UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Obtenção e caracterização de complexos e sistemas ternários contendo pterostilbeno, β-ciclodextrina e polímeros hidrofílicos.

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Obtenção e caracterização de complexos e sistemas ternários contendo pterostilbeno, β-ciclodextrina e polímeros hidrofílicos.

Dissertação apresentada por **Mairique Waszczuk** para obtenção do GRAU DE MESTRE em Ciências Farmacêuticas.

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RESUMO

O pterostilbeno (PTS) é uma fitoalexina, presente principalmente em mirtilos (*Vaccinium* spp), que tem sido objeto de estudos devido às propriedades preventivas e terapêuticas que tem lhe sido atribuídas. Sua baixa hidrossolubilidade constitui-se numa de suas principais limitações farmacêuticas e biofarmacêuticas, assim, sua associação com ciclodextrinas e polímeros hidrofílicos apresenta-se como estratégia promissora para contorná-la. Neste sentido, o presente trabalho propõe obter e caracterizar complexos e sistemas ternários contendo PTS, β-ciclodextrina (βCD) e polímeros hidrofílicos (polivinilpirrolidona, PVP ou hidroxipropilmetilcelulose, HPMC), otimizar os parâmetros que influenciam a complexação, bem como investigar o efeito do uso de etanol como cossolvente no meio complexante. Como subsídio ao estudo foi desenvolvido e validado um método analítico indicativo de estabilidade, por cromatografia líquida de alta eficiência. O PTS mostrou-se instável diante da exposição à oxidação, elevadas temperaturas e à luz UV. O método mostrou-se linear, específico, exato, preciso e robusto, além de ser um método indicativo de estabilidade aplicável à quantificação do PTS em extrato de mirtilo, e nos complexos e sistemas ternários supracitados. A associação do PTS com βCD formou complexos de inclusão e aumentou em 10 vezes sua hidrossolubilidade. Como resultado da otimização da complexação as melhores condições foram: temperatura de 37°C, 2 horas de agitação com 4mM de PTS no meio. Os polímeros demonstraram ser importantes para o incremento da hidrossolubilidade do PTS, sendo o sistema PTS:βCD:HPMC o que apresentou melhores resultados de promoção da solubilidade do PTS (aumento de 56 vezes) e determinou uma redução drástica na quantidade de ciclodextrina utilizada (da razão molar PTS:βCD de 1:9, sem polímero, para 1:2 na presença do polímero). O uso de etanol como cossolvente na obtenção dos sistemas ternários mostrou excelente resultado, aumentando o conteúdo de PTS em 3 vezes nos correspondentes produtos secos por liofilização, comparativamente aos sistemas ternários obtidos em meio aquoso. Em suma, o sistema ternário, PTS:βCD:HPMC obtido com o uso de cossolvente foi o que demonstrou ter o melhor potencial como produto intermediário para desenvolvimento de novos produtos farmacêuticos, cosméticos ou alimentares contendo PTS.

Palavras-chave: Pterostilbeno, HPLC-método indicativo de estabilidade, eficiência de complexação, ciclodextrina, sistemas ternários, HPMC.

ABSTRACT

Pterostilbeno (PTS) is a phytoalexin, present mainly in blueberries (*Vaccinium* spp), which has been the object of studies due to the preventive and therapeutic properties that have been attributed to it. Its low water solubility is one of its main pharmaceutical and biopharmaceutical limitations. Thus its association with cyclodextrins and hydrophilic polymers represent a promising strategy to circumvent these limitations In this constext, the present work proposes to obtain and characterize complexes and ternary systems containing PTS, β-cyclodextrin (βCD) and hydrophilic polymers (polyvinylpyrrolidone, PVP or hydroxypropylmethylcellulose, HPMC), to optimize the parameters that influence the complexation, as well as to investigate the effect of the use of ethanol as co-solvent in the complexing medium. To quantitate PTS in all of samples, an analytical method indicative of stability was developed and validated by high performance liquid chromatography. PTS was unstable against oxidation, high temperatures and exposure to UV light. The method was linear, specific, accurate, precise and robust, besides being an indicative stability method applicable to the quantification of PTS in blueberry extract, and in the aforementioned complexes and ternary systems. The association of PTS with βCD formed inclusion complexes and increased its water solubility 10-fold. As a result of optimization of the complexation the best conditions were: 37°C temperature, 2 hours stirring with 4mM PTS in the medium. Among the systems tested, the PTS: βCD: HPMC ternary system proved to be the most promising solubility of PTS (increase of 56-fold) determining a drastic reduction in the amount of cyclodextrin used. It reduced the PTS molar ratio from 1:9 PTS:βCD, in the absence of polymer, to 1:2 in the presence of the polymer. The use of ethanol as a cosolvent in obtaining the ternary systems showed excellent results, increasing the content of PTS in 3 times the corresponding freeze dried products, compared to ternary systems obtained in aqueous medium. In summary, the ternary system, PTS:βCD:HPMC obtained with the use of ethanol as cosolvent showed excellent PTS solubilizing characteristics being a promise intermediate product for the development of new pharmaceuticals, cosmetics or foods containing PTS.

Keywords: Pterostilbene, HPLC stability-indicating method, complexation efficiency, cyclodextrin, ternary system, HPMC.

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INTRODUÇÃO

O pterostilbeno (3,5-dimetoxi-4’-hidroxiestilbeno) é uma fitoalexina presente em mirtilos (*Vaccinium* spp), uvas (*Vitis* spp), entre outras fontes (KOSURU et al., 2016; PENG et al., 2018). Há um crescente interesse científico nesta fitoalexina devido às numerosas atividades preventivas e terapêuticas que lhes são atribuídas, incluindo atividades antioxidante, antitumoral, anti-inflamatória, anticolesterolemiante, antifúngica, hipolipidêmica, cardioprotetora, neuroprotetora e analgésica (DOS SANTOS LACERDA et al., 2017; KOSURU et al., 2016). Quimicamente o pterostilbeno (PTS) corresponde ao resveratrol dimetilado, em que os grupos funcionais 3,5-dihidroxi do resveratrol são substituídos por grupos metoxi, o que lhe confere maior lipofilia. O PTS apresenta características químicas, físicas e bioquímicas que podem constituir importantes limitações no desenvolvimento de produtos farmacêuticos, tais como sensibilidade a agentes externos como ar, luz e temperatura, susceptibilidade às enzimas oxidativas e metabolização por enzimas conjugativas do metabolismo da fase II (AZZOLINI et al., 2017; SILVA et al., 2014). No entanto, a sua baixa hidrossolubilidade (aproximadamente 21 µg/ml) é uma das mais significativas limitações, tanto para o desenvolvimento de formulações como para sua dissolução nos meios fisiológicos e, por consequência, para a sua biodisponibilidade após administração pela via oral (BETHUNE; SCHULTHEISS; HENCK, 2011; YEO; HO; LIN, 2013).

Como forma de melhorar sua hidrossolubilidade a utilização de ciclodextrinas se apresenta como uma alternativa de interesse, visto que a sua cavidade hidrofóbica é capaz de encapsular fármacos de baixa hidrossolubilidade, aumentando sua solubilidade aparente em água (LOFTSSON; BREWSTER, 1996). Desta característica decorre o seu maior emprego industrial em vários campos, tais como farmacêutico e alimentar. Tal propriedade das ciclodextrinas também tem sido útil para viabilizar a realização de testes biológicos com moléculas de baixa hidrossolubilidade, como é o caso do PTS, evitando o uso de solventes fisiologicamente incompatíveis com a via oral ou outras vias de administração, como é o caso do dimetilsulfóxido (DMSO) (LACERDA et al., 2018). Entre as ciclodextrinas a β-ciclodextrina (βCD) é amplamente utilizada para complexação com diversas classes de fármacos, devido a dimensão de sua cavidade, sendo possível complexar compostos

aromáticos e heterocíclicos (JAMBHEKAR; BREEN, 2016), bem como pelo seu menor custo. No entanto, comparativamente a seus derivados, a βCD apresenta a menor hidrossolubilidade, 18,5 mg/ml (JANSOOK; OGAWA; LOFTSSON, 2018).

Diversos métodos podem ser utilizados para aumentar a eficiência de complexação das ciclodextrinas com moléculas de interesse, como o uso de polímeros hidrofílicos, para formação de sistemas ternários, e a adição de um cossolvente ao meio. De forma geral, o cossolvente visa a aumentar a solubilidade intrínseca do fármaco, enquanto os polímeros aumentam a constante de estabilidade aparente do complexo fármaco:ciclodextrina (JANSOOK; OGAWA; LOFTSSON, 2018). Tendo em vista que o cossolvente é eliminado no processo de obtenção do complexo, esta técnica resulta, no final do processo, na formação de um sistema binário. Polímeros hidrossolúveis têm vasta aplicação como excipientes farmacêuticos e são capazes de interagir com os fármacos, com as ciclodextrinas e também com complexos fármaco:ciclodextrina (VALERO et al., 2003, BORGHETTI et al., 2011). Os quesitos mais importantes na escolha dos polímeros são a hidrossolubilidade, biocompatibilidade e ausência de atividade farmacológica. Entre os polímeros hidrofílicos mais relatados na literatura e frequentemente empregados em formulações farmacêuticas encontram-se a polivinilpirrolidona (PVP) e a hidroxipropilmetilcelulose (HPMC) (KURKOV; LOFTSSON, 2013).

No que se refere ao PTS, diferentes estratégias para aumentar a sua hidrossolubilidade tem sido relatadas. Dentre estas estratégias está a complexação do PTS com ciclodextrinas naturais e modificadas (ZHANG et al., 2013; YEO et al., 2013; NICOLÁS et al., 2009, LACERDA et al., 2017). Porém, não há relatos de estudos verificando a influência do uso de cossolvente sobre a complexação do PTS com ciclodextrina, tampouco sobre a obtenção de sistemas ternários, constituídos por PTS, ciclodextrina e polímero hidrofílico, para aumentar a sua hidrossolubilidade e eficiência de complexação.

Neste contexto, considerando a potencial atividade biológica do PTS, sua baixa hidrossolubilidade, sua instabilidade química e o já evidenciado aumento de sua hidrossolubilidade pelo uso de ciclodextrinas, o presente trabalho visa obter e caracterizar complexos e sistemas ternários contendo PTS, βCD e

polímeros hidrofílicos (PVP ou HPMC), otimizar os parâmetros que influenciam a complexação do PTS com βCD, bem como investigar o efeito do uso de etanol como cossolvente no meio complexante para o aumento do conteúdo de PTS em complexos secos por liofilização. No presente trabalho conjuga-se duas estratégias para o aumento da eficiência de complexação, utilizando simultaneamente cossolvente e polímero hidrofílico. Como subsídio ao estudo foi desenvolvido e validado um método analítico indicativo de estabilidade ante elevadas temperaturas, irradiação de luz ultravioleta, oxidação e hidrólises ácida e alcalina, utilizando cromatografia líquida de alta eficiência (CLAE), para a determinação quantitativa de PTS nos complexos e sistemas ternários supracitados e em extrato de mirtilo.

A dissertação está estruturada em três capítulos como segue:

- O capítulo I apresenta uma revisão da literatura sobre o tema, sumarizando os aspectos relevantes para o desenvolvimento do trabalho, sendo estes: propriedades físico-químicas e atividades biológicas do PTS, as ciclodextrinas, forma de obtenção e caracterização de complexos de inclusão, sistemas ternários, desenho experimental e validação analítica;
- O capítulo II apresenta os resultados experimentais da validação de metodologia analítica indicativa de estabilidade do PTS, por cromatografia líquida de alta eficiência.
- O capítulo III apresenta os resultados obtidos nos estudos de desenvolvimento e caracterização de complexos e sistemas ternários, contendo PTS, βCD e polímeros hidrofílicos, a otimização das condições de complexação e o efeito do uso de cossolvente na obtenção desses sistemas.

OBJETIVOS

Objetivo geral

Obter e caracterizar complexos e sistemas ternários contendo pterostilbeno, β-ciclodextrina e polímeros hidrofílicos.

Objetivos específicos

- Desenvolver e validar um método analítico indicativo de estabilidade, por cromatografia líquida de alta eficiência, para a determinação quantitativa de pterostilbeno (PTS).

- Realizar estudo de solubilidade da associação PTS:βCD e otimizar os parâmetros que influenciam na formação do complexo entre eles, por meio da utilização de desenho experimental do tipo Box-Behnken.

- Associar o PTS com β-ciclodextrina (βCD) e polímeros hidrofílicos para obtenção de complexos (PTS:βCD) e sistemas ternários (PTS:βCD:polímero hidrofílico).

- Avaliar a influência do cossolvente na formação do complexo PTS:βCD e sistema ternário.

- Caracterizar o complexo e o sistema ternário obtido por meio de análises espectrométricas, cromatográfica, térmica e de microscopia.

CAPÍTULO I

Revisão da literatura

Pterostilbeno (PTS)

O PTS (3,5-dimetoxi-4'-hidroxiestilbeno) é uma fitoalexina, da classe dos estilbenos, de estrutura semelhante ao resveratrol (3,5,4'-trihidroxiestilbeno) **(Figura 1).** Foi inicialmente isolado em 1940 a partir do gênero *Pterocarpus* e posteriormente identificado em espécies vegetais como mirtilos (*Vaccinium* spp) e uvas (*Vitis* spp) (KOSURU et al., 2016; PENG et al., 2018; SILVA et al., 2014).

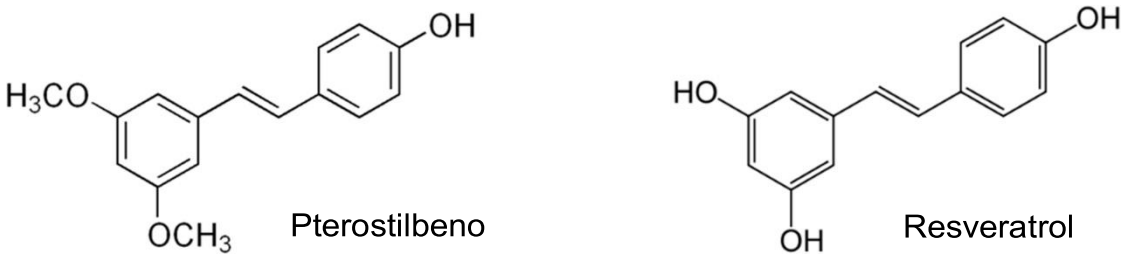

Figura 1: Estrutura química do pterostilbeno e do resveratrol.

Os estilbenos existem tanto como monômeros quanto como oligômeros cada vez mais complexos. A estrutura do estilbeno monomérico é relativamente simples e caracterizada por dois anéis benzênicos unidos por uma ligação dupla *trans* ou *cis* eteno. O *trans*-estilbeno é a configuração mais comum e de maior atividade biológica. Diferentes caminhos que levam à isomerização *trans-cis* têm sido descritos, tais como quebra de ligação dupla por radicais, fotoisomerização direta sob radiação solar ou UV e isomerização térmica (SILVA et al., 2014).

O interesse nesta fitoalexina tem crescido devido às propriedades preventivas e terapêuticas que lhe são atribuídas, tais como atividade antioxidante, anti-inflamatória, antidiabética, antitumoral, hipolipemiante, neuroprotetora, cardioprotetora, entre outras (DOS SANTOS LACERDA et al., 2017; KOSURU et al., 2016). Portanto, o PTS tornou-se uma substância bioativa natural importante, com potenciais aplicações terapêuticas, de especial interesse para as áreas de biotecnologia e de desenvolvimento de produtos farmacêuticos, alimentares e cosméticos (KOSURU et al., 2016; PENG et al., 2018; YEO; HO; LIN, 2013).

Nos últimos anos, tem havido maior consumo de alimentos e suplementos contendo estilbenos, devido aos relatos de seus benefícios à saúde (SILVA et al., 2014). Produtos contendo PTS, considerado um nutracêutico, são

comercializados como suplementos alimentares (TASTEKIN et al., 2018).

