GLÁUCIA VALÉRIA FONSECA LEONEL

CINÉTICA E TERMODINÂMICA DE FORMAÇÃO DE COMPLEXO ENTRE ISOLADO PROTEICO DE BARU E POLIFENÓIS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Magister Scientiae*.

Orientadora: Ana Clarissa dos Santos Pires

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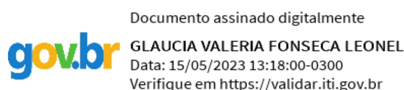

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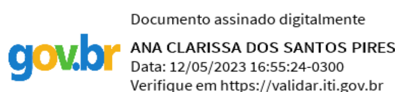

Ana Clarissa dos Santos Pires
Orientadora

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RESUMO

LEONEL, Gláucia Valéria Fonseca, M.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Cinética e termodinâmica de formação de complexo entre isolado proteico de Baru e polifenóis.** Orientadora: Ana Clarissa dos Santos Pires. Coorientadores: Luis Henrique Mendes da Silva e Jaqueline de Paula Rezende.

Os flavonoides são polifenóis amplamente estudados pelo desempenho de diversas atividades biológicas, que incluem atividade antioxidante, anti-inflamatória, redução da incidência de doenças cardiovasculares e câncer. A naringenina (NG) e a naringina (NR) são flavonoides encontrados em frutas cítricas, que apresentam propriedades benéficas à saúde humana. No entanto, o sabor amargo, a baixa solubilidade e biodisponibilidade dessas moléculas limitam o desempenho de suas bioatividades e inserção em matrizes alimentícias. Proteínas são candidatas estratégicas para veicular compostos bioativos, minimizando as limitações de uso desses compostos. Proteínas de origem animal vêm sendo amplamente estudadas para este fim. No entanto, a mudança de hábitos alimentares como a adoção de dietas restritivas (veganos e vegetarianos) tem contribuído para uma nova demanda cientifica, como a exploração de proteínas vegetais como carreadoras de moléculas bioativas. Assim, a formação de complexos entre as proteínas extraídas da amêndoa do Baru (BP) e NG e NR foram caracterizadas termodinamicamente e cineticamente pela técnica de ressonância plasmônica de superfície. Os valores da constante de interação (K_b) reduziram com o aumento da temperatura para ambos os complexos estudados. No entanto, o número de complexos BP-NR^0 formados a 289,15 K foram 17 vezes maiores que BP-NG^0, indicando que a presença do grupo ramnoglicosideo da NR contribuiu para maior afinidade de ligação com BP. A formação de BP-NR^0 foi exotérmica (ΔH^0= -78,54 kJ.mol^{-1}) e ocorreu predominantemente através da energia liberada das novas interações formadas (ΔH^0_{int}<0). A pequena variação da entropia com a temperatura (-50,83 a -52,95 kJ.mol^{-1}) indica que o processo de ligação não requer extensas variações conformacionais. Em temperaturas superiores a 297,15 K, a formação do complexo BP-NG^0 foi entropicamente dirigida. O aumento da entropia do sistema foi compensado pela redução da entalpia, indicando que a formação de BP-NG^0 ocorre com o fenômeno de compensação entálpica-entrópica. Valores negativos de ΔG°

indicaram que a formação de complexos BP-flavonoides foi favorecida no equilíbrio termodinâmico em relação às moléculas livres. Apesar da massa molar da NR ser maior que a da NG, a taxa de associação da NR à BP(k_a = 3,675 a 1,269 x 10^4 $M^{-1}.s^{-1}$) foi de 6 a 12 vezes mais rápida comparada à NG à BP (k_a = 0,291 a 0,196 x 10^4 $M^{-1}.s^{-1}$). A formação de complexos intermediários a partir da associação da proteína BP com NR ($\Delta G_a^{\ddagger}$ = 45,47 a 50,12 $kJ.mol^{-1}$) ocorreu mais rápido do que com NG ($\Delta G_a^{\ddagger}$ = 51,57 a 54,80 $kJ.mol^{-1}$), indicando que a presença do grupo glicosídeo acelera o processo de formação do complexo intermediário obtido a partir da associação de moléculas livres. A caracterização cinética e termodinâmica do complexo BP-flavonoide fornece informações úteis para entender e modular as propriedades físico-químicas dessas nanoestruturas, a fim de otimizar sua inserção em matrizes alimentares.

Palavras-chave: Proteína vegetal não convencional. Baru. Naringenina. Naringina. Ressonância plasmônica de superfície.

ABSTRACT

LEONEL, Gláucia Valéria Fonseca, M.Sc., Universidade Federal de Viçosa, February 2023. **Kinetics and thermodynamics of complex formation between Baru protein isolate and polyphenols.** Adviser: Ana Clarissa dos Santos Pires. Co-advisers: Luis Henrique Mendes da Silva and Jaqueline de Paula Rezende.

Flavonoids are polyphenols widely studied for their performance of various biological activities, including antioxidant and anti-inflammatory activity, and reduction of the incidence of cardiovascular diseases and cancer. Naringenin (NG) and naringin (NR) are flavonoids found in citrus fruits, which have beneficial properties for human health. However, the bitter taste, low solubility, and bioavailability of these molecules limit their bioactivity and incorporation into food matrices. Proteins are strategic candidates for delivering bioactive compounds, minimizing the limitations of using these compounds. Proteins of animal origin have been widely studied for this purpose. However, changes in dietary habits such as adopting restrictive diets (vegan and vegetarian) have contributed to a new scientific demand, such as exploring plant proteins as carriers of bioactive molecules. Thus, the formation of complexes between proteins extracted from the Baru almond (BP) and NG and NR was thermodynamically and kinetically characterized by surface plasmon resonance technique. The interaction constant values (K_b) decreased with increasing temperature for both studied complexes. However, the number of $[BP\text{-}NR]^0$ complexes formed at 289.15 K was 17 times higher than $[BP\text{-}NG]^0$, indicating that the presence of the ramnoglycoside group of NR contributed to a greater binding affinity with BP. The formation of $[BP\text{-}NR]^0$ was exothermic (ΔH^0= -78,54 kJ.mol^{-1}) and occurred predominantly through the energy released from the new intermolecular interactions formed (ΔH^0_{int}<0). The small entropy change (50,83 – 52,95 kJ.mol^{-1}) indicates that the binding process does not require extensive conformational changes. At temperatures higher than 297.15 K, the formation of the $[BP\text{-}NG]^0$ complex was entropically driven. The increase in system entropy was compensated by the reduction in enthalpy, indicating that the formation of $BP\text{-}NG^0$ occurs with the enthalpy-entropy compensation phenomenon. Negative ΔG° values indicated that the formation of BP-flavonoid complexes was favored in thermodynamic equilibrium compared to free molecules. Although the molar mass of NR is higher than that of

NG, the association rate of NR with BP (k_a = 3.675 to 1.269 × 104 $M^{-1}.s^{-1}$) was 6 to 12 times faster compared to NG with BP (k_a = 0.291 to 0.196 × 104 $M^{-1}.s^{-1}$). The formation of intermediate complexes from the association of the BP protein with NR ($\Delta G_a^{\ddagger}$ = 45,47 to 50,12 $kJ.mol^{-1}$) occurred faster than with ($\Delta G_a^{\ddagger}$ = 51,57 to 54,80 $kJ.mol^{-1}$), indicating that the presence of the glycoside group accelerates the intermediate complex formation process obtained from the association of free molecules. The kinetic and thermodynamic characterization of the [BP-flavonoid] complex provides useful information for understanding and modulating the physicochemical properties of these nanostructures, in order to optimize their incorporation into food matrices.

Keywords: Non-traditional plant protein. Baru. Naringenin. Naringin. Surface Plasmon Resonance.

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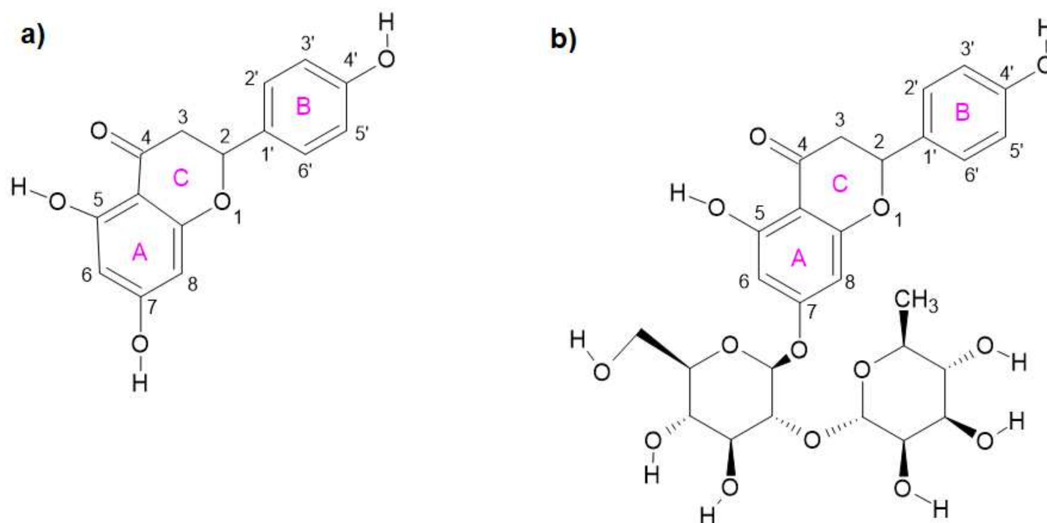
1. Introdução geral

Os polifenóis são metabólitos secundários das plantas, que são sintetizados como processo de defesa desses organismos. Existem, aproximadamente, 10.000 compostos citados na literatura caracterizados pela presença de anéis aromáticos contendo uma ou mais hidroxilas em sua estrutura química. As variações das estruturas dos polifenóis são responsáveis por atribuir a essas moléculas as diferentes funcionalidades biológicas (MOJZER et al., 2016).

Os flavonoides pertencem a uma classe dos polifenóis que são amplamente estudados pelo desempenho de diversas atividades biológicas, que incluem atividade antioxidante (WANG et al., 2017), anti-inflamatória (HÄMÄLÄINEN et al., 2007), redução da incidência de doenças como câncer (LIN et al., 2022) e cardiovasculares (GAO et al., 2018).

A naringenina (NG, 4',5,7-trihydroxyflavanone) e sua forma glicosilada naringina (NR, 4',5,7-trihydroxyflavanone-7-rhamnoglucoside) são flavonoides encontrados em frutas cítricas, principalmente em toranjas. A NG (Fig.1a) possui massa molar de 272 g/mol e está amplamente presente na dieta humana (JOSHI; KULKARNI; WAIRKAR, 2018). Diversos estudos vêm explorando suas propriedades farmacológicas, no entanto sua baixa solubilidade em água (0,24 mg/mL) e biodisponibilidade são os principais obstáculos no desempenho de sua bioatividade e inserção em matrizes alimentícias (ALSAKHAWY et al., 2022; HELMY et al., 2023; NYANE et al., 2017). Por outro lado, sua forma glicosilada, NR (Fig.1b) apresenta massa molar de 580,4 g/mol e boa solubilidade em água (0,69 mg/mL), no entanto, o grupo neohesperosideo é responsável por atribuir à molécula gosto amargo acentuado (MOJZER et al., 2016; NUNES et al., 2020; PACHECO et al., 2020).

Figura 1. Estruturas químicas da naringina (NG) (a) e naringenina (NR) (b).



Fonte: adaptado de LI et al. (2021).

Com o objetivo de reduzir esses fatores limitantes da NG e NR, estudos prévios utilizaram proteínas como veiculadores dessas moléculas. Em sua maioria, esses estudos utilizam proteínas de origem animal, que incluem β-lactoglobulina (SHPIGELMAN et al., 2014), β-caseína (PACHECO et al., 2020), lactoferrina (NUNES et al., 2019) e lisozima (DAS et al., 2018). No entanto, uma mudança nos hábitos alimentares nos últimos anos, tem contribuído para o aumento da demanda por proteínas vegetais, incluindo o seu uso como veiculadoras de compostos bioativos.

Além da adoção de dietas restritivas aos produtos de origem animal (vegetarianismo e veganismo), a preocupação com as questões ambientais relacionadas à alta utilização dos recursos hídricos na cadeia produtiva de produtos de origem animal e a degradação do solo pelos sistemas de plantio (monocultura) de proteínas vegetais convencionais, como a soja, também contribuem para a ascensão das proteínas de origem vegetal. Neste sentido, a busca por proteínas vegetais obtidas de fontes não convencionais tem se tornado objeto de vários estudos.

O Baru é um fruto do cerrado brasileiro com alto valor nutricional, porém é pouco explorado âmbito científico e tecnológico. O fruto do Baru é utilizado principalmente para a retirada de sua amêndoa, que possui em sua composição uma alta concentração de lipídeos (24,4 a 42,4 %, m/m), proteínas (24,0 a 29,9%, m/m) e minerais (2,1% m/m) (CRUZ et al., 2011; SOUSA et al., 2011). Por

apresentar alta concentração de proteínas, a amêndoa do Baru, pode contribuir para o enriquecimento de alimentos e avanços tecnológicos na indústria de alimentos.

Estudos anteriores exploraram a utilização de proteínas de fontes vegetais convencionais, como amendoim (REZENDE et al., 2022), soja (OLIVEIRA JÚNIOR; CUNHA, 2022) e ervilha (ZHANG et al., 2022) como potenciais veículos de pequenos ligantes. No entanto, estas proteínas são amplamente cultivadas em sistemas de monoculturas e são responsáveis por proporcionar degradação do solo. Visando contribuir para a manutenção da biodiversidade dos biomas brasileiros, bem como conhecer as propriedades das proteínas do baru (BP) como carreadoras de compostos bioativos, é importante caracterizar termodinamicamente e cineticamente a interação entre BP e a NG e NR.

A obtenção dos parâmetros cinéticos e termodinâmicos são essenciais para compreender a dinâmica de formação do complexo entre estas proteínas e os compostos bioativos. Além disso, os parâmetros cinéticos são importantes para determinar a viabilidade da utilização dos complexos, já que permitem conhecer a taxa de associação das moléculas e de dissociação do complexo em diferentes condições termodinâmicas.

