NATÁLIA MOREIRA NUNES

TERMODINÂMICA DE INTERAÇÃO ENTRE ÁCIDO CINÂMICO E CINAMATO DE METILA E ALBUMINA DO SORO BOVINO

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.*

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APROVADA: 22 de fevereiro de 2016.

_____	_____
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À Deus e à Virgem Santíssima,

meus pais, familiares e amigos.

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RESUMO

NUNES, Natália Moreira, M. Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Termodinâmica de interação entre ácido cinâmico e cinamato de metila e albumina do soro bovino.** Orientadora: Ana Clarissa dos Santos Pires. Coorientadores: Luis Henrique Mendes da Silva e Maximiliano Soares Pinto.

O ácido cinâmico (AC) e o cinamato de metila (CM) são compostos de estruturas similares que tem despertado interesse de grupos de pesquisa devido às suas amplas funções terapêuticas. Assim, torna-se importante estudar a interação destes compostos com a albumina do soro bovino (BSA), uma vez que essa proteína é uma importante molécula carreadora no sangue e é também um agente interfacial em produtos alimentícios. Neste trabalho, foi estudada, em nível molecular, a interação entre BSA e AC ou CM (em pH 3.5, 5.0 e 7.4) usando espectroscopia de fluorescência, nanocalorimetria diferencial de varredura e medidas de tensão interfacial, tamanho e potencial zeta. As constantes de interação de AC/CM-BSA foram na ordem de 10^4 $L.mol^{-1}$ e a estequiometria de formação dos complexos foram de acordo com a proporção 1:1 de proteína/ligante no pH 7.4. Nesta condição, a formação dos complexos CA-BSA foi regida por interações hidrofóbicas ($\Delta H°$ = 14.44 $kJ.mol^{-1}$) devido a repulsão eletrostática entre a BSA carregada e AC. Nos pH 3.5 e 5.0 a formação dos complexos teve contribuição entálpica ($\Delta H°$ -29.81 $kJ.mol^{-1}$ at pH 3.5 and -21.12 $kJ.mol^{-1}$ at pH 5.0). A presença de sulfato de amônio (pH 7.4) não teve influência no termo entrópico, mas levou a uma redução de $\Delta H°$ ($\Delta H°$ = -17.84 $kJ.mol^{-1}$). Estudos calorimétricos indicaram que o pH desempenha um papel importante na conformação da proteína, já que em condição ácida a proteína estava desnaturada. Quando a BSA foi desnaturada, continuou formando complexo com AC, mas os valores de $\Delta G°$ se tornaram menos negativos. Foi verificada que a presença de AC ou CM não afetou os valores de σ_{eq} em todos os pH estudados. Foi verificado também que a interação da proteína com CM foi mais favorável ($\Delta G°_{AC\text{-}BSA}$ = -26.53 kJ;mol^{-1} and $\Delta G°_{CM\text{-}BSA}$ = -30.32 $kJ.mol^{-1}$ no pH 7.4 e 25 °C) que com AC e foi dirigida pela entalpia ($\Delta H°_{CM}$ = -8.71 $kJ.mol^{-1}$ no pH 7.4) provavelmente porque CM não é ionizável e é mais hidrofóbico. A presença de sulfato de amônio quase não alterou os valores de $\Delta H°$ (-8.71 $kJ.mol^{-1}$, -19.32 $kJ.mol^{-1}$, -11.32 $kJ.mol^{-1}$, -14.06 $kJ.mol^{-1}$, -1.95 $kJ.mol^{-1}$ para 0, 1, 10, 50 e100 mM, respectivamente) e $T\Delta S°$ (21.64 $kJ.mol^{-1}$, 11.29 $kJ.mol^{-1}$, 23.06 $kJ.mol^{-1}$, 17.95 $kJ.mol^{-1}$, 28.4 $kJ.mol^{-1}$ para 10,

50 e 100 mM, respectivamente). Esses dados contribuem para o conhecimento sobre as interações AC/CM-BSA e, desta forma, para futuras aplicações nas áreas alimentícia e farmacêutica.

ABSTRACT

NUNES, Natália Moreira, M. Sc., Universidade Federal de Viçosa, February 2016. **Thermodynamic of interaction between cinnamic acid and methyl cinnamate and bovine serum albumin.** Adviser: Ana Clarissa dos Santos Pires. Co-advisors: Luis Henrique Mendes da Silva and Maximiliano Soares Pinto.

Cinnamic acid (AC) and methyl cinnamate (CM) are compounds with similar structures that have attracted interest of researches because of its larger therapeutic functions. Thus, is important to study its interaction with bovine serum albumin (BSA), once this protein is an important molecule career in blood and also is an interfacial agent in food products. For this paper, it was studied at molecular level the interaction between BSA and AC or CM (at pH 3.5, 5.0 and 7.4) using fluorescence spectroscopy, differential scanning nanocalorimetry and interfacial tension analysis, size and zeta potential measurements. AC/CM-BSA binding constants (K_b) were in order of 10^4 L.mol^{-1} and complexes formation stoichiometry according with 1:1 protein/binding proportion at pH 7.4. At this condition, AC-BSA complex formation was driven by hydrophobic interactions ($\Delta H°$ = 14.44 kJ.mol^{-1}) due to electrostatic repulsion between charged BSA and AC. At pH 3.5 and 5.0 complexes formation had enthalpic contribution ($\Delta H°$ -29.81 kJ.mol^{-1} at pH 3.5 and -21.12 kJ.mol^{-1} at pH 5.0). Ammonium sulfate presence (pH 7.4) had no influence in entropic term but lead to $\Delta H°$ decrease ($\Delta H°$= -17.84 kJ.mol^{-1}). Calorimetric studies indicated that pH plays an important role in protein conformation, since at acid condition protein was denatured. When BSA was denatured, it also formed complex with AC, but $\Delta G°$ values became less negative. It was verified that AC or CM presence did not affect σ_{eq} values in all studied pH. Also, it was verified that protein interaction with CM was more favorable ($\Delta G°_{AC\text{-}BSA}$ = -26.53 kJ;mol^{-1} and $\Delta G°_{CM\text{-}BSA}$ = -30.32 kJ.mol^{-1}at pH 7.4 and 298.15 K) than with AC and it was enthalpically driven ($\Delta H°_{CM}$ = -8.71 kJ.mol^{-1} at pH 7.4) probably because CM is not ionizable and it is more hydrophobic. Ammonium sulfate presence almost did not change $\Delta H°$ values (-8.71 kJ.mol^{-1}, -19.32 kJ.mol^{-1}, -11.32 kJ.mol^{-1}, -14.06 kJ.mol^{-1}, -1.95 kJ.mol^{-1} for 0, 1, 10, 50 and 100 mM, respectively) and $T\Delta S°$ values (21.64 kJ.mol^{-1}, 11.29 kJ.mol^{-1}, 23.06 kJ.mol^{-1}, 17.95 kJ.mol^{-1}, 28.4 kJ.mol^{-1} for 10, 50 and 100 mM, respectively). These data contributed to knowledge about AC/CM-BSA interactions and for future applications in food and pharmaceutical areas.

SUMÁRIO

1. INTRODUÇÃO

As albuminas séricas possuem o importante papel de carrear moléculas na corrente sanguínea. Por isso, diversos estudos visam avaliar a interação dessas proteínas com moléculas presentes em fármacos ou nos alimentos, que atingiriam a corrente sanguínea, a fim de investigar se possíveis interações com as albuminas afetariam seu papel fisiológico. Dentre essas proteínas, destaca-se a albumina sérica bovina (BSA), que é homóloga à albumina sérica humana (HSA)

Por possuírem características carreadoras, estudos também são feitos para avaliar o potencial dessas proteínas como veiculadoras de moléculas de interesse tanto em alimentos (BSA) quanto no organismo humano (BSA ou HSA).

A BSA é importante também na ciência e tecnologia de alimentos, uma vez que possui propriedades interfaciais, podendo atuar como agentes emulsificantes e espumantes.

O ácido cinâmico e o cinamato de metila (éster de ácido cinâmico) tem despertado interesse em grupos de pesquisa pelo mundo devido às amplas atividades terapêuticas, como propriedades antimicrobiana, antioxidante, anticancerígena e imunomodulatória.

Desta forma, torna-se importante estudar a interação entre a BSA e estes compostos para avaliação da influência da estrutura molecular do ligante na formação do complexo.

A determinação de parâmetros termodinâmicos de interação, como constante de ligação, estequiometria de formação de complexo, variações de energia livre de Gibbs, entalpia e entropia padrão de formação de complexo, em diferentes condições de pH e temperatura permite uma avaliação profunda dos mecanismos de formação do complexo. Além disso, o estudo da mudança de propriedades intrínsecas das proteínas (como atividades interfaciais, potencial zeta, tamanho e parâmetros referentes à sua desnaturação) também são interessantes.

Por tudo isso, percebe-se que o estudo de interação entre a BSA e ácido cinâmico e cinamato de metila são relevantes para a comunidade científica e merecem destaque devido à sua importância e potencial de aplicação nas áreas médica e alimentícia.

2. OBJETIVOS

2.1 Objetivo geral

O presente trabalho foi desenvolvido com o objetivo de estudar em nível molecular a termodinâmica de interação entre a albumina sérica bovina e o ácido cinâmico e cinamato de metila em diferentes condições de pH (3,5, 5,0 e 7,4), temperatura (15 °C, 25 °C, 35 °C, 45 °C) e conformação da proteína (nativa e desnaturada).

2.2 Objetivos específicos

Os objetivos específicos trabalhados foram:

- Determinar a constante de interação, a estequiometria da formação do complexo, variação da entalpia, variação da energia livre de Gibbs e variação da entropia de formação do complexo AC/CM-BSA;
- Determinar a variação da entalpia de desnaturação das proteínas e dos complexos em diferentes concentrações de AC ou CM, bem como a temperatura onde ocorre a máxima mudança conformacional na estrutura da BSA;
- Avaliar a influência da interação AC/CM-BSA no tamanho e no potencial zeta dos complexos;
- Estudar a mudança da tensão interfacial entre ar e solução de proteína na ausência e em diferentes concentrações de AC ou CM;
- Avaliar a influência da presença de sulfato de amônio nos parâmetros termodinâmicos de interação.

3. REVISÃO DE LITERATURA

3.1 Albumina sérica bovina

As albuminas são as proteínas mais abundantes no sangue, correspondendo de 52 a 60 % do plasma sanguíneo. Elas têm formato de coração, com dimensões de 80 Å x 80 Å x 30 Å e contém três domínios homólogos ricos em α-hélices, cada um deles contém 10 dessas estruturas e são divididos em um domínio com seis e um com quatro hélices (domínios A e B) ligadas por pontes dissulfeto (Figura 1)(Fanali, Trezza, Marino, Fasano, & Ascenzi, 2012).

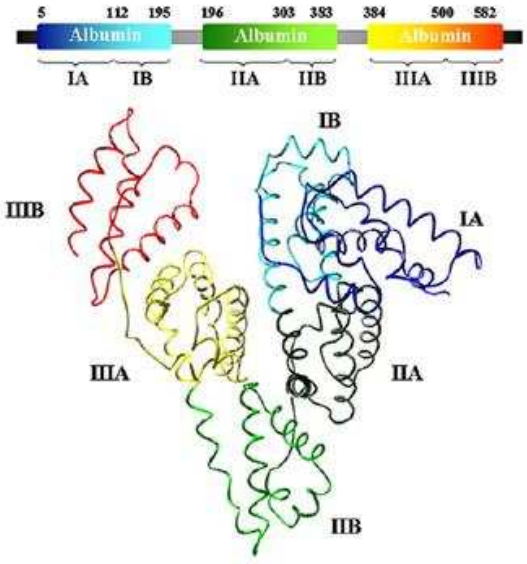


Figura 1 - Estrutura da HSA, destacando-se os domínios I, II e III e subdomínios A e B. Fonte: Fanali et al. 2012.

Essas proteínas são capazes de se ligar a moléculas insolúveis em meio aquoso e sua principal função é o transporte de metabólitos. Entre as albuminas séricas, destacam-se a albumina sérica humana (HSA) e a albumina sérica bovina (BSA) (Naveenraj & Anandan, 2013).

Tanto na BSA quanto na HSA, há dois sítios de ligação principais, que são localizados nos subdomínios IIA e IIIA (Figura 1). No primeiro sítio geralmente ocorrem interações hidrofóbicas, enquanto no segundo podem ocorrer interações eletrostáticas, hidrofóbicas e ligações de hidrogênio. Ambas as proteínas emitem fluorescência próximo a 340 nm quando excitadas a 280 nm (comprimento de onda responsável pela excitação da tirosina, triptofano e

fenilalanina) e 295 nm (comprimento de onda responsável pela emissão de fluorescência do triptofano). Podem ocorrer mudanças no espectro de emissão do triptofano caso ocorram mudanças conformacionais, associação de subunidades, desnaturação ou interação da proteína com algum substrato (Memarpoor-Yazdi & Mahaki, 2013), o que é importante, já que a emissão de fluorescência ocorre principalmente devido a este resíduo de aminoácido, por ter maior rendimento quântico (Naveenraj & Anandan, 2013).