Com base nas características físico-químicas do PTS, como lipofilia moderada, um único grupamento hidroxila capaz de estabelecer ligações de hidrogênio, baixa polaridade, poucas ligações rotativas e massa molecular de 256,299 g/mol, pode-se prever alta permeabilidade em membranas (KOSURU et al., 2016). Porém, a baixa hidrossolubilidade do PTS (aproximadamente 21 µg/mL) representa uma limitação para a sua dissolução nos meios fisiológicos e, por consequência, para a sua biodisponibilidade após administração pela via oral (BETHUNE; SCHULTHEISS; HENCK, 2011; YEO; HO; LIN, 2013). Portanto, o PTS pode se beneficiar de estratégias utilizadas para melhorar a dissolução de compostos de classe II do Sistema de Classificação Biofarmacêutico, onde a solubilidade é o principal limitante de sua biodisponibilidade oral (PENG et al., 2018).

Outras limitações podem ser observadas para o PTS, como o grupo fenólico na posição 4' que se constitui em alvo ideal para as enzimas conjugativas do metabolismo da fase II, e sua sensibilidade a agentes externos como ar, luz e enzimas oxidativas constituem um sério problema para sua estabilidade e biodisponibilidade (AZZOLINI et al., 2017; SILVA et al., 2014),

Entre as estratégias de estabilização e aumento da hidrossolubilidade do PTS encontram-se a formação de cocristal (BETHUNE; SCHULTHEISS; HENCK, 2011), formação de sistemas micelares (SILVA et al., 2014), síntese de pró-fármacos nos quais a porção hidroxila é reversivelmente protegida por meio de ligação éster de carbamato ligado ao terminal N de um aminoácido natural (AZZOLINI et al., 2017), e formação de complexos com ciclodextrinas (LACERDA et al., 2018b; LÓPEZ-NICOLÁS et al., 2009; SILVA et al., 2014; YEO; HO; LIN, 2013; ZHANG et al., 2013). Esta última estratégia vem sendo objeto de estudo de nosso grupo de pesquisa nos últimos anos (DOS SANTOS LACERDA et al., 2017; LACERDA et al., 2018a, 2018b).

Ciclodextrinas (CDs)

As CDs são oligossacarídeos cíclicos compostos por unidades de D-glicopiranose unidas por ligações glicosídica α(1-4). As CDs de ocorrência natural mais relatadas na literatura possuem 6, 7 ou 8 unidades de glicose, o

que confere propriedades particulares a cada uma delas, e são denominadas α, β e γ-ciclodextrina, respectivamente, conforme apresentado na **Tabela 1**(COVA et al., 2018).

Tabela 1: Propriedades das α, β, e γ-ciclodextrinas.

Propriedade	α-BD	β-CD	γ-CD
Nº unidades de glicose	6	7	8
Massa molecular (g.mol-1)	972	1135	1297
Diâmetro da cavidade interna (Å)	5.7	7.8	9.5
Volume da cavidade ($Å^3$)	174	262	427
Solubilidade em água (g.L-1)	145	18,5	232

Fonte: (COVA et al., 2018)

A conformação das unidades de α-D-glicopiranose confere às CDs uma forma de cone truncado, caracterizado pela presença de uma cavidade hidrofóbica, em decorrência das ligações entre carbonos e oxigênios etéreos, e de uma face externa com características hidrofílicas, composta por hidroxilas (BREWSTER; LOFTSSON, 2007; JAMBHEKAR; BREEN, 2016a). Devido a sua natureza anfifílica as CDs são capazes de incluir moléculas hidrofóbicas, ou parte delas, em sua cavidade formando complexos de inclusão, assim como associar-se externamente a moléculas hidrofílicas, formando complexos de não-inclusão resultando no aumento da hidrossolubilidade, estabilidade e biodisponibilidade destas moléculas (JANSOOK; OGAWA; LOFTSSON, 2018). Em condições especiais há ainda a possibilidade de formação de agregados (HE et al., 2008). Geralmente, quanto menor a hidrossolubilidade de uma substância, maior o aumento relativo na sua solubilidade obtido como resultado da complexação com ciclodextrina (CD) (JAMBHEKAR; BREEN, 2016b).

A β-ciclodextrina (βCD) e seus derivados são as mais comumente encontradas em relatos de estudos científicos e em formulações farmacêuticas disponíveis no mercado (KURKOV; LOFTSSON, 2013; MIRANDA et al., 2011). A βCD é a CD natural produzida em maior escala (JANSOOK; OGAWA; LOFTSSON, 2018) e apresenta como vantagens o fato de ser obtida industrialmente com elevado rendimento e qualidade, possuir baixo custo, e

principalmente, possuir uma cavidade interna cujas dimensões são excelentes para incorporar moléculas, sendo especialmente adequada para complexar fármacos que contenham anel aromático em sua estrutura (JAMBHEKAR; BREEN, 2016b). Por outro lado, é a CD que apresenta a menor hidrossolubilidade (JANSOOK; OGAWA; LOFTSSON, 2018).

As CDs, além de promoverem aumento da hidrossolubilidade, podem proteger a molécula complexada contra efeitos oxidativos, alterações físico-químicas, radiação ultravioleta e luz visível, promover o controle da volatilidade e sublimação, diminuir ou eliminar interações e incompatibilidades, reduzir irritação gástrica, modificar sabor e odor, melhorar o perfil de liberação e permeação da substância ativa, bem como promover o incremento da biodisponibilidade desta substância quando administrada por via oral (DEL VALLE, 2004; JAMBHEKAR; BREEN, 2016a; SAOKHAM et al., 2018).

O aumento da biodisponibilidade do insumo ativo, gerado pelas CDs, está vinculado tanto à promoção da hidrossolubilidade como à permeação através da camada de difusão (camada de água estacionária, CAE) e da membrana intestinal. A CAE constitui uma importante barreira física à absorção intestinal de substâncias, pois limita a sua difusão passiva, juntamente com a presença de muco e células na parede intestinal (MUDRA; BORCHARDT, 2010). Na CAE as CDs comportam-se como agente “caotrópico”, que desestabiliza as ligações de hidrogênio da fase aquosa, facilitando a passagem da substância ou complexo através da mesma, aumentando assim a sua concentração na membrana intestinal onde a interação é dependente do tipo de CD. A αCD atua na remoção dos fosfolipídios, enquanto a βCD tem maior afinidade pelo colesterol, processos que podem induzir redução da resistência da membrana, promovendo maior penetração (CARRIER; MILLER; AHMED, 2007). A capacidade de depleção do colesterol da membrana intestinal inibe indiretamente o efluxo de substâncias promovido pela glicoproteína-P e aumenta a permeabilidade de substâncias por via paracelular por alterar a ação das ocludinas (ZIDOVETZKI; LEVITAN, 2007).

A complexação do PTS com CD foi primeiramente relatada por López-Nicolás e colaboradores (2009), que descreveram a formação de complexos de inclusão do PTS com CDs naturais (αCD, βCD, γCD) e derivadas da βCD (hidroxipropil-βCD), metil-βCD e etil-βCD), avaliaram a estequiometria do

complexo PTS:βCD e determinaram o efeito da estrutura da CD sobre as respectivas constantes de associação. Verificaram que entre as CDs naturais, a interação do PTS com o βCD foi a mais eficiente (maior valor de constante de complexação), no entanto, as CDs modificadas apresentaram constantes de complexação mais altas do que a βCD (LÓPEZ-NICOLÁS et al., 2009). Zhang e colaboradores relataram a formação de complexos de inclusão com βCD e avaliaram a influência da temperatura, pH e proporção molar entre PTS e βCD, e determinaram a temperatura como o fator que mais influenciou a formação do complexo (ZHANG et al., 2013). Yeo e colaboradores avaliaram a farmacocinética do PTS, e para isso realizaram a complexação do mesmo com 0,3 M de hidroxipropil-βCD (HPβCD) após estudo de solubilidade de fase (YEO; HO; LIN, 2013). Recentemente, nosso grupo de pesquisa realizou a obtenção de complexos de PTS com HPβCD e caracterizou-os. O aumento da hidrossolubilidade obtido com esta CD mais solúvel viabilizou sua administração por via oral em testes biológicos, evitando o uso de solventes orgânicos como o dimetilsulfóxido. Este trabalho foi realizado em cooperação com o Dr Alex Sander da Rosa Araújo e Drª. Denise Lacerda, que realizaram estudos de toxicidade e de propriedades farmacológicas desses complexos. Os resultados do estudo demonstraram que o complexo apresentou efeito antioxidante dose-dependentes com uso potencial na prevenção de doenças relacionadas a danos oxidativos e sinalização de insulina (LACERDA et al., 2018b). Em modelo animal (ratos) foi demonstrado que, além de reduzir o estresse oxidativo, a administração oral do complexo PTS:HPβCD preveniu a hipertrofia e preservou a função sistólica do ventrículo direito no modelo cor pulmonale, com ausência de efeitos tóxicos (DOS SANTOS LACERDA et al., 2017).

Complexos de inclusão com ciclodextrina: obtenção e caracterização

Existem diversos métodos para a obtenção de complexos de inclusão de moléculas em ciclodextrinas, tais como o método em solução, em suspensão, coprecipitação, coevaporação, malaxagem, moagem, neutralização, liofilização, secagem por pulverização, micro-ondas, fluidização supercrítica e mistura a seco (LOFTSSON; BREWSTER, 2012; MIRANDA et al., 2011; PINHO et al.,

2014; YATSU et al., 2013). A formação do complexo depende da estrutura química e propriedades físico-químicas tanto da substância a ser complexada (hóspede) como da CD (hospedeiro) (JAMBHEKAR; BREEN, 2016b) e do método de complexação empregado (PINHO et al., 2014).

O processo de inclusão de uma molécula hóspede na cavidade da CD ocorre em nível supramolecular, havendo uma substituição de moléculas de água (de elevada entalpia) da cavidade, pela molécula hóspede ou parte desta, sem a quebra de ligações covalentes ou formação de novos compostos. A complexação é mantida por meio de interações hidrofóbicas e forças de Van der Waals, além de outros fatores, como diminuição da tensão do anel, modificações na superfície do solvente e ligações de hidrogênio de grupamentos da molécula hóspede com hidroxilas externas da CD (ancoragem) que tornam o complexo mais estável energeticamente (DEL VALLE, 2004; MIRANDA et al., 2011).

O estudo de solubilidade de fase é um método comumente empregado quando se estuda a complexação entre fármaco e CDs, a solubilidade do fármaco é plotada contra a concentração de CD, obtendo-se uma representação gráfica que permite identificar como o aumento na concentração de CD influencia na solubilidade do fármaco e qual perfil de solubilidade o complexo possui, possibilitando assim calcular parâmetros importantes como o valor da constante de estabilidade aparente (K_s) e a eficiência de complexação (EC) (JANSOOK; OGAWA; LOFTSSON, 2018).

O tipo mais comum de complexo formado é o que possui estequiometria 1:1 fármaco:CD, em que uma molécula de fármaco (F) forma um complexo com uma molécula de CD (JANSOOK; OGAWA; LOFTSSON, 2018):

$$F + CD \overset{Ks}{\leftrightarrow} F{:}\,CD$$

Quando o coeficiente angular obtido no estudo de solubilidade de fase for menor que 1, a K_s do complexo pode ser calculada a partir da inclinação e da solubilidade intrínseca aparente (S_0) do fármaco no meio de complexação aquoso, isto é, solubilidade do fármaco sem a presença de CD (JANSOOK; OGAWA; LOFTSSON, 2018).

$$K_s\ (M^{-1}) = \frac{coeficiente\ angular}{S_0 \text{ x } (1 - coeficiente\ angular)}$$

Já a EC pode ser calculada apenas com o coeficiente angular obtido no estudo de solubilidade de fase, sendo este um método mais confiável para avaliar o efeito solubilizante das CDs em fármacos pouco solúveis, uma vez que não considera a S_0 (JANSOOK; OGAWA; LOFTSSON, 2018).

$$EC = \frac{coeficiente\ angular}{(1 - coeficiente\ angular)}$$

As associações de moléculas com CDs podem ser caracterizadas por meio de técnicas analíticas como: difração de raios-X, análises térmicas, microscopia eletrônica de varredura, métodos espectroscópicos como espectroscopia UV/VIS, espectroscopia de fluorescência, espectroscopia por ressonância magnética nuclear, espectrometria de massas e espectroscopia no infravermelho com transformada de *Fourier*, espectroscopia Raman, estudos de solubilidade de fase, cromatografia líquida, entre outras (JANSOOK; OGAWA; LOFTSSON, 2018; MIRANDA et al., 2011; YATSU et al., 2013).

Métodos de promoção da eficiência de complexação

Além da limitada hidrossolubilidade das CDs naturais, a EC entre fármaco e CD, em geral, é baixa, portanto, é necessário utilizar grandes quantidades de CDs para a formação de complexos. Em suma, a razão molar fármaco:CD para a formação de uma molécula de complexo, frequentemente é maior do que 1:1 (LOFTSSON; BREWSTER, 2012; LOFTSSON; MÁSSON, 2004). Porém, devido às questões toxicológicas, custo de produção e dificuldade de obter formas farmacêuticas devido ao elevado volume dos pós, é importante reduzir ao máximo possível a proporção de CD empregada (JAMBHEKAR; BREEN, 2016b). Para melhorar a EC pode-se lançar mão de estratégias como: 1) utilização de cossolvente, que pode aumentar a S_0 da substância e favorecer a complexação, 2) a utilização de pequenas quantidades de polímeros hidrofílicos, para formar complexos ternários com fármaco e CD, também chamados de sistema multicomponentes; este último tem sido referido por promover o aumento da K_s fármaco:CD (JAMBHEKAR; BREEN, 2016b; LOFTSSON; BREWSTER, 2012, JANSOOK; OGAWA; LOFTSSON, 2018).

Os cossolventes orgânicos aumentam a solubilidade aquosa de fármacos

apolares, reduzindo a auto interação das moléculas de água e a densidade de ligações de hidrogênio na mistura aquosa e, com isso, facilitam a dispersão e solubilização dos solutos nas soluções aquosas (LOFTSSON; BREWSTER, 2012). Cossolventes, como o etanol, podem melhorar a S_0, acarretando um aumento da EC; no entanto, a adição de solventes orgânicos ao meio de complexação aquoso diminui o valor da K_s, devido à diminuição da polaridade do meio (KURKOV; LOFTSSON, 2013; LOFTSSON; BREWSTER, 2012). Estima-se que a polaridade da cavidade da CD seja semelhante à de uma solução hidroetanólica de aproximadamente 55% (v/v) de etanol em água a 25°C. A tendência do fármaco entrar na cavidade da CD diminui com a diminuição da polaridade do meio de complexação (LOFTSSON; BREWSTER, 2012). Adicionalmente, cossolventes podem participar da complexação por meio da formação de complexos ternários fármaco:CD:cossolvente ou dificultar a complexação competindo com o fármaco pelo espaço na cavidade da CD. Porém, quando o cossolvente é eliminado no processo de obtenção do complexo, esta técnica resulta, no final do processo, na formação de um sistema binário fámaco:CD. Assim, os cossolventes podem aumentar e diminuir a solubilização de fármacos por CD e seu efeito é dependente da concentração (LOFTSSON; BREWSTER, 2012; SAOKHAM et al., 2018).

