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Carlos Borges Filho

ANÁLISE DO FLAVONOIDE CRISINA POR QUECHERS MODIFICADO E HPLC-DAD E CO-ENCAPSULAÇÃO COM PROBIÓTICOS

Santa Maria, RS

2024

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM-RS), como requisito parcial para obtenção do grau de **Doutor em Ciência e Tecnologia dos Alimentos**.

Orientador: Prof. Dr. Cristiano Ragagnin de Menezes

Coorientadora: Profa. Dra. Magali Kemmerich

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Declaro, CARLOS BORGES FILHO, para os devidos fins e sob as penas da lei, que a pesquisa constante neste trabalho de conclusão de curso (Tese) foi por mim elaborada e que as informações necessárias objeto de consulta em literatura e outras fontes estão devidamente referenciadas. Declaro, ainda, que este trabalho ou parte dele não foi apresentado anteriormente para obtenção de qualquer outro grau acadêmico, estando ciente de que a inveracidade da presente declaração poderá resultar na anulação da titulação pela Universidade, entre outras consequências legais.

Carlos Borges Filho

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Aprovada em 26 de abril de 2024:

_____

Cristiano Ragagnin de Menezes, Dr. (Universidade Federal de Santa Maria)

(Presidente, orientador)

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André Riago Rossito Goes, Dr. (Faculdades Integradas de Bauru)

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Caroline Tuchtenhagen Rockembach, Dra. (Universidade Federal do Pampa)

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Lucian Del Fabbro, Dr. (Prefeitura Municipal de Itaqui)

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Thaiane Marques da Silva, Dra. (Weecaps)

Santa Maria, RS

2024

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"O Senhor está comigo entre aqueles que me ajudam." (Salmo 118:7a)

"*O verdadeiro conhecimento leva invariavelmente à humildade, e também à santidade e à piedade.*"

(Martyn Lloyd-Jones, 1899-1981)

RESUMO

ANÁLISE DO FLAVONOIDE CRISINA POR QUECHERS MODIFICADO E HPLC-DAD E CO-ENCAPSULAÇÃO COM PROBIÓTICOS

AUTOR: Carlos Borges Filho
ORIENTADOR: Cristiano Ragagnin de Menezes
COORIENTADORA: Magali Kemmerich

O flavonoide crisina possui inúmeros efeitos bioativos. Apesar de estar presente em fontes naturais, a maioria dos bioensaios não utiliza fontes naturais para obtenção da crisina. Isto decorre da falta de trabalhos que demonstrem o teor de crisina em fontes naturais e métodos analíticos adequados. O primeiro objetivo deste trabalho foi adaptar e validar um método QuEChERS (rápido, fácil, barato, efetivo, robusto e seguro), seguido de análise por cromatografia líquida de alta eficiência com detector de arranjo de diodos (HPLC-DAD) para determinação de crisina em espécies de *Passiflora*, e aplicar este método para quantificação de crisina em folhas, polpa e casca de *Passiflora caerulea.* Os parâmetros de exatidão, precisão e precisão intermediária apresentaram resultados adequados, com efeito matriz significativo para polpa e casca. O limite de quantificação foi de 0,08, 0,01 e 0,014 mg kg^{-1} para folha, polpa e casca, respectivamente. As folhas e casca verde apresentaram os maiores teores de crisina e o melhor potencial antioxidante, e a digestão gastrointestinal *in vitro* não alterou a bioacessibilidade da crisina. O segundo objetivo foi avaliar a viabilidade da co-encapsulação de crisina e *Lactiplantibacillus plantarum (L. plantarum)*. Os esforços concentraram-se primeiramente em catalogar as metodologias usadas para encapsulação da crisina já documentadas na literatura. Observou-se que não havia nenhum trabalho associando probióticos e crisina, e verificou-se a escassez do uso de técnicas verdes e tradicionais, como a gelificação iônica externa. A partir disso, cepas de *L. plantarum* foram co-encapsuladas na concentração de 10 UFC mL^{-1} com a crisina nas concentrações de 0,1, 0,25 e 0,5 %. A eficiência de encapsulação para o probiótico foi de 90%, e para a crisina foi de 54 a 84 %. As micropartículas foram submetidas à pasteurização e à simulação da digestão gastrointestinal, obtendo-se contagens de probióticos e níveis de crisina satisfatórios. A estabilidade das cápsulas foi avaliada durante 120 dias sob 3 diferentes condições de temperatura (25, 8 e -18 °C). A estabilidade dos probióticos não foi afetada pela concentração de crisina, e a melhor estabilidade de *L. plantarum* ocorreu na temperatura de -18 °C. O teor de crisina não foi substancialmente modificado ao longo dos 120 dias de estudo em nenhuma das concentrações e temperaturas. Demonstrou-se, pela primeira vez, um método validado de QuEChERS modificado seguido de análise HPLC-DAD que pode ser aplicado para análise de crisina em espécies de *Passiflora*. Observou-se que as folhas de *Passiflora caerulea* são ótimas fontes de crisina e possuem potencial antioxidante, seguidas pela casca verde, e que a bioacessibilidade da crisina nestas matrizes não é alterada pela digestão gastrointestinal. A co-encapsulação de *L. plantarum* e crisina mostrou-se viável, com a crisina não exercendo toxicidade aguda ou crônica nos probióticos, e a encapsulação sendo benéfica para a estabilidade dos agentes encapsulados. Como perspectivas, vislumbra-se estudar a aplicação das folhas e casca verde de *Passiflora caerulea* como fonte de crisina na co-encapsulação com probióticos e como fonte de crisina em formulações nutracêuticas ou alimentares, bem como o estudo do efeito bioativo das microcápsulas em ensaios *in vitro* e *in vivo*.

Palavras-chave: Validação. Cromatografia. Compostos fenólicos. *Passiflora caerulea*. *L. plantarum.*

ABSTRACT

CHRISIN FLAVONOID ANALYSIS BY MODIFIED QUECHERS AND HPLC-DAD AND CO-ENCAPSULATION WITH PROBIOTICS

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ADVISOR: Cristiano Ragagnin de Menezes
CO-ADVISOR: Magali Kemmerich

The flavonoid chrysin has numerous bioactive effects. Despite being present in natural sources, most bioassays do not use natural sources to obtain chrysin. This is due to the lack of studies demonstrating the chrysin content in natural sources and adequate analytical methods. The first objective of this work was to adapt and validate a QuEChERS method (quick, easy, cheap, effective, rugged and safe), followed by analysis by high-performance liquid chromatography with diode array detector (HPLC-DAD) for the determination of chrysin in species of *Passiflora*, and apply this method to quantify chrysin in leaves, pulp and peel of *Passiflora caerulea*. The accuracy, precision and intermediate precision parameters showed adequate results, with a significant matrix effect for pulp and peel. The limit of quantification was 0.08, 0.01 and 0.014 mg kg^{-1} for leaf, pulp and peel, respectively. The leaves and green peel presented the highest chrysin content and the best antioxidant potential, and *in vitro* gastrointestinal digestion did not alter the bioaccessibility of chrysin. The second objective was to evaluate the feasibility of co-encapsulation of chrysin and *Lactiplantibacillus plantarum (L. plantarum)*. Efforts were primarily focused on cataloging the methodologies used for chrysin encapsulation already documented in the literature. It was observed that there was no work associating probiotics and chrysin, and there was a lack of use of green and traditional techniques, such as external ionic gelation. From this, strains of *L. plantarum* were co-encapsulated at a concentration of 10 UFC mL^{-1} with chrysin at concentrations of 0.1, 0.25 and 0.5 %. The encapsulation efficiency for the probiotic was 90%, and for chrysin it was 54 to 84%. The microparticles were subjected to pasteurization and simulation of gastrointestinal digestion, obtaining satisfactory probiotic counts and chrysin levels. The stability of the capsules was evaluated for 120 days under 3 different temperature conditions (25, 8 and -18 °C). The stability of the probiotics was not affected by the concentration of chrysin, and the best stability of *L. plantarum* occurred at a temperature of -18 °C. The chrysin content was not substantially modified over the 120 days of study at any of the concentrations and temperatures. For the first time, a validated modified QuEChERS method followed by HPLC-DAD analysis that can be applied for chrysin analysis in *Passiflora* species was demonstrated. It was observed that the leaves of *Passiflora caerulea* are excellent sources of chrysin and have antioxidant potential, followed by the green peel, and that the bioaccessibility of chrysin in these matrices is not altered by gastrointestinal digestion. The co-encapsulation of *L. plantarum* and chrysin proved to be viable, with chrysin not exerting acute or chronic toxicity in the probiotics, and encapsulation being beneficial for the stability of the encapsulated agents. As perspectives, it is envisioned to study the application of the leaves and green peel of *Passiflora caerulea* as a source of chrysin in co-encapsulation with probiotics and as a source of chrysin in nutraceutical or food formulations, as well as the study of the bioactive effect of microcapsules in *in vitro* and *in vivo* assays.

Key-words: Validation. Chromatography. Phenolic compounds. *Passiflora caerulea. L. plantarum.*

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SUMÁRIO

1 INTRODUÇÃO

A crisina (5,7-Dihidroxiflavona) pertence à classe flavona de flavonoides e tem sido apresentada como tendo inúmeros e promissores efeitos bioativos, como atividade antioxidante (PUSHPAVALLI et al., 2010), anticonvulsivante (MEDINA et al., 1990), anti-hipertensiva (VILAR et al., 2002), anti-inflamatória (BAE et al., 2011), antineoplásica (PICHICHERO et al., 2011), anti-hiperlipidêmica (ZARZECKI et al., 2014) e, especialmente associada a este trabalho, atividade antidepressiva (BORGES FILHO et al. 2015; 2016a; 2016b; BORTOLOTTO et al., 2018). A crisina é encontrada naturalmente em mel, própolis, e várias espécies de plantas, incluindo espécies do gênero *Passiflora* (NABAVI et al., 2015). Apesar disso, a maioria dos bioensaios com crisina não utiliza fontes naturais para sua obtenção, sendo adquirida na forma de pó com alta pureza em empresas especializadas, elevando o custo dos experimentos e deixando de explorar produtos naturais. Entre outros fatores, isso se deve à escassez de trabalhos que demonstrem com precisão o teor de crisina em fontes naturais e meios adequados para sua extração. Isso ocorre porque a maior ênfase nos estudos com crisina é na sua biotividade, ficando muitas vezes em segundo plano a sua extração e quantificação.

O maracujá *Passiflora caerulea* é uma espécie do gênero *Passiflora* abundante e de fácil acesso na América do Sul, ocorrendo em campos, beiras de estradas e bordas de florestas (MONDIN et al., 2011), sendo raramente utilizada para consumo humano ou qualquer outra finalidade. Embora os estudos sobre o *Passiflora caerulea* sejam escassos, já se sabe que suas folhas contém o flavonoide crisina (MEDINA et al., 1990; EL-ASKARY et al., 2017), possuindo potencial bioativo promissor (OZAROWSKI et al., 2018). Assim, estabelecer um método analítico que quantifique os níveis de crisina nas folhas de *Passiflora caerulea* pode ser um grande passo para avanços nos estudos deste produto natural. Além disso, é interessante analisar o teor de crisina e o potencial bioativo das demais partes da planta, que são a polpa e a casca, que ainda não foram estudadas.

Em relação à análise de crisina em matrizes vegetais, uma etapa importante é a preparação da amostra, que geralmente consiste em uma etapa que requer várias horas e/ou alto consumo de energia para execução. Gharari et al. (2020) mostraram a determinação de crisina em raízes e parte aérea de quatro espécies de *Scutellaria*, com secagem do material em temperatura ambiente e moagem seguidas de 48h de extração com metanol. Em seguida, o extrato foi armazenado a 4ºC por 48h antes da injeção no sistema de cromatografia líquida de alta eficiência (HPLC). Hadas et al. (2017) e Ozarowski et al. (2018) avaliaram o perfil químico de folhas de *Passiflora caerulea* e, para isso, as folhas foram secas com circulação de

ar (25°C, 24h), e posteriormente extraídas com metanol por 1h (n=3) e concentradas sob vácuo. Portanto, é necessário buscar alternativas mais rápidas, que também tenham baixo custo e impacto ambiental. Além disso, ainda não existem métodos validados para quantificação da crisina em outras matrizes vegetais, como polpa e casca, e este também é um dos propósitos do presente trabalho.

Em 2003, Anastassiades et al. desenvolveram o método QuEChERS (rápido, fácil, barato, efetivo, robusto e seguro (do inglês *quick, easy, cheap, effective, rugged and safe*)), com o objetivo de minimizar o manuseio de amostras, o tempo e melhorar as recuperações para determinação de pesticidas em matrizes alimentares. Estudos recentes também relataram o uso bem sucedido do método QuEChERS para determinação de compostos fenólicos em diferentes matrizes alimentares (ROTTA et al., 2019; NICÁCIO et al., 2021). Porém, esta metodologia ainda não foi aplicada para a análise da crisina em nenhuma matriz. O QuEChERS destaca-se ainda por possuir uma etapa de limpeza, favorecendo a identificação dos compostos e diminuindo possíveis danos causados pelos co-eluentes ao sistema cromatográfico. Já nos métodos até então usados para análise de crisina, esta etapa de limpeza não ocorre.

Quanto à quantificação de crisina, a cromatografia líquida de alta eficiência com detector de arranjo de diodos (HPLC-DAD) é uma excelente escolha, pois fornece limites de quantificação (LOQ) mais baixos, análise simples e de baixo custo (GHARARI et al., 2020; OROIAN et al. , 2017; GIACOMELI et al., 2020). Além disso, muitos trabalhos relataram o uso de HPLC-DAD para análise de compostos fenólicos em matrizes alimentares (KEBAL et al., 2022; RAHMAN et al., 2022; BUENO-HERRERA et al., 2020).

Além da questão analítica, caminha-se neste trabalho para avanços em uma outra questão associada ao flavonoide crisina, o estudo da sua interação com probióticos, que são uma tendência nos estudos da terapia antidepressiva (TIAN et al., 2022; DIB et al., 2021). O termo probiótico é derivado de uma palavra grega que significa “vida” e é usado para referir organismos vivos não patogênicos com efeitos benéficos nos hospedeiros (PANDEY et al., 2015). Os probióticos podem ser incluídos na composição de uma vasta gama de produtos, que varia entre medicamentos e suplementos alimentares, mas encontram-se frequentemente associados a lacticínios. Neste caso, os gêneros *Bifdobacterium* e *Lactobacillus* são os que possuem os principais micro-organismos utilizados como probióticos (REIS et al., 2019). Vários efeitos benéficos à saúde já foram relatados em estudos que avaliaram a utilização dos probióticos no organismo humano, como: fortalecimento e aumento da imunidade (PALOMAR et al., 2017), capacidade de decomposição dos ácidos biliares (RAMASAMY et al., 2010), diminuição da pressão sanguínea (MAHBOOBI et al., 2014), melhora na absorção

de minerais como o ferro e o cálcio (DUBEY et al., 2018), melhora da digestão e redução da intolerância à lactose (ALMEIDA et al., 2012), e atividade anticarcinogênica (ZEZE et al., 2017).

Tal como o flavonoide crisina, resultados interessantes têm demonstrado o efeito dos probióticos na melhora de quadros de depressão em modelos experimentais, especialmente cepas de *Lactiplantibacillus plantarum (L. plantarum)*. Esta relação entre probióticos e as doenças do sistema nervoso central tem ganhado destaque devido à crescente compreensão do eixo microbiota-intestino-cérebro, demonstrando que a microbiota intestinal tem importante papel na regulação de algumas funções cerebrais (TIAN et al., 2022). Entre outros estudos, Dib et al. (2021) mostraram que os probióticos são capazes de melhorar os sintomas depressivos em pacientes clínicos, e Kim et al. (2019) mostraram que o consumo de probióticos está associado com a redução da ocorrência e severidade da depressão, principalmente em homens.

A viabilidade dos probióticos é essencial para que esses micro-organismos consigam atingir e colonizar o intestino humano, sendo um dos fatores mais importantes para que as bactérias probióticas promovam os benefícios para a saúde do hospedeiro (FERREIRA, 2018). Neste sentido, aumentar a resistência dos micro-organismos ao trato gastrointestinal e sua estabilidade por longos períodos de armazenamento tem sido um objetivo de diversas pesquisas (COLÍN-CRUZ et al., 2019; RADDATZ E MENEZES, 2021; RADDATZ et al., 2022a, 2022b). Para tanto, diferentes técnicas para aumentar a resistência dos micro-organismos têm sido estudadas, dentre estas, destaca-se a microencapsulação (RADDATZ e MENEZES, 2021). Cabe destacar também, que a microencapsulação permite conectar micro-organismos probióticos com diferentes compostos e extratos, através da co-encapsulação, gerando possíveis benefícios destas espécies químicas para as culturas probióticas, possibilitando conjuntamente a maior estabilidade dos probióticos e dos compostos, e gerando cápsulas poli-funcionais com potencial efeito sinérgico na saúde do indivíduo (RADDATZ et al., 2022a). Neste sentido, destaca-se ainda que a interação entre a crisina e culturas probióticas ainda não foi estudada.

Deste modo, teve-se neste trabalho dois objetivos principais: I. Adaptar e validar um método analítico para extração e quantificação do flavonoide crisina em espécies do gênero *Passiflora*; II. Avaliar a viabilidade do desenvolvimento de microcápsulas contendo crisina e micro-organismos probióticos.

2 REVISÃO BIBLIOGRÁFICA

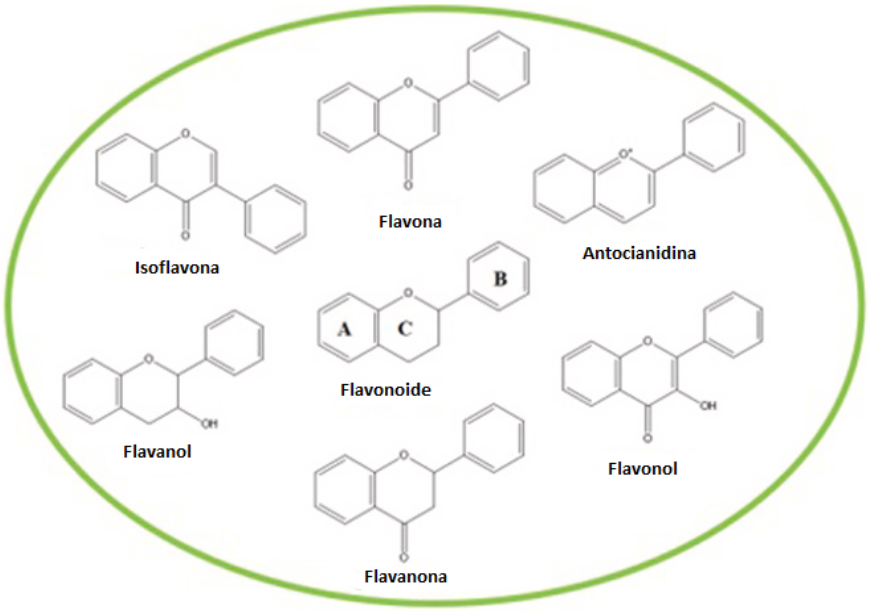
2.1 FLAVONOIDES

Os compostos fenólicos derivados de plantas são divididos em diversas categorias, como fenóis simples, ácidos fenólicos, cumarinas, flavonoides, taninos condensados e hidrolisáveis, lignanas e ligninas (NACZK E SHAHIDI, 2004).

Os flavonoides são divididos em vários grupos, incluindo flavonas, flavanonas, antocianidinas, flavanóis, flavonóis e isoflavonas, de acordo com o grau de oxidação no anel C central, a hidroxilação dos anéis, e a substituição na posição 3 (MAHER et al., 2019) (Figura 1). Dentro de cada grupo, a diversidade é gerada pelo arranjo dos grupos hidroxila combinados com a glicosilação ou alquilação (TALEBI et al., 2021).

Os flavonoides são compostos presentes em alimentos e bebidas de origem vegetal e que têm despertado interesse em decorrência da bioatividade destes compostos. Nesse contexto, flavonoides isolados de plantas, como luteolina, hespiridina, apigenina, rutina, quercetina e crisina têm demonstrado efeitos protetores e terapêuticos em diversos transtornos, como doenças cardíacas, circulatórias, renais, hepáticas, cerebrais, e neoplásicas (SEQUETO et al., 2012; BORGES FILHO et al., 2016a, 2016b; TALEBI et al., 2021).

Figura 1 - Estrutura básica das principais classes de flavonoides.



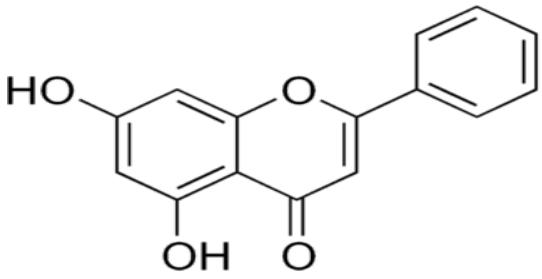
Fonte: Adaptada de Talebi et al. (2021)

2.2 CRISINA

2.2.1 Estrutura

A crisina (5,7-Dihidroxiflavona) pertence à classe flavona de flavonoides, e é formada por dois anéis de benzeno duplos (A e B) e um anel central heterocíclico pirano contendo oxigênio (C) (Figura 2).

Figura 2 - Estrutura química do flavonoide crisina.



Fonte: Adaptada de Talebi et al. (2021)

2.2.2 Fontes Naturais

A crisina é encontrada naturalmente em mel, própolis, e várias espécies de plantas, incluindo espécies do gênero *Pelargonium, Passiflora* e da família *Pinaceae* (NABAVI et al., 2015).

Estudos anteriores avaliaram as concentrações de crisina em vários méis. O teor de crisina é de 0,10 mg/kg em mel de melada, 5,3 mg/kg em méis florestais, e chega a 28g/L no própolis (HADJMOHAMMADI et al., 2010; PICHICHERO et al., 2010). Outra importante fonte de crisina é a planta *Passiflora caerulea*, da qual foi isolada em 1990 e apresentada como um composto com propriedades anticonvulsivantes (MEDINA et al., 1990).

Quanto à *Passiflora caerulea*, é uma espécie do gênero *Passiflora* abundante e de fácil acesso na América do Sul, ocorrendo em campos, beiras de estradas e bordas de florestas (MONDIN et al., 2011), e é considerada uma planta ornamental comestível que possui fruto adocicado. Embora seja raramente utilizada para consumo com fins alimentícios ou medicinais na maioria dos países em que é encontrada, folhas de *Passiflora caerulea* são comercializadas como erva para infusão na Argentina, com a alegação de possuírem efeito ansiolítico (MINTEGUIAGA et al., 2021).

Embora os estudos sobre *Passiflora caerulea* sejam escassos, já se sabe que suas folhas contém o flavonoide crisina (MEDINA et al., 1990; EL-ASKARY et al., 2017), e possuem potencial bioativo promissor (OZAROWSKI et al., 2018). Assim, estabelecer um método analítico que quantifique os níveis de crisina nas folhas de *Passiflora caerulea* pode ser um grande passo para avanços nos estudos deste produto natural. Além disso, é interessante analisar o teor de crisina e o potencial bioativo das demais partes da planta, que são a polpa e a casca, que ainda não foram estudadas.

2.2.3 Análise

Em relação à análise de crisina em matrizes vegetais, uma etapa importante é a preparação da amostra, que geralmente consiste em uma etapa que requer várias horas e/ou alto consumo de energia para execução. Gharari et al. (2020) mostraram a determinação de crisina em raízes e parte aérea de quatro espécies de *Scutellaria*, com secagem do material em temperatura ambiente e moagem seguidas de 48h de extração com metanol. Em seguida, o extrato foi armazenado a 4ºC por 48h antes da injeção no sistema HPLC. Hadas et al. (2017) e Ozarowski et al. (2018) avaliaram o perfil químico de folhas de *Passiflora caerulea* e, para isso, as folhas foram secas com circulação de ar (25°C, 24h), e posteriormente extraídas com metanol por 1h (n=3) e concentradas sob vácuo. Portanto, é necessário buscar alternativas mais rápidas, que também tenham baixo custo e impacto ambiental. Além disso, ainda não existem métodos validados para quantificação da crisina em outras matrizes vegetais, como polpa e casca, e este também é um dos propósitos do presente trabalho.

Procurando reduzir custos, tempo e impacto ambiental na análise de compostos fenólicos, têm sido demonstradas adaptações do método QuEChERS para a quantificação destes compostos em diferentes matrizes. O QuEChERS foi apresentado em 2003, por Anastassiades et al., com o objetivo de minimizar o manuseio de amostras, o tempo e melhorar as recuperações para determinação de pesticidas em matrizes alimentares. O método QuEChERS compreende uma etapa de extração/partição e uma etapa de limpeza, que proporcionam a extração dos compostos da amostra para um solvente orgânico apropriado por meio de extração com sais, onde é promovido um equilíbrio entre uma camada aquosa e uma orgânica, seguida de etapa de limpeza por extração dispersiva em fase sólida (d-SPE), para remoção de pigmentos ou interferentes (ROTTA et al., 2019). Estudos recentes relataram o uso bem sucedido do método QuEChERS para determinação de compostos fenólicos em diferentes matrizes alimentares (ROTTA et al., 2019; NICÁCIO et al., 2021). Porém, esta metodologia ainda não foi aplicada para a análise da crisina em nenhuma matriz. Além dos

benefícios relacionados ao baixo custo e rapidez do método, a possibilidade do uso do QuEChERS para análise de crisina destaca-se pela existência da etapa de limpeza, que diminui substancialmente o potencial de dano ao sistema cromatográfico causado por pigmentos e interferentes. Observe-se que esta etapa de limpeza não ocorre nas metodologias já citadas na literatura para análise de crisina.

