, using a 5 kV acceleration voltage and a large number of images was obtained on different areas of the samples (at least 20 images per sample) to assure the reproducibility of the results.

3.2.5.7 Confocal Laser Scanning Microscopy (CLSM)

The particles were also analyzed in the National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC) at the State University of Campinas, using a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) using a 40× objective. The images were measured at a wavelength of 488 nm, without previous preparation of the samples.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of the emulsions

3.3.1.1 Mean diameter of the emulsion droplets

3.3.1.1.1 Effect of formulation

The models generated for the mean diameter of droplets were properly adjusted considering only the significant terms (Equations (5) and (6)). The adjusted parameters presented correspond to the encoded model, thus the variables do not assume their real values. The determination coefficients for the droplet mean diameters were 98% and 99% for HC and SF, respectively. The F test was highly significant for both, confirming that the models provide statistical significance and can be used for predictive purposes.

$$Y_{(HC)} = 117.3333 - 30.2000\,X_2 - 46.9688\,X_3 + 13.5396\,X_1^2 - 15.5729\,X_2^2 + 54.9896\,X_3^2 - 6.9250\,X_1X_2 - 8.5125\,X_1X_3 + 16.1250\,X_2X_3 \quad (5)$$

$$Y_{(SF)} = 94.9000 + 4.6375\,X_1 - 27.1812\,X_2 - 58.5438\,X_3 + 21.5375\,X_1^2 + 48.2000\,X_3^2 - 13.6875\,X_1X_2 - 5.9315\,X_1X_3 + 10.4250\,X_2X_3 \quad (6)$$

Equations (5) and (6) show how the different factors affect the droplet size. The biopolymer concentration (X_1) had a positive linear effect only for SF, showing that the droplet size increases with the increase of concentration. This could be assigned to the presence of an

excess of biopolymer within the emulsion, which could be deposed over the droplets surface, thus forming droplets with thicker polymer surface layers and increased in size. Oleoresin concentration (X_2) had a negative linear effect, meaning that if the concentration increases, the droplet size decreases. This reinforces the hypothesis suggested for the biopolymer concentration. An addition in oleoresin concentration would require a greater amount of biopolymer to cover the droplet interface and to stabilize the emulsion. Therefore, less biopolymer would be available to be deposed over the droplet surface, resulting in smaller droplets. Regarding the ethyl acetate/water proportion (X_3), a negative effect was found, meaning that if this proportion increases, the droplet size decreases. This effect agrees with the hypotheses related for X_1 and X_2. The interaction effects reveal a similar behavior. The effect X_1X_2 was negative, so both factors must vary in the same direction to reduce the droplet size. This means that if there is not enough biopolymer to encapsulate the oleoresin, the droplet size will increase to reduce the interfacial area and the instability of the emulsion. The interaction effect X_1X_3 was negative, showing that if both factors vary on the same direction, the result will be the droplet reduction. This suggests that when the biopolymer concentration increases, the emulsion will be able to absorb a greater proportion of the oleoresin solution, due to the existence of more biopolymer available to encapsulate the oleoresin. The combined effect X_2X_3 was positive. Therefore, if one of the factors increases and the other decreases the result will be the droplet reduction. If the oleoresin concentration and the ethyl acetate/water proportion increase, the consequence will be an increase of the amount of oleoresin and a reduction of the biopolymer amount in the emulsion, thus there will not be enough biopolymer to encapsulate the oleoresin and the emulsion will be stabilized with a greater droplet size.

Table 1 shows the droplet size obtained experimentally and predicted by the model. The smallest droplet diameter in the emulsions stabilized with HC (experiment 04) and SF (experiment 12) were 80.95 nm (± 2.1) and 71.8 nm (± 2.9), respectively.

The polydispersity indexes (PDI) are also shown in Table 1. The emulsions PDI were smaller than 0.110 ± 0.003, showing little variation in the mean droplet size and thus the formation of a quite homogeneous system. Therefore, PDI below 0.2 indicates homogeneity, whereas values higher than 0.3 indicate heterogeneity of droplet sizes (Dias et al., 2012).

Table 1. Experimental Box-Behnken design and experimental results obtained in the preparation of the emulsions.

Experiment	Independent variables			Droplet mean diameter (nm)					
				Hi-Cap 100®			Snow-Flake® E 6131		
	X_1 (g/L)	X_2 (mg/mL)	X_3 (%, v/v)	Observed ($Y_{(HC)}$)	Predicted	PDI	Observed ($Y_{(SF)}$)	Predicted	PDI
01	-1 (10)	-1 (20)	0 (15)	135.8 ± 6.5	138.58	0.005 ± 0.000	122.4 ± 0.8	125.29	0.005 ± 0.000
02	+1 (20)	-1 (20)	0 (15)	147.9 ± 0.4	152.43	0.059 ± 0.005	172.6 ± 2.9	161.94	0.052 ± 0.005
03	-1 (10)	+1 (40)	0 (15)	96.55 ± 0.8	92.03	0.005 ± 0.000	91.35 ± 1.3	98.31	0.005 ± 0.000
04	+1 (20)	+1 (40)	0 (15)	80.95 ± 2.1	78.18	0.005 ± 0.000	86.85 ± 2.6	80.21	0.083 ± 0.006
05	-1 (10)	0 (30)	-1 (10)	212.9 ± 0.71	224.32	0.033 ± 0.002	217.7 ± 0.6	212.61	0.005 ± 0.000
06	+1 (20)	0 (30)	-1 (10)	235.45 ± 1.8	241.34	0.005 ± 0.000	225.3 ± 2.2	233.76	0.005 ± 0.000
07	-1 (10)	0 (30)	+1 (20)	153.3 ± 0.4	147.41	0.005 ± 0.000	112.2 ± 2.7	107.39	0.110 ± 0.003
08	+1 (20)	0 (30)	+1 (20)	141.8 ± 0.6	130.38	0.005 ± 0.000	96 ± 4	104.79	0.005 ± 0.000
09	0 (15)	-1 (20)	-1 (10)	262.35 ± 1.3	250.04	0.015 ± 0.001	238.95 ± 2.3	239.25	0.037 ± 0.004
10	0 (15)	+1 (40)	-1 (10)	162.4 ± 2.6	157.39	0.005 ± 0.000	167.75 ± 3.3	164.04	0.094 ± 0.002
11	0 (15)	-1 (20)	+1 (20)	118.9 ± 5.3	123.86	0.094 ± 0.007	101.3 ± 1.9	101.31	0.005 ± 0.000
12	0 (15)	+1 (40)	+1 (20)	83.4 ± 2.4	95.71	0.005 ± 0.000	71.8 ± 2.9	67.80	0.005 ± 0.000
13	0 (15)	0 (30)	0 (15)	117.3 ± 0.5	117.33	0.005 ± 0.000	94.9 ± 1.9	94.90	0.005 ± 0.000
14	0 (15)	0 (30)	0 (15)	118.3 ± 0.6	117.33	0.005 ± 0.000	92.25 ± 4.3	94.90	0.005 ± 0.000
15	0 (15)	0 (30)	0 (15)	116.5 ± 2.7	117.33	0.005 ± 0.000	90.2 ± 3.5	94.90	0.005 ± 0.000

Results are expressed as mean ± standard deviation of the analysis. X_1 = biopolymer concentration; X_2 = copaiba oleoresin concentration; X_3 = ethyl acetate/water proportion; PDI = polydispersity index.

3.3.1.1.2 Effect of ultrasonic power and sonication time

Table 2 shows the mean droplet diameters of the emulsions stabilized with HC and SF in different ultrasonic powers and sonication times. The droplet size was significantly influenced ($p < 0.05$) by ultrasonic power, sonication time and their interaction.

Table 2. Effect of ultrasonic power and sonication time on the mean diameter of the emulsion droplets.

Ultrasonic power (W)	Time (min)	Droplet mean diameter (nm)	
		Hi-Cap 100® *(Experiment 04)*	Snow-Flake® E 6131 *(Experiment 12)*
160	2	389.9 ± 2.1	157.8 ± 0.3
	4	294.8 ± 8.3	131.8 ± 0.9
	6	136.8 ± 1.8	111.45 ± 0.21
	8	116.1 ± 5.3	100.5 ± 1.8
320	2	112.5 ± 1.8	81.9 ± 1.5
	4	109.5 ± 2.1	79.15 ± 0.92
	6	95.95 ± 0.07	73.9 ± 1.1
	8	102.95 ± 4.45	75.75 ± 0.21
480	2	99.3 ± 1.5	76.6 ± 1.4
	4	93.4 ± 2.8	76.35 ± 1.91
	6	80.95 ± 2.1	71.8 ± 2.9
	8	101.2 ± 1.3	78.4 ± 0.7
640	2	102.8 ± 0.6	77.4 ± 1.6
	4	88 ± 6.8	74.45 ± 1.34
	6	82.7 ± 4.7	72.4 ± 2.4
	8	113.2 ± 1.7	79.5 ± 0.9

The smallest droplet diameter was observed in the emulsions produced at 480 W and 6 minutes of sonication. According to Kentish et al. (2008), the droplet size decreases with the increase of shear stress generated by the applied power. A decrease in the droplet size was also observed with increasing sonication times, as expected. Similar behavior was observed by Leong et al. (2009) in nano-emulsions from sunflower oil.

As shown in Fig. 2 (a) and (b), the emulsions stabilized with HC and SF show similar behavior in terms of the effect of process time on the droplet diameter.

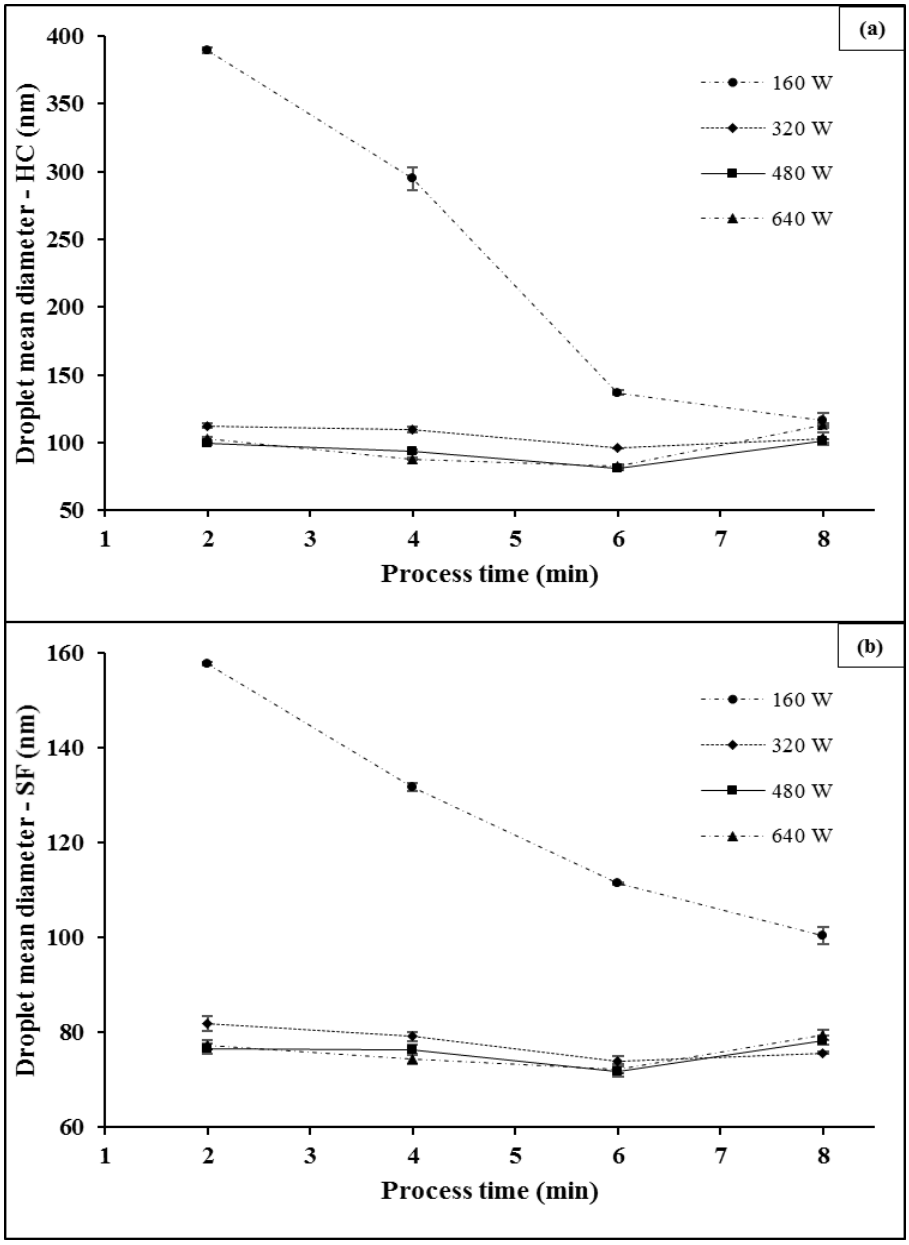


Fig. 2. Influence of ultrasonic power and sonication time on the mean diameter of emulsion droplets stabilized with: (a) HC and (b) SF.

For example, to achieve the smaller diameter of the droplets at 160 W, 8 minutes of sonication were required. On the other hand, when the ultrasonic power is increased to 320, 480 and 640 W, the smallest droplet diameters were achieved in 6 min of sonication. However, with 8 min of sonication the droplet size increased, which means that higher power applied to

the system affects negatively the droplet size. In other words, an excessively intense sonication process can increase the emulsion droplet size due to coalescence of smaller droplets, in a phenomenon known as “excess processing”. Therefore, there is a limit on process conditions to achieve the smallest droplet diameter. According to Pangu and Feke (2004), ultrasonic radiation forces, known as Bjerknes forces, increase with ultrasound power, causing drag of the droplets to nodes (part that does not vibrate) and antinodes (part that vibrates) of the sound field. The proximity of the droplets in these positions increases their coalescence, and therefore the excess processing is observed. These results confirmed that the reduction in emulsion droplet size does not depend only on the energy amount supplied to the system or sonication time. It also depends on the physicochemical properties of the encapsulation material. The results obtained in this work are in agreement with those observed by Abbas et al. (2014) and Jafari et al. (2007) for systems emulsified with ultrasound.

3.3.1.2 Emulsion stability

The modified starches HC and SF exhibit high emulsifying capacity, due to the presence of lipophilic groups in their structures, thus improving the stability of emulsions. The emulsions stabilized with Hi-Cap 100® (experiment 04) did not show phase separation after 24 hours, indicating high stability. The emulsions prepared with Snow-Flake® E 6131 (experiment 12) did not show phase separation after 120 minutes, but after 24 hours phase separation was perceived, evidencing a more instable system (CI = 6.0 ± 0.0%). The higher stability of HC emulsions may be related to the higher concentration of biopolymer, which increases the viscosity of the continuous phase, thus reducing the mobility of the droplets and the probability of coalescence.

These results also suggest that smaller droplet diameters do not necessarily lead to high stability, as noted in the emulsions stabilized with SF. The trend to aggregate can be higher in systems containing smaller droplets, which are more susceptible to the influence of Brownian motion. Besides, the same volume of liquid distributed in small droplets results in a large number of droplets. Both effects increase the probability of collisions that may result in coalescence and instability. Finally, Ostwald ripening also has an important effect on very small droplets, favoring their dissolution and formation of larger droplets. In fact, this statement coincides with the result observed in the emulsion stabilized with SF. Biopolymers, such as proteins and polysaccharides, are good examples of natural emulsifiers currently used to

stabilize emulsions in the pharmaceutical industry (Bouyer et al., 2012), since they help increasing the viscosity of the aqueous phase and prevent creaming of the oil droplets (McClements, 2000).

3.3.1.3 Optical microscopy of the emulsion droplets

Fig. 3 shows the images obtained by optical microscopy of the emulsions before evaporating ethyl acetate ((a) and (b)) and of the suspensions obtained after evaporation ((c) and (d)). The images show that, before evaporation, the emulsion droplets are more dispersed in the aqueous phase, whereas after the solvent evaporation the droplets have smaller diameter and are more concentrated in the aqueous phase.

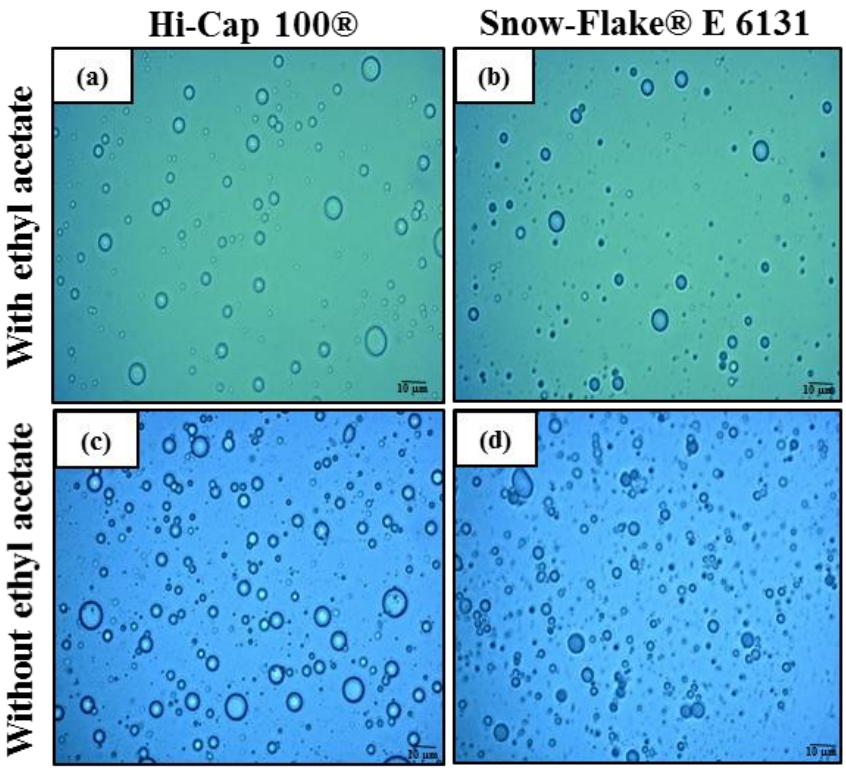


Fig. 3. Optical microscopy of the copaiba oleoresin emulsions before and after evaporation of ethyl acetate.

The objective of the solvent evaporation was to eliminate the ethyl acetate that was present in the emulsion. However, part of the water was also evaporated, leading to a reduction of the continuous phase, which achieves higher concentration of particles.

The high stability of the emulsions may have prevented the phase coalescence after the evaporation of ethyl acetate, thus avoiding the increase of particle size. In fact, the droplet size decreased because of the ethyl acetate evaporation, from 81.0 ± 2.1 to 46.2 ± 1.6 nm for experiment 04 (HC) and from 71.8 ± 2.9 to 18.9 ± 0.1 nm for experiment 12 (SF), indicating that part of the ethyl acetate was inside the particles. Therefore, the particle size is also controlled by the displacement of the solvent and its evaporation.

According to Li et al. (2008), along the droplets solidification of the dispersed phase, the microspheres shrink due to the solvent removal, which includes the diffusion of the solvent from the inner parts of the droplet to the interphase between dispersed and continuous phases. Therefore, the change in droplet size is a direct consequence of the solvent evaporation.

3.3.2 Particle characterization

3.3.2.1 Moisture content

The water content was higher in the particles obtained by spray-drying than with freeze-drying (Table 3), for both biopolymers. This difference may be caused by the high temperature used in the SD process. According to Frascareli et al. (2012), this can occur due to the fast formation of a crust, which limits the water diffusion inside the particle, therefore reducing water evaporation and resulting in particles with higher moisture content. Other works report the same behavior in the encapsulation of annatto seed oil using protein isolate and modified starch (Silva et al., 2016), ginger essential oil using cashew gum and inulin (Fernandes et al., 2016) and encapsulation of annatto seed oil using arabic gum as stabilizing agent (Silva et al., 2015b).

3.3.2.2 Particle size distribution

The drying method had a determining effect on the particle size. Table 3 shows that the particles obtained by SD had smaller mean diameter than those obtained by FD, regardless of the emulsion formulation used. Moreover, there was not significant difference between the mean diameters of the particles obtained by SD.

Table 3. Particle characterization: moisture, particle diameter, encapsulation efficiency and surface oil.

Particle	Moisture (%)(*)	Particle diameter (µm)(*)	Encapsulation efficiency (%)	Surface oil (%)
Hi-Cap 100® - FD	0.62 ± 0.05[a]	246.01 ± 3.40[a]	76.8 ± 0.3	23.2 ± 0.3
Hi-Cap 100® - SD	1.24 ± 0.01[b]	12.21 ± 0.66[b]	83.41 ± 1.01	16.59 ± 1.01
Snow-Flake® E 6131 - FD	0.95 ± 0.08[c]	212.28 ± 6.01[c]	86.8 ± 0.5	13.2 ± 0.5
Snow-Flake® E 6131 - SD	3.29 ± 0.04[d]	12.01 ± 0.07[b]	89.9 ± 0.1	10.1 ± 0.1

Results are presented as mean ± standard deviation. (*) Equal letters equal in the same column indicate no significant difference at the level of 5% by the Tukey test.

Regardless the coating material, the particle size distribution was bimodal for SD and monomodal for FD (Fig. 4). The graphical representation of the distribution allows observing the volume percentage of each size range observed in the particles. Similar result was observed by Silva et al. (2016) in the microencapsulation of annatto seed oil by SD and FD using Snow-Flake® E 6131 and whey protein isolate as encapsulating materials.