Loftsson e Brewster (2012) elucidaram o efeito da concentração de etanol na solubilização de complexos de HPβCD com fluasterona, e verificaram que o valor de K_s do complexo diminui com o aumento da concentração de etanol, porém, embora a solubilidade da fluasterona diminua com o aumento da concentração de etanol quando este está em baixas concentrações, ocorre um aumento de solubilidade do fármaco quando o etanol está presente em concentrações acima de 40% (v/v). Em baixas concentrações deste cossolvente, o valor de K_s diminui mais rápido do que a S_0 aumenta, mas em concentrações mais altas, a S_0 aumenta mais rápido que a mudança de K_s. Os autores enfatizam o fato de que a EC é um indicador melhor da solubilização do complexo do que o K_s (LOFTSSON; BREWSTER, 2012). Tais observações levaram ao uso de métodos que utilizam cossolventes para promover S_0, os quais são posteriormente eliminados, sem prejuízo ao K_s.

Os polímeros hidrossolúveis são capazes de interagir com fármacos,

moléculas de CD e com complexos fármaco:CD (LOFTSSON; BREWSTER, 2012). O mecanismo envolvido no aumento da EC na presença de polímeros ainda não é totalmente compreendido; no entanto, acredita-se que polímeros solúveis em água podem reduzir a mobilidade da CD e seus complexos com as moléculas hóspedes e aumentar a solubilidade do complexo (MIRANDA et al., 2011). Adicionalmente, já se tem relatado a capacidade dos polímeros hidrofílicos solubilizarem a βCD e seus complexos. A βCD, cuja solubilidade aquosa a ±25°C é de apenas 18,5 mg/ml, tem sua solubilidade em formulações aquosas aumentada significativamente com a formação de complexos de inclusão com alguns fármacos e com a formação de complexos com polímeros solúveis em água (LOFTSSON; *FRIDRI*KSDÓTTIR, 1998; MIRANDA et al., 2011).

A adição de polímeros hidrossolúveis pode aumentar a biodisponibilidade do fármaco e reduzir em até 80% a quantidade de CD necessária para adquirir o aumento de solubilidade da substância de interesse (LOFTSSON; FRIDRIKSDÓTTIR, 1998). Os sistemas ternários formados podem promover uma maior solubilização do fármaco do que quando a CD ou o polímero são utilizados separadamente, obtendo, desta forma, um efeito aditivo ou, mesmo, sinérgico (LOFTSSON et al., 1994). De modo geral, polímeros solúveis em água são capazes de melhorar as propriedades farmacêuticas e biológicas dos complexos fármaco:CD (LOFTSSON; MÁSSON, 2004).

Entre os polímeros hidrofílicos mais relatados na literatura e frequentemente empregados em formulações farmacêuticas encontram-se a polivinilpirrolidona (PVP) e a hidroxipropilmetilcelulose (HPMC) (KURKOV; LOFTSSON, 2013; MIRANDA et al., 2011). Estes polímeros são fisiologicamente bem tolerados, apresentam baixa toxicidade, alta hidrossolubilidade, são disponíveis no mercado com diversas massas moleculares e são de baixo custo (ALVES et al., 2012).

A estratégia de formação de sistemas multicomponentes foi aplicada por nosso grupo de pesquisa para a isoflavona daidzeína (DAI) (BORGHETTI et al., 2011), demonstrando que complexos de daidzeína com βCD e HPβCD resultaram num aumento da hidrossolubilidade da mesma, de 5,7 e 9,4 vezes, respectivamente. A simples combinação entre daidzeína e os polímeros HPMC

ou PVP resultaram, respectivamente, num aumento de sua hidrossolubilidade em 2,2 e 4,3 vezes. Os sistemas ternários DAI:βCD:HPMC e DAI:βCD:PVP apresentaram, respectivamente aumentos na solubilidade da daidzeína de 7,1 e 8,0 vezes ($p < 0,05$). Em suma, os autores constataram que, o efeito combinado da complexação da daidzeína com CDs e polímeros hidrofílicos foi capaz de melhorar ainda mais a solubilidade desta molécula do que a complexação com CD unicamente, e que o sistema ternário contendo PVP e HPβCD foi o que demonstrou aportar o maior aumento de hidrossolubilidade (12,7 vezes) (BORGHETTI et al. 2011).

Na literatura diversos são os relatos encontrados sobre a utilização deste artifício para aumentar a solubilidade de moléculas hidrofóbicas. Chowdary e Srinivas investigaram a complexação do celecoxib com HPβCD na presença e ausência de três polímeros hidrofílicos: PVP, HPMC e polietilenoglicol (PEG); e constataram que a adição de polímeros melhorou a complexação e dissolução do fármaco (CHOWDARY; SRINIVASA, 2006). Sami, Philip e Pathak relataram a formação de complexo ternário de genfibrozil, βCD e substâncias auxiliaries (diferentes povidonas, base orgânica e íon metálico), revelando que os sistemas ternários demonstraram superioridade em relação aos sistemas binários em termos de EC, solubilidade do fármaco e redução no volume da formulação (SAMI; PHILIP; PATHAK, 2010). Alexanian e colaboradores estudaram a influência de CDs (βCD, HPβCD e metil-βCD) e polímeros hidrossolúveis (HPMC. PVP, PEG e carboximetilcelulose sódica) na solubilidade aquosa da nimesulida além da capacidade de complexação deste fármaco com as CDs; e demonstraram que a solubilidade da nimesulida foi afetada pelas CDs na ordem: metil-βCD > βCD> HPβCD; e a solubilização e a eficiência de complexação das três CDs foram aumentadas na presença de HPMC, enquanto que os demais polímeros apresentaram efeitos menores na promoção da solubilidade do fármaco (ALEXANIAN et al., 2008). No entanto, não foram encontrados relatos na literatura científica sobre organização exata desses sistemas, tampouco sobre a associação do PTS e CDs a polímeros hidrossolúveis.

Desenho experimental

Devido à necessidade de maximizar a eficiência na investigação científica minimizando tempo e custo, muitos pesquisadores tem buscado realizar experimentos que fornecem o máximo de informações possíveis com um menor número de experimentos (HIBBERT, 2012).

Nesse sentido, a utilização de desenho experimental tornou-se uma ferramenta importante, pois é capaz de otimizar processos, melhorando o seu rendimento, reduzindo o tempo de desenvolvimento, o número de experimentos e os custos globais, além de minimizar a variabilidade, (MONTGOMERY, 2012) tendo como base a análise estatística. Desenho experimental é um resultado específico multivariado de avaliações empíricas, definido por uma matriz composta pelas diferentes combinações de níveis das variáveis estudadas, com caráter linear, quadrático ou de interação, que pode ser usada para promover informações sobre um determinado sistema (HIBBERT, 2012).

Entre outras razões, um desenho experimental pode ser realizado com vistas a:

· Caracterizar fatores ou realizar uma triagem: quando um sistema ou processo é novo, para saber quais fatores têm mais influência sobre a(s) resposta(s) de interesse.

· Otimizar condições experimentais: para encontrar as configurações ou níveis dos fatores importantes que resultam em valores desejáveis da resposta.

· Avaliar a robustez do método empregado: para verificar o efeito das mudanças nos parâmetros do método sobre um analito (MONTGOMERY, 2012)

Alguns passos devem ser considerados para a elaboração de um desenho experimental, sendo eles: definição do objetivo do experimento, detecção dos fatores que podem afetar o processo, planejamento dos experimentos, execução dos experimentos, e análise dos dados obtidos (LEARDI, 2009). Assim que são obtidas as informações necessárias para o planejamento, é possível escolher o tipo de desenho experimental que será aplicado. Dentre os existentes, destaca-se o de superfície de resposta.

A metodologia de superfície de resposta consiste em um conjunto de técnicas matemáticas e estatísticas úteis para a modelagem e análise de

problemas nos quais uma resposta de interesse é influenciada por diversas variáveis; permitindo assim analisar a influência dos parâmetros sobre esta resposta e descrever o comportamento de um conjunto de dados com o objetivo de fazer previsões estatísticas (MONTGOMERY, 2012). Dentre as vantagens da metodologia de superfície de resposta está a simplicidade analítica, pois a metodologia gera polinômios. Após o ajuste do modelo aos dados, é possível estimar a sensibilidade da resposta aos fatores, além de determinar os níveis dos fatores nos quais a resposta é ótima (MONTGOMERY, 2012).

Dentre os desenhos experimentais que se baseiam na metodologia de superfície de resposta encontra-se o proposto por Box e Behnken (BOX; BEHNKEN, 1960). Este desenho pertence ao modelo de segunda ordem e baseia-se no delineamento fatorial incompleto de três níveis, resultando em planejamentos muito eficientes em termos do número de execuções (FERREIRA et al., 2007; MONTGOMERY, 2012). Uma vantagem desse desenho é que ele não possui combinações para cada um dos fatores simultaneamente nos seus níveis altos ou baixos, sendo útil àqueles que desejam evitar pontos extremos de condição. Além disso, o desenho tipo Box-Behnken é capaz de construir sequências experimentais, estimar modelos quadráticos, detectar falta de ajuste de modelo e realizar os experimentos em blocos (FERREIRA et al., 2007), daí sua grande utilidade no desenvolvimento de metodologias analíticas e tecnológicas. Delineamentos fatoriais têm sido utilizados para avaliar e otimizar os parâmetros de complexação do fármaco com CD (BORGHETTI et al., 2009; ZHANG et al., 2013), porém, não há relatos, de nosso conhecimento, da utilização do desenho experimental Box-Behnken para esta finalidade.

Validação de método analítico

A validação de uma metodologia objetiva demonstrar que o método desenvolvido é adequado para o uso pretendido. De acordo com as diretrizes oficiais para validação de procedimento analítico, especificadas pelo Food and Drug Administration (FDA) e International Conference on Harmonization (ICH), parâmetros como especificidade/seletividade, exatidão, precisão, sensibilidade (limites de detecção e quantificação), linearidade e estabilidade devem ser analisados (FDA, 2015; ICH, 2005).

O primeiro critério para um analista avaliar se a metodologia aplicada está adequada é a especificidade, caracterizada pela capacidade de mensurar inequivocamente o analito na presença de componentes que possam estar presentes, tais como impurezas, produtos de degradação, elementos da matriz etc. (ICH, 2005).

Neste sentido, para determinar a ausência de interferência de produtos de degradação, é necessário expor a amostra a estudos de degradação forçada, a fim de permitir a geração de produtos de degradação por meio da exposição da substância-teste a condições de estresse, como luz, temperatura, oxidação, hidrólise ácida e básica, entre outras. A degradação forçada é um processo que envolve condições mais severas do que as condições utilizadas no estudo de estabilidade acelerado e permite o desenvolvimento de métodos indicativos de estabilidade (BLESSY et al., 2014; ICH, 2003)

Um método indicativo de estabilidade é um procedimento analítico usado para determinar a diminuição na quantidade de uma determinada substância devido à degradação, e pode ser usado para detectar como a estabilidade das substâncias muda com o tempo. O desenvolvimento de um método indicativo de estabilidade fornece uma base para os estudos de pré-formulação, estudos de estabilidade e o estabelecimento de condições de armazenamento adequadas (BLESSY et al., 2014). Na literatura há muitos métodos intitulados indicadores de estabilidade originando dois novos termos para designar corretamente o método desenvolvido. O termo “Specific-Stability Indicating Analytical Method” é atribuído a um método que é capaz de mensurar inequivocamente o fármaco, na presença de produtos de degradação, excipientes e aditivos que possam estar presentes na formulação; enquanto que o termo “Selective-Stability Indicating Analytical Method” é atribuído a um método que é capaz de mensurar inequivocamente o fármaco e produtos de degradação, na presença de excipientes e aditivos que possam estar presentes na formulação (MORAES DO CARMO; PEREIRA; GRATIERI, 2018).

Em relação à métodos analíticos aplicados à quantificação de PTS em diferentes matrizes, encontram-se diversos relatos na literatura. Jeandet e colaboradores, em 1997, validaram um método para quantificação de fitoalexinas, entre as quais o PTS, em extratos de videira, por cromatografia

líquida de alta eficiência (CLAE) acoplada a detector de arranjo de diodos e fluorimetria (JEANDET et al., 1997). Rimando e colaboradores utilizaram um método de cromatografia gasosa associada a espectrofotômetro de massas para determinar a presença de resveratrol, PTS e piceatannol em frutos de plantas do gênero *Vaccinium* (RIMANDO et al., 2004). Remsberg e colaboradores descreveram um método para quantificação de PTS em soro de rato por CLAE com detector de fluorescência (REMSBERG et al., 2007). Lin e colaboradores relataram um método para quantificação de PTS em plasma de rato por CLAE com detector UV (LIN; YUE; HO, 2009). Silva e colaboradores relatam o emprego de um método empregando CLAE para quantificação de PTS associado a CD (HPβCD e hidroxipropil-gama-ciclodextrina) e a sais biliares (SILVA et al., 2014). Em 2015, Koritela e colaboradores desenvolveram métodos espectrofotométricos para determinação de PTS em formulações farmacêuticas (cápsulas) (KORITELA et al., 2015).

Um método indicativo de estabilidade utilizando CLAE para determinação de PTS em formulações farmacêuticas (cápsulas) foi proposto, no decorrer da realização do presente trabalho, por Annapurna, Venkatesh e Teja (ANNAPURNA; VENKATESH; TEJA, 2018). Porém, a análise do referido estudo denota a necessidade de esclarecimento dos resultados relativos à degradação forçada desta molécula, pois estes resultados divergem daqueles já conhecidos para a degradação do PTS (SILVA et al., 2014; ZHANG et al., 2013), assim como dos conhecidos para o resveratrol, um estilbeno mais estudado (JITRANGSRI; CHAIDEDGUMJORN; SATIRAPHAN, 2015; KUMAR; LATHER; PANDITA, 2016; PANGENI et al., 2015) e em relação aos estilbenos de forma geral (HENDRICKSON; CRAM; HAMMOND, 1970; KWASNIEWSKI et al., 2003; MALLORY; MALLORY, 1984; OGATA; TOMIZAWA; IKEDA, 1979). Annapurna e colaboradores, neste estudo, afirmam que o PTS não se degrada quando exposto a altas temperaturas, irradiação de luz UV ou ao meio oxidativo; porém é conhecida a instabilidade dos estilbenos frente a oxidação (OGATA; TOMIZAWA; IKEDA, 1979) e irradiação de luz UV, podendo ocorrer fotoisomerização (HENDRICKSON; CRAM; HAMMOND, 1970; MALLORY; MALLORY, 1984) e até mesmo termoisomerização em elevadas temperaturas (KWASNIEWSKI et al., 2003).

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CAPÍTULO II

Development and validation of a specific-stability indicating liquid chromatography method for quantitative analysis of pterostilbene.

Development and validation of a specific-stability indicating liquid chromatography method for quantitative analysis of pterostilbene.

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Abstract

The pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) is a natural constituent which has attracted the attention of researchers due to its numerous preventive and therapeutic properties in a wide range of human diseases. It has been isolated from *Pterocarpus* spp, blueberries, and grapes. The instability of this molecule is the reason why to develop a method of quantification indicative of stability is a requirement to avoid the interference of the degradation products in the quantification of the analyte. Thus, the present study was designed to evaluate the stability of pterostilbene when exposed to forced degradation conditions and to develop and validate a versatile liquid chromatography method to quantify pterostilbene. In the stability-indicating test pterostilbene samples were exposed to stress conditions: temperature, UV light, oxidative, acid and alkaline media. The pterostilbene was stable in acid and alkaline medium but unstable when exposed to oxidation, temperature, and UV light. The robustness of the method was evaluated using a Box-Behnken experimental design that demonstrated how analytical parameters (flow rate, wavelength, concentration of trifluoroacetic acid and acetonitrile in the mobile phase) influenced the peak area. Using the proposed method, no interference of the degradation products in the analyte analysis was observed. The method revealed to be simple, fast, selective, linear, precise and accurate for the quantitative analysis of pterostilbene. Besides being a specific-stability indicating method, it was applied to quantify pterostilbene in a blueberry extract. These findings are relevant for the development of food, pharmaceutical or cosmetic products containing pterostilbene or blueberry extracts.

Key-words: pterostilbene, blueberry, HPLC, stability indicating, stilbenes, validation.