A albumina sérica bovina é de grande importância biológica, uma vez que é muito similar à albumina presente na corrente sanguínea humana, pois 76 % das sequências de aminoácidos da BSA e HSA são idênticas. Sua massa molar é 66.462 g/mol e esta proteína é composta por 583 resíduos de aminoácidos, com dois triptofanos (posições 134 e 212), sendo o primeiro localizado na interface da proteína e o segundo no núcleo hidrofóbico da mesma, em local análogo ao triptofano 214 da HSA (Figura 2) (Bujacz, 2012; Ferreira, 2009; Naveenraj & Anandan, 2013).

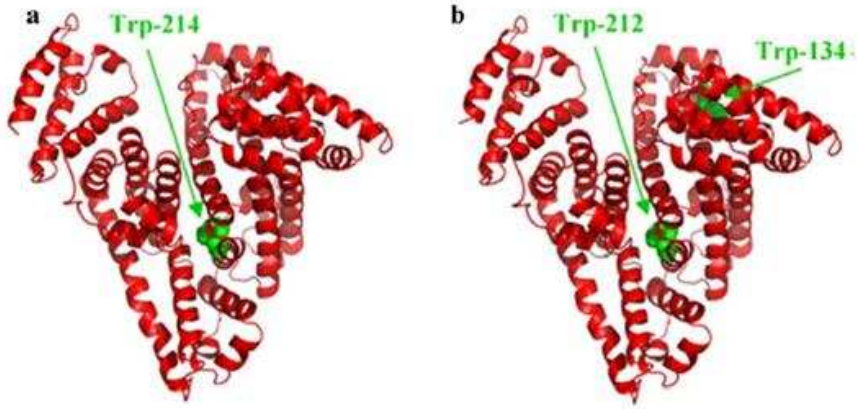


Figura 2 – Estruturas tridimensionais da HSA (a) e BSA (b), evidenciando a localização dos resíduos de triptofano presentes. Fonte: Naveenraj and Anandan 2013.

O ponto isoelétrico da BSA é em torno do pH 4,7 (Patil, Sandberg, Heckert, Self, & Seal, 2007; Schveitzer, 2006) e esta proteína possui diâmetro hidrodinâmico de 5 a 7 nm em pH próximo a 5,0 e seu tamanho aumenta em pH baixo (17,5 nm em pH 2,0) e e alto (8,3 nm em pH 10,0) (Kun, Szekeres, & Dékány, 2009). Sua estrutura sofre mudanças conformacionais no intervalo de pH entre 4 e 8, como mostra a Figura 3 (Ferreira, 2009; Kun et al., 2009).

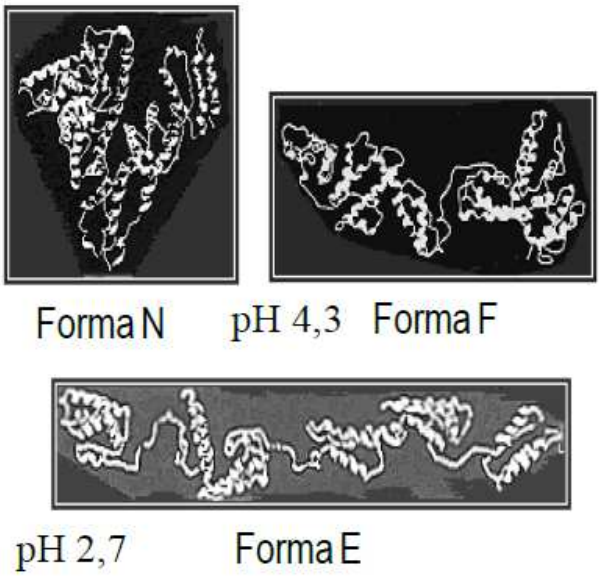


Figura 3 – Isomerização conformacional da BSA. pH 4,3 – transição da forma normal (N) para forma *fast* (F); pH 2,7 – transição da forma F para a forma estendida (E). Fonte: Ferreira 2009.

A transição da forma E da BSA para a F ocorre com o enovelamento das hélices no domínio I e depende da concentração da proteína, uma vez que em concentrações maiores que 0,1 % o pH de transição aumenta. Já o dobramento do domínio III (transição da forma F para N), ocorre no pH 4,8 em solução de BSA 0,1 % e em valores de pH mais elevados quando a concentração passa para 0,2 % (Kun et al., 2009).

Deste modo, estudos de interação que envolvem a BSA são importantes do ponto de vista fisiológico, uma vez que podem indicar se os ligantes estudados afetariam o papel da HSA como carreadora de metabólitos no sangue humano.

Muitos estudos são realizados visando entender possíveis interações entre a albumina sérica bovina e diversas drogas, como antibióticos, antianginosos, hipertensivos e diuréticos, bem como suas consequências no organismo humano (Ni, Su, & Kokot, 2010; Wang & Arntfield, 2015; Zhou, Liang, & Wang, 2008).

3.2 Ácido cinâmico e cinamato de metila

O ácido cinâmico (Figura 4) é encontrado naturalmente em canela e própolis, por exemplo. Ele possui pKa 4,4 (Cherrington, Hinton, Mead, &

Chopra, 1991) e massa molar de 148,16 g/mol (Y.-P. Chen, Chen, & Tang, 2009).

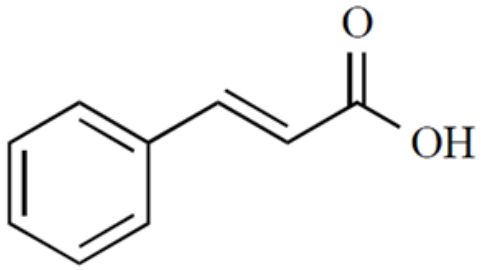


Figura 4 – Estrutura molecular do ácido cinâmico. Fonte: Sova, 2012.

Este composto tem despertado o interesse de diversos grupos de pesquisa por possuir uma ampla atividade terapêutica. Os derivados do ácido cinâmico também possuem diversas propriedades farmacológicas importantes, similares à do AC (Conti, Búfalo, Golim, Bankova, & Sforcin, 2013; De, Baltas, & Bedos-Belval, 2011; Kumazaki et al., 2014; Sova, 2012). Assim, vários estudos têm sido feitos a fim de comprovar ou entender propriedades do AC, entre elas: propriedades antimicrobiana, antioxidante, anticancerígena e imunomodulatória.

Outro composto de interesse na área alimentícia e farmacêutica é o cinamato de metila (Figura 5), um metil éster do ácido cinâmico com aroma frutífero doce que é produzido e liberado durante a maturação do morango, sendo um dos componentes majoritários do aroma deste fruto (Peretto et al., 2014).

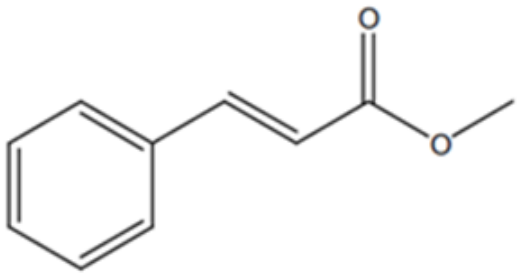


Figura 5 – Estrutura molecular do cinamato de metila. Fonte: Bhatia et al. 2007.

O cinamato de metila é pouco solúvel em água (387,1 mg/L a 25 °C) e tem massa molar igual a 162,19 g/mol. Além de estar presente em matrizes alimentícias que contém morango, este composto é geralmente reconhecido como seguro (GRAS) e pode ser utilizado como aditivo em alimentos. O cinamato de metila é utilizado também como ingrediente de fragrâncias em

cosméticos, xampus e sabonetes. Além disso, ele demonstra possuir atividade antimicrobiana e tem potencial para ser utilizado em revestimento de morangos, a fim de contribuir para a sua conservação (Bhatia et al., 2007; Peretto et al., 2014; Stefanovi, Radojevi, & Ljiljana, 2014).

Este composto também demonstrou possuir atividades anti adipogênica e vasodilatadora, além de ter demonstrado *in vivo* que é capaz de prevenir os efeitos de colite resultante de lesão causada por ácido acético (Y.-Y. Chen, Lee, Hsu, Wei, & Tsai, 2012; Lima et al., 2014; Vasconcelos-Silva, Lima, Brito, Lahlou, & Magalhaes, 2014).

Tendo em vista o potencial farmacológico do ácido cinâmico e também do cinamato de metila, torna-se importante investigar os mecanismos e as energias envolvidas nas interações entre esses componentes e a albumina sérica humana, uma vez que esta proteína desenvolve um papel importante como carreadora de metabólitos no sangue humano (Naveenraj & Anandan, 2013). Deste modo, é importante avaliar se as interações entre esses componentes e tal proteína modificaria seu papel no corpo. Diversas vezes, estudos de interação são realizados utilizando a albumina sérica bovina, pois é homóloga à HSA e possui menor custo de aquisição.

Para estudos de interação envolvendo o fenômeno de fluorescência, é importante ressaltar que apesar da presença de um anel aromático em sua estrutura, o ácido cinâmico é um composto que não emite fluorescência em solução, enquanto o cinamato de metila fluoresce fracamente somente em solução de diclorometano (Lewis, Quillen, Elbert, Schneider, & Geiselhart, 1989).

3.3 Técnicas para estudos de interação intermolecular

Para o estudo das interações entre compostos de aroma e proteínas, faz-se necessária a utilização de técnicas sensíveis, que permitam a determinação de parâmetros termodinâmicos de interação, tais como a constante de ligação (K), estequiometria de formação de complexo (n) e os valores da variação de energia livre de Gibbs (ΔG°), entalpia (ΔH°) e entropia padrão de formação de complexo (ΔS°). É interessante também a determinação de parâmetros que dão informações sobre as mudanças que ocorrem na proteína ao interagir com os ligantes, como variações no tamanho,

potencial zeta, na temperatura de mudança conformacional máxima dessas biomoléculas (T_m) e na variação de entalpia de desnaturação (ΔH_{des}).

As proteínas do soro do leite contêm os resíduos de aminoácidos triptofano, tirosina e fenilalanina, que emitem fluorescência. Porém, cerca de 90 % da emissão de fluorescência emitida é atribuída ao triptofano. Moléculas de proteína emitem fluorescência de maneiras diferentes se estiverem livres ou interagindo com algum ligante. Quando interagem, seja por colisões ou formação de complexo, a fluorescência de uma molécula de proteína pode ser totalmente suprimida, reduzindo a intensidade de fluorescência média emitida por uma amostra proteica. Sabendo disso, pode-se usar a espectroscopia de fluorescência para encontrar os valores de K, n, ΔG°, ΔS° e ΔH° de formação do complexo proteína-ligante (Ferreira, 2009).

Quando há supressão de fluorescência pelo mecanismo colisional, o fenômeno é denominado supressão dinâmica. Por outro lado, quando a supressão ocorre devido à formação de um complexo não fluorescente entre um fluoróforo e um ligante, é chamada supressão estática (Kaboudin, Moradi, Faghihi, & Mohammadi, 2013).

Há diversas relações matemáticas que explicam os fenômenos de supressão de fluorescência. Uma delas é a equação de Stern-Volmer (Equação 1).

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

Onde F_0 e F são as intensidades de fluorescência do fluoróforo na ausência e presença do ligante, respectivamente, K_{SV} é a constante de Stern-Volmer, [Q] é a concentração do ligante, k_q é a constante biomolecular de supressão e τ_0 é o tempo de meia vida do fluoróforo na ausência do ligante.

Deste modo, podem ser encontrados os valores de K_{SV} e k_q se forem plotados gráficos de $F_0/F=f([Q])$, uma vez que sabe-se que para biomoléculas o valor de τ_0 é 10^{-8} s. Se forem feitos gráficos de F_0/F versus $[Q]$ para diversas temperaturas, pode-se comparar a inclinação (K_{SV}) de cada uma delas. Se, ao aumentar a temperatura, o K_{SV} diminuir, o mecanismo de supressão da fluorescência é estático, indicando que há formação de complexo.

Outra forma de verificar o mecanismo de supressão de fluorescência é a partir do valor de k_q. Levando em consideração o tempo de meia vida do biopolímero (10^{-8} s), o valor de k_q pode ser obtido a partir da relação $k_q=K_{SV}/\tau_0$. Se os valores de k_q forem superiores a 2 x 10^{10} L/mol s, que é o valor máximo para a constante biomolecular de supressão colisional, o mecanismo de supressão é classificado como estático.

Pode-se avaliar o mecanismo de supressão também pelos espectros de emissão. Se houver deslocamento do comprimento de onda de máxima emissão de fluorescência, comparando os espectros da proteína sem ligante com os da proteína com o ligante, provavelmente houve formação de complexo, que emite fluorescência em comprimento de onda diferente do da proteína.

Uma vez que houver formação de complexo entre a proteína e o ligante, os parâmetros termodinâmicos de formação deste complexo podem ser determinados. A obtenção da constante de interação é importante porque permite inferir sobre a intensidade da interação entre as espécies que formam o complexo e também em razão da relação com a energia livre de Gibbs padrão de formação do complexo. A determinação da estequiometria da formação do complexo também é um parâmetro de interesse, pois permite saber quantas moléculas de aroma estão interagindo com cada molécula de proteína. Para determinar o K e n, tem-se a Equação 2.

$$log\left(\frac{F_0-F}{F}\right) = logK + nlog\,[Q] \quad (2)$$

Deste modo, ao serem plotados gráficos de log [(F_0-F)/F) versus log [Q], o intercepto da curva é o log K e a inclinação, o n. Diretamente a partir do K, consegue-se obter a variação da energia livre de Gibbs padrão de formação do complexo (ΔG°) através da Equação 3.

$$\Delta G° = -RT\ lnK \quad (3)$$

Em que R é a constante universal dos gases (R = 8,31 J/mol K) e T a temperatura, em Kelvin.

