RICHARD MARINS DA SILVA

CASEÍNA MICELAR COMO NANOCARREADOR PARA CURCUMINA

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.*

VIÇOSA
MINAS GERAIS – BRASIL
2017

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
S586c
2017

Silva, Richard Marins da, 1991-
Caseína micelar como nanocarreador para curcumina / Richard Marins da Silva. – Viçosa, MG, 2017.
vii, 52f. : il. (algumas color.) ; 29 cm.

Orientador: Ana Clarissa dos Santos Pires.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Corantes em alimentos. 2. Caseína. 3. Curcumina. 4. Fluorescência. 5. Termodinâmica. I. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-graduação em Ciência e Tecnologia de Alimentos. II. Título.

CDD 22 ed. 664.06

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APROVADA: 17 de julho de 2017.

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(Orientadora)

À minha avó.

AGRADECIMENTOS

À Deus, primeiramente, pela oportunidade oferecida, por sempre estar presente em todos os momentos da minha vida.

À minha avó, Constância, que sempre acreditou e apoiou todos os meus esforços.

À Universidade Federal de Viçosa e ao Departamento de Tecnologia de Alimentos, pela oportunidade de realização do mestrado.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de estudo.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), assim como ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pelo financiamento das pesquisas realizadas.

À professora Ana Clarissa dos Santos Pires, pela orientação, ensinamento, apoio, dialogo e confiança proporcionados.

Ao professor Luís Henrique Mendes da Silva pela coorientação, conselhos e colaboração.

À Professora Márcia Cristina T. Ribeiro Vidigal pela participação da banca de defesa.

Aos amigos do laboratório de Termodinâmica Molecular Aplicada – Therma, que me acolheram e ajudaram em todos os momentos (Carini, Juninho, Eliara, Otávio, Nathália, Jacqueline, Ana Flávia).

Ao laboratório de embalagens do Departamento de Tecnologia de Alimentos por disponibilizar o uso de equipamentos sempre que necessário.

À todos meus amigos de Goiás: Bheatriz, Tainara e Jessyca.

À todos meus amigos de Minas Gerais, em especial ao meu grande amigo Otávio Lauriano.

À todos os colegas de Pós-Graduação pela agradável convivência.

À todos, que de alguma forma, foram importantes para a realização deste trabalho.

SUMÁRIO

RESUMO

SILVA, Richard Marins, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Caseína micelar como nanocarreador para curcumina**. Orientadora: Ana Clarissa dos Santos Pires. Coorientador: Luis Henrique Mendes da Silva.

A curcumina é um composto fenólico presente no açafrão, com múltiplas atividades biológicas, incluindo anti-câncer, anti-microbiana, anti-inflamatória e antioxidante além de ser um corante natural. Neste estudo, a interação entre a caseína micelar e a curcumina foi investigada em pH 6,6. Através da análise de UV-Vis e fluorescência, demonstrou-se que a interação entre a caseína micelar e curcumina ocorre por um mecanismo predominantemente estático. A constante de ligação variou de $1.28x10^{-5}$ $L.mol^{-1}$ a $9.52x10^{-4}$ $L.mol^{-1}$ para a faixa de temperatura de 295.15 - 323.15 K. A estequiometria de formação de complexo foi aproximadamente unitária pela análise de fluorescência. De acordo com os resultados obtidos pela análise termodinâmica, a interação entre caseína micelar e curcumina é entalpicamente e entropicamente dirigida, através de um processo espontâneo. Ao realizar uma experiência de partição, a presença de concentrações crescentes de caseína micelar na fase aquosa aumentou o número de moléculas de curcumina transferidas da fase orgânica. O diâmetro médio da micela de caseína na presença de curcumina foi de cerca de 290 nm e o potencial zeta, ~ -16 mV. A micela de caseína foi capaz de proteger a curcumina contra a degradação térmica. Os resultados deste estudo contribuem para o conhecimento do processo de interação entre a caseína micelar e a curcumina para futuras aplicações em diversas áreas da indústria de alimentos.

ABSTRACT

SILVA, Richard Marins, M.Sc., Universidade Federal de Viçosa, July, 2017. **Micellar casein as a nanocarrier for curcumin**. Adviser: Ana Clarissa dos Santos Pires. Co-adviser: Luis Henrique Mendes da Silva.

Curcumin is a phenolic compound present in turmeric, with multiple biological activities, including anti-cancer, anti-microbial, anti-inflammatory and antioxidant in addition to natural dye. In this study, the interaction between micellar casein and curcumin was investigated at pH 6.6. Through the analysis of UV-Vis and fluorescence, it was demonstrated that the interaction between micellar casein and curcumin occurs by a predominantly static mechanism. The binding constant ranging from $1.28x10^{-5}$ $L.mol^{-1}$ to $9.52x10^{-4}$ $L.mol^{-1}$ for the temperature range of 295.15 - 323.15 K. The complex-forming stoichiometry was approximately unit by fluorescence analysis. According to the results obtained by the thermodynamic analysis, the interaction between micellar casein and curcumin is enthalphically and entropically directed, through a spontaneous process. By performing a partition experiment, the presence of increasing concentrations of micellar casein in the aqueous phase increased the number of curcumin molecules transferred from the organic phase. The mean diameter of the casein micelle in the presence of curcumin was around 290 nm and the zeta potential, ~ -16 mV. The casein micelle was able to protect curcumin against thermo-degradation. The results of this study contribute to the knowledge of the interaction process between micellar casein and curcumin for future applications in various areas of the food industry.

Capítulo 1

1. Introdução

Os aditivos alimentares fazem parte da composição de alimentos processados, sendo os corantes alimentares um dos principais constituintes das formulações. Os corantes são adicionados a fim de tornar o alimento mais atraente ao consumidor, aprimorar a cor já existente que pode se degradar durante o processamento e/ou armazenamento ou até mesmo, atribuir novas cores ao produto. Esses corantes são divididos entre naturais e sintéticos, sendo os sintéticos mais utilizados.

Alguns corantes vêm sendo estudados por serem compostos naturais e por apresentarem efeitos benéficos especialmente relacionados à saúde do consumidor. A curcumina por exemplo, molécula oriunda do açafrão, é um corante natural muito utilizado na culinária, além de apresentar capacidade antioxidante, antimicrobiana, anti-inflamatória e anticâncer. Entretanto, o emprego desses compostos naturais ainda é limitado, em razão da sua instabilidade à luz, pH alcalino e elevadas temperaturas e, além disso, apresentam baixa solubilidade em meio aquoso.

O estudo de métodos para incorporar essas moléculas em formulações alimentícias, protegendo-as de fatores adversos que poderiam degradá-las bem como o aumento da sua solubilidade, são de extrema importância e necessários. As proteínas do leite, são consideradas excelentes carreadores naturais de componentes. Essas proteínas possuem alto valor nutricional, boas propriedades sensoriais e tecnológicas como estabilizante, emulsificante e na formação de espumas. O balanço de interações hidrofílicas/hidrofóbicas que essas proteínas fazem, podem torná-las um veículo capaz de interagir com diversas moléculas com diferentes graus de intensidade e especificidade, podendo então transportar essas moléculas em diferentes matrizes alimentícias.

Muitos trabalhos têm sido realizados na área de carreamento de compostos, mas são poucos os que utilizam a caseína micelar como veículo de compostos de interesse em alimentos, bem como os que tratam da termodinâmica de interação entre caseína micelar e curcumina. O estudo da interação entre caseína micelar e a curcumina por meio da análise termodinâmica será uma grande fonte de informação a respeito da formação

deste complexo, além do conhecimento sobre a estequiometria de carreamento, tamanho, potencial zeta da caseína micelar na presença de curcumina e estabilidade térmica da curcumina.

2. Objetivos

2.1 Geral

- Estudar a termodinâmica de interação entre caseína micelar e o corante curcumina em diferentes temperaturas e pH 6.6.

2.2 Específicos

- Estudar a influência temperatura nas interações entre caseína micelar e curcumina, determinando a constante de interação (K_a), estequiometria de formação de complexo (*n*) e os parâmetros termodinâmicos de formação de complexo ($\Delta G°$, $\Delta H°$ e $\Delta S°$ (variação da energia livre de Gibbs, entalpia e entropia padrão de formação do complexo, respectivamente));
- Determinar a influência da concentração de curcumina no tamanho e no potencial zeta dos complexos caseína micelar-curcumina formados;
- Avaliar a estabilidade à temperatura da curcumina livre e complexada com a caseína micellar;
- Determinar a estequiometria de formação de complexo (*n*) através da transferência de moléculas entre duas fases.

3. Revisão da Literatura

3.1 Micelas de Caseínas: Estrutura e Função

O leite bovino contém em torno de 3,3 m/v de proteínas totais e as caseínas, representam aproximadamente 80% desse total (Farrell et al., 2004). Elas são proteínas que precipitam em pH 4,6 e são consideradas intrinsicamente desordenadas. No leite, as caseínas formam partículas coloidais altamente hidratadas conhecidas como micela. A micela de caseína é uma associação de frações formada dentro das células secretoras das glândulas mamárias das quatro parcelas de proteínas anfifílicas, κ-, αs1-, αs2, e β-caseína. A fosforilação das caseínas é essencial para ligação de Ca^{2+} e associação das frações (Kjeldsen, Savitski, Nielsen, Shi, & Zubarev, 2007), que ocorre nos resíduos de serina das frações sensíveis ao cálcio, αs1-αs2- e β-caseína, proporcionando um local para a ligação de cálcio e a subsequente formação de nanopartículas de fosfato de cálcio que servem para ligar as regiões hidrofílicas das frações e ajudar na estabilidade global da micela de caseína (Farrell, Malin, Brown, & Qi, 2006).