A ressonância plasmônica de superfície (RPS) é uma técnica que permite obter os parâmetros cinéticos e termodinâmicos em um único experimento e sem o uso de marcadores (*label-free*) (SCHASFOORT, 2017). Além disso, a partir dos resultados obtidos, é possível determinar os parâmetros energéticos relacionados a formação dos complexos de transição, que intermediam a formação dos complexos termodinamicamente estáveis, fornecendo uma caracterização completa da dinâmica molecular.

2. Objetivos

2.1. Objetivo Geral

Avaliar os parâmetros termodinâmicos e cinéticos da interação intermolecular de BP com NR e NG em pH 7,4, sob diferentes temperaturas.

2.2. Objetivos específicos

- Determinar a constante termodinâmica de interação (K_b), a variação da energia livre de Gibbs padrão (ΔG°), a variação da entalpia padrão (ΔH°) e a variação da entropia padrão (ΔS°) de formação dos complexos entre as proteínas do Baru e NG e NR, em diferentes temperaturas utilizando a técnica de ressonância plasmônica de superfície.
- Determinar a constante cinética de associação (k_a) e a constante cinética de dissociação (k_d) dos complexos estáveis termodinâmicos, em diferentes temperaturas (289.15 K; 293.15 K; 297.15 K , 298.15K e 301.15 K).
- Determinar os parâmetros energéticos de formação do complexo ativado para os processos de associação das moléculas livres (a) e dissociação dos complexos termodinamicamente estáveis (d): energia de ativação ($E_a^{\ddagger}, E_d^{\ddagger}$), variação da entalpia de ativação ($\Delta H_a^{\ddagger}, \Delta H_d^{\ddagger}$)variação da entropia de ativação ($\Delta S_a^{\ddagger}$,$\Delta S_d^{\ddagger}$) e variação da energia livre de Gibbs de ativação ($\Delta G_a^{\ddagger}$,$\Delta G_d^{\ddagger}$) dos complexos ativados, por meio da técnica de RPS.

Capítulo 1

3. Referencial teórico

3.1. Baru

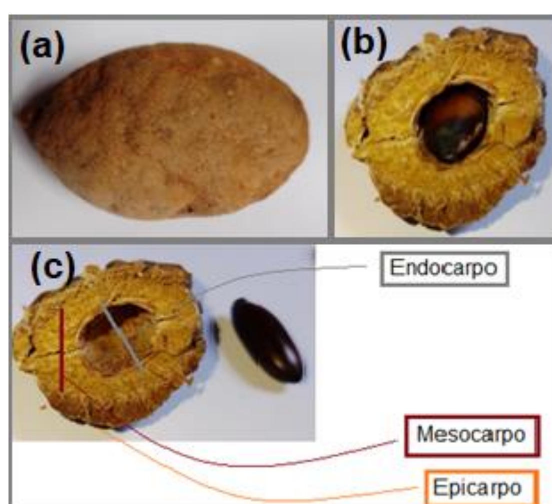
A árvore conhecida como baruero ou baruzeiro é típica do Bioma do cerrado brasileiro com intensa frutificação na fase adulta (VERA et al., 2009). Seu cultivo ocorre principalmente em solos areno-argiloso do Bioma do cerrado com temperaturas variando entre 20 e 30 ºC (SANO; RIBEIRO; DE BRITO, 2004). Devido à facilidade de seu cultivo (alta taxa de germinação e sobrevivência de mudas) e alta produção de massa foliar, o uso dessa espécie é promissor na recuperação de áreas degradadas, contribuindo para o equilíbrio do ecossistema (RINALDI et al., 2021; SANO; RIBEIRO; DE BRITO, 2004).

A produção do fruto é iniciada seis meses após o plantio e cada árvore produz em média 7800 frutos (SANO, 2016; SANO; RIBEIRO; DE BRITO, 2004). O fruto produzido é conhecido como baru, mas, dependendo da localidade, apresenta diferentes nomes como, por exemplo, feijão-coco, combaru, cumarurana e barujo.

De acordo com a sua classificação, o baru pertence à Divisão *Magnoliophyta (Angiospermae*), à Classe *Magnoliopsida (Dicotyledoneae*), à Ordem *Fabales, à Família Fabaceae (Leguminosae*; *Papilionoideae*), gênero *Dipteryx* e à espécie *Dipteryx alata* Vogel (CARVALHO, 2014; LIMA et al., 2022). A ocorrência natural do baruero no Brasil possui maior intensidade nos estados de Minas Gerais, Maranhão, Mato Grosso, Mato Grosso do Sul, e em menor intensidade no Pará, Piauí, Goiás e São Paulo. Ainda podem ser encontrados em países vizinhos, como Venezuela, Paraguai, Colômbia e Bolívia (LIMA et al., 2022; SANO; RIBEIRO; DE BRITO, 2004).

O fruto é classificado como drupa e sua estrutura pode ser dividida em porções (Fig. 2): epicarpo, mesocarpo, endocarpo e semente. O epicarpo corresponde à parte mais externa do fruto (casca), o mesocarpo (polpa), apresenta consistência macia, adocicada e adstringente, com potencial utilização para alimentação animal e humana; o endocarpo com estrutura lenhosa, pode ser utilizado para produção de carvão, e é responsável por envolver e proteger a semente (amêndoa), que possui elevado teor nutricional (ROCHA; CARDOSO SANTIAGO, 2009; SANO, 2016; SANO; RIBEIRO; DE BRITO, 2004).

Figura 2. Fruto do baru (a), corte latitudinal do fruto do baru com a amêndoa (b) e (c) divisão estrutural do fruto sem amêndoa.



Os principais usos do fruto do baru estão relacionados à amêndoa, que possui em sua composição uma alta concentração de lipídeos (24,4 – 42,4%, m/m),

proteínas (24 - 29,9%, m/m) e minerais (2,1%, m/m) (CRUZ et al., 2011; SOUSA et al., 2011). Em relação ao perfil lipídico, o óleo de baru, obtido por prensagem mecânica, possui ácidos graxos insaturados correspondendo a 79,6% m/m da composição total de ácidos graxos, com a presença do ácido oleico (C18:1 ω-9) (48,61%), ácido linoleico (C18:2 ω-6) (28%) e ácido gadoleico (C20:1). Os ácidos graxos saturados são: palmítico (C16:0) (6,7%), esteárico (C18:0) (5,1%), lignocérico (C24:0) (5%) e behênico (C22:0) (3,9%) (Aracava et al., 2022). Além desses, o ácido alfa-linolênico (C18:3 ω-3) (3,14g/100g do total de lipídeos) também já foi encontrado no óleo do baru (ALVES et al., 2016). A relação obtida ω-6: ω-3 é um importante indicador de contribuição para redução dos fatores de riscos associados a doenças cardiovasculares (MARTIN et al., 2006). O óleo obtido da amêndoa do baru apresenta a relação ω-6: ω-3 de 9:1, estando próximo à relação de ingestão recomendada (10:1) (ALVES et al., 2016).

A composição da fração proteica do baru é similar à maioria dos legumes, sendo constituída por 52,88% de globulinas, 14,09% de albuminas, 9,63% de gluteninas e 0,54% de prolaminas. As globulinas apresentam massa molar na faixa de 45-66 kDa, enquanto as albuminas apresentam cerca de 96 kDa (GUIMARÃES et al., 2012). A solubilidade de uma proteína é um fator determinante no desempenho das propriedades técnico-funcionais, e sua inserção nas matrizes alimentícias. As proteínas da amêndoa do baru possuem alta solubilidade em pH alcalino (10) e ácido (1-2), porém a solubilidade máxima é em pH 10 (83% m/m) e a mínima em pH 4-5 (15%). O ponto isoelétrico encontrado para estas proteínas é próximo de pH 4,8, característico de leguminosas, como a soja (CRUZ et al., 2011; NUNES et al., 2016).

Proteínas de fontes vegetais podem desempenhar propriedades tecnológicas interessantes para a indústria de alimentos e são potenciais substitutas para as proteínas de origem animal, principalmente para elaboração de produtos para o público com restrições alimentares (veganos). Propriedades como capacidade emulsificante, capacidade de retenção de água e óleo, atividade emulsificante e estabilidade da espuma, são propriedades consideradas importantes pela indústria de alimentos. BP apresenta capacidade de retenção de água (CRA) e óleo (CRO) de 193,84% e 205,28%, respectivamente (GUIMARÃES et al., 2012). A CRA de BP é

maior que a CRA da caseína (161,3%) e CRO maior que a proteína de soja (60,0%). Além disso, apresenta propriedade emulsificante (54,8%) e estabilidade de espuma (53,9%) similar às proteínas da soja. BP possui capacidade de formação de espuma em pH 7,0 (58,3%) similar às caseínas (53,3%) e menor que as albuminas (98,3%). Essas propriedades são importantes fatores que influenciam diretamente a qualidade final de um produto.

BP apresenta alta qualidade nutricional, com a presença de todos os aminoácidos essenciais em quantidade suficientes para suprir a necessidade recomendada pela organização mundial da saúde (SOUSA et al., 2011; WORLD HEALTH ORGAN, 2007). No entanto, dependendo da localidade de cultivo, os perfis de aminoácidos são variáveis, podendo apresentar aminoácidos sulfurados limitantes (FERNANDES et al., 2010). Além da composição de aminoácidos, a digestibilidade e o conteúdo total de nitrogênio também são parâmetros de qualidade de uma proteína (PIRES et al., 2006). Um importante padrão de medida para avaliar a qualidade proteica é o escore químico de aminoácidos corrigido pela digestibilidade proteica (*Protein digestibility corrected amino acid score* - PDCAAS). O método é baseado na comparação do perfil de aminoácidos de uma proteína de referência e a proteína em questão, levando em consideração a quantidade adequada nutricionalmente para uma criança de dois a cinco anos e multiplicado pela digestibilidade verdadeira (HUGHES et al., 2011). A digestibilidade verdadeira *in vivo* das proteínas do baru é de 86,38 % e PDCAAS de 90,70%, portanto, as proteínas do baru destacam-se por serem proteínas de alta qualidade entre as espécies do cerrado brasileiro (SOUSA et al., 2011).

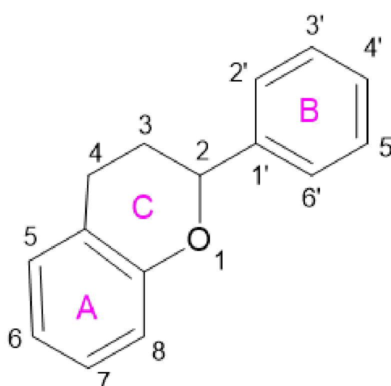
Além das proteínas de alto valor biológico, a amêndoa do baru também pode ser considerada uma boa fonte de minerais, composta principalmente por manganês (8,85 mg/100g), fósforo (832,8 mg/100g), potássio (980,35 mg/100), magnésio (164,81 mg/100g), zinco (4,83 mg/100g), ferro (8,65 mg/100g) e cálcio (110,94 mg/100g) (OLIVEIRA GONÇALVES et al., 2020; SOUSA et al., 2011). Baseada na RDC nº 269 de 22 de setembro de 2005, a porção bioacessível de 100 g de amêndoa é capaz de fornecer 314% de Mn, 55,9% de Zn, 32,2% de Cu e 13,1% de Fe da ingestão diária recomendada para adultos (BRASIL, 2005; DE OLIVEIRA GONÇALVES et al., 2020). A ingestão de micronutrientes é vital para o bom

funcionamento do organismo humano, sendo a amêndoa do baru uma candidata promissora para auxiliar na manutenção das funções biológicas diárias.

3.2. Naringenina

Os flavonoides pertencem à classe dos polifenóis que apresentam dois anéis benzênicos ligados por um anel heterocíclico de pirano ou pirona (Fig. 3) (HÄMÄLÄINEN et al., 2007). Esses polifenóis são amplamente estudados por desempenharem, principalmente, atividade antioxidante (WANG et al., 2017) e atuarem na redução da incidência de doenças como câncer (LIN et al., 2022) e doenças cardiovasculares (GAO et al., 2018).

Fig. 3. Estrutura química geral dos flavonoides.



Fonte: Adaptado de NUNES et al. (2020)

Um dos compostos mais comuns dos flavonoides é a NG, uma aglicona do grupo dos flavonoides amplamente encontrada em frutas cítricas, principalmente em toranjas (14,17- 53 mg/100g). Sua estrutura química apresenta três grupos hidroxilas nas posições 4', 5 e 7 (Fig.1a) e massa molar de 272 g/mol (JOSHI; KULKARNI; WAIRKAR, 2018; SHPIGELMAN et al., 2014).

A NG apresenta propriedades funcionais importantes que são amplamente estudadas por promoverem benefícios à saúde humana, que incluem atividade antioxidante (HERMENEAN et al., 2013) e anti-inflamatória (HÄMÄLÄINEN et al., 2007), redução da incidência de diferentes tipos de câncer (MOTALLEBI et al., 2022) e de doenças cardiovasculares (GAO et al., 2018). No entanto, sua solubilidade limitada em água (0,24 mg/mL) e a baixa biodisponibilidade são os maiores

obstáculos na utilização da NG como composto bioativo e sua inserção em matrizes alimentícias (JOSHI; KULKARNI; WAIRKAR, 2018; NUNES et al., 2020).

Neste sentido, esforços têm sido realizados para aumentar a solubilidade em água e a biodisponibilidade da NG, por meio da formação de complexo com diferentes biomoléculas, como a β-lactoglobulina utilizada para aumentar a solubilidade da NG em até três vezes acima do seu limite (SHPIGELMAN et al., 2014) e o complexo de inclusão entre β-ciclodextrina e NG utilizado para melhorar a estabilidade térmica e a solubilidade desse flavonoide (YANG et al., 2013).

No entanto, apesar das contribuições dos estudos realizados previamente, e diante da necessidade existente acerca do uso de proteínas vegetais, o estudo das interações de complexos formados a partir da NG e proteínas de fonte vegetais, como o baru, são necessários para elucidar o mecanismo de formação do complexo e fornecer informações sobre os parâmetros termodinâmicos e cinéticos de interação para melhor compreensão sobre a magnitude das energias envolvidas e a dinâmica molecular do processo de interação. Estes parâmetros serão fundamentais para o desenvolvimento de complexos proteicos bioativos de fontes vegetais.