Em relação à quantificação de crisina, Gharari et al. (2020) apresentaram um método analítico determinando crisina e outros compostos em plantas por cromatografia líquida de alta eficiência com detector de arranjo de diodos (HPLC-DAD), em 262nm, coluna C8, usando metanol como solvente e água-acetonitrila-metanol-ácido orfofosfórico (60:38:30:1 v/v/v/v) como fase móvel. Oroian et al. (2017) demonstraram a determinação de crisina e outros flavonoides em mel por HPLC, com detector UV na faixa de 200 a 210nm, coluna C18, utilizando acetonitrila-água (48:52 v/v) como eluente. Giacomeli et al. (2020) quantificaram crisina em nanocápsulas lipídicas através de HPLC, com detector UV em 260nm, coluna RP-18, usando acetonitrila/água (70:30 v/v) como fase móvel. Desta forma, o uso de HPLC-DAD é uma excelente escolha para análise de crisina, pois fornece LOQs baixos, análise simples e de baixo custo (GHARARI et al., 2020; OROIAN et al. , 2017; GIACOMELI et al., 2020).

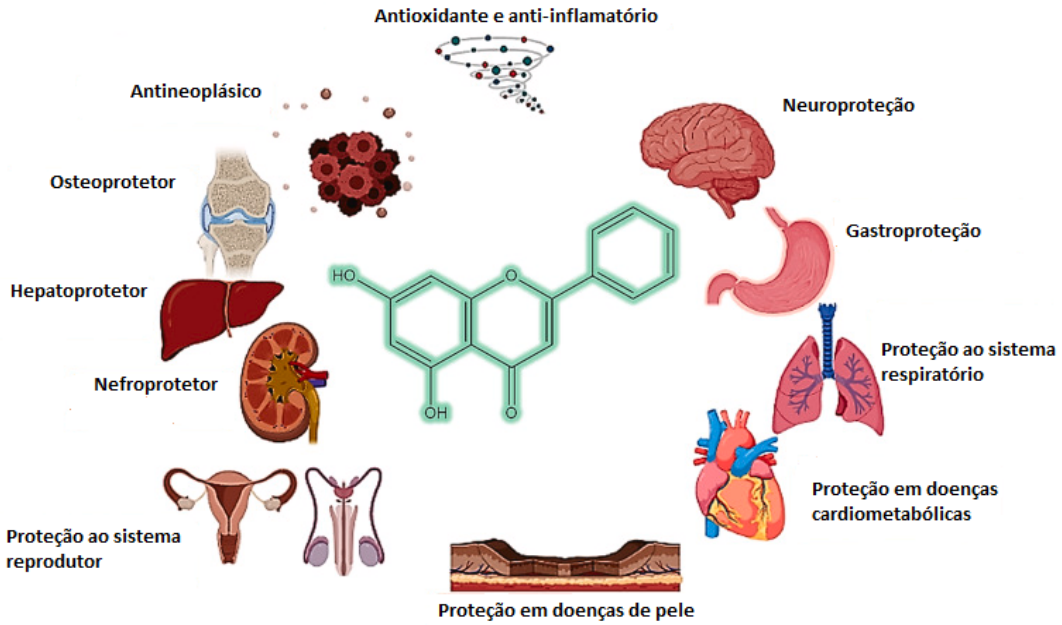
2.2.4 Bioatividade

A crisina já tem sido apresentada como tendo inúmeros e promissores efeitos bioativos, como atividade antioxidante (PUSHPAVALLI et al., 2010), anticonvulsivante (MEDINA et al., 1990), anti-hipertensiva (VILAR et al., 2002), anti-inflamatória (BAE et al., 2011), antineoplásica (PICHICHERO et al., 2011)e anti-hiperlipidêmica (ZARZECKI et al., 2014). Também há relatos que mostram que a crisina aumenta os níveis de testosterona pela inibição da enzima aromatase (KAO et al., 1998), que converte a testosterona em estradiol, e em decorrência disto, a crisina já está disponível no mercado como um suplemento dietético (500 mg por cápsula). A Figura 3 ilustra os principais achados científicos até o momento acerca da bioatividade da crisina.

Quanto à toxicologia, embora existam baixas doses de flavonoides na dieta diária dos indivíduos, a ingestão de doses maiores pode levar a alguma toxicidade (TALEBI et al., 2021). Tsuji e Walle (2008) demonstraram a citotoxicidade de elevadas concentrações de crisina em células hepáticas em estudos *in vitro*. Este efeito tóxico foi atribuído à atividade peroxidase nos hepatócitos, levando à oxidação da crisina para formar produtos tóxicos. Mohos et al. (2018) mostraram que a ingestão exacerbada de crisina pode interferir no

transporte de determinados fármacos na corrente sanguínea, devido à forte interação entre os metabólitos da crisina e a albumina. Yao et al. (2019) verificaram alterações hematológicas e químicas no sangue de ratos tratados com crisina na dose de 1000mg/kg. Neste estudo de Yao, realizado com experimentos crônicos e subcrônicos, o nível sem efeitos adversos observáveis (NOAEL) para a crisina foi de 500mg/kg, o menor nível de efeito adverso observável (LOAEL) foi de 1000mg/kg, e a dose letal 50 (DL_{50}) foi de 4350mg/kg para ambos os sexos.

Figura 3 - Principais atividades farmacológicas da crisina.



Fonte: Adaptada de Talebi et al. (2021)

Estudos sobre a atividade antioxidante de flavonoides sugerem a relação da presença de hidroxilas na bioatividade destes compostos (PUSHPAVALLI et al., 2010). Mais especificamente, a capacidade antioxidante de um flavonoide geralmente está relacionada principalmente com a hidroxilação do anel B. No entanto, as hidroxilas presentes nos carbonos 5 e 7 do anel A da crisina desempenham uma função significativa na atividade antioxidante deste composto (TOREL et al., 1986).

Ainda, o potencial antioxidante da crisina está também associado à existência da dupla ligação C2–C3 e o grupo carbonila que está posicionado no átomo C4 (HARRIS et al., 2006; TSUJI et al., 2008; TALEBI et al., 2021).

Especialmente associado a este trabalho, tem sido demonstrado o efeito antidepressivo da crisina em diferentes modelos experimentais de depressão. Foi demonstrado que a crisina é

capaz de reverter os danos oxidativos, inflamatórios, hormonais, monoaminérgicos e comportamentais em modelos de depressão induzida por estresse, bulbectomia olfatória e hipotireoidismo em camundongos (BORGES FILHO et al. 2015; 2016a; 2016b; BORTOLOTTO et al., 2018). Neste sentido, um importante avanço para os estudos com este flavonoide é o estudo da sua interação com probióticos, que por sua vez são uma tendência nos estudos da terapia antidepressiva.

2.3 MICRO-ORGANISMOS PROBIÓTICOS

2.3.1 Definições

O termo probiótico é derivado de uma palavra grega que significa vida e é usado para referir organismos vivos não patogênicos com efeitos benéficos nos hospedeiros (PANDEY et al., 2015).

A primeira definição de probióticos foi a de Parker, em 1974, que definiu os probióticos como "organismos e substâncias que contribuem para o equilíbrio microbiano intestinal". Décadas mais tarde, Fuller propôs a definição de probióticos como “suplementos alimentares microbianos vivos que afetam beneficamente o animal hospedeiro, melhorando o seu equilíbrio microbiano intestinal” (REIS, 2019).

Só em 2001, surgiu um consenso na definição de probióticos. O termo “probiótico”, tal como definido originalmente pela Organização das Nações Unidas para Agricultura e Alimentação (FAO) / Organização Mundial da Saúde (OMS), tem a seguinte redação: “micro-organismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro” (REIS, 2019).

2.3.2 Propriedades das Bactérias Probióticas

Vários efeitos benéficos à saúde já foram relatados em estudos que avaliaram a utilização dos probióticos no organismo humano, como: fortalecimento e aumento da imunidade (PALOMAR et al., 2017), capacidade de decomposição dos ácidos biliares (RAMASAMY et al., 2010), diminuição nos níveis de colesterol sérico (JONES et al., 2012), diminuição da pressão sanguínea (MAHBOOBI et al., 2014), melhora na absorção de minerais como o ferro e o cálcio (DUBEY et al., 2018), melhora da digestão e redução da intolerância à lactose (ALMEIDA et al., 2012), e atividade anticarcinogênica (ZENE et al., 2017).

Os micro-organismos pertencentes ao gênero dos lactobacilos, como *L. acidophilus, L. rhamnosus, L. paracasei, L. plantarum*, dentre outros, são as bactérias mais comuns e utilizadas para fins probióticos, assim como bactérias do gênero *Bifidobacterium* (SAAD et al., 2013). Os lactobacilos são caracterizados como Gram-positivos, incapazes de formar esporos, com forma bacilar ou cocobacilar e ausência de flagelos, aerotolerantes ou anaeróbios e estritamente fermentativos, produzindo ácido lático (homofermentativo) ou quantidades equimolares de ácido lático e outros compostos (heterofermentativos) (SOCCOL et al., 2010). Estes micro-organismos constituem somente 1% da microbiota fecal e possuem faixa de crescimento ótima em pH entre 5,5 e 6,3 (ANTUNES et al., 2007).

2.3.3 Probióticos, Depressão e Crisina

Tal como o flavonoide crisina, resultados interessantes têm demonstrado o efeito dos probióticos na melhora de quadros de depressão em modelos experimentais. Esta relação entre probióticos e as doenças do sistema nervoso central tem ganhado destaque devido à crescente compreensão do eixo microbiota-intestino-cérebro, demonstrando que a microbiota intestinal tem importante papel na regulação de algumas funções cerebrais (TIAN et al., 2022). Entre outros estudos, Dib et al. (2021) mostraram que os probióticos são capazes de melhorar os sintomas depressivos em pacientes clínicos. Kim et al. (2019) mostraram que o consumo de probióticos está associado com a redução da ocorrência e severidade da depressão, principalmente em homens. Com relação a mecanismos, Aygun et al (2022) demonstraram o efeito tipo-antidepressivo de probióticos em ratos por meio do aumento de fatores neurotróficos e diminuição de citocinas inflamatórias. Freimer et al. (2022) sugeriram que um dos mecanismos envolvidos no efeito tipo-antidepressivo de probióticos em adolescentes é a regulação do eixo hipotálamo-pituitária-adrenal. Tian et al. (2022) mostraram o papel da regulação do sistema serotoninérgico no efeito-tipo antidepressivo de probióticos. De forma interessante, todos os mecanismos supracitados também fazem parte dos mecanismos antidepressivos do flavonoide crisina (BORGES FILHO et al. 2015; 2016a). Apesar disso, a interação entre a crisina e probióticos ainda não foi estudada.

Dentre as cepas probióticas com potencial antidepressivo, destaca-se o *L. plantarum*, objeto deste estudo. Ma et al. (2023) e Zhu et al. (2024) demonstraram os efeitos antidepressivos de cepas de *L. plantarum* em modelos de depressão induzida por estresse em roedores. Quanto a estudos em humanos, Rudzki et al. (2019) estudaram os efeitos do *L. plantarum* em pacientes com depressão maior. Neste estudo, setenta e nove pacientes com depressão maior foram randomizados e alocados para um estudo duplo-cego controlado por

placebo. Os participantes receberam um antidepressivo clássico com o probiótico por um período de 8 semanas ou um antidepressivo com o placebo do probiótico pelo mesmo período. A gravidade dos sintomas psiquiátricos e as funções cognitivas foram avaliadas por meio de inúmeras escalas e testes. Parâmetros bioquímicos como triptofano, quinurenina, interleucinas e concentrações plasmáticas de cortisol foram medidas. Observou-se a melhora das funções cognitivas e a diminuição dos níveis de quinurenina nos indivíduos que receberam *L. plantarum.* De modo similar, já se demonstrou o papel do flavonoide crisina na regulação dos níveis de quinurenina e na melhora de sintomas cognitivos em roedores deprimidos (BORGES FILHO et al. 2016 a, 2016b, BORTOLOTTO et al. (2018).

Cepas de *L. plantarum* têm ainda importante efeito na regulação da microbiota intestinal (GEORGIEVA et al., 2009). Apresentam também característica antimicrobiana frente a diferentes patógenos (BEN SLAMA et al., 2013), atividade imunológica, anti-inflamatória e antitumoral (WANG et al., 2009), antioxidante (ZHANG, et al., 2013), eficácia em neutralizar citotoxicidade induzida por enterotoxinas (ZHANG et al., 2015), além de ser eficaz no tratamento da diabetes (LI et al., 2014).

Estas e outras ações do *L. plantarum* tem atraído a atenção de pesquisadores no desenvolvimento de ingredientes para formulações nutracêuticas e alimentares (DE DEUS et al., 2023; KULEY et al., 2021; VAZIRI et al., 2018). Nestes estudos, buscando elevar a estabilidade e visando o desenvolvimento de produtos polifuncionais, os probióticos, por meio de técnicas de microencapsulação, foram associados ainda a extrato do caule da beterraba vermelha (*Beta vulgaris L.*) (DE DEUS et al., 2023), extrato de própolis (KULEY et al., 2021), e ácido graxo docosahexaenóico, um ácido da família ômega 3 (VAZIRI et al., 2018).

Neste sentido, cabe ressaltar que a interação entre o flavonoide crisina e *L. plantarum* e um possível efeito biológico conjunto ainda não foi investigada, e é um alvo interessante para pesquisas.

2.3.4 Microencapsulação

A viabilidade dos probióticos é essencial para que esses micro-organismos consigam atingir e colonizar o intestino humano, sendo um dos fatores mais importantes para que as bactérias probióticas promovam os benefícios para a saúde do hospedeiro (FERREIRA, 2018). Neste sentido, aumentar a resistência dos micro-organismos ao trato gastrointestinal e sua estabilidade por longos períodos de armazenamento tem sido um objetivo de diversas pesquisas (COLÍN-CRUZ et al., 2019; RADDATZ e MENEZES, 2021; RADDATZ et al., 2022a, 2022b). Para tanto, diferentes técnicas para aumentar a resistência dos micro-

organismos têm sido estudadas, dentre estas, destaca-se a microencapsulação (RADDATZ e MENEZES, 2021).

O processo de microencapsulação consiste no aprisionamento de um material ativo (aromas, óleos essenciais, micro-organismos probióticos, compostos antioxidantes, vitaminas, enzimas, dentre outros) em um material de revestimento natural ou sintético, formando partículas que variam entre micrômetros até milímetros de tamanho (STANCIUC et al., 2018). Esta técnica tem como finalidade básica garantir a proteção do material ativo contra condições desfavoráveis de armazenamento, do alimento em que são inseridos e de seu processamento, bem como frente à passagem pelo trato gastrintestinal, permitindo uma vida útil estendida, com maior estabilidade do composto ativo (WURTH et al., 2015). Além disso, a microencapsulação permite promover a liberação controlada do material ativo em seu local de ação, por meio de diferentes mecanismos, como, alteração de pH, força mecânica, temperatura, ação de enzimas, tempo, entre outros (COOK et al., 2012).

Cabe destacar também, que a microencapsulação permite conectar micro-organismos probióticos com diferentes compostos e extratos, através da co-encapsulação, gerando possíveis benefícios destas espécies químicas para as culturas probióticas, possibilitando conjuntamente a maior estabilidade dos probióticos e dos compostos, e gerando cápsulas poli-funcionais com potencial efeito sinérgico na saúde do indivíduo (RADDATZ et al., 2022a). Chaikham et al. (2015) pesquisaram os impactos de extratos de ervas tailandesas na estabilidade de probióticos em partículas de alginato. Os autores escolheram extratos com altos teores de compostos bioativos e alta atividade antioxidante e conseguiram melhorar a viabilidade dos probióticos com extrato de flor de caju e chá-verde. Da mesma forma, a co-encapsulação de compostos bioativos do suco de amora com *Lactobacillus acidophilus* por spray dryer foi testada por Colín-Cruz et al. (2019), que obtiveram boa viabilidade de probióticos após armazenamento ao usar proteína de soro de leite como material de parede. Raddatz et al. (2022a) demonstraram o aumento da viabilidade e estabilidade de probióticos com a co-encapsulação com extrato de cebola roxa. Neuenfeldt et al. (2022) demonstraram o aumento da viabilidade e estabilidade de probióticos pela co-encapsulação com extrato de mirtilo. Recentemente, de Deus et al. (2023) estudaram a co-encapsulação de *L. plantarum* e compostos bioativos extraídos do caule da beterraba vermelha (*Beta vulgaris L.*) por spray dryer, e verificaram que a encapsulação foi positiva para viabilidade dos micro-organismos e estabilidade dos compostos bioativos por 120 dias em diferentes temperaturas de armazenamento. Deste modo, a co-encapsulação de probióticos com compostos bioativos é uma área promissora nos estudos da interação alimentos e saúde.

Dentre as diversas técnicas de encapsulação existentes, destaca-se a gelificação iônica. A produção de micropartículas por gelificação iônica é baseada na capacidade de polissacarídeos aniônicos sendo os mais utilizados a pectina e alginato que formam um gel na presença de íons como o cálcio (BUREY et al., 2008). A gelificação iônica é convencionalmente descrita em termos do modelo denominado caixa de ovos, que caracteriza a formação desta rede de gel. Nesse modelo, a ligação entre grupos carboxílicos presentes nos ácidos glucurônico ou galacturônico, no alginato e na pectina respectivamente, em pH acima de seus valores de pKa e íons cálcio faz com que ocorra um entrelaçamento de cadeias de alginato ou pectina, promovendo a formação de um hidrogel com uma estrutura de rede tridimensional, conforme mostrado na Figura 4 (WANG et al., 2006).

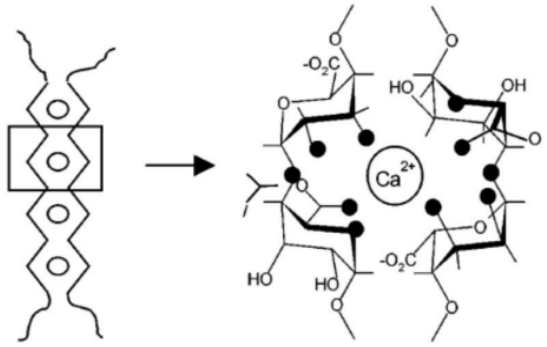
Há dois métodos pelos quais a gelificação iônica pode ocorrer, a gelificação iônica externa e a interna (SILVÉRIO, 2018). Na gelificação interna a produção de partículas se dá pela liberação de íons cálcio, inicialmente na forma de um sal insolúvel, na solução/emulsão do polissacarídeo contendo material ativo. O processo de gelificação é iniciado a partir da mudança de pH do sistema, em que a adição de uma solução ácida diminui o pH, provocando a liberação dos íons cálcio, permitindo assim a complexação do cálcio com os grupos carboxílicos. A gelificação externa (demonstrada na Figura 5), largamente utilizada, envolve a introdução de uma solução polimérica negativamente carregada em uma solução iônica carregada positivamente, sob agitação constante, com a gelificação ocorrendo através da difusão de cátions para dentro da solução do hidrocoloide, formando cápsulas de diferentes formas e tamanhos, em função do correto ajuste das condições de produção, incluindo concentração do íon, do polissacarídeo, do bico atomizador, velocidade de gotejamento e condições de agitação (CHAN et al., 2006).

As partículas de gel são geradas em duas etapas, o desenvolvimento da gota (por extrusão ou atomização) e o endurecimento da mesma. Quando formadas, as partículas permanecem sob agitação por um período denominado tempo de maturação, no qual, lenta e progressivamente as ligações cruzadas desenvolvem-se da superfície para o interior da partícula gelificada (ROCHA, 2001). Segundo Racovita et al. (2009) a espessura da parede da cápsula, e consequentemente a sua resistência à ruptura mecânica está diretamente relacionada com o tempo de permanência das partículas na solução iônica. Para que as partículas obtenham resistência mecânica constante e estabilidade, faz-se necessário aproximadamente 20 minutos de contato com a solução iônica.

A gelificação iônica externa destaca-se ainda por ser uma técnica verde, com baixo custo, baixo consumo energético, baixa geração de resíduos tóxicos e capacidade de produção em escala (RADDATZ et al., 2022a).

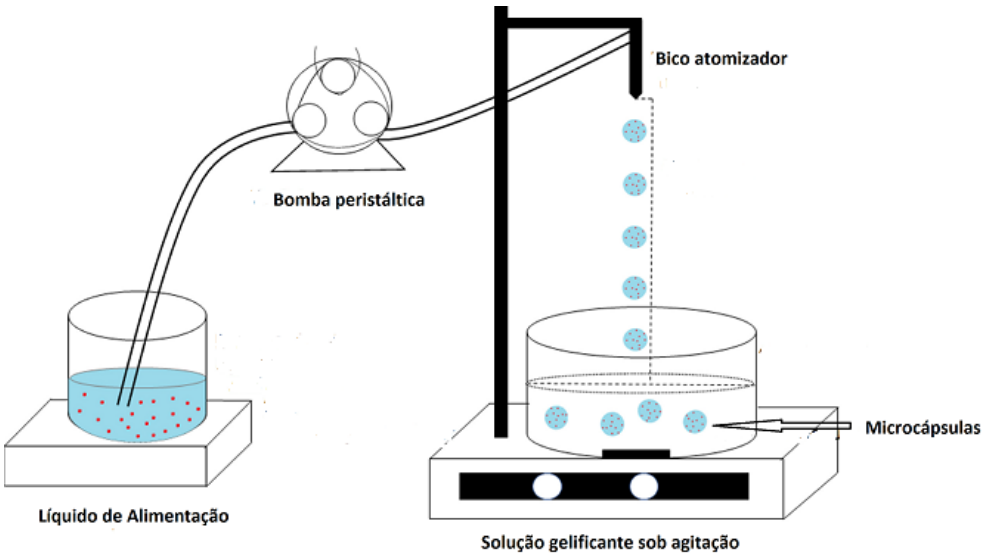
Pelos aspectos supracitados, a técnica de gelificação iônica externa vem sendo utilizada para a co-encapsulação de probióticos e extratos bioativos, mostrando efeito promissor no aumento da viabilidade dos micro-organismos e estabilidade de compostos bioativos frente às condições do trato gastrointestinal e armazenamento (RADDATZ et al., 2022a, 2022b; SOMACAL et al., 2022).

Figura 4 - Esquema representativo do modelo “caixa de ovos”. Os pontos negros representam os átomos de oxigênio envolvidos na coordenação com os íons cálcio.



Fonte: Adaptado de Braccini e Pérez (2001).

Figura 5 - Aparato para gelificação iônica externa.



Fonte: Adaptado de Sultana et al. (2022).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Desenvolver e validar uma metodologia para análise de crisina em espécies de *Passiflora,* e avaliar a viabilidade da co-encapsulação de crisina e micro-organismos probióticos.

3.2 OBJETIVOS ESPECÍFICOS

-Otimizar e validar uma metodologia QuEChERS seguido de HPLC-DAD para análise de crisina em espécies de *Passiflora*;

-Quantificar crisina nas folhas, polpa e casca de *Passiflora caerulea*;

-Avaliar o potencial antioxidante e o comportamento das partes de *Passiflora caerulea* frente à digestão gastrointestinal *in vitro*;

-Estudar a viabilidade da co-encapsulação da crisina e micro-organismos probióticos por gelificação iônica externa, suas características gerais, sua resistência ao tratamento térmico e ao trato gastrointestinal *in vitro*, e sua estabilidade durante o armazenamento.

4 RESULTADOS OBTIDOS

Os resultados desta tese estão apresentados sob a forma de 1 artigo de revisão publicado, contendo a pesquisa bibliográfica sobre a encapsulação do flavonoide crisina, e 2 manuscritos com os resultados obtidos. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências encontram-se nos próprios documentos e representam a íntegra deste estudo. Os documentos estão dispostos da mesma forma que foram submetidos para as respectivas revistas. O artigo de revisão foi publicado na revista "*Ciência Rural*". O manuscrito I está em revisão na revista "*Food Chemistry"*, e o manuscrito II será submetido à revista "*Food Research International*".

4.1 ARTIGO DE REVISÃO

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FOOD TECHNOLOGY

Chrysin flavonoid encapsulation: a review about methodologies used and biological potential

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ABSTRACT: Chrysin flavonoid has been presented as having numerous and promising bioactive effects, such as antioxidant, anticonvulsant, antihypertensive, anti-inflammatory, antineoplastic, antihyperlipidemic, and antidepressant. However, one of the main challenges for advances in studies on the bioactivity of chrysin is its low bioavailability in humans. Thus, aiming to overcome this barrier, several studies have demonstrated the bioactive potential of capsules containing chrysin. The objective of this review was to present the main methodologies used for the encapsulation of chrysin, and its main biological effects demonstrated so far. Our intention is to offer ways to advancement of research in the area of flavonoid encapsulation. As for the encapsulation techniques, it was found that they are diverse, and the most recurrent encapsulating agents were PEG, PLGA polymers and their derivatives, in addition to other agents, such as PCL, albumin, lipids and chitosan.The bioactive effects of the capsules are also numerous, and anticarcinogenic effects are the most frequent, in addition to other effects such as antioxidant, antidiabetic, antimicrobial and neuroprotective. In conclusion, we verified a lack of use of green techniques for the encapsulation of chrysin, and the production of lipid-water emulsions and the dissolution of chrysin in ethanol seems to be alternatives in this regard. In addition, bioactive potential of these capsules can be evaluated in other experimental models, and should advance to clinical trials and application in food formulations.
Key words: Anticarcinogenic, nanocapsules, bioavailability, PEG-PLGA.