Muitos modelos para a estrutura das micelas de caseína têm sido propostos e ainda, no que diz respeito às interações envolvidas na sua montagem e manutenção da integridade destas partículas, controvérsia entre os pesquisadores permanece. No entanto, há um consenso geral de que as caseínas são mantidas por um equilíbrio entre interações eletrostáticas e hidrofóbicas (Horne & Horne, 2006).

O tamanho da micela de caseína encontrado na literatura é variado, isto é, elas são altamente poli-dispersas. A distribuição de tamanhos varia de 80 – 800 nm, com um diametro médio de 200 nm (Dalgleish, 2011). Contudo, o tamanho das micelas de caseina ainda é muito discutido, alguns autores relataram populações de mini-micelas, com diametro entre 20 e 60 nm, enquanto outros consideram seu tamanho entre 20 e 40 nm (Müller-Buschbaum, Gebhardt, Roth, Metwalli, & Doster, 2007). O tamanho da micela ainda é contestado, variando de acordo com a técnica analítica utilizada.

A micela de caseína é constituída por quatro frações principais de proteína: αs1-, αs2-, β- e κ-caseína, representando cerca de 38%, 10%, 35% e 15%, respectivamente, as quais são constituídas por 199, 207, 209 e 169 resíduos de aminoácidos, com massas moleculares de 23, 25, 24 e 19 kDa,

respectivamente (VEGA, 2009). Além disso, cerca de 6% da massa, em base seca, da micela de caseína é composta por fosfato de cálcio coloidal (Dalgleish, 2011).

De um ponto de vista industrial, tecnológico e de produtos lácteos, as caseínas são, de longe, o componente mais importante e valioso do leite. Os principais produtos lácteos como leite líquido, queijo e iogurte derivam suas propriedades texturais, sensoriais e nutricionais das caseínas (De Kruif, Huppertz, Urban, & Petukhov, 2012). Para o recém-nascido, elas são facilmente metabolizadas e carregam substanciais quantidades dos elementos importantes de nutrição cálcio, magnésio e fósforo (como fosfato). A sua hidratação e fortes interações entre elas e com vários outros componentes, tornaram-nas valorizados ingredientes em alimentos (Farrell, 2011).

3.2 Corantes

A cor é um caráter sensorial muitas vezes esquecido, que certamente influencia a percepção de sabor. Para manter ou restabelecer a uniformidade da cor do produto, agentes, considerado mundialmente como aditivos alimentares de coloração, são intencionalmente adicionados aos produtos alimentares (Solymosi, Latruffe, Morant-Manceau, & Schoefs, 2015).

Os consumidores exigem alimentos que são atraentes e apetitosos. Tal fatores estimulam o apetite e sistema digestivo e contribuem para o prazer de comer e beber. A cor é frequentemente associada com qualidade. Por exemplo, tomates verdes e bananas seriam rejeitados pelos consumidores como sendo imaturos pelo fato de serem de cor verde. Muitos alimentos são produtos sazonais e, como tal, estão sujeitos a variação em cor (por exemplo, produtos lácteos). Tal variação da cor pode ser minimizada pela adição de corantes alimentares para o produto para produzir um material uniforme, durante todo o tempo de prateleira (Damant, 2011).

Segundo a FDA, um corante alimentar é: “qualquer corante, pigmento ou substância que, quando adicionado ou aplicado a um alimento, medicamento ou cosmético, ou para o corpo humano, é capaz (isoladamente ou através de reacções com outras substâncias) de transmitir cor” (“Food and Drug Administration,” 2010).

Não é de hoje que várias condições externas, tais como condições de luz, ar, temperatura, umidade e armazenamento desempenham um papel crucial na perda de cor dos alimentos (Cejudo-Bastante, Hurtado, Mosquera, & Heredia, 2014). Assim, corantes alimentares são aplicados principalmente para compensar e superar essas características desagradáveis, como também para homogeneizar a cor dos alimentos, através da correção de variações de cor e/ou melhoria dos que ocorrem naturalmente cor dos alimentos.

Corantes alimentares podem ser classificados de acordo com vários critérios: origem (natural, idêntico ao natural ou sintético; orgânico e inorgânico), solubilidade (solúveis e insolúveis) e de cobertura (transparente e opaco). No entanto, essas categorias muitas vezes se sobrepõem. A classificação mais utilizada é a distinção entre substâncias solúveis e insolúveis (Amchova, Kotolova, & Ruda-Kucerova, 2015):

3.2.1 Curcumina

A curcumina (Figura 1), uma das cores amarela naturais mais comuns, é derivada a partir da raiz da *Curcuma longa* L. com o constituinte principal coletivamente conhecido como curcumina, juntamente com pequenas quantidades de desmetoxicurcumina e bisdemetoxicurcumina (Mirjalili & Karimi, 2013) geralmente utilizado como especiaria e corante alimentar. Quimicamente, tem massa molar de em pH <1, as soluções aquosas de curcumina têm uma cor vermelha que indica a forma protonada. Na faixa de pH 1-7, a maioria das espécies está na forma neutra. A solubilidade em água é muito baixa nesta gama de pH e as soluções são amarelas. A pH> 7.5, a cor muda para vermelho. Os valores de pKa para a dissociação foram determinados como sendo de 7,8, 8,5 e 9,0, respectivamente (Stankovic, 2004). Apresenta baixa solubilidade em água, em pH acido e neutro, mas é solúvel em soluções aquosas básicas e em óleo (SUETH-SANTIAGO et al., 2015; STANKOVIC, 2004).

.

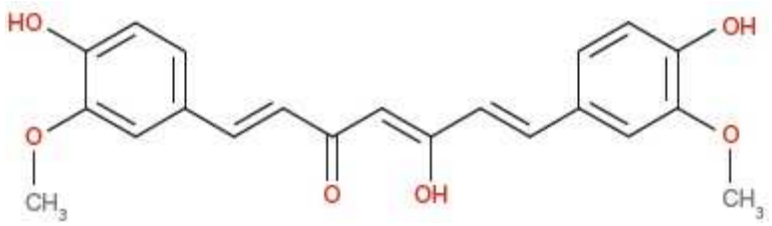

Figura 1: Estrutura química da curcumina

Corantes derivados de alimentos naturais, incluindo especiarias, frutas e legumes têm atraído recentemente grande interesse devido às suas atividades de promoção da saúde (Moussa, Hmadeh, Abiad, Dib, & Patra, 2016). Um aspecto importante sobre estudos com curcumina é sua utilização como substituto de corantes artificiais, tais como o amarelo #5 e amarelo #6, os quais já são conhecidos como um potencial alergênico, particularmente em crianças (Borrin, Georges, Moraes, & Pinho, 2016). Estudos recentes têm demonstrado que a curcumina tem um amplo espectro de atividades terapêuticas, incluindo antioxidantes, anti-inflamatória, anticâncer, antimicrobiano, cicatrização de feridas e a capacidade potencial de prevenção de doenças neuro-degenerativas (LI et al., 2016; LIU et al., 2016; LIU et al., 2015).

A curcumina é listada para uso em produtos lácteos, óleos e gorduras, emulsões, gelados comestíveis, frutas e produtos hortícolas, produtos de confeitaria, derivados de cereais, produtos de panificação, carne e produtos cárneos, peixe e seus derivados, ovos e seus derivados, temperos, sopas, molhos e produtos de proteína, gêneros alimentícios destinados a uma alimentação especial, bebidas e salgados. Níveis de utilização de curcumina está entre de 5 a 500 mg/kg (Stankovic, 2004), dependendo da categoria de alimentos. Apesar dos vários benefícios que esse corante natural apresenta, ele apresenta uma baixa biodisponibilidade, tendo uma fraca solubilidade em meio aquoso, hidrólise rápida seguida por fragmentação molecular em pH fisiológico (Lin, Pan, & Lin-Shiau, 2000), alcalino e na presença de luz (Stankovic, 2004).

3.3 Interação Proteínas e Corantes

O uso de proteínas como veículos naturais para compostos hidrofóbicos apresenta-se com grande potencial de aplicação na indústria de alimentos (Sekhon & Singh Sekhon, 2010). Há muitos aspectos sobre as proteínas que

estão diretamente relacionados com as suas interações com outras moléculas. As micelas de caseína por exemplo, são aglomerados proteicos de estrutura aberta e de natureza anfifílica, que contêm numerosos resíduos de prolina distribuídos uniformemente ao longo da sua estrutura, proporcionando maior interação com compostos fenólicos (Faridi Esfanjani & Jafari, 2016). Estas interações conferem a capacidade de atuar como carreadores de compostos fenólicos e de outros diferentes compostos de natureza hidrofóbica, por exemplo.

As proteínas do leite são veículos naturais, que evoluíram para entregar micronutrientes essenciais (por exemplo, de cálcio e fosfato) e blocos de construção (por exemplo, aminoácidos), bem como os componentes do sistema imune (por exemplo, imunoglobulinas e lactoferrina), da mãe para o recém-nascido. Algumas delas (por exemplo, β-lactoglobulina e albumina do soro de bovino) podem interagir com uma variedade de pequenas moléculas (LIVNEY, 2010; LELIS et al., 2017;ZHANG; ZHONG, 2012).

As nanoestruturas de proteína têm a capacidade de interagir com uma grande variedade de produtos, em resposta a alterações de condições ambientais, além de proteger os compostos sensíveis da degradação e controlar a sua biodisponibilidade (Ramos et al., 2015). As interações entre proteínas e outros compostos são dirigidas e estabilizadas de forma reversível por interações eletrostáticas, hidrofóbicas, pontes de hidrogênio e forças de van der Walls, e muitas vezes ocorrem de forma espontânea. Os parâmetros de ligação vão depender da natureza química do ligante, condições do meio e a conformação da proteína (Tavares, Croguennec, Carvalho, & Bouhallab, 2014).