1 Introduction

Pterostilbene (3,5-dimethoxy-4'-hydroxystilbene, $C_{16}H_{16}O_3$); is a phytoalexin that also belongs to the stilbene family of phenolic compounds, naturally found in *Pterocarpus* spp. *Vaccinium* spp *and Vitis* spp. genus, among others. In *Vaccinium myrtillus* (blueberry), pterostilbene is present in high concentration, around 0.15 mg per 100 g of the fruits (PENG et al., 2018). Pterosilbene is available in the market as the main constituent of several nutraceuticals (BETHUNE; SCHULTHEISS; HENCK, 2011). The pterostilbene has attracted the attention of researchers due to its numerous preventive and therapeutic properties for treating a wide range of human diseases. Among these properties are the antioxidant, anti-inflammatory, antitumor, hypolipidemic, neuroprotective, antidiabetic and cardioprotective activities (DOS SANTOS LACERDA et al., 2017; KOSURU et al., 2016; LACERDA et al., 2018b; PENG et al., 2018).

Pterostilbene has poor aqueous solubility, which is a problem for its dissolution in physiological media and, consequently, for its oral bioavailability (KOSURU et al., 2016). Due to this, several strategies have been adopted to increase its solubility in water, among them is the association with cyclodextrins (DOS SANTOS LACERDA et al., 2017; LACERDA et al., 2018a, 2018b) which has shown to be a promising strategy.

Several analytical techniques have been published for quantification of pterostilbene in different complex matrices, grapevine leaf extracts, *Vaccinium* berries, rat serum and plasma, associated with cyclodextrins, bile acids or in pharmaceutical formulations (Capsules) (JEANDET et al., 1997; KORITELA et al., 2015; LIN; YUE; HO, 2009; REMSBERG et al., 2007; RIMANDO et al., 2004; SILVA et al., 2014). More recently, a study on the stability of pterostilbene was reported by Annapurna; Venkatesh and Teja, (2018), however when its results are compared to others previously reported in the literature, especially regarding to stilbenes instability against UV light and oxidation (HENDRICKSON; CRAM; HAMMOND, 1970; KWASNIEWSKI et al., 2003; MALLORY; MALLORY, 1984; OGATA; TOMIZAWA; IKEDA, 1979), some discrepancies are detected what reveals the need of new studies.

Thus a specific-stability indicating analytical method was designed to measure unequivocally the active ingredient in the presence of all degradation products,

excipients, and additives expected to be present in a formulation or matrix. According to ICH guideline, the stability testing of drug substances should be carried out under different stress conditions such as hydrolysis, oxidation, photolysis, and thermal degradation (BLESSY et al., 2014; MORAES DO CARMO; PEREIRA; GRATIERI, 2018).

Thus, the aim of the present study was to develop and validate an HPLC isocratic reversed-phase specific-stability indicating method for the quantitation of pterostilbene in several types of samples, pterostilbene:β-cyclodextrin complexes, pterostilbene:β-cyclodextrin:hydrophilic polymers (polyvinylpyrrolidone, PVP or hydroxypropylmethylcellulose, HPMC) ternary systems and also in a dried blueberry extract, following the ICH guidelines.

2 Material and methods

2.1 Chemical and materials

Pterostilbene (≥ 98% of purity) was purchase from Changsha Organic Herb (China). Acetonitrile liquid chromatography grade was provided from Tedia (USA), trifluoroacetic acid was purchased from Vetec (Brazil), and purified water was obtained from a Milli-Q apparatus (Milli-QTM system, Millipore, USA). All other reagents used were of analytical grade. Blueberry extract was purchase from a local pharmacy (Rio Grande do Sul, Brazil).

2.2 Stock and reference solutions

A stock solution (50 µg/mL) of pterostilbene was prepared in acetonitrile by weighing accurately 5 mg of pterostilbene into a 100 mL calibrated volumetric flask and diluting to volume. This stock solution was transferred to glass flask protected from light and stored under refrigeration (2-8°C).

The reference solutions were prepared in appropriate concentrations by the stock solution dilution with acetonitrile 60% (v/v).

2.3 Apparatus and procedures

2.3.1 HPLC analysis

The development and validation of the HPLC method were performed using a Shimadzu HPLC-20A equipment (Kyoto, Japan) composed of HPLC-20AT pump, a

SIL-20A autosampler, and a SPD-20AV photodiode array detector. Data acquisition and treatment were performed with Shimadzu HPLC Solution GPC software (Shimadzu, Japan). The stationary phase employed was a C18 Phenomenex Gemini column (150 × 4.6 mm, i.d., 5 µm) guarded by a C18 pre-column (20 × 3.9 mm i.d., 10 mm) (Waters, USA). The mobile phase was composed of acetonitrile and water with 0.1% trifluoroacetic acid (60:40 v/v), filtered through a 0.45 µm pore size membrane filter and degassed for 30 minutes, eluted in isocratic flow. The flow rate, detection wavelength, injection volume, and column temperature were, respectively, 0.8 mL/minutes, 320 nm, 20 µL and room temperature. All the samples were filtered through Millipore PTFE membrane (0.45 µm of pore diameter) before injected.

2.4 Validation of the analytical method

The HPLC method was validated according to recognized international recommendations and guidelines in the concentration range of 1 – 20 µg/mL. It was used the ICH specification and the FDA recommendations (FDA, 2015; ICH, 2005). Analysis of variance (ANOVA) was used to analyze the results using a significance level of $\alpha = 0.05$. The possible interference of degradation products generated from forced degradation test of pterostilbene was also evaluated in the validation.

2.4.1 Linearity

Three standard curves were obtained in triplicate, in three consecutive days, by plotting the measured peak area *versus* pterostilbene concentration. The following pterostilbene reference solutions were used: 1.0, 2.5, 5.0, 10.0, 15.0, and 20.0 µg/mL, which were prepared by dilution of the pterostilbene stock solution in acetonitrile:water (60:40, v/v). The linearity of the method was evaluated by regression analysis using the least square method.

2.4.2 Determination of the limit of quantitation (LOQ) and detection (LOD)

The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated from the reference curves, using the values of standard deviation (SD) between the interception of axis Y of three linearity curves and slope of the linearity mean curve using the following formulas:

$LOD = 3{:}3 \cdot SD/slope$ $LOQ = 10 \cdot SD/slope$

2.4.3 Specificity and forced degradation

These studies were performed to verify the stability property and selectivity of the proposed method. The study followed the ICH guidelines (ICH, 2003). The details of the stress testing procedure were outlined according to the review by Blessy and cols. (2014) that discusses the current trends in the performance of forced degradation studies.

The specificity of the HPLC method was first evaluated by comparing chromatograms obtained for pure standard pterostilbene solution (2.5 µg/mL), solutions containing only the matrices (blank samples) and blueberry extract solution, due to the impossibility of obtaining a blank matrix of this sample. To verify the presence or absence of interferences, the peak corresponding to pterostilbene was analyzed and identified by UV spectra between 210 and 400 nm, peak purity and retention time.

For the specificity test regarding degradation products, pterostilbene stock solutions were previously submitted to acid and alkaline hydrolysis, oxidative stress, photolytic and temperature degradation. All samples were compared with a sample of pterostilbene solution with 10 µg/mL prepared protected from light (control) and injected in three replicates.

To assess thermal stability, a part of pterostilbene stock solution with a part of diluent (acetonitrile:water, 60:40 v/v) was exposed, in a closed glass flask, to dry heat at 80°C for 30 hours. The effect of UVA light was analyzed by conditioning a part of stock solution pterostilbene with a part of diluent in transparent and closed quartz buckets fixed to the chamber in a horizontal position and exposed to radiation (360 nm, 30 W) lined with mirrors during 10 minutes. Acid and alkaline hydrolysis were investigated by adding hydrochloric acid (HCl) or sodium hydroxide (NaOH) in different glass flask containing pterostilbene stock solutions to achieve the final concentration of 1.0 M. After 30 hours of exposure, both solutions were neutralized with base and acid, respectively. For evaluating oxidative stress condition, hydrogen peroxide (H_2O_2) 29% was added in the stock solutions to achieve the final concentration of 1.5% H_2O_2 during 10 hours. All solutions were kept at room temperature (25°C) and protected from light. After removal from degradation conditions in pre-established times (not exceeding 30 hours), the samples were prepared for analysis by diluting to 10 µg/mL with the mobile phase and analyzed

determining the peak purity of pterostilbene using a photodiode array detector (PDA). All solutions were filtered through PTFE membrane of 0.45 µm nominal pore diameter (Millipore).

2.4.4 Precision and accuracy

The precision was determined as both repeatability and intermediate precision and expressed as the relative standard deviations (% RSD) for each level of pterostilbene. The intra-day precision (repeatability) of the method was evaluated by analyzing pterostilbene at three levels (2.5, 10.0 and 20.0 µg/mL), with three determinations per concentration within a day. The inter-day precision (intermediate precision) was evaluated by assaying pterostilbene samples of the same concentration levels on three consecutive days. The accuracy of the method was determined by analyzing pterostilbene at three levels (2.5, 10.0 and 20.0 µg/mL). Samples were analyzed in nine replicates at each level. The accuracy was evaluated as the standardized correlation between the experimental value and the theoretical value, as follows formula: Accuracy % = (mean experimental concentration × 100 / mean theoretical concentration).

2.4.5 Robustness

A response surface methodology was performed using a Box-Behnken design (BBD) to analyze the robustness of the analytical method, evaluating the effect of four different experimental factors (X_1, organic phase proportion in mobile phase; X_2, solvent flow rate; X_3, TFA concentration in mobile phase; X_4 wavelength detection) in three factor levels (−1; 0; 1) **(Table 1)** on the pterostilbene peak area (Y_1) obtained from the HPLC analysis. The assay was performed with a reference stock solution of pterostilbene at 10 µg/mL. The full design comprised 27 experiments with three replicates at the central point, in order to estimate the pure error, and was carried out in triplicate, in three different days, totalizing 81 experiments. Minitab® 17.0.1 software and Design Expert® 10 were used to perform the study and for further statistical analyses. The results were evaluated based on RSD (%) values, and the influence of each parameter was verified from the statistical and graphical analysis generated by the software.

Table 1. Independent variables used in Box–Behnken design to evaluate robustness.

	Factor	Uncoded level (coded level)		
	Independent Variables	Low (-1)	Medium (0)	High (+1)
X_1	Organic phase (%)	59	60	61
X_2	Flow rate (mL/minutes)	0.78	0.80	0.82
X_3	TFA concentration (%)	0.09	0.10	0.11
X_4	Wavelength (nm)	319	320	321

TFA = trifluoroacetic acid

2.4.6 Stability

The stability of the samples was determined after 48 hours of storage at ambient temperature in the HPLC vials, intending to overestimate the time consumed during routine analysis. The stability was determined by performing the analysis of peak area and observing any change in the chromatographic pattern of the stored samples compared with a freshly prepared sample.

2.4.7 System suitability

The system suitability test was carried out to verify the adequacy of the system for the analysis. The measured and evaluated parameters were peak area, retention time, plates and tailing factor of pterostilbene. This analysis was performed with 9 replicates.

2.5 Method application

The determination of pterostilbene in samples of dried blueberry extract, pterostilbene:β-cyclodextrin complexes and pterostilbene:β-cyclodextrin:hydrophilic polymers (PVP or HPMC) ternary systems was performed as described below:

The dried blueberry extract (150 mg) was extracted in acetonitrile (1 mL) in a closed glass vial, maintained in an ultrasonic bath for 15 minutes, filtered through 0.45 µm membrane (PTFE, Millipore) and analyzed. The dried blueberry extract is available as food supplement. The pterostilbene:β-cyclodextrin complexes were prepared in aqueous solution in the molar ratio of 1:1, stirred at 37 ± 1°C for 2 hours, protected from light. After this period, the dispersion was filtered through 0.45 µm PTFE membrane (Millipore, USA), and the supernatant was analyzed. For obtaining the ternary system, an aqueous dispersion containing pterostilbene, β-cyclodextrin, and HPMC or PVP (1% w/w) was employed, stirred at 37 ± 1°C for 2 hours,

protected from light. After this period, the dispersion was filtered through 0.45 µm PTFE membrane (Millipore, USA), and the supernatant was analyzed.

Samples were analyzed in six replicates the according to the previously described method. Peak purity was analyzed using a PDA detector, and the peak absorption maxima present in samples was compared to the absorption maxima of the pterostilbene peak of the reference solution (10 µg/mL).

3 Results and discussion

3.1 HPLC method development and advantages

During the development of HPLC method different proposals, mobile phase and flow rate were preliminarily tested. A mixture of acetonitrile/water was selected for further studies once showed high chromatographic performance (average peak high, peak symmetry). Trifluoroacetic acid (TFA) was used to acidify the mobile phase, to ensure the ionization suppression of pterostilbene during the analysis and improve peak symmetry. The optimum mobile phase composition, which yielded a sharp pterostilbene peak, consisted of a 60:40 mixture of acetonitrile/water (the aqueous phase containing 0.1% of TFA). The wavelength at 320 nm was selected since it is in the region of maximum UV absorption for pterostilbene under these conditions. The pterostilbene chromatogram obtained by the developed method is shown in **Figure 1** and the most important chromatography parameters are presented in **Table 2**.

Several scientific reports on validated methods used to determine pterostilbene in different matrices have been published (JEANDET et al., 1997; KORITELA et al., 2015; LIN; YUE; HO, 2009; REMSBERG et al., 2007; RIMANDO et al., 2004; SILVA et al., 2014) and more recently a stability-indicating method by HPLC, for determination of pterostilbene in pharmaceutical formulations, was proposed by (ANNAPURNA; VENKATESH; TEJA, 2018). However, when the findings on pterostilbene stability are compared with that previously reported to similar molecules such as resveratrol and stilbenes (JITRANGSRI; CHAIDEDGUMJORN; SATIRAPHAN, 2015; KUMAR; LATHER; PANDITA, 2016; KWASNIEWSKI et al., 2003; MALLORY; MALLORY, 1984; OGATA; TOMIZAWA; IKEDA, 1979; PANGENI et al., 2015) or even to the pterostilbene stability (SILVA et al., 2014; ZHANG et al., 2013)**,** some crucial questions arise. In this recent study (ANNAPURNA; VENKATESH; TEJA, 2018), the authors state that pterostilbene does not degrade

when exposed to oxidation, UV light and high temperatures, and degrades in acidic and alkaline media. However, it is well known that stilbenes are unstable against oxidation and may undergo photoisomerization and thermoisomerization (HENDRICKSON; CRAM; HAMMOND, 1970; KWASNIEWSKI et al., 2003; MALLORY; MALLORY, 1984; OGATA; TOMIZAWA; IKEDA, 1979).

Thus, the present work was designed to develop a specific-stability indicating method and validate an HPLC method for quantifying pterostilbene in samples of pterostilbene:β-cyclodextrin complexes, pterostilbene:β-cyclodextrin:hydrophilic polymers ternary systems and blueberry extract.