O valor de ΔG° é devido a duas componentes, uma entálpica (ΔH°) e outra entrópica (TΔS°). Para determinar a contribuição dessas duas componentes para o processo de formação do complexo, pode-se determinar a variação da entalpia padrão de formação do complexo por meio da aproximação de van't Hoff (Equação 4).

$$\ln \frac{K_2}{K_1} = -\frac{\Delta H^0}{R}\left(\frac{1}{T_1} + \frac{1}{T_2}\right) \quad (4)$$

Ao fazer um gráfico de ln K *versus* 1/T, a inclinação será o valor de -ΔH°/R. Como o R é uma constante conhecida, é possível calcular o valor de ΔH°.

Por fim, a equação fundamental de Gibbs (Equação 5) permite o cálculo do termo entrópico.

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

Outra técnica que pode ser utilizada é a nanocalorimetria diferencial de varredura (Nano DSC). O Nano DSC foi desenvolvido para determinar a estabilidade térmica e o calor específico de proteínas e outras macromoléculas em soluções diluídas, com versatilidade e precisão (da ordem de nanojoules), além de avaliar as mudanças conformacionais e interações entre biomoléculas e ligantes. O calorímetro mede a quantidade de calor absorvida ou liberada por soluções diluídas de biomoléculas quando elas são aquecidas ou resfriadas. A partir dos experimentos de Nano DSC, pode-se encontrar a variação da entalpia de mudança conformacional (ΔH_{des}) e a temperatura que leva à máxima mudança conformacional das proteínas na presença e na ausência dos ligantes. Assim, pode-se avaliar se a formação do complexo proteína-ligante altera a estabilidade térmica dessas biomoléculas (TA Instruments, 2012).

Da análise de Nano DSC obtém-se os termogramas de onde são extraídos os parâmetros termodinâmicos citados (Figura 6). A T_m é dada pela temperatura correspondente ao valor máximo da variação do calor específico (ΔCp) e a $\Delta H_{dês}$ obtém-se através da integração da área abaixo do pico obtido (Equação 6).

$$\Delta H_{des} = \int_{T_i}^{T_f} Cp^{\circ}\, dT \quad (6)$$

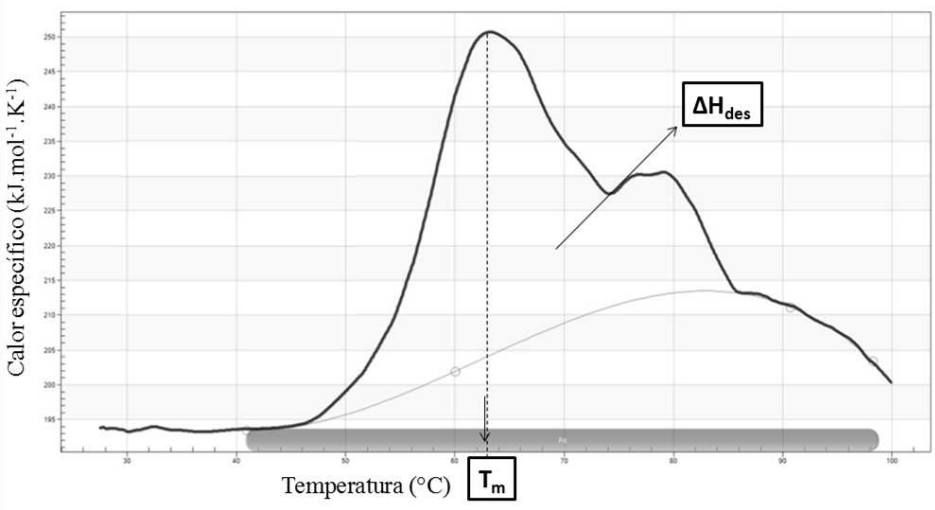


Figura 6 – Esquema de termograma obtido pelo Nano DSC destacando-se os valores de T_m e ΔH_{des}.

Para avaliar os efeitos da formação de complexos na estrutura e carga das proteínas podemos utilizar de medidas de potencial zeta e tamanho. Assim, consegue-se perceber se a formação de complexo leva a mudanças estruturais nessas biomoléculas, bem como se a interação ocorre próxima ou não à superfície da proteína.

A medida de tamanho é realizada através da determinação do coeficiente de difusão da proteína no meio em que se encontra dispersa, através da equação 7.

$$D = \frac{K\,T}{6\,\pi\,\eta\,r} \quad (7)$$

Em que D é o coeficiente de difusão, K a constante de Boltzman, T a temperatura, η o coeficiente de viscosidade e r o raio da partícula.

Assim, ao serem fornecidas ao equipamento as informações sobre o solvente e a temperatura de leitura, este determina o coeficiente de difusão das partículas sob movimento Browniano por meio de espalhamento de luz dinâmico e determina o tamanho delas através da relação de Stokes-Einstein (Equação 7).

Para a medição do potencial zeta é utilizada a microeletroforese Doppler a laser. Aplica-se um campo elétrico sobre a amostra, de modo que as partículas com carga positiva e negativa se movam para o eletrodo negativo e positivo, respectivamente. Através de uma técnica interferométrica a laser é medida a velocidade com que essas partículas se movimentam e determinar o potencial zeta a partir da equação 8.

$$\Delta\varphi = -\eta \frac{V_m}{\varepsilon \varepsilon_0 E} \quad (8)$$

Em que Δφ é o potencial zeta, η é a viscosidade do meio, V_m é a velocidade média da partícula, ε é a constante dielétrica do meio e ε_0 a constante dielétrica no vácuo.

Outra medida da qual se extrai resultados interessantes é a de tensão interfacial. Através dela podem ser descritos o estado termodinâmico e a estrutura de uma interface (Anastasiadis et al., 1987). Proteínas do soro do leite são conhecidas, por exemplo, por apresentarem atividade interfacial, tornando-se importante avaliar se a interação dessas proteínas com ligantes altera as propriedades interfaciais das mesmas, além de serem fornecidas informações relevantes sobre o mecanismo de interação que está acontecendo.

A tensão interfacial líquido-ar pode ser medida através do método da gota pendente, que consiste na avaliação do perfil de uma gota suspensa no ar. Quando esta gota líquida alcança o equilíbrio hidrodinâmico e mecânico (governado pela gravidade e tensão interfacial), seu perfil é resultado do balanço entre a força gravitacional e as forças de superfície (Figura 7) e pode ser descrito pelas Equações 9 a 11 (Song & Springer, 1996).

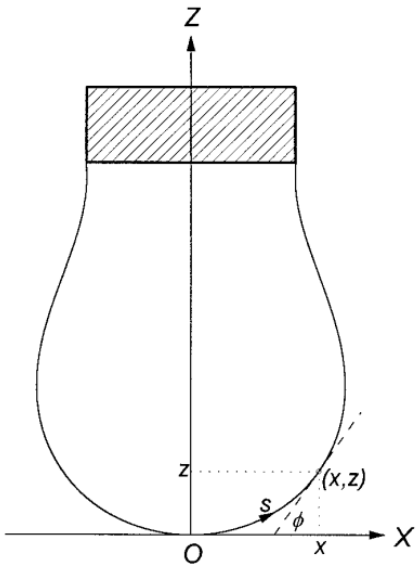


Figura 7 – Geometria e notação de símbolos de um perfil de gota pendente. Fonte: Song & Springer 1996.

$$\frac{d\varphi}{dS} = \frac{2}{B} - Z - \frac{sen\,\varphi}{X} \tag{9}$$

$$\frac{dX}{dS} = \cos\varphi \tag{10}$$

$$\frac{dZ}{dS} = sen\,\varphi \tag{11}$$

Nas condições do limite do ápice da gota temos que:

$$X = Z = S = \varphi = 0 \tag{12}$$

$$\frac{sen\,\varphi}{X} = \frac{1}{B} \tag{13}$$

Onde

$$B = \frac{\Delta\rho\, g\, a^2}{\gamma} \tag{14}$$

As variáveis X, Z e S são formas adimensionais das variáveis x, z e s (Figura 7), que estão relacionadas do seguinte modo:

$$x = X\,a, \quad z = Z\,a, \quad s = S\,a \tag{15}$$

Onde *a* é a constante de capilaridade. *B* é denominado como parâmetro de forma da gota, que determina o perfil adimensional da gota *P (X, Z)*. O perfil dimensional da gota, por sua vez, é determinado por B juntamente com a *p (x, z). Δρ* é a diferença de densidade entre os dois meios (líquido e ar), *g* é a aceleração da gravidade e *γ* a tensão interfacial a ser encontrada.

Por tudo isso, nota-se que através da utilização dos métodos descritos pode-se investigar molecularmente o que acontece quando uma proteína interage com um ligante de interesse.

3.4 Estudos de interação entre BSA e outros compostos

Diversos são os estudos de interação entre a BSA e outros compostos. Há estudos envolvendo moléculas com capacidade antioxidante (Nafisi, Bagheri Sadeghi, & Panahyab, 2011; Roy, 2010; G. Zhang, Ma, Wang, Zhang, & Zhou, 2012), moléculas com potencial anticancerígeno (Y. Liu, Chen, & Song, 2013; Shen, Liu, Wang, Jiang, & Shi, 2015; Shi et al., 2012) e moléculas que tem características antimicrobianas (Naik et al. 2009; Dawoud Bani-Yaseen 2011).

A partir de trabalhos envolvendo a BSA e moléculas similares estruturalmente com os compostos estudados nesta dissertação, pode-se inferir sobre os potenciais resultados do presente trabalho.

Em 2010, Li e colaboradores estudaram a interação da BSA com ácido caféico, clorogênico e felúrico (Figura 8).

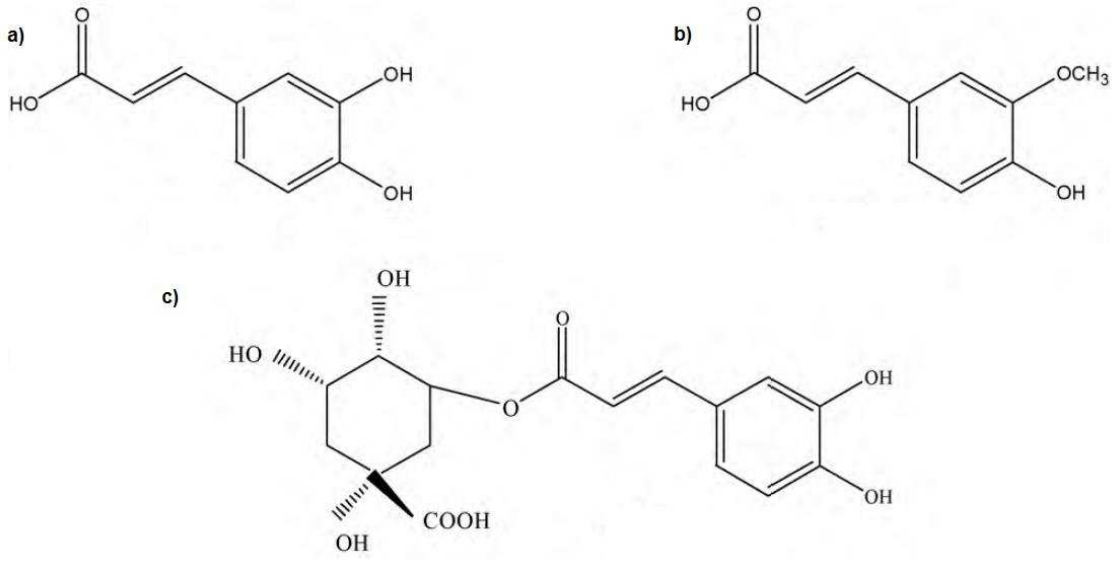


Figura 8 – Estrutura molecular do: a) ácido caféico; b) ácido ferúlico e c) ácido clorogênico. Fonte: adaptado de Li et al. 2010.

A interação desses compostos com a BSA foi investigada utilizando as técnicas de espectroscopia de absorção UV, espectroscopia de fluorescência e espectroscopia de fluorescência sincrônica. Os maiores valores de constante de ligação (K) encontrados foram para o ácido clorogênico, seguido do ácido caféico e do ácido felúrico (Valores: 63,44 x 10^4 L/mol, 42,64 x 10^4 L/mol e 1,79 x 10^4 L/mol, respectivamente). Para todos os ligantes, com o aumento da temperatura houve aumento nos valores de K, porém, a formação de complexos entre ácido clorogênico e o ácido felúrico com a BSA foi mais sensível à temperatura do que o ácido caféico com a proteína. As diferentes afinidades entre os fenóis e a proteína devem-se às diferenças estruturais das moléculas. Também para todos os compostos estudados a estequiometria de formação de complexo manteve a relação 1:1 (Li et al., 2010).

Também em 2010 foi publicado um trabalho por Trnkova e coladoradores, que estudaram a interação da BSA com diversos ácidos hidroxicinâmicos, são eles: o-ácido cumárico, m-ácido cumárico, p-ácido cumárico, ácido caféico, ácido felúrico, ácido sinápico, ácido clorogênico e ácido rosmarínico (Figura 9).