A complexação entre proteínas e compostos fenólicos (especialmente polifenóis) tem atraído o interesse da indústria de alimentos, uma vez que pode fornecer uma estratégia eficaz para melhorar a prestação e biodisponibilidade de muitos compostos ativos insolúveis em água, como a curcumina (Livney, 2010).

Muitos trabalhos têm sido realizados a respeito do uso de proteínas como veículos de compostos em alimentos afim de determinar os parâmetros de interação com ligantes. Ying et al. (2015) realizaram estudo de interação entre curcumina e pepsina através de técnicas espectrofotométricas, obtendo resultados relacionados ao tipo de mecanismo de interação (predominantemente dinâmico), constante de associação (10^5-10^7 $L.mol^{-1}$ com o aumento da temperatura), número de sítios de interação (~1) e elucidando o local de interação e as energias de interação; Penalva et al. (2015) utilizaram caseínas

como carreador de ácido fólico, encapsulando 25 µg de ácido fólico por mg de caseína, com uma eficiência de 40%, e prevenindo a degradação do mesmo em meio ácido e aumentando sua biodisponibilidade oral em 50% em relação a solução aquosa ; Nadi et al. (2014) estudaram a interação entre curcumina com BSA e caseínas, concluindo que a interação foi capaz de estabilizar a curcumina mediante fatores externos de degradação.

3.4 Métodos Instrumentais de Análise

Diversas técnicas são capazes de avaliar a interação intermolecular entre proteínas e corantes. Técnicas espectroscópicas são ferramentas úteis para obter informações importantes sobre as interações intermoleculares. Associação do ligante com as proteínas normalmente envolve mudanças nas interações intramoleculares e intermoleculares. As mudanças nas interações são refletidas na variação da absorção de energia, emissão de fluorescência, variação de entalpia e entropia de interação, que por sua vez determinam a variação da energia livre de interação.

3.4.1 Espectroscopia do UV-Vis

Os espectros de absorção de UV próximos aos resíduos de aminoácidos aromáticos em proteínas contêm informações abundantes relacionadas às conformações protéicas. A espectroscopia UV usa algoritmos para topografia de superfície molecular e para a acessibilidade de certos grupos de átomos ao solvente. Os algoritmos também permitem a análise da disposição tridimensional de átomos e grupos dentro do ambiente de grupos cromóforos. Os picos espectrais na faixa de 250-265 nm correspondem aos resíduos de fenilalanina e aqueles no atributo da região 265-280 nm às interações eletrônicas de tirosina-triptofano, enquanto que os picos acima de 285 nm são identificados exclusivamente como contribuições de triptofano (Wang, Sun, Pu, & Wei, 2017)

Medidas de absorção realizados em UV-VIS é um método simples para analisar mudanças estruturais complexos no estado fundamental (Xu et al., 2009). Alterações conformacionais da proteína irão conduzir às mudanças no microambiente e nos espectros de absorção de UV-Vis dos cromóforos. Se existir alguma diferença nos espectros de absorção de macromoléculas antes e

depois da adição de algum ligante, é considerado que a conformação da proteína foi alterada (H. Bi, Tang, Gao, Jia, & Lv, 2016). O sentido e a magnitude do deslocamento do espectro de absorção de um cromóforo indicará que este pode ter sido transferido para um ambiente mais hidrofílico ou hidrofóbico (Wen et al., 2009).

3.4.2 Espectroscopia de Fluorescencia

A fluorescência é a emissão de fótons devido à absorção de luz UV ou visível de cromóforos que podem emitir fótons. Os princípios da geração de fluorescência podem ser elucidados por um diagrama de Jablonski. Em geral, um espectrofluorímetro compreende seis componentes: uma fonte de luz, que normalmente é uma lâmpada de mercúrio ou xenônio para a emissão de luz UV ou visivel, um suporte de amostra, dois monocromadores e/ou filtro (s), com um para selecionar os comprimentos de onda de excitação e o outro para selecionar os comprimentos de onda de emissão, um detector para converter a luz emitida para o sinal eletrônico e uma unidade de aquisição de dados (Wang et al., 2017).

As proteínas contem três resíduos de aminoácidos que contribuem com sua fluorescência intrínseca: triptofano, tirosina e fenilalanina (Lakowicz, 2006). A micela de caseína possui vários desses resíduos de aminoácidos (30000 – 225000 residuos de triptofano) que vão permitir o estudo de interações entre ligantes e a micela por meio da técnica de espectroscopia de fluorescência. A extinção de fluorescência é qualquer processo que resulta numa diminuição da intensidade de fluorescência de um fluoróforo. Uma variedade de interações moleculares pode resultar em extinção, incluindo reações no estado excitado, rearranjos moleculares, transferência de energia, a formação do complexo estado fundamental e extinção colisional (Lakowicz, 2006).

A redução da intensidade de fluorescência pode ser descrita pela Equação 1, conhecida como equação de Stern-Volmer (Lakowicz, 2006):

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

Em que F_0 e F são as intensidades de fluorescência na ausência e na presença de diferentes concentrações de ligantes, respectivamente. K_{sv} é a constante de supressão de Stern-Volmer ($L.mol^{-1}$), [Q] é a concentração ($mol.L^{-1}$) do agente de supressão (ligante) da fluorescência, k_q é a constante de taxa de supressão

($L.mol^{-1}.s^{-1}$) e T_0 é o tempo de meia-vida da proteína na ausência do supressor (cerca de 10^{-8} s para proteínas).

O estudo de extinção de fluorescência fornece informações sobre as interações do ligante, como corantes naturais, e a proteína e seus sítios de ligação. Se ligantes interagem com uma proteína próximo à região de um fluoróforo, isso induzirá há uma mudança no microambiente destes fluoróforos, então o comportamento da emissão de fluorescência da proteína será afetado.

Se o mecanismo de supressão é estático, isto é, devido à formação de um complexo entre a proteína e o ligante no estado fundamental, a constante de ligação (K_a) e o número de sítios de ligação fluoróforo (a estequiometria de formação do complexo (n)) poderão ser obtidos por meio da Equação 2.

$$Log\frac{(F_0-F)}{F} = nLogK_a - nLog\left(\frac{1}{[Q_t]-\frac{(F_0-F)[P_t]}{F_0}}\right) \quad (2)$$

em que K_a é a constante aparente de interação ($L.mol^{-1}$) ; *n* é a estequiometria média de formação de complexo; $[Q_t]$ e $[P_t]$ são as concentrações totais de ligante e proteína, respectivamente em $mol.L^{-1}$.

Através da análise termodinâmica, é possível obter informações importantes a respeito da estabilidade do complexo, bem como as energias que regem a formação deste complexo. Diretamente a partir do K_a, consegue-se obter a variação da energia livre de Gibbs padrão de formação do complexo ($\Delta G°$) através da Equação 3. Os valores de $\Delta H°$ (variação da entalpia padrão de formação de complexo) serão obtidos a partir da aproximação de van't Hoff, por meio do ajuste linear entre os valores de ln K_a versus o inverso da temperatura (Equação 4), R é a constante universal dos gases (8,314 $J.mol^{-1}.K^{-1}$) e T é a temperatura em Kelvin. Com os valores de $\Delta G°$ e $\Delta H°$ foi possível obter os valores de $\Delta S°$ (variação da entropia padrão de formação de complexo) através da equação fundamental de Gibbs (Equação 5).

$$\Delta G° = -RTlnK_a \quad (3)$$

$$ln\frac{ka_2}{ka_1} = \frac{\Delta H°}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right) \quad (4)$$

$$\Delta G° = \Delta H° - T\Delta S° \quad (5)$$

3.4.3 Medidas de Tamanho e Potencial Zeta

Para avaliar os efeitos da formação de complexos na estrutura e carga dos aglomerados proteicos, podemos utilizar de medidas de potencial zeta e tamanho. É importante investigar estes dois parâmetros durante o desenvolvimento de complexos, especialmente dado o fato de que matrizes biológicas são conhecidas por alterar estas duas características quando ocorrer a formação de complexos (por exemplo, a adsorção de proteínas) (Bhattacharjee, 2016).

Com a medida do potencial zeta, consegue-se perceber os efeitos da formação do complexo na estrutura e carga da proteína ou se a interação pode mudar a distribuição dos aminoácidos e, com isso, alterar o potencial zeta. E junto com o potencial zeta, a medida de tamanho pode nos dar ideia se a formação do complexo está causando agregação da proteína ou até mesmo a desestruturação da mesma.

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Capítulo 2

Curcumin-casein micelle binding: thermodynamic of interaction and kinectic of curcumin thermodegradation

Abstract – Curcumin is a phenolic compound present in turmeric, with multiple biological activities, including anti-cancer, anti-microbial, anti-inflammatory and antioxidant in addition to natural dye. In this study, the interaction between micellar casein and curcumin was investigated at pH 6.6. Through the analysis of UV-Vis and fluorescence, it was demonstrated that the interaction between micellar casein and curcumin occurs by a predominantly static mechanism. The binding constant ranging from $1.28x10^{-5}$ $L.mol^{-1}$ to $9.52x10^{-4}$ $L.mol^{-1}$ for the temperature range of 295.15 - 323.15 K. The complex-forming stoichiometry was approximately unit by fluorescence analysis. According to the results obtained by the thermodynamic analysis, the interaction between micellar casein and curcumin is enthalphically and entropically directed, through a spontaneous process. By performing a partition experiment, the presence of increasing concentrations of micellar casein in the aqueous phase increased the number of curcumin molecules transferred from the organic phase. The mean diameter of the casein micelle in the presence of curcumin was around 290 nm and the zeta potential, ~ -16 mV. The casein micelle was able to protect curcumin against thermo-degradation. The results of this study contribute to the knowledge of the interaction process between micellar casein and curcumin for future applications in various areas of the food industry.