3.3. Naringina

A forma glicosilada da naringenina é conhecida como naringina (NR, 4',5,7-trihidroxiflavanona-7-ramnoglicosídeo), com massa molar de 580,4 $g.mol^{-1}$ e sua estrutura química é apresentada na Fig.1b (SHARMA; BHARDWAJ; ARYA, 2021).

Assim como sua aglicona (NG), a NR é comumente encontrada em frutas cítricas, variando sua concentração entre 0,02 e 0,07% (m/m) nos sucos cítricos e 1% na casca (RAJAN; CHAND; GUPTA, 2006). Também é destacada por apresentar desempenhar atividades antioxidantes e anti-inflamatórias (NI et al., 2021), efeitos neuroprotetores (HELMY et al., 2023) e cicatrização de lesões na pele (ALSAKHAWY et al., 2022). Em contraste com a sua aglicona, a NR apresenta maior solubilidade em água (0,69 mg/mL), no entanto, apresenta amargor acentuado (GOLLAVILLI et al., 2020; RAJAN; CHAND; GUPTA, 2006). Além disso, apresenta baixa absorção no trato gastrointestinal, sendo em sua maioria, convertida em NG por hidrólise enzimática (MEMARIANI et al., 2021). Uma das formas de reduzir as

limitações de uso da NR e NG, foi estudada por NUNES et al., (2020), que utilizaram a formação de complexo entre a lactoferrina e NG para redução do amargor.

3.4. Interações intermoleculares entre proteínas e a NG e NR

O estudo das interações intermoleculares entre as proteínas e os compostos bioativos permite a compreensão dos mecanismos de interações e a determinação dos parâmetros termodinâmicos de interação entre as moléculas de interesse. Durante o processo de interação intermolecular, a energia envolvida pode ser resultante de interações eletrostáticas, ligações de hidrogênio, ligações de van der Waals e interações hidrofóbicas (GHEONEA et al., 2018). A variação de energia permite a determinação das forças motrizes que dirigem o processo de interação intermolecular e as variáveis termodinâmicas, como a variação da energia livre de Gibbs padrão (ΔG^0), variação da entropia padrão (ΔS^0) e variação da entalpia padrão (ΔH^0).

A interação entre a lactoferrina (LF) e diferentes concentrações de NR (1-6 µM) estudada por RPS resultou em constante de associação das moléculas livres (k_a) e de dissociação do complexo termodinamicamente estável (k_d) de 5,00 x $10^4 M^{-1}.s^{-1}$ e 0,36 s^{-1}, respectivamente, a 298,15K. A partir da plotagem do gráfico da variação de entropia ($T\Delta S_a^{\ddagger}$) *vs.* variação de entalpia ($\Delta H_a^{\ddagger}$) para o processo de associação do complexo de transição foi observado um ajuste linear. Esse comportamento indica compensação isocinética, ou seja, a mudança conformacional no sítio da LF e o processo de dessolvatação das moléculas interagentes ocorreram simultaneamente e ambos os processos moleculares determinaram $\Delta H_a^{\ddagger}$ e $T\Delta S_a^{\ddagger}$. Os parâmetros termodinâmicos obtidos para LF-NR^0 indicaram que em temperaturas entre 293,15 e 298,15K os complexos foram mais estáveis ($\Delta G^0_{293,15} = -28,78$ kJ.mol.$^{-1}$, $\Delta G^0_{297,15} = -29,30$ kJ.mol.$^{-1}$ e $\Delta G^0_{298,15} = -29,35$ kJ.mol.$^{-1}$) (NUNES et al., 2019).

Quando a interação da NR foi estudada com outra proteína (α-amilase), os valores de k_a ($7,05.M^{-1}.s^{-1}$) e k_d ($0,016.s^{-1}$) foram, respectivamente, 7100 e 20 vezes menores do que os mesmos parâmetros encontrados para a interação com LF. Estes resultados indicam que o processo de formação do complexo termodinamicamente estável ocorre mais rápido com a LF, no entanto, também se dissocia mais rápido desta proteína.

A interação da β-lactoglobulina (β-LG) nas formas nativa e desnaturada com a NG e a NR foi estudada por espectroscopia de fluorescência e ultravioleta na região do visível (UV-vis). A β-Lg não interagiu com a NR, provavelmente pela presença do grupo glicosídeo na NR, que pode ter impedido estericamente seu acesso ao sítio de interação da β-Lg. Além disso, a natureza hidrofílica da NR pode ter interferido na interação com o sítio hidrofóbico da β-Lg. Por outro lado, foi observada a supressão da fluorescência da proteína pelo mecanismo estático com a adição de concentrações crescentes de NG. O valor da constante termodinâmica de interação (k_b) obtido no comprimento de onda de excitação de 280 nm para a β-Lg desnaturada com a NG ($5,4x10^4$ M^{-1}) foi aproximadamente duas vezes maior que o encontrado para β-Lg nativa-NG ($2,4x10^4$ M^{-1}). No entanto não houve efeito significativo da desnaturação proteica na estequiometria de ligação (2:1 β-Lg- NG) (SHPIGELMAN et al., 2014).

Os parâmetros cinéticos e termodinâmicos de interação entre a β-caseína (β-CN) e a NR foram elucidados por PACHECO et al. (2020) utilizando a espectroscopia de fluorescência e RPS. A constante de extinção biomolecular (k_q) foi da ordem de 10^{12}, indicando a ocorrência do mecanismo estático com a formação do complexo. Os valores de k_b aumentaram com o aumento da temperatura (1,90 a 4,46 x 10^4 M^{-1}), demonstrando que mais complexos são formados com o aumento da temperatura, caracterizado por um processo endotérmico. Além disso, os valores positivos de ΔH^0 e $T\Delta S^0$ indicaram que a formação do complexo β-CN-NR^0 é entropicamente favorável. Os parâmetros energéticos do complexo de transição β-CN-$NR^‡$ tiveram variação muito pequena com a temperatura, indicando que ocorrem mínimas alterações conformacionais e dessolvatação das moléculas interagentes para o processo de ligação.

Como pôde ser observado, esforços científicos vêm sendo conduzidos visando compreender a energética e a dinâmica molecular de formação de complexos bioativos envolvendo NG e NR. Estes estudos abordam, essencialmente, proteínas de origem animal. No entanto, não foram encontrados estudos que investigassem o processo de interação entre proteínas de fontes vegetais alternativas, como as proteínas do baru, e moléculas bioativas. Deste modo, determinar os parâmetros termodinâmicos e cinéticos de interação entre as

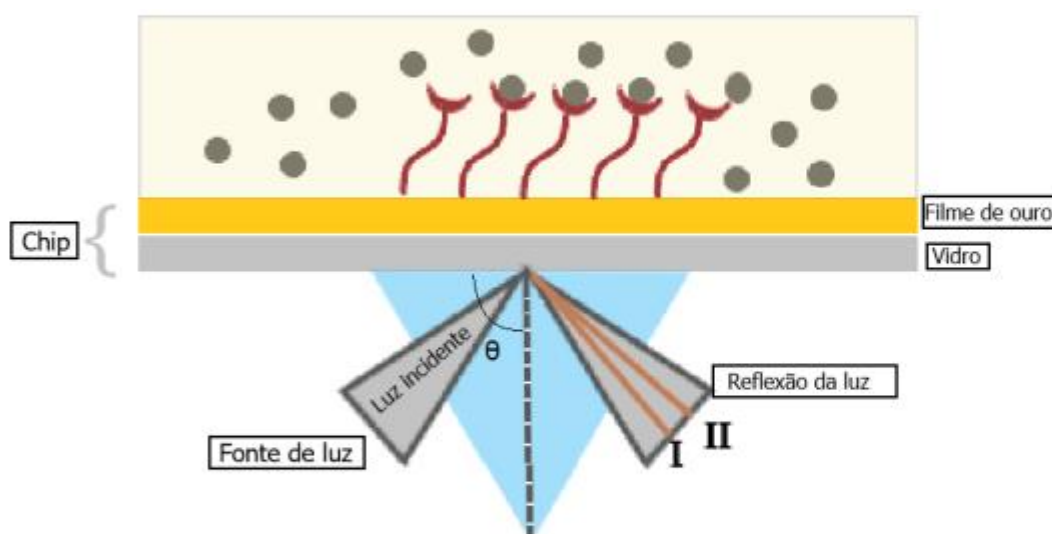
proteínas em questão e compostos bioativos são necessários para elucidar o mecanismo de formação do complexo entre estas moléculas.

3.4.1. Ressonância plasmônica de superfície (RPS)

A técnica de RPS permite o monitoramento da cinética de interação entre duas moléculas em tempo real, e ainda, possui a capacidade de detectar os processos de formação de complexos altamente dinâmicos, com constantes cinéticas (k_a e k_d) muito pequenas (DOUZI, 2017). A partir desta técnica, é possível obter os parâmetros cinéticos e termodinâmicos de formação de complexos moleculares, os quais fornecem informações relevantes para a compreensão dos mecanismos de formação de complexo entre as proteínas e moléculas bioativas.

O princípio da técnica é baseado na variação do índice de refração decorrente das interações entre o analito e a molécula imobilizada na superfície de um chip sensor (REZENDE et al., 2020). A Figura 4 ilustra como ocorre o fenômeno de ressonância plasmônica.

Figura 4: Esquema de funcionamento do RPS.

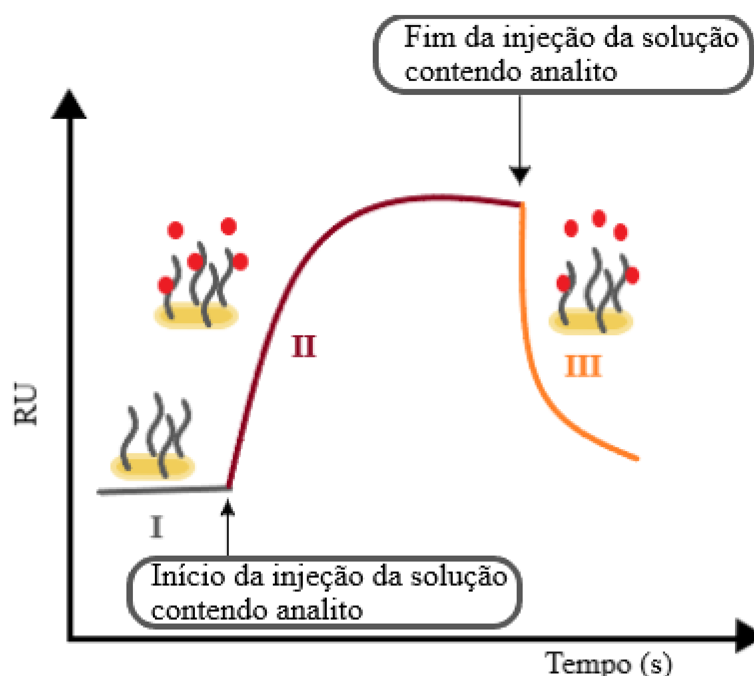


Fonte: adaptado de NAGATA & HANDA (2000).

Um feixe de luz polarizada é incidido sobre um material dielétrico (vidro), revestido com uma película fina de metal (ouro ou prata) através de um prisma, em

um ângulo de incidência específico θ. Parte da radiação incidida é refratada e outra parte é absorvida pelos elétrons livres oscilantes (plasmons) presentes no filme metálico, que promovem uma oscilação de carga longitudinal, ao longo da interface do metal e do dielétrico, conhecida como ressonância. Este processo produz um campo eletromagnético evanescente na superfície do ouro, que altera o ângulo de refração (de I para II). Em consequência da transferência de energia para a ressonância, a intensidade da luz refletida é reduzida no ângulo em que o RPS ocorre (ATKINS; DE PAULA, 2011; REZENDE et al., 2020a). A realização do experimento de RPS requer a utilização de chip sensor, que possui dois canais de fluxo, um contendo a molécula previamente imobilizada (canal de amostra) e o outro sem a molécula ligante, denominado canal de referência. Para a imobilização da molécula ligante na superfície do chip sensor, são utilizadas moléculas que possuem grupos carboxílicos, como a carboximetildextrana, e esta interação ocorre por meio de ligação covalente. A outra molécula a ser estudada (analito) flui sobre a superfície do chip e pode se ligar à molécula imobilizada, formando um complexo e alterando o índice de refração. O equipamento detecta estas alterações e expressa os resultados em um sensorgrama relacionando as unidades ressonantes (RU) com o tempo (Fig.5) (DE MOL; FISCHER, 2008).

Figura 5: Sensorgrama característico do RPS dividido em três etapas.



Fonte: adaptado de RITZEFELD; SEWALD (2012).

Na primeira etapa, a injeção inicial do tampão flui na superfície do chip sensor e estabelece a linha de base. Depois, à medida que a solução contendo o analito passa pelos canais de amostra e de referência, os processos de associação e dissociação ocorrem, caracterizando o aumento da RU na segunda etapa. Ainda nesta etapa, à medida que o fluxo continua, a taxa de ligação observada diminui à medida que menos sítios de ligação estão disponíveis na superfície do ligante. Na terceira etapa a solução contendo o analito é substituída pelo tampão, e o analito fracamente ligado, é dissociado, provocando a diminuição do sinal (HUDSON et al., 2018).