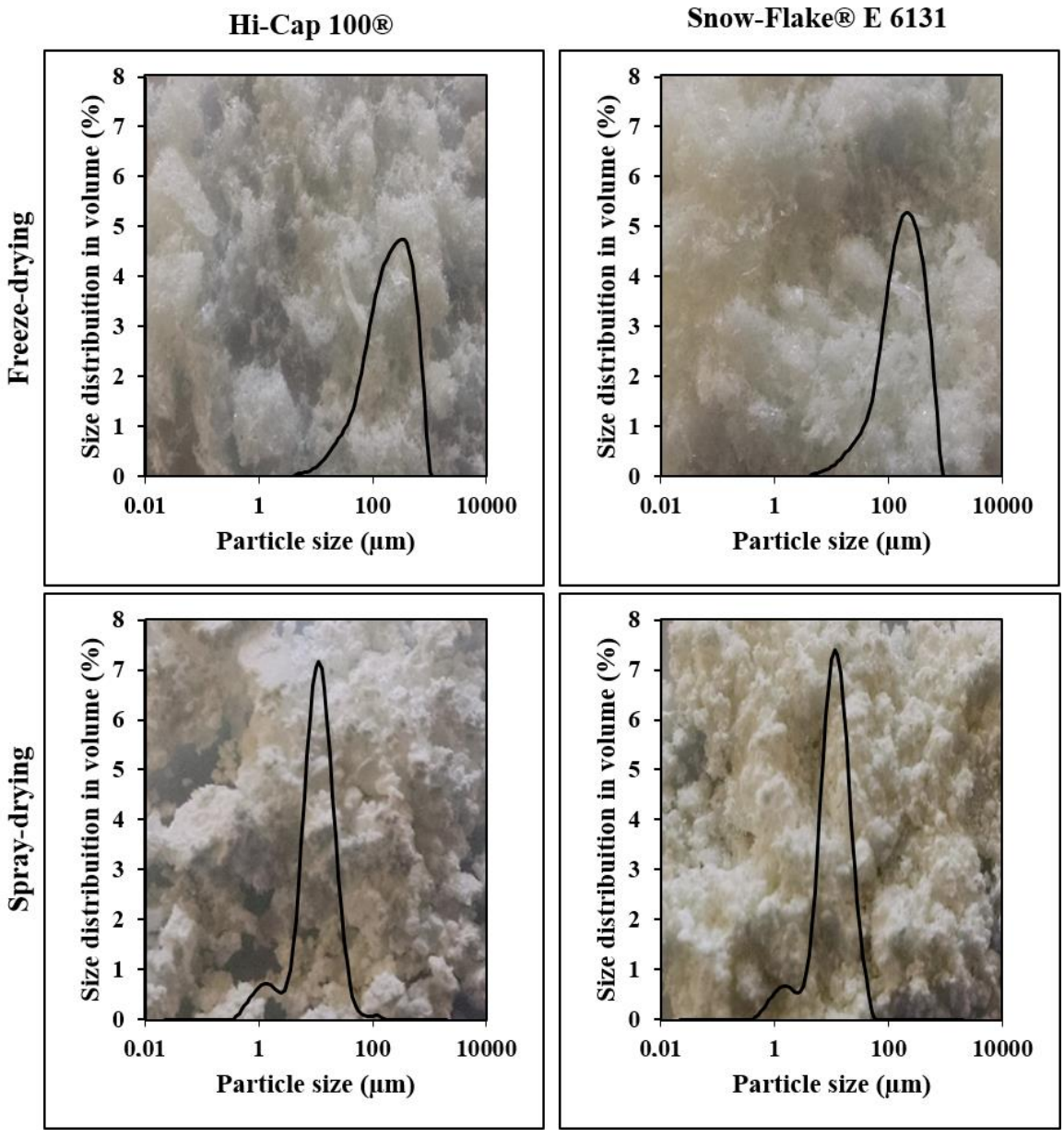


Fig. 4. Particle size distributions obtained by freeze-drying and spray-drying using Hi-Cap 100® and Snow-Flake® E 6131 as encapsulating agents.

3.3.2.3 Encapsulation efficiency and surface oil

Table 3 shows the surface oleoresin percentages and the encapsulation efficiencies as functions of the polymer type and drying technique. The particles obtained from the emulsions stabilized with Hi-Cap 100® had more free oleoresin on the surface and a lower encapsulation efficiency than those obtained from the emulsions stabilized with Snow-Flake® E 6131. However, when comparing both drying techniques, the particles obtained by FD showed higher free oil content and lower encapsulation efficiency than those obtained by SD. This can be related to the formation of ice crystals during freezing, which contribute to the rupture of the emulsion droplets, thus releasing oil from the particle to the surface (Silva et al., 2016). Similar results were found by Anwar and Kunz (2011) and Silva et al. (2015b) when comparing both techniques

3.3.2.4 Oxidative Stability

The oxidation curves obtained by the Rancimat method for the particles and pure copaiba oleoresin are expressed as conductivity against time (Fig. 5).

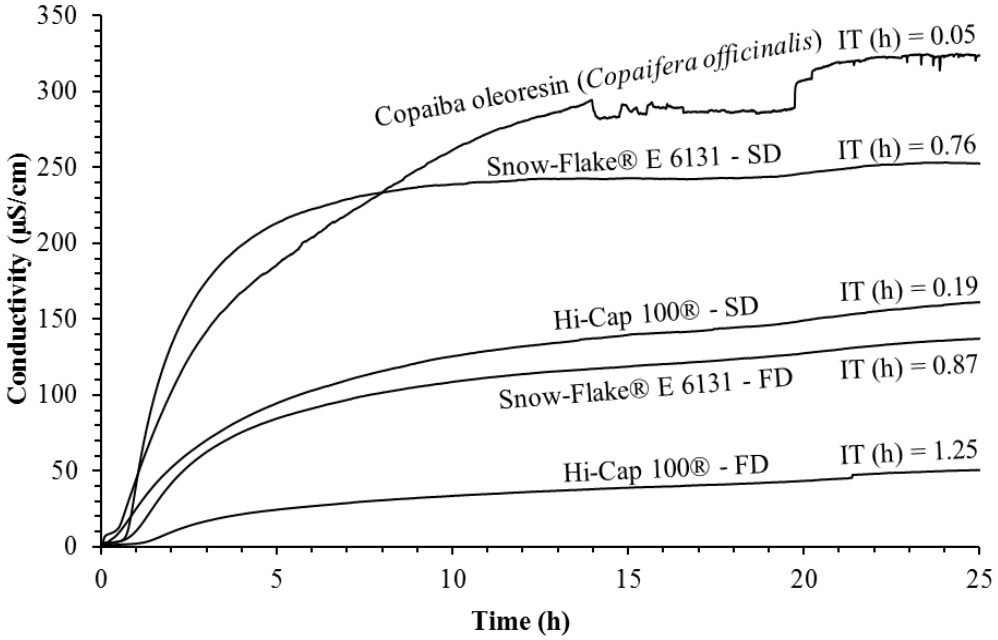


Fig. 5. Oxidation curves (conductivity versus time) obtained from the Rancimat before and after encapsulation the copaiba oleoresin.

The conductivity was influenced by the release of volatile compounds present in the copaiba oleoresin and, therefore, the observed induction times (IT) were low. The induction time can be defined as the time required to achieve the inflection point of the curve of conductivity versus time, and greater oxidative stability is related to higher induction time (Velasco et al., 2009). Greater induction times result in increased oxidative stability, since this time corresponds to a response of the process of formation of oil oxidation compounds due to air flow and temperature. It can be noted that the pure copaiba oleoresin has higher oxidation rate than the encapsulated oleoresin, reaching conductivities of 310 µS/cm. The particles obtained by FD were more stable than those obtained by SD, regardless of the biopolymer used. Similar result was observed by Silva et al. (2015b) in the encapsulation of annatto seed oil. This was already expected due to the aggressive heat treatment suffered by the oleoresin during SD, which accelerates the oxidative processes. Overall, it can be concluded that the encapsulation process of copaiba oleoresin was effective to provide greater oxidative stability to the particles.

3.3.2.5 X-ray diffraction (XRD)

X-ray diffraction was used to evaluate the crystallinity degree of the copaiba oleoresin particles in comparison to the pure encapsulation agents. In general, crystalline materials present well defined sharp XRD peaks, whereas amorphous materials generally have wide bands, because the molecules in the amorphous state are disordered (Wu et al., 2011). Amorphous materials are usually more soluble and, therefore, more hygroscopic than crystalline materials (Yu, 2001). According to Zobel (1988), the starch crystalline region is related uniquely to the provision of the double helix of amylopectin, whereas the amorphous region can be mostly attributed to amylose. Fig. 6 shows that, at their natural state, HC and SF behave as amorphous and semi-crystalline materials, respectively. After the drying process, both particles showed characteristics of amorphous materials. So, the particles produced with HC exhibited the same characteristic of pure HC, while the particles prepared by SF were amorphized during drying. Such change may reflect the interaction between modified starch and copaiba oleoresin, water and ethyl acetate. However, most water and ethyl acetate are evaporated during the drying process. Therefore, copaiba oleoresin is the main responsible for the changes on the morphological state of SF. Thus, we could assume that the copaiba oleoresin was present and interacting with the biopolymer inside the dried particle. Similar results were obtained during encapsulation of annatto seed oil (Silva et al., 2016) and oregano extract (Zabot

et al., 2016). The change from crystalline to amorphous state may also be affected by the drying process. When the modified starches are solubilized in water and then dried, they may change their structure according to the drying conditions, producing solids with different characteristics (Ahmed and Lelièvre, 1978).

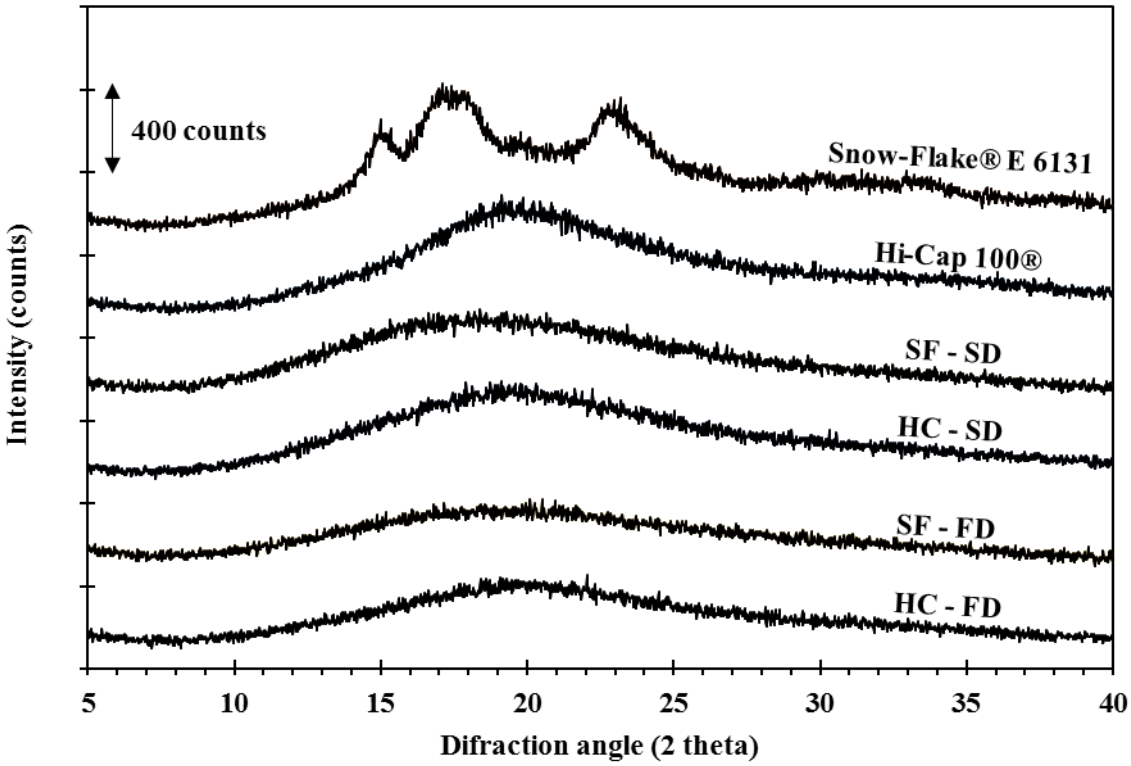


Fig. 6. X-ray diffraction of Hi-Cap 100® (HC), Snow-Flake® E 6131 (SF) and the copaiba oleoresin particles produced by SD and FD.

3.3.2.6 Field Emission Scanning Electron Microscopy (FESEM)

3.3.2.6.1 Particle external microstructure

Fig. 7 (a) and (b) shows the surface morphology of the structures obtained by freeze-drying. Regardless of the encapsulation material, irregular and lightweight structures, similar to sheets, are observed. These structures are formed by agglomeration and coalescence of particles during the freeze-drying process. Similar result was observed in particles of pepper oleoresin (Aguiar et al., 2016) and annatto seed oil (Silva et al., 2016) after freeze-drying.

Fig. 7 (c-d) shows the surface morphology of the particles obtained by SD, which is notably different from those obtained by FD. In general, the particles produced by SD had spherical shape of different sizes and rough surface without fissures, which is important to provide lower permeability to gases, better protection and core retention. Moreover, wrinkled surfaces result from solvent evaporation, through the contraction of the droplets during the drying process. The roughness observed on the surface of the microcapsules is greater in smaller particles, suggesting that the solidification of the walls takes place before the expansion of the microcapsules (Gharsallaoui et al., 2011). Similar results concerning the morphological analysis were reported in the microencapsulation of green coffee oil (Carvalho et al., 2014), beet juice (Janiszewska, 2014), D-limonene (Soottitantawat et al., 2005), jussara pulp (da Silva Carvalho et al., 2016), cumin oleoresin (Kanakdande et al., 2007) and in the microencapsulation of monoterpenes (Bertolini et al., 2001), all produced by spray drying. Some particles produced by SD showed small holes on their surfaces, indicated by the arrows in Fig. 7 (c) and (d). According to Jafari et al. (2007), these small holes are probably formed in the late drying stages and may result from low viscoelastic properties of encapsulation materials.

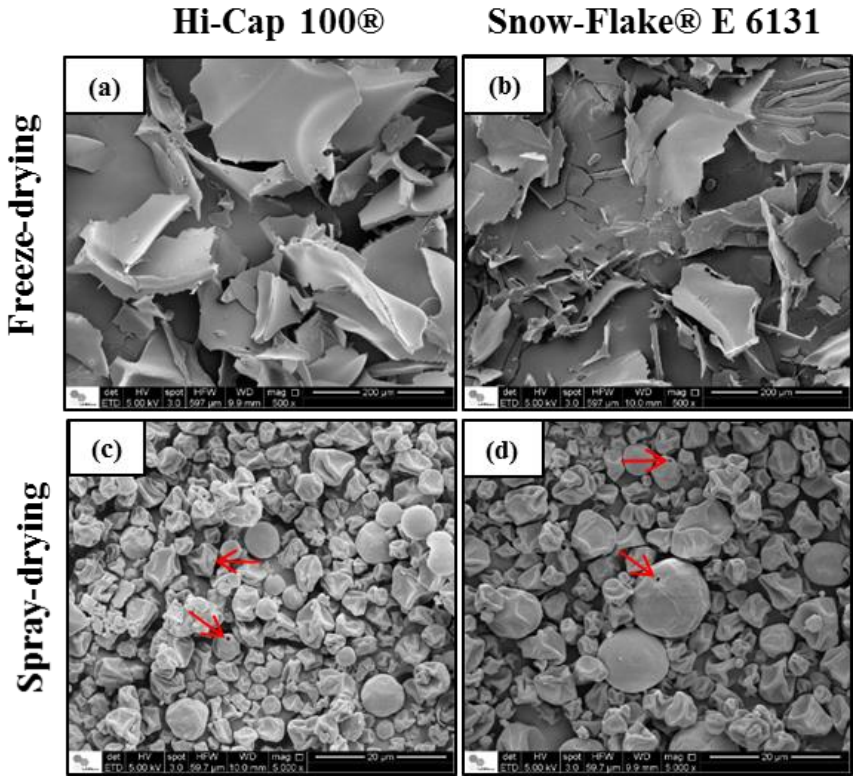


Fig. 7. External microstructure the copaiba oleoresin particles obtained by freeze-drying (500× (a) and (b)), and spray-drying (5000× (c) and (d)) of magnification.

3.3.2.6.2 Particle internal microstructure

The particles obtained by FD presented cracks in the walls, revealing small pores (Fig. 8 (a) and (b)). These pores seem to be reminiscent of the previous droplets forming the emulsion and those agglomerated during the freezing and drying processes. The inner parts of the droplets, previously containing copaiba oil, appear to be empty now, but the droplet remains spherical, as can be observed in Fig. 8 (a) and (b). Similar morphologies were observed for microcapsules of annatto seed oil (Silva et al., 2016), and during the encapsulation of limonene with Arabic gum, gelatin, and sucrose (Kaushik and Roos, 2007). The internal structures of the particles obtained by SD reveal empty cavities where the active compound possibly became trapped, leading to a reservoir type microencapsulated system (Fig. 8 (c) and (d)). These empty cavities could be the result of the desorption of gases present in the emulsion during drying and subsequent expansion of these gases. Other reasons may be the formation of vapor bubbles inside the particles after the formation of the solid surface, or even the air incorporation inside the emulsion droplets during SD (Rosenberg et al., 1990).

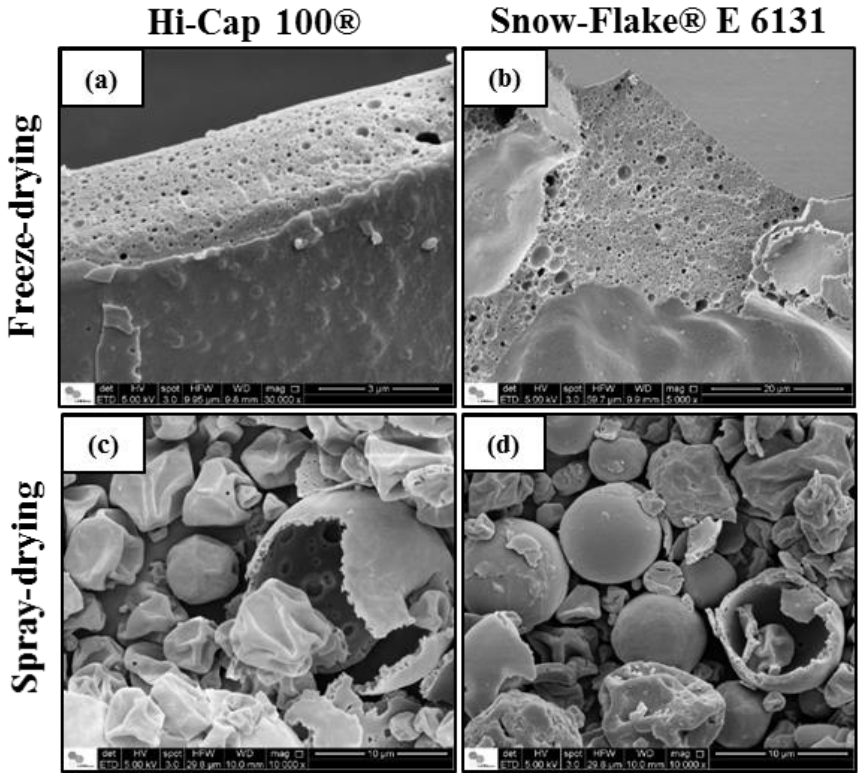


Fig. 8. Internal microstructure the copaiba oleoresin particles, obtained by freeze-drying (30000× (a) and 5000× (b)), and spray-drying (10000× (c) and (d)) of magnification.

The particles, possibly containing the active material, have small pores uniformly embedded in the matrix of the encapsulation material, suggesting that the active material is dispersed and retained in the form of small spherical droplets on the inner surface. Researches conducted by Teixeira et al. (2004) and Carvalho et al. (2014) have also reported the presence of oil inside the microsphere walls in the form of small holes, besides empty cavities inside the particles, which typically result from the expansion of air bubbles incorporated within the microsphere droplets.

3.3.2.7 Confocal laser scanning microscopy (CLSM)

Fig. 9 shows the images of the copaiba oleoresin particles obtained by CLSM. This technique is not destructive, since it allows the observation of the internal structure of the particles without causing fragmentation of the active material (Strand et al., 2003). The CLSM images confirm the morphological results obtained by FESEM. The green regions represent the distribution of active material (copaiba oleoresin) within the structures of the particulate systems, thus confirming the encapsulation of copaiba oleoresin in polymeric matrices by FD and SD.

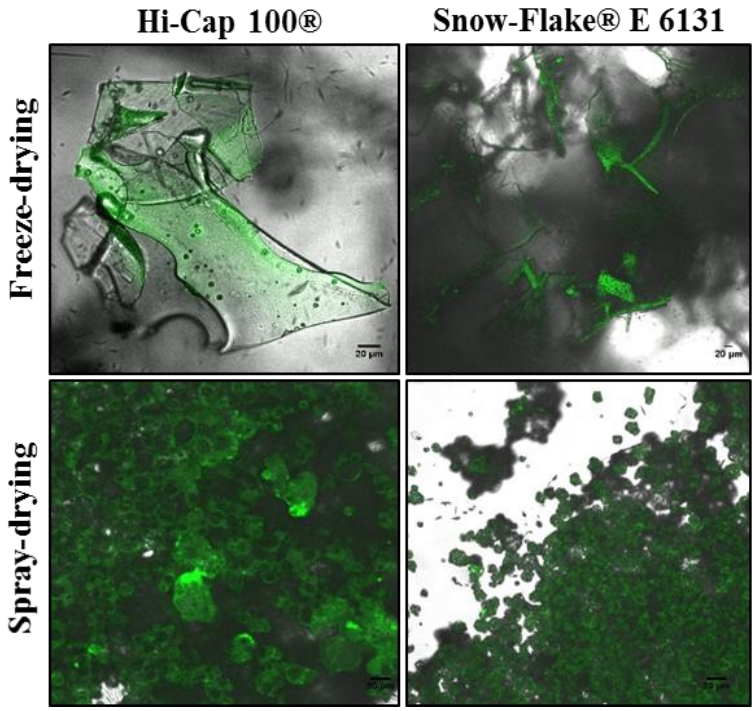


Fig. 9. Confocal micrographs of copaiba oleoresin particles obtained by freeze-drying and spray-drying. Scale bar – 20 μm.