Keywords: UV-Vis, fluorescence, micellar casein, curcumin, thermodynamic.

Introdution

Curcumin (diferuloylmethane) is the main bioactive phenolic (Figure S1) compound derived from the root of *Curcuma longa* L., which may be used as a natural dye. In addition to the dye function, recent studies have shown that curcumin has a broad spectrum of therapeutic activities, including antioxidants, anti-inflammatory, anti-cancer, antimicrobial, wound healing and potential prevention of neurodegenerative diseases (Li, Hwang, Chen, & Park, 2016; Fang et al., 2016; Y. Liu, Cai, Jiang, Wu, & Le, 2015).

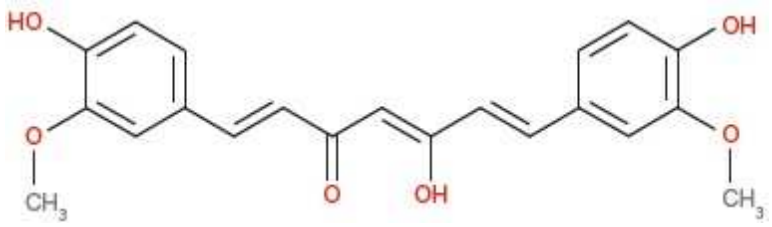


Figure S1. Chemical structure of Curcumin

The curcumin can be used as a substitute for natural dyes, such as yellow # 5 and yellow # 6 that are known as potential allergens, particularly in children (Borrin et al., 2016). In spite of the several benefic properties of curcumin, it presents low bioavailability and water solubility and fast hydrolysis followed by molecular fragmentation in physiological and alkaline pH (Lin et al., 2000) and in the presence of light (Stankovic, 2004). These characteristics limmitate its successful application in different áreas, therefore, strategies are needed to improve the solubility, bioactivity and bioavailability of lipophilic phytochemicals.

Food industry has been focused on the development of nanometric delivery systems for functional ingredients and additives (e.g., vitamins, antimicrobials, antioxidants, flavorings, dyes and preservatives) (Nunes et al., 2017). Casein micelle (CM), for example, show potential for nanodelivery systems because they are natural carriers of essential nutrients from the mother

to the newborn. In addition, its open structure, due to the high content of proline, together with calcium phosphate bridges, makes this protein a nanocarrier with excellent mechanism of controlled release of compounds in the stomach (Livney, 2010).

The interaction between milk proteins and different compounds of interest in the food area has been studied, representing great potential in the area of product innovation and development (Penalva et al., 2015; Arroyo-Maya, Campos-Terán, Hernández-Arana, & McClements, 2016; Sneharani, Singh, & Rao, 2009). Nadi et al. (2014) studied the interaction between casein from bovine milk – technical grade and curcumin, as a food grade biopolymer and a safe drug delivery system, through spectrophotometric and fluorescence analyzes at pH 7.0, thus obtaining the interaction parameters (binding constant ($13.3x10^5$ $L.mol^{-1}$), binding sites (~1) and thermodynamics parameters). Elucidating that casein may be a good matrix to increase the stability of curcumin in water. Khanji et al. (2015) studied the interaction between phosphocaseins micelles and curcumin by fluorescence spectroscopy in pH in the range of 5.0 – 7.4 (298.15 K and K^b 7.5 – $3.9x10^4$ $L.mol^{-1}$), showing that higher temperatures (293.15 – 308.15 K - K^b in the range of 0.6 – $6.6x10^4$ $L.mol^{-1}$) favored curcumin – micelle casein interactions. The micelle casein zeta potential remained unchanged (-12.0 ± 0.7 mV – 293.15 K) after curcumin addition.

However, there is still little information about native casein micelles as carrier. Native casein micelles stand out as potential nanocarriers due to maintainance of natural properties found in milk or dairy product as the natural delivery mechanism and they are remarkably more stable to thermal processing compared to other proteins such as whey proteins (Tavares et al., 2014).

Therefore, the objective of this study was to use techniques such as UV-Vis and fluorescence spectroscopy to characterize the interaction between micellar casein and curcumin at different temperatures and pH 6.6. We used native casein micelle at simulated milk pH in vitro. We present, for the first time, a partition experiment, characterizing the interaction stoichiometry between micellar casein and curcumin. We evaluated the size and zeta potential as well as the thermal degradation kinetics of curcumin in the absence and presence of casein micelle.

Material and Methods

Material

Curcumin (≥ 94% curcuminoids and ≥80% curcumin - Sigma Aldrich), sodium phosphate monohydrate, bibasic, tribasic, sodium azide and DMSO (analytical grade reagents). Micellar casein was obtained by centrifugation according to Sahu, Kasoju, & Bora (2008).

Methods

UV-Visible Absorption Spectroscopy

Absorption spectra of the micellar casein and curcumin blend were obtained on a PerkinElmer UV-Vis spectrometer, model Lambda 35 (Chalfont Road, Seer Green, Beaconsfield, United Kingdom). For absorption measurements, approximately 3.0 mL of micellar casein suspension ($4x10^{-10}$ $mol.L^{-1}$) containing different concentrations of curcumin ($1x10^{-6}$ to $25x10^{-6}$ $mol.L^{-1}$)at pH 6.6 were added in a quartz cuvette. The scanning spectrum was obtained between 250-550 nm and 298.15 K.

Fluorescence Experiments

Fluorescence spectra of casein suspension were obtained with a PerkinElmer spectrofluorimeter, model LS 55 (Chalfont Road, Seer Green,

Beaconsfield, United Kingdom). For fluorescence measurements, approximately 3.0 mL of micellar casein suspension ($8x10^{-10}$ mol.L^{-1}) containing different concentrations of curcumin ($1x10^{-6}$ to $10x10^{-6}$) at pH 6.6 were added in a quartz cuvette. Fluorescence spectra were obtained at different temperatures (293.15, 303.15, 313.15 and 323.15 K) in the range 281-450 nm (excitation wavelength 280 nm).

Partition experiment

Curcumin was solubilized in chloroform with a fixed concentration of $5x10^{-4}$ mol.L^{-1} and stored in tubes in the absence of light. Micellar casein at different concentrations (0 – $1x10^{-8}$ mol.L^{-1}) was suspended in buffer at pH 6.6 and subsequently added to the same tube containing the curcumin solution. A 10-point curve containing 5 ml of each component was then prepared and stored in the bath for 24 hours at different temperatures (298.15, 303.15 and 308.15 K). After 24 h, the concentration of curcumin in the aqueous phase was read in PerkinElmer UV-Vis spectrometer, model Lambda 35 (Chalfont Road, Seer Green, Beaconsfield, United Kingdom).

Zeta Size and Potential Measurements

Measurements of hydrodynamic diameter (nm) and the polydispersity index (PdI) were obtained by the light scattering technique dynamic-DLS using detection angle of 173 °. The zeta potential was obtained from the electrophoretic mobility of casein micelles. The experiment was performed at 25 ° C and pH 6.6 on Malvern Zetasizer Nano ZS according to Yuan, Lin, Zhang, Gao, & Yao (2016) with modifications. Five-point curves were prepared by keeping fixed the micellar casein concentration ($8x10^{-10}$ mol.L^{-1}) and ranging the curcumin concentration ($1x10^{-6}$ at $10x10^{-6}$ mol.L^{-1}).

Thermal Degradation Kinetics

Curcumin solutions ($1x10^{-5}$ $mol.L^{-1}$) in the absence and presence of increasing concentration of micellar casein at pH 6.6 were added to flasks, with oxygen removal by nitrogen ablation, and conditioned in baths with different temperatures (303.15, 313.15, 323.15 and 333.15 K). Aliquots were withdrawn at different time intervals (0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.5, 3.5, 4.5 and 5.5 hours). The scanning spectra was obtained between 180-880 nm using 1.0 cm quartz cuvettes in a UV-vis spectrophotometer (Lambda 35, PerkinElmer). The values of the kinetic degradation constant (K_d, h^{-1}) and the half-life time of curcumin ($t_{1/2}$, h) were determined by equations 1 and 2 (Chung, Rojanasasithara, Mutilangi, & McClements, 2016), respectively. The plot of $\ln K_d$ versus 1/T with van't Hoff approach (Eq. 6) was used to calculate the enthalpic energy of degradation activation ($\Delta H^{\ddagger}_d$).

$$-ln\frac{ABS_f}{ABS_0} = K_d t \quad (1)$$

$$t_{\frac{1}{2}} = \frac{ln2}{K_d} \quad (2)$$

where ABS_f and ABS_0 are the absorbances at each time interval and at time 0, respectively, Kd is the degradation constant, t is the time, $t_{1/2}$ is the half-life time.

Results and discussion

UV-Vis Absorption Spectroscopy

UV-visible spectroscopy is a simple technique that provides information on interaction between proteins and ligands in the ground state (Xu et al., 2009). If the interaction between the ligand and the protein changes the conformation in the interaction environment or the conformation of the protein, the UV-Vis absorption spectra of protein is altered (H. Bi et al., 2016), and the direction and magnitude of the absorption spectrum shift of a chromophore indicate that it may

have been transferred to a more hydrophilic or hydrophobic environment (Wen et al., 2009).

Figure 1 shows the absorption spectra of the micellar casein and curcumin system at pH 6.6 in the absence and presence of increasing concentrations of curcumin. The spectra show two distinct absorption peaks, one around 276 nm for protein agglomerate and another around 426 nm for curcumin. A change in the wavelength of maximum absorption also indicates that the addition of curcumin leads to a conformational change in the interaction environment, however, in this study we did not obtain a considerable wavelength shift (~ 1 nm), and can not state that the interaction causes changes in the interaction environment.