Encapsulação do flavonoide crisina: uma revisão sobre as metodologias utilizadas e potencial biológico

RESUMO: O flavonoide crisina tem sido apresentado como tendo numerosos e promissores efeitos bioativos, como antioxidante, anticonvulsivante, anti-hipertensivo, anti-inflamatório, antineoplásico, anti-hiperlipidêmico e antidepressivo. No entanto, um dos principais desafios para o avanço dos estudos sobre a bioatividade da crisina é sua baixa biodisponibilidade em humanos. Assim, visando superar essa barreira, diversos estudos têm demonstrado o potencial bioativo de cápsulas contendo crisina. O objetivo desta revisão é apresentar as principais metodologias utilizadas para o encapsulamento da crisina e seus principais efeitos biológicos demonstrados até o momento. Nossa intenção é oferecer caminhos para o avanço das pesquisas na área de encapsulação de flavonoides. Quanto às técnicas de encapsulamento, verificou-se que são diversas, e os agentes encapsulantes mais recorrentes são os polímeros PEG, PLGA e seus derivados, além de outros agentes como PCL, albumina, lipídeos e quitosana. Os efeitos bioativos das cápsulas também são numerosos, sendo os efeitos anticarcinogênicos os mais frequentes, além de outros efeitos como antioxidante, antidiabético, antimicrobiano e neuroprotetor. Em conclusão, verificamos a falta de utilização de técnicas verdes para o encapsulamento de crisina, e a produção de emulsões lipídeo-água e dissolução da crisina em etanol parecem ser alternativas neste aspecto. Além disso, o potencial bioativo destas cápsulas pode ainda ser avaliado em outros modelos experimentais, e deve-se avançar para ensaios clínicos e aplicação em formulações alimentícias.
Palavras-chave: anticarcinogênico, nanocápsulas, biodisponibilidade, PEG-PLGA.

INTRODUCTION

Chrysin (5,7-Dihydroxyflavone) belongs to the flavone class of flavonoids, and is found naturally in honey, propolis, and several species of plants, including species of the genus *Pelargonium, Passiflora*, and the family Pinaceae (NABAVI et al., 2015). Chrysin has already been presented as having numerous and promising bioactive effects, such as antioxidant activity (PUSHPAVALLI et al., 2010), anticonvulsant (MEDINA et al., 1990), antihypertensive (VILLAR et al., 2002), anti-inflammatory (BAE et al., 2011), antineoplastic (PICHICHERO et al., 2011), antihyperlipidemic (ZARZECKI et al., 2014), and antidepressant (BORGES FILHO et al. 2015; 2016a; 2016b).

One of the main challenges for advances in studies on the bioactivity of the chrysin flavonoid is its low bioavailability in humans. Studies showed that chrysin is poorly solubilized, poorly absorbed, quickly

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metabolized and quickly eliminated in the human body, and it is estimated that its oral bioavailability is in the range of 0.003-0.02% (WALLE et al., 2001; JUNG et al., 2016; TALEBI et al., 2021).

An important way to overcome the low bioavailability of chrysin and phenolic compounds in general is the use of encapsulation techniques (KHALIB et al., 2022). So, several studies have demonstrated the development of capsules containing chrysin, and bioactive potential of these capsules. There are numerous methodologies used to produce the capsules, as well as a diversity of effects in different experimental models.

Thus, the objective of this review is to present the main methodologies used for the encapsulation of chrysin, and its main biological effects demonstrated so far. Our perspective is to offer information and alternatives for researchers who study ways to increase the bioavailability of chrysin and other flavonoids, and explore its bioactive potential. For this, we intend to describe and evaluate the methodologies and bioactivity studies presented so far, pointing out the most recurrent materials and techniques and discussing possible advances to be made in this area.

METHODOLOGY

The search was carried on the "science direct" platform, in the advanced search field. In the "title" field, the word "chrysin" was typed and in the "title, abstract or author-specified keywords" field, "nano" or "encapsulation" or "nanocapsules" words were typed.

In the search with "nano", 6 articles were found, being 6 associated with this review. In the search with "encapsulation", 9 papers were found, being 9 associated with this review. However, out of 9, 3 were also found in the first search. In the search with "nanocapsules", 2 papers associated with this review were found, but 1 had already appeared in other searches.

Thus, 13 articles of science direct platform were thoroughly evaluated for this review.

The "scopus" platform was also used for research. In the "article title, abstract, keywords" field, "chrysin nano" was used for research selecting the "article" filter. In this search, 27 articles were found, 19 of which are associated with the theme of this review. Among these 19, 6 appeared in searches on the science direct platform. Thus, scopus platform added 13 articles to this review.

Finally, "PubMed" platform was also used for research. In the "Search" field, "chrysin encapsulation" was used for research. In this search, 42 articles were found, 34 of which are associated with the theme of this review. Among these 34, 16 were not found in previous searches and were evaluated in the review.

For the preparation of this review, the encapsulation methodologies and the biological potential were evaluated separately, and are arranged this way in the article. As this is an area that has only recent studies, we did not limit the search period.

RESULTS AND DISCUSSION

Encapsulation methods

KHALID & NASEEM (2022), dissolved chrysin in organic phases which were acetone and dichloromethane (DCM). The solution was then emulsified with aqueous solution of polyvinyl alcohol (PVA) (used as surface stabilizer) by sonication and stirred over a magnetic stirrer at room temperature for 6, 7h. This is followed by centrifugation at 10,000 rpm for 30min. Supernatant was discarded and the synthesized nanoparticles were obtained as pellet, dried in hot air oven and stored.

In the EATEMADI et al. (2016) study, 20 mg chrysin and 200 mg of ε-caprolacton-poly-ethylene-glycol)-ε-caprolacton (PCL-PEG-PCL) triblock copolymers were added to DCM solvent. The mixture was added dropwise into 20 mL of H_2O. A probe-type sonicator at 80 W was used. The solution was sonicated at 16 interval and the one-minute pulse was turned off for 1s at 15s interval. For solvent evaporation and micelles formation, the beaker was opened to air during the night and in rotary evaporator the residual solvent was evaporated. For PCL-PEG-PCL synthesis, PEG and ε-caprolacton was the initiator in the presence of stannous octoate (Sn(Oct)2) as catalyst. Briefly, by utilizing mPEG, polymerization of ε-caprolacton was initiated. 3G ethylene glycol and 7.4g ε-caprolacton was added to a dry three-necked flask and under vacuum for 10min which was heated at 130 °C to dissolve the materials and remove moisture. By this mean, Sn (Oct) 2 were added into three-necked flask under a nitrogen atmosphere. A heating device was used to heat the compounds at 180 °C under stirring condition for 6h. After 12h cooling at room temperature, a firm and milky mixture was obtained. DCM was used for dissolving the mixture. After 30min, cold diethyl ether was added under stirring condition for purification. After 24h the solution was precipitated. The precipitate was stored in a desiccator.

JABBARI et al. (2018) presented doxorubicin and chrysin combination with novel

pH-responsive poly [(lactide-co-glycolic acid)-block-methacrylic acid] (PLGA-co-PMAA) nanoparticle. For this, 200 mg of PLGA-co-PMAA copolymer was dissolved in dimethyl sulfoxide (DMSO) (4 mL) under stirring at room temperature. Then, doxorubicin solution (10 mg) added to the mixture of the flask and after stirring for 24 h under dark conditions, chrysin solution (10 mg dissolved in 2 mL DMSO) was added to dozorubicin /(PLGA-co-PMAA) copolymer mixture and dispersed with the aid of ultra-sonication for 5 min. The PVA (1 wt %) solution was added dropwise (with the rate of 1 drop per 6 s) to vigorously stirring polymer/drug solution. Doxorubicin/chrysin-loaded PLGA-co-PMAA nanoparticles were collected from unloaded drugs and organic solvent using the Amicon centrifugal filters at 5000 rpm for 15 min. Drug-loaded nanoparticles were washed twice with distilled water and centrifuged at the same condition to remove any trace of the organic solvent or unbounded drugs.

FIROUZI-AMANDI et al. (2018) showed chrysin-encapsulated PLGA-PEG. PLGA-PEG tri-block copolymer was synthesized through ring opening polymerization of DL-lactide and glycolide in presence of PEG6000. PEG6000 and PLGA were copolymerized under vacuum using Sn(Oct)2 as the catalyst. The combination of DL-lactide (2.882 g), PEG6000 (1.44 g) and glycolide (0. 270 g) was completely melted in bottleneck flask in 140 °C under a nitrogen atmosphere. Then, 0.05% (w/w) Sn(Oct)2 was added and the temperature of the reaction mixture was raised to 180 °C for 5 h. The produced copolymer was dissolved in DCM and precipitated in ice-cold diethyl ether. Chrysin-loaded PLGA-PEG nanocapsule was obtained using oil-in-water (O/W) emulsion-solvent evaporation technique. Briefly, 200 mg of PLGA-PEG dissolved in 5 mL of DCM-methanol co-z solvent (4:1) and 2 mg of chrysin were moved to a centrifuge tube, and the solution mixture was gently stirred for 15 min at room temperature and emulsified using sonication in 50 mL of PVA aqueous solution (0.5%, w/v). After vacuum vaporization of the solvent, the nanocapsules were gathered by centrifugation at 12,000 rpm for 10 min at room temperature and washed three times using dH_2O. The obtained nanocapsules loaded suspensions were lyophilized and stored at 4 °C until further use. In addition, MOHAMMADIAN et al. (2016a, 2016b) also developed and evaluated bioactive potential of chrysin-PLGA-PEG nanoparticles in gastric cancer cell lines. KHALEDI et al. (2020) also showed the preparation and characterization of PLGA-PEG-PLGA polymeric nanoparticles for co-delivery of 5-fluorouracil and chrysin.

EL-HUSSIEN et al. (2021) prepared polymeric chrysin nanocapsules based on polylactic-glycolic acid PLGA. In this research, organic phase of the system composed of PLGA in different amounts, chrysin, Labrafac PG (propylene glycol dicaprylocaprate EP/propylene glycol dicaprylate/ dicaprate NF) and phosphatidylcholine was dissolved in 10mL acetone. The organic phase was added drop-wise to an aqueous solution of tween 80 as a stabilizer (20mL), placed on a magnetic stirrer at room temperature to aid the evaporation of the organic solvent.

GIACOMELI et al. (2020) investigated the effects of chrysin loaded lipid-core nanocapsules. In the methodology, loaded lipid-core nanocapsules suspensions were prepared by interfacial deposition of polymer. An organic phase containing chrysin (0.005 g), poly (ε-caprolactone) (0.100 g), pomegranate oil (0.33 mL), and sorbitan monostearate (0.077 g) were dissolved in acetone (27 mL) at 40 °C. In a separate flask, P80 (0.077 g) was added to 53 mL of water (Aqueous phase). Organic solution was poured into the aqueous phase under magnetic stirring at room temperature. After 10 min, a rotary evaporator was used to remove acetone and the suspensions were concentrated under reduced pressure. The final volume was adjusted to 10 mL for a drug concentration of 0.5 $mg.ml^{-1}$.

FERRADO et al. (2019) presented formation and characterization of self-assembled bovine serum albumin nanoparticles (BSAnp) as chrysin delivery systems. Chrysin-loaded BSAnp (BSAnp-Chrys) formation was monitored by intrinsic and extrinsic fluorescence measurements. Forthis, both native BSA and BSAnp solutions were diluted to a final concentration of 0.01%wt. in PBS buffer at pH 7.4. Chrys stock solution (2 mM) was prepared by dissolving the compound in DMSO. To perform intrinsic fluorescence binding experiments a volume of 2.0 ml containing 0.01%wt. BSA in phosphate buffered saline (PBS) was titrated by successive additions of Chrys stock solution until to reach a final concentration in the range of 0–140 μM. After Chrys addition, tubes were vigorously stirred. DMSO was chosen as solvent for binding experiments due to its properties: it is an organic solvent, water miscible, nontoxic, and widely used in biologic assays (acompound that precipitates in DMSO is a compound that cannot be biologically tested). Since it was reported that a final concentration of 10% v/v produces structural changes on BSA, it was contemplated that DMSO final concentration must not exceed 2.5%. In order to evaluate possible changes of BSA structure in presence of DMSO, a

control sample was prepared by adding a volume of 50 µl of DMSO to 2.0 ml of BSA solution (0.01% wt.). It represents the maximal concentration of DMSO used in all experiments. Intrinsic fluorescence emission spectra were recorded in triplicate at room temperature (25 °C). Finally, in order to know the mode in which chrysin is bond to BSAnp, extrinsic fluorescence measurements were performed.

ZHU et al. (2016) showed inclusion of chrysin in β-cyclodextrin complex. In this paper, an amount of recrystallizated β-cyclodextrin was completely dissolved in H_2O in accordance with the rule of 1g β-cyclodextrin adding 25mL dH_2O, and then added a certain amount of chrysin ethanol solution in some time with continuous stirring and by maintaining the temperature. The suspension was then slowly cooled at room temperature, the crystallization process was perfected in refrigerator over night, and the complex crystals were filtered in vacuum, dried at room temperature and weighed. Similarly, CHAKRABORTY et al. (2010) and SUNDARARAJAN et al. (2017) also showed inclusion of chrysin in β-cyclodextrin capsules. CHAKRABORTY et al. (2010) demonstrated antioxidant potencial, while SUNDARARAJAN et al. (2017) evaluated antioxidant and antitumorous effect *in vitro*.

RASOULI et al. (2020) developed electrospun nanofiber-mediated codelivery of curcumin andchrysin. First, PLGA/PEG copolymers were synthesized through ring-opening polymerization procedure. For fabrication of drug-loaded electrospun nanofibers (NFs), PLGA/PEG copolymers were dissolved in DCM: Methanol at a ratio of 4:1 (v/v) to prepare a 10%w/v solution. Gel permeation chromatography was used to determine the number of molecular weight (Mn) and polydispersity index (PDI) of the copolymer. To obtain drug-loaded PLGA/PEG solutions, different weight ratios of curcumin and chrysin (5:0, 10:0, 0:5, 0:10, 5:10, 5:5 and 10:5 wt:wt%, respectively, with respect to the PLGA/PEG content) were added to PLGA/PEG solution and stirred magnetically for 8h at 25 °C. The obtained solutions were fed in a 5mL plastic syringe with a blunted 22-gauge needle, and the flow rate of solution maintained at 2ml/h. The electrospun NFs were collected by a foil□coated rotating collector. The electrospinning was carried out at a range of 22–25kV and needle-to-collector distance of 200mm. The gained NFs were dried for 24h under vacuum oven to remove the residual solvent. Similarly, BAGHERI et al. (2018), TAVAKOLI et al. (2018) and LOFTI-ATTARI et al. (2017) developed nano-encapsulated chrysin-curcumin with PLGA-PEG copolymers, demonstrating anticancer activity in different models. JAVAN et al. (2019) demonstrated synergistic antiproliferative effects of co-nanoencapsulated curcumin and chrysin on MDA-MB-231 breast cancer cells.

VEDAGIRI & THANGARAHAN (2016) developed solid lipid nanoparticles of chrysin with stearic acid, lecithin and taurocholate. For this, stearic acid was maintained at ~75 °C to melt completely, simultaneously distilled water was heated up to ~75 °C in a separate beaker. Typically, surfactants were added to distilled water on a magnetic stirrer and allowed to equilibrate at ~75 °C. The water-surfactant solution containing chrysin was then added to the melted lipid and again allowed to equilibrate at ~75 °C. The mixture was then homogenized at 24,000rpm for 150s to form the emulsion. Then the aliquot was continuously stirred near ice cold water (~2 °C), at a ratio of 1:20 (warm microemulsion/cold water) resulting in the formation of solidified solid lipid nanoparticles. The final product was centrifuged at 20,000×*g* for 15min, and nanoparticle pellet was resuspended in distilled water. The preparation was stored in a sterile vial at 4 °C, until use. Similarly, PANDEY et al. (2021) and KOMATH et al. (2018) demonstrated in vitro anti-cancer activity of solid lipid nanoparticles of chrysin.

JASIM et al. (2022) showed gold nanoparticles conjugated chrysin. In this article, chrysin was dissolved in 5 mL of DMSO and stirred at 1,000 rpm for 15 min under room temperature to obtain a homogeneous solution with complete and clear visible solubility. The chrysin suspension was added to the solution of Au nanocapsules (1:9 mL) and stirred for 20 h, through overnight at room temperature. The color of the solution changed to light violet, and the excess chrysin was removed by ultracentrifugation. Furthermore, SATHISHKUMAR et al. (2015) report a new approach to formulate biofunctionalized metallic silver (chrysin-Ag), and gold (chrysin-Au) nanoparticles.

LUO et al. (2022) presented methoxy poly(ethylene glycol)-b-poly(e-caprolactone) (MPEG-PCL) nanomicelles platform for synergistic metformin and chrysin delivery to breast cancer in mice. For this, chrysin and metformin drug-loaded micelles were prepared by modified thin-film hydration. The specific operation was as follows: 50, 70, and 90 mg of polymer with different molecular weights were accurately weighed, mixed with chrysin and metformin, and placed in a rotary flask. About 2 mL of DCM was added and dissolved completely

by ultrasound. The organic solvent was removed by 0.07 MPa spin evaporation, and the bottle wall was covered with a film evenly distributed between drug and polymerdrug carrier material. Subsequently, 10 mL of re-distilled water was added to hydrate at 40 °C, 60 °C, and 80 °C and then cooled to room temperature after hydration. A 0.22 μm microporous filtration membrane was used to remove the uncoated chrysin and metformin and insoluble impurities. The solution of chrysin and metformin nano-micelles was obtained and stored in a refrigerator at 4 °C for future use. Similarly, KIM et al. (2017) showed improved chemotherapeutic efficacy of injectable chrysin encapsulated by MPEG-PCL nanoparticles.

ROY et al. (2020) developed chrysin loaded nanoparticle by the solvent displacement method. In this research, 100 mg of PLGA and 10 mg of chrysin were dissolved in 20 ml of acetone. This solution was poured dropwise overnight under magnetic stirring on an aqueous solution of 1% PVA. The solution was then centrifuged, and the precipitate was lyophilized.

MENON et al. (2018) evaluated the sustained release of chrysin from chitosan-based scaffolds. In this paper, scaffolds were prepared using simple ionic gelation method. Briefly, carboxymethyl cellulose (1% w/v) was dissolved in distilled water with continuous agitation. Chrysin, at different concentrations (2, 5, and 10 μM), in DMSO was added drop-wise. Chitosan (1% w/v) was added subsequently and stirred for 10 min, followed by addition of nano-hydroxyapatite (1% w/v). After 3 h of stirring, acetic acid (0.3% v/v) was added spontaneously, and the solution was cast into plates. The plates were maintained at −20 °C overnight, followed by lyophilization. The lyophilized scaffolds were crosslinked using 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and maintained at 4 °C overnight, followed by lyophilization for 24 h. Similarly, SIDDHARDHA et al. (2020) showed that chrysin-Loaded chitosan nanoparticles potentiates antibiofilm activity against Staphylococcus aureus. FARHADI et al. (2023) presented anticancer effects of chrysin-loaded chitosan-folic acid coated solid lipid nanoparticles in pancreatic malignant cells.

Thus, there are so far a variety of methodologies used to encapsulate chrysin (summarized in Table 1), and it is up to scientists to choose the methodology to be used in their experiment, always seeking to optimize techniques, seeking to increase the bioavailability of the chrysin combined with the reduction of costs and time. We also found that there is a strong need the development of green methodologies for chrysin encapsulation, as most methods use highly toxic reagents such as DMSO, DCM and methanol. In this sense, some studies have have pointed to green alternatives, such as the use of lipid-water emulsions (VEDAGIRI & THANGARAHAN., 2016), dissolution of chrysin in ethanol (ZHU et al., 2016), and others. In addition, other techniques currently used can also be tested, such as spray drying, ionic gelation and coacervation.

Bioactive potencial of capsules

KHALID & NASEEM (2022) showed the antidiabetic and antiglycating potential of chrysin nanocapsules on *in vitro* studies. For this, antioxidant potential was determined and *in vitro* anti-diabetic activity was assessed by α-amylase and α-glycosidase inhibition assays and the results showed a dose-dependent increase in percent inhibition of the enzyme. Glycation was reduced to a high extent in presence of chrysin nanoparticles as compared to its bulk form and this was estimated by decrease in synthesis of Amadori products as well as advanced glycation end products. The results were further confirmed by spectroscopic techniques showing structural changes in human serum albumin glycated in the absence and presence of chrysin or its nanoparticle. The antiglycating effect was also evident by estimating free lysine residues, and protein oxidation.

EATEMADI et al. (2016) evaluated the effect of nano-chrysin on breast cancer cell line. Dataanalysis from MTT ((3(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide) assay showed that chrysin has a time-dependent cytotoxic effect on T47D cell line. Furthermore, the results of Real-time PCR suggested that encapsulated chrysin has higher antitumor effect on gene expression of FTO, BRCA1 and hTERT than free chrysin. Thus, combined nano-chrysin therapy will not only improve cancer cell cytotoxicity, but also be a complementary and potential complex in breast cancer therapy.

JABBARI et al. (2018) showed that viability of human lung epithelial cancer cell lines (A549) was significantly decreased upon interaction doxorubicin and chrysin-loaded (PLGA-co-PMAA) nano-formulation.

FIROUZI-AMANDI et al. (2018) investigated the efficiency of chrysin encapsulated in PLGA-PEG nanoparticles for the modulation of macrophage polarity from the pro-inflammatory M1 to anti-inflammatory M2 phenotype. Findings revealed that the chrysin-encapsulated were considerably less toxic to the macrophages. Additionally, the nano-

Table 1 - Summary of encapsulation methods and bioactive potential of capsules containing chrysin. The works are organized in chronological order.

Summarized Methodology	Biological Potential	Reference
Dissolution in PCL-PEG-PCL in DCM + dH_2O + Sonication + Solvent evaporation	Anticancer in breast cancer cell line	EATEMADI et al. (2016)
Mix of water-surfactant solution containing chrysin and stearic acid completely melted + Equilibrate at ~75 °C + Homogenization + Agitation near ice cold water + Centrifugation + Resuspension of pellet in distilled water	Ameliorates neurobehavioral alterations of Alzheimer's	VEDAGIRI & THANGARAHAN (2016)
Dissolution of chrysin in ethanol + Recrystallizated β-cyclodextrin dissolved in H_2O with continuous stirring + Crystallization in refrigerator + Vacuum filtration + Drying	Antioxidant, antimicrobial and anti-tumor activity	ZHU et al. (2016)
Dissolution with PLGA-PEG tri-block copolymer in DCM-methanol + Agitation + PVA + sonication + Vacuum vaporization of the solvent + Centrifugation + Washing with dH_2O + Lyophilization	Possible aplicattion in tissue regeneration	FIROUZI-AMANDI et al. (2018)
Addition in dozorubicin/(PLGA-co-PMAA) copolymer + Sonication + PVA and agitation + Centrifugation + Washing with dH_2O and centrifugation	Anticancer effect in lung epithelial cancer cell lines	JABBARI et al. (2018)
Dissolution of carboxymethyl cellulose in dH_2O + Addition of chrysin dissolved in DMSO + Addition of Chitosan + Agitation + Addition of nano-hydroxyapatite + Agitation + Addition of acetic acid + Solution cast into plate + Lyophilization	Mesenchymal stem cell proliferation and osteoblast differentiation	MENON et al. (2018)
Dissolution of chrysin in DMSO + Addition of chrysin solution to BSAnp in PBS + Agitation	Antitumor therapies	FERRADO et al. (2019)
Dissolution with poly(ε-caprolactone), pomegranate oil, and sorbitan monostearate in acetone + Addition to the aqueous phase (polysorbate H_2O) with magnetic stirring + Acetone evaporation by rotary evaporator + Concentration of suspensions under reduced pressure	Ameliorates neurobehavioral alterations of Alzheimer's disease in mice	GIACOMELI et al. (2020)
Dissolution of chrysin and curcumine in PLGA/PEG + Magnetic stirring + Electrospinning + Nfs drying in a vacuum oven	Effect on T47D breast cancer cells	RASOULI et al. (2020)
Dissolution of chrysin and PLGA in acetone + Mix with PVA + Centrifugation + Lyophilization	Attenuation of allergic asthma	ROY et al. (2020)
Dissolution with PLGA, Labrafac PG and phosphatidylcholine in acetone + Addition dropwise to an aqueous solution of tween 80 placed on a magnetic stirrer + Evaporation of the organic solvent	Anti-glycemic and anti-hyperlipidemic	EL-HUSSIEN et al. (2021)
Dissolution of chrysin in DMSO + Agitation + Addition to Au nanocapsules solution + Agitation + Centrifugation	Antioxidant, anti-microbial and cytotoxic	JASIM et al. (2022)
Dissolution in DCM and acetone + Emulsification with PVA + Centrifugation + Supernadant discard + Drying and pelletizing	Antioxidant, antidiabetic and antiglycating	KHALID & NASEEM (2022)
Chrysin and metformin mixed with polymer + Placed in rotary flask + Addition of DCM + Sonication + Solvent evaporation + Hydration + Filtration	Effect in breast cancer in mice	LUO et al. (2022)

formulated chrysin efficiently showed a reduction in M1 markers and an increase in M2 markers levels than free chrysin. Furthermore, macrophage phenotype switching by PLGA-PEG encapsulated chrysin significantly suppressed LPS/IFN-γ induced inflammation by a remarkable reduction in pro-inflammatory cytokine levels, TNF-α, IL-1β, and IL-6. Results revealed that PLGA-PEG encapsulated chrysin based drug delivery system might be introduced into biomaterials to fabricate bioactive smart multifunctional nanocomposites with macrophage repolarization activities for regenerative medicine purposes.

EL-HUSSIEN et al. (2021) showed anti-glycemic and anti-hyperlipidemic effects of chrysin nanocapsules in rats. Diabetes was induced in an animal model using strptozotocin to assess the anti-hyperglycemic activity, and hyperlipidemia was induced using a high fat diet to assess its anti-hyperlipidemic activity.