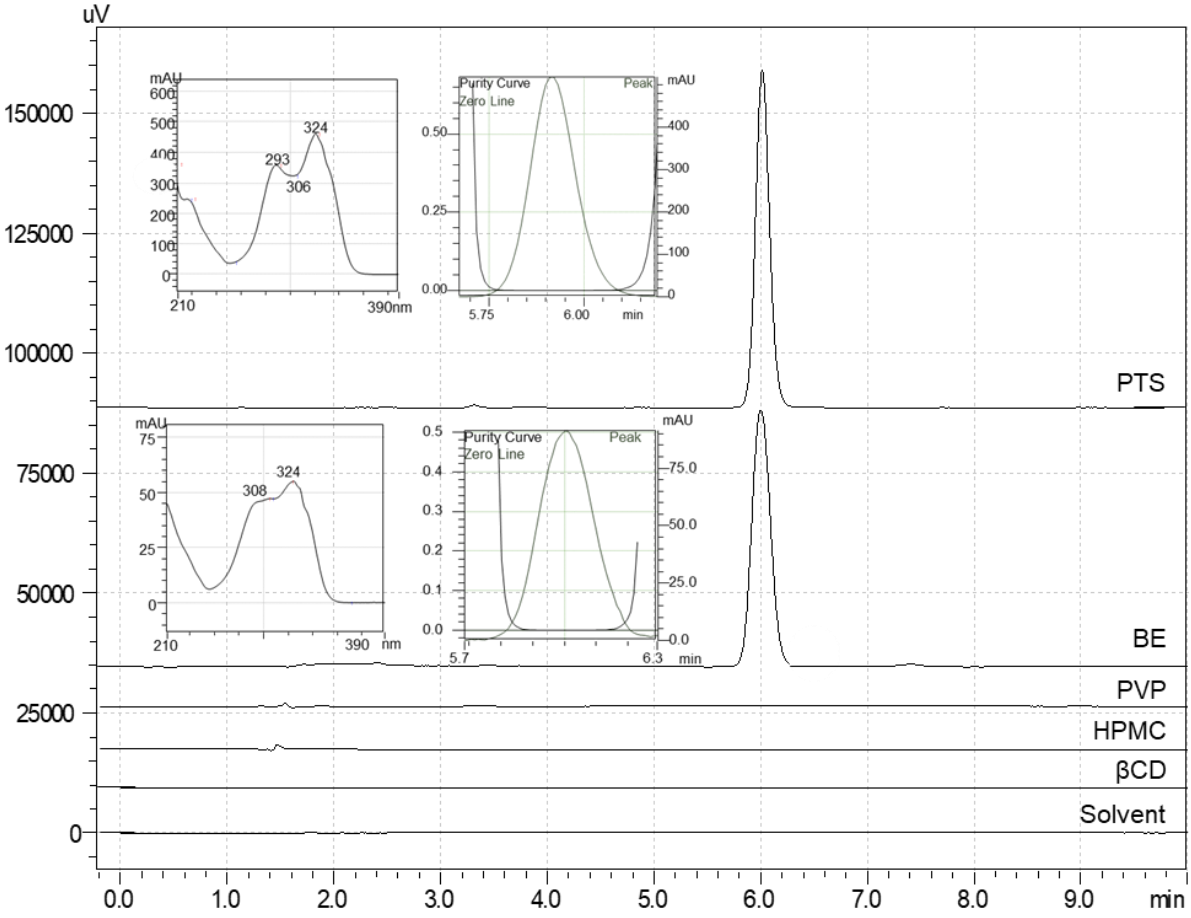
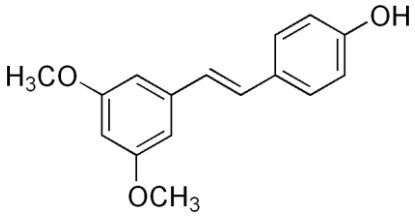


Figure 1: Chromatograms obtained by HPLC at 320 nm in pterostilbene (PTS) reference solution at 2.5 µg/mL and blank samples (specificity in matrices), with purity curve and UV spectrum of the peaks. Where, BE = blueberry extract; PVP = polyvinylpyrrolidone; HPMC = Hydroxypropyl methylcellulose; βCD = β-cyclodextrin; Solvent: mobile phase.

Table 2. Suitability testing of chromatographic **s**ystem of pterostilbene reference.

Compound	Chemical Structure	RT	N	T
E-Pterostilbene		5.91	9594.85	1.14

RT: retention time (minutes); T: tailing factor; N: plates

3.2 Validation of the analytical method

3.2.1 Linearity, limit of quantitation (LOQ) and detection (LOD)

The results for linearity were obtained using standard analytical curves, determined in three different days. The HPLC method showed good linearity between response (peak area) and the corresponding concentration of pterostilbene in the tested range from 1.0 to 20.0 µg/mL. The standard curve obtained was y = 387869.96x - 208188.47. The correlation coefficient obtained was satisfactory ($r > 0.999$). The statistical evaluation for regression residues did not reveal linearity deviation ($p > 0.05$). Constant systematic error was not observed after analysis of the graphic of the residuals (data not shown). The LOD and LOQ results for pterostilbene were 0.09 and 0.29 µg/mL, respectively, and show that the method presents good sensitivity.

3.2.2 Precision and accuracy

The repeatability, intermediate precision, and accuracy data for the pterostilbene assay are presented in **Table 3**. The results obtained for pterostilbene were analyzed at concentrations 2.5 µg/mL (lowest concentration), 10 µg/mL (medium concentration), and 20 µg/mL (highest concentration). The intra-day precision results demonstrated a relative standard deviation (RSD, %) lower than 1.65% and the inter-day precision RSD results were lower than 1.48%. The accuracy data for pterostilbene were within 96.88 to 100.77% range. Taken together, the analytical HPLC method can be considered precise and accurate according to official guidelines.

Table 3. Repeatability, intermediate precision, accuracy, and stability evaluation of pterostilbene standard.

Level	Precision (RSD)				Accuracy	SE
(µg/mL)	First day	Second day	Third day	Inter-day[a]	(%)	(%)
2.5	0.56	0.64	0.59	0.69	100.77	102.82
10.0	1.65	1.19	0.71	1.48	96.88	103.91
20.0	0.91	0.45	0.20	0.91	99.90	102.86

SE: stability evaluation (48 hours); RSD=relative standard deviation (%); [a] n=3 days;

3.2.3 Specificity and forced degradation

HPLC chromatographic conditions resulted in pterostilbene retention time of 5.9 minutes (RSD 1.13%), λ max (maximum UV absorption) peak at 293/306/324, and peak purity index > 0,999, as shown in **Figure 1**. The short run time makes the chromatographic conditions suitable for the routine analysis of a large number of samples. No co-eluting substances from matrices were detected at the same retention time of molecule of interest, and the peak purity data from pterostilbene evaluation in solution blueberry extract demonstrated that the method was specific for the pterostilbene assay **(Figure 1)**. Thus, these results show that the method is specific for pterostilbene determination in samples of pterostilbene:β-cyclodextrin complexes, pterostilbene:β-cyclodextrin:hydrophilic polymers and blueberry extract.

Stability tests are important because they provide a basis for pre-formulation and stability studies and for the development of adequate storage requirements (BLESSY et al., 2014), thus ensuring that quality attributes are maintained over a period under the influence of a variety of environmental factors, thus maintaining product efficacy and safety (MORAES DO CARMO; PEREIRA; GRATIERI, 2018). In this sense, the development of a stability-indicating method can be used to detect how the stability of the substances and products changes with time. A stability-indicating method accurately measures the changes in active ingredients concentration without interference from other degradation products, impurities, and excipients (BLESSY et al., 2014). Therefore, the stability-indicating properties of HPLC method were also evaluated. In this paper, pterostilbene was exposed to acid, alkaline, oxidative, photolytic and thermal stress conditions and further analyzed.

No significant changes were observed after exposure of pterostilbene in acid or alkaline media at 25°C during 30 hours. In contrast, in oxidative (10 hours) and high

temperature (30 hours) conditions, pterostilbene degraded 9.71% and 9.17%, respectively, and when exposed to UVA light (10 minutes) degraded 71.84%, proving to be highly photosensitive. The relative standard deviations of the analysis, performed in triplicate, were less than 5%.

When pterostilbene was exposed to the oxidative medium, the HPLC analysis showed that different new peaks could be observed, but they also did not interfere in the peak of interest, as is shown in **Figure 2**. In heat conditions, besides the pterostilbene peak, a new small peak was observed, as is shown in **Figure 3**, which did not interfere in the response of interest. **Figure 4** shows the HPLC chromatogram of the pterostilbene sample after it was irradiated by UVA light. A new well-defined peak with retention time close to the peak of interest was observed, but did not interfere in its quantification (resolution 1.9).To verify if this peak correspond to the *cis*-isomer of pterostilbene, the UV max of both peaks was determined. *Trans* isomer has maximum absorption of about 310-320 nm while the maximum absorption of the *cis* isomers shifts towards higher energy wavelengths, around 280 nm (BEJARANO et al., 2017), what is coincident with the two peaks showed in **Figure 4**. The PDA detector showed that the pterostilbene peak presented high purity (peak purity index >0.999) even the presence of these degradation products.

It is important to emphasize that stability of pterostilbene has been a controversial issue. While Annapurna and cols. (ANNAPURNA; VENKATESH; TEJA, 2018) have reported that pterostilbene is unstable against acidic and alkaline but stable under other stress conditions, other studies on stilbene stability have demonstrated that these molecules undergo *cis-trans* isomerization under UV light irradiation, by exposure to high temperatures, besides oxidation (BUSCH; YIN; LEE, 2008; KWASNIEWSKI et al., 2003; MALLORY; MALLORY, 1984; OGATA; TOMIZAWA; IKEDA, 1979). Silva *et al* have already demonstrated the reduction of the *trans*-isomer area of pterostilbene when exposed to fluorescent light (SILVA et al., 2014).

Our results, in general, are in agreement with the most reports on stability of stilbenes, with small differences in relation to what has already been described for resveratrol due to little differences in the experimental stress conditions. (JITRANGSRI; CHAIDEDGUMJORN; SATIRAPHAN, 2015; KUMAR; LATHER; PANDITA, 2016; PANGENI et al., 2015). **Table 2** shows the chemical structure of

pterostilbene (3,5-dimethoxy-4'-hydroxystilbene), an olefinic compound, which is characterized by classical *cis-trans* isomerization in light exposure (HENDRICKSON; CRAM; HAMMOND, 1970; MALLORY; MALLORY, 1984) which may also occur under high temperatures (KWASNIEWSKI et al., 2003). In addition, the double bonds and the phenolic moiety are susceptible to oxidation reactions (OGATA; TOMIZAWA; IKEDA, 1979). These characteristics may explain and support our degradation results when pterostilbene was exposed to UV light, high temperature and hydrogen peroxide.

In summary, regarding the specificity the results of the present work reveal that the proposed method is appropriate for studies concerning pterostilbene even in the presence of degradation products. In this case, it is able to separate and differentiate the peaks of the analyte from that corresponding to the degradation products.

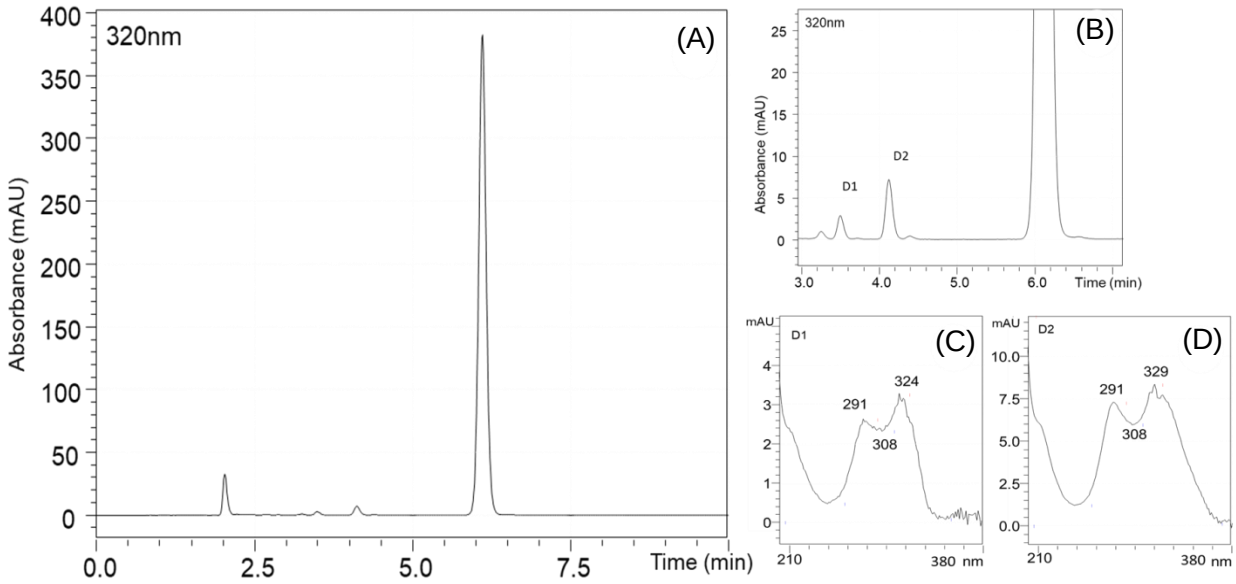


Figure 2: (A) HPLC chromatogram, detection at 320 nm (B) zoom on the chromatogram, (C) and (D) UV spectrum of pterostilbene degraded after oxidative stress (10 hours) of standard solutions.

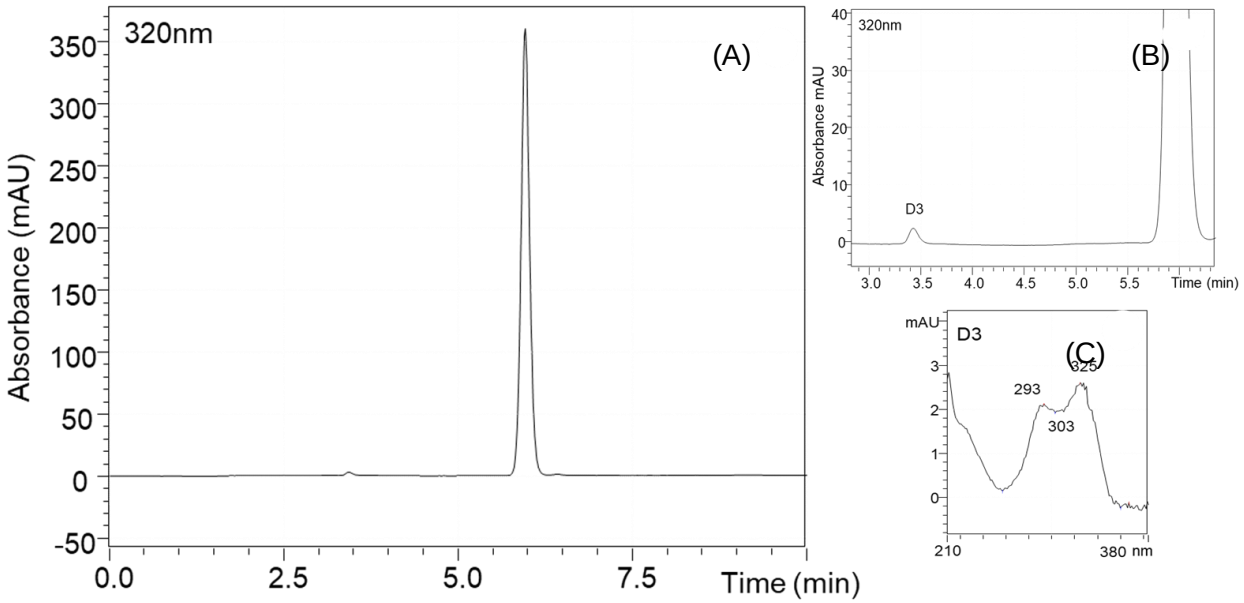


Figure 3: (A) HPLC chromatogram, detection at 320 nm (B) zoom on the chromatogram, and (C) UV spectrum of pterostilbene degraded after dry heat at 80°C (30 hours) of standard solutions

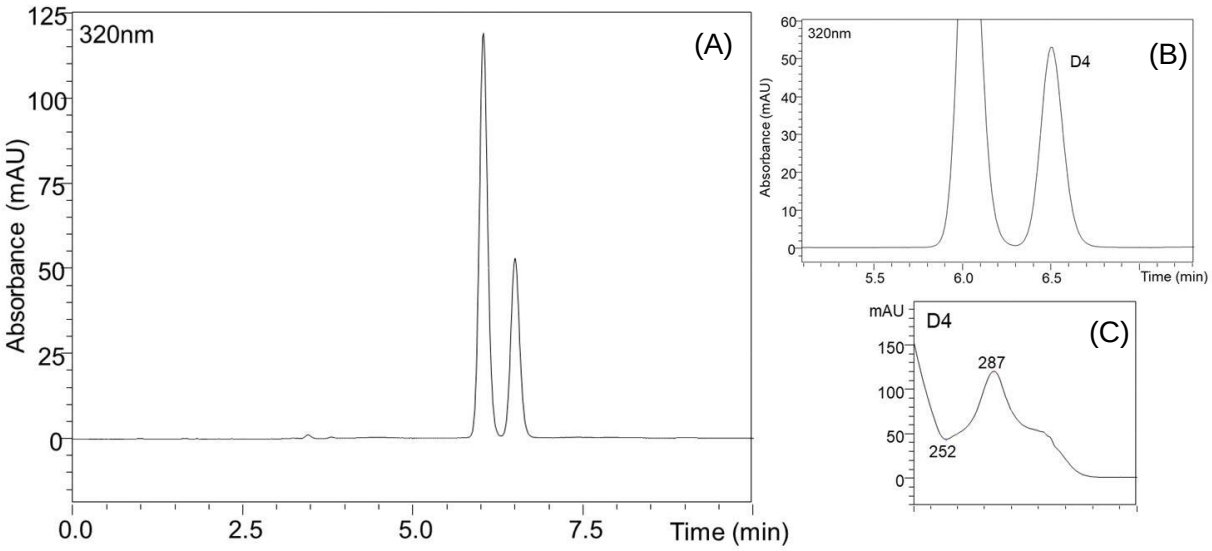


Figure 4: (A) HPLC chromatogram, detection at 320 nm (B) zoom on the chromatogram, and (C) UV spectrum of pterostilbene degraded after irradiation by UVA light (10 minutes) of standard.