3.4 CONCLUSIONS

This paper addresses the production of copaiba oleoresin particles from emulsions generated by emulsification assisted by ultrasound and stabilized by modified starch. The tested polymers showed similar characteristics, since both derived from corn. However, the emulsions produced with these biopolymers presented different stability characteristics: HC provided more stable emulsions. The emulsion droplet size was significantly influenced by ultrasonic power and sonication time. Spray-drying and freeze-drying were applied to obtain dry particles, which characterization allowed observing that the oleoresin was effectively encapsulated. The produced particles had characteristics of amorphous materials, as well as high encapsulation efficiency. Moreover, the encapsulation process of copaiba oleoresin increased its oxidative stability against thermal degradation. In general, the particles obtained by FD and SD showed different morphological characteristics. However, the used biopolymers (HC and SF) did not influence the particles microstructure. CLSM analysis of the particles confirmed the encapsulation of the copaiba oleoresin in polymeric matrices. Overall, the use of modified starches (HC and SF) and the emulsification assisted by ultrasound may be considered as a good alternative to encapsulate copaiba oleoresin. Regardless of the advances developed in this work, more studies must be performed in order to replace the organic solvent (ethyl acetate) by food grade solvents and reduce the environmental impact. The present work is the starting point for further investigations of different techniques to remove the organic solvent, such as supercritical fluid extraction of emulsions.

3.5 ACKNOWLEDGMENTS

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CAPÍTULO 4 - PRODUÇÃO DE PARTÍCULAS DE ÓLEO-RESINA DE COPAÍBA (*Copaifera officinalis*) POR EXTRAÇÃO COM FLUIDO SUPERCRÍTICO DE EMULSÕES

Production of copaiba (*Copaifera officinalis*) oleoresin particles by supercritical fluid extraction of emulsions

José Luis Pasquel Reátegui[a], Flavia P. Fernandes[a], Philipe dos Santos[a], Camila A. Rezende[b], Adilson Sartoratto[c], Carmen Lucia Queiroga[c], Julian Martínez[a].

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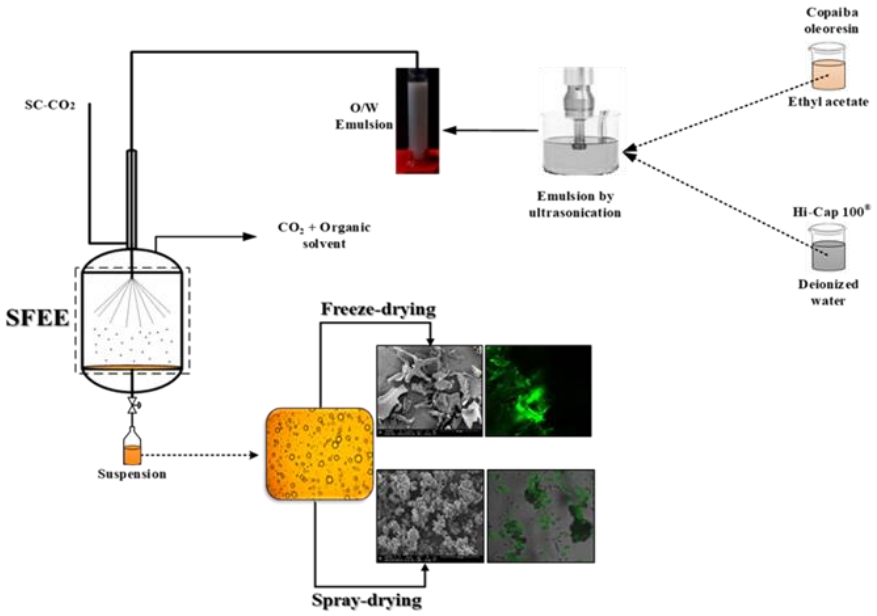
Highlights

1- SFEE encapsulated copaiba oleoresin and reduced ethyl acetate to below 5000 ppm.
2- The best condition of β-caryophyllene recovery (7.3 %) and ethyl acetate content (4117 ppm) was obtained at 0.5 mL/min of emulsion, 12.48 g/min of SC-CO_2, with extraction time of 30 minutes.
3- The mean droplets size in the dispersed systems is not modified in SFEE.
4- The dried particles by spray-drying and freeze-drying showed monomodal size distribution.
5- The micrographs obtained by CLSM confirm the encapsulation the copaiba oleoresin in the modified starch.



ABSTRACT. To enhance the properties of copaiba (*Copaifera officinalis*) oleoresin, suspended particles were produced by supercritical fluid extraction of emulsions (SFEE), using modified starch Hi-Cap 100® as core material. First, ultrasound was applied to produce oil in water (O/W) emulsions with droplet diameter of 261.7 ± 2.2 nm after 24 h. In SFEE, CO_2 flow rate, emulsion flow rate and solvent extraction time were evaluated in terms of residual ethyl acetate content (REA) and β-caryophyllene recovery in the produced suspension. SFEE achieved about 94.1% (REA = 4117 ppm) reduction of ethyl acetate in the suspension and a β-caryophyllene recovery of 7.3%. This REA is within the exposure limit of ethyl acetate (5000 ppm per day). The size of the suspended nanoparticles showed little variation from the mean emulsion droplet diameter. Finally, the suspensions were dried to obtain powder particles with low moisture content. Morphological analyses by field emission scanning electron microscopy (FESEM) showed different structures depending on the drying method (freeze-drying or spray-drying). Moreover, confocal laser scanning microscopy (CLSM) confirmed that the copaiba oleoresin was encapsulated in the particles.

Keywords: Encapsulation; Copaiba; SFEE process; Supercritical CO_2; X-ray diffraction; Confocal microscopy.

4.1 INTRODUCTION

Copaiba (*Copaifera officinalis*) is a tree, native to South America, which produces an oleoresin with medicinal properties, such as anti-inflammatory [1], anticancer [2], antitetanic and antiseptic, besides being indicated to treat syphilis, bronchitis and wounds [3]. Copaiba oleoresin is mainly composed by hydrocarbon sesquiterpenes (about 90%), among them, β-caryophyllene is the major component and considered responsible for its biological activity [4, 5]. However, copaiba oleoresin cannot be directly ingested, because of its unpleasant taste [6]. Moreover, it has low solubility in water but is soluble in some organic solvents.

Encapsulation of active compounds has attracted the attention of researchers in this field, and techniques using supercritical fluids, mainly carbon dioxide (CO_2), have been developed to face the disadvantages of conventional techniques like nanoprecipitation, spray-drying, freeze-drying and solvent evaporation of emulsions. According to Santos, Martín, Meireles and Cocero [7] one of the most widespread and simple encapsulation techniques is the evaporation of the solvent from an oil-in-water emulsion (O/W). Organic solvents can generally be removed by evaporation, allowing the production of micro or nanometric particles [8]. However, the production of gas bubbles during solvent evaporation can modify the structure of the emulsion, resulting in low encapsulation efficiency [9]. Supercritical fluid technologies can contribute to eliminating this problem since it avoids the exposure of the product to high temperatures for extended times. Besides, because of the high solubility of organic solvents in supercritical CO_2, this technology is appropriate to reduce the concentration of residual organic solvents in the final suspension.

Supercritical fluid extraction of emulsions (SFEE) is based on the extraction of the organic solvent from emulsion droplet using supercritical CO_2 (SC-CO_2), which has advantages such as moderate critical point, non-toxicity and environmental safety [10]. SFEE combines emulsion techniques and the supercritical antisolvent precipitation process (SAS). According to Della Porta, Campardelli and Reverchon [11], with this combination, the particles size can be controlled from the initial emulsion droplet size. Several works have applied SFEE to natural compounds and polymers, obtaining a correspondence between the droplet and the particle size distributions [12]. This technique can also reduce the time for solvent removal and polymer precipitation [13]. SFEE has been successfully applied in the formation of β-carotene suspended particles from an O/W emulsion using dichloromethane as a solvent [14], particles containing capsaicinoids from red pepper [15], encapsulated carotenoids (lycopene and β-carotene) in

modified starch to be used as antioxidants and dyes [7] and other compounds with pharmaceutical applications [16, 17, 18].

In this work SFEE was applied for the encapsulation of copaiba oleoresin, focusing on β-caryophyllene as the target compound. The influence of CO_2 flow rate, emulsion flow rate and organic solvent extraction time were evaluated in terms of residual ethyl acetate content, concentration of β-caryophyllene in the particles, mean particle diameter and oleoresin loss. The suspension obtained after SFF were dried by *freeze-drying and spray-drying* and then characterized for their moisture content, water activity, particle size distribution, X-ray diffraction and particle morphology by field emission scanning electron microscopy (FESEM) and confocal laser scanning microscopy (CLSM).

4.2 MATERIAL AND METHODS

4.2.1 Material

Modified starch Hi-Cap 100® donated by Ingredion Brazil Industrial Ingredients Ltda. (Mogi Guaçu-SP, Brazil) was used as the core material. Ethyl acetate 99.5% (Dinâmica, São Paulo, Brazil) was used as organic solvent in the emulsions. Carbon dioxide (CO_2) with 99.9% purity (White Martins, Campinas, Brazil) was used as supercritical antisolvent in SFEE. The standard used in gas chromatography coupled to mass spectrometry (GC-MS) was (−)-*trans*-caryophyllene (98.5% purity), which was purchased from Sigma-Aldrich (Sao Paulo, Brazil).

Copaiba (*Copaifera officinalis*) oleoresin was purchased from Ferquima Indústria Comercio LTDA (Vargem Grande Paulista, Brazil) and stored at 5 °C until emulsion preparation.

4.2.2 Preparation of the emulsion

The emulsification condition that provided droplets with the smallest size and the greatest kinetic stability for the encapsulation of copaiba oleoresin by SFEE were selected from a previous work [19]. Briefly, 200 mL of emulsion were prepared for each SFEE experiment with the following procedure: 1.2 g of copaiba oleoresin was dissolved in 30 mL of ethyl acetate, and 3.4 g of modified starch (Hi-Cap 100®) was separately dissolved in 170 mL of

deionized water. The modified starch solution was let to rest for two hours at room temperature to ensure its complete saturation. The oleoresin and starch solutions were mixed with a magnetic stirrer for 3 minutes to obtain a homogeneous dispersion. Finally, the dispersion was emulsified at 480 W for 6 minutes with an ultrasound probe (Unique, Model DES500, Campinas-SP, Brazil). The variation of the droplet mean diameter with time after emulsification was investigated to establish the appropriate moment to inject the emulsion in the SFEE unit.

4.2.3 Supercritical fluid extraction of emulsion

The extraction of the organic solvent from the emulsion was carried out in a homemade SFEE unit (Fig. 1), which contains a CO_2 supply system, an emulsion injection system and a high-pressure stainless-steel column. The supercritical solvent used in SFEE must have high affinity to the organic solvent and low affinity to the target compound to achieve proper extraction. Briefly, CO_2 was initially cooled in a thermostatic bath (MA184, Marconi, Campinas, Brazil) to -5 °C, then pressurized using a pneumatic pump (PP 111-VE MBR, Maximator, Nordhausen, Germany) and subsequently heated to the operating temperature in a heating bath (MA184, Marconi, Campinas, Brazil). Then, supercritical CO_2 was injected into the high-pressure column (712 ml of internal volume) at a flow rate controlled by a micrometer valve. After stabilization of temperature and pressure, the emulsion was injected into the high-pressure column through two different coaxial nozzles with internal diameter of 177.8 and 122 μm, using a HPLC pump (PU-2080, Jasco, Tokyo, Japan). After the injection of the emulsion, the system was maintained under the operating conditions to remove the organic solvent. Then, the column was slowly depressurized, keeping the CO_2 flow rate constant, and the suspension was collected and stored in amber glasses at -18 °C until the analyses.

To maximize the extraction of ethyl acetate, three CO_2 flow rates (Q_{CO2}, 12.48, 18.60 and 24.42 g/min) and two emulsion flow rates (Q_{em}, 0.5 and 1.0 mL/min) were tested. According to Della Porta and Reverchon [12], the mixture of ethyl acetate and CO_2 has critical pressure of 8.5 MPa at 38 °C, and 0.9 molar fraction of CO_2. Under these operating conditions, water is only slightly soluble in supercritical CO_2, whereas ethyl acetate is completely soluble [20]. Therefore, temperature and pressure of SFEE were set at 40 °C and 9 MPa, respectively, to ensure that the system would be at the supercritical state. Also, the temperature of 40 °C is mild enough to prevent the organic solvent from reaching the boiling point, in which bubbles

could be formed, thus damaging the emulsion droplets and affecting the particle characteristics [21].

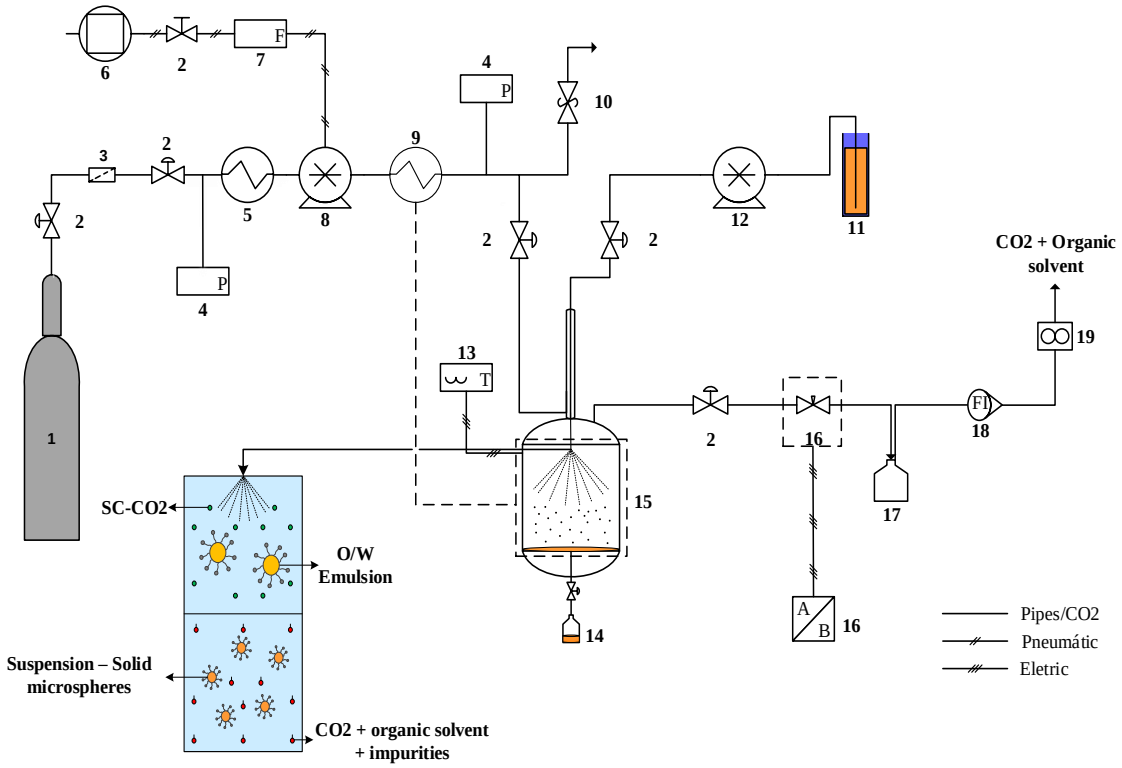


Fig. 1. Schematic diagram of the SFEE unit. (1) CO_2 cylinder; (2) Control valves; (3) CO_2 filter; (4) Pressure indicators (manometers); (5) Cooling bath; (6) Compressor; (7) Compressed air filter; (8) CO_2 pump; (9) Heating bath; (10) Safety valve; (11) Emulsion reservoir (solute/solvent); (12) HPLC pump; (13) Temperature controllers; (14) Suspension; (15) Precipitation column; (16) Micrometric valve with a heating system; (17) Glass flask; (18) Flow meter; (19) Gas totalizer.

4.2.4 Characterization of the suspensions

4.2.4.1 Residual ethyl acetate content

The residual ethyl acetate content (REA) of the suspensions was determined using a gas chromatograph with a flame ionization detector (GC-FID, Shimadzu, CG17A, Kyoto/Japan) equipped with a capillary column ZB-Wax plus (Phenomenex, 30 m × 0.18 mm × 0.18 μm). Each sample was filtered (Chormafil Xtra PA-20/25, Macherey-Nagel, Düren/Germany) and 1 μL was injected in the chromatograph. The sample split ratio was 1:100. The carrier gas (Helium, 99.9% purity, White Martins, Campinas/Brazil) flow rate was 2.2

mL/min. The injector and the detector temperatures were 180 and 220 °C, respectively. The column was heated from 35 °C to 200 °C at 7 °C/min, each one with a hold time of 5 min (35 °C) and 2 min (200 °C), respectively. The retention time of ethyl acetate peak was 2.07 min and quantification was performed using an external standard calibration curve ($R^2 = 0.9999$).

4.2.4.2 Optical microscopy

The suspensions were analyzed in an optical microscope (Carl Zeiss, model Axio Scope A1, Göttingen, Germany). using 100x magnification lens, operating under oil immersion. Aliquots of the suspensions were placed on glass slides and covered with coverslips to be imaged.

4.2.4.3 Particle diameter

The mean diameters of the suspended particles were determined after 24 hours by light scattering (PCS) using a Zeta Potential Analyzer (Brookhaven Instruments Corporation, USA), which is a gauge of particle size by spreading of light, with a 15 mW power solid-state laser and wavelength of 675 nm. The diameters were measured in triplicate.

4.2.4.4 β-caryophyllene recovery and oleoresin loss

The determination of β-caryophyllene in the suspension was performed by gas chromatography (Agilent Technologies model 6890 N, Santa Clara, USA) coupled to a selective mass detector (model MSD 5975, Santa Clara, USA). The compounds were separated in a capillary column HP-5 MS (30 m x 0.25 mm x 0.25 μm) operating at 120 °C, 2 °C/min, 160 °C, 10 °C/min, 300 °C (3 min). Injector split-splitless was used. Helium was the carrier gas with flow rate of 1.0 mL/min. The injector and detector temperatures were 220 and 290 °C, respectively. 1 μL of the suspension was injected into the chromatograph and the quantification of β-caryophyllene (Tr = 23.82 min) was performed using an external standard calibration curve. The identification was based on the retention time of the standard used and the comparison of its mass spectra with those found in the NIST (National Institute of Standards and Technology) electronic database.

The β-caryophyllene recovery was defined as the ratio between the initial mass of β-caryophyllene in the emulsion before SFEE ($M_{initial}$) and the mass of β-caryophyllene in the suspension after SFEE (M_{Final}), as shown in Eq. (1).

$$R_{\beta C} = \frac{M_{Initial}}{M_{Final}}\ x\ 100 \quad (1)$$

The oleoresin loss was calculated as the ratio between the mass of oleoresin injected in the SFEE system and the residual mass of oleoresin in the collecting flask at the end of the process.

4.2.5 Characterization of the dried suspensions

After the SFEE, the oleoresin particles that were produced remained suspended in the aqueous phase. The best SFEE condition selected for drying was the one that resulted in the lowest REA in the suspension. Water was removed from the suspension by two drying techniques (*freeze-drying* and *spray-drying*), following the conditions previously established by Pasquel-Reátegui et al. [19]. The dried particles were stored at -18° C in amber glass vials to be protected from light and oxygen, before characterization by their moisture content, water activity, particle size distribution, x-ray diffraction and morphology by FESEM and CLSM.

4.2.5.1 Moisture content and water activity

The moisture content of the particles was gravimetrically determined according to the AOAC method 925.10 [22]. The particle samples were placed on previously weighed glass plates and oven dried under forced air circulation (model NT 395-1, Tecnal, São Paulo) at 60 °C for 4 hours. Water activity (aw) was measured at 25 °C in a water activity meter equipment (AquaLab Series 3TE, Decagon, Pullman, USA).

4.2.5.2 Particle size and distribution

The particle size distributions and their mean diameter were determined by laser diffraction on a Mastersizer 2000 equipment (Malvern Instruments Ltd., Malvern, United Kingdom). The mean particle diameter was based on the mean diameter of a sphere of the same

volume, named diameter of De Brouckere (D_([4,3])), calculated with Eq. (2). The samples were analysed in quintuplicate by the wet method, using ethanol (99.5%).

$$D_{[4,3]} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (2)$$

Where d_i is the particle diameter and n_i is the number of particles.

4.2.5.3 X-ray diffraction

The X-ray diffraction patterns of the particles and polymers were obtained in a diffractometer (Shimadzu XRD-7000, Tokyo, Japan). The analyses were carried out with radiation source from Cu-Kα (λ = 1.5406 Å), operating at 40 kV/30 mA, and the angle (2 θ) was scanned from 5 to 50° at a 2°/min rate.

4.2.5.4 Field emission scanning electron microscopy

The morphology of the particles was analysed in a scanning electron microscope equipped with a field emission gun (FESEM - FEI Quanta 650, USA). Before analysis, the samples were coated with iridium (about. 30 min) in a SCD 050 sputter coater (Oerlikon-Balzers, Balzers, Liechtenstein). Analyses of the sample surfaces were carried out under vacuum, using a 5 kV acceleration voltage.

4.2.5.5 Confocal Laser Scanning Microscopy

The confocal laser scanning microscopy (CLSM) analyses were performed on the copaiba oleoresin particles without any previous preparation. The particles were analyzed in a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany), equipped with a 40× objective. The images were obtained at a wavelength of 488 nm, as described by Pasquel-Reátegui et al. [19].