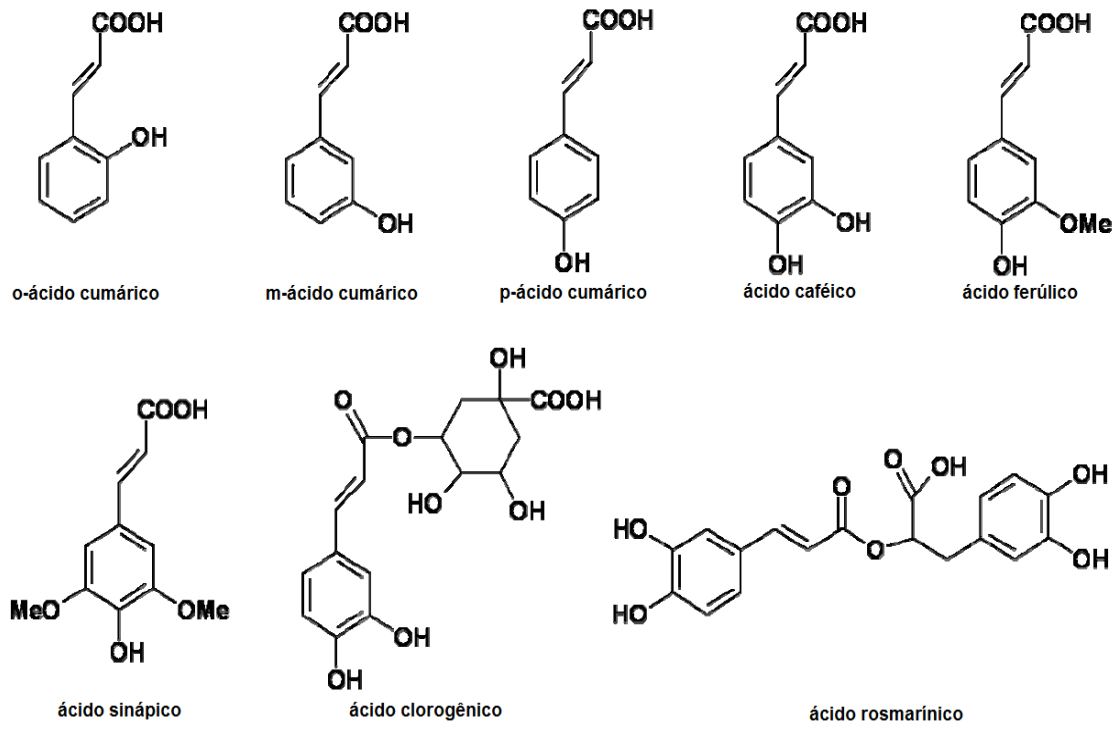
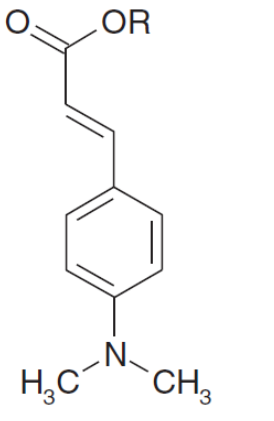


Figura 9 – Estruturas moleculares dos ácidos hidrocinâmicos estudados por Trnkova et al. (2010). Fonte: adaptado de Trnkova et al. 2010.

No trabalho citado, foram utilizadas a espectroscopia de absorção UV e a espectroscopia de fluorescência. Todos os compostos suprimiram a fluorescência da proteína em pH fisiológico, exceto o ácido rosmarínico. A ordem decrescente das constantes de ligação foi: ácido clorogênico, ácido sinápico, ácido caféico, ácido ferúlico, o-ácido cumárico, p-ácido cumárico e m-ácido cumárico, ressaltanto que os valores de K para os dois últimos foram iguais, assim como para os ácidos sinápico e caféico. Os valores encontrados foram na ordem de 10^5, variando de 1,31 x 10^5 L/mol a 6,67 x 10^5 L/mol. Para todos os ligantes a estequiometria de formação de complexo foi 1:1. Os autores concluíram que os ácidos hidrocinâmicos estudados (exceto o ácido rosmarínico) podem ser transportados no sangue pela proteína, o que pode influenciar nas suas atividades farmacológicas. Além disso, as funções da proteína no sangue podem ser alteradas pela formação dos complexos (Trnkova et al., 2010).

Singh & Mitra (2011) estudaram a interação entre BSA e HSA e dois derivados do ácido cinâmico (Figura 10) através da espectroscopia de fluorescência. As constantes de ligação tiveram valores na ordem de 10^4 L/mol e foram similares tanto para os complexos formados com BSA quanto para os com HSA, indicando que a interação ocorre com o triptofano localizado no sítio hidrofóbico das proteínas. Para todos os ligantes e proteínas a estequiometria de formação de complexo foi de 1:1.



R = H : DMACA
= $-C_2H_5$: EDAC

Figura 10 – Estrutura molecular dos derivados do ácido cinâmico: 4-(dimetilamino) ácido cinâmico (DMACA) e *trans*-etil p-(dimetilamino) ácido cinâmico (EDAC). Fonte: Singh & Mitra, 2011.

Outro estudo tratou da interação entre o ácido cinâmico e a BSA em pH 7,4 a 23 °C, 30 °C e 37 °C utilizando espectroscopia de fluorescência em conjunto com espectroscopia de infravermelho com transformada de Fourrier e espectroscopia de dicroísmo circular. Foi comprovado que o composto de aroma interage com a BSA e determinado que a 23 °C a constante de ligação foi de 7,9 x 10^4 L/mol e a variação de energia livre de Gibbs, de complexação foi – 27,71 kJ/mol. As variações de entropia e entalpia de formação de complexo foram 38,03 J/mol/K e -16,46 kJ/mol, respectivamente. Foi determinado também que o ácido cinâmico se liga ao sítio I da BSA e a formação do complexo possivelmente afeta a estrutura de α-hélice da proteína. Neste mesmo trabalho foram determinadas as constantes de ligação do complexo ácido cinâmico-BSA na presença de vários íons, que diminuíram quando comparadas à formação de complexo na ausência de íons. Os valores se compreenderam entre 1,05 x 10^4 L/mol e 5,12 x 10^4 L/mol (Bian et al., 2007).

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INTERACTION OF CINNAMIC ACID AND METHYL CINNAMATE WITH BOVINE SERUM ALBUMIN: A THERMODYNAMIC APPROACH

Abstract – Cinnamic acid (CA) and methyl cinnamate (MC) have attracted interest of researches because of its larger therapeutic functions. Thus, is important to study its interaction with bovine serum albumin (BSA), once this protein is an important molecule career in blood and also is an interfacial agent in food products. For this paper, it was studied at molecular level the interaction between BSA and CA or MC (at pH 3.5, 5.0 and 7.4) using fluorescence spectroscopy, differential scanning nanocalorimetry, interfacial tension, size and zeta potential measurements. CA/MC-BSA binding constants (K_b) were in order of 10^4 L.mol^{-1} and complexes formation stoichiometry according with 1:1 protein/binding proportion at pH 7.4. At this condition, CA-BSA complex formation was driven by hydrophobic interactions ($\Delta H°$ = 14.44 kJ.mol^{-1}) due to electrostatic repulsion between charged BSA and CA. At pH 3.5 and 5.0 complexe formation had enthalpic contribution ($\Delta H°$ -29.81 kJ.mol^{-1} at pH 3.5 and -21.12 kJ.mol^{-1} at pH 5.0). Ammonium sulfate presence (pH 7.4) had no influence in entropic term but lead to $\Delta H°$ decrease ($\Delta H°$ -17.84 kJ.mol^{-1}). Calorimetric studies indicated that pH plays an important role in protein conformation, since at acid condition protein was denatured. When BSA was denatured, it also formed complex with CA, but $\Delta G°$ values became less negative. It was verified that CA or MC presence did not affect σ_{eq} values in all studied pH. Also, it was verified that protein interaction with MC was more favorable ($\Delta G°_{CA\text{-}BSA}$ = -26.53 kJ;mol^{-1} and $\Delta G°_{MC\text{-}BSA}$ = -30.32 kJ.mol^{-1}at pH 7.4 and 298.15 K) than with CA and it was enthalpically driven ($\Delta H°_{MC}$ = -8.71 kJ.mol^{-1} at pH 7.4) probably because MC is not ionizable and it is more hydrophobic. Ammonium sulfate presence almost did not change $\Delta H°$ values (-8.71 kJ.mol^{-1}, -19.32 kJ.mol^{-1}, -11.32 kJ.mol^{-1}, -14.06 kJ.mol^{-1}, -1.95 kJ.mol^{-1} for 0, 1, 10, 50 and 100 mM, respectively) and $T\Delta S°$ values (21.64 kJ.mol^{-1}, 11.29 kJ.mol^{-1}, 23.06 kJ.mol^{-1}, 17.95 kJ.mol^{-1}, 28.4 kJ.mol^{-1} for 10, 50 and 100 mM, respectively). These data contributed to knowledge about CA/MC-BSA interactions and, therefore, for future applications in food and pharmaceutical areas.

Keywords: Cinnamic acid, methyl cinnamate, interfacial tension, BSA conformation, pH, intermolecular interaction

1. INTRODUCTION

Cinnamic acid (CA), like other phenolic acids, is a secondary plant metabolite and can be widely found in nature (Bian et al., 2007; Singh & Mitra, 2011), mainly in cinnamon, cloves, cranberries and prunes (Patel, Ray, Aswal, & Bahadur, 2014). It is approved by FDA for use in flavor composition (H.-M. Zhang, Chen, Zhou, Shi, & Wang, 2011), perfumes and synthetic indigo (Singh & Mitra, 2011).

CA also has broad possible therapeutic functions, including antimicrobial, antifungal (Patel et al., 2014) and antiparasitic activities (Cury et al., 2015). The interest in CA has continuously increasing because it has been recognized that it also shows antitumor activity against human malignant tumors, such as melanoma, gioblastoma and adenocarcinoma of the prostate and lung (Bian et al., 2007; H.-M. Zhang et al., 2011); in addition CA is also known as an anti-diabetic agent (Ganugapati & Swarna, 2014; Hafizur et al., 2015; Singh & Mitra, 2011).

Methyl cinnamate (MC) is a cinnamic acid ester, produced and released by strawberries during maturation. MC is generally recognized as safe (GRAS) and can be used as food additive. MC also has importance as antimicrobial agent and was studied to act as an antimicrobial agent to recover strawberries (Bhatia et al., 2007; Peretto et al., 2014; Stefanovi, Radojevi, & Ljiljana, 2014). MC demonstrated anti adipogenic and vasodilatatory activity, and was demonstrated *in vivo* that it is able to prevent the effects of colitis resulted of injury caused by acetic acid. (Y.-Y. Chen, Lee, Hsu, Wei, & Tsai, 2012; Lima et al., 2014; Vasconcelos-Silva, Lima, Brito, Lahlou, & Magalhaes, 2014).

Because CA contains a propenoic chain, it shows low solubility in water (B. Liu et al., 2016), around 0.002 M (Patel et al., 2014). Thus, enhanced solubility of this compound would improve its application in food, pharmaceutical and cosmetic industries. Some studies have shown that CA can be carried by different compounds, such as surfactant micelles, carbohydrates and proteins. Patel et al. (2014) found that Triton X-100 micelles enhanced CA solubility by about 25 times at physiological pH. Cyclodextrins (CDs) were also efficient to improve solubility of CA, forming 1:1 stoichiometric inclusion complex with β-CD, determined by fluorescence (B. Liu et al., 2016). The carrier of drugs and bio-active small molecules by proteins such serum albumins and the study of

intermolecular interaction between them can have immense physiological and industrial importance because these molecules can bind reversibly to albumins, significantly affecting its solubility, stability, absorption, distribution and metabolism (Kamat, Seetharamappa, & Melwanki, 2004; G. Zhang et al., 2012).

Bovine serum albumin (BSA) consists in a single polypeptide chain of 583 amino acids and no carbohydrate residue. It is made up of three homologous domains (I, II and III) that, in turn, are divided in two sub-domains (IA, IB, etc). BSA contains two tryptophan residues, Trp-134 located in the first domain on the surface of the molecule and Trp-212 entrapped within the hydrophobic pocket of the protein (second domain) (Bolel, Mahapatra, Datta, & Halder, 2013; Bourassa, Kanakis, Tarantilis, Pollissiou, & Tajmir-Riahi, 2010).

Although some spectroscopic studies on the interaction of cinnamic acid with BSA have been reported (Bian et al., 2007; Min et al., 2004; Singh & Mitra, 2011), there is lack of information about the effect of CA-BSA interaction on surface properties and conformation of the protein, as well as the influence of pH on the formation of CA-BSA complex. This knowledge may contribute to gain key insights for CA application either in food or in pharmaceutical areas. Therefore, in this work we report a thermodynamic approach of interaction between BSA and CA and methyl cinnamate (MC) at different pHs and presence of salt by using fluorescence spectroscopy, differential scanning nanocalorimetry and interfacial tension and electrokinetic measurements.

2. MATERIAL AND METHODS

2.1. Materials

Bovine serum albumin (heat shock fraction, pH 7, ≥ 98 %) and methyl cinnamate (≥ 99.0 %) and cinnamic acid (> 99.0 %) were purchased from Sigma-Aldrich (St Louis, US). Was used distilled water to prepare buffer solutions pH 3.5, pH 5.0 (sodium citrate and citric acid for both pH) and pH 7.4 (sodium phosphate monobasic and bibasic), where BSA was diluted. Cinnamic acid was diluted directly using BSA solution, whereas to prepare methyl cinnamate solutions, was used a stock solution of 1.0×10^{-2} mol.L^{-1} in ethanol, because of its water insolubility. Curves containing MC were prepared in a way that never had more than 1 % (v/v) of ethanol.