A partir dos dados obtidos no sensorgrama, a cinética de formação de complexo pode ser descrita pela Equação 1 (DOUZI, 2017):

$$\mathrm{P} + \mathrm{A} \underset{k_d}{\overset{k_a}{\rightleftarrows}} \mathrm{P-A} \qquad (1)$$

Em que P é a proteína ligante, A o analito, k_a é a constante de associação e k_d a constante de dissociação. Durante a etapa II do sensorgrama ocorre a formação de complexo e a taxa de formação é descrita pela Equação 2:

$$\frac{d[\mathrm{P-A}]}{dt} = k_a\,[P]_f[A]_f - k_d[\mathrm{P-A}] \qquad (2)$$

Em que $[P]_f$ e $[A]_f$ são as concentrações de proteína e analito livres (*f*), respectivamente, e $[\mathrm{P-A}]$ é a concentração do complexo formado. A variação na concentração de moléculas na superfície do sensor devido à formação de complexo proteína-analito é medida em RU (RITZEFELD; SEWALD, 2012). Deste modo podemos reescrever a Equação 2 da seguinte forma:

$$\frac{d[RU(t)]}{dt} = k_a[RU_{max}(t_\infty)] - [RU(t)] \cdot [A]_f - k_d[RU(t)] \qquad (3)$$

Em que $[RU(t)]$ é o sinal de RPS obtido no sensorgrama no tempo t, e $[RU_{max}(t_\infty)]$ é a resposta do RPS na saturação da proteína pelo analito no tempo t_∞. Durante a

fase II também é possível obter a constante observada (K_{obs}), obtida pela Equação 4 (REZENDE et al., 2019):

$$k_{obs} = k_a \cdot [\mathrm{A}] + k_d \qquad (4)$$

Resolvendo a equação diferencial 3 e substituindo a Equação 4 em (3), obtemos:

$$RU\ (t) = \ RU_{máx}(t_\infty)[1 - \ e^{-K_{obs}(t)}] \qquad (5)$$

Durante a fase III a alteração do sinal ressonante ocorre principalmente devido à dissociação do complexo P-A, e a taxa deste processo pode ser obtida através da seguinte equação:

$$\frac{d[RU(t)]}{dt} = -k_d[RU(t)] \qquad (6)$$

Resolvendo a diferencial da equação 6, temos:

$$RU\ (t) = \ RU\ (t_m)\ e^{-k_d(t-t_m)} \qquad (7)$$

Em que, t_m é o tempo em que o processo de dissociação prevalece (fase III).

Uma abordagem de Arrhenius (ln k_x (x = a ou d) versus 1 / T) permite a determinação da energia de ativação ($E_x^{\ddagger}$) para formação do complexo ativado por meio de associação das moléculas livres (a) ou dissociação dos complexos termodinamicamente estáveis (d) (Equação 8). O complexo ativado é um complexo intermediário formado a partir da associação das moléculas livres (P e A) ou da dissociação do complexo $\mathrm{P - A}$.

$$E_x^{\ddagger} = -R\ \left(\frac{d \ln k_x}{d\ T}\right) \qquad (8)$$

Em que k_x (T) é a constante cinética para associação (x = a) ou dissociação (x = d) em uma determinada temperatura T e R é a constante universal dos gases (8,3145 J.mol $^{-1}$.K $^{-1}$).

A determinação da força motriz para formação do complexo ativado pode ser obtida através das Equações 9-11:

$$\Delta G_x^{\ddagger}(T) = -RTln(k_x h/K_B T) \quad (9)$$

$$\Delta H_x^{\ddagger}(T) = E_x^{\ddagger}(T) - RT \quad (10)$$

$$T\Delta S_x^{\ddagger}(T) = \Delta H_x^{\ddagger}(T) - \Delta G_x^{\ddagger}(T) \quad (11)$$

Em que $\Delta G_x^{\ddagger}(T)$ é a variação da energia livre de Gibbs de ativação, $\Delta H_x^{\ddagger}(T)$ é a variação da entalpia de ativação, $\Delta S_x^{\ddagger}(T)$ é a variação de entropia de ativação, h é a constante de Planck ($6,62608 \times 10^{-34}$ J.s) e K_B é a constante de Boltzmann ($1,38066 \times 10^{-23}$ $J.K^{-1}$) na temperatura T (K) (REZENDE et al., 2019).

A realização do experimento em temperaturas diferentes permite a obtenção dos parâmetros termodinâmicos de formação do complexo termodinamicamente estável a partir da determinação da constante de interação (K_b) para cada temperatura estudada (REZENDE et al., 2019).

$$K_b = \frac{k_a}{k_d} \quad (12)$$

A variação da energia livre de Gibbs padrão (ΔG°) pode ser obtida através da equação 13.

$$\Delta G^0 = -RT \ ln \ K_b \quad (13)$$

Em que R é a constante universal dos gases ($8,3145$ $J.mol^{-1}.K^{-1}$) e T é a temperatura (K).

Além disso, a obtenção do K_b em diferentes temperaturas permite calcular os demais parâmetros termodinâmicos, como a variação da entalpia padrão de formação do complexo (ΔH°), obtido pela aproximação de Van't Hoff, a partir da plotagem do gráfico de lnK_b *versus* $1/T$, onde a inclinação da curva será $-\Delta H^0/R$.

$$\frac{\ln K_{b2}}{ln \ K_{b1}} = -\frac{\Delta H^0}{R}(\frac{1}{T_1} + \frac{1}{T_2}) \quad (14)$$

Onde R é a constante universal dos gases (8,3145 J.mol-1.K-1) e T é a temperatura (K).

Através da equação fundamental de Gibbs (Equação 15) pode-se obter a contribuição entrópica ($T\Delta S^0$), que fornecem informações sobre as forças motrizes que impulsionam a formação do complexo.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (15)$$

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Capítulo 2

Formation of nanostructures of baru (*Dipteryx alata*) proteins and flavonoids: evaluation of binding kinetics and thermodynamics

Abstract

The adoption of new eating habits and concern for environmental sustainability highlights the need to search for alternative sources of plant-based proteins. Therefore, we used the surface plasmonic resonance to evaluate the baru proteins (BP) as carrier of naringin (NR) and naringenin (NG). The NR ($\Delta G°_{298.15K}$= -26.22 $kJ.mol^{-1}$) formed more stable complexes with BP compared to NG ($\Delta G°_{298.15K}$= -20.87 $kJ.mol^{-1}$). The binding constant value at 298.15 K was 8.6 times higher for $BP\text{-}NR^0$ compared to $BP\text{-}NG^0$, suggesting that the rhamminoglycoside group of NR contributes to greater interaction with BP. Although NR has a higher molecular volume, its association rate (3.675 to 1.269 x 10^4 $M^{-1}.s^{-1}$) with BP was 6 to 12 times higher compared to that with NG (0.291 to 0.196 x 10^4 $M^{-1}.s^{-1}$). The formation of hydrophilic interactions was crucial to form the $BP\text{-}NR^0$ complex regardless of temperature ($\Delta H°_{BP-NR^0}$ = -78.54 $kJ.mol^{-1}$; $T\Delta S°_{BP-NR^0}$ = ~ -52 $kJ.mol^{-1}$), while NG accessed more hydrophobic protein binding sites as temperature increased ($\Delta H°_{BP-NG^0}$ = -44.61 to -2.27 $kJ.mol^{-1}$; $T\Delta S°_{BP-NG^0}$ = -23.72 to 18.76 $kJ.mol^{-1}$). Our results provide useful information about molecular recognition mechanisms, which allow designing optimized plant-based nanocarriers for insertion of flavonoids into food matrices.

Keywords: Biodiversity. Non-traditional plant protein. Naringin. Naringenin. Surface plasmonic resonance.

1. Introduction

Proteins are biopolymers with nutritional and techno functional properties of great relevance to several areas, such as food, pharmacology, and medicine (CHAKRABORTY et al., 2022; COSTA et al., 2022; NILSEN et al., 2020; ZHANG et al., 2023). In the last decade, the changes in the eating habits of consumers have increasingly stimulated scientific investigations involving the search for proteins from plant sources that replace, totally or partially, animal proteins in various applications. In addition, socio-environmental demands have also encouraged this research (LUSK et al., 2022; MAZAC et al., 2022; NEUHOFER; LUSK, 2022).

The current high demand for plant-based proteins is associated with religious issues, dietary restrictions for health reasons, and concerns about animal mistreatment and environmental sustainability (NEUHOFER; LUSK, 2022; POINTKE et al., 2022). In this sense, the choice of plant sources to meet this demand must be done carefully, since the traditional monoculture systems, such as soy and corn, can also promote unsustainable production. The large-scale cultivation of these traditional plant sources leads to losses in the biodiversity of the environment around where they are due to the extensive land-use, depletion of freshwater resources, and over application of chemical fertilizers (ESCOBAR et al., 2020; FAO, 2022b, 2022c; POORE; NEMECEK, 2018; SPRINGMANN et al., 2018). In addition, the socioeconomic effects resulting from these commodities must also be considered, such as the concentration of power and income in the hands of a few people. Therefore, it is evident the need to search for alternative sources of plant-based proteins to strengthen social, economic, and environmental sustainability. In view of all these effects, the Food and Agriculture Organization (FAO) has constantly published on the need for actions that promote the diversification of the types of plant sources explored to ensure the biodiversity recovery and land and water resources and building inclusive and sustainable economies (FAO, 2022a, 2022b, 2022c, 2022d).

The use of proteins from traditional vegetable sources, such as soy (OLIVEIRA JÚNIOR; CUNHA, 2022), peas (ZHANG et al., 2022), and peanuts (REZENDE et al., 2022), as carriers of small molecules is well investigated. On the other hand, we believe that the technofunctional potential of proteins from alternative

plant sources, such as baru, still lacks research to enable the successful application of these biopolymers in nanoscience from several areas. Baru (*Dipteryx alata*) is a native plant present in the Brazilian Savannah (Cerrado) biome that has an almond with a high protein content (~ 30% w/w on wet basis) (SÁ; MORENO; CARCIOFI, 2020). Baru proteins are considered of high nutritional quality since they have all the essential amino acids in sufficient quantity to meet the quality requirements established by FAO (FAO, 2013; SOUSA et al., 2011). To the best of our knowledge, baru protein has not yet been evaluated as a carrier nanostructure for bioactive compounds. The discovery of the baru protein's technofunctional properties is important to encourage the cultivation of this plant and prevent native areas from being deforested to be replaced by extensive plantations of traditional vegetables.

Flavonoids are a class of polyphenolic compounds that have a wide range of pharmacological functions, including antioxidant activity (WANG et al., 2017), cancer treatment and prevention (LIN et al., 2022), neuroprotective effects (HELMY et al., 2023) and reduction of dyslipidemia (CAI et al., 2023). Naringin (NR, 4',5,7-trihydroxyflavanone-7-rhamnoglucoside, Fig. 1a) and its aglycone form naringenin (NG, 4',5,7-trihydroxyflavanone, Fig.1b) are flavonoids found mainly in citrus fruits (SHPIGELMAN et al., 2014). However, the limited water solubility of NG (0.24 mg/mL) can affect its gastrointestinal absorption and bioavailability, and hence their bioactivity (GERA et al., 2017; NUNES et al., 2020). On the other hand, NR has good solubility in water (0,69mg/mL), however, it presents undesirable bitterness (GOLLAVILLI et al., 2020; GUPTA et al., 2021), which limits its use in food matrices, as a form of nutritional enrichment. Therefore, strategies have been studied to increase NG bioavailability through the formation of complexes with different molecules such as lysozyme (DAS et al., 2018), beta-lactoglobulin (SHPIGELMAN et al., 2014), human serum albumin (TU et al., 2015) and beta-cyclodextrin (YANG et al., 2013). Complex formation has also been reported to reduce the intensity of the bitterness of bioactive molecules (NUNES et al., 2020; SANTA ROSA et al., 2021), making it possible to increase their consumption. Despite the contribution of these previous studies with different animal proteins, there is still a gap about the kinetic and thermodynamic e mechanism of intermolecular interaction between NR and NG, and proteins from plant sources.

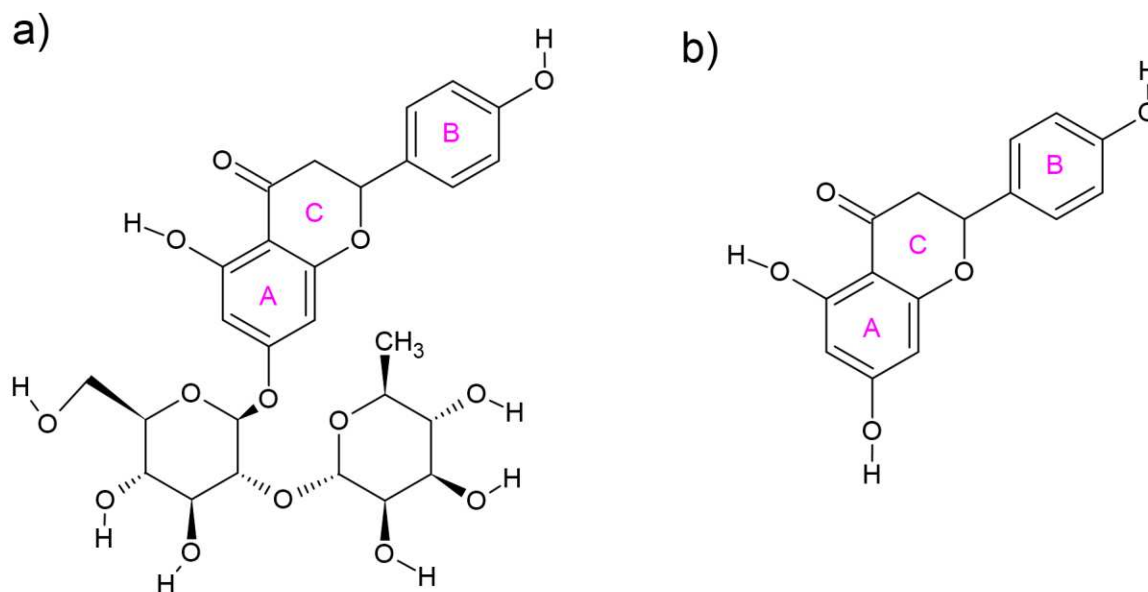


Fig. 1. Chemical structures of naringin (NR) (a) and naringenin (NG) (b).

In this context, the aim of this study was to investigate whether baru protein has the potential to carry bioactive compounds. For this, we evaluated the thermodynamics and kinetics of complex formation between baru protein and NR and NG at different temperatures. The interaction studies were performed by the surface plasmonic resonance (SPR) technique that provides analytical measurements related to the association and dissociation processes of interacting molecules in real time.

2. Material and methods

2.1. Materials

Naringin (71162) and naringenin (N5893) were obtained from Sigma Aldrich (USA). The CM5 chips and the reagents used for protein immobilization (3-(N,N-dimethylamino) propyl-N-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine-HCl (1M, pH 8.5) and sodium acetate (pH 4.0)) were purchased from General Electric Healthcare Company (Sweden). All reagents used for the extraction of baru proteins were of analytical grade and were acquired from Vetec (Brazil) (sodium phosphate) and Dinâmica Química Contemporânea (Brazil) (N-hexane, 99%).