With increasing colorant concentration, the absorbance of peak referred to the protein agglomerate also increased about 12.0%. The increase in absorption intensity indicates that there is an interaction between micellar casein and curcumin by a predominantly static process, indicating that the interaction of protein agglomerate and curcumin may cause conformational change in some caseins within CM and / or complex formation increases the molar absorptivity of the aromatic amino acids. (Ye, Fan, Xu, & Liang, 2013).

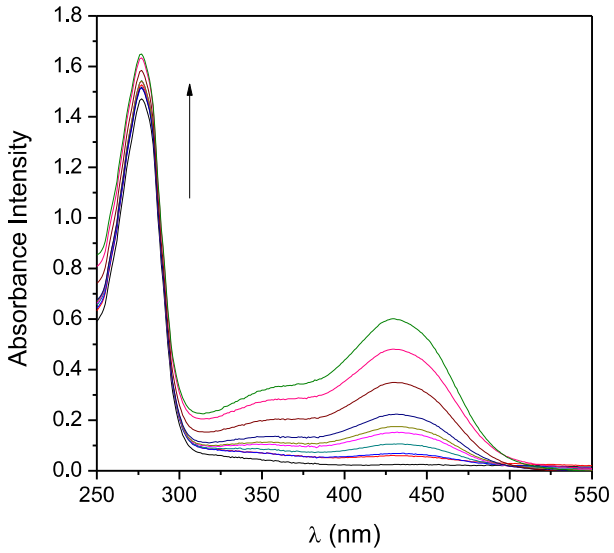


Figure 1. UV-vis spectra of casein micelle at pH 6.6 and 298.15 K with increasing concentration of curcumin. The concentration of casein micelle was 4.0×10^{-9} mol.l^{-1} and the curcumin concentration varied from 0 – 25 x 10^{-6} mol.l^{-1}. The arrow indicates increase in absorption intensity at the wavelength of maximum absorption of casein with increasing additions of curcumin.

Fluorescence Measurements

Fluorescence Quenching Spectra

Fluorescence quenching is any process that results in a decrease in the fluorescence intensity of a fluorophore. A variety of molecular interactions may result in fluorescence extinction, including excited state reactions, molecular rearrangements, energy transfer, ground state complex formation and collision extinction (Lakowicz, 2006). Fluorescence is considered a convenient tool to investigate binding between small molecules and proteins because it provides enough information to characterize the binding mechanism and thermodynamic binding parameters (Lelis et al., 2017). If ligands interact with a protein, in the vicinity of aromatic amino acid residues, the microenvironment of these fluorophores may change, and so the fluorescence emission behavior of the protein will be affected.

189 The casein micelle contains around 20,000 to 150,000 casein molecules
190 (Antonov, Moldenaers, & Cardinaels, 2017). The intrinsic fluorescence of the
191 casein molecule is mainly due to the presence of the amino acids tryptophan and
192 tyrosine. Each α-casein molecule contains the two tryptophan residues placed at
193 positions 164-199 and 109-193, for α_{s1}- and α_{s2}-caseins, respectively; while β-
194 and κ-casein have one tryptophan residue at positions 143 and 76, respectively
195 (Farrell et al., 2004). Considering that a casein micelle is formed by 39%, 11%,
196 36% and 14% of α_{s1}, α_{s2}, β and k-casein, respectively (Farrell, 2011) , there are
197 between 30,000 and 225,000 tryptophan residues contributing to fluorescence of
198 casein micelle. If curcumim molecules bind to some of these tryptophans, then
199 the fluorescence of casein micelle will quench.

200 The fluorescence spectra of micellar casein at pH 6.6 and 293.15 K in
201 the presence of increasing curcumin concentration is shown in Figure 2. With
202 increase in the concentration of curcumin, added at a fixed concentration of
203 micellar casein, the fluorescence intensity of the protein decreases with a slight
204 blue-shift, from 341 to 339 nm, indicating that curcumin interacts with micellar
205 casein. This displacement on emission wavelength pointed to fluorophores
206 changed to a more hydrophobic environment due to interaction with curcumin
207 (Bourassa, Bariyanga, & Tajmir-Riahi, 2013). With temperature increasing, a
208 similar behavior on the fluorescence quenching of casein was obtained (Figure
209 S2).

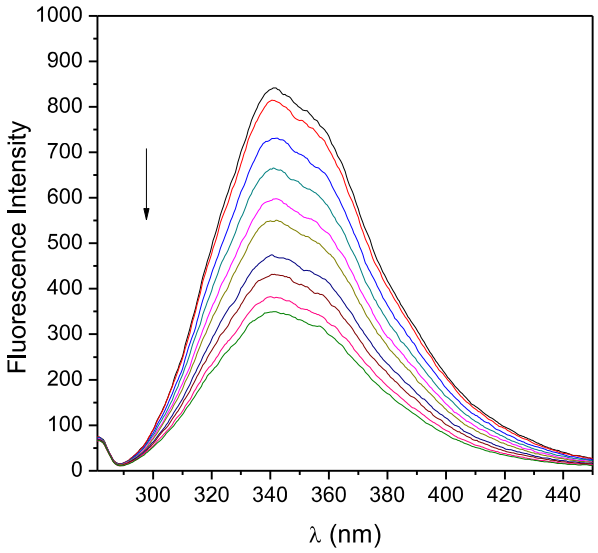


Figure 2. The fluorescence quenching spectra of casein micelle by curcumin (0 – $10x10^{-6}$ $mol.L^{-1}$) at T=293.15 K, pH 6.6 and λ_{ex}=280 nm. The arrow indicates that fluorescence intensity of casein micelle reduces as curcumin concentration increases.

Casein micelles have multiple tryptophan residues, and the total fluorescence emission of these proteins naturally produces only average information on the structure of the protein or the environment where molecules may be interacting (Tcherkasskaya, Bychkova, Uversky, & Gronenborn, 2000). Considering the high hydrophobicity of curcumin, is expected that it interacts closely with the tryptophan residues, since they are in the most hydrophobic part of the micellar casein, resulting in the extinction of the fluorescence of this protein.

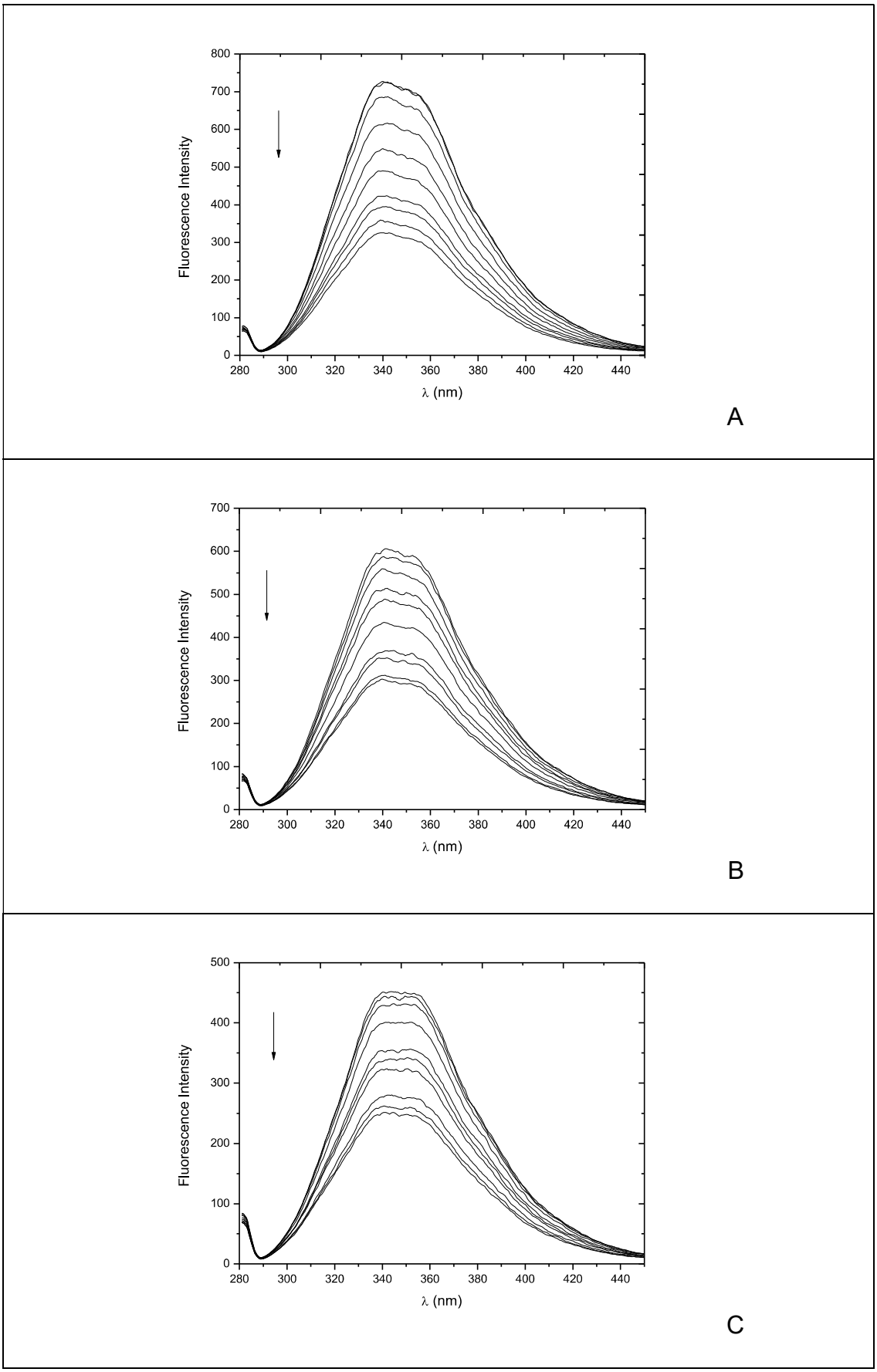


Figure S2. The fluorescence quenching spectra of casein micelle by curcumin (0 – $10x10^{-6}$ $mol.L^{-1}$) at T=303.15 K (A), 313.15 K (B) and 323.15 (C) in pH 6.6 and λ_{ex}=280 nm. The arrow indicates that fluorescence intensity of casein micelle reduces as curcumin concentration increases.