2.2. Fluorescence quenching studies

For fluorescence experiments were prepared 8 samples containing BSA 3.01 x 10^{-5} mol.L^{-1}. The first sample hadn't binding solution and the others had 1.0 x10^{-5} mol.L^{-1} to 1.0 x 10^{-6} mol.L^{-1} of methyl cinnamate or cinnamic acid and they were left at 298.15 K for 12 hours in Ultra thermostatic bath MA-184 (Marconi, Piracicaba, BR). It was done at pH 3.5, 5.0 and 7.4 for native BSA and at pH 7.4 for denatured BSA. To denature BSA, the protein solution was submitted to 358.15 K for 15 minutes using Ultra thermostatic bath MA-184. Also, at pH 7.4 were prepared samples containing 1 mM, 10 mM, 50 mM and 100 mM of ammonium sulfate. It was lead to Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, USA) in quartz cells and excited at wavelength 295 nm and excitation slit of 2.5 nm and emission slit of 5.0 nm, 650 V PMT voltage and scan rate at 600.00 nm/min to obtain emission spectra from 296 nm to 450 nm. The spectra were obtained at 298.15 K, 308.15 K, 318.15 K and 328.15 K.

2.3. Zeta potential and size measurements

For zeta potential and size measurements, were prepared 6 samples containing 3.01 x 10^{-5} mol.L^{-1} of BSA. The first one without methyl cinnamate or cinnamic acid and the followings with 1.0 x 10^{-5} mol.L^{-1} to 1.0 x 10^{-4} mol.L^{-1} of MC or CA. Similarly to fluorescence experiments, it was done at 3 pH values for native BSA and at pH 7.4 for denatured BSA and also, at pH 7.4 were prepared samples with 1 mM, 10 mM and 100 mM of the same salts used in that experiment. The samples were diluted 10 times in water and left at 298.15 K for 12 hours in the same ultra thermostatic bath and then were lead to Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). At the equipment, the dispersant chosen was water at 298.15 K and was fixed the viscosity of 0.8872 cP, refraction index 1.330 and dielectric constant 78.5.

2.4. Interfacial tension analysis

Was used the pendant drop method to determine interfacial tension of the samples. For this, were prepared 8 samples containing 7.5 x 10^{-6} mol.L^{-1} of BSA, for better curve shape. The first sample just contained BSA and buffer (pH

3.5, 5.0 or 7.4) and the followings had 2.5 x 10^{-6} mol.L^{-1} to 2.5 x 10^{-5} mol.L^{-1} of MC or AC, to maintain the protein:binding proportion of the other experiments. For denatured BSA the experiment also was carried at pH 3.5 and 7.4. All the samples were kept in ultra thermostatic bath for 12 hours at 298.15 K. For the purpose of obtain the behavior of samples drop/air interfacial tension during the time, was used Easy Drop goniometer (Kruss, Hamburg, DE), where was measured interfacial tension in each second during 20 minutes at 298.15 K.

2.5. Differential scanning calorimetric experiments

Nano DSC (TA Instruments, New Castle, USA) calorimeter with two platinum cells was used to carry the experiments in the following conditions: one heating and cooling at 3 atm and rate of 274.15 K/min from 293.15 K to 383.15 K. Besides, were lead to Nano DSC samples containing 1.0 x 10^{-5} mol.L^{-1}, 5.0 x 10^{-5} mol.L^{-1} or 1.0 x10^{-4} mol.L^{-1} of MC or CA maintaining BSA concentration constant (3.01 x 10^{-5} mol.L^{-1}) at 3 studied pH. Was studied native BSA at pH 3.5, 5.0 and 7.4 and also denatured BSA at pH 3.5 and 7.4.

3. RESULTS AND DISCUSSION

3.1. Fluorescence spectroscopy for analyze cinnamic acid-BSA interaction

Fluorescence is a powerful technique to evaluate interaction between small ligands and proteins (Wei, Xiao, Wang, & Bai, 2010). BSA molecules presents in aqueous solution and excited at 295 nm emits fluorescence mainly due to tryptophan residues. When other molecules interact with BSA, Trp fluorescence spectrum may change (Bourassa et al., 2010; Han et al., 2012). The gradual addition of cinnamic acid (CA) into a fixed concentration of BSA at pH 7.4 and 298.15 K decreased fluorescence intensity of Trp (quenching) (Fig 1). BSA fluorescence quenching was verified for other temperatures in this physiological pH.

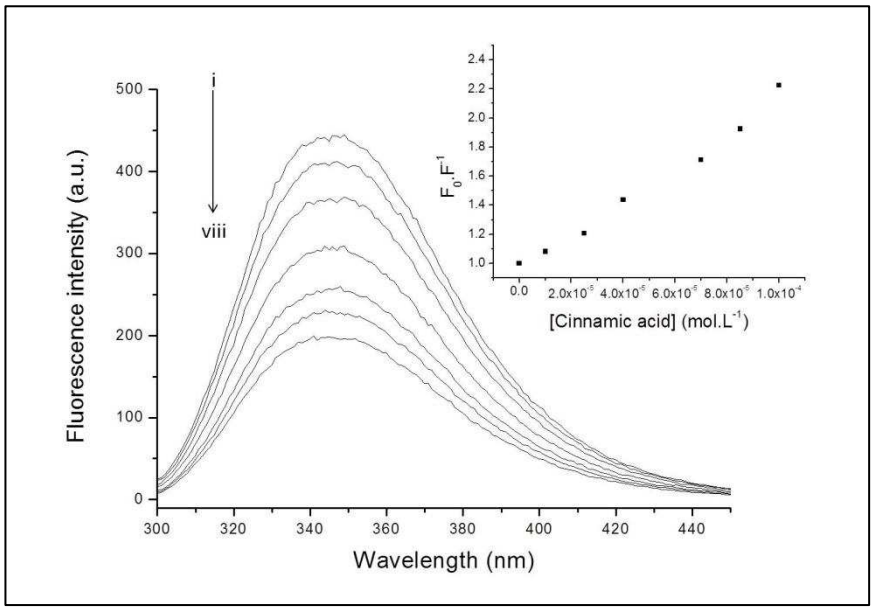


Fig 1 – BSA fluorescence spectra in pH 7.4 at 298.15 K. CA concentrations varied from 0 to 1.0 x 10^{-4} mol.L^{-1}. The inset represents the Stern-Volmer plot at the same temperature.

The maximum emission wavelength was around 345 nm and it did not change as CA concentration enhanced, which indicates that CA binding to BSA did not modify Trp microenvironment.

Fluorescence quenching could proceed by two mechanisms, classified as dynamic and static quenching, which can be distinguished mainly by their dependence of temperature (Zhang et al., 2012). The quenching rate constants decrease with increased temperature for the static quenching, while the reversed temperature effect occurs for the dynamic quenching (C. Chen, Ma, Zhang, Wang, & Xiang, 2008). The possible quenching mechanism can be interpreted by Stern-Volmer curves of titration of BSA aqueous solution by CA, which are shown in Fig 1.

For all studied temperatures, the linear plot indicated the existence of a single type of quenching (Kaboudin et al., 2013). The slope (Stern-Volmer constant - K_{SV}) at each temperature is presented in Table 1.

Table 1 – Thermodynamic parameters obtained from fluorescence studies of CA-BSA systems at physiological pH.

T	K_{SV}	r^2	K_q	n	K_b	r^2	ΔG°	TΔS°	ΔH°	r^2
(K)	(10^4.L.mol^{-1})		(10^{12}.L.mol^{-1}.s^{-1})		(10^4.L.mol^{-1})		(kJ.mol^{-1})			
288.15	1.19	0.97	1.19	1.14	4.08	0.98	-25.42	40.33		
298.15	1.17	0.96	1.17	1.15	4.46	0.98	-26.53	41.43		
308.15	1.13	0.97	1.13	1.19	6.13	0.98	-28.23	43.14	14.22	0.99
318.15	1.07	0.96	1.07	1.15	3.87	0.98	-27.93	42.84		
328.15	0.97	0.97	0.97	1.18	4.69	0.98	-29.33	44.24		

It was found that K_{SV} values inversely correlated with temperature indicating that quenching mechanism was due to CA-BSA complex formation. In order to confirm this point, we assumed the process as a dynamic procedure, whose quenching equation is:

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

where: F_0 and F are the fluorescence intensity in the absence and in the presence of the quencher (in this case, CA), respectively; K_q is the quenching rate constant of the biomolecule (in this case, BSA); K_{SV} is the Stern-Volmer constant, τ_0 is the average lifetime of BSA without quencher and [Q] is the concentration of CA. Obviously, K_{SV} can be written as:

$$K_{SV} = K_q \tau_0 \quad (2)$$

Since K_{SV} was determined and τ_0 for biopolymers (such as BSA) is around 10^{-8} s, K_q values were obtained and were of the order of 10^{12} L.mol.s^1 for all studied temperatures. However, the maximum K_q value of various dynamic quenchers with biopolymers found in literature is 2.0 x 10^{10} L.mol.s^1 (C. Chen et al., 2008), thus it means that quenching process initiated by CA was due to formation of a CA-BSA complex.

Since a complex was formed, results from fluorescence measurements allow estimate the binding constant of CA-BSA complex (Equation 3).

$$\log[(F_0 - F)/F] = \log K_b + n \log [Q] \quad (3)$$

where: F_0 and F are the fluorescence intensity in the absence and in the presence of the CA; K_b is the biding constant; n is the stoichiometry of complex formation and [Q] is the concentration of CA.

From the intercept and slope of regression curve plotted using Eq. 3, the values of K_b and n were obtained at five different temperatures (Table 1). A representative plot is shown in Fig. 2.

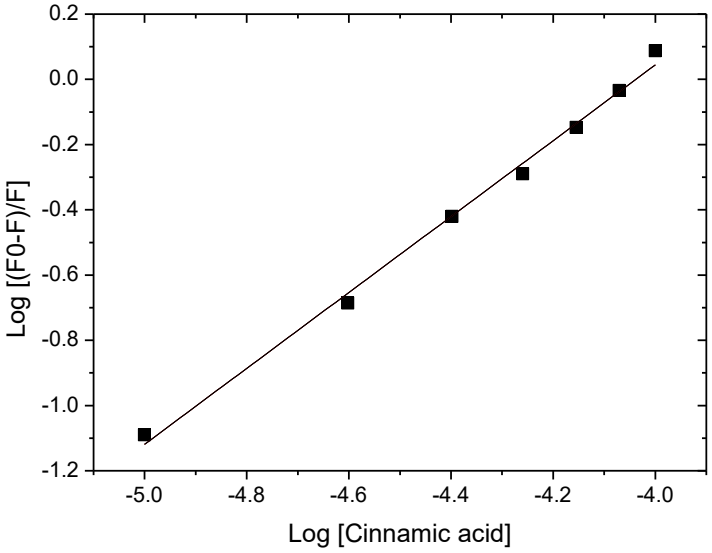


Fig 2 – Plot to find binding constant and complex stoichiometry of CA-BSA pH 7.4 at 25 °C.

We found that the constants were in the order of magnitude of 10^4 $mol.L^{-1}$ for CA-BSA interaction and n values around 1 for all studied temperatures, suggesting that there is just a single biding site in BSA for CA.

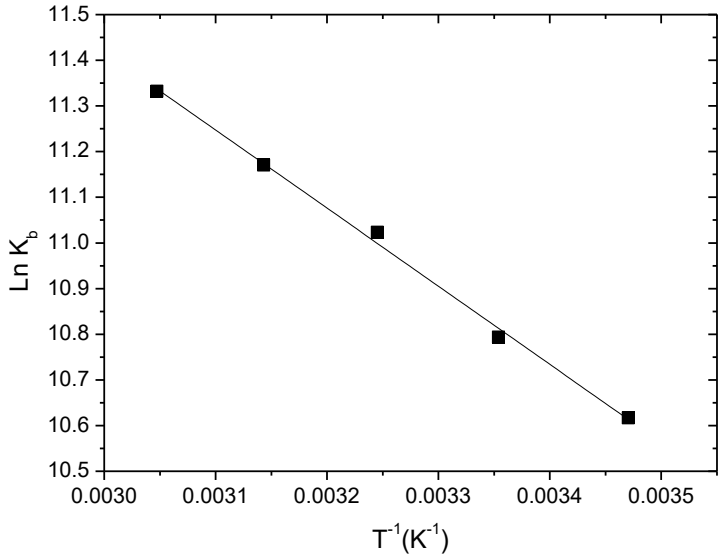
The interaction forces between small molecules and proteins may include electrostatic interactions, formation of hydrogen bounds, van der Waals and hydrophobic interactions (Han et al., 2012; Zhang et al., 2011); and the sign and magnitude of the thermodynamic parameters provide information towards the stability of a complex.

Thus, in order to further identify the nature of the interaction between CA and BSA, the thermodynamic parameters were calculated using van't Hoff approach. Considering that the standard enthalpy change of complex formation (∆H°) for CA-BSA is constant over the temperature range studied (Supplementary material S1) the relation between temperature, k_b and change in other thermodynamic parameters can be written by following relations:

$$\ln \frac{K_2}{K_1} = -\frac{\Delta H^0}{R}\left(\frac{1}{T_1} + \frac{1}{T_2}\right) \quad (4)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (5)$$

where *R* is the universal gas constant (8.3145 J.mol^{-1}.K^{-1}), *T* is the temperature (K), K_a is binding constant (L.mol^{-1}), ∆G° is the standard Gibbs free energy change (kJ.mol^{-1}), ∆H° is the standard enthalpy change (kJ.mol^{-1}), and ∆S° is the standard entropy change (kJ.mol^{-1}) for the formation of CA-BSA complex.