2.2. Baru protein extraction procedure

2.2.1 Obtaining defatted flour baru

Roasted baru almonds were obtained from local market in Viçosa, Minas Gerais state, Brazil. After manually removing the peel, the almonds were milled and homogenized in knife mills to obtain the flour (NUNES et al., 2016). In sequence, the flour was defatted with hexane (1:5 w/v flour to solvent ratio) with agitation each 30 minutes at 25 ºC for 12 hours. The fat extraction process was repeated once more with a change in the proportion of solvent to 1:6 w/v flour to solvent ratio (CRUZ et al., 2011). Finally, the solvent was evaporated in a fume hood for 48 hours at room temperature.

2.2.2 Obtaining the baru protein isolate

Proteins were extracted from baru almonds as procedure described by NUNES et al., (2016) with some modifications. Initially, 1 g of flour was dispersed in 20 mL of distilled water at pH 10 followed by centrifugation at 1509 g/30min (Heraeus Fresco 21 Centrifuge, Thermo Scientific). The supernatant was collected and its pH adjusted to 4.8, then centrifuged at 1509 g/ 40min.The pellet was resuspended in water distilled and pH adjusted to 7.0. Finally, to obtain baru protein isolate (BP), this suspension was lyophilized using a SL-404 lyophilizer (SOLAB Laboratory Equipment, Brazil).

2.3. Determination of interaction parameters by surface plasmon resonance

To analyze the binding process between BP and NG or NR by SPR technique, the Biacore X100 instrument (GE Healthcare, Pittsburgh, PA, USA) apparatus was used. The CM5 chips used in the SPR experiment have two flow channels on their surface: sample channel and reference channel.

2.3.1. Protein immobilization on the chip surface by amine coupling

First, a BP suspension (30 $\mu g.mL^{-1}$) was prepared in sodium acetate (10 mM, pH 4.0). The protein immobilization process was performed using the Biacore amine coupling kit, whose method is characterized by the formation of covalent bonds

between the amine groups of the biopolymer and the carboxyl groups of the carboxymethyl dextran present on the CM5 chip surface (NH_2-COOH). Before forming these covalent bonds, it is necessary to activate the carboxyl groups on the surface of the chip. For this, an EDC/NHS mixture (1:1 (v/v), 0.4 M and 0.1 M, respectively) was injected for 7 min (20 μL.min^{-1}) into the two flow channels. Then, the protein suspension was injected into the flow system so that it only flowed into the sample channel, where NH_2-COOH bonds were formed until obtaining a response signal from 3061 resonant units (RU). Lastly, all active COOH groups of the reference channel and the sample channel (in this case, those that did not form binding with protein) were blocked with ethanolamine (1 M, pH 8.5, at 10 μL.min^{-1} during 7 min). The output signal (raw data, RU *versus* time) corresponded to the difference between the resonant signal generated in the sample channel and in the reference channel, so that the effect of non-specific bindings was disregarded (MAGALHÃES et al., 2021; REZENDE et al., 2022).

2.3.2. Investigation of protein-analyte interaction in real-time

A series of NR (7, 8, 9, 10, 11, 12 and 13 μM) or NG (80, 90, 100, 110, 120, 130 and 140 μM) solutions were prepared in HBS-P running buffer (0.01 M HEPES, 0.15 M NaCl, and 0.005%, v/v, surfactant P20 at pH 7.4) and DMSO (2%, v/v). The flavonoids concentrations were determined from flow tests using different analyte concentrations and different flow rates to ensure that the effect of mass transport on association and dissociation rates were negligible. The binding experiments were performed in triplicate (using three different sensor chips) at temperatures of 289.15, 293.15, 297.15, 298.15 and 301.15 K. Each flavonoid solutions were injected into the flow system (both channels) for 50 s so that the complex formation with the protein immobilized in the sample channel could occur (flow rate of 10 μL.min^{-1}). Chip regeneration between the different flavonoid solutions was performed by injecting HBS-P flow buffer for 30 s (enough time for the resonant signal obtained to return to baseline). The formation of BP-flavonoid complexes promotes small changes in the refractive index in the region immediately above the chip. These changes are measured in real-time by the optical system of the equipment and expressed in sensorgrams (RU versus time).

3. Results and discussion

3.1 Evaluation of the formation of BP-flavonoid complexes

In the molecular dynamics involved in the formation of supramolecular structures, the thermodynamic stable complex formation occurs simultaneously with the interaction of free molecules and the dissociation of the stable complex. The kinetic parameters determination, mainly the constants of association (k_a) and dissociation (k_d), as well as its dependence of temperature change, make possible to describe the molecular mechanism of molecular interaction. The RU versus time data, obtained at different temperatures and concentrations, allow us to calculate the k_a and k_d values. Figure 2 shown the sensorgram (RU x t) results of the interaction between immobilized baru proteins on the sensor chip surface and the NR (a) or NG (b) molecules present in the solution that flows over the sensor surface at a temperature of 298.15K and pH 7.4. The sensorgrams of the other temperatures studied are shown in Fig. S1 and Fig. S2 in Supporting Information, for NR and NG, respectively.

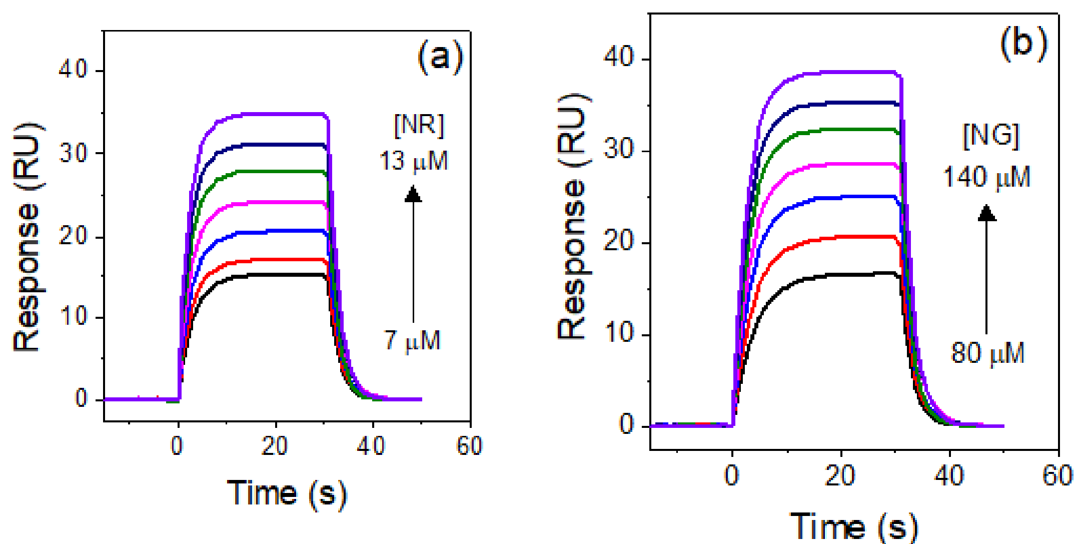


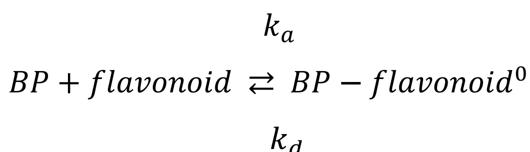
Fig. 2. Sensorgrams of immobilized baru protein (BP) interaction with different concentrations of (a) naringin (NR) (7, 8, 9, 10, 11, 12 and 13 μM) and (b) naringenin (NG) (80, 90, 100, 110, 120, 130 and 140 μM) at 298.15 K. The direction of the arrows indicates the rise in concentrations of either NR or NG.

The sensorgram profile confirms that there is an interaction between BP and bioactive molecules (NG or NR). This interaction is detected, in real time, by observing exponential increase in the RU signal over time.

The sensorgram showed an initial baseline, followed by a rapid increase in the intensity of the RU signal as the analyte solution (NR or NG) was injected and contacted the immobilized proteins on the surface of the sensor chip. Finally, there was an exponential decay in the RU values due to the substitution of the analyte solution flowing on the sensor chip surface by a buffer solution, indicating that the dissociation processes of thermodynamically stable complexes prevailed at this stage (FATHI et al., 2018). $\partial RU/\partial T$ >0 indicates that despite association and dissociation processes occur simultaneously, the former prevail. However, the rate of RU depends on the time, showing that association and dissociation velocity became equal as the time increase.

3.2. Kinetic parameters for association and dissociation processes related to BP-flavonoid complexes.

Using data extracted from sensorgrams, it was possible to determine k_a and k_d values, applying the one-to-one binding model expressed in the following equilibrium:



$$BP + flavonoid \underset{k_d}{\overset{k_a}{\rightleftarrows}} BP - flavonoid^0$$

For that, the observed rate constant ($k_{obs} = k_a \cdot [F] + k_d$, where, $[F]$ is the concentration of flavonoid) and k_d rate were obtained by global fitting of the integrated rate equations (Eqs. (1) and (2)) to RU *versus* time data (COELHO et al., 2022; SCHASFOORT, 2017).

$$RU(t) = RU_{max}(t_\infty)[1 - e^{-k_{obs}(t)}] \quad (1)$$

$$RU(t) = RU(t_m)\, e^{-k_d(t-t_m)} \quad (2)$$

Where, $RU(t)$ is the SPR signal obtained from the sensorgram at time t, $RU_{max}(t_\infty)$ is the response on BP saturation by the flavonoid at t = ∞, $RU(t_m)$ is the SPR signal in the time (t_m) that descendant curve started. Based on the relationship established

in for k_{obs}, was possible to obtain k_a by the slope of the curve k_{obs} vs. [flavonoid] (Fig. S3 in Supporting Information).

Table 1 shows the k_a and k_d values for the BP-NR and BP-NG interactions at the different temperatures.

Table 1. Association (k_a) and dissociation (k_d) constants for the BP-NR and BP-NG interactions at different temperatures.

T (K)	$[BP – NR]^0$		$[BP – NG]^0$	
	k_a (10^4 $M.^{-1}.s^{-1}$)	k_d (s^{-1})	k_a (10^4 $M^{-1}.s^{-1}$)	k_d (s^{-1})
289.15	3.67	0.36	0.29	0.49
293.15	2.97	0.44	0.23	0.47
297.15	2.04	0.47	0.21	0.45
298.15	1.85	0.47	0.20	0.45
301.15	1.27	0.46	0.20	0.44

* For all parameters, the standard deviation did not exceed 5%.

The k_a values for both complexes decreased with increasing temperature, indicating that, despite the increase in average kinetic energy of interacting molecules probably promoting favorable molecular collisions for complex formation, collisions between water/ions molecules and the formed complex are also favored, causing their dissociation, resulting in a reduction in k_a values. The flavonoid with the highest molecular volume, that is, NR, associated 6 to 12 times faster with protein. The k_a values for the BP-flavonoid complex suggest that the glycosidic group present in NR interacts with hydrophilic binding sites close to the BP surface, accelerating the formation of the complex, while NG accesses internal more hydrophobic sites of the protein, making the process slower. Regarding the dissociation, $BP-NR^0$ complexes was favored by increasing temperature ($0.362 \leq k_d \leq 0.462$ s^{-1}) while the $BP-NG^0$ dissociation ($0.490 \leq k_d \leq 0.439$ s^{-1}) decreased with increasing temperature.

Other studies also used the SPR technique to evaluate the interaction kinetics between NR and different proteins, such as α-amylase (αam) (LIU et al., 2017), lactoferrin (LF) (NUNES et al., 2019) and β-casein (βcas) (PACHECO et al., 2020).

At 298.15 K, the number of complexes formed per second between BP and NR (1.84×10^4 $M^{-1}.s^{-1}$) was close to the order of magnitude observed for LF (5.00×10^4 $M^{-1}.s^{-1}$) and βcas (0.255×10^4 $M^{-1}.s^{-1}$) milk proteins. Furthermore, the dissociation rate also occurred at similar speeds ($k_{d\ (BP\text{-}NR)} = 0.472\ s^{-1}$; $k_{d\ (LF\text{-}NR)} = 0.360\ s^{-1}$; $k_{d\ (\beta cas\text{-}NR)} = 0.360\ s^{-1}$). On the other hand, the association between the vegetable protein of the present study and the NR occurred 2600 times faster than for the αam ($k_{a\ (\alpha am\text{-}NR)} = 7.05\ M^{-1}.s^{-1}$), but the dissociation of the αam-NR complex was slower ($k_{d\ (\alpha am\text{-}NR)} = 0.016\ s^{-1}$). This variation in the rate values may be related to different molecular processes, such as desolvation of interacting molecules, conformational changes, release of ions from the electrical double layer, which were necessary for the flavonoid to access the protein binding site (LELIS et al., 2020). In addition to molecular processes, protein amino acid composition, especially of the protein interface, and binding specificity can also describe these differences (NUNES et al., 2019). In order to discovery the origin these differences between vegetable and animal protein interaction with NR and NG, an analysis of energetic and thermodynamic parameters of interaction will be done.

3.3. Energetic kinetic parameters of intermediate complex formation

In the binding path from free molecules that interact to form a thermodynamically stable complex ($[BP\text{-}flavonoid]^0$), there is the formation of an unstable chemical structure ($[BP\text{-}flavonoid]^{\ddagger}$) known as an intermediate complex (IC). The IC can be formed by the association of NR or NG with BP free molecules or by the dissociation of the TSC. Due to potential energy barrier ($E^{\ddagger}$, activation energy) associated with the IC formation it is highly unstable and has a very short lifetime. To form the IC, $E^{\ddagger}$ must be overcome through the association of BP and flavonoids free molecules ($E_a^{\ddagger}$) or through the dissociation of the $[BP\text{-}flavonoid]^0$ ($E_d^{\ddagger}$) (SCHASFOORT, 2017). The $E_a^{\ddagger}$ is an energy barrier arising from the desolvation of interacting molecules ($E_{des(a)}^{\ddagger}$), formation of new interactions ($E_{int}^{\ddagger}$) and molecular conformational changes ($E_{conf}^{\ddagger}$) (FRISCH; FERSHT; SCHREIBER, 2001; VALE et al., 2022).