GIACOMELI et al. (2020) studied chrysin loaded lipid-core nanocapsules effect in neurobehavioral alterations induced by β-amyloid$_{1-42}$ in aged female mice. Results support that chrysin displayed significant effect against Aβ_{1-42}, via attenuation of oxidative stress and neuroinflammation, modulation of neurochemical and behavioral changes in a model of Alzheimer's disease.

FERRADO et al. (2019) did not perform bioactive potential assessments, but suggested that their results highlighted the ability of self-assembled BSAnp for chrysin vehiculization in an aqueous

medium, which could found potential application in antitumor therapies.

ZHU et al. (2016) presented inclusion of chrysin in β-cyclodextrin and its biological activities. The process of inclusion not only increased the solubility of chrysin but also its antioxidant potential, antimicrobial activity and anti-tumor activity on mouse hepatoma H22 cells.

RASOULI et al. (2020) showed that co-delivery of curcumin and chrysin through a polymeric electrospun nanofibrous scaffold exerts a synergistic anti-proliferative and pro-apoptotic effect on T47D breast cancer cells. In this study, dual drug-loaded nanofibrous showed an excellent capacity to inhibit T47D breast cancer cells *in vitro* than the single drug-encapsulated nanofibrous. Therefore, the fabricated dual drug-encapsulated nanofibrous may achieve a safe and suitable application for breast cancer relapse rate after surgery.

VEDAGIRI & THANGARAHAN (2016) demonstrated effect of chrysin loaded solid lipid nanoparticles against Amyloid β_{25-35} induced oxidative stress in rat hippocampal region. In this paper, all the antioxidant enzymes and non-antioxidant enzyme in hippocampus were reduced in the Aβ25–35 injected group, whereas lipid peroxidation and acetylcholine were increased. In addition, Aβ25–35 also resulted in poor memory retention in behavioral tasks and histopathological sections of the hippocampal region showed the extent of neuronal loss. These changes were restored significantly by chrysin nanoparticles.

JASIM et al. (2022) presented an assessment of antioxidant, anti-microbial, and in vitro cytotoxic activities of the gold nanoparticles conjugated chrysin. The chrysin-Au nanocapsules effectively scavenged the 2,2- diphenyl-1-picrylhydrazyl free radicals, and exhibited potential cytotoxic effects in a dose-dependent manner and demonstrated significant reduction of the cells proliferation, and growth of the human breast cancer celllines, AMJ13. Furthermore, chrysin-Au nanocapsules exerted highest anti-microbial bioactivity against.

Staphylococcus aureus and *Escherichia coli*.

LUO et al. (2022) showed MPEG-PCL nanomicelles platform for synergistic metformin and chrysin delivery to breast cancer in mice. In this paper, metformin/chrysin co-delivery micelles showed a good synergistic effect on inhibiting proliferation in T47D cells by suppressing hTERT and cyclin D1 gene expression. The tumour volume and tumour weight of the metformin/chrysin group increased more slowly than that of the single-drug treatment group.

ROY et al. (2020) demonstrated that chrysin-loaded PLGA attenuates ovoalbumin-induced allergic asthma by modulating TLR/NF-κB/NLRP3 axis. In this research, the spherical nanosized particles showed slow, sustained release in vitro. Moreover, nanocapsules dramatically reduced the serum IgE, ovoalbumin-induced lung histological alteration, as well as Th2 (T-helper 2) cytokines in the bronchoalveolar lavage fluid. It also suppressed the elevated serum pro-inflammatory cytokines and their upstream TLR/NF-κB/NLRP3 pathway activation in lung superior to chrysin and almost identical to dexamethasone.

MENON et al. (2018) showed that sustained release of chrysin from chitosan-based scaffolds promotes mesenchymal stem cell proliferation and osteoblast differentiation. In this article, chrysin-containing scaffolds were not cytotoxic to mouse mesenchymal stem cells. Chrysin released from scaffolds stimulated cell proliferation and promoted osteoblast differentiation. Osteoblast differentiation enhanced by chrysin from scaffolds could be due to downregulation of co-repressors of the osteoblast differentiation transcription factor Runx2 in these cells.

In view of afore mentioned, the main biological potential of capsules containing chrysin, in order to facilitate visualization by readers, are depicted in table 1.

In addition, other authors have also developed and evaluated biologic potential of capsules containing chrysin. NOSRATI et al. (2018) studied activity of L-phenyl alanine-coated iron oxide magnetic nanoparticles as potential chrysin delivery system. WANG et al. (2015) developed chrysin-nanosuspension composed of chrysin and poloxamer 188 prepared by high pressure homogenization technique. The *in vitro* anti-hepatocarcinoma effect was showed. KAMAT et al. (2022) demonstrated apoptotic effect of nano-chrysin (using PLGA polymer) in HeLa cells. TING et al. (2021) developed chrysin-loaded oil-in-water nanoemulsions and demonstrated in vitro antioxidant and anti- Alzheimer's disease. KHOSHRAVAN et al. (2022) showed the development of nanostructured co delivery of artemisinin and chrysin for targeting hTERT gene expression in breast cancer cell line. HALEVAS et al. (2021) presented the evaluation of the hemocompatibility and anticancer potential of poly (ε-Caprolactone) and poly(3-Hydroxybutyrate) microcarriers with encapsulated chrysin. BAIDYA et al. (2019) showed chrysin-loaded folate conjugated PF127-F68 mixed micelles with enhanced oral bioavailability and anticancer activity against human breast cancer cells.

TARAHOMI et al. (2023) demonstrated niosomes nanoparticles as a novel approach in drug delivery enhances anticancer properties of chrysin in human ovarian carcinoma cells (SKOV3). ABDELHAKM et al. (2023) presented that chrysin encapsulated copper nanoparticles with low dose of gamma radiation elicit tumor cell death through p38 MAPK/NF-κB pathways. DESHMUKH et al. (2021) showed chrysin liposomes for breast cancer treatment. Chrysin liposomes were developed by electrostatic deposition assisted film hydration method using chitosan/ lecithin to protect chrysin in the nano-lipoidal shell. Some other works also presented the bioactive effects of lipossomal chrysin, as HUANG et al. (2022) and BEYRAMI et al. (2020).

CONCLUSION

In conclusion, we observed that encapsulation methods of chrysin are diverse, and the most recurrent encapsulating agents were PEG, PLGA polymers and their derivatives, in addition to other agents, such as PCL, albumin, lipids and chitosan.

We believed that the information presented in this article will point the way for researchers in choosing flavonoid encapsulation methodologies, considering their possibilities and working conditions, always seeking to optimize techniques, seeking to increase the bioavailability of the chrysin combined with the reduction of costs and time. We also found that there is a strong need the development of green methodologies for chrysin encapsulation, as most methods use highly toxic reagents such as DMSO, DCM and methanol. In this sense, some studies have have pointed to green alternatives, such as the use of lipid-water emulsions and dissolution of chrysin in ethanol. In addition, other techniques currently used can also be tested, such as spray drying, ionic gelation and coacervation.

The bioactive effects of the capsules are also numerous, and anticarcinogenic effects are the most frequent, in addition to other effects, such as antioxidant, antidiabetic, antimicrobial and neuroprotective. Furthermore, bioactive potential of these capsules can be evaluated in other experimental models, and should advance to clinical trials and application in food formulations.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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4.2 MANUSCRITO I

Chrysin analysis by QuEChERS modified method and HPLC-DAD, antioxidant potential and *in vitro* gastrointestinal digestion of *Passiflora caerulea*

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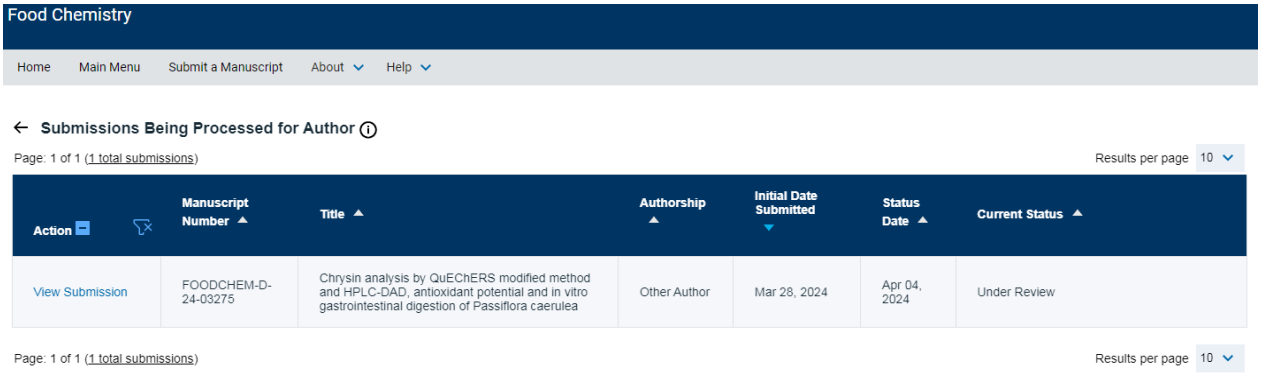

ABSTRACT

This work modified and validated a QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) followed by analysis by High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD), for quantification of chrysin in leaf, pulp and peel of *Passiflora* species, applying to *Passiflora caerulea*, and evaluated the *in vitro* antioxidant potential and gastrointestinal digestion. The QuEChERS method was successfully employed for the first time to determine chrysin, and the method presented excellent results in all validation parameters. The highest levels of chrysin and the best performance in the antioxidant evaluations were found in leaves and green peel. The gastrointestinal digestion was not able to alter the chrysin bioaccessibility. Results showed that QuEChERS modified method followed by HPLC-DAD is suitable for chrysin analysis in different parts of *Passiflora* species. Also, it demonstrates that leaves and green peel of *Passiflora caerulea* may be chrysin source for bioassays and food or nutraceutical formulations.

Keywords: Bioaccessibility; Chromatography; Flavonoids; *Passiflora caerulea*; Sample preparation; Validation.

1 INTRODUCTION

Chrysin (5,7-Dihydroxyflavone) belongs to the flavone class of flavonoids and presents numerous and promising bioactive effects, such as antioxidant activity (Pushpavalli et al., 2010), anticonvulsant (Medina et al., 1990), antihypertensive (Vilar et al., 2002), anti-inflammatory (Bae et al., 2011), antineoplastic (Pichichero et al., 2011), and antihyperlipidemic (Zarzecki et al., 2014) action. Also, some works have demonstrated the neuroprotective role of chrysin, through protective and/or therapeutic effects in models of depression (Borges Filho et al. 2015; 2016a; 2016b), Parkinson (Del Fabbro et al., 2019; De Gomes et al., 2022), autoimmune encephalomyelitis (Del Fabbro et al., 2019b and 2021), Alzheimer (Souza et al., 2018) and hypothyroidism (Bortolotto et al., 2021).

Chrysin is found naturally in honey, propolis and several species of plants, including species of the *Pelargonium and Passiflora* genus, and the *Pinaceae* family (Nabavi et al., 2015). Despite this, most bioassays with chrysin do not use natural sources to obtain it, being acquired in the form of powder with high purity from specialized companies, raising the cost of experiments and failing to explore natural products. Among other factors, this is due to the scarcity of works that demonstrate accurately the content of chrysin in natural sources and suitable means for its extraction.

Passiflora caerulea is a species of the *Passiflora* genus that is abundant and easily accessible in South America, occurring in fields, roadsides and forest edges (Mondin et al., 2011), and it is rarely used for human consumption or any other purpose. Although studies on *Passiflora caerulea* are scarce, it is already known that its leaves present the flavonoid chrysin (Medina et al., 1990; El-Askary et al., 2017), and have promising bioactive potential (Ozarowski et al., 2018). Thus, establishing an analytical method that quantifies the levels of chrysin in *Passiflora caerulea* leaves can be a big step towards advances in the studies of this natural product. Furthermore, it is interesting to analyze the chrysin content and bioactive potential of the other parts of the plant, which are the pulp and the peel, that have not been studied yet.

Regarding chrysin analysis, an important step is sample preparation, which generally consists of a step that requires several hours to execute. Gharari et al. (2020) showed the determination of chrysin in roots and shoots of four species of *Scutellaria*, with drying of the material followed by 48h of extraction with methanol. Hadas et al. (2017) and Ozarowski et al. (2018) evaluated the chemical profile of *Passiflora caerulea* leaves and, for this, the leaves were dried with air circulation (24h), and subsequently extracted with methanol (1h) and

concentrated under vacuum. Therefore, it is necessary to look for faster alternatives that also have low cost and environmental impact.

In 2003, Anastassiades et al. developed the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method with the purpose of minimizing sample handling, time and improving recoveries for determination of pesticides in food matrices. The QuEChERS method comprises an extraction/partitioning step and a clean-up step, which provide the extraction of the compounds from the sample to an appropriate organic solvent *via* salting-out extraction, where an equilibrium between an aqueous and an organic layer is promoted, followed by a dispersive solid-phase extraction (d-SPE) clean-up step, for removal of pigments or interferents (Rotta et al., 2019).

Recent studies have also reported the successful use of the QuEChERS method for phenolic compound determination in different food matrices such as in fruits, vegetables, herbs, spices, honey, and beverages (Rotta et al., 2019; Nicácio et al., 2021). However, this methodology has not yet been applied for the analysis of chrysin in any matrix.

Regarding chrysin determination, high performance liquid chromatography with diode array detector (HPLC-DAD) is an excelent choice, since it provides lower limits of quantification (LOQ), simple and low cost analysis (Gharari et al., 2020; Oroian et al., 2017; Giacomeli et al., 2020). In addition, many works have reported the use of HPLC-DAD for phenolic compounds analysis in food matrices (Kebal et al., 2022; Rahman et al., 2022; Bueno-Herrera et al., 2020).

In addition to the analysis of the phenolic content in plant matrices, an important current trend in the field of functional foods is to verify the behavior of these phenolics in the conditions of the digestive system. Through *in vitro* simulation of gastrointestinal digestion, it has been shown that digestion can alter the phenolic content and bioactivity of different extracts (Farias et al., 2021; Wu et al., 2022; Zhao et al., 2023). This evaluation is extremely important, as it helps to predict the bioaccessibility of the phenolic compounds present in a matrix, as well as their bioactivity. Although it has already been examined in its pure form (Ting et al., 2021), the behavior of chrysin present in plant matrices against the gastrointestinal tract has not yet been evaluated. Furthermore, the effects of *in vitro* digestion on the bioactivity of *Passiflora caerulea* has never been explored.

Thus, the main goal of this work is to adapt and validate a QuEChERS method and HPLC-DAD analysis for chrysin determination in *Passiflora* species, and to apply this method for chrysin quantification in leaves, mature and green pulp, and mature and green peel of *Passiflora caerulea* and, also, to evaluate the antioxidant potential of its parts, analyzing the effect of *in vitro* gastrointestinal digestion.

2 METHODOLOGY

2.1 Reagents and Solutions

Chrysin analytical standard (98 %), pepsine, pancreatine, bile, 2,2 diphenyl 1 picrylhydrazyl (DPPH) and acetonitrile HPLC grade were purchased from Sigma-Aldrich (Brazil). Anydrous Mg_2SO_4 and $K_2S_2O_8$ were purchased from Cinética (Brazil). Methanol HPLC grade, Folin Ciocauteau reagent, Gallic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ethanol, NaCl, SiO_2 and Na_2CO_3 were purchased from Proquímios, Dinâmica, Êxodo, MP Biomedicals, PROC9, Alphatec, Synth and Vetec respectively, all in Brazil.

For the stock solution, chrysin was solubilized in methanol at a concentration of 100 mg L^{-1} (Gharari et al., 2020). Folin Ciocauteau reagent, Gallic acid, ABTS, $K_2S_2O_8$, HCl, NaCl, KCl, $NaHCO_3$ and Na_2CO_3 were solubilized in ultrapure water (ELGA Purelab Ultra Mk 2). DPPH was solubilized in ethanol.

2.2 Definition of HPLC-DAD system conditions

The initial conditions employed in the chromatographic system (Thermo Scientific Dionex UltiMate 3000 Series, equipped with Autosampler Column Compartment ACC-3000, Diode Array Detector - DAD and a Dionex HPLC Column Acclaim 120 C18 5µm 4.6 x 150mm) were obtained from literature (Gharari et al., 2020; Giacomeli et al., 2020; Oroian et al., 2017). Initially, chrysin solution at 2,5 mg L^{-1} concentration was injected in the HPLC-DAD at isocratic mode, with mobile phase containing acetonitrile and ultrapure water (60:40, v/v), with a flow rate of 0.5 mL/min, injection volume of 20 µL, and running time of 30 min (Giacomeli et al., 2020). Then, ideal wavelength was evaluated by the highest absorbance between 192 and 400 nm. The injection volumes from 20 to 50 µL were tested, and the smallest volume with the highest peak was chosen. The temperature of the autosampler and the column were maintained according to the literature at 25°C (Oroian et al., 2017). C8 and C18 columns with different lengths were tested, and the column that provided the best peak with the shortest running time was chosen. For the mobile phase, isocratic modes with different proportions between water and acetonitrile were tested, as well as gradient modes with the same solvents. The chosen mobile phase was the one with the best peak definition in the fortified blank sample.

2.3 Samples

Leaves and fruits of *Passiflora alata* (blank sample*)* and *Passiflora caerulea* were collected in south of Brazil (Itaqui, RS, Brazil, latitude -29.1854853 and longitude -56.5183362), in November of 2022. The leaves, mature and green pulp, and mature and green peel were processed separately in a blender (Philips Walita 600W) and stored at -4°C until the analysis.

2.4 Development of sample preparation for determination of chrysin

For sample preparation, a version of QuEChERS method (Anastassiades et al., 2003), adapted from recent works (Rotta et al., 2019; Nicácio et al., 2021; Rodrigues et al., 2022) was tested. The method development was divided into extraction/partition and clean-up, and the evaluation was carried out based on visual analysis, chromatograms, recovery results and absorption spectra.

In the extraction/partition tests, mass variations were made (5 or 10 g) and also the use of an ultrasound bath (15 min), setting the parameters of acetonitrile volume (10 mL), vortex agitation (1 min) and centrifugation (3,000 rpm for 10 min). For clean-up, sorbents C_{18} and SiO_2 were tested, with a mass of 25 mg for 1 mL of supernatant, 150 mg of Mg_2SO_4, 1 min at vortex and centrifugation at 3,000 rpm for 10 min. The supernatants were filtered through a nylon syringe filter (25 mm, 0.22 μm) prior to the chromatographic analysis. For leaf analysis, the filtred sample was further diluted in acetonitrile at 1:4 (v/v), due to the high presence of pigments in the sample.

The same samples analysed in the HPLC-DAD system, was also used in the evaluation of the *in vitro* antioxidant potential and *in vitro* gastrointestinal digestion.

2.5 Final sample preparation for determination of chrysin

The final methodology consists on weighing samples (5 g of leaves; 10 g of pulp/peel) and transfer to a 50 mL PP tube, add 10 mL of acetonitrile and vortex agitation for 1 min. Pulp and peel samples were still submitted to an ultrasonic bath during 15 min. Afterwards, 4 g of Mg_2SO_4 and 1 g of NaCl were added to all samples. Next, the tubes were agitated (vórtex, 1 min) and centrifuged at 3,000 rpm for 10 min. Clean-up step was performed using 1 mL of supernatant, 25 mg of SiO_2 and 150 mg of Mg_2SO_4, with vortex mixing (1 min) and centrifugation (3,000 rpm, 10 min). The supernatants were filtered

through a nylon syringe filter (25 mm, 0.22 μm) prior to the analysis. For leaf analysis, the filtred sample was further diluted in acetonitrile at 1:4 (v/v).

2.6 Method Validation

The evaluated parameters for the proposed method were selectivity, linearity (by determination coefficient, $r^2 \geq 0.99$), matrix effect (ME), limit of detection (LOD) and limit of quantification (LOQ), accuracy (by recovery assay), repeatability and intermediate precision (by relative standard deviation, RSD%).

Selectivity was evaluated by comparing the chromatograms obtained from blank and spiked samples. Linearity was checked considering the levels of 0.08, 0.8, 3.2, 6.4, 12.8, 19.2, 25.6 and 32 mg kg^{-1} for leaves (sample was diluted 8 times) and 0.01, 0.1, 0.4, 0.8, 1.6, 2.4, 3.2 and 4 mg $kg^{-1}$for pulp and peel. Matrix effect was estimated comparing the slopes of calibration curves prepared in matrix blank extract and in pure solvent (methanol), considering the matrix effect significant when higher than ±20% (Sante, 2019; ANVISA, 2017). Accuracy was evaluated trough the average recovery results for spike levels tested, with criteria of 70–120% for acceptance (Sante, 2019). In this study, for accuracy and precision (repeatability) evaluation, recovery assays at the levels 3.2, 19.2 and 32 mg kg^{-1} for leaves and 0.4, 2.4 and 4 mg kg^{-1} (n =3) for pulp and peel were performed. Intermediate precision (RSDip) was performed on a different day, with recovery assays at 19.2 mg kg^{-1} for leaves and 2.4 mg kg^{-1} for pulp and peel (n =3). Method LOQ was established as the lowest calibration curve concentration level with signal/noise of 10 (Sante, 2019). Method LOD was obtained dividing the respective LOQ values by 3.33 (Kruve et al., 2008). Leaves, pulp and peel of *Passiflora alata* were used as blank sample. After validation, the method was used to quantify chrysin (mg kg^{-1}) in leaves, green and mature pulp, and green and mature peel of *Passiflora caerulea.*

2.7 *In vitro* essay of antioxidant potential

The total phenolic content (TPC) was measured according to Singleton et al. (1999), with adaptations. The sample extract (250 μL) and 3% (v/v) of Folin-Ciocalteu reagent (2.75 mL) were mixed in test tubes in shelter from light, vortexed and kept in the dark for 5 min, followed by adding $NaCO_3$ (10%, 250 μL); the mixture was then kept in the dark for another 60 min at room temperature before recording the absorbance at 765 nm (AAKER BEL 2000UV). A standard curve with gallic acid was performed (20, 40, 60, 80 and 100 mg L^{-1}) and the results was expressed as gallic acid equivalents (GAE) per kg of sample.

DPPH free radical scavenging activity was measured according to Brand-Williams et al. (1995), with adaptations. A DPPH standard curve dissolved in ethanol (10, 20, 30, 40, 50 and 60 μM) was performed in a spectrophotometer at 515 nm for identification of absorbance and mass equivalent to 50% of reduction of DPPH. Subsequently, 3 dilutions of the extract (300 μL) were mixed in test tubes with 3.9 ml of DPPH (60 μM) and the absorbance at 515 nm was measured. Results were expressed as sample mass (g) required to reduce 1 mg of DPPH. In this analysis, high values mean low antioxidant potential and vice versa.

ABTS free radical scavenging activity was measured according to Ozgen et al. (2006), with adaptations. Initially, for the formation of the radical $ABTS^+$, 5 mL of ABTS (7 mM) was mixed with 88 μL of $K_2S_2O_8$ (140 mM). The mixture was kept in the dark for 16 h. Then, 1 mL of the mixture was diluted in ethanol until obtain an absorbance of 0.70 ± 0.05 at 734 nm, equivalent to 45 mg/L of $ABTS^+$. Posteriorly, a $ABTS^+$ standard curve dissolved in ethanol (9, 11, 15, 22, 30 and 45 mg L^{-1}) was performed in a spectrophotometer at 734 nm, for identification of absorbance and mass equivalent to 50% of reduction of $ABTS^+$. Subsequently, 3 dilutions of the extract (300 μL) were mixed in test tubes with 3 mL of $ABTS^+$ (45 mg L^{-1}) and the absorbance at 734 nm was measured. Results were expressed as sample mass (g) required to reduce 1 mg of $ABTS^+$. In this analysis, high values mean low antioxidant potential and vice versa.

2.8 *In vitro* gastrointestinal digestion

In this analysis, only the leaf and green pulp extracts were evaluated, since they presented higher concentrations of chrysin. An evaporation step was performed, because the use of acetonitrile could not be suitable for gastrointestinal simulation. So, 1mL of the sample extracts were evaporated under $N_{2(g)}$ gentle flow and re-suspended with 1 mL of ultrapure water.

The *in vitro* gastrointestinal digestion assay was performed according to the method described by Sancho et al. (2017) and Farias et al. (2021). Briefly, 1 mL of each extract was homogenized in 3.5 mL of saline solution (140 mmol NaCl + 5 mmol KCl) using a tube shaker and the pH of the mixture was adjusted to 2.0 with HCl (1 mol L^{-1}). Right after that, 125 μL of porcine pepsin solution (200 mg pepsin 424 U mg^{-1} in 5 mL of 0.1 mol L^{-1} HCl) was added and the samples were incubated in a water bath with shaking at 130 rpm for 1 h at 37 °C. A 2 mL aliquot from this phase was collected and stored at -8 °C for further analysis. After the gastric digestion step, the pH of the sample was raised to 6.8 by adding 1 mol L^{-1} $NaHCO_3$. Then, 625 μL of pancreatin and bile solution (225 mg of bile extract and 37 mg of pancreatin diluted in 18.7 mL of 0.1 mol L^{-1} $NaHCO_3$) were added and the samples were

incubated at 37 °C at 65 rpm for 2 h. At the end of the intestinal phase, the samples were cooled in an ice bath and the digested volume was adjusted to 5.5 mL with saline solution and stored at -8°C until analysis. A sample with saline solution at pH 7.0 and no addition of enzymes was submitted to digestion procedures to eliminate any interferences from the reagents. This was the blank sample (undigested group).