S1 – Plot of T^{-1} as function of Ln K_b for ΔH° determination for CA-BSA complex at pH 7.4.

The ∆H° value was obtained from slope of the van't Hoff plot (ln K_b x T^{-1}), while ∆G° and ∆S° values were calculated from fundamental Gibbs equation (Eq. 5). All thermodynamic parameters are shown in Table 1.

The negative values of ∆G° indicate that the interaction process favored the formation of CA-BSA complex in all temperatures. The positive ∆H° value associated to large and positive values of ∆S° point to the process interaction between CA and BSA is entropy driven probably due to hydrophobic interactions, but it may also have contribution from repulsive electrostatic interaction. Since pKa of CA is about 4.4, its carboxylic group is partly dissociated at physiological pH showing negative charge (Zhang et al., 2011), as well as BSA whose isoelectric point (IP) is around 4.7 (Mandal, Bardhan, & Ganguly, 2010). Therefore, probably an electrostatic repulsion occurred between groups located at surface of both chemical species, while hydrophobic interaction between benzene ring of CA and hydrophobic site entrapped into BSA driven the complex formation. The entropy increasing is due to the releasing of water molecules that were solvating both chemical species interacting, named hydrophobic effect (Mandal et al., 2010).

3.1.1. The effect of pH on CA-BSA interaction

In order to elucidate the effect of surface charge of CA and BSA on complex formation, the fluorescence experiments were repeated at pH 3.5 (bellow pKa of CA and IP of BSA) and pH 5.0 (very close to pKa of CA and IP of BSA).

When surface negative charge was reduced by pH decrease to 5.0 and 3.5, the stoichiometry of complex formation was maintained in one CA for 1 BSA molecule, and K_b ranged between the order of 10^4 and 10^5 $L.mol^{-1}$. However, even though $\Delta G°$ values almost did not change compared to pH 7.4, the contributions of enthalpy and entropy to the process of formation of CA-BSA complex changed. It was remarkable the enthalpic contribution caused by reduction of negative electrostatic repulsion at pH 5.0 (which allowed the formation of hydrogen bounds between OH group pH cinnamic acid and carboxylic groups of BSA) and by electrostatic attraction at pH 3.5, since in the last condition protein is positively charged. The results of thermodynamic parameters for pHs 3.5 and 5.0 are shown in Table 2.

Table 2 – Thermodynamic parameters obtained for CA-BSA complexes at pH 3.5 and 5.0.

pH	T (K)	K_{SV} (10^4.L.mol^{-1})	r^2	K_q (10^{12}.L.mol^{-1}.s^{-1})	n	K_b (10^4.L.mol^{-1})	r^2	$\Delta G°$	$T\Delta S°$ (kJ.mol^{-1})	$\Delta H°$	r^2
3.5	288.15	2.59	0.95	2.59	1.13	7.95	0.97	- 27.02	- 2.80	- 29.81	0.93
	298.15	1.93	0.99	1.93	1.03	2.44	0.99	- 25.03	- 4.79		
	308.15	1.88	0.99	1.88	1.02	2.16	0.99	- 25.56	- 4.25		
	318.15	1.73	0.99	1.73	0.99	1.62	0.99	- 25.63	- 4.19		
	328.15	1.65	0.99	1.65	0.99	1.51	0.99	- 26.24	- 3.57		
5.0	288.15	1.44	0.99	1.44	1.33	31.7	0.99	- 30.33	9.21	-21.12	0.95
	298.15	1.35	0.99	1.35	1.29	19.1	0.99	- 30.13	9.00		
	308.15	1.27	0.99	1.27	1.31	21.8	0.99	- 31.48	10.36		
	318.15	1.20	0.99	1.20	1.25	12.2	0.99	- 30.95	9.83		
	328.15	1.15	0.99	1.15	1.24	10.5	0.99	- 31.52	10.39		

3.1.2. The effect of ionic strength on CA-BSA interaction

Since it was found that surface charge of protein is important to the formation of CA-BSA complex, we repeated the fluorescence experiments with BSA at pH 7.4, however in the presence of 1, 10, 50 and 100 mM ammonium sulfate.

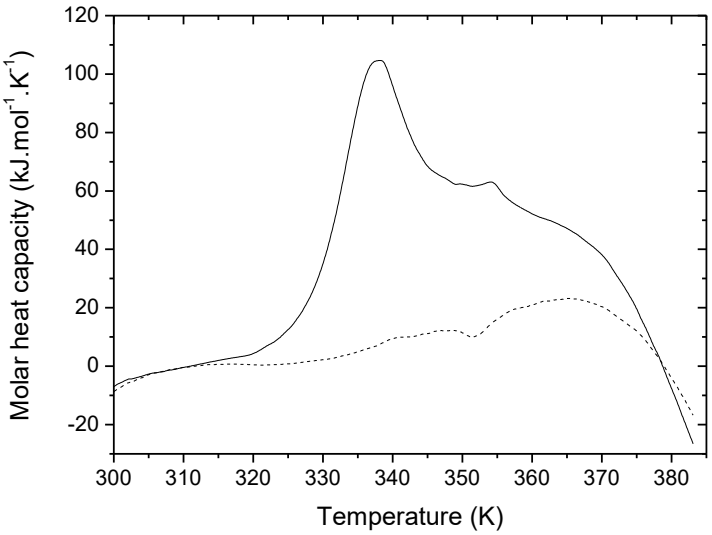
The addition ammonium sulfate did not avoid the formation of CA-BSA complexes with stoichiometry 1:1; however thermodynamic parameters of

complex formation were changed. The salt caused a pronounced decreasing in $\Delta H°$ values which went from 14.22 kJ.mol^{-1} (without salt) to -17.84 kJ.mol^{-1} (with salt concentration of 1 mM), probably due to specific bounds occurring between NH_4^+ and amine groups of BSA (Scrutton & Raine, 1996). Higher salt concentrations did not cause continuous decreasing on $\Delta H°$, which were -16.90, -12.91 and -17.89 kJ.mol^{-1} for 10, 50 and 100 mM of ammonium sulfate.

In relation to entropic term, there was very similar values for each studied temperature, so we analyzed the $T\Delta S°$ values only at 298.15 K. The salt addition resulted in $T\Delta S°$ reduction (ranging from 41.43 kJ.mol^{-1} without salt from 6.35 kJ.mol^{-1} with ammonium sulfate at 1mM), but as salt concentration increased, entropic term did not continued to reduce (values of 6.84, 32.38 and 3.07 kJ.mol^{-1} for 10, 50 and 100 mM of salt). This $T\Delta S°$ decrease can be attributed of the fact that NH_4^+ is considered a kosmotropic cation, according with Hofmeister serie.

3.1.3. The effect of BSA conformation on CA-BSA interaction

The results obtained for the effect of surface properties of BSA on the formation of a complex with CA lead us to believe that the conformation of protein may also influence the interaction between CA and BSA, probably because of the importance of BSA hydrophobic sites on interaction. The study of the role of protein conformation on its interaction with small molecules could provide important information for its future application, as some steps in food and pharmaceutical industries may cause the denaturation of the biomolecule. Therefore, BSA solution at pH 7.4 was submitted to heat (358.15 K/15 min) aiming to achieve protein denaturation. In order to ensure that BSA was unfolded after heat treatment, thermograms of unheated and heated BSA solution was recorded using nano-DSC equipment (Supplementary material, S2).



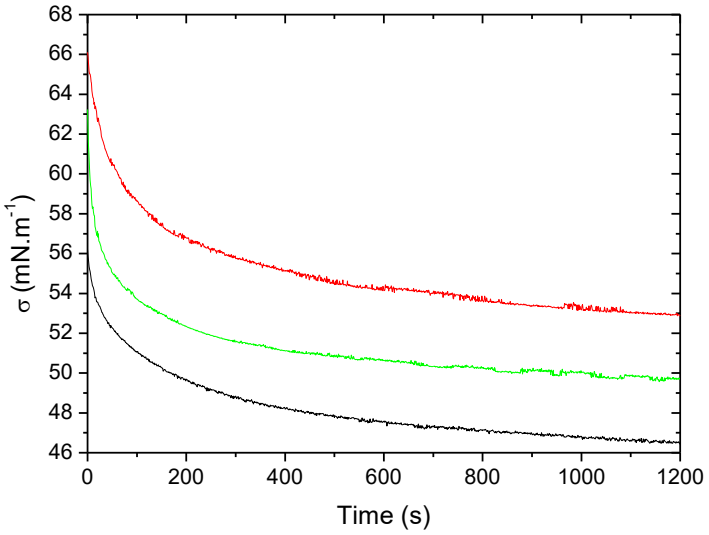
S2- Thermograms of native (__) and denatured (- - -) BSA at pH 7.4.

The change in BSA conformation did not avoid the complex formation and maintained its stoichiometry; however it resulted in a reduction of K_b and $\Delta G°$ values became less negative (K_b = 1.06 x 10^4 L.mol^{-1} and $\Delta G°$ = - 22.96 kJ.mol^{-1} at 298.15 K), suggesting that the presence of hydrophobic sites of BSA play an important role in its interaction with CA. In addition, there was a change in enthalpic and entropic contributions. While the process of formation of CA-BSA complex was endothermic for native protein ($\Delta H°$ = 14.22 kJ.mol^{-1}), occurring driven by entropy increasing; for the denatured biomolecule the process was slightly exothermic ($\Delta H°$ = -3.95 kJ.mol^{-1}) and with lower entropic contribution ($T\Delta S°$ = 19.01 kJ.mol^{-1} at 295 K), indicating that denaturation exposed different amino acid residues at the surface unfolded BSA, which formed interactions with CA, releasing energy.

3.2. Surface proprieties of BSA interacting with CA

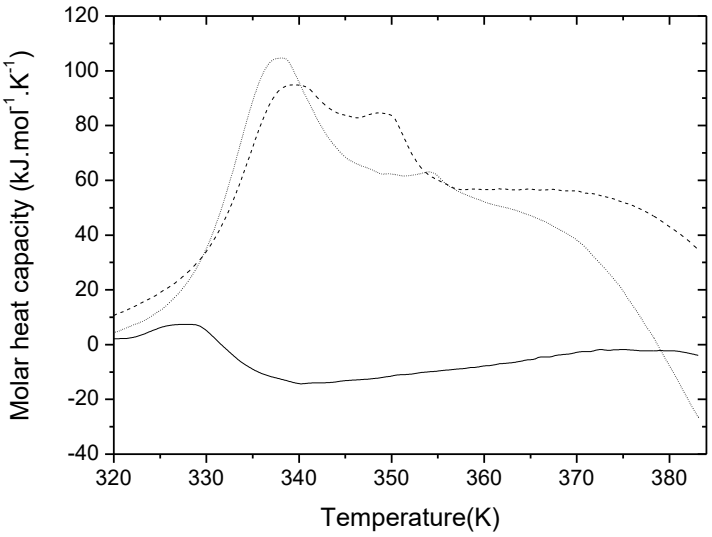
In addition to quenching the fluorescence of Trp present in BSA, the formation of CA-BSA complexes could modify other important properties of the protein, such as its surface and conformational properties. Thus, in order to evaluate the impact of the complex formation on these properties, measurements of the equilibrium surface tension, zeta potential and size of BSA in the presence and absence of CA were carried out.

As most proteins, BSA adsorbs at different interfaces which have many applications such as stabilizing emulsions and foams in food, biotechnological, pharmaceutical and medical areas (Berthold, Schubert, Brandes, Kroh, & Miller, 2007). Surface tension (σ) of water immediately dropped in presence of BSA until it reached steady-state values of 46.8, 48.8 and 53.4 $mN.m^{-1}$ at pH 3.5, 5.0 and 7.4, respectively (see supplementary material, S3).



S3 – Interfacial tension between BSA solution and air at 298.15 K in pH 3.5 (——), 5.0 (——) and 7.4 (——) as function of time.

This difference in the behavior of protein adsorption at water/air interface could be attributed to a larger fraction of amino acid residues in trains, rather than loops, in pH 3.5 and 5.0 compared to pH 7.4 (McClellan & Franses, 2003). In addition, associating results of tension surface with nano-DSC results we could verify that at pH plays an important role on the tertiary structure of BSA (Supplementary material S4). At pH 5.0 the value of denaturation enthalpy change ($\Delta_{den}H$) was smaller than at pH 7.4 (803.3 and 943.5 $kJ.mol^{-1}$, for pH 5.0 and 7.4, respectively), pointing to a modification of protein conformation caused by reduction of pH.



S4 – Thermograms of native BSA at pH 3.5 (——), 5.0 (····) and 7.4 (----).

At pH 3.5 there was a pronounced change in the protein conformation, since $\Delta_{den}H$ value for BSA at this pH was only 86.18 kJ.mol^{-1}, which was completely different from value obtained for native protein (943.5 kJ.mol^{-1}), but very close to value verified for unfolded BSA (62.60 kJ.mol^{-1}) at pH 7.4. Thus, at acid pH protein is denatured and it exhibits more defined hydrophobic and hydrophilic regions, therefore it was more capable to adsorb at water/air interface promoting greater reduction in surface tension compared to BSA at pH 7.4.