4.2.6 Statistical analyses

All results are presented as a mean ± standard deviation. In order to evaluate the statistical differences between the experiments, the Tukey test was performed at a significance level of 5% ($p < 0.05$). Statistical analyses were executed using Minitab statistical software (version 16.2.1, 2016, Minitab Inc.). The results of XRD were analyzed descriptively.

4.3 RESULTS AND DISCUSSION

4.3.1 Droplet size variation

Fig. 2 presents: a) the dispersion (non-emulsified) with a mean droplet diameter of 716 ± 3 nm, and b) the emulsified dispersion, which achieved a 71-fold reduction in the droplet diameter in 10 minutes. It is worth mentioning that the droplet diameter achieved 262 ±2 nm after 24 h, and previous works showed that this emulsion has high kinetic stability after 24 h [19]. Based on these results, the emulsion was injected in the SFEE unit before 30 min, since its droplet diameter at this time is below 100 nm. The required time for the stabilization of the SFEE conditions was also considered for this choice.

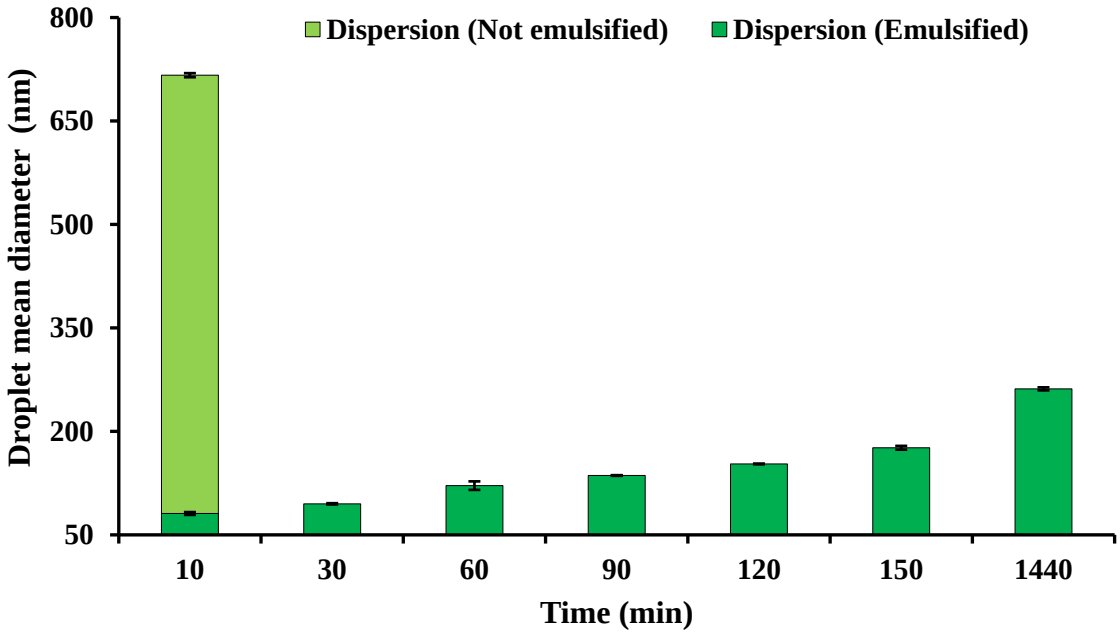


Fig. 2. Influence of time on the mean droplet diameter of the emulsion of copaiba oleoresin produced with ultrasonic power of 480 W and sonication time of 6 min.

4.3.2 SFEE process

The SFEE process consisted on the polymer precipitation since the organic solvent (ethyl acetate) was extracted from the emulsion with supercritical CO_2. Initially, the extraction time was fixed in 30 min. During the SFEE process, some oleoresin was collected in the glass flasks, indicating some solubility in supercritical CO_2 under these process conditions. The lowest oleoresin loss was achieved with the highest emulsion flow rate (1.0 mL/min) for all CO_2 flow rates, as shown in Table 1. A similar trend was found in SFEE for the encapsulation of capsaicinoids from red pepper [15]. The loss of oleoresin by co-extraction is related to the solubility of the oleoresin of copaiba in ethyl acetate and subsequent affinity with the SC-CO_2, causing the ethyl acetate to act as co-solvent in CO_2, thus removing compounds of copaiba oleoresin. Moreover, possible instability of the emulsion during SFEE can lead to the release of oleoresin to the aqueous phase and its further dissolution in supercritical CO_2.

Table 1 shows that the highest reduction in the residual ethyl acetate content was achieved with an emulsion flow rate of 0.5 mL/min, independently of Q_{CO2}. The increase in Q_{CO2} and the reduction of Qem enhanced the removal of ethyl acetate up to 97.1%, achieving REA of 2325 ± 225 ppm in the suspension. The velocity of CO_2 increases with its flow rate, intensifying turbulence and mass transfer, thus resulting in a decrease of ethyl acetate concentration in the suspension. From the results exposed in Table 1 and considering the lowest CO_2 consumption and the lowest REA in the suspension, three experimental conditions (E-1, E-2 and E-5) were selected for additional experiments to evaluate the effect of extraction time. The extraction time may affect the removal rate of ethyl acetate and, therefore, the technical and economic viability of the process. The results of these experiments are presented in Table 2.

The highest reduction of solvent (98.2%) was observed at 60 min of extraction for Q_{em} = 0.5 mL/min and Q_{CO2} = 12.48 g/min, as shown in Table 2. Moreover, REA in the suspension decreases as extraction time increases, but no statistical difference was found between REA at 45 and 60 min for Q_{em} = 0.5 mL/min and both tested SC-CO_2 flow rates (12.48 and 24.42 g/min). High CO_2 flow rates reduce the contact time between supercritical CO_2 and emulsion, resulting in an inefficient extraction of the solvent. Furthermore, the best RAE condition (E-1D) resulted in a suspension with an ethyl acetate content (1484.5 ± 16.0 ppm) legally permitted by the pharmaceutic industry, which must be below 5000 ppm for ethyl acetate [23].

Table 1. Influence of CO_2 and emulsion flow rates in the SFEE of copaiba oleoresin.

Experiment	SFEE – Coaxial nozzle (177.8 μm)								
	T (°C)	P (MPa)	Q_{CO2} (g/min)	Q_{em} (mL/min)	REA (ppm)(*)	SR (%)	$D_{[4.3]}$ (nm)	Oleoresin loss (%)	β-caryophyllene recovery (%)(*)
E-1	40	90	12.48	0.5	4117±161^{C}	94.1	244.20±3.42	10.06	7.3±0.2^{E}
E-2	40	90	12.48	1.0	13619±490^{A}	83.1	240.72±2.43	1.83	12.9±0.2^{A}
E-3	40	90	18.60	0.5	3264.3±30.8^{C}	95.9	239.27±2.33	1.39	8.3±0.3^{D}
E-4	40	90	18.60	1.0	10554±609^{B}	86.9	281.98±3.99	0.22	10.8±0.1^{B}
E-5	40	90	24.42	0.5	2325±225^{C}	97.1	231.35±1.35	6.28	6.8±0.1^{E}
E-6	40	90	24.42	1.0	11801±904^{AB}	85.8	249.87±2.63	0.33	9.8±0.2^{C}

Results are expressed as mean ± standard deviation of the analysis. T = temperature; P = pressure; Q_{CO2} = carbon dioxide flow rate; Q_{em} = emulsion flow rate; REA = residual ethyl acetate; SR = solvent reduction; $D_{[4.3]}$ = suspended particles diameter. (*) Equal letters in the same column indicate no significant difference at the level of 5% according to Tukey test.

Table 2. Influence of solvent extraction time in the SFEE of copaiba oleoresin.

SFEE – Coaxial nozzle (177.8 μm)									
Experiment	t (min)	T (°C)	P (bar)	Q_{CO2} (g/min)	Q_{em} (mL/min)	REA (ppm)(*)	SR (%)	$D_{[4.3]}$ (nm)	β-caryophyllene recovery (%)(*)
E-1A	15	40	90	12.48	0.5	5567.6 ± 66.3^{CD}	93.1	242.60±4.38	9.1 ± 0.2^{C}
E-1B	30	40	90	12.48	0.5	4117 ± 161^{D}	94.1	244.20±3.42	7.3 ± 0.2^{D}
E-1C	45	40	90	12.48	0.5	1757.7 ± 47.8^{EF}	97.8	251.53±4.61	6.9 ± 0.3^{DE}
E-1D	60	40	90	12.48	0.5	1485.5 ± 16.0^{EF}	98.2	262.27±2.01	5.7 ± 0.6^{EF}
Experiment	t (min)	T (°C)	P (bar)	Q_{CO2} (g/min)	Q_{em} (mL/min)	REA (ppm)	SR (%)	$D_{[4.3]}$ (nm)	β-caryophyllene recovery (%)
E-2A	15	40	90	12.48	1.0	14091 ± 948^{A}	82.5	241.23±2.58	13.8 ± 1.1^{A}
E-2B	30	40	90	12.48	1.0	13619 ± 490^{A}	83.1	240.72±2.43	12.9 ± 0.2^{A}
E-2C	45	40	90	12.48	1.0	7525 ± 606^{B}	90.6	261.57±0.42	10.8 ± 0.2^{B}
E-2D	60	40	90	12.48	1.0	7009 ± 542^{BC}	91.3	294.70±4.93	7.6 ± 0.1^{D}
Experiment	t (min)	T (°C)	P (bar)	Q_{CO2} (g/min)	Q_{em} (mL/min)	REA (ppm)	SR (%)	$D_{[4.3]}$ (nm)	β-caryophyllene recovery (%)
E-5A	15	40	90	24.42	0.5	4425 ± 381^{D}	94.5	245.40±0.52	7.2 ± 0.5^{D}
E-5B	30	40	90	24.42	0.5	2325 ± 225^{E}	97.1	231.35±1.35	6.8 ± 0.1^{DE}
E-5C	45	40	90	24.42	0.5	2129.1 ± 160.1^{EF}	97.3	266.30±1.70	5.2 ± 0.2^{FG}
E-5D	60	40	90	24.42	0.5	2006.3 ± 100.5^{EF}	97.5	282.37±3.01	4.2 ± 0.1^{G}
SFEE – Coaxial nozzle (127 μm)									
Experiment	t (min)	T (°C)	P (bar)	Q_{CO2} (g/min)	Q_{em} (mL/min)	REA (ppm)	SR (%)	$D_{[4.3]}$ (nm)	β-caryophyllene recovery (%)
E-1D	60	40	90	12.48	0.5	662.9 ± 49.5^{G}	99.2	532.87±6.17	2.0 ± 0.1^{H}
Evaporation of ethyl acetate - rotary evaporator									
Experiment	t (min)	T (°C)	P (bar)	Q_{CO2} (g/min)	Q_{em} (mL/min)	REA (ppm)	SR (%)	β-caryophyllene recovery (%)	
E-6	60	45	-	-	-	7865.0 ± 129.3^{B}	90.2	5.1 ± 0.1^{FG}	

Results are expressed as mean ± standard deviation. t = solvent extraction time; T = temperature; P = pressure; Q_{CO2} = carbon dioxide flow rate; Q_{em} = emulsion flow rate; REA = residual ethyl acetate; SR = solvent reduction; $D_{[4.3]}$ = suspended particles diameter. (*) Equal letters equal in the same column indicate no significant difference at the level of 5% according to Tukey test.

The removal of ethyl acetate in rotary evaporation at 45 ºC achieved REA of 7865.0 ± 129.3 ppm in the suspension (Table 2), thus higher than in SFEE. The difference in these methods is the contact area between the suspension and the extraction solvent, which is larger in SFEE, enhancing the elimination rate of ethyl acetate.

It is noted in Tables 1 and 2 that the particle size of the suspensions had small variation (approximately ±20 nm), using the coaxial nozzle of 177.8 µm at all experimental conditions. Therefore, neither SC-CO_2 or emulsion flow rates affect the particle size [13, 15]. Similar behaviour were observed by Della Porta and Reverchon [12] in the production of PLGA/Piroxicam microspheres and by Mezzomo, Paz, Maraschin, Martín, Cocero and Ferreira [24] in the encapsulation of emulsions from pink shrimp wastes. The particles produced by SFEE presented monodisperse distribution, which is important for the controlled delivery of the target compounds. The same trend was found in the production of chitosan-based nanosupensions [25] and encapsulated carotenoids [7].

The influence of the coaxial nozzle diameter was also investigated at the condition of experiment E-1D, which achieved the lowest REA. The results show that, as the nozzle diameter decreases, higher is the reduction in REA, achieving 99.2% (662.9 ± 49.5 ppm of ethyl acetate in the suspension), as shown in Table 2. The emulsions injected into the SFEE system with the smallest coaxial nozzle had an increase in droplet size after 24 hours as compared to the droplet size of the injected emulsion, as shown in Fig. 3. According to Li, Rouaud and Poncelet [21], the distance between droplets increases with their size, thus avoiding coalescence. Even so, the droplet size must remain small, since big droplets are less stable in microspheres and risk to leak the target compounds. No flocculation or coalescence was noticed after 24 h, as can be observed in Fig. 3 (b). So, the droplets may have expanded due to the *Ostwald ripening* phenomenon - for instance, smaller droplets not covered with starch may have been coalesced into larger droplets because of pressure gradients. Smaller droplets can appear because the emulsion droplet diameter is lower than the coaxial nozzle. The particles are reduced under pressure to flow through the nozzle and have their surface areas increased. Thus, larger Hi-Cap 100® amount, which may not be available, is needed to cover their surface. This behavior can explain the lower REA in the suspension (662.9 ± 49.5 ppm), since larger surface area and less starch core enhance the contact between supercritical CO_2 and ethyl acetate, intensifying its removal.

The β-caryophyllene recovery in the suspensions after SFEE is higher for Q_{em} = 1.0 mL/min for all CO_2 flow rates. Moreover, the highest oleoresin losses coincide with the lowest

β-caryophyllene recoveries in the suspensions, as presented in Table 1. Regarding REA, the increase in extraction time intensified the loss of β-caryophyllene, as shown in Table 2. This profile can probably be explained by the dissolution of β-caryophyllene in supercritical CO_2, which might have been enhanced when low content of ethyl acetate was available. Summarizing, the SFEE process with Q_{em} = 0.5 mL/min, Q_{CO2} = 12.48 g/min and 30 min of solvent extraction achieved REA within the limit required by FDA and the highest recovery of β-caryophyllene, being recommended for further investigations.

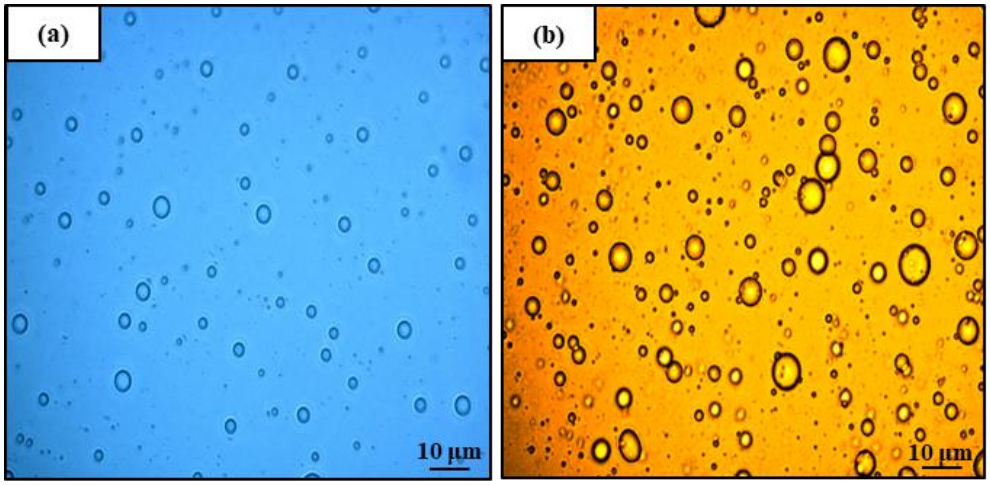


Fig. 3. Image of optical microscopy: (a) emulsion before the SFEE process, and (b) suspension after 24 hours, obtained with coaxial nozzle of smaller diameter (127 µm) after removal of ethyl acetate.

4.3.3 Characterization of the suspended particles after drying

4.3.3.1 Moisture content and water activity

The spray-dried particles presented higher moisture and water activity than those obtained by freeze-drying, as can be noted in Table 3. The high temperature in spray-drying (170 ºC) may induce the formation of a barrier on the particle surface, thus hampering the water diffusion and reducing its evaporation rate [26]. Damodaran, Parkin and Fennema [27] report that particles with low moisture and water activity are stable in terms of their physical properties, besides presenting slow deterioration reactions and microbial growth, thus increasing their shelf-life. According to Quek, Chok and Swedlund [28], a particle can be

considered microbiologically stable with a_w below 0.6. Therefore, the particles produced by freeze-drying and those obtained by spray-drying with the 127 μm nozzle are resistant to microorganisms. Pasquel-Reátegui et al. [19] observed that the freeze-dried particles present higher oxidative stability than the non-encapsulated copaiba oleoresin. Moreover, the particle moistures were quite low for all methods, which assures them small decomposition rates, which are typical in moistures below 5% [29].

Table 3. Characterization of the suspended particles after freeze-drying (FD) and spray-drying (SD).

Particle	Moisture (%)(*)	a_w (*)	Particle diameter (μm)(*)
SFEE – Coaxial nozzle (177.8 μm) - FD	0.88 ± 0.05^{C}	0.15 ± 0.01^{BC}	238.72 ± 0.79^{B}
SFEE – Coaxial nozzle (127 μm) - FD	0.73 ± 0.06^{C}	0.09 ± 0.01^{C}	277.75 ± 5.81^{A}
SFEE – Coaxial nozzle (177.8 μm) - SD	2.36 ± 0.12^{A}	0.62 ± 0.04^{A}	10.52 ± 0.64^{C}
SFEE – Coaxial nozzle (127 μm) - SD	1.80 ± 0.08^{B}	0.17 ± 0.01^{B}	14.68 ± 1.44^{C}

Results are presented as mean ± standard deviation. a_w = water activity. (*) Equal letters in the same column indicate no significant difference at the level of 5% according to Tukey test.

4.3.3.2 Particle size distribution

Spray-drying produced particles around 10-15 μm with no statistical effect of the nozzle diameter in SFEE. The achieved particle size was expected for this method, as well as those of freeze-drying, which can reach 300 μm [30, 31]. The large particles produced in freeze-drying are a consequence of the low temperature and energy available to break the frozen

droplets. Drying by spray-drying and freeze-drying presented bimodal and monomodal size distribution, respectively, as shown in Fig. 4. Similar result was observed by Pasquel-Reátegui et al. [19] in copaiba oleoresin particles encapsulated in modified starch.

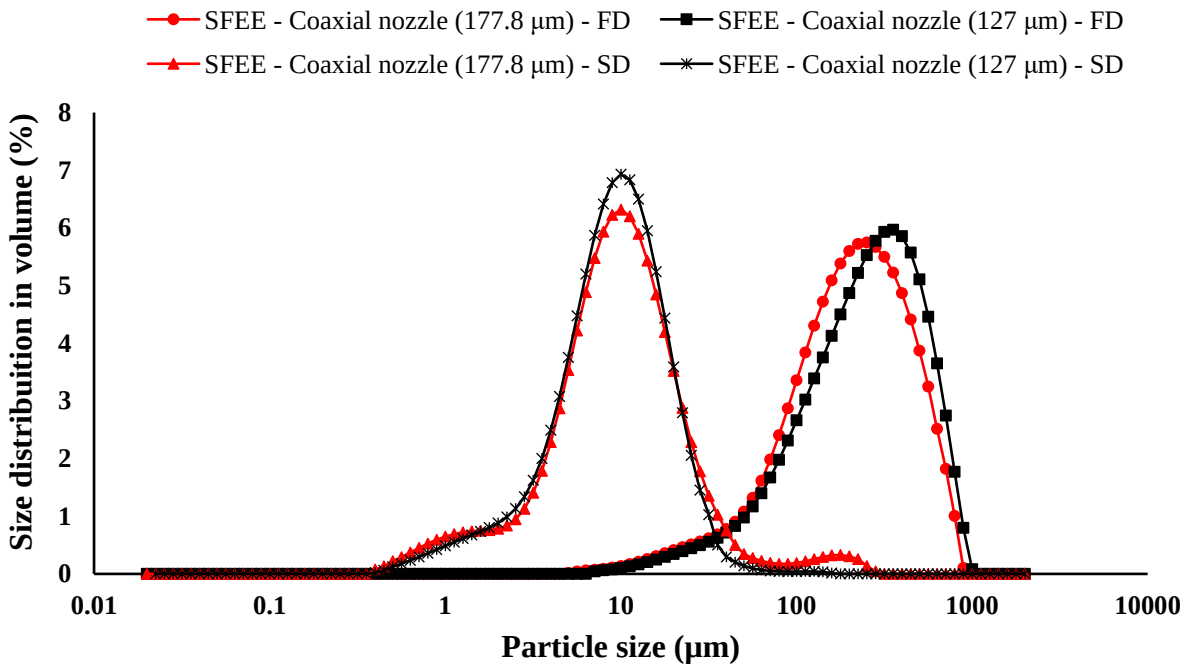


Fig. 4. Particle size distribution obtained after freeze-drying (FD) and spray-drying (SD) of the suspensions obtained by SFEE.

4.3.3.3 X-ray diffraction

Fig. 5 presents the diffractograms of pure Hi-Cap 100® and particles obtained by spray-drying and freeze-drying. The dried particles and the starch behave as amorphous materials, indicating that neither SFEE nor the drying methods affected the starch's structural properties. Amorphous materials exhibit amorphous halos in XRD and are usually more soluble and hygroscopic than the crystalline ones, since the molecules in the amorphous state are disordered [32, 33]. Powders with amorphous characteristics hydrate rapidly due to the low energy levels of the bonds between molecules when compared to the crystalline state [34]. Similar behavior was observed in copaiba oleoresin particles encapsulated in modified starch, using Hi-Cap 100® and Snow-Flake® E 6131 as polymer, and dried by freeze-drying and spray-drying [19].