After digestion, the samples were submitted to the sample method validated at this work, with minor adaptations in the extraction/partition step, due to the low sample volume obtained. The gastric digestion solution (2 mL), and the intestinal digestion solution (5.5 mL) were mixed 1:1 (v/v) with acetonitrile into a 15 mL Falcon tube and vortexed for 1 min. Afterwards, Mg_2SO_4 (0.4 g/mL of acetonitrile) and NaCl (0.1 g/mL of acetonitrile) were added to all samples, vortex agitated and then centrifugated, as described in item 2.5. No changes were made to the clean-up and filtration stage.

After analyzing the chrysin and TPC content, we also evaluated the bioaccessibility index, calculated by the following equation: % Bioaccessibility = 100 x [A/B], where:

A = total chrysin content or TPC after *in vitro* digestion;

B = total chrysin content or TPC before *in vitro* digestion (obtained from the undigested sample).

In this regard, bioaccessibility represents the amount of a nutrient that is released from the solid matrix being potentially available for absorption after digestion (Pinto et al., 2023).

2.9 Statistical analysis

The results of the chrysin content, antioxidant potential and *in vitro* gastrointestinal digestion were compared by one-way ANOVA, and mean difference was determined by Newman-Keuls Multiple Comparison Test at $p < 0.05$. All data were expressed as mean $\pm$ standard deviation for replicate analyses ($n \geq 3$). When appropriate, Person's correlation test was also realized to verify the possible statistic relation between the evaluated parameters. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 RESULTS AND DISCUSSION

3.1 HPLC-DAD system conditions

The wavelength with maximum absorbance adopted for chrysin analysis was 265 nm, as showed by the center of the absorption spectrum in **Figure 1**.

The injection volume adopted was 40 µL, and Dionex HPLC Column Acclaim 120 C18 5 µm 4.6 x 150 mm was chosen. The most suitable mobile phase was in the form of a gradient, containing ultrapure water (A) and acetonitrile (B). The isocratic mode and with shorter running time were tested. However, given the complexity of the matrices, it was necessary to use the gradient and run for 20 min. The gradient was defined as follows: 0-2min (10% B); 3-5min (30% B); 6-8min (50% B); 9-14min (65% B); 15-18min (80% B); 20min (10% B). In these conditions, the running time of 20 min was adopted, with acquisition between 14.6 min and 16.6 min and chrysin retention time at 15.6 min. The use of HPLC-DAD for chrysin analysis is an excellent option due to its low cost and low detection limits. Furthermore, in many laboratories, this is the only chromatographic system available, making the presented methodology accessible to many researchers.

3.2 Sample preparation

Initially, samples (leaves, pulp and peel of *Passiflora alata*) were extracted employing 10g of sample. When injecting these initial extracts, it was observed that the leaf chromatogram presented peaks that overlapped chrysin. Thus, with the aim of reducing co-extractives, mainly other phenolic compounds, widely found in the leaf, for this type of matrix the extraction started to be conducted with 5 g of sample. Even presenting more adequate chromatograms for the peel and pulp samples (**Figure 2A and 2B)**, the recovery results for chrysin were below expectations (<70%) and, therefore, samples were subjected to an ultrasonic bath for 15 min after extraction with salts, thus, achieving accuracy for the method.

In the clean-up stage, C_{18} and SiO_2 sorbents were tested. As can be seen in **Figures 2C and 2D**, the use of both sorbents had a similar effect on the interferents in leaf samples, and visually no differences were observed between the two extracts. Therefore, the use of SiO_2 is more suitable for this research, as it is a low-cost and easy-to-acquire sorbent. Furthermore, due to the high levels of pigments, the final leaf sample extracts were diluted 1:4 (v/v) in ultrapure water before injection.

In addition to issues related to cost, time and environmental impact, QuEChERS method presents excellent versatility, making it possible, with few adaptations, to carry out sample preparation in three different matrices. Another important factor to be highlighted is that QuEChERS has a clean-up step, which substantially reduces damage to the chromatographic system. As demonstrated in the scan performed between 190 and 1100 nm on a Shimadzu UV-1800 UV-Vis spectrophotometer **(Figure 3)**, the QuEChERS method presented in this work proved to be considerably efficient for cleaning the extract, in comparison to a method available in the literature for analyzing chrysin in vegetables showed by Gharari et al. (2020) (48h of extraction with methanol followed by filtration and injection into the system).

3.3 Method validation

The method selectivity is shown in **Figure 4**, where it is possible to observe the absence of a peak for the solvent **(Fig. 4A)**, and also for the blank samples **(Figs. 4C, 4E, 4G)**, in contrast to the presence of a peak for the samples containing chrysin in solvent **(Fig. 2B)** and in the different matrix extracts **(Figs. 4D, 4F, 4H)**. Other validation results are shown in **Table 1.**

Linearity was confirmed in the range evaluated (0.08 to 36 mg kg^{-1} for leaves and 0.01 to 4 mg kg^{-1} for pulp and peel, with determination coefficient (r^2) ≥ 0.9926 for all samples. Matrix effect showed negative values for leaves and positive values for pulp and peel, being significant (≥ ± 20%, according to Sante, 2019) for the latest ones. Thus, only for pulp and peel matrix matched curves were used. The high matrix effect for pulp and peel is probably due to the complexity of these matrices, causing the co-extraction of several substances capable of absorbing at this wavelength and interfering with the peaks of the compound of interest. Accuracy and precision presented adequate results for all evaluated samples, with recovery ranging from 73 to 101%, repeatability with RSD ≤ 15% and intermediate precision with RSD ≤ 7%. Method LOQ was established as the lowest calibration curve concentration level with signal/noise of 10, being 0.08 mg kg^{-1} for leaves, 0.01 mg kg^{-1} for pulp and 0.014 mg kg^{-1} for peel. Method LOD was obtained dividing the respective LOQ values by 3.33, being 0.024 mg kg^{-1} for leaves, 0.003 mg kg^{-1} for pulp, and 0.0042 mg kg^{-1} for peel.

Gharari et al. (2020) showed the determination of chrysin in roots and shoots of four Iranian *Scutellaria* species by HPLC-UV, trough drying method and 48h of extraction with methanol, it presented LOD of 0.15 mg L^{-1} and LOQ of 0.45 mg L^{-1}. Hadas et al. (2017) and

Ozarowski et al. (2018) evaluated the chemical profile of *Passiflora caerulea* leaves by HPLC-MS and, for this, leaves were dried with air circulation (25°C, 24h), extracted with methanol for 1h (n=3) and concentrated under vacuum. Among all these methods, the method developed in this work can be considered simple and fast, with good accuracy and precision. Furthermore, comparing with the use of QuEChERS for extracting phenolics (Rotta et al., 2019; Nicácio et al., 2021; Rodrigues et al., 2022), it should be noticed that this method employes a low-cost and easily accessible sorbent for clean-up, SiO_2.

3.4 Application of the proposed method

Although naturally abundant, easily accessible, and with an interesting bioactive potential, this specie of *Passiflora* is underused in human food. In this way, exploring its chrysin content is important to support ways for its greater inclusion in food or nutraceutical formulations. In addition, evaluating the levels of chrysin in the leaves, green and mature pulp, and green and mature peel is an important way to observe the distribution of this flavonoid in the plant and the behavior of chrysin in the maturation process. In this work, chrysin levels in leaf, green and mature pulp, and green and mature peel of *Passiflora caerulea,* were evaluated as shown in **Table 2**.

Although it is already known that this passion fruit species contains chrysin flavonoid (Medina et al., 1990), this is the first work that quantifies this flavonoid in the entire structure of the plant (leaf, pulp and peel). The highest levels of chrysin were found in the leaf (5 mg kg^{-1}), followed by green peel (0.35 mg kg^{-1}). Mature and green pulp, and mature peel showed low levels of chrysin (<0.044 mg kg^{-1}), but all analyzed samples were above the method LOQ. The significant decrease in chrysin levels in the mature peel compared to the green peel is due to the fact that in the maturation process, the phenolics undergo oxidation and/or polymerization processes, which decreases the phenolic content (Murata et al., 1995).

Gharari et al. (2020) determinated chrysin in four Iranian *Scutellaria* species, demonstrating that *S. orientalis* is a good source of chrysin, with roots and shoots showing 0.107 and 0.087 μg by 100 μg, respectively. In addition, previous studies have evaluated chrysin concentrations in honeys, being 0.10 mg kg^{-1} in honeydew honey and 5.3 mg kg^{-1} in forest honeys (Hadjmohammadi *et al*., 2010; Pichichero *et al*., 2010).

The results obtained in this work showed that the highest chrysin content in *Passiflora caerulea* is in the leaves, a normally unused part of fruit, and this demonstrates that studies should advance towards the use of leaves as a source for chrysin extraction, and also

for obtaining extracts with bioactive potential. Also, the green peel of *Passiflora caerulea*, the second matrix with the highest chrysin content, is an interesting subject for future studies.

3.5 *In vitro* essay of antioxidant potential

It was possible to verify the *in vitro* antioxidant potential of leaves, pulp and peel of *Passiflora caerulea*, trough TPC, ability to reduce DPPH and ABTS radical evaluations. The results are presented in **Table 3.**

Like chrysin content, the highest TPC were found in leaves, followed by green peel, mature peel, and green and mature pulp. Hadas et al. (2017) found similar results of leaves TPC, of 623 mg kg^{-1} in dry leaves of *Passiflora caerulea*. These results show that the distribution of chrysin in the different parts of the plant is like that of other phenolic compounds, and this fact is corroborated by the positive correlation (0.92) between chrysin content and TPC in *Passiflora caerulea* parts (**Table 4**). Hadas et al. (2017) and Ozarowski et al. (2018) evaluated the chemical profile of *Passiflora caerulea* leaves by HPLC-MS and, in addition to chrysin, other phenolics were observed, such as luteonin, apigenin, vitexin, orientin and their respective metabolites.

Regarding pulp and peel, Rotta et al. (2019) evaluated the TPC of other *Passiflora* species, such as *Passiflora edulis, Passiflora alata and Passiflora ligularis*, and found results from 200 to 400 mg kg^{-1}. Domíngues-Rodrígues et al. (2019) evaluated the TPC of lyophilized peel of *Passiflora edulis, Passiflora ligularis* and *Passiflora molissima*, and observed results from 5,000 to 2,000 mg kg^{-1} of dried extract. This work is the first to evaluated TPC of *Passiflora caerulea* pulp and peel. The higher levels of TPC obtained in green pulp and peel compared to mature pulp and peel, is associated with the oxidation and polymerization reactions of phenolics during the maturing process.

The antioxidant potential of extracts obtained from *Passiflora caerulea* were analyzed using DPPH and ABTS tests (**Table 3**). The best performance in the DPPH and ABTS tests came from the leaf samples, certainly due to the higher levels of chrysin and TPC, as corroborated by the negative correlation trend between chrysin and the DPPH and ABTS tests, and by the negative significant correlation between TPC levels and the DPPH and ABTS tests (**Table 4**). Reis et al. (2018), also demonstrated antioxidant activity through the ABTS test of fresh and pasteurized juice of *Passiflora caerulea* pulp during cold storage. The antioxidant activity of different solvent extracts and fresh pulp of *Passiflora subpeltata* was showed by DPPH and ABTS methods by Shanmugam et al. (2018). Gomes et al. (2022), demonstrated the antioxidant effect of the hydroalcoholic extract of *Passiflora cincinnata* by the DPPH test.

In addition to the leaf, the green and mature peel of *Passiflora caerulea* also showed interesting antioxidant activity in this work, presenting significant difference in relation to the pulp. Mature and green peel of the passion fruit is usually not used in human food, and is often discarded. Thus, these findings indicate the potential of *Passiflora caerulea* leaves and peel for future studies aiming at its inclusion in food or nutraceutical formulations.

3.6 *In vitro* gastrointestinal digestion

Studies have demonstrated the effect of the digestive tract in altering the content of phenolic compounds and the bioactive potential of several samples (Farias et al., 2021; Wu et al., 2022; Zhao et al., 2023). Thus, this analysis is important to demonstrate whether the digestive tract modifies the levels of chrysin and TPC of *Passiflora caerulea* and alters their antioxidant potential.

It is possible to observe that gastric and intestinal digestion did not significantly alter chrysin levels (**Figure 5**) and bioaccessibility (**Table 5**), in relation to the undigested sample in leaf extract.

For green peel, there is a reduction in the content and bioaccessibility of chrysin in the gastric phase of the digestive tract, but in the intestinal phase the values were equivalent to the undigested sample. These findings are interesting, as in many cases the simulation of the gastrointestinal tract decreases the phenolic content, reducing the bioaccessibility of these compounds. Ma et al. (2023) demonstrated that gastrointestinal digestion decreases the flavonoids content of *Lycium barbarum* fruit extract. Likewise, Farias et al. (2021) observed a reduction in the flavonoid content in fractions of *Eugenia pyriformis* fruit. Ting et al. (2021), simulating gastrointestinal digestion of pure chrysin at a concentration of 232 mg L^{-1}, showed that *in vitro* digestion decreases the content of chrysin in 70% in the gastric phase and in the intestinal phase it decreases even more.

The results of this work differ from that presented by Ting et al. (2021), and this is probably due to the fact that in this experiment the chrysin is not pure, but is inserted in a matrix, which may somehow protect from the effect of the digestive tract on this compound. Furthermore, the concentration of chrysin in Ting's work is much higher than that present in the leaf and green peel, which may accentuate a possible effect.

Also, the *in vitro* digestion did not change the chrysin content and bioaccessibility in leaf extract, and in the green peel extract it reduced the content and bioaccessibility only in the gastric phase, normalizing in the intestinal phase. This result is important, as it demonstrates that in a possible application of the leaves and green peel in a food or nutraceutical formulation, the chrysin content would not be reduced by the digestive tract.

Similarly to these results, Pinto et al. (2023) demonstrated that the gastrointestinal tract did not decrease the bioaccessibility on flavonoids in functional cookies enriched with chestnut shells extract. Tian et al. (2021) showed that the gastrointestinal tract progressively increased the content and bioaccessibility of hydroxybenzoic acid and vanillic acid, two phenolic acids, in whole wheat products. Thus, the chrysin flavonoid present in the leaf and green peel of *Passiflora caerulea* showed a positive behavior in the simulation of the gastrointestinal tract, suggesting that digestion is not capable of reducing its content and bioaccessibility.

Besides, similar to Pinto et al. (2023) and Tian et al. (2021) results, simulation of digestion increased TPC in *Passiflora caerulea* leaves (**Fig. 6A**) and increased the bioaccessibility of TPC (**Tab. 5**). This can be explained by the fact that phenolics in general have greater solubility in acidic pH, causing the gastric phase to increase the content and bioaccessibility of TPC. In the green peel, however, the increase of TPC occurred only in the gastric phase (**Fig. 6B**), as well as the bioaccessibility of TPC (**Tab. 5**), with a marked decrease in the intestinal phase. Ma et al. (2023) observed a similar behavior in the phenolics of *Lycium barbarum* fruit extract. Furthermore, this behavior of TPC of green peel is certainly not associated with changes in chrysin levels, as the intestinal phase presented a chrysin content equivalent to the undigested sample. This is reinforced by the fact that there was no significant correlation between the chrysin content and the TPC of the digested green peel (**Tab. 4**).

In the DPPH and ABTS tests on the leaf (**Figs. 6C and 6E**), the gastrointestinal tract increased antioxidant activity, especially in the gastric phase for ABTS, and in the intestinal phase for DPPH. As for the peel (**Figs. 6D and 6F**), there was a decrease in antioxidant activity in the ABTS test and an increase in antioxidant activity in the DPPH test, in the intestinal phase. Differences in the results of these tests certainly cannot be attributed to levels of chrysin, as seen, showed a uniform behavior and showed no significant correlation with these parameters (**Tab. 4**). Thus, the characteristics of each test and the type of compound that is preferentially active in each test probably explain these differences. in order to clarify these questions, future studies may evaluate other tests and present a more complete profile of the chemical composition of the leaf and green peel of *Passiflora caerulea*.

4 CONCLUSION

This work presented for the first time a QuEChERS modified method and HPLC-DAD analysis for quantification of chrysin in *Passiflora* species. Compared to the few methods already presented in literature, this method showed excellent selectivity, accuracy, precision, being faster, cheaper and simpler than others. The methodology presented is an accessible technique for many researchers and is versatile, as it can be applied, with few adaptations, to three different matrices (leaves, pulp and peel).

Also, it was demonstrated that the leaves and green peel of *Passiflora caerulea* are interesting sources of chrysin, in addition to having excellent levels of phenolics and good antioxidant activity. Furthermore, except for TPC of green peel, the digestive tract is not able to decrease the content and bioaccessibility of chrysin and phenolics in these matrices. Thus, leaves and green peel of *Passiflora caerulea* may be chrysin source for bioassays, and more details about its chemical composition and application in food or nutraceutical formulations must be evaluated.

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GRAPHICAL ABSTRACT

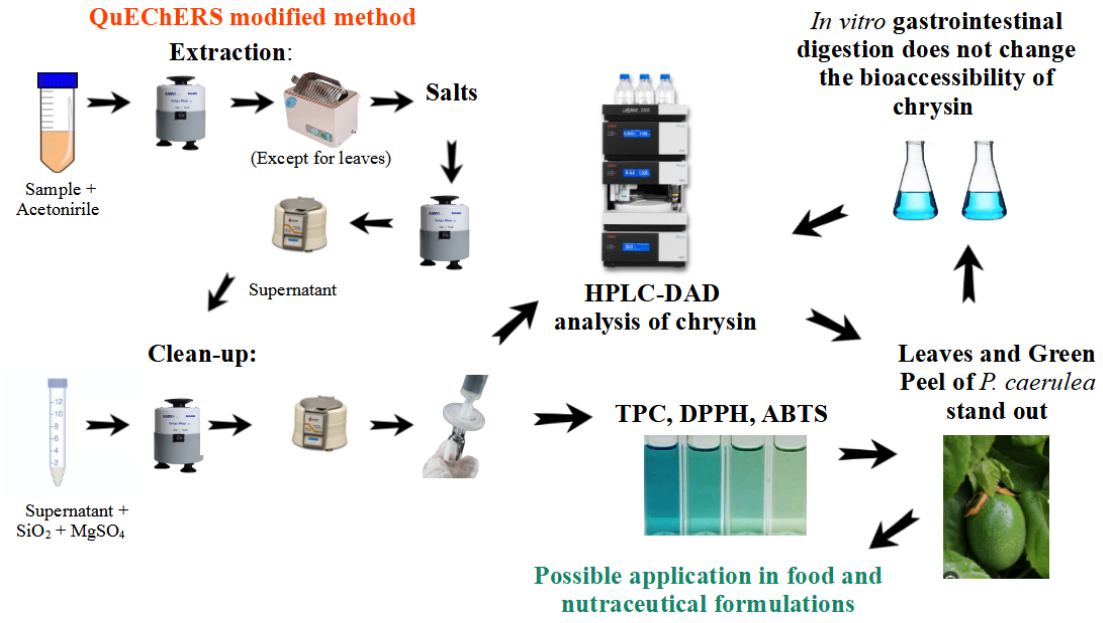


Figure 1. 3D representation of the chrysin flavonoid absorption spectrum. Data were obtained from a Thermo Scientific Dionex UltiMate 3000 Series, equipped with Autosampler Column Compartment ACC-3000 and Diode Array Detector (DAD).

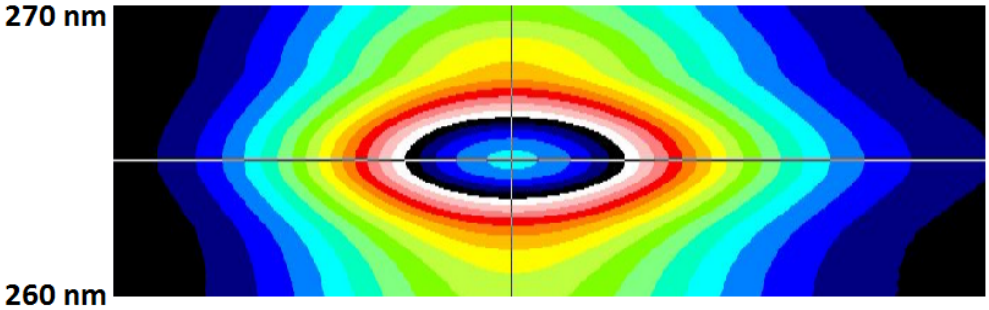


Figure 2. Chromatograms of recovery of QuEChERS without the use of ultrasound (A) and with the use of ultrasound (B) in the extraction step, and with the use of C18 (C) or SiO_2 (D) as sorbents in the clean-up step. Tests A and B were carried out on the pulp, and tests C and D were carried out on the leaves.

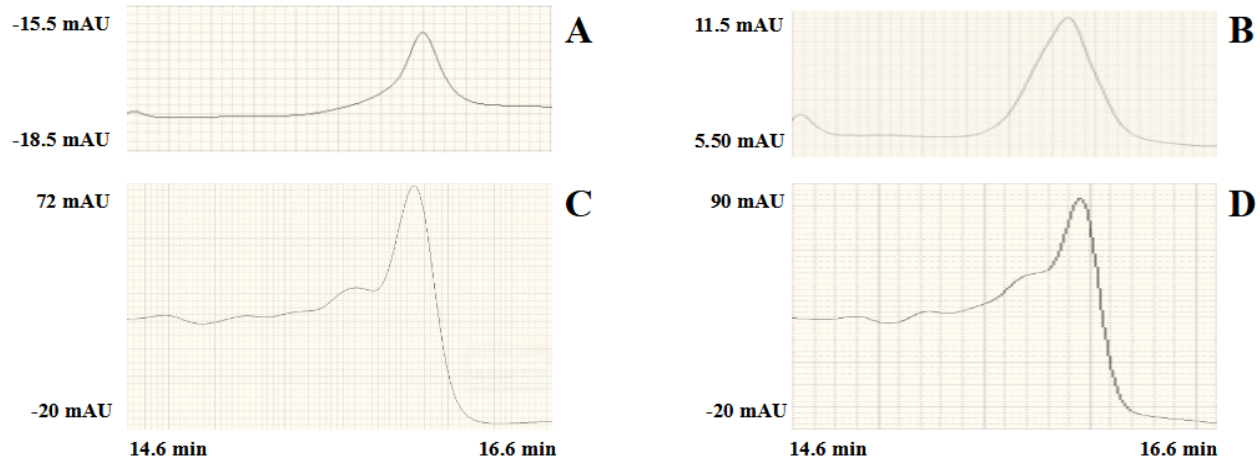


Figure 3. Scanning between 190 and 1100 nm for comparison between extracts obtained by 48h of extraction with methanol and QuEChERS. Extraction was performed on blank sample leaves and methanol was used as blank. Scanning was performed on a Shimadzu UV-1800 UV-Vis spectrophotometer.

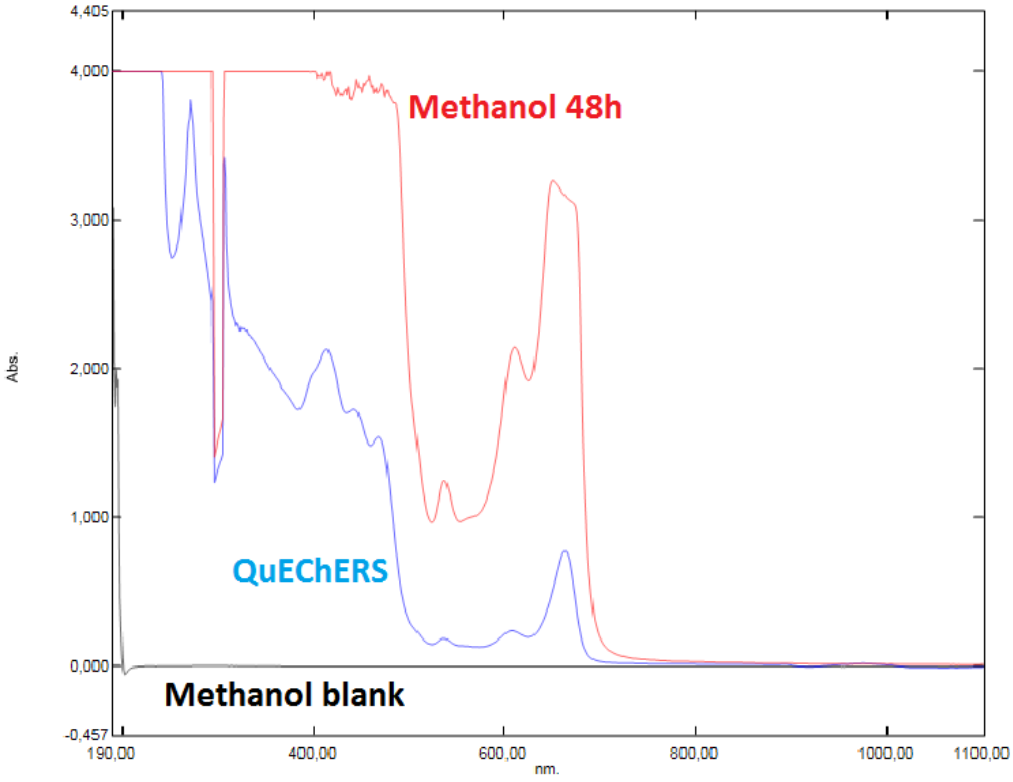


Figure 4. Method selectivity for QuEChERS and HPLC-DAD for chrysin analysis in *Passiflora* species. Chromatogram of methanol (A), chrysin in methanol 2.4 mg L^{-1} (B), blank of leaves (C), blank of leaves with chrysin 2.4 mg L^{-1} (D), blank of pulp (E), blank of pulp with chrysin 2.4 mg L^{-1} (F), blank of peel (G), and blank of peel with chrysin 2.4 mg L^{-1} (H). *Passiflora alata* was used as blank sample. Acquisition time: 14.6 to 16.6 min.