To evaluated the effect of the formation of CA-BSA complex on adsorbing behavior of protein, we repeated the experiments with BSA (at different pHs) in the presence of increasing concentrations of CA, and surface tension at equilibrium (σ_{eq}) was calculated by adjusting the experimental data to the exponential model: $\sigma_{(t)} = \sigma_0\, e^{\left(\frac{-\sigma_t}{A_0}\right)} - \sigma_\infty$, were $\sigma_{(t)}$ is the experimental surface tension, σ_0 is the surface tension of BSA without CA, A_0 is an exponential adjustment parameter and σ_∞ is the surface tension at infinite time.

As can be observed, the presence of increasing concentrations of CA did not affect the σ_{eq} values (Fig. 3).

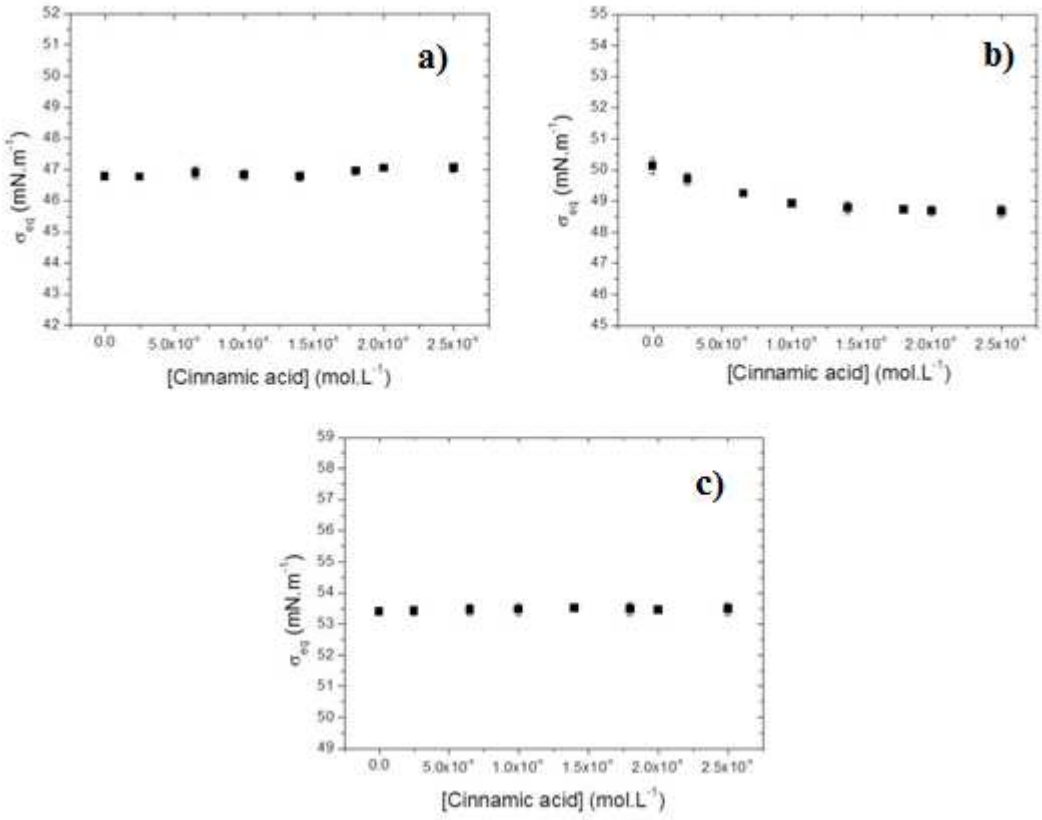


Fig. 3 – σ_{eq} values in function of CA concentration at pH 3.5 (a), 5.0 (b) and 7.4 (c).

These results point to the complexes formed by CA and BSA at different pHs did not modify surface properties of the protein and/or did not promote protein aggregation changing the surface characteristics. Results obtained from measurements of zeta potential and size of BSA added from increasing concentrations of CA corroborate these findings. The size of BSA added by different concentrations of CA was found to be constant at pH 3.5 (9.2 ± 0.4 nm) 5.0 (8.3 ± 0.4 nm) and 7.4 (7.0 ± 0.2 nm); and zeta potential of the protein also did not change in the presence of increasing concentrations of CA, being + 2.7 ± 0.7 mV, - 7.5 ± 1.0 mV and - 17.6 ± 2.0 mV at pH 3.5, 5.0 and 7.4, respectively.

As discussed above, native or unfolded conformation may influence surface properties of protein. In order to confirm this hypothesis, measurements of surface tension, zeta potential and size were performed using BSA solution at pH 7.4 after it underwent heat treatment to promote denaturation.

There was a reduction in the σ_{eq} from 53.4 $mN.m^{-1}$ (when BSA was in native form) to 48.0 $mN.m^{-1}$ (when BSA was denatured). Additionally, zeta

potential of unfolded BSA became less negative (- 11.5 mV) and its size increased to 19.1 nm. These results show that denaturation promoted aggregation of around three molecules of BSA, which probably interacted by hydrophobic interaction since residues of hydrophobic amino acids were exposed by denaturation (Moro, Báez, Ballerini, Busti, & Delorenzi, 2013). Another impact of heat treatment was the distribution of less charged amino acid residues at aggregate surface, decreasing (in modulus) BSA zeta potential; and probably these changes became this new proteinous nanoaggregate a more effective tensoactive agent.

As it was verified for native BSA, the addition of CA did not change properties (surface tension, zeta potential and size) of nanoaggregate of denatured BSA (Fig. 4). Perhaps, hydrophobic sites were formed inside aggregate, in which CA molecules interacted, not changing the surface of BSA nanoaggregate.

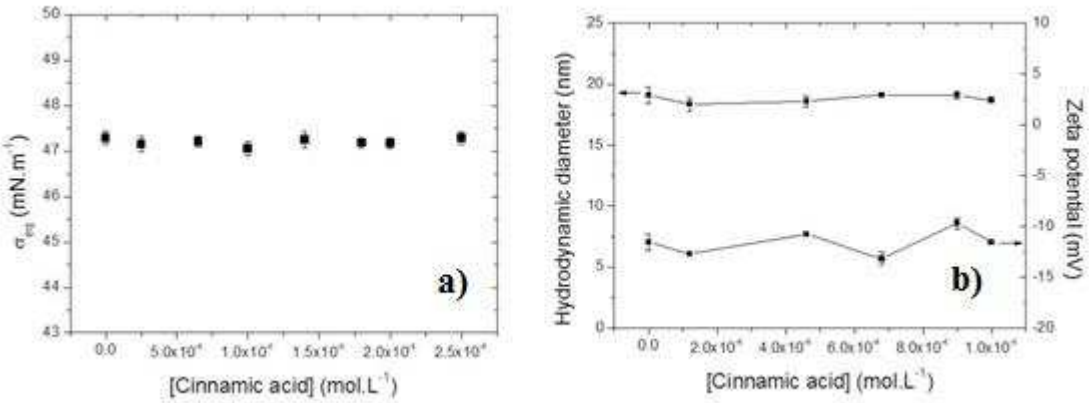


Fig. 4 – σ_{eq} values as function of CA concentration at pH 7.4 for solutions with denatured BSA (a) and hydrodynamic diameter and zeta potential of denatured BSA in different CA concentrations (b).

3.3. Nano-DSC analysis of BSA interacting with CA

It is known that BSA conformation may change due to its interaction with low molecular compounds (Datta, Mahapatra, & Halder, 2013; Shahabadi, Maghsudi, & Rouhani, 2012; Sinisi, Forzato, Cefarin, Navarini, & Berti, 2015). Thus, study the effect of interaction between BSA and small molecules in protein conformation is very important from the point of view of chemistry, food, pharmaceutical and medical areas.

Differential scanning nanocalorimetry (nano-DSC) is a very sensitive technique (in order of nanojoujes) to investigate the thermal stability of proteins, giving us information about the energy involved in the protein denaturation process (Basu & Kumar, 2015). In order to explore the effect of CA on thermal stability of BSA, nano-DSC experiments were carried out.

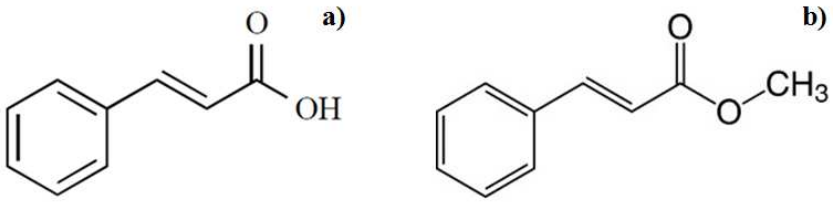
Protein denatured cooperatively exhibiting a single endothermic peak, whose melting temperature was 337.9 ± 1.7 K at pH 5.0 and 7.4. At pH 3.5 it was also verified a small endothermic peak, but the melting temperature was lower (329.1 ± 1.0 K). Regarding the enthalpy change due to unfolding process ($\Delta_{den}H$), at pH 7.4 it was 943.5 ± 4.2 kJ.mol^{-1}, similar to value found by (Giancola, De sena, Fessas, Graziano, & Barone, 1997). At pH 5.0 the value of this property was slightly smaller (803. 3 ± 3.9 kJ.mol^{-1}), while at pH 3.5 there was a remarkable decreasing in $\Delta_{den}H$ (86.16 ± 0.45 kJ.mol^{-1}), suggesting that at acid condition studied BSA was almost unfolded.

Thus, we choose pHs 5.0 and 7.4 to investigate the effect of ligand on the conformation of BSA. The addition of increasing concentrations of CA into BSA solution did not affect the protein stability at both pH, since the T_m value was maintained constant for CA concentration ranging between 1 x 10^{-5} to 1 x 10^{-4} mol.L^{-1}. At pH 5.0, increasing concentrations of CA did not change $\Delta_{den}H$; however, upon complexation with CA the magnitude of $\Delta_{den}H$ decreased about 100 kJ.mol^{-1} at pH 7.4, suggesting that in the presence of ligand BSA at physiological pH became less enthalpically stable (Celej, Montich, & Fidelio, 2003), which could influence its transport functions in bloody system (Fanali et al., 2012).

3.4. Interaction between methyl cinnamate and BSA

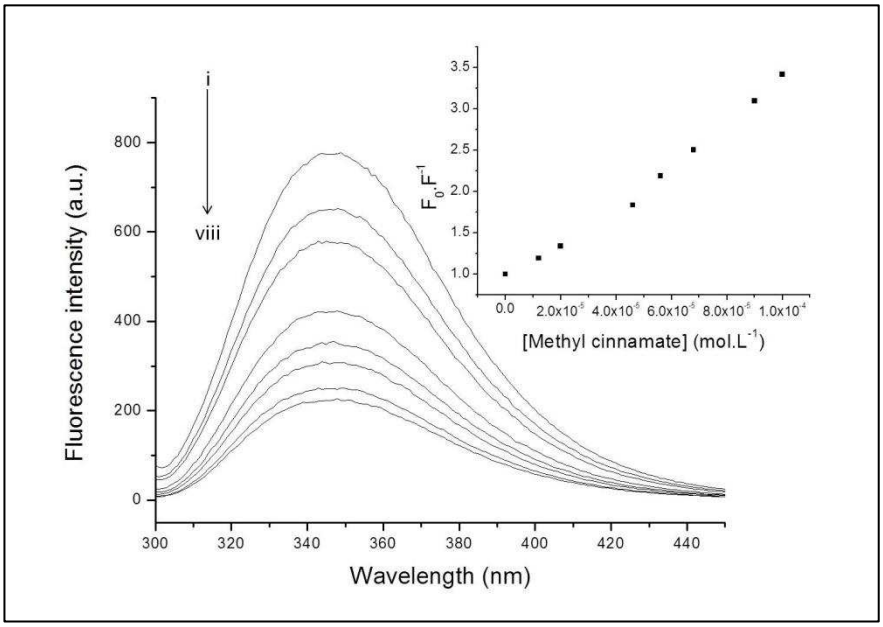
3.4.1. Fluorescence experiments at pH 7.4

Up to now we studied the interaction between a small molecule and BSA modifying aspects mainly related to the protein. However, we believe that small changes in the ligand structure may also be important in binding parameters. Thus, we repeated the experiments replacing CA for methyl cinnamate (MC); since CA and MC molecules differ themselves by changing the hydrogen atom by methyl group (Supplementary material S5).



S5 – Cinnamic acid (a) and methyl cinnamate (b) structures.

In the fluorescence experiments, at pH 7.4, as it was found for CA interacting with BSA, increasing concentrations of MC also quenched BSA fluorescence without changes in the maximum emission wavelength (346 nm) (Supplementary material S6).



S6 - Fluorescence spectra for MC-BSA in pH 7.4 at 298.15 K. MC concentrations varied from 0 to 1.0 x 10^{-4} mol.L^{-1}. The inset represents the Stern-Volmer plot at the same temperature.