3.2.4 Robustness

In validation studies, multivariate optimization techniques can be used for determination of robustness, which is defined as the capacity of an analytical method to reproduce results when the procedure is performed under small changes in the nominal values of the experimental factors previously established (FERREIRA et al., 2007). In this sense, the Box-Behnken design has been used due to its advantages over other methods, such as: be slightly more efficient than the central composite design but much more efficient than the three-level full factorial designs, have low cost, not contain combinations for which all factors are simultaneously at their highest or lowest levels, being useful in avoiding experiments performed under extreme conditions (FERREIRA et al., 2007). Therefore, the Box-Behnken design was used to evaluate not only whether changes in the parameters of the method can affect the result, but also to evaluate the effect of each independent variable (X_1, organic phase proportion in mobile phase; X_2, solvent flow rate; X_3, TFA concentration in mobile phase; X_4 wavelength detection) on the dependent variable (Y_1, peak area).

The value of the determination coefficient (R^2 = 0.92) indicates that the experimental data were well adjusted by the second-order polynomial model. According to the ANOVA of the regression model, **Table 4**, the linear terms for all independent variables were significant ($p < 0.05$).

Table 4: ANOVA from Box-Behnken design for response pterostilbene peak area.

Constant	Coefficient	(*f*-value)	(*p*-value)
X_1	12856	8.12	0.006
X_2	-79712	312.06	0.000
X_3	15827	12.30	0.001
X_4	79285	308.73	0.000
X_1^2	-3231	0.23	0.635
X_2^2	-5072	0.56	0.456
X_3^2	-42506	39.44	0.000
X_4^2	-28967	18.32	0.000
X_1 X_2	-2692	0.12	0.732
X_1 X_3	13185	2.85	0.096
X_1 X_4	-221	0.00	0.978
X_2 X_3	-770	0.01	0.922
X_2 X_4	-1919	0.06	0.807
X_3 X_4	461	0.00	0.953

Flow rate (X_2), and wavelength detection (X_4) are the most influential factors in the response (Y_1, peak area). The negative linear term of X_2 indicated an inverse relationship with Y_1, so faster flow rates (X_2) determine smaller the peak area (Y_1) **(Figures 5A, 5D and 5E).** In contrast, the positive linear term of X_4 indicated a direct relationship with Y_1, meaning that in the higher tested wavelength, the peak area is larger than in the lower tested wavelength, within range evaluated **(Figures 5C, 5D and 5F)**. Regarding the organic phase proportion in the mobile phase (X_1), its higher proportion resulted in higher peak area (Y_1), due to the positive linear term of X_1 **(Figures 5A, 5B and 5C).** Only the quadratic terms of X_3 (TFA concentration) and X_4 (wavelength detection) were significant ($p < 0.05$), but for X_4 the corresponding linear factor prevails (according to the value of *f*). With respect to X_3, the quadratic term is responsible for the curvature observed in the response surface graph. The positive linear term of X_3 indicated an increase of Y_1 (peak area), but the negative quadratic term revealed a nonlinear trend, indicating a decrease of Y_1 after a certain concentration of TFA **(Figures 5B, 5D and 5F).** Regarding the interaction terms between the variables, all were not significant ($p > 0.05$), indicating that the factors do not interfere with each other. Thus, changes in one factor do not affect the other factors, and hence the response (Y_1).

Despite the changes in the experimental parameters performed in the method, no significant differences related to the quantification of pterostilbene (RSD < 5.0%) were observed, as shown in **Table 5**, indicating the robustness of the method. Thus, although no significant differences had been observed in the pterostilbene peak area, it is noticed that changes in the parameters established for the method cause changes in the result. Therefore, one should caution with all variables of the method.

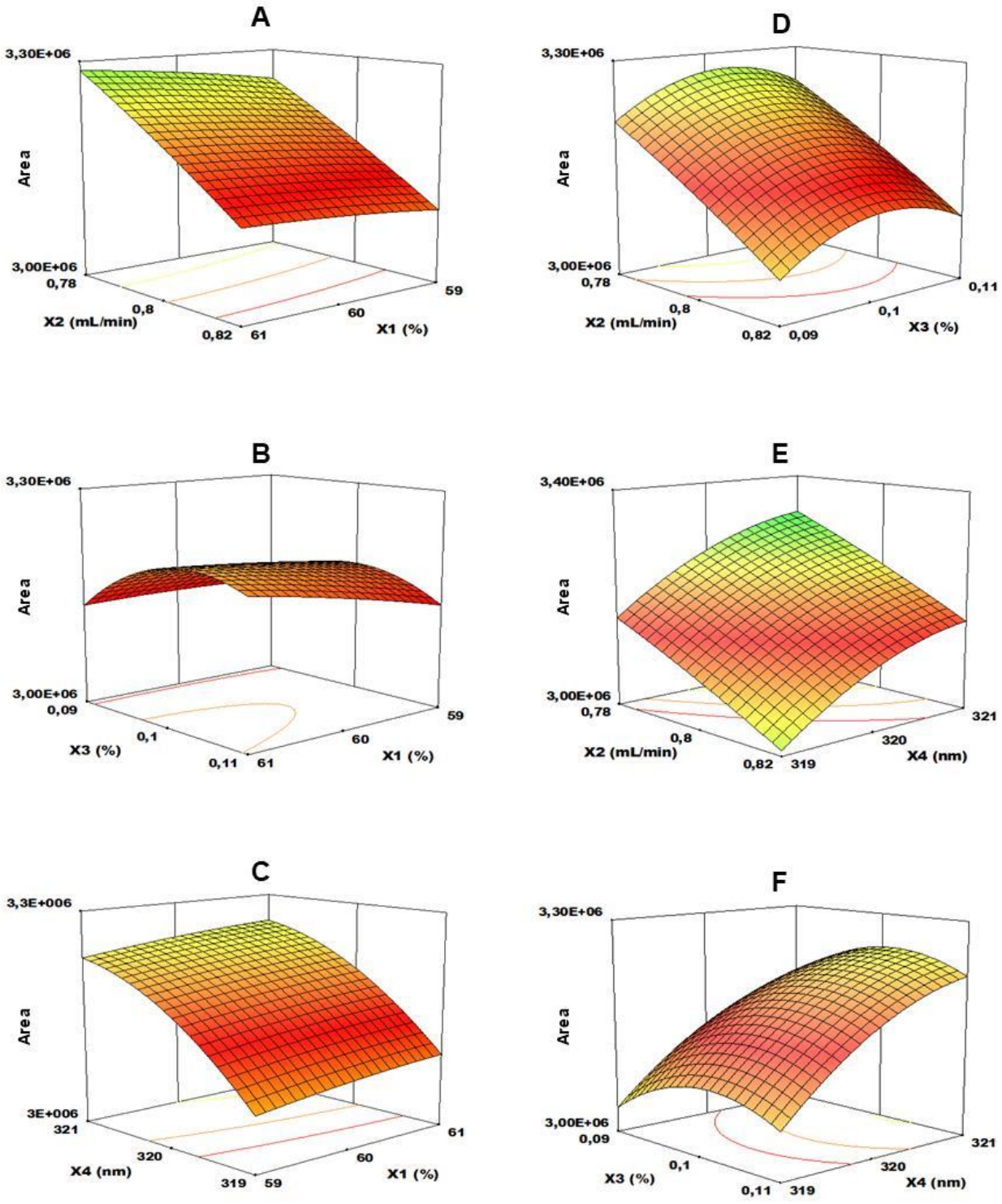


Figure 5: Response surface plots (three-dimensional) showing the effect of the organic composition (X_1; % v/v), flow rate (X_2; mL/minutes), trifluoroacetic acid concentration (X_3; %) and Wavelength (X_4; nm) on pterostilbene area (Y_1, mAU).

Table 5: Box-Behnken design arrangement and experimental responses for pterostilbene peak area.

Run	X_1	X_2	X_3	X_4	Y_1[a]
1	59	0,78	0,1	320	3252961 ± 0.39
2	61	0,78	0,1	320	3292481 ± 0.30
3	59	0,82	0,1	320	3095284 ± 0.28
4	61	0,82	0,1	320	3124037 ± 0.38
5	60	0,8	0,09	319	3029151 ± 2.55
6	60	0,8	0,11	319	3069366 ± 0.13
7	60	0,8	0,09	321	3185754 ± 2.52
8	60	0,8	0,11	321	3227813 ± 0.13
9	59	0,8	0,1	319	3069729 ± 0.32
10	61	0,8	0,1	319	3101184 ± 0.39
11	59	0,8	0,1	321	3228981 ± 0.32
12	61	0,8	0,1	321	3259553 ± 0.38
13	60	0,78	0,09	320	3208662 ± 2.74
14	60	0,82	0,09	320	3052129 ± 2.41
15	60	0,78	0,11	320	3248376 ± 0.04
16	60	0,82	0,11	320	3088762 ± 0.04
17	59	0,8	0,09	320	3158915 ± 0.48
18	61	0,8	0,09	320	3144529 ± 2.37
19	59	0,8	0,11	320	3148195 ± 0.16
20	61	0,8	0,11	320	3186549 ± 0.05
21	60	0,78	0,1	319	3168205 ± 0.45
22	60	0,82	0,1	319	3014906 ± 0.37
23	60	0,78	0,1	321	3331421 ± 0.44
24	60	0,82	0,1	321	3170444 ± 0.36
25	60	0,8	0,1	320	3202943 ± 0.39
26	60	0,8	0,1	320	3199879 ± 0.32
27	60	0,8	0,1	320	3199015 ± 0.35
Total mean	-	-	-	-	3165156 ± 2.69

[a]Mean ± relative standard deviation (%) of three determinations.

3.2.5 Stability

The stability of analytical samples showed that the concentration of pterostilbene remained constant after 48 hours of their storage at room temperature. The amounts of pterostilbene were within 102.8 % and 103.9 % range after this period of time as showed in **Table 3**.

3.2.6 System suitability

Routine analyses of the standard pterostilbene were performed under the developed experimental conditions. The parameters values and their relative standard deviation (RSD, %) for pterostilbene analysis (**Table 2**) were: 5.91 (1.13) minutes, 9594.85 (4.11), and 1.14 (0.34) for retention time, plates and tailing factor, respectively. These parameters indicated that the chromatographic system is suitable for the analysis. This short run time makes the chromatographic conditions suitable for the routine analysis of a large number of samples.

3.3 Method application

The HPLC method was applied to determine the pterostilbene amount in a dried blueberry extract, pterostilbene:β-cyclodextrin complexes, pterostilbene:β-cyclodextrin:hydrophilic polymers (PVP or HPMC) ternary systems. The results indicate the precision of the method in all samples, with a standard deviation lower than 5.0%.

The concentration of pterostilbene in the blueberry extract was 19.14 µg per gram of dried extract, with a relative standard deviation (RSD) of 1.04%, indicating adequate precision. The pterostilbene quantification in the complexes (pterostilbene:β-cyclodextrin) resulted in RSD of 4.48% (range of 12.25 - 13.35 µm/mL). Finally, in the pterostilbene:β-cyclodextrin:PVP ternary system the quantification resulted in RSD of 0.63% (range of 10.05 - 10.16 µm/mL) and in the pterostilbene:β-cyclodextrin:HPMC ternary system resulted in RSD of 3.47% (range of 10.09 - 10.81 µm/mL). The analysis performed using PDA detector showed the purity of the pterostilbene peak in the samples (> 0.999), as shown **Figure 6.**

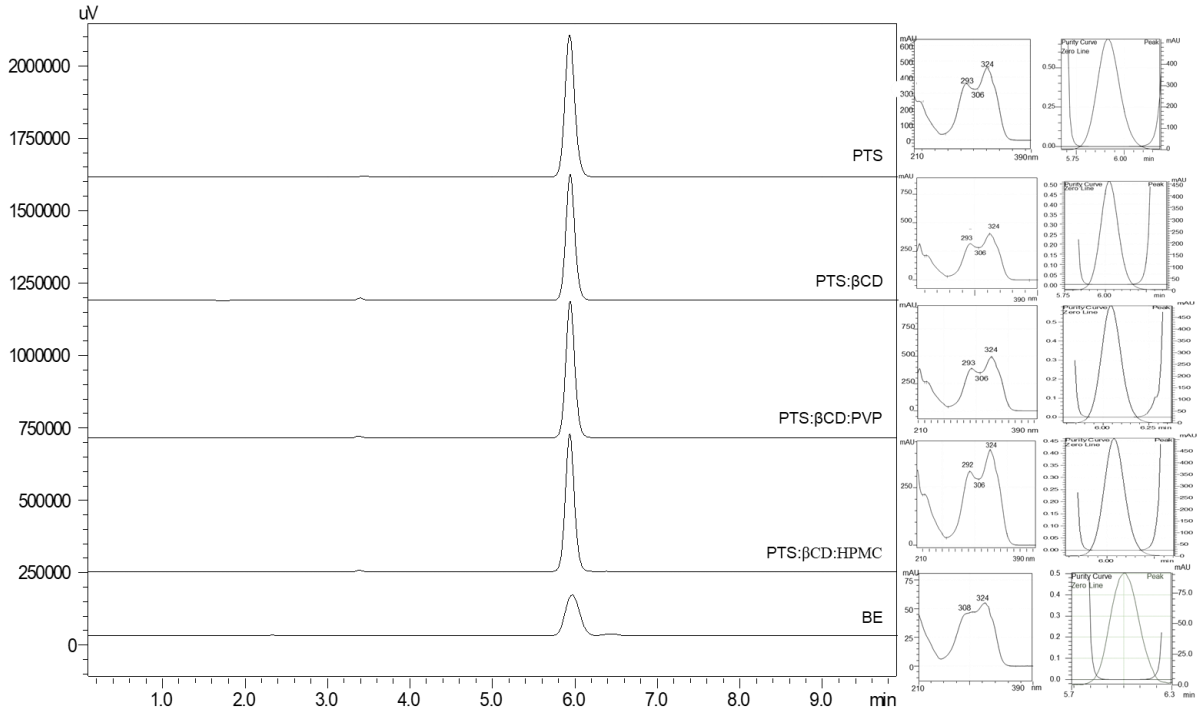


Figure 6: Chromatograms obtained by HPLC at 320 nm, purity curve and UV spectrum in pterostilbene reference solution at 10 µg/mL (PTS); pterostilbene:β-cyclodextrin complexes (PTS:βCD); pterostilbene:β-cyclodextrin:polyvinylpyrrolidone ternary systems (PTS:βCD:PVP); pterostilbene:β-cyclodextrin:hydroxypropyl methylcellulose ternary systems (PTS:βCD:HPMC) and blueberry extract (BE).