From the dependence of k_a and k_d on temperature, was possible to calculate the energetic parameters of the binding mechanism of protein-flavonoid interactions

(CASTRO et al., 2021). Using a nonlinear Arrhenius approach ($ln\, k_x vs. 1/T$), the activation energies ($E_a^{\ddagger}$ or $E_d^{\ddagger}$) for the formation of the [BP-flavonoid]‡ were obtained from the Eq. (3) (Fig. S4 in Supporting Information).

$$E_x^{\ddagger}\,(T) = -\,R\left(\frac{d \ln k_x}{d\,T}\right) \quad (3)$$

Where k_x is the association (x = a) or dissociation (x = d) rate constants, R is the universal gas constant (8.3145 J.mol^{-1}.K^{-1}), and T is the temperature (K).

The other activation energy parameters ($\Delta G_x^{\ddagger}$, $\Delta H_x^{\ddagger}$ and $\Delta S_x^{\ddagger}$) involved in the formation of the intermediate complex [BP-flavonoid] ‡ were determined by the Eqs. (4), (5), and (6) (COELHO et al., 2022).

$$\Delta G_x^{\ddagger}(T) = -RTln(k_x h/K_B T) \quad (4)$$

$$\Delta H_x^{\ddagger}(T) = E_x^{\ddagger}(T) - RT \quad (5)$$

$$T\Delta S_x^{\ddagger}(T) = \Delta H_x^{\ddagger}(T) - \Delta G_x^{\ddagger}(T) \quad (6)$$

Where $\Delta G_x^{\ddagger}(T)$ is activation Gibbs free energy change, $\Delta H_x^{\ddagger}(T)$ activation enthalpy change, $T\Delta S_x^{\ddagger}(T)$ activation entropy change, *h* is the Planck constant (6.62608 × 10^{-34} J.s), and K_B is the Boltzmann constant (1.38066 × 10^{-23} J.K^{-1}) at temperature T (K) (REZENDE et al., 2019).

The formation of IC from the association of free BP with NR ($\Delta G_a^{\ddagger}$ from 45.47 to 50.12 kJ.mol^{-1}) occurred faster than with NG ($\Delta G_a^{\ddagger}$ from 51.57 to 54.80 kJ.mol^{-1}). To better understand the activation energies involved in the formation process of intermediate complexes, we can divide them into three components with their respective molecular events: $E_{des}^{\ddagger}$ > 0, energy absorbed to desolvate the molecules; $E_{int}^{\ddagger}$ < 0, energy released in the formation of new BP-flavonoid interactions; and $E_{\mathrm{conf}}^{\ddagger}$ > 0, absorbed energy for conformational changes at the interaction site. These same molecular processes also determine the values of the entropic terms as follows: $T\Delta S_{des}^{\ddagger}$ > 0, entropy gain due to the release of water molecules from the solvation layers of the interacting molecules the bulk of the system; $T\Delta S_{int}^{\ddagger}$ < 0, reduction in

entropy due to the decrease in the degree of freedom of the protein and flavonoid due to complex formation; and $T\Delta S^{\ddagger}_{conf}$ < 0 or $T\Delta S^{\ddagger}_{conf}$ > 0 depending on the change in flexibility of the protein interaction site after [BP-flavonoid]‡ complex formation.

For the process of association of free molecules, at all temperatures studied, $|E^{\ddagger}_{int}|$ > $|E^{\ddagger}_{des} + E^{\ddagger}_{\mathrm{conf}}|$ resulting in negative values for $E^{\ddagger}_{a}$ e $\Delta H^{\ddagger}_{a}$ for both [BP-flavonoid]‡ complexes (Fig. 3 (a) and (b)).

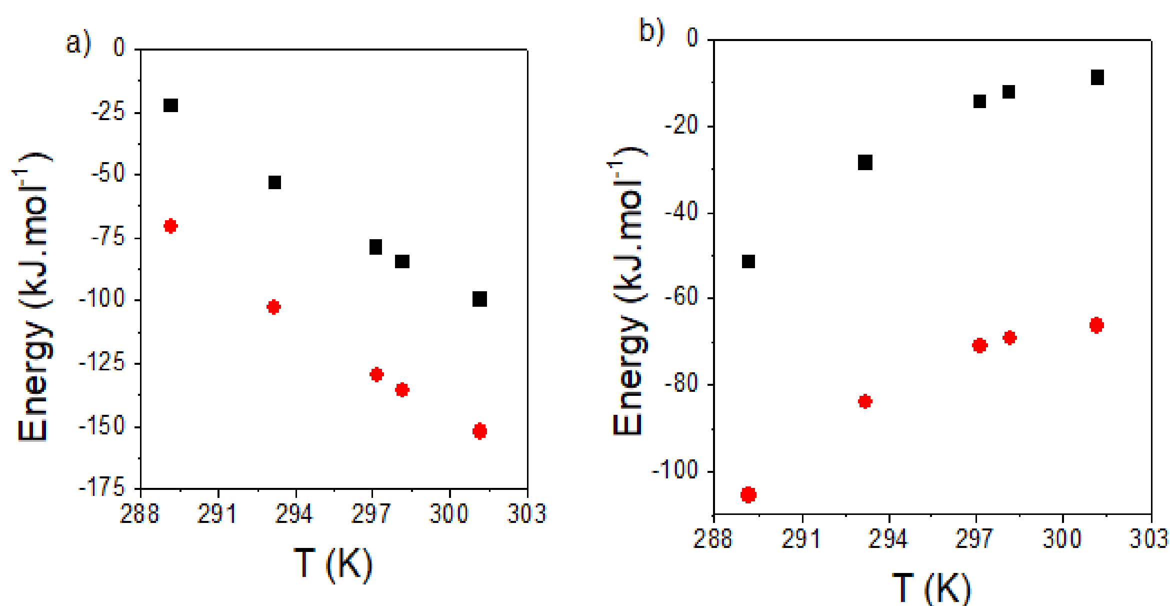


Fig.3. Plots of energetic kinetic parameters, $E^{\ddagger}_{a}$ (■) and $T\Delta S^{\ddagger}_{a}$ (●), *versus* T for the formation of (a) [BP-NR]‡ and (b) [BP-NG]‡ complexes.

For the [BP-NR]‡ complex, the $E^{\ddagger}_{a}$ became more negative as the temperature increased, demonstrating that the interactions between the BP and the NR intensified ($E^{\ddagger}_{int}$ < 0). For these interactions to occur, it is necessary to desolvate the interacting molecules, which would lead to an increase in the entropy of the system ($T\Delta S^{\ddagger}_{des}$ > 0). However, the $T\Delta S^{\ddagger}_{a}$ of the system reduced with increasing kinetic energy, indicating that the formation of the [BP-NR]‡ complex from the association of free molecules made the binding site protein more structured ($T\Delta S^{\ddagger}_{conf}$ < 0). The graphic relationship between $\Delta H^{\ddagger}_{a}$ and $T\Delta S^{\ddagger}_{a}$ for [BP-NR]‡ was linear (R = 0,999) with a slope close to 1 (1.06), indicating the occurrence of isokinetic compensation (IKC)

(Fig.S5 in Supporting Information). The IKC demonstrates that the process of intensification the protein-flavonoid interaction and the process of conformational change that increases the rigidity of the protein binding site occur simultaneously, leading to a reduction in enthalpy and entropy as the temperature increases.

On the other hand, for the formation of [BP-NG]‡ from the association of free BP and NG, the parameters $E_a^{‡}$ and $\Delta H_a^{‡}$ were less negative and $T\Delta S_a^{‡}$ increased with increasing temperature (Fig. 3 (b)). This behavior suggests that at lower temperatures hydrophilic interactions, such as hydrogen bonds and Van der Waals forces, were predominant. However, as the temperature increased, the desolvation process and binding site fitting were intensified so that NG could access more hydrophobic regions of the protein, making the activation energy and entropic term less negative ($E_{des}^{‡}$ > 0; $E_{\mathrm{conf}}^{‡}$ > 0; $T\Delta S_{des}^{‡}$ > 0; and $T\Delta S_{conf}^{‡}$ > 0).

The intermediate [BP-flavonoid]‡ complexes can be formed from the dissociation process of thermodynamically stable BP-flavonoid^{0} complexes. The energy parameters related to this process are shown in Table 2.

Table 2. Energetic parameters for [BP-flavonoid]‡ intermediate complex formation via dissociation of the BP-flavonoid^{0} complex, at pH 7.4.

T	Dissociation process (d)							
	[BP-NR]‡				[BP-NG]‡			
	$E^{‡}$	$\Delta H^{‡}$	$\Delta G^{‡}$	$T\Delta S^{‡}$	$E^{‡}$	$\Delta H^{‡}$	$\Delta G^{‡}$	$T\Delta S^{‡}$
K	kJ.mol^{-1}							
289.15	49.26	46.86	73.18	-26.32		-8.94	72.46	-81.40
293.15	21.90	19.47	73.74	-54.28		-8.97	73.58	-82.56
297.15	2.82	0.35	74.63	-74.27	-6.53	-9.01	74.71	-83.71
298.15	-0.73	-3.21	74.88	-78.08		-9.01	74.99	-84.01
301.15	-8.61	-11.11	75.71	-86.82		-9.04	75.84	-84.88

The data in Table 2 indicate that the passage of the [BP-NR]‡ complex to form BP-NR^{0} was accompanied by the formation of BP-NR interactions that increased the flexibility of the binding site protein. The increase in entropy shows that the protein

binding site in the intermediate complex is more rigid than in the stable BP-NR^0 complex.

The Arrhenius plot (Fig. S4 in Supporting Information) showed a linear dependence between ln k_d and temperature for the [BP-NG]^0 complex dissociation suggesting that the intermediate complex was formed via single-step process. This behavior shows that the processes of binding sites fitting and desolvation necessary to form the BP-NG^0 complex occurred to a greater extent in the process of association of free molecules.

3.4. Thermodynamic study of BP interaction with flavonoids

The intermolecular interaction process between BP and flavonoids involves energy that can result from different acting forces, including electrostatic interactions, hydrogen bonds, van der Waals bonds and hydrophobic interactions (MALEKI et al., 2020). The determination of thermodynamic binding parameters contributed to elucidate which molecular processes are predominant in the formation of the thermodynamically stable BP-flavonoid^0 complex. In that sense, it is well known that the relationship between the kinetic constants (k_a and k_d) described in Eq. 7 allows to determine the thermodynamic binding constant (K_b, L.mol^{-1}).

$$K_b = k_a/k_d \tag{7}$$

The dependence of K_b on temperature allows to determine the forces driving the BP-flavonoid interaction process through thermodynamic parameters such as the standard Gibbs free energy change (ΔG^0), standard entropy change (ΔS^0) and standard enthalpy change (ΔH^0). Firstly, the ΔH^0 values were obtained from the Van't Hoff equation (Eq. 8), followed by plotting the graph ln K_b *versus* 1/T (Fig. S6 in Supporting Information).

$$\frac{lnK_{b2}}{lnK_{b1}} = -\frac{\Delta H^0}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{8}$$

Where R is the universal gas constant (8.3145 J. mol^{-1}.K^{-1}) and T is the temperature studied. Then, the ΔG^0 and ΔS^0 parameters were obtained using Eqs. (9) and (10), respectively:

$$\Delta G^0 = -RTlnK_b \tag{9}$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{10}$$

Where, ΔH^0 is the standard enthalpy change (kJ.mol^{-1}), ΔG^0 is the standard Gibbs free energy change (kJ.mol^{-1}) and ΔS^0 is the standard entropy change (kJ.mol^{-1}.K^{-1}). The thermodynamics parameters of complex formation are presented in Table 3.

Table 3. Standard changes in Gibbs free energy (ΔG°), enthalpy (ΔH°), and entropy (TΔS°) for the formation of the BP-NR^0 or BP-NG^0 complex at different temperatures (T), at pH 7.4.

T	$[BP – NR]^0$				$[BP – NG]^0$			
	K_b	ΔH°	ΔG°	TΔS°	K_b	ΔH°	ΔG°	TΔS°
K	10^3 L.mol^{-1}		kJ.mol^{-1}		10^3 L.mol^{-1}		kJ.mol^{-1}	
289.15	101.52		-27.71	-50.83	5.94	-44.61	-20.89	-23.72
293.15	67.13		-27.09	-51.45	4.94	-21.91	-20.73	-1.18
297.15	43.25	-78.54	-26.37	-52.17	4.57	-8.02	-20.82	12.80
298.15	39.26		-26.22	-52.32	4.53	-5.85	-20.87	15.02
301.15	27.47		-25.59	-52.95	4.46	-2.27	-21.04	18.76

Negative values of ΔG° indicate that the formation of [BP-flavonoid]^0 complexes was favored in thermodynamic equilibrium in relation to free molecules. Therefore, the NR ($\Delta G°_{298.15\ K}$ = - 26.22 kJ.mol^{-1}) formed more stable complexes with BP compared to NG ($\Delta G°_{298.15\ K}$ = - 20.87 kJ.mol^{-1}).

At equilibrium, the number of [BP-NR]^0 complexes formed is 17 times greater than [BP-NG]^0, at 289.15 K. On the other hand, as the temperature increased (289.15 to 301.15 K) this difference was 6 times, since K_b values for [BP-NR]^0 decreased ~ 73% while K_b values for [BP-NG]^0 decreased ~ 25%. These results show that NR has a higher binding affinity with BP than NG. The structural difference between NR and NG is due to the presence of a rhamnoglycoside group on carbon 7 of NR (Fig. 1), which probably contributed in some way to increase this affinity with BP. The analysis of the enthalpic (ΔH°) and entropic (TΔS°) components related to the [BP-flavonoid]^0 interaction can elucidate this behavior.

The BP-NR interaction was an exothermic process ($\Delta H° = -78.54$ kJ.mol^{-1}), whose enthalpic energy was independent of the temperature variation and the entropy of the system reduced with the formation of the complex (~ -52 kJ.mol^{-1}). This behavior suggests that the formation of the [BP-NR]^0 complex occurred predominantly through the release of energy from the formation of hydrophilic interactions ($\Delta H°_{int} < 0$), such as hydrogen bonds and van der Walls interactions, between the flavonoid and amino acids hydrophilic compounds present in the surface region of the protein. Electrostatic interactions are also hydrophilic, but do not contribute to the BP-NG interaction, since the isoelectric point of BP is close to 4.8 (CRUZ et al., 2011) and the pKa of NR is 8.83 (GOLLAVILLI et al., 2020), that is, the flavonoid is neutral at the pH used in the study (pH 7.4) while the protein is negatively charged. This binding process does not require extensive conformational changes that would cost large amounts of enthalpic energy ($\Delta H°_{conf} > 0$). In addition, the small entropy variation as the temperature increased also corroborates this hypothesis.