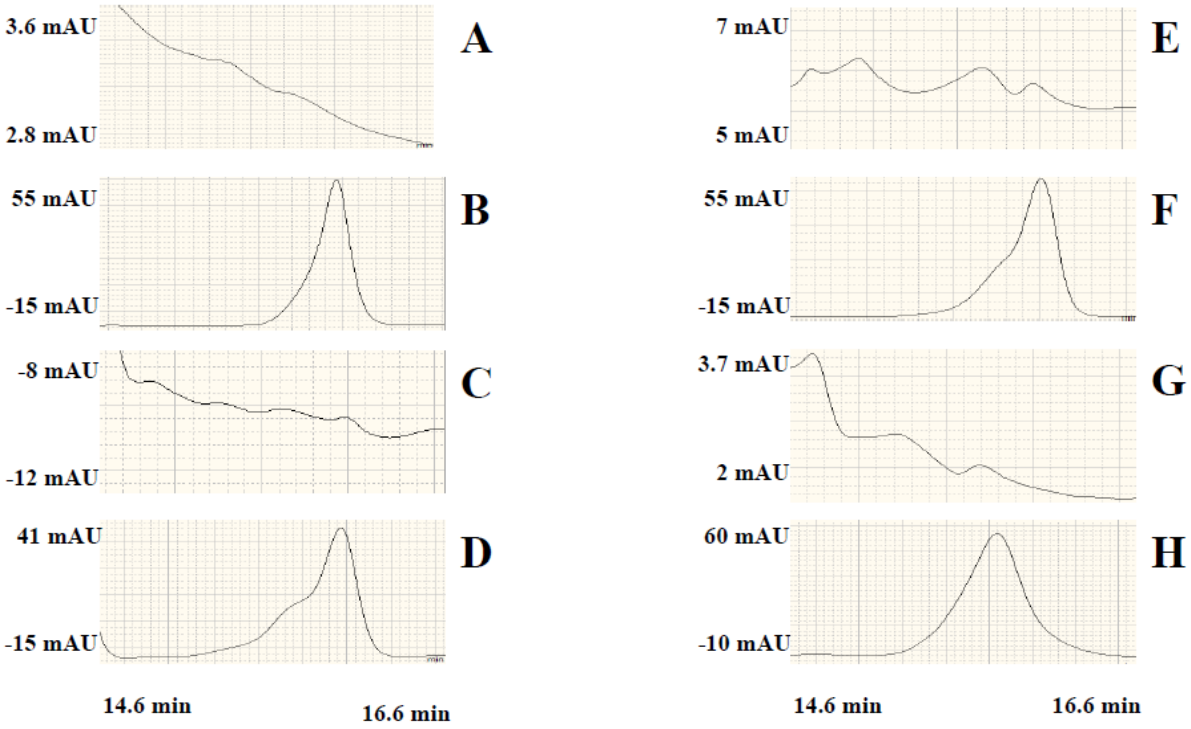
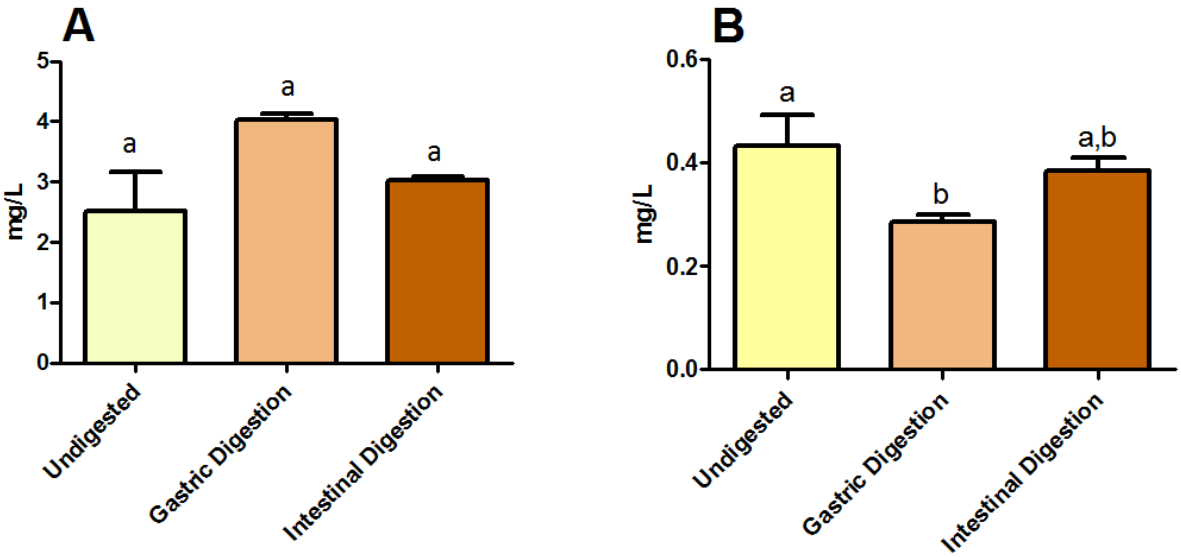
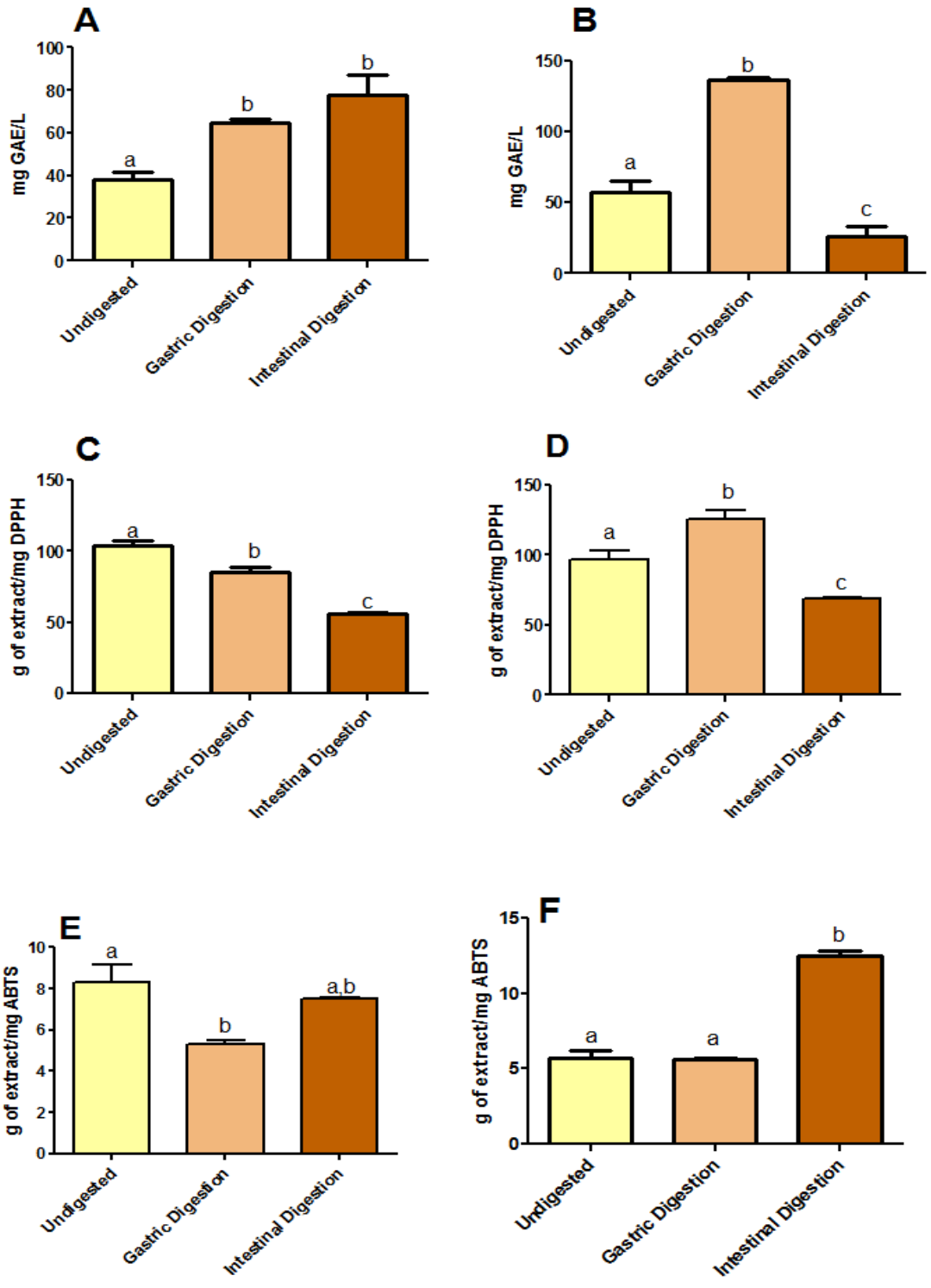


Figure 5. Chrysin content in the extract of leaves (A) and greel peel (B) of *Passiflora caerulea* after *in vitro* gastrointestinal digestion.



Different letters mean significant difference ($p < 0.05$). Values represent the mean ± standard deviation.

Figure 6. Results of TPC, DPPH and ABTS analysis for extract of leaves (A, C and E, respectively) and green peel (B, D and F, respectively) of *Passiflora caerulea* after *in vitro* gastrointestinal digestion.



Different letters mean significant difference ($p < 0.05$). Values represent the mean ± standard deviation.

Table 1. Validation results for chrysin analysis by QuEChERS and HPLC-DAD in *Passiflora* species.

Parameter/Matrice	**Leaf**			**Pulp**			**Peel**		
Concentration (mg kg^{-1})	3.2	19.2	32	0.4	2.4	4	0.4	2.4	4
Recovery (%)	101.40	83.08	73,18	86.89	73.75	73.15	78.56	70.53	74.95
Precision (RSD %)	8.47	2.28	3.32	9.09	7.34	4.40	15.08	3.97	2.64
Intermediate Precision (RSDip %)	-	4.17	-	-	7.30	-	-	2.36	-
Linear Range (mg kg^{-1}) (r^2)	0.08-32 (0.9938)			0.01-4 (0.9986)			0.01-4 (0.9926)		
LOQ (mg kg^{-1})	0.08			0.01			0.014		
LOD (mg kg^{-1})	0.024			0.003			0.0042		
Matrice Effect (%)	-15,26			+32,82			+54,12		

Table 2. Chrysin concentration in leaf, mature and green pulp, and mature and green peel of *Passiflora caerulea.*

Sample	Chrysin (mg/kg sample)
Leaf	5.01 ± 0.55^{a}
Mature Pulp	0.043 ± 0.002^{b}
Green Pulp	0.033 ± 0.003^{b}
Mature Peel	0.014 ± 0.007^{c}
Green Peel	0.35 ± 0.01^{d}

Different letters mean significant difference ($p < 0.05$). Values represent the mean ± standard deviation.

Table 3. *In vitro* antioxidant potential of leaf, mature and green pulp, and mature and green peel of *Passiflora caerulea*.

Sample/ Analysis	TPC (mg GAE/kg sample)	DPPH (g sample/mg DPPH)	ABTS (g sample/mg ABTS)
Leaf	533.83 ± 81.13^{a}	0.71 ± 0.05^{a}	0.12 ± 0.008^{a}
Mature Pulp	41.01 ± 7.50^{b}	6.34 ± 1.76^{b}	0.96 ± 0.04^{b}
Green Pulp	65.85 ± 2.73^{b}	4.93 ± 0.79^{b}	0.95 ± 0.1^{b}
Mature Peel	$125.38 \pm 0.88^{b,c}$	$1.12 \pm 0.15^{a,c}$	0.16 ± 0.04^{a}
Green Peel	190.44 ± 8.69^{c}	$1.96 \pm 0.13^{a,c}$	0.33 ± 0.008^{c}

Different letters mean significant difference ($p < 0.05$). Values represent the mean ± standard deviation.

Table 4. Bioaccessibility of chrysin and TPC of leaf and green peel of *Passiflora caerulea* after *in vitro* gastrointestinal digestion.

Sample/ Bioaccessibility	**Bioaccessibility of chrysin (%)**		**Bioaccessibility of TPC (%)**	
	Gastric Digestion	Intestinal Digestion	Gastric Digestion	Intestinal Digestion
Leaf	160.34 ± 6.51	120.79 ± 4.78	170.86 ± 8.66[a]	205.67 ± 49.09[a]
Green Peel	66.59 ± 7.32[a]	89.34 ± 14.66	236.88 ± 5.69[a]	45.02 ± 24.32[a]

[a] indicates significant difference ($p < 0.05$) compared to the undigested sample. Values represent the mean ± standard deviation.

Table 5. r values resulting from Pearson's correlation test for chrysin and TPC X DPPH, ABTS and TPC.

	TPC			DPPH			ABTS		
Digestion status	Und	Digested Leaf	Digested Green Peel	Und	Digested Leaf	Digested Green Peel	Und	Digested Leaf	Digested Green Peel
Chrysin Content	0.92[a]	0.10	-0.28	-0.51	-0.22	-0.39	-0.52	0.54	-0.04
TPC				-0.66[a]	-0.67[a]	-0.12	-0.67[a]	0.02	0.67[a]

[a] denoted $p < 0.05$. In this analysis, the undigested (Und) sample is the initial analysis on the leaf, pulp and peel of *Passiflora caerulea*.

4.3 Manuscrito II

Co-encapsulation of *Lactiplantibacillus plantarum* and chrysin flavonoid by external ionic gelation

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ABSTRACT

In this work, co-encapsulation of *Lactiplantibacillus plantarum* LP-01 (*L. plantarum*) and chrysin flavonoid by external ionic gelation was evaluated. *L. plantarum* was encapsulated at a concentration of 10 CFU mL^{-1}, and chrysin at concentrations of 0.1, 0.25 and 0.5 % (m/m). The encapsulation efficiency for the probiotic was around 90%, and for chrysin there was a difference in efficiency according to the percentage of flavonoid, showing efficiency of 84, 72 and 54 % for concentrations of 0.1, 0.25 and 0.5 %, respectively. The capsules were subjected to slow (63 °C, 30 min) and fast (72 °C, 15 sec) pasteurization, and simulation of gastrointestinal digestion *in vitro*, obtaining satisfactory probiotic counts and chrysin levels in all analyses. The stability of the capsules was evaluated for 120 days under 3 different temperature conditions (25, 8 and -18 °C). It was observed that the stability of the probiotics was not unaffected by the concentration of chrysin, and that the best stability of *L. plantarum* occurred at a temperature of -18 °C, followed by 8 and 25 °C. At a temperature of -18 °C, the probiotics showed good viability until day 105. The chrysin content was not substantially modified over the 120 days of study at any of the concentrations and at none of the temperatures. In short, this work showed the feasibility of producing of polyfunctional capsules containing *L. plantarum* and chrysin, and future studies should focus on the application of these capsules in food or nutraceutical formulations and in bioassays.

Key-words: Probiotics, flavonoids, nutraceutical formulations, polyfunctional capsules.

1 INTRODUCTION

The term probiotic is derived from a greek word meaning “life” and is used to refer to non-pathogenic living organisms with beneficial effects on hosts (Pandey et al., 2015). Probiotics can be included in the composition of a wide range of products, ranging from medicines to dietary supplements, but they are often associated with dairy products. In this case, the genera *Bifdobacterium* and *Lactobacillus* are those that contain the main microorganisms used as probiotics (Reis et al., 2019).

Strains of *Lactiplantibacillus plantarum* (*L. plantarum*) have an important effect on the regulation of the intestinal microbiota (Georgieva et al., 2009). They also present antimicrobial characteristics against different pathogens (Ben Slama et al., 2013), immunological, anti-inflammatory and antitumor activity (Wang et al., 2009), antioxidant (Zhang, et al., 2013), efficacy in neutralizing cytotoxicity induced by enterotoxins (Zhang et al., 2015), in addition to being effective in the treatment of diabetes (Li et al., 2014). The *L. plantarum* also presented antidepressant-like effect. Rudzki et al. (2019) studied the effects of *L. plantarum* on patients with major depression. In this study, seventy-nine patients with major depression were randomized and allocated to a double-blind, placebo-controlled study. Participants received a classic antidepressant with the probiotic for a period of 8 weeks or an antidepressant with the probiotic placebo for the same period. The severity of psychiatric symptoms and cognitive functions were assessed using numerous scales and tests. An improvement in cognitive functions and a decrease in kynurenine levels were observed in individuals who received *L. plantarum.* These and other actions of *L. plantarum* have attracted the attention of researchers in the development of ingredients for nutraceutical and food formulations (Bakhtiyari et al., 2022; de Deus et al., 2023; Du et al., 2023; Xie et al., 2024).

The viability of probiotics is essential for these microorganisms to reach and colonize the human intestine, being one of the most important factors for probiotic bacteria to promote health benefits for the host (Ferreira, 2018). In this sense, increasing the resistance of

microorganisms to the gastrointestinal tract and their stability for long periods of storage has been an objective of several researches (Colín-Cruz et al., 2019; Du et al., 2023; Xie et al., 2024). Furthermore, ways are being sought to enable the application of probiotics in food or nutraceutical formulations, reducing the influence of the thermo and mechanical processes to which the product is subjected, in addition to controlling the interaction between the probiotic and the product. To this end, an important path followed recently is the encapsulation of probiotics (Bakhtiyari et al., 2022; de Deus et al., 2023). Among the encapsulation techniques, external ionic gelation stands out, which has low cost, simplicity and low environmental impact (Raddatz and Menezes, 2021; Raddatz et al., 2022a and 2022b; Xie et al., 2024). Encapsulation also allows probiotics to be connected to other bioactive agents, generating polyfunctional capsules, in addition to possible benefits of the compounds to the probiotic and/or synergistic biological effect (Raddatz and Menezes, 2021).

Chrysin (5,7-Dihydroxyflavone) belongs to the flavone class of flavonoids and presents numerous and promising bioactive effects, such as antioxidant activity (Pushpavalli et al., 2010), anticonvulsant (Medina et al., 1990), antihypertensive (Vilar et al., 2002), anti-inflammatory (Bae et al., 2011), antineoplastic (Pichichero et al., 2011), antihyperlipidemic (Zarzecki et al., 2014) action. Recent studies have demonstrated the neuroprotective role of chrysin, through protective and/or therapeutic effects in models of Parkinson (Del Fabbro et al., 2019a; De Gomes et al., 2018), autoimmune encephalomyelitis (Del Fabbro et al., 2019b and 2021), Alzheimer (Souza et al., 2018), and hypothyroidism (Bortolotto et al., 2021). Also, similarly to *L. plantarum*, the antidepressant-like effect of chrysin in models of depression was presented, showing the role of hormonal, neurotrophic, monoaminergic, oxidative and inflammatory regulation on this effect (Borges Filho et al., 2015, 2016a, 2016b, Bortoloto et al., 2018).

Despite its relevant bioactivity, the therapeutic use of chrysin has some limitations, one of which is the effect of gastric conditions on the levels of this flavonoid. Tian et al. (2021) demonstrated the effect of gastric conditions in the reduction of levels and

bioacessibility of chrysin. One of the main ways to overcome this problem is encapsulation techniques, which have been widely applied in chrysin studies. However, most techniques have used reagents that have toxicity, such as methanol, dichloromethane and dimethyl sulfoxide (Borges Filho et al., 2024).

Thus, considering the relevant bioactivity of *L. plantarum* and chrysin, especially in antidepressant therapy, considering the importance of using green techniques for chrysin encapsulation, and considering the importance of encapsulation to increase the stability and to promote the union of these two bioactive agents, the aim of this work is to evaluate the co-encapsulation of *L. plantarum* and chrysin by external ionic gelation, evaluating the viability of capsule production and their stability.

2 METHODOLOGY

2.1 Probiotics and reagents

Probiotic culture of *Lactiplantibacillus Plantarum* LP-01 (*Lactobacillus plantarum (L. plantarum)*) was donated by Coana Probiotic Solution (Santa Catarina, Brazil). Chrysin analytical standard (98 %), chrysin (97 %), pepsine, pancreatine, and acetonitrile HPLC grade were purchased from Sigma-Aldrich (Brazil). Anydrous $MgSO_4$ was purchased from Cinética (Brazil). Methanol HPLC grade, $CaCl_2$, ethanol, NaCl, SiO_2 and Na_2CO_3 were purchased from Proquímios, Vetec, PROC9, Alphatec, Synth and Vetec, respectively, all in Brazil. MRS broth and sodium alginate were purchased by Merck (Germany) and FMC-Biopolymer (USA), respectivelly.

2.2 Preparation of the inoculum

The lyophilized strain of *L. plantarum* was activated prior to the microencapsulation process by adding 1 g of the strain in 100 mL of MRS broth and incubated for 16 h at 37 °C. The bacteria were harvested in the exponential growth phase by centrifuged at 2470 × g for 15 min at 4 °C, and the probiotic mass was washed with NaCl solution (0.85 %) and collected for later addition to the process. The initial concentration of this activated probiotic culture was approximately ~ 10 log CFU mL^{-1} (Etchepare et al., 2016; de Deus et al., 2023).

2.3 Co-encapsulation of *L. plantarum* and chrysin

The microcapsules were produced using the external ionic gelation method of Etchepare et al. (2016), with adaptations demonstrated by Somacal et al. (2022). The treatments used are shown in Table 1. The complete dissolution of the *L. plantarum,* chrysin and sodium alginate in water was initially performed using an orbital shaker for 30 min. Agitation was maintained throughout the microencapsulation process. The solution was sprayed onto a 0.1 M $CaCl_2$ solution using a dual fluid atomizer nozzle (0.1 mm) held 12 cm

away from the solution and under 12,258 KPa air pressure. The microcapsules were kept in the 0.1 M $CaCl_2$ solution under constant stirring (30 min) and later filtered. The microparticles were collected and transferred to sterile vials and stored.

2.4 Morphology, average size and color of microcapsules

The morphology of the microcapsules was observed under a optical microscope. The average particle size distribution was measured using Mastersizer 3000 laser diffraction equipment (Malvern, Germany), using water as dispersion means. Color parameters (Luminosity (L) and a* and b* chromatics) were evaluated on a Konica Minolta CR-400 colorimeter (Japan).

2.5 Probiotic viability

The quantification of bacteria from the unencapsulated microcapsules was performed according to the method proposed by Sheu et al. (1993), with modifications. The unencapsulation procedure consisted in weighing 1 g of sample, adding 9 mL of sterile phosphate buffer solution (pH 7.5) and homogenizing for 10 min in a magnetic stirrer. From this first dilution, serial decimal dilutions were made in 0.1 % peptone water. For the *L. plantarum* count, 1.0 mL aliquots of the chosen dilutions were transferred in triplicates to petri dishes. Over each sample, the MRS Agar culture medium with overlay were added. Colony counts were performed will be performed on an electronic colony counter (LOGEN - LS6000) after incubation for 72 h under anaerobiosis at 37 °C. Probiotic viability was presented as CFU mL^{-1}.

2.6 Chrysin content

For analysis of chrysin content, a previously validated technique was used (Borges Filho et al., 2023), using a modified QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) followed by analysis by High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD). Initially, the microcapsules were ruptured, weighing 1 g

of sample, adding 9 mL of sterile phosphate buffer solution (pH 7.5) and homogenizing for 60 min in a magnetic stirrer.

After that, the content was transferred to a 50 mL PP tube containing 10 mL of acetonitrile. Then, the samples were shaken by vortex for 1 min. Afterwards, 4 g of $MgSO_4$ and 1 g of NaCl were added to all samples. Next, the tubes were vortexed for 1 min and immediately centrifuged at 3,000 rpm for 10 min. Then, supernatant was collected and added into a 15 mL PP tube to proceed to the clean-up step.

Clean-up was performed using 1 mL of supernatant, 25 mg of SiO_2 and 150 mg of $MgSO_4$. The tubes were then vortexed for 1 min and immediately centrifuged (3,000 rpm, 10 min). The supernatants were filtered through a nylon syringe filter (25 mm, 0.22 μm) prior to the chromatographic analysis.

Chromatographic analysis were performed in an Thermo Scientific Dionex UltiMate 3000 Series, equipped with Autosampler Column Compartment ACC-3000, Diode Array Detector - DAD and a Dionex HPLC Column Acclaim 120 C18 5μm 4.6 x 150mm. The absorbance was evaluated in 265 nm, temperature of the autosampler and the column were maintained at 25°C, injection volume was 40 μL, mobile phase flow rate 0.5 mL/min, in a gradient containing water (A) and acetonitrile (B). The gradient was defined as follows: 0-2min (10% B); 3-5min (30% B); 6-8min (50% B); 9-14min (65% B); 15-18min (80% B); 20min (10% B). In this conditions, the peak of chrysin occurs in approximately 15.6 min, and the running time of 20 min was adopted, with acquisition between 14.6 min and 16.6 min (Borges Filho et al., 2023). The content of chrysin was presented as mg 100 g^{-1}.

2.7 Encapsulation efficiency (EE%)

Encapsulation efficiency (EE%) was performed for probiotics and chrysin following the methodology suggested by Petraityte and Sipailiene (2019), where an equation (Equation 1) was developed that represents the survival rate of microorganisms and the chrysin content during the microencapsulation process.

Equation 1:

$$EE\% = (N/ N0) \times 100$$

Where N is the number of viable cells (log CFU mL^{-1}) or chrysin (mg 100 g^{-1}) released from the microcapsules, and N0 is the number of viable cells (log CFU mL^{-1}) or chrysin (mg 100 g^{-1}) in the pre-encapsulation concentrate.