227 Stern-Volmer Analysis

228 The main mechanisms of extinction of fluorescence are classified as
229 static or dynamic. Static extinction is due to the formation of complex in the
230 ground state between the protein and the ligand while the dynamic extinction is
231 due to the collisions between the electrons in the excited state. For static
232 mechanism, the extinction constant decreases with increasing temperature
233 whereas, the opposite occurs for dynamic extinction (Lakowicz, 2006).

234 To determine the mechanism of interaction between micellar casein and
235 curuminin, fluorescence intensity data were analyzed by the Stern-Volmer
236 equation below:

237 $$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (3)$$

238 where F_0 and F represent the fluorescence intensity of the protein in the absence
239 and presence of different concentrations of the ligand [Q] respectivelly, and Ksv
240 is the Stern-Volmer extinction constant. By plotting F_0/F versus [Q] it is possible
241 to obtain the value of K_{sv}, which is the slope of the line.

242 Figure 3 shows the plot of F_0/F versus [Q] at different temperatures. As
243 the temperature increased, the Ksv values decreased (Table 1), indicating that
244 the extinguishing mechanism is predominantly static, i.e., a complex formation
245 occurred between micellar casein and curcumin. Results of the same order were
246 obtained by Sahu et al., (2008) (K_{sv} = 1.13x10^5 L.mol^{-1}) and by Khanji et al.,
247 (2015) (Ks_v = 3.25x10^5 – 18.8x10^5 L.mol^{-1}), studying the interaction between
248 curcumin-micellar casein at pH 7.4 and at room temperature and in the range of
249 pH 5.0 and 7.4 ate room temperature, respectively, corroborating the results of
250 this study.

251

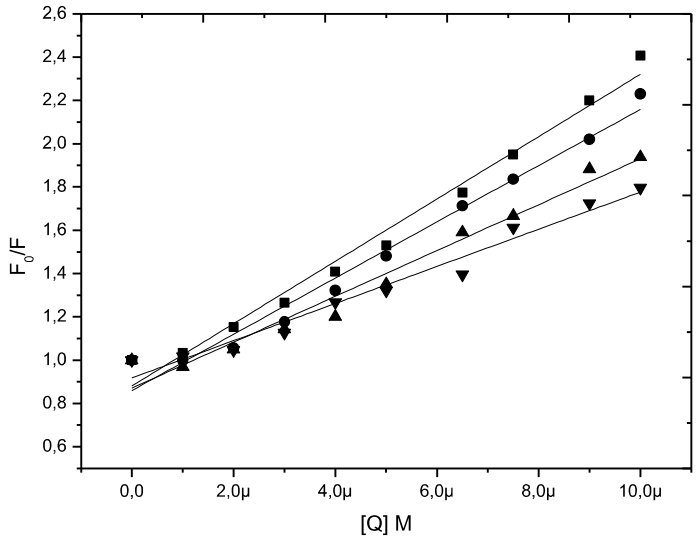


Figure 3. Stern-Volmer plots for casein micelle fluorescence quenching due to the binding of curcumin to casein micelle at pH 6.6 at four different temperatures: (■) 293.15 K; (●) 303.15 K; (▲) 313.15 K and (▼) 323.15 K.

Interaction parameters: interaction constant (Kb) and stoichiometry of complex formation (n)

For static extinction processes, when small molecules interact with a macromolecule, the binding constant (K_b) as well as the stoichiometry of complex formation (n) can be obtained by equation 4.

$$Log\,\frac{(F_0-F)}{F} = nLogK_b - nLog\left(\frac{1}{[Q_t]-\frac{(F_0-F)[P_t]}{F_0}}\right) \quad (4)$$

where K_b (L.mol^{-1}) is the apparent constant of interaction; *n* is the stoichiometry of complex formation; [Q_t] and [P_t] are the total concentrations of ligand and protein (mol.L^{-1}), respectively.

There are different equations for the calculation of the binding constant and the stoichiometry of complex formation (Wei, Xiao, Wang, & Bai, 2010) and some of these equations take into account that the concentration of the interacting binder is much smaller than the total concentration, which results in approximate data. (Bi et al., 2004). Through this consideration, we used Equation

4 (Modified double logarithm regression curve) for the calculations of K^b and *n* (Figure 4).

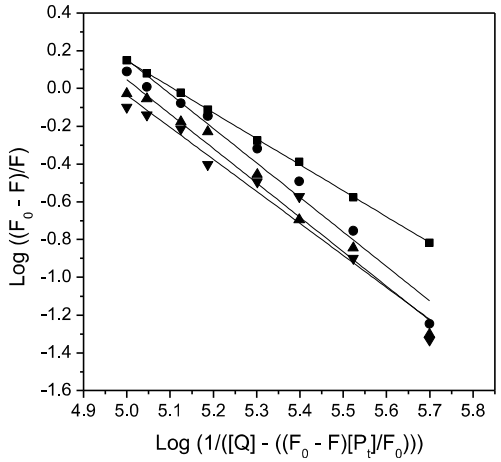


Figure 4. The double logarithm modified regression plots of log ($(F_0 - F)/F$) versus Log ($1/([Q] - ((F_0 - F)[P_t]/F_0))$) of casein micelle at pH 6.6 and different temperatures: (■) 293.15 K; (●) 303.15 K; (▲) 313.15 K and (▼) 323.15 K.

As shown in Table 1, the values for K_b were of the order of 10^5-10^4 L.mol^{-1} for the complex between micellar casein and curcumin. The stoichiometry of complex formation was between one and two, at all temperatures, indicating that for each binding site on casein micelle there are at least one curcumin molecule bonded.

Table 1. Stern-Volmer extinction constant (K_{sv}), binding constant (K^b) and number of binding sites (n) between curcumin and micellar casein at pH 6.6 and four temperatures.

T (K)	K_{sv} (L.mol^{-1})	R^2	K^b (L.mol^{-1})	*n*	R^2
293.15	1.44x10^5	0.98	1.28x10^5	1.38	0.99
303.15	1.30x10^5	0.98	1.21x10^5	1.83	0.97
313.15	1.06x10^5	0.97	1.06x10^5	1.82	0.98
323.15	8.58x10^4	0.97	9.52x10^4	1.70	0.96

When studying the interaction of any molecule with micellar casein, the stoichiometric analysis should take into account the amount of casein present per micelle. Thus, our results suggest that one casein micelle may carry many curcumin molecules; considering the number of caseins per micelle, and if all tryptophan residues are availiable to interact with the bioactive molecule, the number of curcumin molecules per casein micelle range from 30,000 to 225,000.

Different studies estimated the stoichiometry of interaction between curcumin and casein molecules or sodium caseinate (Bourassa et al., 2013; Benzaria, Maresca, Taieb, & Dumay, 2013), but few with micellar casein. Nadi et al. (2014) studied interaction between whole casein and curcumin at pH 7.0, obtaining a unitary stoichiometry and Khanji et al. (2015) studying interaction between micellar casein and curcumin at different pH (5.0-7.4 and 298.15 K), obtained a stoichiometry of approximately 1.5, similar results with this study.

In order to certificate the amount of curcumin molecules carried by each casein micelle, we performed a partition experiment of curcumin between two phases, an organic phase (chlorofom) and an aqueous phase, containing increasing casein concentration, trough 24 h at different temperatures. The slope of the linear region of [curcumin] at aqueous phase *versus* [casein] plot (Figure S3) provided the number of curcumin molecules is carried inside each casein micelle.

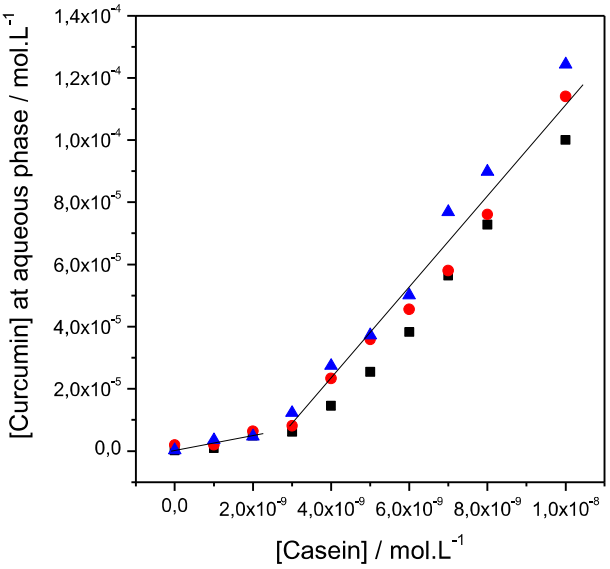


Figure S3. Plot of curcumin concentration on aqueous phase *versus* casein concentration, at: (■) 298.15 K, (●) 303.15 K, and (▲) 308.15 K.