On the other hand, the driving forces driving the formation of the [BP-NG]^0 complex were temperature dependent. At lower temperatures, enthalpic energy was released ($\Delta H° = -44.61$ kJ.mol^{-1}) from the BP-NG interaction and entropy reduction ($T\Delta S° = -23.72$ kJ.mol^{-1}). With increasing temperature, the energy barrier to protein conformational changes was overcome and the flavonoid accessed more hydrophobic sites in BP. In this condition, the binding process became entropically directed ($T\Delta S° = 18.76$ kJ.mol^{-1}), due to the release of water molecules to the bulk of the system, with a very small negative enthalpic energetic contribution ($\Delta H° = -2.27$ kJ.mol^{-1}) as a function to increased energy cost with conformational changes and desolvation of interacting molecules ($\Delta H°_{conf} > 0$ and $\Delta H°_{dess} > 0$). Therefore, the increase in entropy of the system was compensated by the reduction in enthalpy, demonstrating that the formation of the [BP-NG]^0 complex occurs through the phenomenon of enthalpic-entropic compensation (Fig. S7. in Supporting Information). This phenomenon justifies the small variation in the total energy of the system with temperature variation ($^{d\Delta G°}/_{dT} \cong 0$) despite the enthalpic and entropic energies showing great changes.

The formation process of the [BP-NG]^0 complex was accompanied by a considerable variation in enthalpic energy as the temperature increased. This

behavior suggests that there were variations in the transfer of energy in the form of heat to the potential energy components (Rezende et al., 2020). To verify these changes, we calculate the standard heat capacity parameter (ΔC_p^0). The relationship between ΔH° and T in Eq. (11) allows obtaining the ΔC_p^0) from the polynomial fitting of the ΔH° *vs*. T plot (Fig. S8. in Supporting Information).

$$\Delta C_p^0 = \frac{\partial \Delta H^0}{\partial T} \tag{11}$$

The ΔCp^0 values were temperature dependent, ranging from 6.69 to 0.35 $kJ.mol^{-1}.K^{-1}$ (Table S1, in Supporting Information). The ΔCp^0 provides information on the magnitude of the forces involved in the BP-NG interaction process, related to structural changes and energy from the solvation and desolvation events of the interacting molecules (PAIVA et al., 2020). The ΔCp^0 represents the difference between the standard heat capacity of the $BP\text{-}NG^0$ complex ($Cp^0_{BP-NG^o}$) and standard heat capacity of free molecules ($Cp^0_{BP} + Cp^0_{NG}$) ($\Delta Cp^0 = Cp^0_{BP-NG} - (Cp^0_{BP} + Cp^0_{NG})$). At all studied temperatures ΔCp^0 exhibited positive values, indicating that the magnitude and number of intermolecular interactions formed in the complex are greater than what existed when the molecules were free, i.e. as temperature increased energy in the form of heat was transferred to potential form. This can be explained by the desolvation process that occurred for the $[BP\text{-}NG]^0$ complex to be formed, since the water-water interactions are stronger than the water-flavonoid and water-amino acid residues interactions (COELHO et al., 2019; PAIVA et al., 2020).

Nunes et al. (2019) studied the LF-NR interaction by SPR and HUANG et al., (2018) evaluated the formation of the $[LF\text{-}NG]^0$ complex using the fluorescence spectroscopy technique and obtained behaviors similar to those observed in the present study. The K_b for LF-NR ($K_{b\ (LF\text{-}NR)}$ = 91.90 x 10^3 $L.mol^{-1}$) was higher than that observed for LF-NG ($K_{b\ (LF\text{-}NG)}$ = 30.50 x 10^3 $L.mol^{-1}$), at temperatures ~ 300 K. Furthermore, the interaction LF-NR was enthalpically driven (ΔH° = -141.19 $kJ.mol^{-1}$; TΔS° = -112.67 $kJ.mol^{-1}$) while NG interacted with LF by increasing the entropy of the system (ΔH° = 3.57 $kJ.mol^{-1}$; TΔS° = 22.38 $kJ.mol^{-1}$). LF is a glycoprotein, therefore the presence of the glycosidic group of the protein contributed to intensify the hydrophilic interactions with the NR and to make the enthalpic energy more negative when compared to the BP-NR interaction. On the other hand, when LF interacted

with NG, there was a greater expenditure of energy for the desolvation of the interacting molecules so that the flavonoid could access the protein's hydrophobic sites.

4. Conclusions

The data obtained by SPR demonstrated that BP and flavonoids, NR and NG, interact resulting in the formation of a thermodynamically stable complexes, at pH 7.4. However, the hydrophilicity of the flavonoid influenced stability, with NR containing a hydrophilic carbohydrate group forming a more stable complex ($\Delta G°_{BP-NR^0}$ = ~ -26 kJ.mol^{-1}) with BP than NG ($\Delta G°_{BP-NG^0}$ = ~ -20 kJ.mol^{-1}). Furthermore, in chemical equilibrium, the number of [BP-NR]^0 complexes was greater than [BP-NG]^0, indicating greater affinity of NR with BP. For the formation of the [BP-NR]^0 complex, the hydrophilic interactions were determinant regardless of the temperature at which the interaction occurred. On the other hand, at low temperatures, the formation of the [BP-NG]^0 complex was enthalpically driven, but as the temperature increased, the flavonoid accessed more hydrophobic sites of the protein leading to an entropic gain that also contributed to the formation of the complex.

The association rate between NR and BP was faster than NG, however, increasing temperature accelerated the dissociation process for the [BP-NR]^0 and slowed it down for [BP-NG]^0. Furthermore, the formation of the intermediate complex from the association of BP with NR was also faster than with NG ($\Delta G_a^{\ddagger}$ from 45.47 to 50.12 kJ.mol^{-1} and $\Delta G_a^{\ddagger}$ from 51.57 to 54.80 kJ.mol^{-1}, respectively), indicating that the presence of the glycoside group accelerates the thermodynamically stable complex formation process and the formation of the intermediate complex obtained from the association of free molecules.

The use of SPR provided the complete characterization of the BP-flavonoid intermolecular interactions, showing that baru proteins are macromolecules with potential to be applied as plant-based carriers for flavonoids. Kinetic and thermodynamic data provide useful information to understand and modulate the physicochemical properties of these nanostructures in order to optimize their insertion in food matrices.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supporting Information

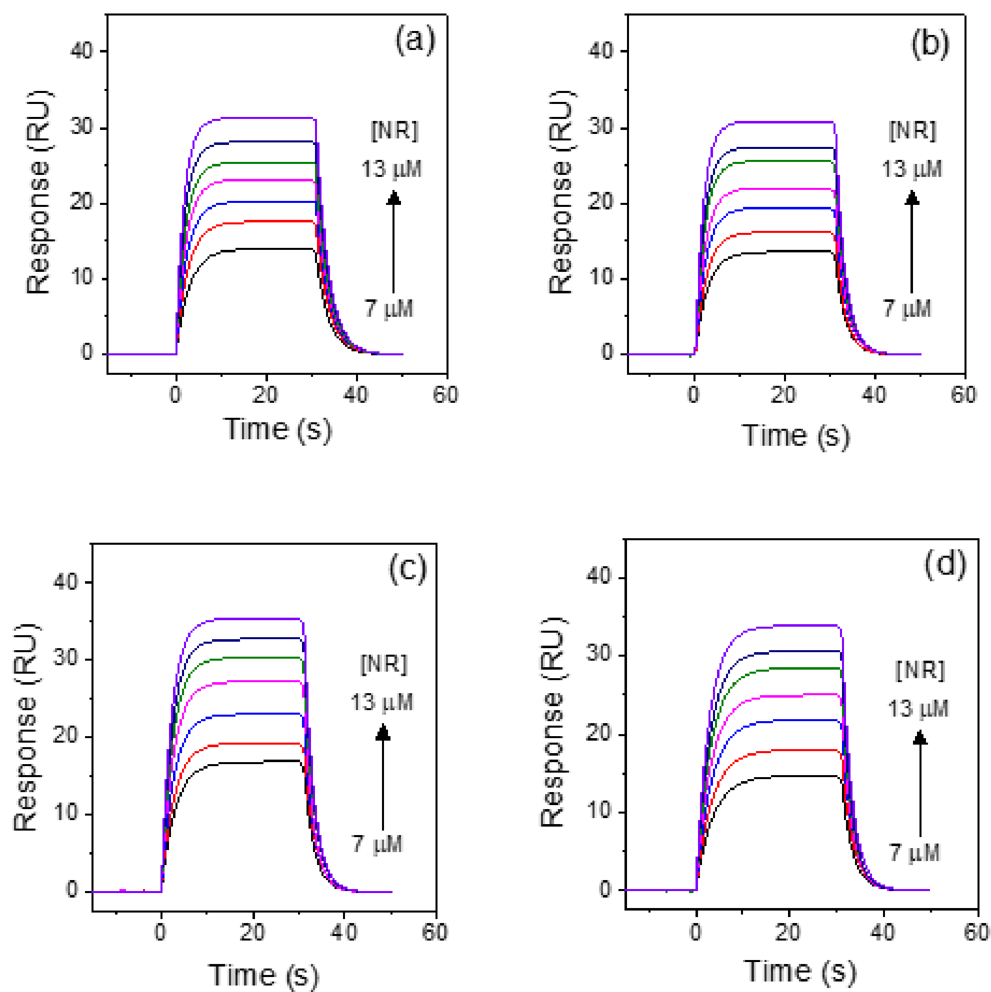

Fig. S1. SPR sensorgrams for naringin (NR) interactions with the CM5 low-density Baru protein-immobilized sensor-chip surface (3061 RU) at four different temperatures (a) 289.15 K, (b) 293.15 K, (c) 297.15 K, and (d) 301.15 K. The arrows indicate increasing NR concentration (7, 8, 9, 10, 11, 12 and 13 μM).

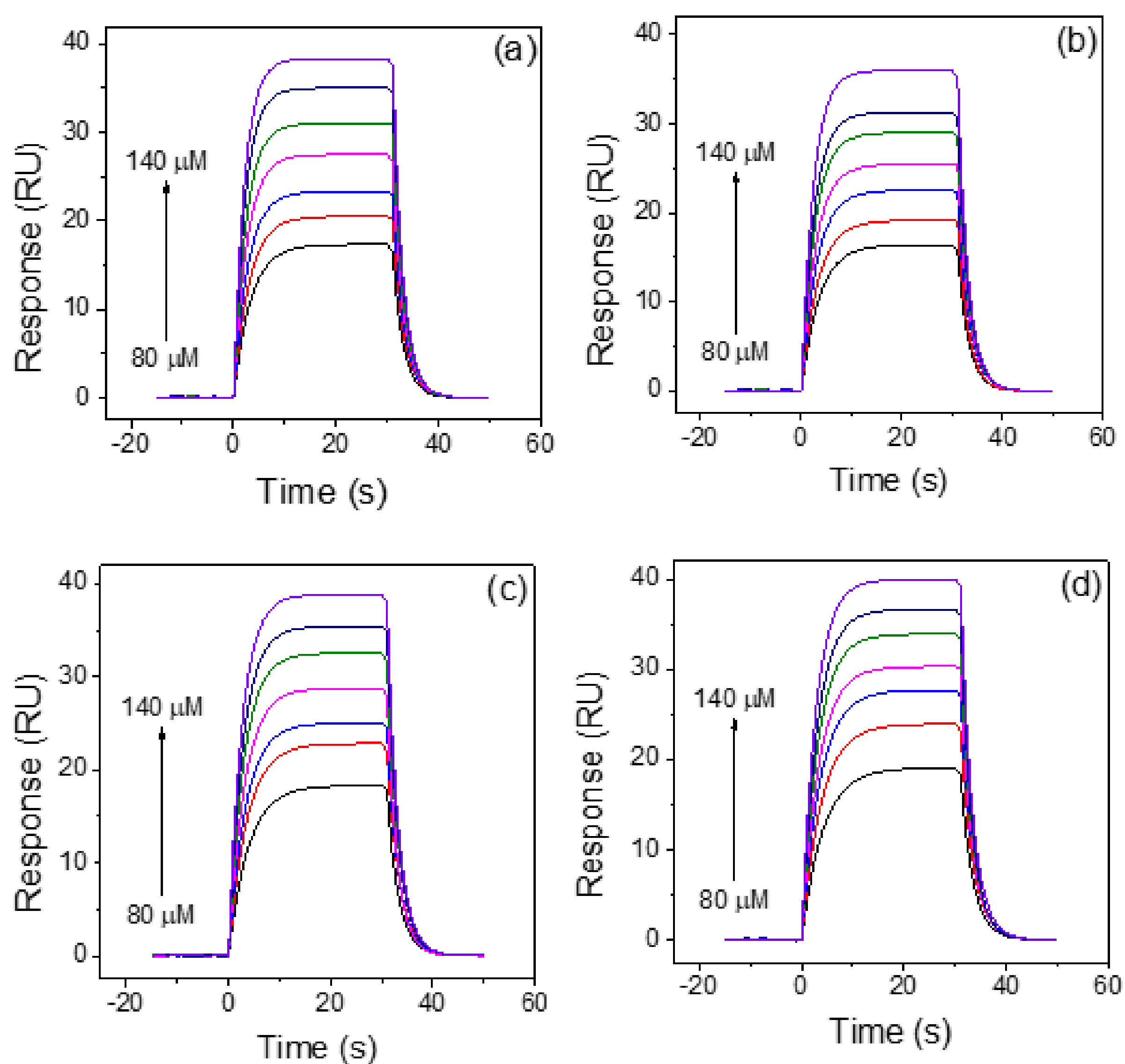

Fig. S2. SPR sensorgrams for naringenin (NG) interactions with the CM5 low-density Baru protein-immobilized sensor-chip surface (3061 RU) at four different temperatures (a) 289.15 K, (b) 293.15 K, (c) 297.15 K, and (d) 301.15 K. The arrows indicate increasing NG concentration (80, 90, 100, 110, 120, 130 and 140 µM).