2.8 Resistance to heat treatment

Resistance to heat treatment was evaluated as proposed by Zhang et al. (2015), with some modifications. For this, 1 g of microcapsules and 1 mL of free culture were transferred to test tubes containing 9 mL of sterile peptone water (0.1 %). The contents were then subjected to different thermal conditions, being 72 ± 1 °C for 15 sec and 63 ± 1 °C for 30 min, simulating fast and slow pasteurization, respectively. After the pasteurization process, the tubes were slightly cooled by immersion in ice for 15 min. Finally, aliquots were collected and cell viability was determined according to item 2.5, and chrysin levels was determined according to item 2.6.

2.9 Simulation of gastrointestinal digestion

To evaluate the behavior of probiotics and chrysin in gastrointestinal digestion, an *in vitro* simulation of these conditions was performed following the method proposed by Madureira et al. (2011), with modifications. The viability of the bacteria and chrysin levels were evaluated (esophagus/stomach, duodenum, and ileum). During the procedure, aliquots will be taken after 0 min, 90 min (esophagus/stomach), 110 min (duodenum), and 200 min (ileum), analyzing the survival of free and microencapsulated *L. plantarum* by enumeration on MRS agar according to item 2.5, and chrysin content according item 2.6.

In the step where the esophagus/stomach phase was simulated, 1 g of microcapsules were weighed followed by homogenization with 25 mg mL^{-1} pepsin in 0.1 M HCl at a

concentration of 0.05 mL for 90 min, where 300 µl of fractionated enzyme was added equally in six moments (10, 20, 30, 50, 70 and 90 min), and the pH adjusted to 2.0 with 1 M HCl. In the step where the duodenum was simulated, a solution containing 2 g pancreatin and 12 g/L bovine bile salts in 0.1 M $NaHCO_3$ at pH 5.0 was used at a concentration of 0.25 mL, where 125 µl of each enzyme in the sample were added. Finally, in the step where the ileum was simulated, the pH was adjusted to 6.5 using 0.1 M $NaHCO_3$. The solutions were prepared at the time of analysis and sterilized by membrane filtration (0.20 µm pore size). The analysis was performed in a refrigerated incubator with agitation (TE-421, Tecnal, Piracicaba, SP, Brazil) at 37 °C simulating body temperature and agitation conditions to simulate the peristaltic movements of the digestive tract, using 130 rpm in the gastric phase, and 65 rpm in the intestinal phases (de Deus et al., 2023). Finally, aliquots were collected and cell viability was determined according to item 2.5, and chrysin levels was determined according to item 2.6, excluding the stage of breaking the capsules.

2.10 Viability of *L. Plantarum* and chrysin content on storage

In order to determine the viability of *L. plantarum* and the stability of the chrysin, analyses were performed every 15 days, with the samples stored in sterile vials under the following conditions: 25 ± 1 °C, 8 ± 1 °C and −18 ± 1 °C, for a period of 120 days, according to Oliveira et al. (2007).

2.11 Statistical analysis

The results of the average size, color, probiotic viability, chrysin content, encapsulation efficiency, resistance to heat treatment and gastrointestinal digestion were compared by one-way ANOVA, and mean difference was determined by Tukey's Multiple Comparison Test at $p < 0.05$. All data were expressed as mean ± standard deviation for replicate analyses ($n \geq 3$). This statistical analysis was performed using the software GraphPad Prism version 10.1.1 (GraphPad Software, Inc., La Jolla, CA, USA). For analysis of viability

of *L. Plantarum* and chrysin content in 120 days of storage and 3 temperature conditions, results were submitted to analysis of variance (ANOVA-Friedman Test) and significant differences identified by comparison test between means (Duncan´s Test) at $p < 0.05$. For data analysis TIBCO STATISTICA® software (version 14.0, StatSoft, Inc., Palo Alto, CA, U.S.) was used.

3 RESULTS AND DISCUSSION

3.1 Morphology, average size and color of microcapsules

The morphology of the microcapsules was elucidated by optical microscope (Figure 1), with magnification of 20 x, presenting uniform spherical morphology. The encapsulated material showed good distribution. Also, no cracking was observed on the surfaces of treatments, being a positive aspect, as it makes it impossible for oxygen to enter, which could degrade compounds (Raddatz et al., 2022a). The microcapsules obtained by this technique generally have varying particle sizes (25 μm to 2 mm) (Martín et al., 2015), and the mean diameter of microparticles produced in our work was 131 μm to 154 μm (Table 2). Due to the absence of probiotics, T3 had the smallest average size, as expected. Microcapsule size is influenced by the method used for capsule formation, the type of encapsulating agent, encapsulation temperature, sample inlet pressure, and agitation speed, which also influence the variation in the distribution of the size, encapsulation efficiency, and yield (Somacal et al., 2022). In the work of Somacal et al. (2022), microcapsules obtained by ionic gelation using sodium alginate as encapsulant presented an average size of 105 μm, and another study that used the same technique had an average size of 158 μm (Raddatz et al., 2022a and 2022b). In work of de Deus et al. (2023), which carried out encapsulation of *L. plantarum* by spray dryer, the average size was approximately 14 μm.

Regarding the evaluations of color parameters (Table 2), the only treatment that significantly differed in Luminosity (L*) was T6, and this is probably due to the higher concentration of chrysin in this treatment. As for the a* parameter, it was observed that with an increase in chrysin concentration, the a* value became more negative. This indicates that the increase in chrysin moved the color away from red and closer to green. Regarding the b* parameter, the addition of chrysin increased the values, indicating that the addition of chrysin made the capsules more yellowish. This is consistent with the characteristic of this flavonoid, which in powder form has a yellowish color. Chrysin thus provided a better visual appearance to the capsules.

3.2 Probiotic viability, chrysin content and encapsulation efficiency

Probiotic viability, chrysin content and EE are presented in Table 3. The viability and EE of probiotics showed excellent results, with an average EE of 90%. This demonstrates the excellent efficiency of external ionic gelation in the encapsulation of probiotics. Using the same encapsulation technique, Raddatz et al. (2022a) demonstrated an EE of 92% for *Lactobacillus casei LC03*. Using the same probiotic as in this work, *L. plantarum*, de Deus et al. (2023) obtained an EE of 71%, using the spray dryer technique. These results also demonstrate a very important fact, that chrysin did not present acute toxicity to probiotics. Although some studies have already demonstrated the antimicrobial effect of this flavonoid (Khasteband et al., 2024; Adesina et al., 2024), at the concentrations of chrysin and with the probiotic that we used, chrysin did not present any toxicity.

Regarding content and EE of chrysin, the concentration of 0.1 % presented the best values, with the EE decreasing at concentrations of 0.25 and 0.5 %. This result was expected, as increasing the concentration of encapsulated material commonly decreases EE. Raddatz et al. (2022a) demonstrated that the EE for anthocyanins decreases from 85 to 69 % when the A*llium cepa L.* peel extract concentration increases from 5 to 20%. In another work, Raddatz et al. (2022b) showed the decrease in the EE of reducing compounds from 50 to 28%, resulting from an increase from 20 to 40 % in the concentration of *Allium cepa L*. residue extract. The EE we found for chrysin concentrations are very interesting results because, as we have already demonstrated in a previous review (Borges Filho et al., 2024), most of the techniques already presented in the literature for chrysin encapsulation use toxic reagents, such as dimethyl sulfoxide, methanol and dichloromethane, making the execution of the technique more harmful in environmental aspects. However, the technique we used in this work is a green technique, with no environmental impact, besides presenting low cost and simplicity of execution. Considering the possibility of future studies on the application of microcapsules to food or nutraceutical products, the most appropriate concentration according to EE would be 0.1 %, aiming for better use of the flavonoid applied. Furthermore, in possible

in vivo studies, if this concentration is administered at a dose of 10 μL g^{-1} of body weight (Ma et al., 2023), it would correspond to the therapeutic dose of chrysin, 10 mg kg^{-1} (Borges Filho et al. 2015; 2016a; 2016b).

3.3 Resistance to heat treatment and *in vitro* gastrointestinal digestion

The heat resistance of the microencapsulated probiotic and chrysin was tested (Table 4), in order to verify their application in foods that undergo previous processing as performed. Regarding the viability of probiotics, we saw that the most harmful heat treatment was 63 °C for 30 minutes. Therefore, if applied to food in the future, the most appropriate treatment would be 72 °C for 15 seconds. However, we observed that, even in the 63°C treatment, cell viability remained at interesting values in all treatments. These results were still similar to those found by de Deus et al. (2023), who obtained a result of approximately 4 CFU g^{-1} of *L. plantarum* after identical heat treatment conditions. As for chrysin levels, especially for T4 and T6, the most harmful treatment was 63 °C for 30 min. This suggests that, for possible future applications in food products, the most appropriate heat treatment would be 72 °C for 15 seconds. This is probably the first work that demonstrates the behavior of microencapsuled chrysin in the face of pasteurization heat treatment, and points to the possibility of applying of capsules of this flavonoid in pasteurized products. Together, the data obtained from heat treatment are interesting, as they indicate that the capsules produced in this work could be incorporated into foods subjected to pasteurization heat treatment.

Regarding *in vitro* gastrointestinal digestion, it was observed that in the gastric phase the microorganism count was zero, and chrysin levels were also low, when compared to the intestinal phases (Table 5). This result demonstrates the efficiency of the capsules in protecting the encapsulated agents from adverse gastric conditions. As the capsule rupture occurs at a pH close to neutrality, it is believed that during the gastric phase, there was no or very little rupture, which caused cell viability and chrysin levels to be so low. The fact that the capsules had not ruptured caused low levels in the chrysin analysis because the capsule did

not allow chrysin to migrate to the organic phase in the QuEChERS extraction step. In the intestinal phases, the microorganism count and chrysin content were much higher than in the gastric phase. Furthermore, regarding the viability of the probiotic, we observed that the results found in the ileum were superior to those found in the duodenum. These results demonstrates that the capsules were efficient in releasing the encapsulated agents in a controlled manner in the intestine. Recent work has demonstrated that the gastric phase is capable of decreasing chrysin levels (Tian et al., 2021). In this sense, our result shows that encapsulation was effective in inhibiting gastric degradation of chrysin.

Regarding the results found for free bacteria, we observed equal or higher values compared to other treatments, both in heat treatment and in gastrointestinal digestion. This event can be explained in parts, as the encapsulated treatments underwent mechanical stress from the encapsulation process, situation that did not reach the free cells (de Deus et al., 2023). In addition, our objective when encapsulating a probiotic is its application in nutraceutical or food formulations. In this case, the use of free bacteria would be greatly hampered, due to all the thermal and mechanical processes to which the product is subjected, in addition to the problem of the interaction of the probiotic with the product (Marins et al., 2022). It is also worth noting that resistance to the gastrointestinal tract is a requirement for a microorganism to be considered a probiotic (FAO, 2002), which makes the result observed for free bacteria in this analysis expected.

3.4 Viability of *L. Plantarum* and chrysin content on storage

Aiming to evaluate the stability of probiotics and chrysin during storage, the capsules were evaluated every two weeks for 120 days, at 3 different temperatures. As for cell viability, we observed that this parameter was not substantially affected by different chrysin concentrations (Figure 2). This result is very interesting, as it demonstrates that chrysin did not exert chronic toxicity on the probiotics during storage. In a similar work, de Deus et al. (2023) demonstrated that the addition of the extract from red beet stem (*Beta vulgaris* L.) to

capsules obtained by spray dryer and containing *L. plantarum* decreased the viability of probiotics from day 30 onwards, especially at a temperature of 25 °C. As for temperature, it was found that the best temperature was -18°C, followed by temperatures of 8°C and 25°C (Figure 3). The temperature of -18°C showed a greater number of viable cells (9.0 CFU mL^{-1}) and for a longer period, showing good stability until day 105. From day 105 onwards, all treatments in all temperatures had substantial decreases. At a temperature of 8 °C, cell viability began to decrease from day 75, reaching day 105 with an average value of 8.0 CFU mL^{-1}. As for the temperature of 25 °C, cell viability has already had a substantial decrease since day 15, reaching day 105 with an average of 6.0 CFU mL^{-1}. These results are similar to those found by Raddatz et al. (2022a), that showed the viability and stability evaluation of *Lactobacillus casei* LC03 co-encapsulated with red onion (*Allium cepa* L.) peel extract. In Raddatz work, the temperature that presented the best viability was also -18°C, with an average value of 8 CFU mL^{-1} on day 90. At temperatures of 8° and 25°C, viability on day 90 was 6.6 CFU mL^{-1} and 5.2 CFU mL^{-1}, respectively. The behavior of free bacteria (T1) throughout storage was also interesting. In general, we observed that in the 3 temperature conditions, T1 presented equivalent or superior results to the other treatments (Figure 2). Unlike our result, Neuenfeldt et al. (2022) demonstrated that free *Lactobacillus rhamnosus* showed a drastic decrease in cell viability from day 30 onwards, reaching almost zero CFU mL^{-1} on day 120, especially at temperatures of 8 °C and 25 °C. Similarly, Raddatz et al. (2022a) showed a drastic decrease in the viability of free *Lactobacillus casei LC03* from day 15 onwards, reaching around 1.4 CFU mL^{-1} on day 90, especially at a temperature of 8 °C. However, similar to our work, de Deus et al. (2023) showed that the viability of free *L. plantarum* was equivalent or superior to the probiotic encapsulated by spray dryer, in the 120 days of experiment and at the 3 temperatures evaluated. Therefore, we understand that this good stability may be a peculiarity of *L. plantarum*. Furthermore, our objective when encapsulating a probiotic is its application in nutraceutical or food formulations. In this case, the use of free bacteria would be greatly hampered, due to all the thermal and mechanical

processes to which the product is subjected, in addition to the problem of the interaction of the probiotic with the product during storage (Marins et al., 2022).

As for chrysin levels, they were not substantially altered by chrysin concentration (Figure 4) as well as storage temperature (Figure 5). This is possibly the first work to evaluate the stability of the flavonoid chrysin for 120 days, and shows the feasibility of applying this flavonoid in products that are subjected to long periods of storage. In treatment T3, at 8 °C, a significant decrease was observed comparing day 1 to day 120, and the same occurred at a temperature of -18 °C. However, for all other cases there was no decrease in chrysin levels within 120 days. As for temperature, the most suitable temperature for T3 at the end of storage was 25 °C. For the other treatments, the most appropriate was -18 °C. Although some discrepancies can be observed at specific points on the lines, a clear effect of concentration or temperature is not observed. These discrepancies can be explained by the limitations of the flavonoid quantification method, or even due to an inhomogeneous distribution of the capsules at the time of transfer to the storage tubes.

These data demonstrate that encapsulation was effective in providing good stability to microorganisms and the flavonoid chrysin, being promising for the addition of these materials to food or nutraceutical products.

4 CONCLUSION

This work demonstrated the encapsulation of *L. plantarum* by external ionic gelation, obtaining excellent EE and providing resistance to the gastrointestinal tract and stability during storage for the probiotic. We also observed that the greatest cell viability and for a longer period was obtained when stored at -18 °C.

In an unprecedented way, we demonstrated the co-encapsulation of *L. plantarum* with the flavonoid chrysin. We verified that the concentrations of chrysin used did not pose acute or chronic toxicity to the probiotics, and that encapsulation favored the resistance of chrysin to the gastric phase of digestion and its stability during storage.

Finally, we suggest that the possible application of capsules produced in food or nutraceutical formulations could be investigated, and that the bioactivity of capsules should be tested.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest.

CONSENT TO PARTICIPATE

Consent to Participate All authors have given their full consent to participate.

CONSENT FOR PUBLICATION

All authors have given their full consent for publication.

AUTHORS' CONTRIBUTIONS

C.B.F.: conceptualization, methodology, investigation, validation, formal analysis, writing-original draft preparation; M.K.: conceptualization, methodology, investigation, validation, formal analysis, writing-original draft preparation; J.M.F., C. de D., V.S.P., C.P.R., U. da S.R., V.C.B., M.P., and C.R. de M.: review and editing, final draft supervision and monitoring. All authors read and approved the final manuscript.

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GRAPHICAL ABSTRACT

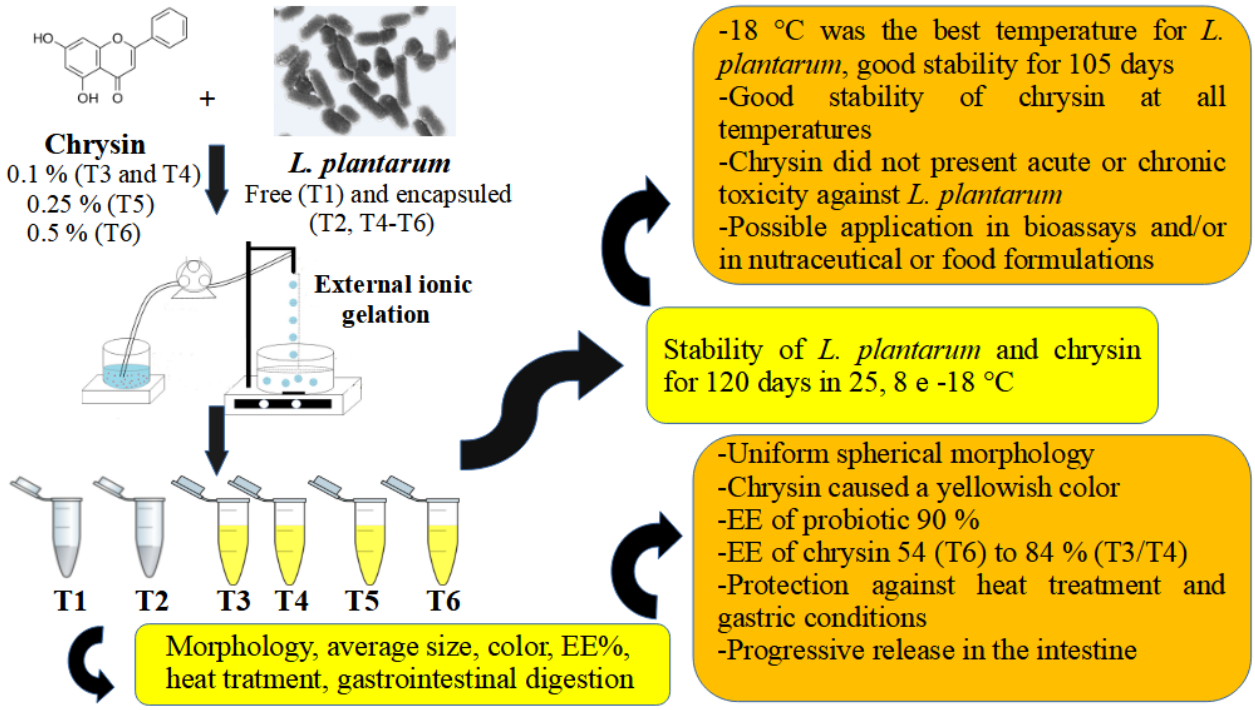
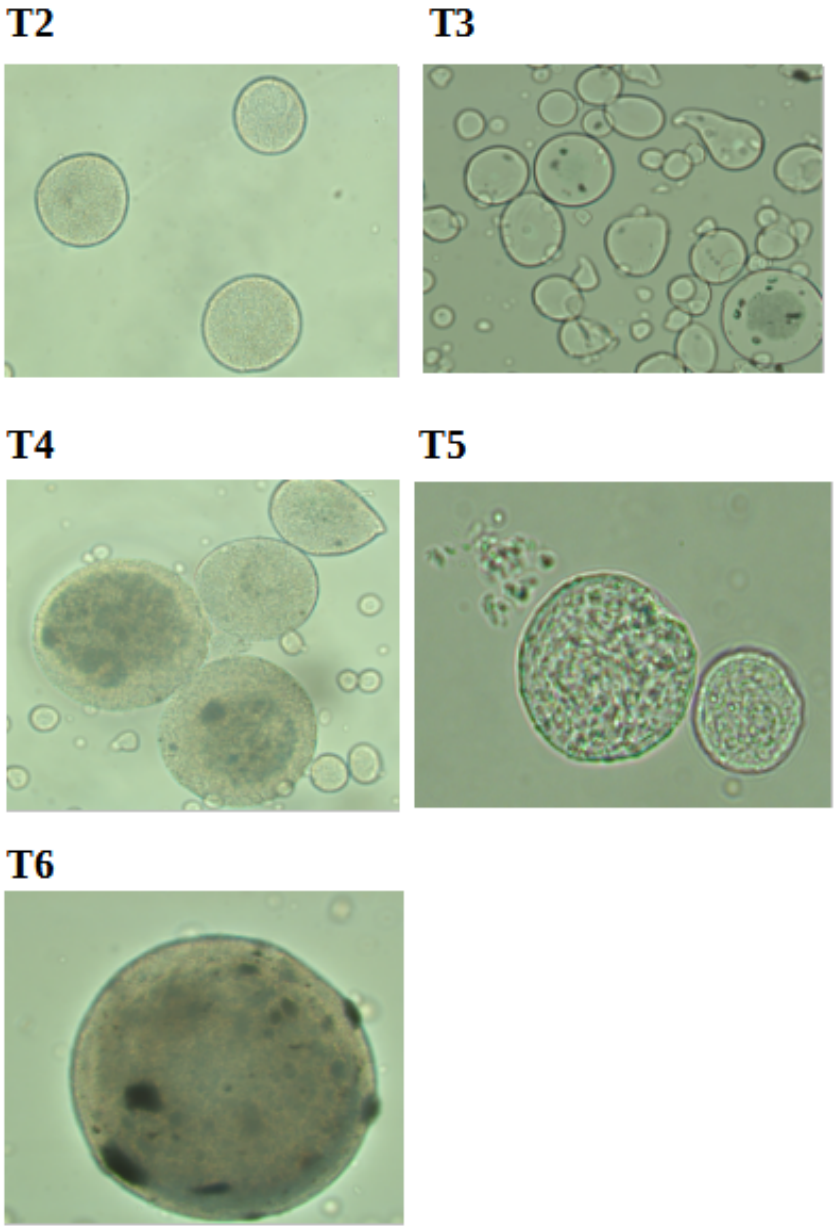
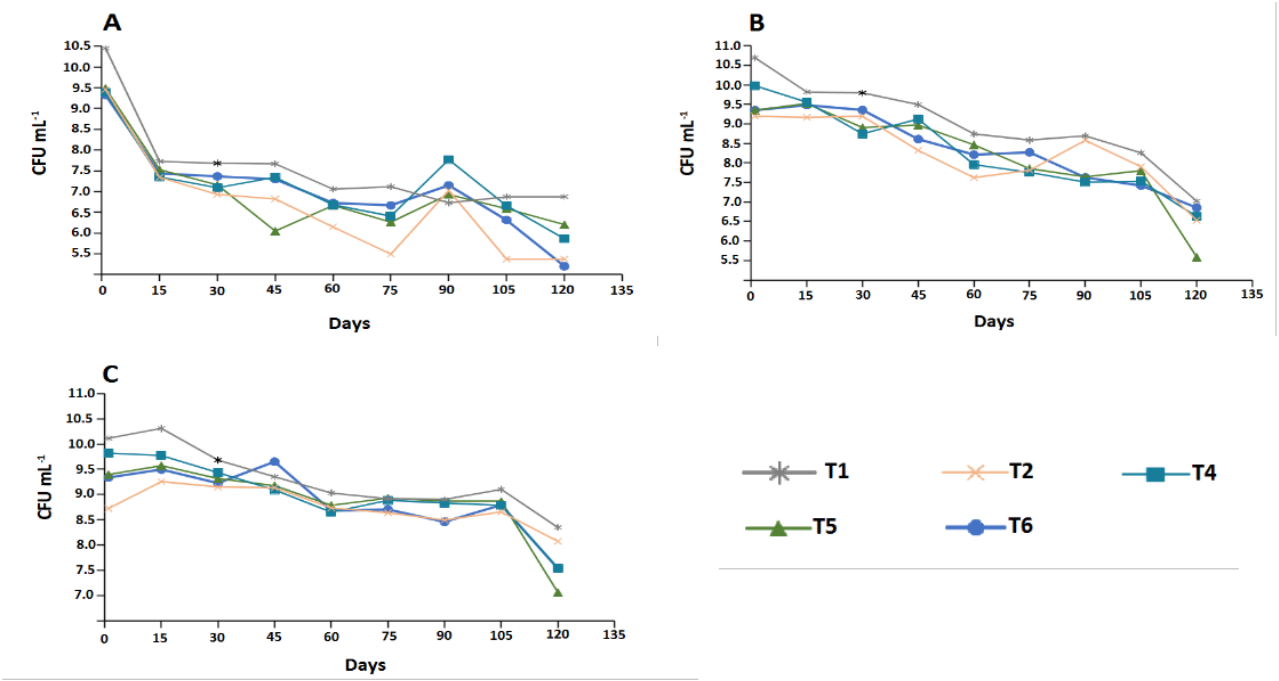


Figure 1.Optical microscopy of microcapsules. The morphology of the microcapsules was observed under a scanning electron microscope. All images are presented at 20x zoom.