The temperature seemed to had no effect on the transferrence of curcumin between the organic and the aqueous phase containing casein. The curves were divided in two regions, suggesting that there are two groups of binding sites for curcumim in casein micelle. At the first region (up to 2.0 x 10^{-9} mol.L^{-1} of casein), the average slopes at three temperatures (25, 30 and 35 ºC) indicated that around 2,273 curcumin molecules per casein micelle; and at the second region (above 4.0 x 10^{-9} mol.L^{-1} of casein), the higher average slope (15,838), suggests that about 15,838 curcumin molecules were carried by a casein micelle. Considering the model of casein micelle, in which α_{s1}, α_{s2}, and β-caseins are placed in the core of casein micelle, while the k-casein is on the interface of the protein aggregate, we could propose that at small casein concentration (up to 2.0 x 10^{-9} mol.L^{-1}), curcumim interacted with the strong binding sites, probably present at k-caseins. However when the casein concentration increased (above 4.0 x 10^{-9} mol.L^{-1}), more sites (the weaker binding sites) are available for interacting with curcumin, probably those present

326 at α_{s1}, α_{s2}, and β-caseins, and a higher number of curcumim molecules were
327 transferred to the casein micelle.

328 The amount of curcumin carried by a casein micelle determined by the
329 partition experiment was remarkable smaller than that estimated by complex
330 stoichiometry determined by fluorescence. This difference can be easily
331 explained mainly considering that even casein micelle has many tryptophan
332 residues, some of them are not available to bind to ligand molecules (Lakowicz,
333 2006), and thus the amount of curcumin per casein micelle calculated based on
334 the complex stoichiometry found by fluorescence was overestimated. In addition,
335 the range of casein molecules per micelle is very wide (Antonov et al., 2017), and
336 so is the number of tryptophan residues, which could contribute for the difference
337 found.

338 Thermodynamics Parameters of Curmin-Casein Micelle Binding

339 Basically, four types of bonding energy are involved in the interaction
340 between biomolecules and small ligands: hydrophobic forces, hydrogen bonds,
341 van der Waals forces and electrostatic interactions (He, Xu, Zeng, Qin, & Chen,
342 2016). In order to determine the nature of the interaction between micellar casein
343 and curcumin, the thermodynamic parameters of this interaction were
344 determined.

345 From the binding constant K_{ab} it was possible to determine the values of
346 standard Gibbs free energy change (ΔG°) of complex formation (Equation 5).

347 $$\Delta G° = -RTlnK_b \quad (5)$$

348 where R is the universal gas constant (8.314 $J.mol^{-1}.K^{-1}$) and T is the temperature
349 in Kelvin.

350 Since there are two components contributing for ΔG° values: the
351 enthalpic and the entropic terms, the values of standard enthalpy change (ΔH°)

and standard entropy change (ΔS°) of complex formation were obtained. The ΔH° values were calculated using the van't Hoff approximation, by linear adjustment (Figure S4) between the values of ln K_a *versus* the inverse of the temperature (Equation 6). With the values of ΔG ° and ΔH ° it was possible to obtain the values of the entropic term (TΔS°) through the Gibbs fundamental equation (Equation 7). The data of thermodynamic parameters for curcumin-casein micelle is presented in Table 3.

$$ln\frac{K_2}{K_1} = -\frac{\Delta H^\circ}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \quad (6)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (7)$$

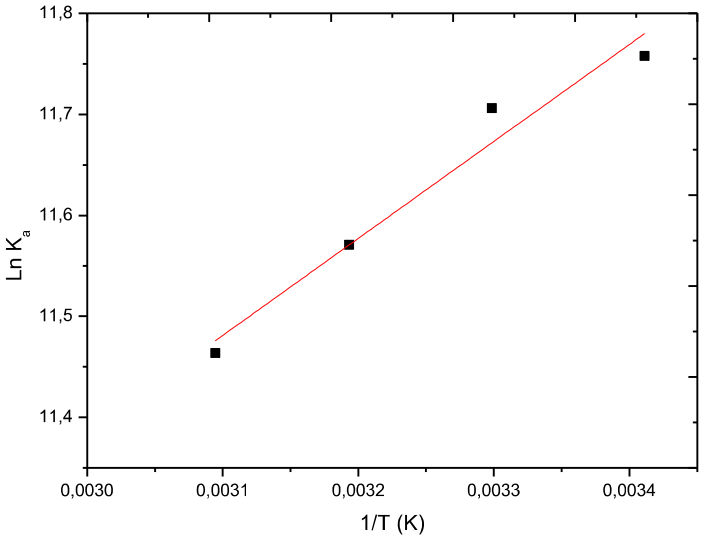


Figure S4. van't Hoff plot for the interaction between casein micelle and curcumin at pH 6.6.

The ΔG ° values were negative, indicating that the reaction equilibrium favors the formation of a complex between micellar casein and curcumin. In addition, the values of ΔG ° have become more negative at higher temperatures, which indicates that temperature increasing became more stable curmin-casein complexes.

According to the signs and magnitude of the ΔH° and TΔS° values, it is possible to discuss wich interaction forces are mainly responsible for curcumin-casein micelle binding. As shown in Table 2, the values of TΔS° were positive while ΔH° was negative, indicating an entropically and enthalpically driven process. The ΔH° value obtained for curcumin-casein micelle complex is an average of ΔH° at each binding site present in the casein micelle. The negative ΔH° value indicated that probably two forces predomined in the ligand-protein aggregate interaction: attractive electrostic interaction and hydrogen bonding (J. Zhang, Tian, Liang, Subirade, & Chen, 2013). Since pH 6.6 micellar casein is negatively charged and curcumin neutral (in the pH range 1-7, the majority of diferuloylmethane species are in the neutral form and pKa values: 7.8, 8.5 and 9.0), the formation of the complex is enthalphically favorable, probably due to the predominance of hydrogen bonds occurring between the OH groups of curcumin and OH groups close to the residues of tryptophan in the interaction environment of casein micelle.

Furthermore, the negative values of ΔH° indicate that the complex formation is through an exothermic process (formation of energetically favorable non-covalent interactions between atoms, thus reflecting the system's energy change (Du et al., 2016) when curcumin binding with micellar casein). The positive entropic term is the result of the hydrophobic effect, due to the release of water molecules from the solvation layer of the species that formed the complex. In this way, the freedom degree of the solvent molecules becomes larger and the entropy of the system increases.

Table 3. Thermodynamic parameters of interaction between micellar curcumin casein at pH 6.6 at four temperatures.

T (K)	ΔH° (kJ.mol^{-1})	R^2	TΔS° (kJ.mol^{-1})	ΔG° (kJ.mol^{-1})
293.15	-7.99	0.97	20.67	-28.66
303.15			21.52	-29.51
313.15			22.14	-30.13
323.15			22.82	-30.80

Size and Zeta Potential

To evaluate the effects of the formation of complexes in the size and charge of the casein micelle, we measure the potential zeta and size of the protein agglomerate. It is important to investigate these two parameters during the development of complexes, especially given the fact that biological matrices are known to change these two characteristics when complex formation occurs (eg protein adsorption) (Bhattacharjee, 2016). Table 4 shows the hydrodynamic diameter and zeta potential of casein micelles in the absence and presence of curcumin.

Table 4. Hydrodynamic diameter, polydispersity index and zeta potential of casein micelles in the presence and absence of different concentrations of curcumin at pH 6.6 and 298.15 K.

Curcumin concentration (mol.L^{-1})	Average Diameter (nm)	PdI	Zeta Potential (mV)
0x10^{-6}	275.15 ± 23.35^{a}	0.410^{a}	-17.72 ± 1.95^{a}
2.5x10^{-6}	291.15 ± 51.89^{a}	0.537^{a}	-17.16 ± 1.63^{a}
5.0x10^{-6}	290.40 ± 40.59^{a}	0.459^{a}	-16.98 ± 1.33^{a}
7.5x10^{-6}	299.77 ± 40.15^{a}	0.440^{a}	-15.68 ± 2.12^{a}
10.0x10^{-6}	298.77 ± 65.36^{a}	0.514^{a}	-15.18 ± 0.81^{a}

The mean diameter of the casein micelles reported in the literature is around 200 nm (Silva et al., 2013). The mean diameter (z-average) of the micellar casein in the absence and presence of curcumin was constant. The PdI of ≤ 0.1 is considered to be highly monodisperse, values of 0.1 – 0.4 and >0.4 are considered to be moderately and highly polydisperse, respectively (Bhattacharjee, 2016). The values of standard deviation obtained indicate a

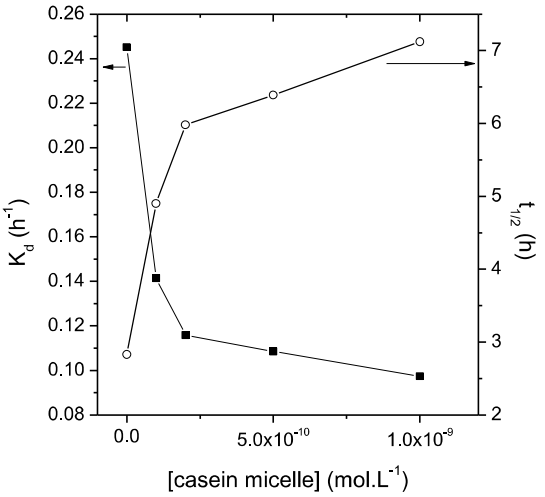
418 higher polydispersity index of casein micelles, in agreement with the existence of
419 different sizes of the casein micelle.

420 At pH 6.6, the micelles are negatively charged with higher electrostatic
421 repulsion. In the case of pure micellar casein and with addition of curcumin, the
422 zeta potential mean was –16.54 mV. The presence of curcumin had no significant
423 effect on zeta potential ($p < 0.05$). The obtained results in this study are in good
424 agreement with current knowledge of the casein micelle.