4 Conclusions

A specific-stability indicating analytical HPLC method for pterostilbene quantification was successfully developed and validated. The method revealed to be linear, specific, accurate, precise and robust according to official guidelines. The developed methodology was able to separate pterostilbene of other compounds coming from its degradation. Statistical tools were essential to verify the robustness of the method and to know chromatographic conditions that should be controlled during HPLC analysis. This investigation also demonstrated good stability of pterostilbene in acidic and alkaline media, and sensitivity of the molecule to hydrogen peroxide, irradiation of UVA light and high temperatures. Taken together the findings are highly relevant for quantitate pterostilbene in samples of further research studies or for the development of food, pharmaceutical or cosmetic products or extracts containing this biologically active constituent.

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CAPÍTULO III

Simultaneos use of hydrophilic polymer and cosolvent significantly reduces the amount of cyclodextrin in the pterostilbene solubilization.

Simultaneos use of hydrophilic polymer and cosolvent significantly reduces the amount of cyclodextrin in the pterostilbene solubilization.

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Abstract

The pterostilbene (PTS) is a natural phenolic compound found especially in blueberries (*Vaccinium* spp.) and grapes (*Vitis* spp). Several pharmacological actions have been reported for this stilbene, including antioxidant, anti-inflammatory, antitumor, neuroprotective, antidiabetic and cardioprotective activities. However, its low aqueous solubility and its physical and chemical unstability are the main limitations for its dissolution, bioavailability and for the development of pharmaceutical, food or cosmetic products. The present work was designed to improve the aqueous solubility of PTS by means of its complexation with β-cyclodextrin (βCD) and to investigate the use of two strategies for promoting the complexation efficiency and reducing the amount of cyclodextrin: (i) using hydrophilic polymers (Hydroxypropylmethylcellulose, HPMC, or polyvinylpyrrolydone, PVP) to obtain ternary systems and (ii) using a cosolvent (ethanol). The parameters involved in the formation of the binary complex PTS:βCD were firstly optimized with the use of the Box-Behnken design, and the influence of the cosolvent on the formation of the complex was verified. The complexes were characterized regarding their chemical and physical properties. According to the Box-Behnken design, temperature is the only factor studied that is statistically significant ($p < 0,05$) in the formation of binary complexes. Thus, the ideal conditions for complexation were 37°C, 2 hours stirring, 4 mM of PTS. The apparent stability constant (K_s = 2568.86 M^{-1}) of the PTS:βCD complex were determined using phase-solubility diagrams. The H^1 NMR analysis indicated the formation of inclusion complex between PTS and βCD, where PTS B-ring is inside of cyclodextrin cavity and the mass spectrometry revealed that the

stoichiometry of the complex PTS:βCD is predominantly 1:1. The PTS:βCD complexation showed to increase the apparent solubility of PTS by more than 10-fold using 4 mM βCD. When ternary systems composed of PTS:βCD:hydrophilic polymer were tested, the water solubility of PTS increased. The highest solubility increase was observed to PTS:βCD:HPMC ternary system (56-fold) reducing the ratio of PTS:βCD from 1:9 (without polymer) to 1:2. The cosolvent influence was evaluated carring out the obtaining of ternary systems in aqueous solution or hydroethanol solution followed by freeze drying. The use of ethanol as cosolvent in the complexation medium showed a positive influence on the formation of ternary systems, essentially improving the PTS content in the freeze dried powder more than 3 times when compared to the ternary system obtained in aqueous solution. This is the first report of obtaining ternary systems using a cosolvent, demonstrating that the simultaneous use of the two methods to improve complexation efficiency and reducing of the cyclodextrin amount can be successfully employed.

Key Words: Complexation; cosolvent; cyclodextrins; drug formulation; hydrophilic polymers; pterostilbene; ternary systems.

1. Introduction

Pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) **(Figure 1)**; is a phenolic compound belonging to the class of stilbenes, initially isolated from *Pterocarpus* spp and later identified in plant species such as blueberries (*Vaccinium* spp) and grapes (*Vitis* spp) (KOSURU et al., 2016; PENG et al., 2018; SILVA et al., 2014). Pterostilbene (PTS) has attracted the attention of researchers because of its numerous preventative and therapeutic properties against a wide range of human diseases. Among the biological activities already reported for the PTS are the antioxidant, anti-inflammatory, antitumor, hypolipidemic, neuroprotective, antidiabetic and cardioprotective activities (DOS SANTOS LACERDA et al., 2017; KOSURU et al., 2016; LACERDA et al., 2018; PENG et al., 2018).

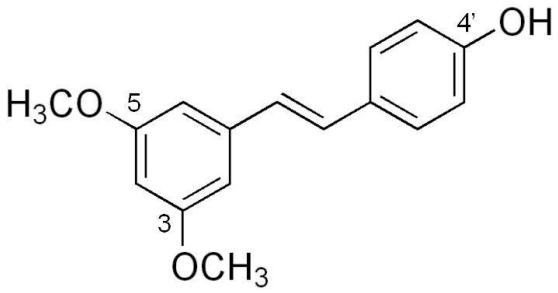

Figure 1: Chemical structure of pterostilbene.

Based on the physicochemical characteristics of PTS, such as moderate lipophilicity, few groups capable of hydrogen bonding, small polar surface area, few rotational bonds and molecular mass of 256,299 g/mol, high permeability in membranes can be predicted (KOSURU et al., 2016). However, the low aqueous solubility of PTS, as well as the phenolic group at the 4' position, the ideal target for the conjugative enzymes of phase II metabolism, and its sensitivity to external agents such as air, light and oxidative enzymes constitute the main limitations of PTS for formulation, dissolution in physiological media and bioavailability (BETHUNE; SCHULTHEISS; HENCK, 2011; YEO; HO; LIN, 2013).

Several technological strategies that improve the aqueous solubility and bioavailability of PTS have been reported in the literature. Among them are the formation of cocrystall (BETHUNE; SCHULTHEISS; HENCK, 2011), formation of micellar systems (SILVA et al., 2014), synthesis of prodrugs (AZZOLINI et al., 2017), and the formation of complexes with cyclodextrins (LACERDA et al., 2018; LÓPEZ-NICOLÁS et al., 2009; SILVA et al., 2014; YEO; HO; LIN, 2013; ZHANG et al., 2013).

These strategies are of potential interest to the pharmaceutical industry as well as to the cosmetics and food supplement industry.

Cyclodextrins are well known to be capable of forming inclusion complexes with lipophilic drugs in their hydrophobic cavity. Its hydrophilic outer surface is responsible for the aqueous solubility of the complexes (JANSOOK; OGAWA; LOFTSSON, 2018). The formation of an inclusion complex promotes several changes in the physicochemical properties of the drug, increasing its apparent aqueous solubility (SAOKHAM et al., 2018). The β-cyclodextrin (βCD) and its derivatives are widely reported in the literature and commonly found in pharmaceutical formulations available on the market (KURKOV; LOFTSSON, 2013; MIRANDA et al., 2011). The advantage of the βCD is that it is obtained industrially with high yield and quality, has low cost, and mainly has an internal cavity whose dimensions are excellent for incorporation of molecules, being suitable to complex drugs containing aromatic ring in its chemical structure (JAMBHEKAR; BREEN, 2016). In order to reduce the amount of cyclodextrin (CD) used, eliminating toxicity problems, reducing production costs, as well as making it possible to obtain solid or semi-solid pharmaceutical forms containing CDs, several strategies have been reported. Among them is the use of small amounts of water-soluble polymers and the use of cosolvent. The first one has been used as a promising strategy, forming ternary complexes with drug and CD, also called multicomponent systems, which are referred as increasing of the stability constant of the drug:CD complex, and consequently increases the efficiency of complexation (JAMBHEKAR; BREEN, 2016; LOFTSSON; BREWSTER, 2012). The use of cosolvent, has been recommended for increasing the intrinsic solubility (S_0) of the drug as well as for helping in the solubility of the CD, thus the more soluble molecules, the more molecules are available for complexation (DEL VALLE, 2004; KURKOV; LOFTSSON, 2013).

The objective of the present study was to promote the increasing in the aqueous solubility of PTS by complexation with βCD, optimizing the parameters involved in the formation of the binary complex with the use of Box-Behnken design, and the formation of ternary systems PTS:βCD:hydrophilic polymer, verifying the influence of a cosolvent on the obtaining of the systems and PTS solubility. The solvent will be eliminated by freeze drying and the PTS solubility will be measured

and compared among the binary and ternary systems, obtained in absence and presence of the cosolvent

2. Materials and methods

2.1 Materials

PTS reference substance (≥ 98% purity) was purchased from Changsha Organic Herb (Changha, China). βCD was kindly supplied by Roquette Frères (Lestrem, France). The hydrophilic polymers hydroxypropylmethylcellulose (Methocel E15LV) and polyvinylpyrrolidone K-30 were obtained from Colorcon (São Paulo, Brazil) and Henrifarma (São Paulo, Brazil) respectively. Acetonitrile (HPLC grade, Tedia, Fairfield, USA), trifluoracetic acid (Vetec, Rio de Janeiro, BRA) and water purified by Milli-Q water system (Millipore, Bedford, MA, USA) were used in the liquid chromatography analysis. All other reagents used were of analytical grade.

2.2 Liquid Chromatography analysis

The high performance liquid chromatography analyzes (HPLC) were performed using a Shimadzu HPLC-20A equipment (Kyoto, Japan) composed of HPLC-20AT pump, a SIL-20A autosampler and a SPD-20AV photodiode array detector. Data acquisition and treatment were performed with Shimadzu HPLC Solution GPC software (Shimadzu, Japan). The stationary phase employed was a C18 Phenomenex Gemini column (150 × 4.6 mm, i.d., 5 µm) guarded by a C18 pre-column (20 × 3.9 mm i.d., 10 mm) (Waters, USA). The mobile phase was composed of acetonitrile and water with 0.1% trifluoroacetic acid (60:40 v/v), filtered through a 0.45 µm pore size membrane filter and degassed for 30 minutes, eluted in isocratic flow. The flow rate, detection wavelength, injection volume and column temperature were, respectively, 0.8 mL.minutes^{-1}, 320 nm, 20 µL and room temperature. All the samples were filtered through Millipore PTFE membrane (0.45 µm of pore diameter) before injected. The method was validated over the concentration range of 1.0–20.0 µg.mL^{-1} according to ICH guidelines (ICH, 2005).

2.3 Box-Behnken design

A response surface methodology was applied to evaluate the effect of different experimental conditions and to optimize the formation of complexes between PTS and βCD. For this, a Box-Behnken design (BBD) with three different experimental

factors (X_1, X_2, X_3) at three levels (-1; 0; 1) (**Table 1**) was employed. The full design comprised 15 experiments with three replicates at central point to estimate the pure error, and carried out in randomized order. PTS solubility was the dependent variable expressed in µg/mL.

The assay was performed based on the phase-solubility study. Thus, excess amounts of PTS were added to 2 mL of aqueous solutions containing 2mM of βCD. Flasks were closed and protected from light. The resulting dispersions were magnetically stirred in temperature-controlled water bath. The different experimental conditions of PTS excess, temperature, and time were evaluated according to the experimental design (**Table 1**). Dispersions were then filtered through a 0.45 µm membrane (Millipore PTFE). Aliquots of the supernatant were diluted with acetonitrile:water (60:40 v/v) and PTS content was measured in duplicate by HPLC at 320 nm. The assay was performed with a reference stock solution of βCD at 4mM.

Experimental data were analyzed by multiple linear regression and the results were fitted using the following second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where, Y is the predicted response; X_1, X_2 and X_3 the independent variables; β_0 represent the constant; $\beta_{1..3}$, $\beta_{11...33}$ and $\beta_{12...23}$ are the linear, quadratic and interactive coefficients, respectively.

The least squares method and analysis of variance (ANOVA) were used to determine linear, quadratic and interaction coefficients, *p*-values less than 0.05 were considered significant. To optimize the parameters, equal weight and importance were adopted for independent variables while maximum solubility was desired for dependent variable. The optimized conditions were applied to phase-solubility study and to solubility study of the PTS and of PTS:βCD complex with hydrophilic polymers. Data analysis and optimization were calculated by the Minitab® 17.0.1 software and Design Expert®.

Table 1: Independent variables used in Box–Behnken design to investigate and optimize the main factors involved in the formation of the PTS:βCD complex. PTS = pterostilbene

	Factor	Uncoded level (coded level)		
	Independent Variables	**Low (-1)**	**Medium (0)**	**High (+1)**
X_1	Temperature of heating bath (°C)	25	31	37
X_2	Stirring time (hours)	2	25	48
X_3	PTS excess (mM)	4	6	8

2.4 Phase-solubility study

The phase-solubility study was performed according to Higuchi and Connors (HIGUCHI; CONNORS, 1965), however, following the parameters optimized by the experimental design. Excess amount of PTS (4mM) was added to 2 mL of aqueous solutions (fresh purified water) containing increasing concentrations of βCD (0.0 – 4.0 mM concentration range). Vials were closed and shaking for 2 hours, at the temperature of 37 ± 1°C using a magnetic stirrer and a temperature-controlled bath. The experiment was carried out protected from light to prevent degradation of PTS. After this time, the resulting dispersions were filtered through 0.45 µm PTFE membranes (Millipore). Aliquots of the supernatant were diluted when necessary and PTS content was measured in triplicate by HPLC.

The concentrations of PTS in the supernatant *versus* cyclodextrin concentrations were plotted and parameters such as apparent stability constant (K_s), complexation efficiency (CE) and molar ratio were calculated. The K_s of a drug:cyclodextrin complex represents the binding strength between the drug and the cyclodextrin. The K_s of PTS:βCD complex was calculated based on the phase-solubility diagrams according to the following equation (LOFTSSON; BREWSTER, 2010):

$$K_s\ (M^{-1}) = \frac{slope}{S_0 \text{ x } (1 - slope)}$$

where S_o is the intrinsic solubility of PTS in water (solubility of PTS in the absence of cyclodextrin).

A more reliable method for evaluating the solubilizing effect of cyclodextrins, for poorly-soluble drugs, is to determine their CE. For 1:1 drug:cyclodextrin complexes, CE is calculated from according to the following equation (LOFTSSON; BREWSTER, 2012):

$$CE = \frac{slope}{(1-slope)} \text{ or } CE = S_0 \text{ x } K_s$$

The molar ratio between PTS and βCD was calculated according to the following equation (LOFTSSON; BREWSTER, 2010):

$$Drug{:}\,CD = 1{:}\,(1 + \frac{1}{CE})$$

2.5 Ternary systems: pterostilbene:cyclodextrin:hydrophilic polymers

To obtain the ternary systems an excess of the PTS (4mM) was added to 2 mL of a solution containing 1% (w/w) hydrophilic polymer (hydroxypropylmethylcellulose E-15 (HPMC) or polyvinylpyrrolidone K-30 (PVP). The tests were carried out in triplicates, and stored in vials with cap sealed and protected from light. Increasing concentrations of βCD (0.0 - 4.0 mM) were added to these vials. The flasks were kept under stirring at 37 ± 1°C for 2 hours. The resulting dispersions were filtered (0.45 µm, PTFE, Millipore) and the PTS contents were determined in the supernatant by HPLC analysis.