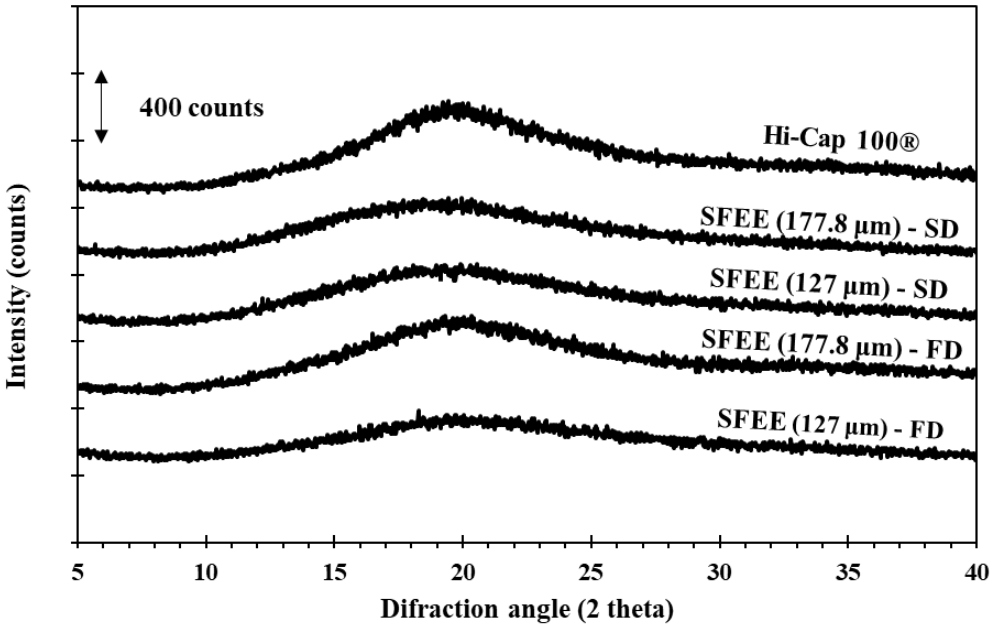


Fig. 5. X-ray diffraction of Hi-Cap 100® and suspensions after freeze-drying (FD) and spray-drying (SD) drying.

4.3.3.4 Microstructural analysis of the particles

The freeze-dried particles presented irregular alongated structures, similar to flat sheets (Fig. 6 (a) and (b)), as typically found in freeze-dried samples [30, 35]. Small pores are also observed on the inner particle walls (Fig. 6 (c) and (d)), which may have been formed by the agglomeration of emulsion droplets during freezing and drying [19] which remain attached by the starch layer. Similar microstructures were also reported in the freeze-drying of annatto seed oil [36] pepper oleoresin [15], and limonene particles [37].

The spray-dried particles have spherical shapes with different sizes. Their surfaces do not present fissures or cracks, thus providing enhanced protection and retention of the target compounds within their nuclei, as shown in Fig. 6 (g) and (h). Nevertheless, rough surfaces are also observed, possibly originated from water evaporation that leads to the core solidification before the expansion of particles, and thus their shrinkage in drying [7, 19, 38].

The CLSM images of the particles obtained by freeze-drying (Fig. 6 (e) and (f)) and spray-drying (Fig. 6 (i) and (j)) reveal the impregnation of the active material (green colour) in the particles, thus confirming the encapsulation of copaiba oleoresin in Hi-Cap 100®. Unlike modified starch, copaiba oleoresin has some fluorescence that allows observing the internal particle structure without damaging the target compounds [39].

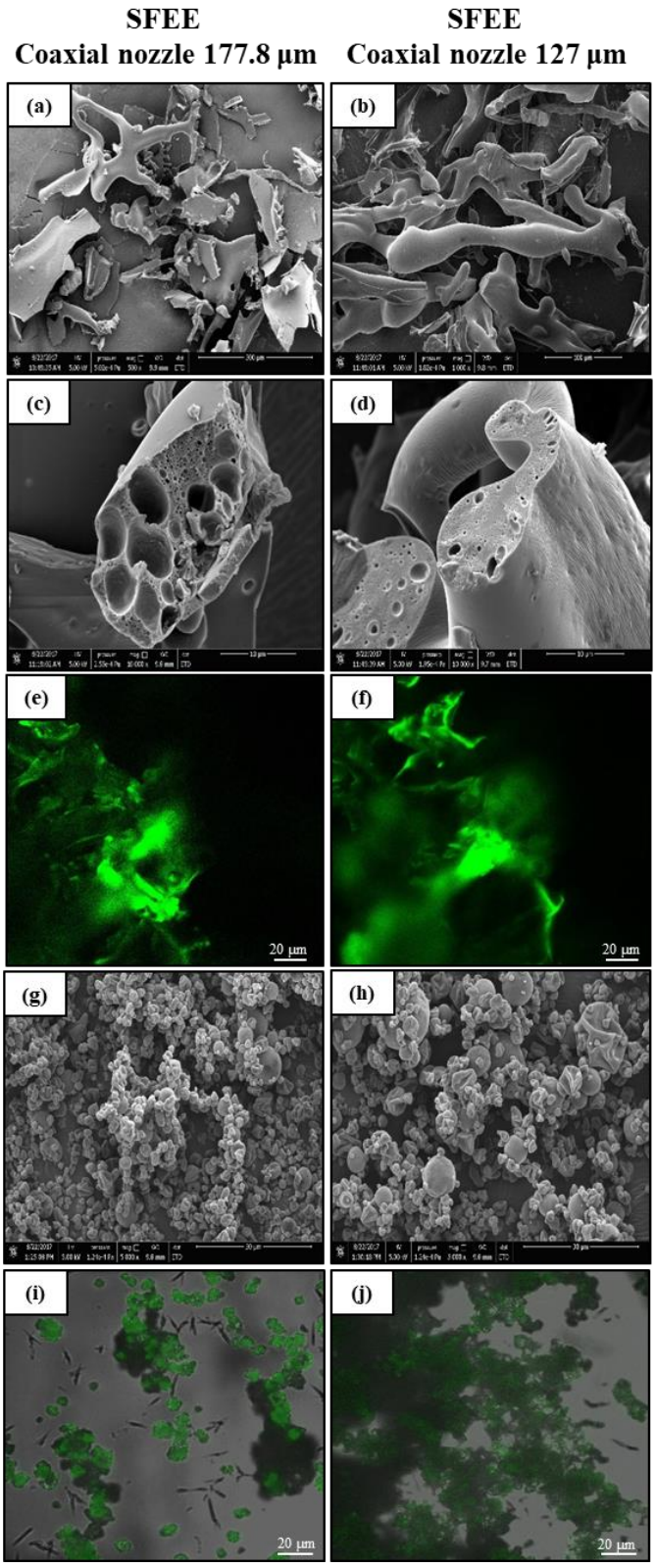


Fig. 6. FESEM and CLSM images of the particles produced after freeze-drying (500X (a), 1000X (b) and 10000X (c) and (d) magnification – CLSM (e) and (f)) and spray-drying (5000X (g) and (h) magnification - CLSM (i) and (j)) of the suspension obtained by SFEE (Q_{CO2} = 12.48 g/min, Qem = 0.5 mL/min and 60 min of solvent extraction time).

4.4 CONCLUSIONS

Supercritical fluid extraction of emulsions was successfully applied to produce copaiba oleoresin particles with residual ethyl acetate content within the legal limits. The SFEE process at flow rates of 0.5 mL/min and 12.48 g/min for emulsion and SC-CO_2, respectively, with 30 minutes of solvent extraction provided the highest β-caryophyllene recovery in the particles. This recovery decreased at low ethyl acetate concentration, indicating a negative influence of solvent extraction time due to the solubilization of β-caryophyllene in supercritical CO_2. The particle size of the suspensions produced in SFEE had small variation from the emulsion droplet diameter, indicating that both dimensions are strongly correlated.

Freeze-drying and spray-drying were applied to dry the suspensions obtained by SFEE and resulted in particles with different morphologies and sizes. The CLSM images confirmed the encapsulation of copaiba oleoresin in the modified starch Hi-Cap 100®. Therefore, SFEE is capable of encapsulating copaiba oleoresin and leaving an acceptable residual ethyl acetate amount in the product. The diameter of the SFEE coaxial nozzle affects the particle size, but additional investigations are needed to elucidate such influence and propose the scale-up of the process, for its economic evaluation. Several applications of copaiba oleoresin particles can be suggested in cosmetic and pharmaceutic industries, where the properties of its components can provide high added-value products. Depending on the application, either suspended or dried particles may be feasible.

4.5 ACKNOWLEDGEMENTS

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CAPÍTULO 5 - FRACIONAMENTO COM DIOXIDO DE CARBONO SUPERCRÍTICO DE SESQUITERPENOS E ÁCIDOS DITERPÊNICOS DO ÓLEO-RESINA DE COPAÍBA (*Copaifera officinalis*)

Supercritical carbon dioxide fractionation of sesquiterpenes and diterpenic acids from copaiba (*Copaifera officinalis*) oleoresin

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Artigo submetido ao periódico "Separation and Purification Technology"

Supercritical carbon dioxide fractionation of sesquiterpenes and diterpenic acids from copaiba (*Copaifera officinalis*) oleoresin

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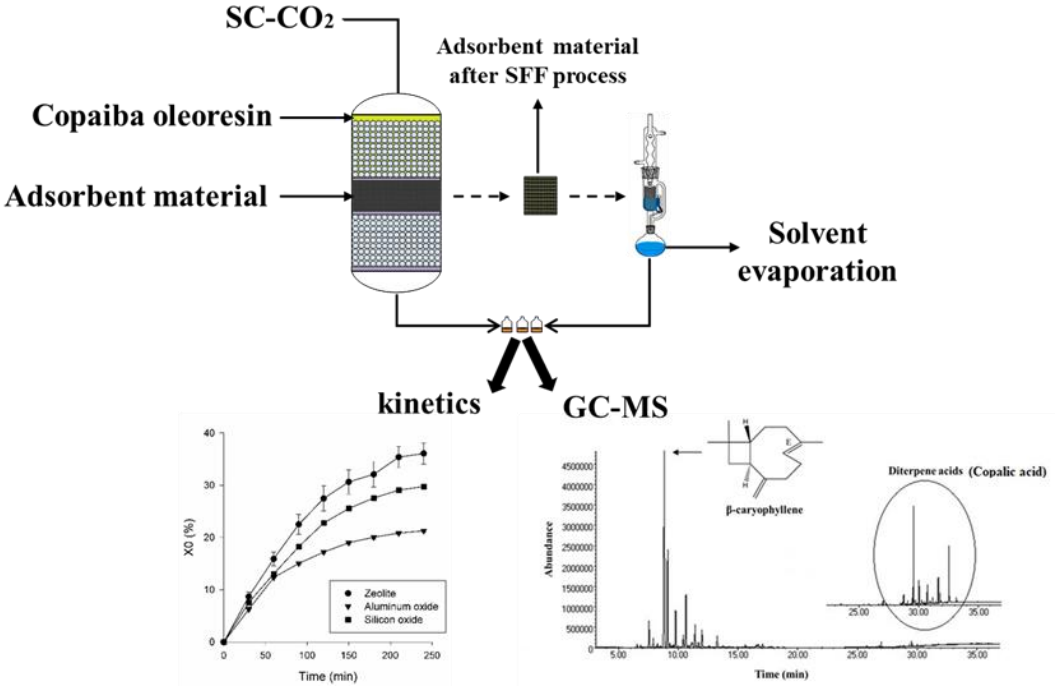
Highlights

1- Supercritical CO_2 fractionation (SFF) at 9 MPa provided the best separation of β-caryophyllene and copalic acid.
2- Ethyl acetate was able to recover copalic acid retained in the adsorbents.
3- Silicon oxide at 9 MPa obtained the most concentrated fractions of β-caryophyllene.
4- Sequential SFF processes are proposed to obtain fractions rich in β-caryophyllene or copalic acid.



ABSTRACT. Supercritical carbon dioxide (SC-CO_2) fractionation was applied to separate sesquiterpenes and diterpenic acids from the copaiba (*Copaifera officinalis*) oleoresin. Natural zeolite type 13X, aluminum oxide and silicon oxide were tested as adsorbent materials at three different pressures (9, 19 and 29 MPa). At the highest pressure all the adsorbents behaved similarly and SC-CO_2 was capable of desorbing the copaiba oleoresin without modifying its original composition. At 9 MPa the zeolite 13X retained β-caryophyllene, achieving concentration factors (CF_c) below 0.48. Meanwhile, the purification factor (PF_c) greater than CF_c indicated the purification of this compound. On the other side, both aluminum oxide and silicon oxide were able to selectively adsorb the polar and oxygenated compounds from the oleoresin, resulting in CF_c above 1 for β-caryophyllene (for silicon oxide, CF_c was around 2). Therefore, a two-step adsorption-desorption process can be proposed to obtain β-caryophyllene fractions with high concentrations. Copalic acid was also identified in the fractions obtained using zeolite 13X, which remained adsorbed in the adsorbent material at the lower pressure used of 9 MPa. On this condition, it was possible to obtain CF_{ca} and PF_{ca} over 2 and 5 respectively, for the fraction recovered with ethyl acetate after the supercritical fluid fractionation.

***Keywords*:** β-caryophyllene; copalic acid; adsorption/desorption; zeolites; aluminum oxide; silicon oxide.

5.1 INTRODUCTION

Brazilian rainforest in known for its exuberance and variety of tropical plants, which provide alternative sources of therapeutic agents for certain diseases. Copaiba trees (*Copaifera* sp.) exude a smelly transparent liquid with widely described biological properties [1, 2]. The exuded oleoresin has been used by perfume, cosmetic and pharmaceutic industries.

Copaiba oleoresin contains a large amount of hydrocarbon sesquiterpenes, which represent about 90% of its total composition, followed by a small content of diterpenic acids [3]. Pharmacologic studies of copaiba oleoresin confirm its anti-inflammatory [4], anticancer [5] and antioxidant properties [6], besides the ability to treat sexually transmitted diseases, bronchitis, skin diseases and act as insect repellent [3, 7]. Literature mentions β-caryophyllene as the major sesquiterpene in copaiba oleoresin, so this compound can be considered a chemical marker of copaiba volatile oils, besides being related to the abovementioned therapeutic properties [8]. According to Sköld, Karlberg, Matura and Börje [9], β-caryophyllene can also be used as aroma agent, due to its woody and spicy odor.

Fractionation of vegetable oils has been performed mainly for the following reasons: 1) search for new fragrances or aromatic raw materials; 2) isolation of compounds to be used as intermediates in the production of other components; 3) obtaining various oil fractions to specific applications, with their consequent valorization. The conventional fractionation methods are vacuum and steam distillation, solvent extraction and adsorption. However, such methods have low yields, generally cause thermal degradation of some compounds or require solvents that must be furtherly removed. Crude vegetable oils must usually be refined to remove certain undesirable compounds, but some valuable components may be lost during the refining process [10]. An alternative process to oil fractionation uses supercritical solvents, which have gained increasing attention in recent years due to its advantages over the conventional techniques. Since some solvents have high solvation power at the supercritical state, they can be attractive to selectively remove components from complex mixtures. Supercritical carbon dioxide (SC-CO_2) is currently used because of its several advantages over the organic solvents used in conventional methods [11]. Supercritical fluid fractionation (SFF) has been applied in mixtures, edible and essential oils and alcoholic beverages [12, 13].

Many authors have investigated strategies to improve the selectivity of new products, and adsorption/desorption is one of them [14, 15]. Reverchon and De Marco [16]

stated that adsorption/desorption can be applied to liquids in continuous mode. Adsorption/desorption explores the ability of some materials to induce the concentration of specific components of a solution in their surface, resulting in their concentration or purification. In this context, SFF can be used to concentrate and fractionate natural essences [13]. A product concentrated by SFF may have high added value, making this technique attractive for industrial applications.

This work aimed obtaining fractions with high concentration of sesquiterpenes and diterpenic acids from copaiba oleoresin using SFF. The effects of pressure and three adsorbent materials on the yield and composition of the recovered fractions were investigated.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and plant material

Carbon dioxide with 99.9% purity (White Martins, Campinas, Brazil) was used as solvent in the SFF process. Ethyl acetate 99.5% (Dinâmica, São Paulo, Brazil) and ethanol 99.5% (Synth, Diadema, Brazil) were used as low pressure extraction solvents after SFF. The quantification of β-caryophyllene in the copaiba oleoresin was performed through gas chromatography coupled to mass spectrometry (GC-MS), and (−)-*trans*-Caryophyllene (98.5% purity) (Sigma-Aldrich, São Paulo, Brazil) was used as standard.

Copaiba (*Copaifera officinalis*) oleoresin was purchased from the company Ferquima Indústria and Comércio LTDA, located in Vargem Grande Paulista, Brazil. The oleoresin was stored at 5 ^{o}C immediately after reception until the experiments.

The investigated adsorbent materials were natural zeolite type 13X (40-170 Mesh) (Celta Brasil, São Paulo, Brazil), aluminum oxide P. A. (Lot: 70179 - Dinâmica, São Paulo, Brazil) and silicon oxide (Lot: 107738 - Synth, São Paulo, Brazil).

5.2.2 Characterization of the adsorbents

5.2.2.1 Particle size, apparent density, real density and porosity

The particle size distribution of the adsorbents was analyzed in a set of standardized sieves of *Tyler* series, and their mean particle diameter (D_p) was calculated according to Eq.

(1), where x_i is the mass fraction retained in the sieve i and a_i is the mean opening size between the sieves i and i+1. The particles had also their real density (ρ_r) measured by Helium picnometry (Micrometrics, Multivolume Pycnometer 1305, Norcross, USA). The adsorption bed had its apparent density (ρ_a) calculated by dividing the sample mass used to pack the bed by its volume. With real and apparent densities, the bed porosity (ε) was calculated with Eq. (2). All the analyses mentioned in this section were performed in triplicate and the results are shown on Table 1.

$$D_p = \left(\sum_1^n \frac{x_i}{a_i}\right)^{-1} \quad (1)$$

$$\varepsilon = 1 - \left({}^{\rho_a}/_{\rho_r}\right) \quad (2)$$

Table 1. Characterization of the adsorbent materials.

Properties	***Zeolite***	***Aluminum oxide***	***Silicon oxide***
Apparent density (g/cm^3)	0.72 ± 0.02	1.10 ± 0.03	0.33 ± 0.01
Real density (g/cm^3)	2.04 ± 0.01	3.79 ± 0.01	2.29 ± 0.01
Porosity	0.65	0.71	0.86
Mean diameter (mm)	0.126	0.079	0.050
Moisture (%)	0.67 ± 0.02	0.009 ± 0.001	0.0050 ± 0.0001

5.2.2.2 Moisture

The adsorbents' moisture was determined according to the AOAC method (925.10) [17] through drying in oven (Model TE 395-1, Tecnal, São Paulo, Brazil) at 105 ºC for 5 h, until constant sample mass. The results are shown on Table 1.

5.2.3 Supercritical Fluid Fractionation (SFF)

Adsorption/desorption was performed in a SFF homemade unit illustrated in Fig. 1 (A). The experiments were performed at three pressures (9, 19 and 29 MPa) with three adsorbent materials (zeolite 13X, aluminum oxide and silicon oxide) in duplicate. In each

experiment a 50 mL stainless steel column was filled with 20 g of the adsorbent. The remaining volume was completed with glass spheres and the extremities were covered with glass wool to avoid the passage of adsorbent particles to the line, as shown in Fig. 1 (B).

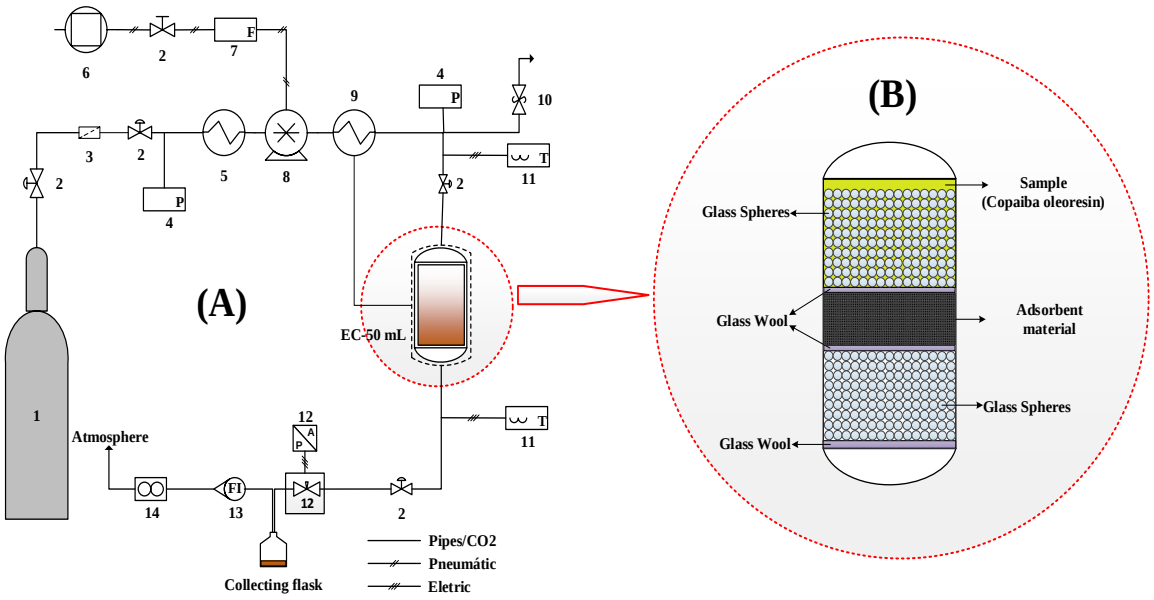


Fig. 1. **(A)** Schematic diagram of the supercritical fluid fractionation –(SFF) unit. (EC-50 mL) Adsorption-desorption cell; (1) CO_2 reservoir; (2) Control valves; (3) CO_2 filter; (4) Pressure indicators (manometers); (5) Cooling bath; (6) Compressor; (7) Compressed air filter; (8) CO_2 pump; (9) Heating bath; (10) Safety valve; (11) Temperature controllers; (12) Micrometer valve with heating system; (13) Flow meter; (14) Gas totalizer. **(B)** Internal scheme of the adsorption-desorption cell.