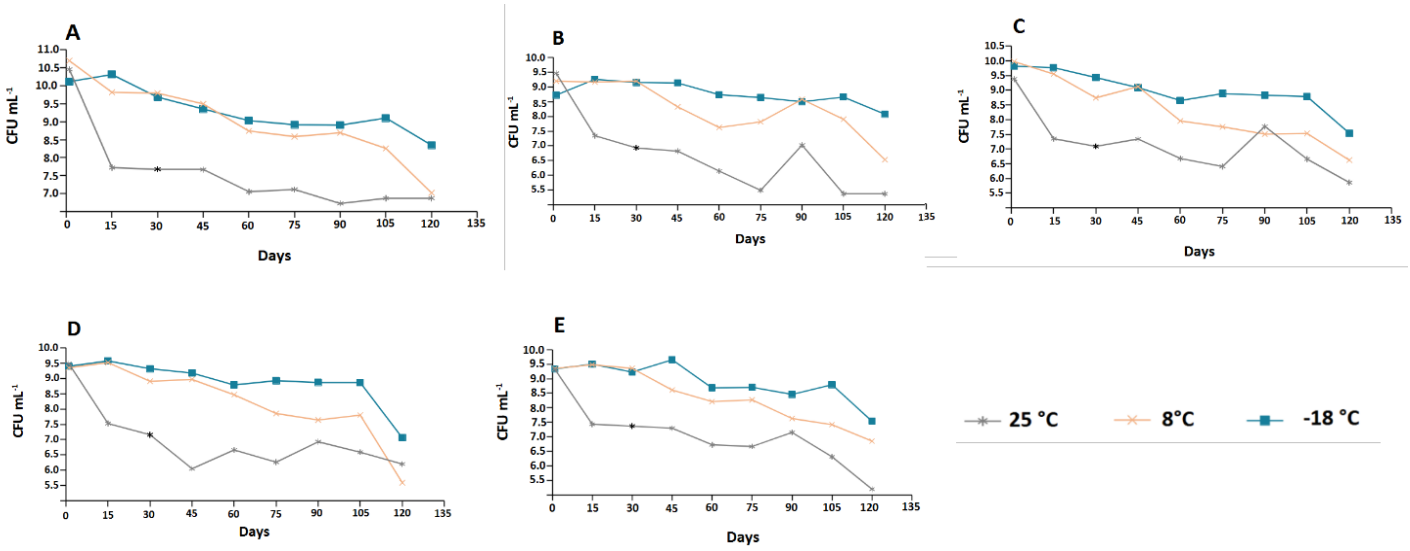
T2 = microcapsules with *L. plantarum* without chrysin; T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

Figure 2. Probiotic viability in T1, T2, T4, T5 and T6 treatments during 120 days of storage at 25 °C (A), 8 °C (B) and -18 °C (C). Comparison between treatments.



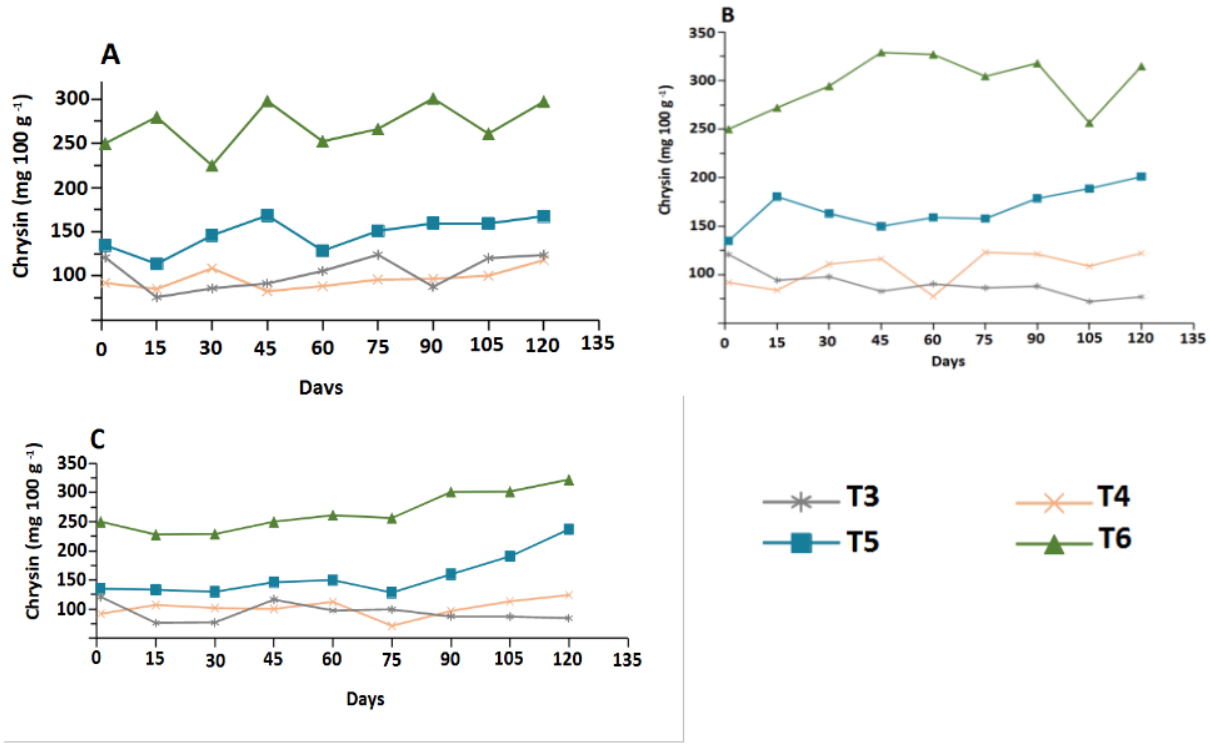
T1 = cell culture in free form of *L. plantarum;* T2 = microcapsules with *L. plantarum* without chrysin; T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

Figure 3. Probiotic viability in T1 (A), T2 (B), T4 (C), T5 (D) and T6 (E) treatments during 120 days of storage at different temperatures (25, 8 and -18°C). Comparison between temperatures.



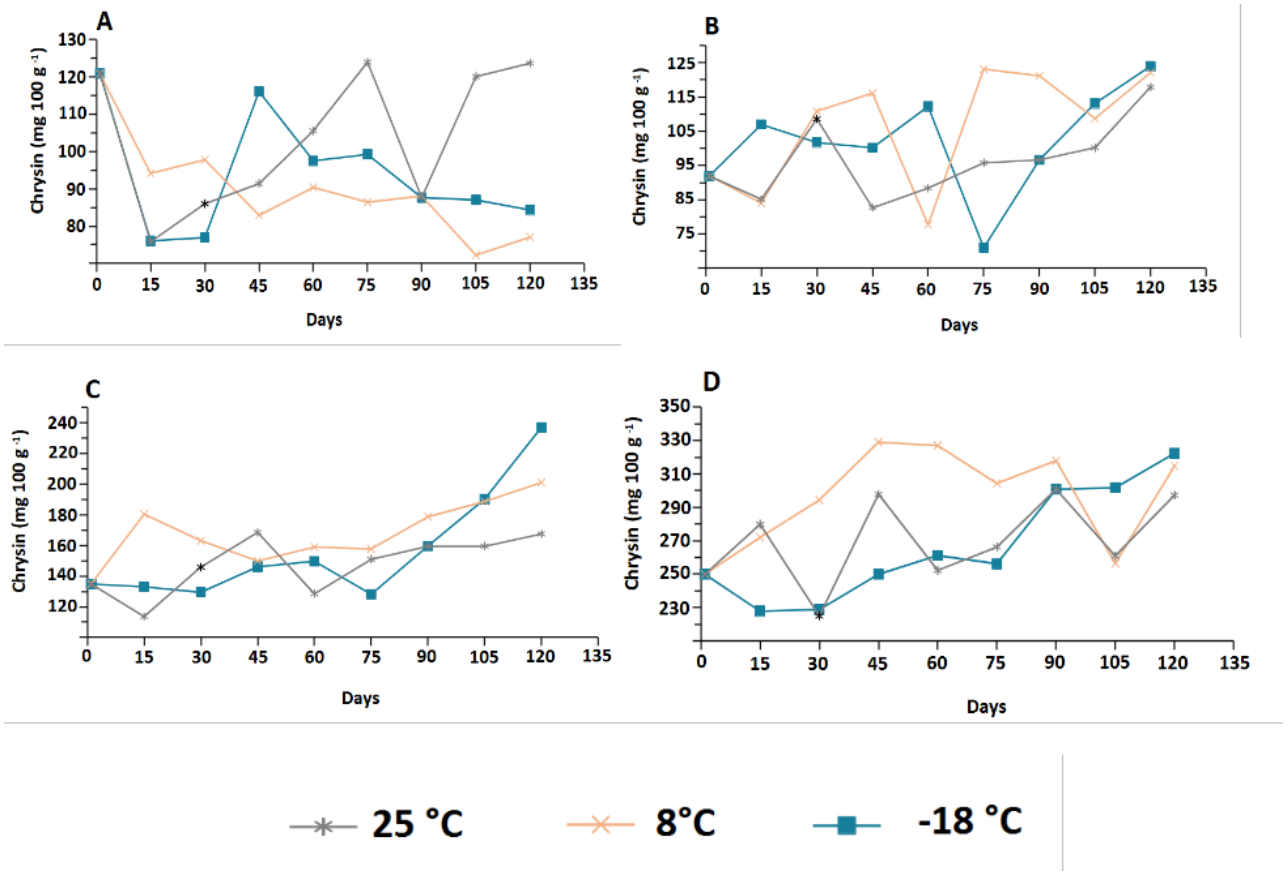
T1 = cell culture in free form of *L. plantarum;* T2 = microcapsules with *L. plantarum* without chrysin; T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

Figure 4. Chrysin content in T3, T4, T5 and T6 treatments during 120 days of storage at 25 °C (A), 8 °C (B) and -18 °C (C). Comparison between treatments.



T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

Figure 5. Chrysin content in T3 (A), T4 (B), T5 (C) and T6 (D) treatments during 120 days of storage at different temperatures (25, 8 and -18°C). Comparison between temperatures.



T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

Table 1. Composition of treatments.

Reagents	T1	T2	T3	T4	T5	T6
Sodium alginate	-	2 g	2 g	2 g	2 g	2 g
Probiotic culture	1 g	1 g	-	1 g	1 g	1 g
Chrysin	-	-	0.1 g	0.1 g	0.25 g	0.5 g
Water	99 g	97 g	97.9 g	96.9 g	96.75 g	96.5 g

T1 = cell culture in free form of *L. plantarum;* T2 = microcapsules with *L. plantarum* without chrysin; T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

Table 2. Average size and color parameters of microcapsules.

Parameter/ Treatment	**T2**	**T3**	**T4**	**T5**	**T6**
Average size (μm)	149.6±0.57	131.33±0.57^{ef}	138.33±1.52	150.33±14.04	154.66±0.57
L*	39.66±2.51	32.01±3.38^{f}	35.59±3.75^{f}	37.26±0.15^{f}	46.01±3.75
a*	-0.61±0.04^{cdef}	-1.61±0.17^{ef}	-1.79±0.19^{ef}	-2.57±0.04^{f}	-3.71±0.24
b*	5.57±0.19^{cdef}	7.88±0.56^{ef}	8.75±0.63^{ef}	10.9±0.07^{f}	15.10±1.05

T2 = microcapsules with *L. plantarum* without chrysin; T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

^{c}denotes significant difference in relation to T3 (comparison within lines); ^{d}denotes significant difference in relation to T4 (comparison within lines); ^{e}denotes significant difference in relation to T5 (comparison within lines); ^{f}denotes significant difference in relation to T6 (comparison within lines).

Table 3. Probiotic viability, chrysin content and encapsulation efficiency.

Parameter/ Treatment	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Probiotic viability (CFU mL^{-1})	10.45 ± 0.07^{bdef}	9.45 ± 0.04^{f}	-	9.38 ± 0.04	9.48 ± 0.01^{f}	9.32 ± 0.02
Chrysin content (mg 100 g^{-1})	-	-	94.12 ± 6.40	83.99 ± 5.91	134.78 ± 0.1	249.41 ± 1.17
EE of probiotic (%)	-	90.50 ± 0.43^{f}	-	89.77 ± 0.47^{e}	90.75 ± 0.19^{f}	89.19 ± 0.27
EE of chrysin (%)	-	-	94.12 ± 6.40^{ef}	83.99 ± 5.91^{f}	72.15 ± 5.97^{f}	54.40 ± 6.96

T1 = cell culture in free form of *L. plantarum;* T2 = microcapsules with *L. plantarum* without chrysin; T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

[b]denotes significant difference in relation to T2 (comparison within lines); [d]denotes significant difference in relation to T4 (comparison within lines); [e]denotes significant difference in relation to T5 (comparison within lines); [f]denotes significant difference in relation to T6 (comparison within lines).

Table 4. Resistance to heat treatment.

Parameter/ Treatment	T1	T2	T3	T4	T5	T6
Probiotic viability (CFU mL^{-1}) 72 ºC, 15 sec	6.35±0.04^{ef}	6.34±0.35^{ef}	-	5.88±0.07^{f}	5.32±0.61	4.95±0.14
Probiotic viability (CFU mL^{-1}) 63 ºC, 30 min	6.34±0.06^{bdef}	4.98±0.85^{e}*	-	4.48±0.24*	3.66±0.25*	4.54±0.05
Chrysin content (mg 100 g^{-1}) 72 ºC, 15 sec	-	-	60.40±13.65	112.70±0.1	99.94±7.88	270.53±2.44
Chrysin content (mg 100 g^{-1}) 63 ºC, 30 min	-	-	78.66±0.36	66.72±0.28*	117.89±10.52	163.56±14.19*

T1 = cell culture in free form of *L. plantarum;* T2 = microcapsules with *L. plantarum* without chrysin; T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

^{b}denotes significant difference in relation to T2 (comparison within lines); ^{d}denotes significant difference in relation to T4 (comparison within lines); ^{e}denotes significant difference in relation to T5 (comparison within lines); ^{f}denotes significant difference in relation to T6 (comparison within lines); *denotes significant difference in relation to the same treatment subjected to 72 °C, 15 sec (comparison within columns).

Table 5. *In vitro* gastrointestinal digestion.

Parameter	Probiotic viability (CFU mL^{-1})			Chrysin content (mg $100\ g^{-1}$)		
Digestive phase/ Treatment	**Stomach**	**Duodenum**	**Ileum**	**Stomach**	**Duodenum**	**Ileum**
T1	ND	$6.34\pm0.06^{bdef*}$	6.35 ± 0.04^{ef}	-	-	-
T2	ND	$4.98\pm0.85^{e*}$	$6.34\pm0.35^{ef*\#}$	-	-	-
T3	-	-	-	24.76±5.79	76.47±0.52*	73.78±5.67*
T4	ND	4.48±0.24*	5.88±0.07f*#	25.09±0.54	72.64±2.20*	72.80±2.21*
T5	ND	3.66±0.25*	5.32±0.61*#	76.18±8.02	319.22±1.42*	320.06±1.33*
T6	ND	4.54±0.05*	4.95±0.14*#	106.19±11.74	456.36±3.90*	457.57±3.91*

ND means not detected.

T1 = cell culture in free form of *L. plantarum;* T2 = microcapsules with *L. plantarum* without chrysin; T3 = microcapsules with chrysin 0.1 % without *L. plantarum;* T4 = microcapsules with *L. plantarum* and chrysin 0.1 %; T5 = microcapsules with *L. plantarum* and chrysin 0.25 %; T6 = microcapsules with *L. plantarum* and chrysin 0.5 %.

[b]denotes significant difference in relation to T2 (comparison within columns); [d]denotes significant difference in relation to T4 (comparison within columns); [e]denotes significant difference in relation to T5 (comparison within columns); [f]denotes significant difference in relation to T6 (comparison within columns); *denotes significant difference in relation to the gastric phase (comparison within lines); [#]denotes significant difference in relation to the duodenal phase (comparison within lines).

5 DISCUSSÃO INTEGRADA

Neste trabalho, objetivou-se desenvolver e validar uma metodologia para análise de crisina em espécies de *Passiflora,* e avaliar a viabilidade da co-encapsulação de crisina e micro-organismos probióticos.

O desenvolvimento de um método QuEChERS modificado seguido de análise HPLC-DAD foi bem sucedido, mostrando excelentes valores de LOQ e bons resultados em todos os parâmetros de validação. Quanto aos métodos já utilizados para análise de crisina por cromatografia em matrizes vegetais (GHARARI et al., 2020), o método apresentou rapidez e menor potencial de danos ao sistema cromatográfico, em decorrência da etapa de limpeza. Comparado às aplicações do QuEChERS para análise de fenólicos (ROTTA et al., 2019; NICÁCIO et al., 2021), o método destaca-se pelo uso de um sorvente mais acessível e de menor custo, o SiO_2, além de versatilidade para aplicação em folha, polpa e casca. Além disso, o método destaca-se pela possibilidade de ser aplicado a muitos laboratórios de análises de HPLC, uma vez que em muitos casos o detector DAD é a única opção disponível em decorrência do seu baixo custo.

O método desenvolvido foi aplicado para avaliação dos níveis de crisina nas folhas, polpa verde e madura, e casca verde e madura de *Passiflora caerulea*, uma fruta abundante e subutilizada na América do Sul. Junto a isso, foi avaliado o potencial antioxidante de cada uma dessas partes da fruta. O melhor potencial antioxidante e melhores níveis de crisina foram observados nas folhas e na casca verde. Provavelmente, a casca verde destacou-se em relação à casca madura pelo fato de que durante o processo de amadurecimento os compostos fenólicos podem sofrer processos de oxidação e polimerização, tendo suas concentrações diminuídas (MURATA et al., 1995). Subsequentemente, as folhas e casca verde foram submetidas à simulação da digestão gastrointestinal *in vitro*, buscando observar o efeito da digestão nos níveis de crisina e na bioatividade destas matrizes. Verificamos que a digestão *in vitro* não foi capaz de alterar os níveis de crisina nas matrizes avaliadas. Este resultado é muito relevante, uma vez que Ting et al. (2021) demonstraram que as condições gástricas degradam e diminuem substancialmente o conteúdo de crisina. No experimento desta tese, acredita-se que o fato de a crisina estar inserida em uma matriz vegetal possa ter contribuído em sua proteção frente às condições gástricas, já que no trabalho de Ting et al. (2021) a crisina foi avaliada em sua forma pura. Já para parâmetros de potencial antioxidante, estes sofreram alterações decorrentes da digestão, mas que não parecem estar atreladas a alterações no conteúdo de crisina, uma vez que este não foi alterado e que os resultados das análises de correlação entre os níveis de crisina e potencial antioxidante pós-digestão não foram significativos.

Quanto à viabilidade da co-encapsulação de crisina e micro-organismos probióticos, realizou-se inicialmente uma revisão catalogando e descrevendo as metodologias já disponibilizadas na literatura para a encapsulação deste flavonoide, e seu respectivo potencial biológico (BORGES FILHO et al., 2024). Em linhas gerais, verificou-se que os métodos usados para encapsulação da crisina utilizavam reagentes com elevada toxicidade, como metanol, diclorometano e dimetilsulfóxido. Ainda, observou-se que a co-encapsulação de crisina e probióticos ainda não havia sido estudada.

Neste sentido, buscou-se uma metodologia verde para a encapsulação de crisina e estudou-se a viabilidade da sua encapsulação com *L. plantarum*, um probiótico conhecidamente antidepressivo, assim como a crisina. As cápsulas foram produzidas por gelificação iônica externa, uma técnica verde, de baixo custo, e ainda não usada para este flavonoide.

A união de substâncias bioativas e probióticos por meio da co-encapsulação é uma importante tendência científica que tem sido alvo de vários estudos, buscando avaliar possíveis benefícios das substâncias bioativas na atividade e estabilidade dos probióticos, ou ainda a ação biológica sinérgica de ambos, gerando cápsulas polifuncionais (DE DEUS et al., 2023; RADDATZ et al., 2022a, 2022b; NEUENFELDT et al., 2022).

Reforçando a versatilidade do método analítico desenvolvido nesta tese, o mesmo foi aplicado com êxito na avaliação dos níveis de crisina nas microcápsulas desenvolvidas, sendo necessárias poucas adaptações, mesmo se tratando de uma matriz substancialmente diferente. A encapsulação da crisina por gelificação iônica foi bem sucedida, apresentando excelente eficiência de encapsulação, sobretudo para a menor concentração, a de 0,1 % (m/m). Ter uma boa eficiência de encapsulação para a crisina nesta técnica é algo muito positivo, já que se trata de uma técnica verde, de baixo custo, e fácil execução. A encapsulação também foi eficiente para o probiótico, e verificou-se que a crisina não exerceu nenhuma atividade antimicrobiana aguda nas concentrações estudadas. Este resultado é muito relevante e positivo, uma vez que a crisina é conhecida pelo seu efeito antimicrobiano (ADESINA et al., 2024; KHASTEBAND et al., 2024). Mas, nas concentrações estudadas e com o probiótico avaliado, este efeito não ocorreu.

Além da avaliação da eficiência de encapsulação, as cápsulas foram inicialmente submetidas a testes de resistência ao tratamento térmico e simulação do trato gastrointestinal. Verificamos que a encapsulação foi eficaz para proteger os probióticos e a crisina contra os efeitos deletérios das altas temperaturas do tratamento térmico, apontando para uma potencial aplicação em produtos que são submetidos a estes métodos de conservação. Nas avaliações da simulação do trato digestivo, a encapsulação foi efetiva na proteção contra as condições

adversas da fase gástrica, e promoveu uma liberação controlada dos agentes encapsulados ao longo da fase intestinal. A proteção contra a degradação gástrica da crisina é um resultado muito positivo, já que na sua forma livre este flavonoide é substancialmente degradado nesta fase digestiva (TING et al., 2021). Quanto aos probióticos, além da proteção contra as condições gástricas, observou-se ainda a maior liberação de células no íleo em comparação com o duodeno, demonstrando a liberação controlada exercida pelo sistema de encapsulação (DE DEUS et al., 2023).

A estabilidade das microcápsulas foi estudada quinzenalmente por 120 dias, em 3 condições diferentes de armazenamento (25, 8 e -18 °C). Observou-se que a crisina não influenciou negativamente na estabilidade dos probióticos durante todo o período de testes. Em outro trabalho com encapsulação de *L. plantarum*, de Deus et al. (2023) demonstraram que a adição de extrato do caule de beterraba vermelha (*Beta vulgaris* L.) diminuiu a viabilidade do probiótico a partir do dia 30, sobretudo na temperatura de 25°C. Deste modo, o resultado desta tese é interessante, pois demonstra que a crisina não exerceu efeito antimicrobiano crônico ao longo de todo o período de armazenamento estudado e em nenhuma das temperaturas.

A melhor temperatura de armazenamento para os probióticos foi a de -18 °C, apresentando excelente viabilidade até o dia 105. Este resultado é semelhante ao já observado por outros autores que avaliaram as mesmas condições de armazenamento para outros probióticos (RADDATZ et al., 2022a; NEUENFELDT et al., 2022). Já para a estabilidade da crisina, verificou-se que esta não foi substancialmente influenciada pela concentração do flavonoide, mostrando também excelente estabilidade em todas as temperaturas de armazenamento estudadas. Embora algumas alterações possam ser observadas ao longo das retas do período de armazenamento, não se pode observar um claro efeito da temperatura na estabilidade do flavonoide. Estas alterações pontuais podem ser decorrentes de eventuais limitações do detector utilizado na análise, ou ainda de uma distribuição não uniforme das cápsulas entre os tubos de armazenamento.

Em linhas gerais, demonstrou-se a encapsulação de *L. plantarum* por gelificação iônica externa, obtendo excelente eficiência de encapsulação e proporcionando resistência e liberação progressiva no trato gastrointestinal, bem como estabilidade durante o armazenamento, especialmente na temperatura de -18 °C. Ainda, de forma inédita, mostrou-se a co-encapsulação de *L. plantarum* com o flavonoide crisina. Verificou-se que as concentrações de crisina utilizadas não apresentaram toxicidade aos probióticos e que a encapsulação favoreceu a resistência da crisina à fase gástrica da digestão e sua estabilidade durante o armazenamento.

6 CONCLUSÕES

-Demonstrou-se, pela primeira vez, um método QuEChERS modificado seguido de análise HPLC-DAD que pode ser aplicado para análise de crisina em espécies de *Passiflora,* apresentando excelente comportamento nos parâmetros de validação, e sendo mais rápido que os métodos já descritos na literatura, além de menos prejudicial ao sistema cromatográfico;

-Comparado a outras aplicações do QuEChERS para análise de fenólicos, o presente estudo utilizou um sorvente mais acessível e de menor custo, o SiO_2;

-O método QuEChERS modificado seguido de análise HPLC-DAD também apresentou versatilidade, podendo ser aplicado a três diferentes matrizes vegetais (folha, polpa e casca), e sendo usado, com poucas adaptações, nas dosagens de crisina nas microcápsulas produzidas;

-As folhas de *Passiflora caerulea* são ótimas fontes de crisina e possuem bom potencial antioxidante, seguidas pela casca verde;

-A bioacessibilidade do flavonoide crisina presente nas folhas e casca verde de *Passiflora caerulea* não é alterada pela digestão gastrointestinal *in vitro;*

-A co-encapsulação de *L. plantarum* e crisina por gelificação iônica externa mostrou-se viável, apresentando excelente eficiência de encapsulação e com a crisina não exercendo toxicidade aguda ou crônica para com os probióticos;

-A co-encapsulação foi benéfica para a resistência da crisina e dos probióticos à fase gástrica da digestão, e para a liberação progressiva dos probióticos ao longo do trato intestinal;

-A melhor temperatura para armazenamento dos probióticos foi de -18 °C, mantendo excelente viabilidade até o dia 105, enquanto a crisina apresentou boa estabilidade em todas as temperaturas e períodos estudados.

7 PERSPECTIVAS

-Estudar a possibilidade da aplicação das folhas e casca verde de *Passiflora caerulea* como fonte de crisina no desenvolvimento de microcápsulas com probióticos, explorando metodologias verdes de extração, como uso de ultrassom, micro-ondas e biosolventes;

-Aplicar folhas e casca verde de *Passiflora caerulea* como fonte de crisina em formulações nutracêuticas ou alimentares;

-Aplicar as microcápsulas desenvolvidas em formulações nutracêuticas ou alimentares;

-Verificar o efeito de microcápsulas contendo crisina e probióticos em modelos experimentais de depressão.

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