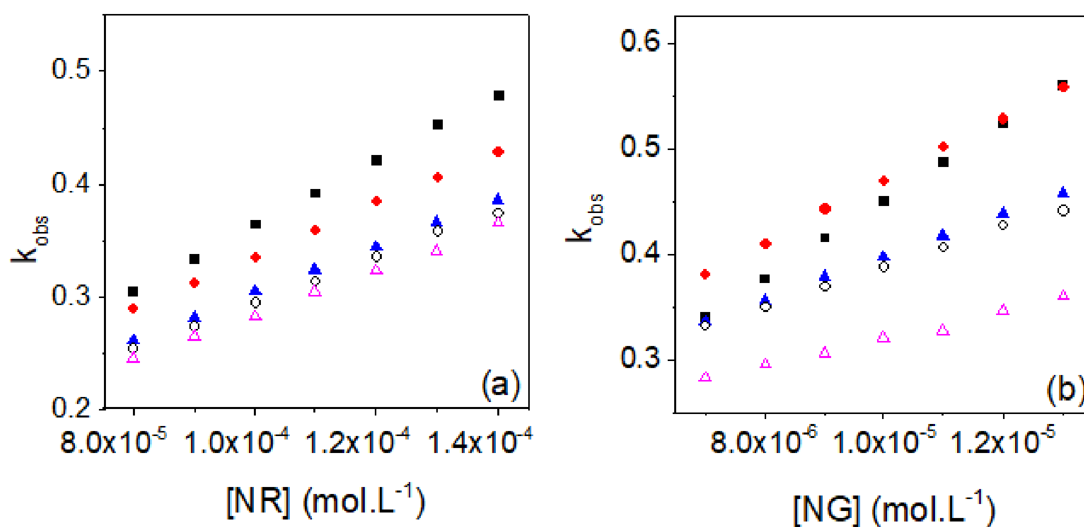

Fig.S3. The Plot of k_{obs} as a function of a) naringin (NR) and b) naringenin (NG) concentration used to determine k_a at temperatures: (■) 289.15 K, (●) 293.15 K, (▲) 297.15 K, (○) 298.15 K, and (Δ) 301.15 K. (a) BP-NR, and (b) BP-NG.

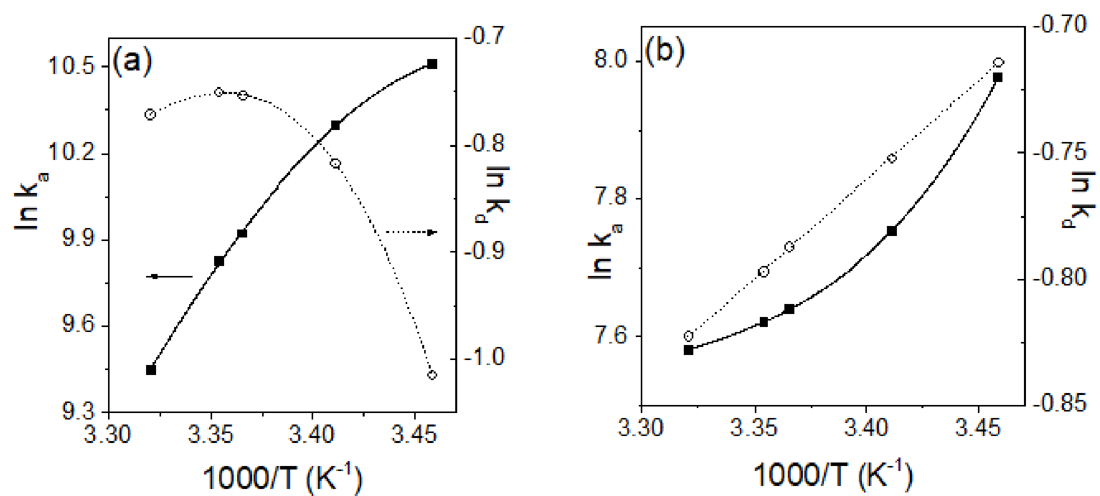

Fig. S4. The Arrhenius plots for the association (k_a, ■) and dissociation (k_d, ○) process of the interactions: (a) Baru protein-naringin and (b) Baru protein-naringenin.

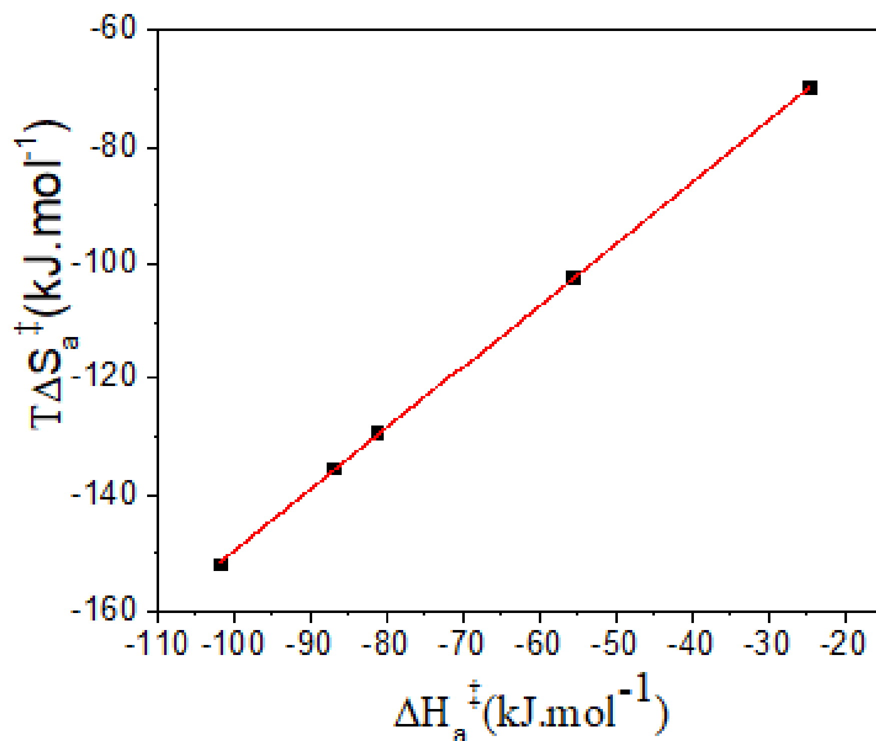

Fig.S5. Enthalpy-Entropy compensation plot for the formation of Baru protein (BP) – naringin (NR)‡ complex via the association between BP and NR free molecules

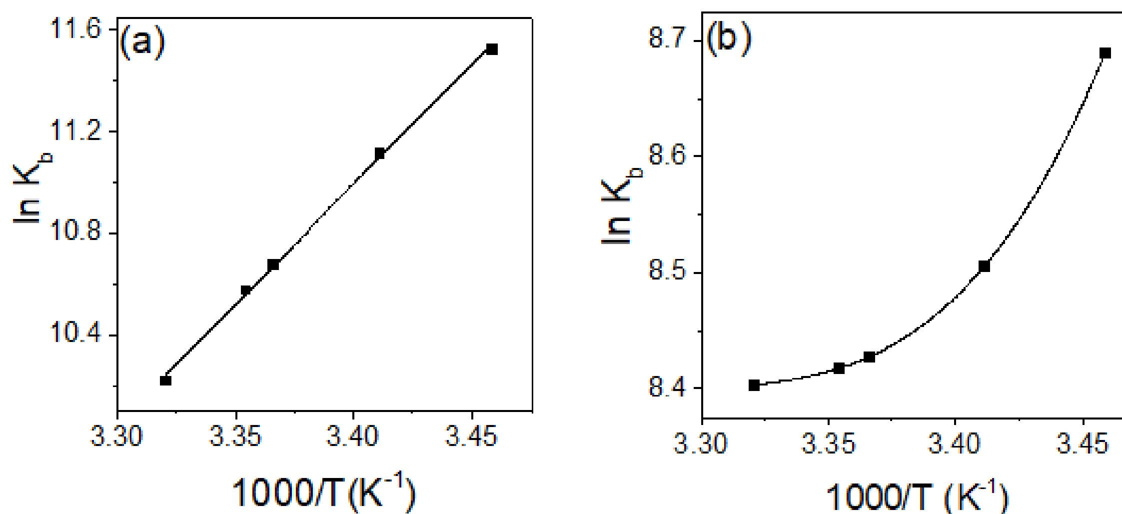

Fig. S6. The van't Hoff plots of Baru protein interaction with (a) naringin (NR) and (b) naringenin (NG).

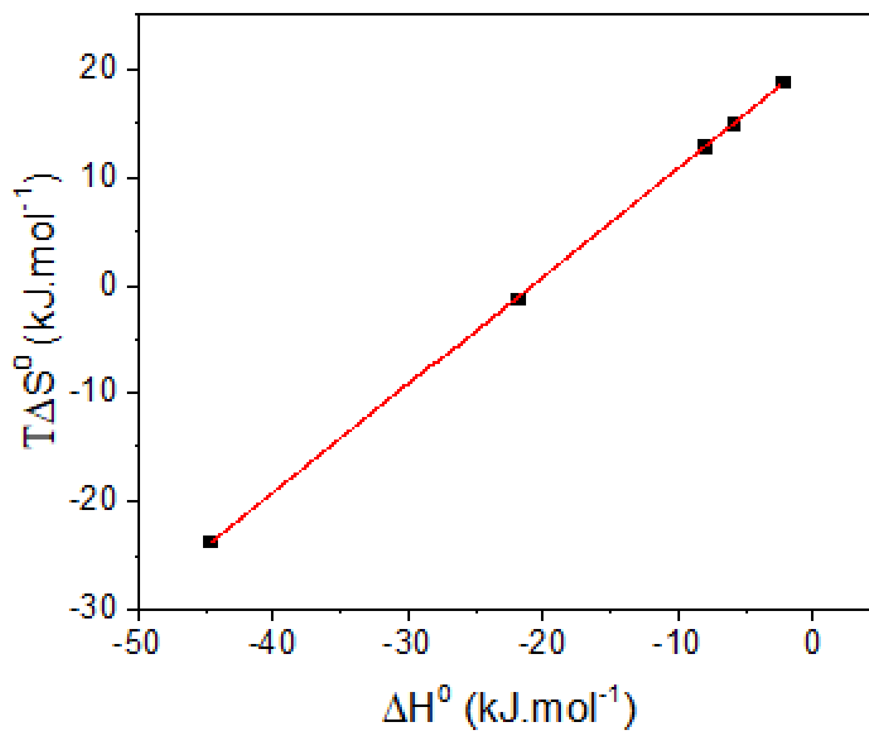

Fig. S7. Enthalpy-Entropy compensation plot for Baru protein (BP) – naringenin (NG) binding at pH 7.4.

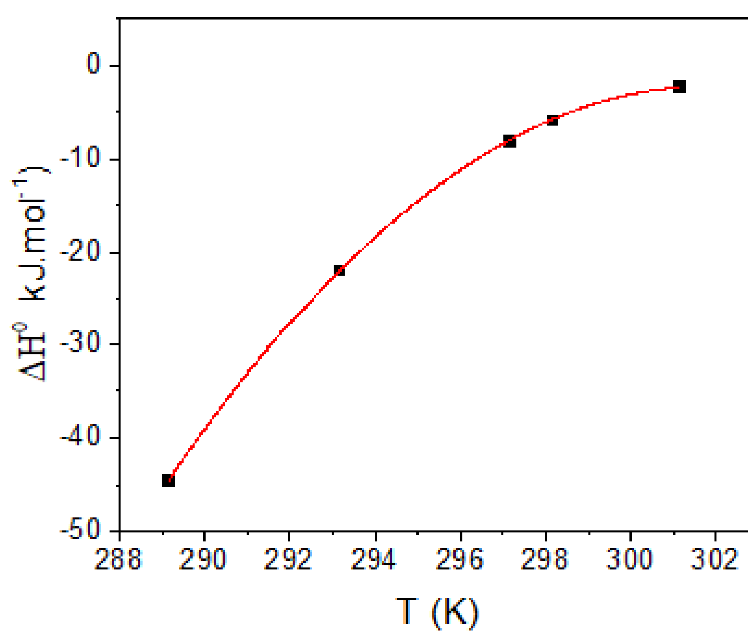

Fig. S8. Temperature dependence of ΔH^0 values for BP-NG binding.

Table S1. Heat Capacity change (ΔCp^0) associated with BP-NG^0 complex formation.

T	ΔC_p^0
K	$kJ.mol^{-1}.K^{-1}$
289.15	6.69
293.15	4.57
297.15	2.46
298.15	1.93
301.15	0.35

6. Conclusões gerais

O surgimento da demanda de produtos *plant-based,* impulsionado pela adoção de novos hábitos alimentares, tem contribuído para a exploração de proteínas de fontes vegetais alternativas, visando o aumento da biodiversidade e diversificação de produtos. A amêndoa do Baru contém alta concentração proteica de excelente qualidade nutricional, porém, a sua utilização como carreadora de moléculas bioativas ainda não foi explorada, tornando necessária a execução do presente trabalho. Moléculas bioativas como a naringina (NR) e naringenina (NG) apresentam limitações para sua inserção em matrizes alimentícias e no desempenho de suas propriedades funcionais, sendo necessário o uso de estratégias para aumentar a sua biodisponibilidade e facilitar sua incorporação em sistemas alimentares, através da formação de complexos com BP.

Por meio da técnica de ressonância plasmônica de superfície (RPS) foi observado que BP é capaz de formar complexo termodinamicamente estável com a NR e NG. A NR apresenta um grupo glicosídeo ligado ao carbono 7 do anel C, responsável por aumentar a estabilidade do complexo termodinamicamente estável. Além disso, no equilíbrio químico, NR forma mais complexos com BP do que NG, indicando maior afinidade da NR com BP.

A taxa de associação entre NR e BP foi mais rápida que NG. Ademais, a formação do complexo intermediário a partir da associação de BP com NR também foi mais rápida do que com NG, indicando que a presença do grupo glicosídeo acelera o processo de formação do complexo intermediário obtido a partir da

associação de moléculas livres. Portanto, foi observado que a característica mais hidrofílica da NR pela presença do grupo glicosídeo influenciou o mecanismo de ligação, a estabilidade e a velocidade da formação do complexo.

Em geral, a caracterização completa das interações intermoleculares BP-flavonoides contribui para a melhor compreensão dos mecanismos de interação, possibilitando o aumento de sua biodisponibilidade e o seu desempenho de suas propriedades funcionais.