Finally, 4 mL (3.71 ± 0.09 g) of copaiba oleoresin were injected into the column, which was coupled to the SFF unit and heated for about 20 min to achieve the process temperature (50 °C). Next, SC-CO_2 was pumped into the column to achieve the process pressure. The column was kept closed for 10 min to stabilize the operation conditions, and the kinetic assays began with a SC-CO_2 flow rate of 1.75×10^{-4} kg/s. The samples leaving the column were collected at each 30 min during 4 h, and the initial time was defined as the moment when the first desorbed drop was collected. The fractions were weighed in analytical balance (Bel Engineering Piracicaba-SP, Brazil) and stored at -18 °C until the analyses. The SFF global yield (X_f) was calculated with Eq. (3).

$$X_f = \frac{m_{fraction}}{m_{sample}} \; x \; 100 \qquad (3)$$

where: $m_{fraction}$ is the mass of the product obtained after fractionation and m_{sample} is the oleoresin mass used in each experiment.

5.2.4 Evaluation of fractions by gas chromatography coupled to mass spectrometry (GC-MS)

5.2.4.1 Sesquiterpenes

GC–MS analyses were carried out using a gas chromatograph (Agilent Technologies model 6890 N, Santa Clara, USA) coupled with a mass spectrometer detector (HP5975) equipped with a fused silica capillary column HP5-MS (30 m × 0.25 mm × 0.25 μm). The oven temperature was programmed from 60 to 240 °C at 3 °C/minute, and from 120 to 160 °C (2 °C/minute) to 280 °C (10 °C/minute, 5min). The carrier gas was He (1 mL/minute). The temperatures of both injector and detector were 220 and 290 °C; 1 μL injection volume. For the preparation of the samples, the crude copaiba oleoresin and their fractions obtained in SFF were dissolved in 1 mL ethyl acetate, to obtain a solution with concentration from 15 to 20 mg/mL. MS spectra were taken at 70 eV with an electron impact (EI) source, source interface at 230 °C, quadrupole temperature at 150 °C; mass scan 40–500 amu; frequency of scanning 3.15 scan/minute. From the GC-MS results, the compounds were identified from their retention indexes (RIs) on the database of the library NIST (National Institute of Standards and Technology), using a homologous series of n-alkanes (C8–C22) [18].

5.2.4.2 Diterpene acids

To analyze the composition of diterpenic acids, a copaiba oleoresin sample was esterified according to the methodology described by Migowska, Stepnowski, Paszkiewicz, Golebiowski and Kumirska [19]. The diterpenic acids in the form of methyl esters were analyzed by GC-MS, as described in Section 5.2.4.1.

5.2.5 Concentration and purification factors

The separation was evaluated according to the concentration (CF) and purification (PF) factors, both in terms of β-caryophyllene (CF_c and PF_c) and copalic acid (CF_{ca} and PF_{ca}).

Eq. (4) was used to calculate CF. Note that CF > 1 indicates that the target compound was concentrated in the desorbed fraction, whereas CF < 1 shows that SC-CO_2 dissolved preferentially other components from the copaiba oleoresin. PF indicates if it was possible to separate the target compound from other similar compounds in the matrix, which appear in the same chromatogram. In this case, PF_c will indicate if it was possible to separate β-caryophyllene from the other sesquiterpenes present in the oleoresin. PF is calculated with Eq. (5). If $PF_c > CF_c$, a purification of β-caryophyllene is evidenced. If not, β-caryophyllene could not be separated from the other sesquiterpenes. The same reasoning is valid for the separation of copalic acid from the other diterpenic acids. In some cases the process may dilute the product and at the same time purify it [20].

$$CF = \frac{Ai_F}{Ai_0} \tag{4}$$

$$PF = \frac{Ai_F}{Ai_0} * \frac{(AT_0 - Ai_0)}{(AT_F - Ai_F)} \tag{5}$$

where:

Ai_F = area of the β-caryophyllene or copalic acid peak in the SFF fraction; Ai_0 = area of the β-caryophyllene or copalic acid peak in the crude copaiba oleoresin; AT_F = total peak area in the SFF fraction; AT_0 = total peak area in the crude copaiba oleoresin. To calculate CF_c and PF_c the peak areas of the non-esterified chromatogram were considered, and to calculate CF_{ca} and PF_{ca}, the peak areas of the esterified chromatogram were considered.

5.2.6 Treatment of the adsorbent after SFF

The adsorbent materials used in SFF were subjected to a two-step Soxhlet extraction to recover the copaiba oleoresin that had remained adsorbed, as shown in Fig. 2. The first step used ethyl acetate 99.5% (Dinâmica, São Paulo, Brazil) as solvent to recover the low polarity compounds. The polar fraction was removed in the second step with ethanol 99.5% (Synth, Diadema-SP, Brazil). Both Soxhlet steps were performed for 5 h. The fractions obtained in this process from zeolite were esterified and analyzed by gas chromatography, as described in Section 5.2.4.2.

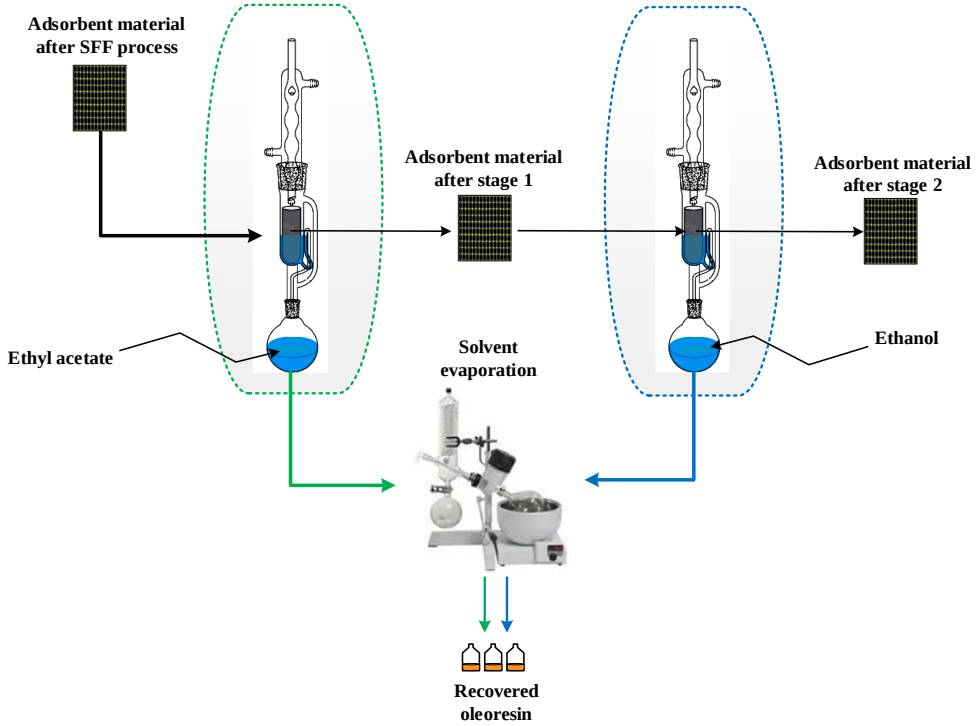


Fig. 2. Schematic diagram for the treatment of the adsorbent material: (Stage 1) extraction with ethyl acetate, and (Stage 2) extraction with ethanol.

5.2.7 Statistical analyses

The results obtained in this work were subjected to mean comparison analyses by Tukey's test with 5% significance level, using the *software* Minitab (version 16.2.1, 2016, Minitab Inc.).

5.3 RESULTS AND DISCUSSION

5.3.1 Chemical characterization of copaiba oleoresin

Table 2 presents the sesquiterpenes and diterpenic acids identified in the copaiba oleoresin by GC-MS. The major sesquiterpene found was β-caryophyllene with 0.31 ± 0.01 g/g oleoresin, followed by α-bergamotene, germacrene D and α-humulene. The major diterpenic acid found was copalic acid. The chemical composition observed in this work agrees with former researches [21, 22], with little variations that may be consequence of external factors, such as injuries caused by insects and fungi, environmental conditions, light incidence, soil nutrients and tree maturation [1, 22, 23].

Table 2. Chemical composition of volatile compounds and diterpenic acids identified by GC-MS in copaiba oleoresin.

Identified Component	RT (min)	RI_{exp}	RI_{lit}	Relative area (%)
Cycloisosativene	7.56	1366	1368	2.85
β-elemene	7.92	1387	1391	1.66
Cyperene	8.27	1400	1398	0.65
β-caryophyllene	8.85	1421	1418	39.61
α-bergamotene	9.13	1430	1432	14.07
α-guaiene	9.30	1438	1439	0.71
α-humulene	9.79	1456	1454	4.35
γ-muurolene	10.43	1478	1477	2.57
Germacrene D	10.65	1487	1480	7.75
α-bulnesene	11.13	1504	1505	0.21
β-bisabolene	11.42	1511	1509	2.04
δ-cadinene	11.70	1526	1524	2.10
∑ Sesquiterpenes (%)				78.57
Epeuric acid**	28.72	-	-	0.81
Kaurenoic acid**	29.47	-	-	0.41
Copalic acid**	29.56	-	-	7.82
Polyalthic acid**	30.07	-	-	3.51
Hardwickii acid**	30.73	-	-	1.49
Pinifolic acid**	31.18	-	-	0.41
Agathic acid**	31.64	-	-	2.00
n.i**	32.53	-	-	4.98
∑ diterpenic acids (%)				21.43

RT = Retention time; RI_{exp} = Retention index determined relative to n-alkanes (C8-C22); RI_{lit} = Retention index (Adams); n.i = Not identified; ** = methyl ester.

5.3.2 Fractionation

5.3.2.1 Fractionation kinetics

The SFF kinetics, in terms of global yield, are show on Fig. 3. Pressure had significant effect on the fractionation kinetics, which was similar for all the adsorbent materials. The affinity of SC-CO_2 to the oleoresin increases with pressure, leading to higher yields. In general, SC-CO_2 at low pressure has poor solvation power, which results in high selectivity. Therefore, as pressure increases the selectivity decreases, and SC-CO_2 dissolves a larger variety of compounds from the samples [24]. The selectivity of SC-CO_2 could be used for fractionation using different pressures and temperatures, although this strategy works only for the separation

of compounds with big differences in their molecular structure, mass and polarity [16]. Since copaiba oleoresin is mainly composed by sesquiterpenes with similar chemical properties, fractionation in this sense would be inefficient.

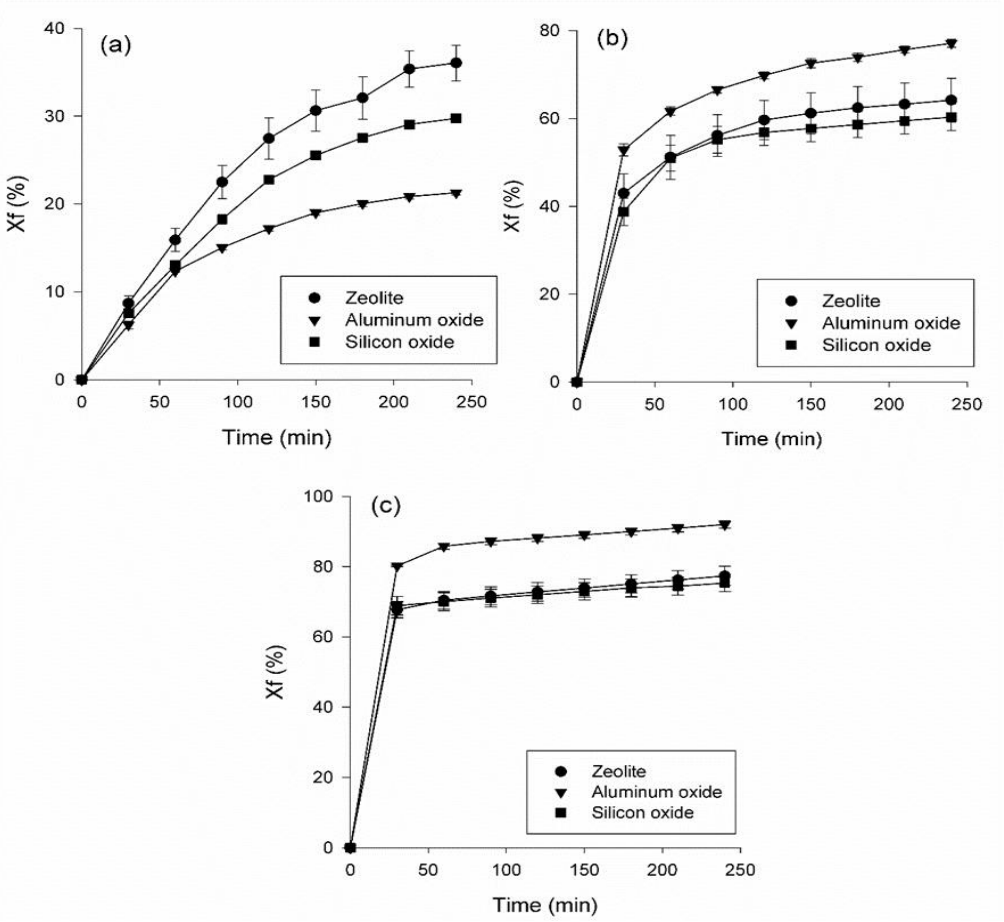


Fig. 3. Desorption kinetics of copaiba oleoresin from zeolite, aluminum oxide and silicon oxide, using supercritical CO_2 at 50 °C and pressures of: (a) 9 MPa; (b) 19 MPa; and (c) 29 MPa.

As can be noted in Fig. 3 (a), the adsorbent material has effect on the SFF kinetics at 9 MPa. The three adsorbent materials presented different behaviors, and those differences are due to their chemical nature, which lead to different separation mechanisms. Silicon oxide (SiO_2) has acid characteristics, low polarity and is applied in the separation of lipophilic compounds, although the presence of oxygen in its molecule suggests some affinity to polar components and those with –OH bonds in their structure. Aluminum oxide (Al_2O_3) is a non-polar solid with alkaline characteristic, thus lipophilic and acid substances tend to be adsorbed in it [25, 26]. These two materials provide separation of molecules according to their polarity

and acid or alkaline characteristic. On the other hand, zeolite is a microporous material, mainly composed by aluminum and silicon oxide molecules in a thetraedric coordinated structure. There are tortuous channels inside the zeolite particles, with uniform diameter and size defined by the thetraedric structure's composition. Therefore, this adsorbent is known as "molecular sieve", in which size separation is the fractionation mechanism [27, 28]. Copaiba oleoresin is mainly composed by low polarity sesquiterpenes and diterpenic acids, which have all similar molecular masses and, thus, similar molecular size. According to the fractionation kinetics in Fig. 3 (a), zeolite offers the lowest resistance to the flow of fractioned oleoresin. That is probably because the molecules in the oleoresin are bigger than the micropores. Therefore, only a weak surface attraction would adsorb the non-polar compounds on the particles' surface. SC-CO_2 is a non-polar fluid, but its ability to dissolve polar molecules increases at higher pressures. Therefore, at 9 MPa mostly non-polar molecules (sesquiterpenes) are desorbed and the polar fraction is retained in the adsorbent material, according to the adsorbent characteristics. That is why aluminum oxide, which has high affinity for acids, retains the diterpenic acids. In the case of silicon oxide, the adsorption of diterpenic acids is poor, leading to higher yields than aluminum oxide. The small particle diameter and the high porosity of silicon oxide leads to a surface area higher than that of zeolite, offering more space for the molecules to be adsorbed.

At 19 and 29 MPa (Fig. 3 (b) and (c)) the opposite behavior is observed, and aluminum oxide provides the highest fractionation yields. When SFF pressure is increased, SC-CO_2 becomes capable of dissolving more polar molecules. In addition, the increase in density with pressure enhances its solvation power, modifying the concentration gradient between the solid and supercritical phases, which is the driving force of mass transfer and controls the desorption of the adsorbed compounds. Therefore, sesquiterpenes and diterpenic acids are more desorbed at high pressures. Since zeolite and silicon oxide have the greatest surface areas, these materials achieved lower yields, whereas aluminum oxide, which has the smallest surface area, obtained the highest yield. The SFF global yield did not achieve 100%, so the adsorbents were subjected to the Soxhlet extractions described in Section 5.2.6. The results of these extractions are presented on Table 3. The highest Soxhlet yields were obtained with ethyl acetate, due to the high solubility of copaiba oleoresin. The sums of the yields achieved in the entire process vary from 80 to 100%. The volatility of sesquiterpenes may explain the reduction in global yield, since these compounds might be dragged with gaseous CO_2 after depressurization, as well as losses in the SFF processing line.

Table 3. Global yield of low-pressure extraction of the adsorbent materials with ethyl acetate and ethanol, after SFF of copaiba oleoresin.

Solvent	Zeolite			Aluminum oxide			Silicon oxide		
	9MPa	19 MPa	29 MPa	9MPa	19MPa	29MPa	9 MPa	19 MPa	29 MPa
Ethyl Acetate	58 ± 5^{aA}	29 ± 3^{aB}	6.4 ± 0.3^{aC}	55.2 ± 0.4^{aA}	0.57 ± 0.03^{aC}	0.47 ± 0.05^{aC}	64 ± 4^{aA}	38.34 ± 0.01^{aB}	1.3 ± 0.1^{aC}
Ethanol	4.6 ± 0.5^{bB}	2.6 ± 0.3^{bCDE}	4.9 ± 0.5^{aB}	2.76 ± 0.01^{bCD}	6.2 ± 0.1^{bA}	0.29 ± 0.02^{aF}	3.0 ± 0.2^{bC}	1.87 ± 0.04^{bDE}	1.68 ± 0.03^{aE}

Same lowercase letters on the same column and same uppercase letters on the same row indicate no significant difference ($p < 0.05$). Data expressed by mean ± standard deviation obtained from duplicate.

5.3.2.2 β-caryophyllene Concentration Factor

The concentration factor of β-caryophyllene was affected by pressure and adsorbent material, as can be observed in Table 4. For zeolite, CFc was lower or equal to 1 at all pressures, indicating that there was not concentration of β-caryophyllene. Molecules with size below the pore diameter have their flow through the particle bed deaccelerated due to the tortuous path. Larger molecules cannot flow inside the pores, so they pass faster out of the particles. Moreover, the presence of aluminum and silicon oxides in the zeolite's composition may provide adsorption sites by van der Waals forces [25].

At 9 MPa CFc ranged from 0.45 ± 0.02 to 0.29 ± 0.01 for 30 and 120 min, respectively. The CFc below 1 obtained at this pressure indicate that β-caryophyllene was retained in the adsorbent and SC-CO2 was not able to remove it from the zeolite. At higher pressures the increased SC-CO2 density enhanced its solvation power, overcoming the van der Waals forces. Therefore, the removed oleoresin's composition was very close to that entering the adsorption column, and there was not fractionation at 19 and 29 MPa.

A sequential fractionation process can be suggested with zeolite, with an initial process at 9 MPa to separate the undesirable compounds from the oleoresin, followed by a step at 29 MPa to desorb β-caryophyllene. This sequence could obtain one product with diluted β-caryophyllene (CF_c = 0.43) and another concentrated in such compound (CF_c = 1.22).

Regarding aluminum oxide, at 9 MPa, a fraction with high β-caryophyllene concentration (CF_c = 1.51 ± 0.05) was obtained at the first 30 min, and CF_c fell to 0.19 ± 0.02 at 60 min. After this time, β-caryophyllene was not detected in the recovered fractions. Therefore, β-caryophyllene seems to have been adsorbed by aluminum oxide. An excess of β-caryophyllene might have initially saturated the active surface of the adsorbent, enhancing its removal by SC-CO_2. The same process seems to have occurred at 19 MPa, since CF_c was 1.29 ± 0.05 in 30 min. The solubility of the compounds from copaiba oleoresin increases with pressure. Therefore, the concentration gradient between the fluid next to the adsorbent surface and SC-CO_2 is higher, enhancing the driving force for mass transfer. This effect is more evident at 29 MPa, in which the β-caryophyllene concentration at the column outlet is close to that in the crude oleoresin, leading to CF_c = 0.96 ± 0.05. This behavior may also be attributed to the increase of solubility of the more polar compounds, like diterpenic acids, in SC-CO_2, with pressure.

Table 4. β-caryophyllene concentration factor (CF_c) and purification factor (PF_c) of the fractions obtained with supercritical CO_2 fractionation using zeolite, aluminum oxide and silicon oxide.

Adsorbent material		Zeolite		Aluminum oxide		Silicon oxide	
P (MPa)	Time (min)	CF_c	PF_c	CF_c	PF_c	CF_c	PF_c
9	30	0.45 ± 0.02^{bC}	1.25 ± 0.02^{aA}	1.51 ± 0.05^{aB}	0.792 ± 0.004^{cC}	2.226 ± 0.001^{aA}	0.904 ± 0.002^{aB}
	60	0.48 ± 0.06^{bB}	1.20 ± 0.03^{abA}	0.19 ± 0.02^{dC}	0.54 ± 0.01^{dC}	1.62 ± 0.03^{bA}	0.83 ± 0.01^{abB}
	90	0.45 ± 0.06^{bB}	1.15 ± 0.02^{bA}	Nd.	Nd.	2.02 ± 0.07^{aA}	0.766 ± 0.003^{bB}
	120	0.29 ± 0.01^{cB}	0.804 ± 0.001^{dA}	Nd.	Nd.	0.995 ± 0.003^{cA}	0.476 ± 0.001^{cB}
	150-240	Nd.	Nd.	Nd.	Nd.	Nd.	Nd.
19	30	0.96 ± 0.03^{aB}	1.0179 ± 0.0003^{cA}	1.29 ± 0.05^{bA}	0.925 ± 0.006^{bA}	1.03 ± 0.09^{cAB}	1.0 ± 0.1^{aA}
	60-240	Nd.	Nd.	Nd.	Nd.	Nd.	Nd.
29	30	1.0 ± 0.1^{aA}	1.01 ± 0.02^{cA}	0.96 ± 0.05^{cA}	1.01 ± 0.01^{aA}	0.8 ± 0.1^{cA}	0.90 ± 0.01^{aB}
	60-240	Nd.	Nd.	Nd.	Nd.	Nd.	Nd.