Similar to it was verified for CA-BSA interaction, K_{SV} values decreased as MC concentration increased and K_q values were also in the order of 10^{13} mol.L^{-1}, pointing to a static mechanism of quenching (Table 3). Even stoichiometries of MC-BSA complex were also 1:1 (MC:BSA) in all studied temperatures, the K_b values were superior for MC than for CA interacting with the protein; and therefore $\Delta G°$ values were more negative for the former (Table 3). These results

indicate that interaction between MC and BSA was more favorable than CA-BSA interaction, probably because MC is more hydrophobic than CA due to methyl group (Peretto et al., 2014), which interacts by hydrophobic interaction inside hydrophobic site of BSA; and mainly because on the opposite of CA, MC is a uncharged molecule at pH 7.4 thus there was no electrostatic repulsion, which can be confirmed by negative value of $\Delta H°$ (Table 3). Therefore, MC-BSA binding was enthalpic and entropic driven.

Table 3 – Thermodynamic parameters obtained from fluorescence studies of MC-BSA systems at physiological pH.

T (K)	K_{SV} (10^4.L.mol^{-1})	r^2	K_q (10^{12}.L.mol^{-1}.s^{-1})	n	K_b (10^5.L.mol^{-1})	r^2	$\Delta G°$	$T\Delta S°$ (kJ.mol^{-1})	$\Delta H°$	r^2
288.15	3.07	0.99	3.07	1.23	2.47	0.99	-29.74	21.02		
298.15	2.94	0.99	2.94	1.21	2.07	0.99	-30.32	21.61		
308.15	2.90	0.99	2.90	1.22	2.27	0.99	-31.58	22.87	-8.71	0.98
318.15	2.79	0.99	2.79	1.20	1.70	0.99	-31.84	23.13		
328.15	2.66	0.99	2.66	1.20	1.57	0.99	-32.62	23.91		

3.4.2. The effect of pH on MC-BSA interaction

At pHs 5.0 and 3.5, the stoichiometry of MC-BSA complexes were maintained 1:1, however K_b values were about 10 times higher than at physiological pH (Table 4). As MC-BSA interaction do not have contribution from electrostatic interaction, these results could be explained due to the changes in BSA conformation as a function of pH (Kun et al., 2009) (Supplementary material S5); probably some amino acid residues were exposed at lower pHs contributing to MC-BSA interaction. Measurements of protein size and zeta potential also supported this hypothesis, since modifying pH caused changes in BSA size (7.0 ± 0.2; 8.3 ± 0.4 and 9.2 ± 0.4, for pH 7.4, 5.0 and 3.5, respectively) and zeta potential (-17.6 ± 2.0, -7.5 ± 1.0 and +2.7 ± 0.7, for pH 7.4, 5.0 and 3.5, respectively).

Table 4 – Thermodynamic parameters for MC-BSA complexes at pH 3.5 and 5.0.

pH	T (K)	K_{SV} (10^4.L.mol^{-1})	r^2	K_q (10^{12}.L.mol^{-1}.s^{-1})	n	K_b (10^5.L.mol^{-1})	r^2	$\Delta G°$	$T\Delta S°$ (kJ.mol^{-1})	$\Delta H°$	r^2
3.5	288.15	2.78	0.98	2.78	1.22	1.95	0.99	- 29.18	20.69		
	298.15	2.68	0.98	2.68	1.84	1.39	0.99	- 29.36	20.87		
	308.15	2.63	0.98	2.63	1.19	1.42	0.99	- 30.40	21.92		
	318.15	2.49	0.98	2.49	1.19	1.35	0.99	- 31.25	22.77	- 8.49	0.98
	328.15	2.20	0.99	2.20	1.20	1.29	0.99	- 32.09	23.60		
5.0	288.15	2.67	0.99	2.67	1.28	3.37	0.99	- 30.49	18.00		
	298.15	2.62	0.99	2.62	1.28	3.54	0.99	- 31.67	19.18		
	308.15	2.50	0.99	2.50	1.29	3.37	0.99	- 32.60	20.11	- 12.50	0.95
	318.15	2.37	0.99	2.37	1.15	0.88	0.99	- 30.12	17.63		
	328.15	2.17	0.99	2.17	1.24	1.82	0.98	- 33.05	20.55		

3.4.3. The effect of ionic strength on MC-BSA interaction

As it was done for CA interacting with BSA, we evaluated the MC-BSA interaction in the presence of ammonium sulfate at 1, 10, 50 and 100 mM and pH 7.4. Similarly to CA-BSA systems, the salt did not disturb the formation of MC-BSA complexes, whose stoichiometry and K_b values were almost the same that those determined in the absence of cations, for all studied concentrations.

The enthalpic and entropic contributions for interactions occurred in the presence of ammonium had just small changes, once ΔH° values (-8.71 kJ.mol^{-1}, -19.32 kJ.mol^{-1}, -11.32 kJ.mol^{-1}, -14.06 kJ.mol^{-1}, -1.95 kJ.mol^{-1} for 0, 1, 10, 50 and 100 mM, respectively) and TΔS° values (21.64 kJ.mol^{-1}, 11.29 kJ.mol^{-1}, 23.06 kJ.mol^{-1}, 17.95 kJ.mol^{-1}, 28.4 kJ.mol^{-1} for 10, 50 and 100 mM, respectively) were close to every salt concentrations. It may have occurred because changes on BSA electric double layer do not alter methyl cinnamate and BSA approximation, once MC is not an ionizable compound.

3.5. Surface proprieties of BSA interacting with MC

The effect of interaction between MC and BSA on the surface properties of protein was also evaluated. The presence of MC also did not change the surface tension between BSA solution/air in all studied pHs (Fig. 5), similarly of what occurred with CA, indicating that MC-BSA complexes are not less hydrophilic than CA-BSA complexes.

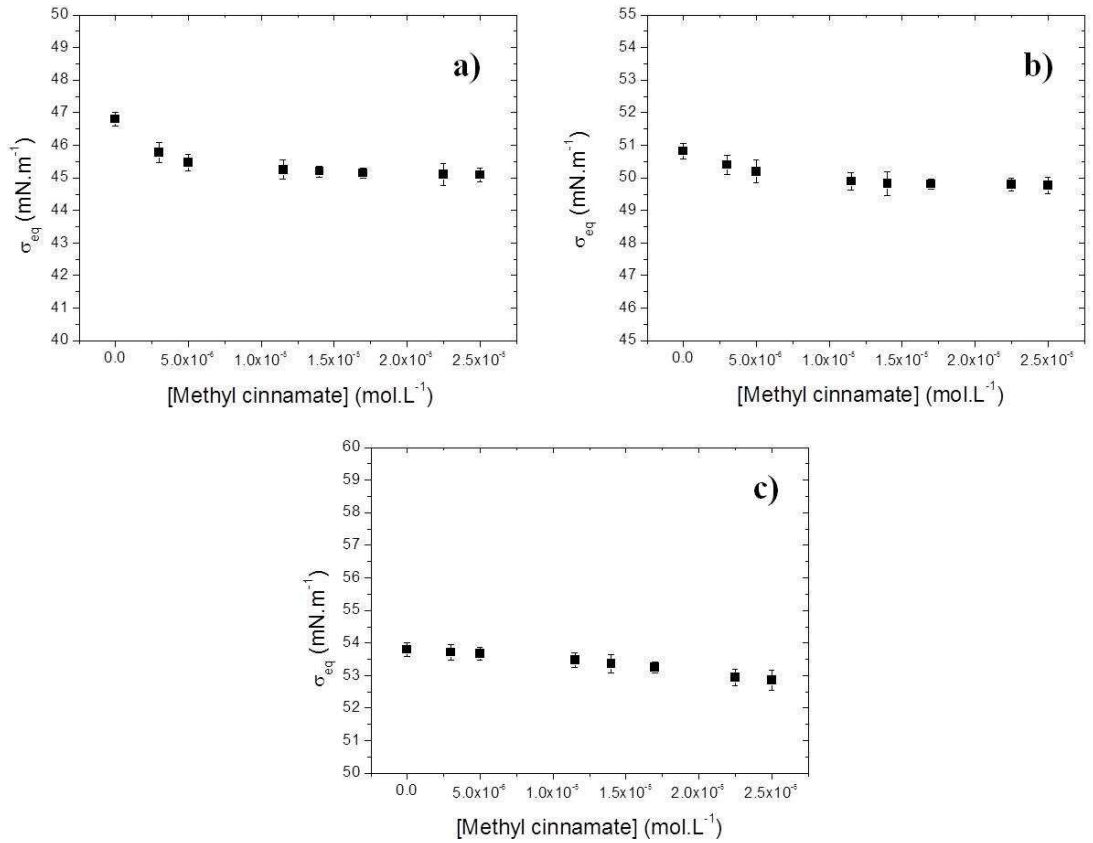
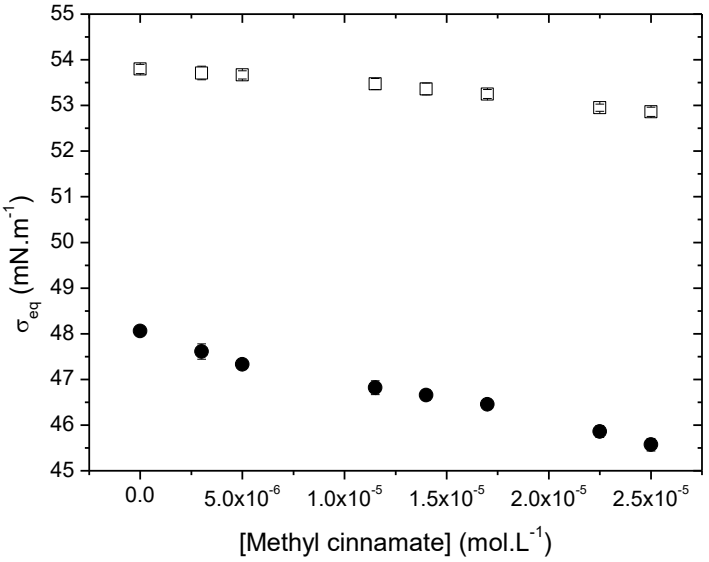


Fig. 5 - σ_{eq} of air/BSA solution varying according MC concentration at pH 3.5 (a), 5.0 (b) and 7.4 (c).

When the BSA was unfolded at pH 7.4, the adsorption behavior was similar that was obtained for native protein (Supplementary Material S7), but σ_{eq} values were smaller, ranging from 48.1 to 45.6 $mN.m^{-1}$ for increasing concentrations of MC. The denaturation process of protein exposes more defined hydrophobic and hydrophilic regions, improving its tensoactivity (Moro et al., 2013); however different that was found CA-BSA complex, whose CA concentration did not affect σ_{eq} value, increasing concentrations of MC forming complexes with unfolded BSA make σ_{eq} lower.



S7 – σ_{eq} values as function of MC concentration for native (□) and denatured (●) BSA.

3.6. Nano-DSC analysis of BSA interacting with MC

Analyzing the effect of MC on the conformation of BSA, we verified that for all pHs studied, the stability of protein was maintained in the presence of MC molecules, since T_m values did not change (337,15 K, 338,15 K and 328,15 K for pH 7.4, 5.0 and 3.5, respectively). However, the $\Delta_{den}H$ values reduced when increasing concentrations of MC was added, being this reduction more pronounced than it was found for CA (Fig. 6). These results suggest that MC affected more the BSA tertiary structure than CA, as verified by Li et al., 2010, who verified by fluorescence that a most hydrophobic binding affects more BSA tertiary structure.

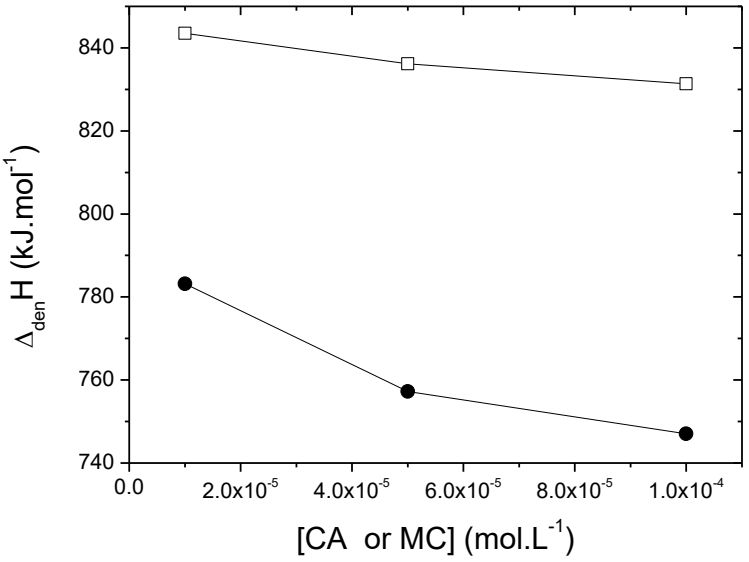


Fig. 6 –$\Delta_{den}H$ values increasing CA (□) or MC (●) concentrations at pH 7.4.

4. CONCLUSION

The interaction between BSA and CA or MC was evaluated. For the first time, was demonstrated the pH effect on CA/MC-BSA interaction, since pH can modify conformation and surface charge distribution of BSA, which affects the interaction forces and mechanisms. Protein conformation also played an important role on the studied interactions once complex formation became less favorable when BSA was denatured which evidenced the importance of the hydrophobic site of the protein on interaction. The ligand structure (either because of its ionization or hydrophobicity) influenced on complex formation with BSA, changing thermodynamic parameters of interaction. For both CA-BSA and MC-BSA complexes, protein surface properties did not change, demonstrating that the complexes have not different hydrophobicity than BSA. The results of the present work provide important information on small ligands and BSA interaction and this knowledge may contribute to improve CA/MC application in pharmaceutical and food areas.

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