425 Curcumin Thermal Degradation Kinetics

426 Food processing generally includes heat treatments that effectively
427 preserve food and also provide desirable sensory properties. However, current
428 knowledge indicates that thermal processing, especially under severe conditions,
429 can affect the levels of active compounds in food products (Mercali, Jaeschke,
430 Tessaro, & Marczak, 2013). Kinetic analysis, which is widely used technique for
431 studying thermal decomposition, has received considerable attention (Chen et
432 al., 2014; Sarkis, Jaeschke, Tessaro, & Marczak, 2013). An important practical
433 application is the prediction of process rates, thermal stability and the half-life of
434 materials (Chen et al., 2014).

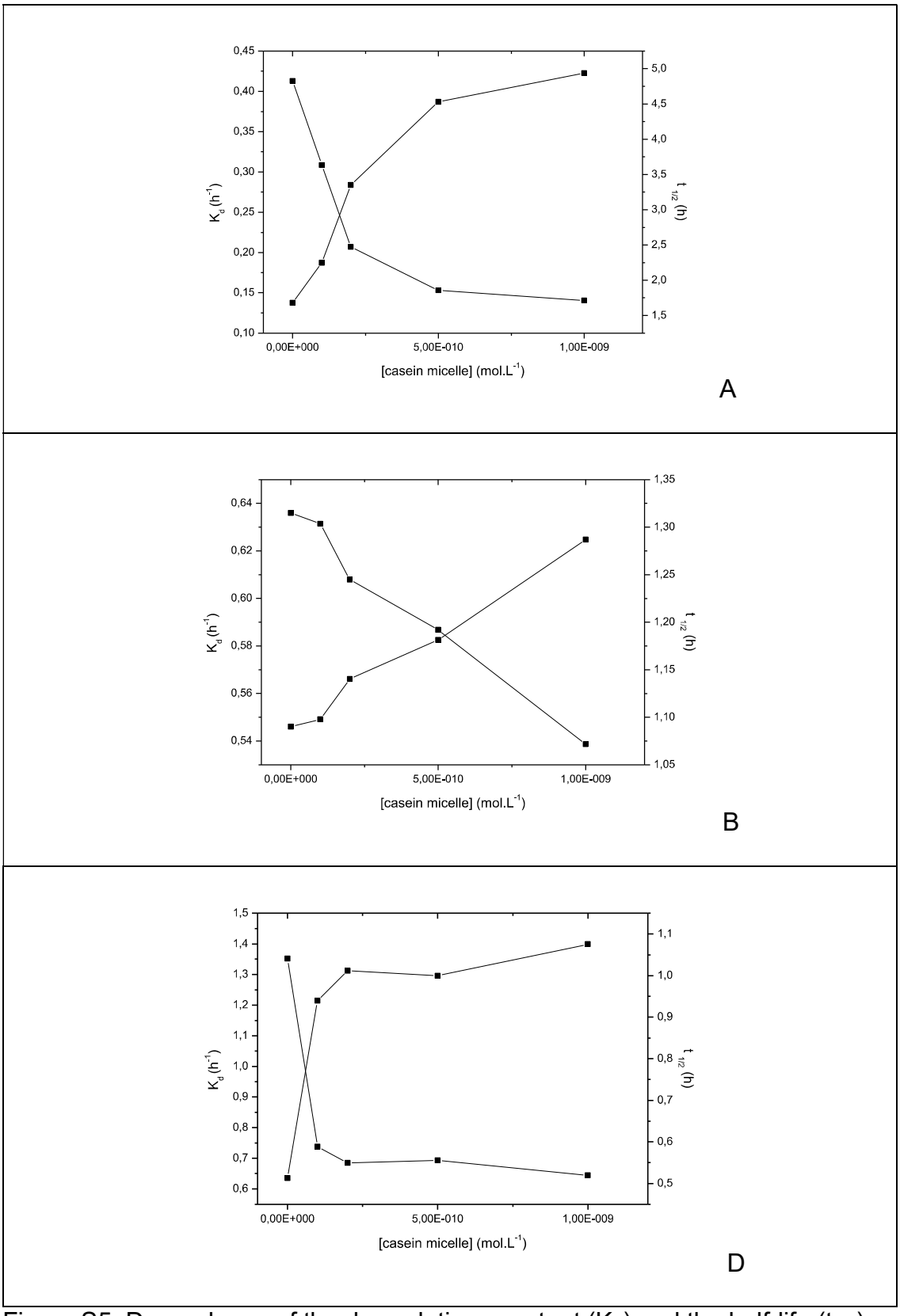
435 Figure 8 summarizes the kinetic parameters of the degradation constant
436 and half-life time obtained for the temperature of 303.15 K evaluated at different
437 concentrations of micellar casein. The same profile shown in Figure 8 was
438 observed for the temperatures of 313.15, 323.15 and 333.15 K (Figure S5). The
439 kinetic constants increased with increasing temperature for all casein
440 concentrations.



441

442 Figure 8. Dependence of the degradation constant (K_d) and the half-life ($t_{1/2}$) as
443 a function of micellar casein concentration at pH 6.6 and 303.15 K.

444 At each temperature, the increasing casein concentration provided a
445 reduction in the kinetic degradation constant (k_d) of curcumin, suggesting the
446 protective effect of micellar casein. Chen et al. (2014) suggests that the thermal
447 degradation of curcumin occurs in two stages: (I) the stage I is due to the
448 decomposition of substituent groups of curcumin; (II) the stage II is due to the
449 decomposition of two benzene rings of curcumin. The same trend presented by
450 the kinetic degradation constant occurred in relation to the half-life of curcumin in
451 the presence of micellar casein, increasing the half-life ($t_{1/2}$) of curcumin in the
452 presence of micellar casein. He et al. (2016) found small thermal degradation of
453 anthocyanins in the presence of casein fractions (pH 6.3 and 353.15 K / 2h),
454 suggesting the protective effect of this protein and corroborating with this study.



455 Figure S5. Dependence of the degradation constant (K_d) and the half-life ($t_{1/2}$) as
456 a function of micellar casein concentration at pH 6.6 and 313.15 K (A), 323.15 K
457 (B) and 333.15 K (C).

458

Trough Arrhenius approach (Figure 9A), the activation enthalpic energy of degradation ($\Delta H^{\ddagger}_{d}$) for each casein concentration was calculated (Figure 9B).

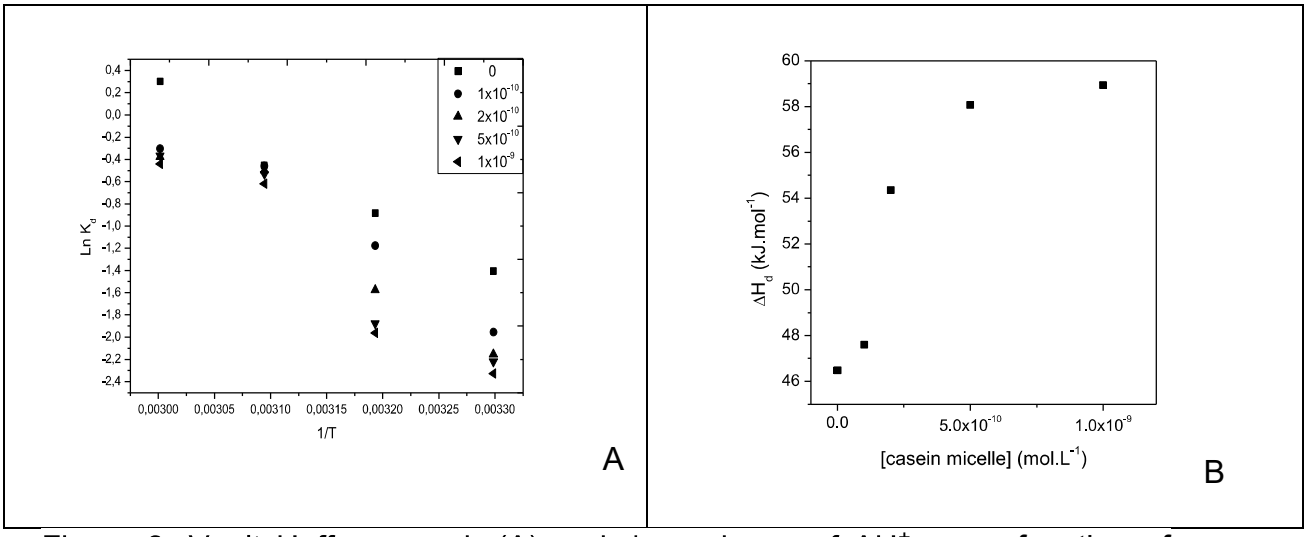


Figure 9. Van't Hoff approach (A) and dependence of $\Delta H^{\ddagger}_{d}$ as a function of concentration of micellar casein (B).

As the concentration of micellar casein increased the $\Delta H^{\ddagger}_{d}$ also enhanced, indicating that it is necessary a greater energy for occurring the thermal degradation of curcumin. This behavior is problably due to at small concentration of casein micelle the curcumin interact with binding sites present in the protein aggregate, saturating these sites; however when the casein concentration increased, there are more casein micelle available to bind with curcumin, stabilizing the bioactive molecule, and thus, it is necessary more energy to degradate curcumim.

Conclusion

The interaction between micellar casein and curcumin was studied using UV-vis and fluorescence spectroscopy. The results obtained demonstrate that curcumin interacts with micellar casein through a predominantly static mechanism, that is, the formation of complex in the ground state. The process of interaction, through thermodynamic analysis, is enthalphically and entropically

478 directed. The presence of curcumin showed no significant difference in relation
479 to the mean size and zeta potential of the casein micelle. Through the kinetic
480 analysis of degradation, the results suggest the protective effect of micellar
481 casein against thermal degration processes on curcumin. The results of this study
482 present a better understanding of how micellar casein can be used as an
483 interaction vehicle, transport and protection for other molecules.

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