P = Pressure; Nd = Not detected. Same lowercase letters on the same column and same uppercase letters on the same row indicate no significant difference ($p < 0.05$). Results expressed as mean ± standard deviation obtained from duplicate.

Regarding silicon oxide, the best CF_c results were achieved at 9 MPa, ranging from 2.226 ± 0.001 to 0.995 ± 0.003 for 30 and 120 min, respectively. The diterpenic acids of copaiba oleoresin have carboxyl groups (-COOH), which have great affinity with the oxygen from the adsorbent [26]. On the other side, the high porosity of silicon oxide provides a large surface area with abundant adsorption sites. Therefore, both sesquiterpenes and diterpenic acids are attracted, having their desorption hampered. At 19 and 29 MPa, CF_c were 1.03 ± 0.09 and 0.8 ± 0.1 after 30 min of fractionation, respectively. These fractions did not differ significantly from the crude oleoresin. Thus, at high pressures desorption is enhanced, and the resulting oleoresin is similar to the crude one. Once again, a sequential procedure can be proposed, operating at 9 MPa for the first 90 min to selectively desorb β-caryophyllene, with a theoretical CF_c of 1.99, and then raising pressure up to 29 MPa to achieve CF_c = 0.82.

5.3.2.3 β-caryophyllene Purification Factor

The PF_c presented on Table 4 indicates possible differences between the GC profile of the fractions and that of the crude oleoresin. PF_c evaluates the separation efficiency of β-caryophyllene from the other identified compounds. There was no difference in the PF_c behavior of the fractions produced with aluminum and silicon oxides. These adsorbents were incapable to purify β-caryophyllene, possibly due to the chemical similarity of the sesquiterpenes present in the copaiba oleoresin, which have close molecular mass and double bonds (C=C). The adsorption-desorption process with zeolite at 9 MPa achieved $PF_c > CF_c$, indicating the purification of β-caryophyllene, probably because of the separation principle of zeolite, which is size exclusion. All the sesquiterpenes molecules have the same molecular mass but varied spatial distributions that confer them different molecular sizes. These differences may have favored the purification of β-caryophyllene. When pressure increases to 19 and 29 MPa the effect of molecular size is compensated by the high desorption rate, so the fraction profile is similar to the crude oleoresin and PF_c is near 1.

5.3.2.4 Evaluation of Copalic acid during the fractionation of the copaiba oleoresin

The presence of diterpenic acids in the copaiba oleoresin and its fractions was determined by esterification followed by GC-MS analysis. Table 5 presents the CF_{ca} and PF_{ca} obtained in SFF.

Table 5. Copalic acid concentration factor (CF_{ca}) and purification factor (PF_{ca}) of the fractions obtained with supercritical CO_2 fractionation using zeolite, aluminum oxide and silicon oxide.

Adsorbent material		Zeolite		Aluminum Oxide		Silicon oxide	
P (MPa)	Time (min)	CF_{ca}	PF_{ca}	CF_{ca}	PF_{ca}	CF_{ca}	PF_{ca}
9	30	Nd	Nd	Nd	Nd	Nd	Nd
	60	Nd	Nd	0.01	0.05	Nd	Nd
	90	Nd	Nd	0.05	0.16	Nd	Nd
	120	Nd	Nd	0.03	0.31	Nd	Nd
	150	Nd	Nd	0.05	1.02	0.03	0.03
	180	0.02	0.04	0.08	1.79	0.27	0.23
	210	0.03	0.09	0.16	0.32	0.07	0.09
	240	0.02	0.57	0.18	0.57	0.64	0.62
19	30	1.97	1.31	1.00	0.64	1.12	0.78
	60	3.80	6.06	3.96	5.55	2.40	4.36
	90	0.31	4.04	3.58	5.02	Na	Na
	120	0.11	4.22	0.99	3.23	Na	Na
29	30	1.21	0.87	1.06	0.85	1.29	0.99
	60	3.38	6.57	3.52	4.84	0.17	1.30
	90	1.54	6.47	1.11	3.59	Na	Na
	120	0.05	4.49	0.04	3.91	Na	Na

P = Pressure; Nd = Not detected; Na = Not analyzed because there was not fraction recovered at the given time.

Low pressure (9 MPa) provided the best performance to separate copalic acid from copaiba oleoresin. All the adsorbent materials were able to separate copalic acid, although with different efficiencies. As noted in Table 5, copalic acid was not detected until 180 min of SFF for zeolite, 150 min for silicon oxide and 60 min for aluminum oxide. After those times, the corresponding CF_{ca} were far below 1, so the concentration of copalic acid in those fractions is lower than in the copaiba oleoresin. The total masses corresponding to those last fractions are the smallest, so copalic acid must have been retained in the adsorbent material.

The higher pressures, 19 and 29 MPa, did not present the same behavior. For the three adsorbent materials, CF_{ca} and PF_{ca} are high in the first minutes. The change in SFF behavior is due to the increase of pressure, which increases the solvation power of SC-CO_2 for diterpenic acids.

5.3.2.5 Evaluation of the copaiba oleoresin constituents retained in the zeolite

Table 6 shows CF_{ca} and PF_{ca} for the fractions obtained after low pressure extraction performed in two steps, first using ethyl acetate and then ethanol in a Soxhlet apparatus. Ethyl acetate was able to remove the copalic acid that remained in the zeolite. When the zeolite had been previously treated with SC-CO_2 at 9 MPa, the best separation of diterpenic acids was achieved, as confirmed by the highest CF_{ca}, PF_{ca}, and global yields. This finding suggests another sequential process, first using SFF at 9 MPa for 150 min to separate other compounds, followed by a low-pressure treatment with ethyl acetate to obtain a fraction rich in copalic acid. The ethanolic fraction also succeeded to concentrate copalic acid, with CF_{ca} above 1, but the small fraction amount obtained in this step made us disregard it.

Table 6. Concentration (CF_{ca}) and purification (PF_{ca}) factors of copalic acid obtained from the low-pressure extraction of zeolite, using ethyl acetate and ethanol, after SFF from copaiba oleoresin performed at 9, 19 and 29 MPa.

Solvent	9MPa		19 MPa		29 MPa	
	CF_{ca}	PF_{ca}	CF_{ca}	PF_{ca}	CF_{ca}	PF_{ca}
Ethyl Acetate	2.0±0.1	5.4±0.2	0.9±0.2	3.9±0.2	0.8±0.1	5.4±0.5
Ethanol	1.1±0.2	2.5±0.3	2.2±0.2	3.1±0.1	1.2±0.2	3.2±0.3

5.4 CONCLUSIONS

The chromatographic analyses revealed that β-caryophyllene is the major compound of copaiba oleoresin, followed by α-bergamotene among the sesquiterpenes, whereas the major diterpenic acid was copalic acid. Both pressure and adsorbent material had significant effects on the SC-CO_2 fractionation kinetics and chemical composition of the fractions.

Zeolite presented the highest resistance to the β-caryophyllene flow through the adsorption column, providing desorbed fractions with low β-caryophyllene concentrations at 9 MPa. At higher pressures the concentrations were close to the crude oleoresin. Therefore, a sequential procedure is suggested to obtain fractions concentrated in β-caryophyllene. Moreover, PF_c indicates that more purified fractions of β-caryophyllene were produced with zeolite at 9 MPa. This treatment also allowed concentrating and purifying copalic acid, which remained adsorbed even after SC-CO_2 desorption, and then might be recovered with another solvent, like ethyl acetate. Thus, a second product with high concentration of copalic acid can be obtained.

Aluminum and silicon oxides had similar behaviors as adsorbents. The surface properties of these materials allowed retaining oxygenated and polar compounds, such as diterpenic acids. Thus, SC-CO_2 desorption at 9 MPa succeeded to remove sesquiterpenes, which are non-oxygenated compounds with large affinity to CO_2. This effect was more pronounced for silicon oxide, but in none of the adsorbents β-caryophyllene could be purified. For every adsorbent, pressures from 19 MPa resulted in the complete desorption of copaiba oleoresin, with no differences in the GC profile of the fractions.

To sum up, zeolite and silicone oxide had good performance and allow proposing sequential procedures: a) For zeolite, a prior step at 9 MPa is suggested to desorb undesirable compounds, and then pressure can be increased to 29 MPa to desorb the remaining oleoresin, with increased β-caryophyllene concentration; b) With silicon oxide, a first step is indicated to obtain a highly β-caryophyllene-concentrated fraction (CF~2), followed by desorption at 29 MPa to recover the adsorbed copalic acid and the adsorbent material.

5.5 ACKNOWLEDGEMENTS

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CAPÍTULO 6 - DISCUSSÃO GERAL

Como mencionado na revisão bibliográfica apresentada no **Capitulo 2**, o óleo-resina de copaíba é extraído de árvores de várias espécies do gênero *Copaifera* e é muito utilizado em cosméticos e na medicina popular pelas suas propriedades farmacológicas já confirmadas. Além disso, o óleo-resina de copaíba é constituído por uma mistura de sesquiterpenos e diterpenos, cujas concentrações podem variar entre espécies. O β-cariofileno é o principal sesquiterpeno encontrado na fração volátil de várias espécies de copaíba. A natureza insolúvel do óleo de copaíba em água transmite uma sensação desagradável quando este é utilizado de forma direta, o que reforça a necessidade de desenvolver novas abordagens para sua aplicação.

Neste contexto, para o melhor aproveitamento das propriedades farmacológicas do óleo-resina de copaíba, este trabalho teve como um de seus principais objetivos a produção de partículas de óleo-resina de copaíba a partir de emulsões estabilizadas com amidos modificados (Hi-Cap 100® e Snow-Flake® E 6131), como relatado no **Capitulo 3**.

A encapsulação foi realizada por meio da emulsificação assistida por ultrassom seguida pelo processo de secagem por *freeze-drying* ou *spray-drying*. Para cumprir com o objetivo proposto, inicialmente foram preparadas emulsões com diferentes formulações, variando concentração de amido modificado (g/L), concentração de óleo-resina (mg/mL) e proporção acetato de etila/água (%, v/v), com a finalidade de avaliar o diâmetro médio das gotas. A seguir foi selecionada a formulação onde foi observado o menor diâmetro da gota e estudou-se o efeito da potência ultrassônica e do tempo de sonicação sobre o diâmetro médio da gota na emulsão. O menor diâmetro da gota foi observado nas emulsões preparadas a 480 W e 6 minutos de tempo de sonicação. Além disso, as emulsões produzidas nessas condições apresentaram estabilidade cinética. Isto evidencia que não houve efeito gravitacional de separação de fases, não sendo observada cremeação na superfície. Finalmente, as emulsões com menor diâmetro de gota e maior estabilidade cinética foram submetidas à secagem para obtenção de partículas solidas. A secagem foi realizada em duas etapas: 1) evaporação do acetato de etila em evaporador rotativo sob vácuo a 45 °C, na qual se observou que a evaporação do solvente ajudou a reduzir o tamanho das gotas; e 2) remoção da água através de freeze-drying e spray-drying, que permitiram gerar partículas com diâmetro micrométrico e baixos teores de umidade. Além disso, as partículas secas por *spray-drying* foram esféricas, enquanto as obtidas por *freeze-drying* apresentaram estruturas irregulares semelhantes a folhas.

O trabalho desenvolvido no **Capitulo 3** foi o ponto de partida para estudar técnicas adicionais para remover o solvente orgânico da emulsão, como a extração com fluido

supercrítico de emulsões (SFEE). Esta técnica consiste em extrair o solvente orgânico a partir de uma gota emulsionada de óleo em água pelo CO_2 supercrítico.

Nesse sentido, no **Capitulo 4** foi estudada a produção de partículas suspensas de óleo-resina de copaíba usando tecnologia supercrítica. A emulsão selecionada para os experimentos de SFEE foi a determinada com base nos resultados do **Capitulo 3** (menor diâmetro da gota e maior estabilidade cinética). Verificou-se que as menores concentrações residuais de acetato de etila na suspensão foram obtidas com uma vazão de emulsão de 0,5 mL/min, independentemente da vazão de CO_2 utilizada. Por outro lado, observou-se que, com maior vazão de CO_2 e menor vazão de emulsão, maior será a taxa de remoção de acetato de etila, aproximadamente 97,08% (2325 ppm). A perda de óleo-resina observada no frasco de coleta ao final do processo foi relacionada com a solubilidade do óleo-resina de copaíba em acetato de etila e subsequente afinidade com CO_2 supercrítico, fazendo que o acetato de etila atue como co-solvente em CO_2, removendo desta forma alguns compostos do óleo-resina de copaíba. Além disso, a possível instabilidade da emulsão durante a SFEE pode levar à liberação de óleo-resina para a fase aquosa e à sua posterior dissolução em CO_2 supercrítico.

Os resultados obtidos nesta etapa foram importantes para a seleção de condições de processo para a seguinte etapa do trabalho, na qual foram estudados diferentes tempos de extração do acetato de etila, pois o tempo influencia diretamente na taxa de remoção do solvente utilizado. Verificou-se que o conteúdo residual de acetato de etila na suspensão após o processo SFEE diminui à medida que o tempo de extração do solvente aumenta. Tal comportamento pode ser explicado pelo maior tempo de contato entre o acetato de etila e o CO_2 supercrítico, além da afinidade existente entre ambos. Isto é, a área de contato entre a suspensão e o solvente de extração é maior, aumentando desta forma a taxa de eliminação do acetato de etila presente na suspensão. Foi possível uma redução na concentração de acetato de etila de 79588 ppm (concentração de acetato de etila no início da emulsão) para 1484,5 ppm (aproximadamente 98,15%), atingindo desta forma o nível permitido pela FDA que é de 5000 ppm por dia. Verificou-se também que o tamanho das partículas suspensas apresentou pouca variação em relação ao diâmetro médio das gotas da emulsão injetada ao sistema SFEE, indicando que o tamanho da partícula está mais relacionado com o tamanho e natureza das gotículas da emulsão do que com as condições de transferência de massa do processo como a vazão de CO2 e vazão de emulsão. Observou-se também que a maior recuperação de β-cariofileno foi a 0,5 mL/min e 12,48 g/min, para vazão de emulsão e vazão de CO_2 supercrítico, respectivamente, e 30 minutos de extração do solvente. Finalmente as partículas suspensas foram submetidas à secagem

(*freeze-drying* e *spray-drying*) com o intuito de obter partículas em pó. As partículas mostraram características de materiais amorfos com tamanho em escala micrométrica e distribuição monomodal, o que sugere maior homogeneidade do tamanho das partículas. Em relação com a morfologia das partículas, notou-se microestruturas com características semelhantes ao vidro quebrado, para as partículas obtidas por *freeze-drying*. Observaram-se também pequenos poros nas paredes internas das partículas, possivelmente formadas pela aglomeração das gotículas da emulsão ao longo da congelação e secagem. Por outro lado, as partículas obtidas por *spray-drying* apresentam formato esférico com superfícies rugosas, originado possivelmente pela evaporação da água. Além disso, as micrografias obtidas por CLSM confirmaram a encapsulação do óleo-resina de copaíba na matriz polimérica.

O óleo-resina de copaíba é amplamente utilizado na indústria farmacêutica devido às propriedades medicinais que apresenta. No entanto, sua aplicação ainda encontra limitações quando a intenção é obter compostos com alto valor agregado e pureza. Nesse sentido, a adsorção/dessorção com fluido supercrítico se apresenta como uma alternativa interessante para fracionar diferentes tipos de óleos e, assim, concentrar os compostos de interesse.

Neste contexto, no **Capitulo 5** foi estudado o fracionamento com CO_2 supercrítico de sesquiterpenos e ácidos diterpênicos do óleo-resina de copaíba. O fracionamento com CO_2 supercrítico proporciona vantagens interessantes em relação aos métodos de fracionamento tradicionais como a ausência de solvente no produto final, seletividade do CO_2 com pequenas mudanças de temperatura e pressão. Verificou-se que a pressão e os materiais adsorventes utilizados tiveram efeito significativo na cinética de fracionamento e na composição química das frações. Além disso, o comportamento em relação à pressão foi semelhante para os três materiais adsorventes, sendo que, a maiores pressões, a afinidade do CO_2 supercrítico pelo óleo-resina de copaíba aumenta, resultando em maiores rendimentos, o que leva a uma extração indiscriminada de compostos presentes no substrato. Nesse sentido, comportamento diferente foi observado com a zeólita 13X a 9 MPa, que mostrou capacidade de reter o β-cariofileno, ou seja, a zeólita ofereceu uma maior resistência à passagem do β-cariofileno através da coluna de adsorção, fornecendo desta forma frações com baixa concentração de β-cariofileno. Notou-se que o óxido de alumínio teve comportamento similar ao óxido de silício, ou seja, a característica superficial destes materiais permitiu reter com maior força compostos com maior polaridade e oxigenados, como os ácidos diterpênicos presentes no óleo-resina de copaíba. Desta forma, quando foi utilizado 9 MPa, obteve-se a dessorção dos sesquiterpenos em geral, compostos não oxigenados com elevada afinidade pelo CO_2.

Finalmente, os materiais adsorventes empregados na SFF foram submetidos a uma extração *Soxhlet*, utilizando como solventes acetato de etila e etanol, com o intuito de recuperar o óleo-resina residual retido nos adsorventes. Nesse sentido, verificou-se a presença de ácido copálico em concentrações elevadas na fração recuperada do material adsorvente zeólita 13X com acetato de etila. Tal comportamento pode ser explicado pelo fato de que o CO_2 supercrítico não é capaz de dissolver o ácido copálico, devido à baixa polaridade do CO_2 nas condições utilizadas. No entanto o emprego de um solvente mais polar como o acetato de etila faz com que seja possível a separação do ácido copálico do material adsorvente.

CAPÍTULO 7 - CONCLUSÃO GERAL E SUGESTÕES PARA TRABALHOS FUTUROS

7.1 CONCLUSÃO GERAL

O trabalho desenvolvido no Capitulo 3 permitiu concluir que os amidos modificados (Hi-Cap 100® e Snow-Flake® E 6131) apresentam características semelhantes, uma vez que são derivados do milho. As emulsões que originaram as partículas foram geradas usando a emulsificação assistida por ultrassom, confirmando a capacidade desta técnica de gerar emulsões com elevada estabilidade cinética. Além disso, as emulsões produzidas com estes amidos mostraram estabilidades cinéticas diferentes, sendo mais estáveis aquelas produzidas com Hi-Cap 100®. O diâmetro médio da gota é influenciado positivamente pela potência ultrassônica e pelo tempo de sonicação. Também foi confirmado o efeito de excesso de processamento quando elevadas potências de ultrassom foram utilizadas em tempos prolongados. As técnicas *spray-drying* e *freeze-drying* foram empregadas para a secagem das emulsões, permitindo a obtenção de partículas secas com diâmetro micrométrico. A caracterização destas partículas permitiu evidenciar que o óleo-resina de copaíba foi efetivamente encapsulado, e a análise de estabilidade oxidativa demostrou que as partículas secas são mais estáveis ao longo do tempo que o óleo-resina de copaíba não encapsulado. Verificou-se também que os diferentes tipos de amidos modificados utilizados não influenciaram nas características morfológicas das partículas secas, ao contrário do que ocorreu com as técnicas de secagem.

Com base nos resultados obtidos no Capítulo 3, partículas suspensas de óleo-resina de copaíba foram produzidas por extração supercrítica de emulsões (SFEE), conforme apresentado no Capítulo 4. As condições experimentais de SFEE aplicadas permitiram produzir partículas suspensas com teor residual de acetato de etila dentro dos limites permitidos (< 5000 ppm). A maior perda de óleo-resina no processo SFEE está relacionada com a menor recuperação de β-cariofileno nas suspensões. Além disso, o tempo de extração do solvente influencia positivamente na remoção do acetato de etila, mas intensifica a perda de β-cariofileno, devido à dissolução deste sesquiterpeno em CO_2 supercrítico. O tamanho das nanopartículas suspensas apresentou pouca variação em relação às gotas das emulsões injetadas no sistema SFEE, reforçando a forte relação existente entre estas duas propriedades. A secagem das suspensões por *freeze-drying* e *spray-drying* formou partículas com características morfológicas distintas de tamanho micrométrico. As partículas apresentaram características de matérias amorfos e as análises por CLSM confirmaram novamente que o óleo-resina de copaíba foi encapsulado no amido modificado Hi-Cap 100®. Neste contexto, foram levantadas

informações relevantes, principalmente com relação à remoção do acetato de etila e à recuperação do β-cariofileno das suspensões. Estas informações permitem sugerir aplicações industriais da SFEE nos setores cosmético e farmacêutico, nos quais as propriedades dos componentes do óleo-resina de copaíba podem fornecer produtos de alto valor agregado. Dependendo da aplicação, as partículas suspensas ou secas podem ser viáveis.

O estudo de fracionamento do óleo-resina de copaíba com adsorção e dessorção por CO_2 supercrítico, mostrado no Capitulo 5, permite concluir que pressão e tipo de material adsorventes têm efeitos significativos na cinética de fracionamento e na composição química das frações. As cromatografias revelaram que o β-cariofileno é o principal sesquiterpeno do óleo-resina de copaíba, enquanto o principal ácido diterpênico é o ácido copálico. As frações obtidas usando a zeólita 13X a 9 MPa atingiram uma significativa purificação do β-cariofileno. Além disso, óxido de alumínio e silício foram capazes de adsorver os compostos polares e oxigenados do óleo-resina de copaíba, resultando também na concentração de β-cariofileno. No entanto, em pressões elevadas obteve-se a dessorção de todos os compostos do óleo-resina de copaíba. Nesse sentido, para zeólita, propõe-se realizar uma primeira etapa a 9 MPa, para dessorção de compostos que não são de interesse, e posteriormente, incrementar a pressão do sistema, para 29 MPa, para efetuar a dessorção do material remanescente, com concentração incrementada de β-cariofileno. Por outro lado, para óxido de silício, pode se propor uma primeira etapa a 9 MPa, para a obtenção de uma primeira fração muito concentrada em β-cariofileno, seguida de uma segunda etapa a 29 MPa, para dessorver os compostos adsorvidos e recuperar o material adsorvente. A fração retida na zeólita 13X após SFF a 9 MPa permitiu concentrar e purificar o ácido copálico, que foi recuperado usando acetato de etila. Além de aumentar a concentração de ácido copálico e diminuir a concentração dos contaminantes, o elevado rendimento desta fração proporciona um produto com características únicas para aplicações na indústria farmacêutica.

De modo geral, este trabalho permite propor diversos processos para a valorização e aplicação do óleo-resina de copaíba. Foi possível a produção de emulsões contendo óleo-resina de copaíba, com auxílio de acetato de etila (fase dispersa), além da aplicação de ultrassom para diminuir o tamanho das gotículas e obter estabilização cinética. A partir destas emulsões, os caminhos podem ser diversos ainda para chegar a produtos finais: é possível remover o solvente orgânico por evaporação ou ainda aplicar um processo em alta pressão para o mesmo fim, como a SFEE. Este processo apresentou melhor desempenho em termos de eliminação de solvente orgânico e retenção de composto de interesse, como também nas características das

partículas formadas. Além disso, o estudo do efeito de diferentes tempos de extração de solvente orgânico permitiu estabelecer a relação de massa de CO_2 usada por volume de emulsão para obter a máxima redução do teor de acetato de etila sem uma perda significativa de β-cariofileno. Assim, o primeiro produto foi produzido, uma suspensão de óleo-resina de copaíba encapsulado em amido modificado, que pode ser matéria prima para produtos de uso tópico ou ainda ser submetida a processos de secagem por *freeze-drying* e *spray-drying*, para obter partículas secas contendo óleo-resina de copaíba. Além disso, todos estes processos podem ser antecedidos por um fracionamento do óleo-resina de copaíba por adsorção/dessorção, com o qual seria possível concentrar compostos específicos como β-cariofileno e ácido copálico.

A abrangência do presente trabalho mostra como a combinação de diferentes técnicas emergentes pode ser aproveitada para produzir e aprimorar produtos de interesse para o setor farmacêutico e cosmético. Ao mesmo tempo, esta tese serve como ponto de partida para trabalhos futuros que visem ao melhor aproveitamento das propriedades farmacológicas do óleo-resina de copaíba.

7.2 SUGESTÕES PARA TRABALHOS FUTUROS

Levando em conta os avanços desenvolvidos neste trabalho, propõem-se as seguintes sugestões para futuros trabalhos:

i. Realizar mais estudos para substituir o solvente orgânico (acetato de etila) usado na SFEE por solventes de grau alimentício, e assim reduzir o impacto do processo no meio ambiente;
ii. O diâmetro do bico coaxial utilizado para injetar a emulsão no SFEE afeta o tamanho das nanopartículas suspensas. Nesse sentido, pesquisas adicionais são necessárias para elucidar essa influência;
iii. Estudar o aumento da escala dos processos SFEE e SFF, para sua avalição econômica;
iv. Aprofundar os estudos de SFF avaliando o efeito de outras variáveis como temperatura, vazão de CO_2, diâmetro de coluna, entre outras.

CAPÍTULO 8 - REFERÊNCIAS

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APÊNDICES

APÊNDICE A - MATERIAL SUPLEMENTAR DO ARTIGO PRODUCTION OF COPAIBA OLEORESIN PARTICLES FROM EMULSIONS STABILIZED WITH MODIFIED STARCHES

Tabela 1. Dados dos ensaios experimentais do planejamento Box-Behnken correspondente aos resultados da preparação das emulsões.

Experimento	Variáveis independentes			Diâmetro médio da gota (nm)					
				Hi-Cap 100®			Snow-Flake® E 6131		
	X_1 (g/L)	X_2 (mg/mL)	X_3 (%, v/v)	Observado (1)	Observado (2)	Observado (Média)	Observado (1)	Observado (2)	Observado (Média)
01	-1 (10)	-1 (20)	0 (15)	131,2	140,4	135,8	121,8	122,9	122,4
02	+1 (20)	-1 (20)	0 (15)	147,6	148,2	147,9	170,5	174,7	172,6
03	-1 (10)	+1 (40)	0 (15)	96,0	97,1	96,55	90,4	92,3	91,35
04	+1 (20)	+1 (40)	0 (15)	79,5	82,4	80,95	85,0	88,7	86,85
05	-1 (10)	0 (30)	-1 (10)	212,4	213,4	212,9	217,3	218,1	217,7
06	+1 (20)	0 (30)	-1 (10)	234,2	236,7	235,45	223,7	226,8	225,3
07	-1 (10)	0 (30)	+1 (20)	153,0	153,6	153,3	110,3	114,1	112,2
08	+1 (20)	0 (30)	+1 (20)	141,4	1442,2	141,8	93,2	98,8	96,0
09	0 (15)	-1 (20)	-1 (10)	261,4	263,3	262,35	237,3	240,6	238,95
10	0 (15)	+1 (40)	-1 (10)	160,6	164,2	162,4	165,4	170,1	167,75
11	0 (15)	-1 (20)	+1 (20)	115,1	122,6	118,9	99,9	102,7	101,3
12	0 (15)	+1 (40)	+1 (20)	81,7	85,1	83,4	69,7	73,9	71,8
13	0 (15)	0 (30)	0 (15)	116,9	117,6	117,3	93,5	96,3	94,9
14	0 (15)	0 (30)	0 (15)	117,8	118,7	118,3	89,2	95,3	92,25
15	0 (15)	0 (30)	0 (15)	114,6	118,4	116,5	87,7	92,6	90,2

Tabela 2. Dados experimentais correspondente a influência da potência ultrassónica e tempo de sonicação sobre o diâmetro médio das gotas da emulsão.

Potência ultrassónica (W)	Tempo (min)	Diâmetro médio da gota (nm)					
		Hi-Cap 100® *(Experimento 04)*			Snow-Flake® E 6131 *(Experimento 12)*		
		Observado (1)	Observado (2)	Observado (Média)	Observado (1)	Observado (2)	Observado (Média)
160	2	388,4	391,4	389,9	157,6	158,0	157,8
	4	288,9	300,6	294,8	131,2	132,4	131,8
	6	135,5	138,0	136,8	111,3	111,6	111,45
	8	112,3	119,8	116,1	99,2	101,7	100,5
320	2	111,2	113,7	112,5	80,8	82,9	81,9
	4	108,0	111,0	109,5	78,5	79,8	79,15
	6	95,9	96,0	95,95	73,1	74,6	73,9
	8	99,8	106,1	102,95	75,6	75,9	75,75
480	2	98,2	100,3	99,3	75,6	77,6	76,6
	4	91,4	95,4	93,4	75,0	77,7	76,35
	6	79,5	82,4	80,95	69,7	73,9	71,8
	8	100,3	102,1	101,2	77,9	78,9	78,4
640	2	102,3	103,2	102,8	76,2	78,5	77,4
	4	83,2	92,8	88,0	73,5	75,4	74,45
	6	79,4	86,0	82,7	70,7	74,1	72,4
	8	112,0	114,4	113,2	78,8	80,1	79,5

Tabela 3. Dados experimentais correspondente a caracterização das partículas após secagem.

Partícula	Umidade (%)			Diâmetro da partícula (µm)			Eficiência de encapsulação (%)			Óleo superficial (%)		
	A	B	Média	A	B	Média	A	B	Média	A	B	Média
Hi-Cap 100® - FD	0,57	0,67	0,62	250,79	241,30	246,01	76,6	76,9	76,8	23,40	23,03	23,22
Hi-Cap 100® - SD	1,23	1,24	1,24	12,37	11,98	12,21	84,1	82,6	83,4	15,88	17,31	16,59
Snow-Flake® E 6131 - FD	0,87	1,03	0,95	218,24	206,25	212,28	87,1	86,4	86,8	12,86	13,60	13,23
Snow-Flake® E 6131 - SD	3,25	3,32	3,29	12,14	11,98	12,01	89,8	89,9	89,9	10,18	10,02	10,10

APÊNDICE B - MATERIAL SUPLEMENTAR DO ARTIGO PRODUCTION OF COPAIBA (*Copaifera officinalis*) OLEORESIN PARTICLES BY SUPERCRITICAL FLUID EXTRACTION OF EMULSIONS

Curvas de Calibração

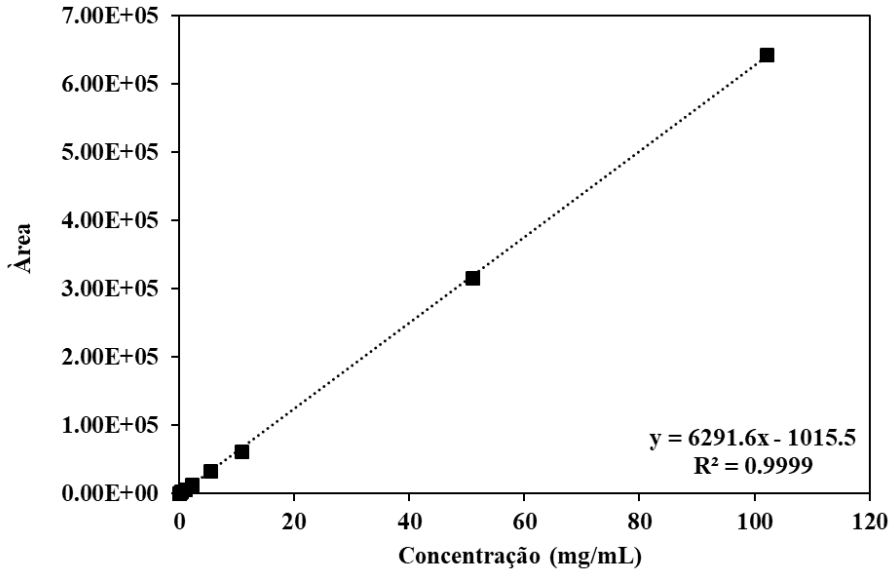

Figura 1. Curva padrão de acetato de etila para a determinação do teor residual de acetato de etila na suspensão após o processo SFEE.

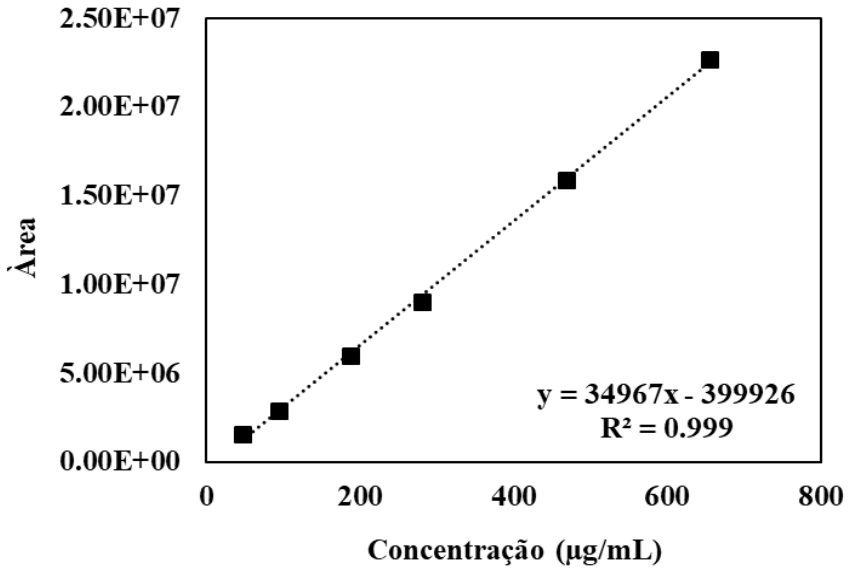

Figura 2. Curva padrão de trans-cariofileno para a determinação de β-cariofileno na suspensão após o processo SFEE.

Tabela 4. Dados experimentais da influência do tempo no diâmetro médio da gota da emulsão produzida com potência ultrassônica de 480 W e tempo de sonicação de 6 min.

Dispersão	Time (min)	Diâmetro médio da gota (nm)		
		A	B	Média
emulsionada	10	79,5	82,4	81,0
	30	95,6	94,3	95,0
	60	125,7	117,0	121,4
	90	136,4	135,7	136,1
	120	152,4	153,2	152,8
	150	178,2	174,3	176,3
	1440	260,1	263,2	261,7
Não emulsionada	-	714,3	718,5	716,4

Tabela 5. Dados experimentais correspondente com a caracterização das partículas suspensas após freeze-drying (FD) e spray-drying (SD).

Particle	Umidade (%)			a_w			Diâmetro da partícula (μm)		
	A	B	Média	A	B	Média	A	B	Média
SFEE – Coaxial nozzle (177.8 μm) - FD	0,91	0,84	0,88	0,16	0,14	0,15	238,20	239,25	238.72
SFEE – Coaxial nozzle (127 μm) - FD	0,69	0,77	0,73	0,08	0,09	0,09	283,42	272,08	277,75
SFEE – Coaxial nozzle (177.8 μm) - SD	2,27	2,44	2,36	0,59	0,64	0,62	10,18	10,86	10,52
SFEE – Coaxial nozzle (127 μm) - SD	1,85	1,74	1,80	0,16	0,18	0,17	15,32	14,02	14,68

APÊNDICE C - MATERIAL SUPLEMENTAR DO ARTIGO SUPERCRITICAL CARBON DIOXIDE FRACTIONATION OF SESQUITERPENES AND DITERPENIC ACIDS FROM COPAIBA (*Copaifera officinalis*) OLEORESIN

Tabela 6. Dados experimentais dos rendimentos correspondente a cinética de SFF (T = 50 °C, V_{CO2} = 1,75x10-4 kg/s) e extração a baixa pressão sob refluxo (Soxhlet) para o material adsorvente zeólita 13X.

Experimento	tempo (min)	9 MPa		19 MPa		29 MPa	
		$M_{fração}$ (g)	Rendimento -X_f (%)	$M_{fração}$ (g)	Rendimento -X_f (%)	$M_{fração}$ (g)	Rendimento -X_f (%)
0	0	0,000	0,000	0,000	0,000	0,000	0,000
1	30	0,3229	8,70	1,5950	42,99	2,5131	67,74
2	60	0,2676	7,21	0,3038	8,19	0,1002	2,70
3	90	0,2445	6,59	0,1837	4,95	0,0457	1,23
4	120	0,1840	4,96	0,1304	3,51	0,0421	1,13
5	150	0,1173	3,16	0,0569	1,53	0,0410	1,11
6	180	0,0539	1,45	0,0461	1,24	0,0432	1,16
7	210	0,1225	3,30	0,0307	0,83	0,0438	1,18
8-Dp	240	0,0254	0,69	0,0340	0,92	0,0414	1,12
Extração a baixa pressão sob refluxo (Soxhlet)							
Tratamento		9 MPa		19 MPa		29 MPa	
		$M_{recuperda}$	Rendimento (%)	$M_{recuperda}$	Rendimento (%)	$M_{recuperda}$	Rendimento (%)
Acetato de etila		2,1670	58,41	1,0749	28,97	0,2366	6,38
Etanol		0,1707	4,60	0,0967	2,61	0,1831	4,94

Tabela 7. Dados experimentais dos rendimentos correspondente a cinética de SFF (T = 50 °C, V_{CO2} = 1,75x10^{-4} kg/s) e extração a baixa pressão sob refluxo (Soxhlet) para o material adsorvente óxido de alumínio.

Experimento	tempo (min)	9 MPa		19 MPa		29 MPa	
		$M_{fração}$ (g)	Rendimento -X_f (%)	$M_{fração}$ (g)	Rendimento -X_f (%)	$M_{fração}$ (g)	Rendimento -X_f (%)
0	0	0,000	0,000	0,000	0,000	0,000	0,000
1	30	0,2318	6,25	1,9604	52,84	2,9758	80,21
2	60	0,2265	6,11	0,3268	8,81	0,2079	5,60
3	90	0,0997	2,69	0,1810	4,88	0,0524	1,41
4	120	0,0806	2,17	0,1211	3,26	0,0335	0,90
5	150	0,0666	1,80	0,1035	2,79	0,0342	0,92
6	180	0,0390	1,05	0,0502	1,35	0,0346	0,93
7	210	0,0297	0,80	0,0641	1,73	0,0363	0,98
8-Dp	240	0,0157	0,42	0,0542	1,46	0,0382	1,03

Extração a baixa pressão sob refluxo (Soxhlet)

Tratamento	9 MPa		19 MPa		29 MPa	
	$M_{recuperda}$	Rendimento (%)	$M_{recuperda}$	Rendimento (%)	$M_{recuperda}$	Rendimento (%)
Acetato de etila	2,0467	55,17	0,0211	0,57	0.0174	0,47
Etanol	0,1026	2,77	0,2309	6,22	0,0109	0,30

Tabela 8. Dados experimentais dos rendimentos correspondente a cinética de SFF (T = 50 °C, V_{CO2} = 1,75x10-4 kg/s) e extração a baixa pressão sob refluxo (Soxhlet) para o material adsorvente óxido de silício.

Experimento	tempo (min)	9 MPa		19 MPa		29 MPa	
		$M_{fração}$ (g)	Rendimento -X_f (%)	$M_{fração}$ (g)	Rendimento -X_f (%)	$M_{fração}$ (g)	Rendimento -X_f (%)
0	0	0,000	0,000	0,000	0,000	0,000	0,000
1	30	0,2806	7,56	1,4389	38,78	2,5562	68,90
2	60	0,2031	5,47	0,4517	12,17	0,0422	1,14
3	90	0,1940	5,23	0,1557	4,20	0,0378	1,02
4	120	0,1674	4,51	0,0609	1,64	0,0355	0,96
5	150	0,1022	2,76	0,0344	0,93	0,0354	0,95
6	180	0,0752	2,03	0,0335	0,90	0,0370	1,00
7	210	0,0556	1,50	0,0314	0,85	0,0181	0,49
8-Dp	240	0,0255	0,69	0,0309	0,83	0,0328	0,88
Extração a baixa pressão sob refluxo (Soxhlet)							
Tratamento		9 MPa		19 MPa		29 MPa	
		$M_{recuperda}$	Rendimento (%)	$M_{recuperda}$	Rendimento (%)	$M_{recuperda}$	Rendimento (%)
Acetato de etila		2,3572	63,54	1,4226	38,34	0,0487	1,31
Etanol		0,1127	3,04	0,0693	1,89	0,0622	1,68

APÊNDICE D – MEMÓRIA DO PERÍODO DE DOUTORADO

José Luis Pasquel Reátegui é engenheiro Agroindustrial, graduado pela Universidade Nacional de San Martín (UNSM - Peru) e Mestre em Engenharia de Alimentos pela Universidade Estadual de Campinas (UNICAMP). Em 2014, iniciou o curso de Doutorado em Engenharia de Alimentos da Faculdade de Engenharia de Alimentos (FEA/UNICAMP), realizando as atividades de pesquisa no Laboratório de Alta Pressão em Engenharia de Alimentos (LAPEA), com bolsa concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), com vigência de março de 2014 a fevereiro 2018.

O trabalho de doutorado foi realizado sob orientação do Prof. Dr. Julian Martínez (FEA/UNICAMP) e co-orientação da Dra. Carmen Lucia Queiroga, do Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA/UNIAMP).

Foram cursados 18 créditos, que incluem duas participações no Estágio de Capacitação Docente – PED C (CD003) e as disciplinas: Reologia (TP143), Tecnologia de Café e Cacau (TP240), Seminários (TP199), Novos processos em Engenharia de Alimentos (TP360) e Encapsulação de Agentes Bioativos (IQ064).

A participação no Programa de Estágio Docente (PED C) ocorreu do segundo semestre de 2015 ao primeiro semestre de 2016, com atividades de apoio parcial à docência na disciplina de Instalações Industriais (TA734).

Em 2015, participou do III Congresso brasileiro de Reologia, realizado na UNICAMP – Campinas – SP. Os resultados desta tese de doutorado renderam três artigos: 1) "Production of Copaiba oleoresin particles from emulsions stabilized with modified starches", publicado no periódico "*Industrial Crops and Products*"; 2) "Production of copaiba (*Copaifera officinalis*) oleoresin particles by supercritical fluid extraction of emulsions", submetido à revista "*Journal of Supercritical Fluids*"; 3) "Supercritical carbon dioxide fractionation of sesquiterpenes and diterpenic acids from copaiba (*Copaifera officinalis*) oleoresin", submetido ao periódico "*Separation and Purification Technology*". Além disso, foram publicados três trabalhos em anais de eventos, sendo um trabalho completo no "IV Iberoamerican Conference on Supercritical Fluids – Prosciba 2016" e dois resumos no "XI Congreso Iberoamericano de Ingeniería de Alimentos - CIBIA 2017".

Parte dos resultados deste trabalho foi obtida com a participação de duas estudantes de iniciação cientifica (Flavia Pavanel Fernandes e Vanessa Laís Grober), ambas do curso de graduação em Engenharia de Alimentos (FEA/UNICAMP).

ANEXO

PERMISSÃO PARA O USO DO ARTIGO CORRESPONDENTE AO CAPÍTULO 3