The solubility of PTS in binary mixtures (PTS:βCD, PTS:PVP and PTS:HPMC) and ternary combinations (PTS:PVP:βCD and PTS:HPMC:βCD) were then determined. The K_s, CE and the molar ratio and the complexing efficiency calculated using the equations described in section Phase-solubility study following the method described by Loftsson (LOFTSSON; BREWSTER, 2012; LOFTSSON; HREINSDÓTTIR; MÁSSON, 2005).

2.6 Preparation of PTS:βCD solid complex and pterostilbene:βCD:HPMC ternary system

2.6.1 Preparation in aqueous media

To prepare the PTS:βCD solid complex an aqueous dispersion containing PTS:βCD at a molar ratio of 1:1 was stirred at 37 ± 1°C for 2 hours, protected from light, following the same conditions employed in the phase-solubility study. After this period, the dispersion was filtered through 0.45 µm PTFE membrane (Millipore, USA), and the supernatant was frozen at −18°C for 48 hours and then freeze dried 72 hours (Edwards EF4 Modulyo freeze dryer system).

For obtaining the ternary system, an aqueous dispersion containing PTS, βCD, and HPMC (1% w/w) was employed. The other conditions of the process were the same as that reported to PTS:βCD solid complex reported in this section, including the freeze drying step.

2.6.2 Preparation using ethanol as cosolvent

Accurately weighed amounts of PTS and βCD at the molar ratio of 1:1 were dissolved in a mixture of ethanol:water 40:60 (v/v) and stirred at 37 ± 1°C for 2 hours protected from light. The ethanol was evaporated under vacuum (Heidolph, Germany) and the collected dispersion was filtered through a 0.45 um PTFE membrane (Millipore, USA), frozen at −18°C for 48 hours and then dried 72 hours by lyophilization in an Edwards EF4 Modulyo freeze dryer (Edwards Modulyo, UK).

To obtain the ternary systems, the HPMC (1% w/w) was also added to the initial solution. The next steps of the method followed the technique described to obtain the PTS:βCD solid complex.

2.7 Physical mixtures preparation

PTS:βCD physical mixture was prepared for comparative purpose in a glass mortar. For comparison with the PTS:βCD solid complex, a physical mixture of PTS and βCD were accurately weighed at a molar ratio of 1:1 and carefully mixed for 30 minutes. For the comparison with the ternary system, a physical mixture containing PTS, βCD and HPMC were accurately weighed, PTS and βCD in a molar ratio of 1:1 and the last one at the ratio of 1% (w/w). The three components were then carefully mixed in a mortar for 30 minutes.

2.8 Characterization of PTS:βCD complex and PTS:βCD:hydrophilic polymer ternary systems.

2.8.1 Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) analysis of PTS, βCD, polymer, binary and ternary systems, as well as physical mixing were performed using a Shimadzu DSC-60 calorimeter. The samples were accurately weighed (approximately 2 mg) in aluminum pan and crimped. The operating conditions were 10°C.minutes^{-1} of heating rate (25°C to 350°C) and 50 mL.minutes^{-1} of nitrogen gas flow. The thermograms were evaluated by TA Analysis software using an empty sealed aluminum pan as reference.

2.8.2 Scanning electron microscopy

The photomicrographs obtained for PTS, βCD, polymer, binary and ternary systems, and physical mixing were taken at a voltage of 10 kV, using a Jeol JSM 6060 microscope (Tokyo, Japan). The samples were previously mounted on aluminum stubs using double-sided adhesive tape and vacuum-coated with a thin layer of gold.

2.8.3 *Fourier* transformed-infrared (FTIR) spectroscopy

Fourier transformed infrared spectra obtained for PTS, βCD, polymer, binary and ternary systems, and for physical mixing were recorded in scanning range between 4000–400 cm^{-1}, at a resolution of 4 cm^{-1}, using a Thermo Scientific Nicolet IS10 (United States) spectrometer equipped with ATR accessory.

2.8.4 Nuclear magnetic resonance spectroscopy

^1H nuclear magnetic resonance (^1H NMR) analysis for PTS, βCD and PTS:βCD complex were recorded on a Bruker ASCEND 400 spectrometer operating at 400 MHz, using D_2O as solvent, except for PTS, where CD_4O was used due to its low aqueous solubility. One-dimensional ^1H NMR spectra were acquired under standard conditions. Two-dimensional ^1H homonuclear 2D-ROESY spectra were obtained to get insights on the supramolecular geometry of the PTS:βCD complex.

2.8.5 Mass Spectrometry

For mass spectrometry (MS) the PTS, βCD and PTS:βCD complex were diluted in Milli-Q water and analyzed by direct injection in a Bruker micrOTOF-Q II spectrometer using an ESI (electrospray ionization source). The mass-to-charge ratio (m/z) data were processed and the results were analyzed using Bruker Daltonics softwares - Compass Data Analysis and Isotope Pattern.

2.8.6 Content of pterostilbene in the solid complex or ternary system

Approximately 2 mg of each sample of solid complex were dissolved, separately, in 25 mL of acetonitrile:water (60:40 v/v) in a glass vial. The glass vial was capped and attached to a wrist shaker in a water bath at 25 ± 1°C for 2 hours with agitation. The samples were filtered through a 0.45 µm membrane (Millipore PTFE), and the PTS content was determined in the supernatant using the previous mentioned by HPLC method at 320 nm. The test was carried out in triplicate.

2.9 Statistical analysis

A one-way analysis of variance (ANOVA) was used to evaluate the significance of the results obtained in the evaluation of the isolated or combined effect of cyclodextrins and hydrophilic polymers on the solubility of PTS. Multiple comparisons were then performed by Tukey's test for significance at *p*-values < 0.05.

3. Results and discussion

3.1 Box-Behnken design

A Box-Behnken design was employed to investigate and optimize variables that may affect the PTS:βCD complexation. Three factors were investigated (X_1: Temperature of heating bath; X_2: Stirring time; X_3: PTS excess) on the aqueous solubility of PTS (Y_1). The Box-Behnken design allows linear, quadratic and interaction analysis of variables.

Experimental data were adequately adjusted by the second-order polynomial model (R_2 > 0.88). Considering the complexity of the assay, where a constant dynamic equilibrium is established between the free and associated forms of the molecules, the R^2 value was satisfactory, so that the quadratic model was considered good and acceptable, with a random distribution of residuals (data not shown), without evidence of deviation from linearity (lack-of-fit > 0.05).

The analysis of ANOVA is presented in **Table 2**, and the results indicate that the linear term is significant ($p < 0.05$), whereas the quadratic and interaction terms are not ($p > 0.05$). Regression coefficient analysis showed that the linear term of X_1 (temperature) was significant ($p < 0.05$) and positive, indicating that an increase in temperature resulted in a linear increase in PTS concentration. This can be explained by the fact that higher temperatures increase the intrinsic solubility of the PTS, making it more available to be complexed. This variable proved to have a relevant role in the response variable (Y_1), considering that its *f* value was higher than the other variables. This result contrasts with that reported by López-Nicolás et al., who investigated the influence of temperature in the range of 15 to 37°C in the K_s of the PTS:HPβCD complex, and found that a lower degree of interaction between PTS and HPβCD (lower K_s) was obtained at the higher temperatures, 37°C (LÓPEZ-NICOLÁS et al., 2009). The different chemical structure of this cyclodextrin derivative (Hydroxypropyl-β-cyclodextrin, HPβCD) may be related to this different behavior.

Table 2: ANOVA from Box-Behnken design for response pterostilbene solubility.

Constant	Coefficient	(*f*-value)	(*p*-value)
X_1	10.753	133.55	0.000
X_2	0.066	0.01	0.944
X_3	1.454	2.44	0.135
X_1^2	-1.26	0.85	0.369
X_2^2	-0.96	0.49	0.494
X_3^2	-2.76	4.06	0.058
X_1 X_2	3.20	5.93	0.025
X_1 X_3	1.61	1.49	0.237
X_2 X_3	0.19	0.02	0.888

A linear profile can be observed in the response surface graphs (**Figure 2**). Although the response surface plots suggest an apparent influence of the terms X_2 and X_3 on Y_1, the non-significant linear and quadratic coefficients show the opposite ($p > 0.05$). The interaction effect between variables X_1 and X_2 was significant, but not substantially when compared to the linear term of X_1 (weighted the *f* value, which is small).

Based on these observations, the optimum conditions for the phase-solubility study and to solubility study of the PTS and of PTS:βCD complex with hydrophilic polymers were determined, aiming at a maximum increase in PTS solubilization. Thus, considering that time and excess have no significant influence on the response of interest, due to practical and economic feasibility, the assay was performed using temperature of 37°C, PTS excess of 4 mM and stirring time of 2 hours. The calculated composite desirability index (D) was greater than 0.94.

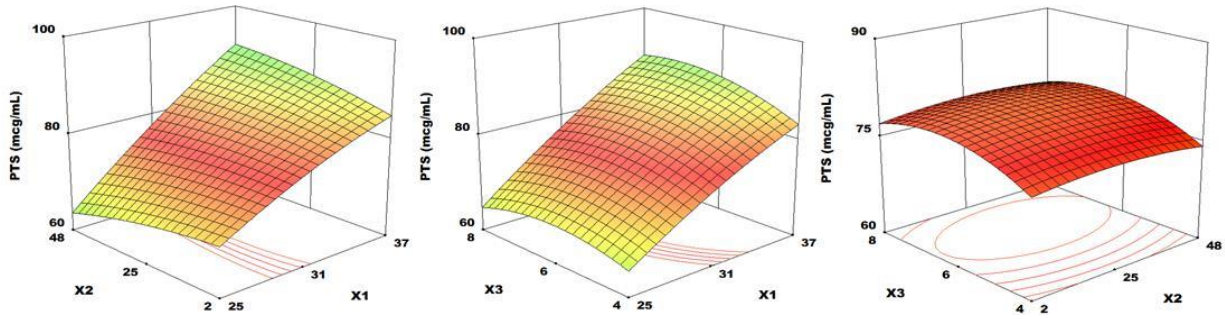

Figure 2: 3D response surface plot showing the effects of (X_1) temperature of heating bath (°C), (X_2) stirring time (hours) and (X_3) PTS concentration (mM) on PTS solubility in the complex formed with 2mM of βCD (µg/mL) (Y_1).

3.2 Phase-solubility study

The phase-solubility diagram obtained for the PTS:βCD complex **(Figure 3)** showed a linear relationship in increase of the solubility of PTS and βCD concentration (R^2 > 0.999). According to Higuchi and Connors, the curve obtained can be classified as A_L type profile, and that the association is first order (HIGUCHI; CONNORS, 1965).

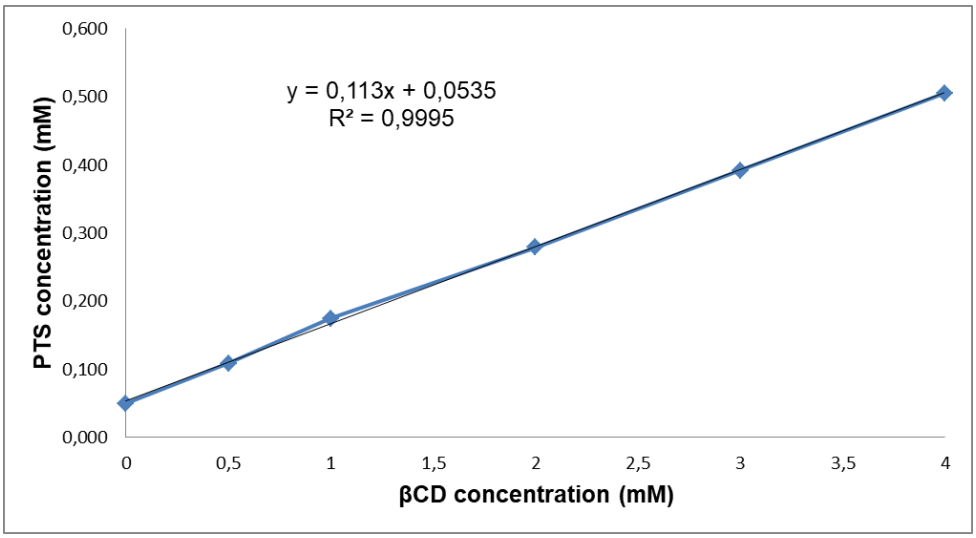

Figure 3: Phase-solubility diagram of PTS:βCD at 37.0 ± 1°C (PTS = pterostilbene; βCD = β-cyclodextrin)

The intrinsic solubility (S_0) of PTS in water (at 37°C ± 1°C) was 12.7 μg.mL^{-1}. The PTS aqueous solubility was increased by 10-fold when associated to βCB in a molar ratio of 1:1. The K_s of a drug:cyclodextrin complex represents the binding strength between the drug and cyclodextrin and has an important influence on the extent of drug release (STELLA et al., 1999). The K_s determined for PTS:βCD complex was 2569 M^{-1} suggesting a moderate interaction between PTS and βCD (CARRIER; MILLER; AHMED, 2007). Considering that the K_s value is more frequent between 50 and 2000 M^{-1} for the natural CDs, with an average value of 130, 490 and 350 M^{-1} for αCD, βCD and γCD, respectively (BREWSTER; LOFTSSON, 2007), an excellent K_s for PTS and βCD was obtained. The CE value observed for the association was of 0.127, indicating a high solubilizing power of the βCD. Based on the CE values, the complex to free cyclodextrin concentration ratios were determined: one out of every 9 cyclodextrin molecules is able to form a water-soluble complex with PTS in the medium. Thus for each PTS molecule (MM= 256.30 g/mol) it is necessary that nine βCD molecules are present (9 mols x 1134.98 g/mol =

10214.82 g) to obtain a molecule of PTS:βCD complex. This observation denotes the relevance to use strategies for of increasing the EC.

3.3 Solubility study of the pterostilbene and of pterostilbene:cyclodextrin complex with hydrophilic polymers

The effect of the hydrophilic polymers, HPMC or PVP, on the PTS solubility was also tested. A significant increase in the aqueous solubility of the PTS was observed for both polymers compared to the PTS intrinsic solubility (S_0), being this effect more pronounced when HPMC was used **(Figure 4).** It is worth mentioning that the polymers provided a PTS solubilizing effect superior to that observed to CD alone in the complexes PTS:CD (10-fold). The binary system HPMC:PTS increased 48-fold the PTS solubility, while the PTS:PVP binary system demonstrated an increment of only 15-fold.

The ternary systems PTS:βCD:hydrophilic polymer determined the highest increases in the water solubility of PTS, comparatively to both binary system (PTS:polymer or PTS:βCD), revealing an additive effect. The highest solubility increase was observed for the ternary system obtained with the association PTS, βCD and HPMC (56-fold).

The nature of the interaction and the chemical structure resulting from the interaction between drug, CD and hydrophilic polymers is still little known, but it is recognized that in aqueous solutions, the polymers stabilize micelles and other types of aggregates, reduce the mobility of the complexes, and increase solubility of the same, altering the hydration properties of the CD molecules (MIRANDA et al., 2011). The polymers are capable of increasing the K_s and by consequence, the CE of the complexes (JAMBHEKAR; BREEN, 2016). **Table 3** shows the values of K_s, CE and molar ratio of ternary systems. It is found that with both polymers significantly the K_s and CE values were higher than that observed to binary system as well as the PTS:βCD molar ratio, required to form a complex, is significantly smaller. This effect showed to be more pronounced with the use of HPMC in comparison to PVP. Even the binary system containing HPMC (PTS:HPMC), improved the water solubility of the PTS more than the ternary system PTS:βCD:PVP, denoting that among the tested adjuvants, the HPMC is the most important adjuvant for improving PTS solubility, being selected for further tests.

. It is not known how exactly these ternary systems influence the PTS water solubility and how it is related to the physico-chemical characteristics of the polymers. However, there are reports in the literature demonstrating that PVP is able to form clusters (COSENTINO et al., 2019), whereas HPMC is a matrix-forming polymer denoting that they are differently organized (JOSHI, 2011).

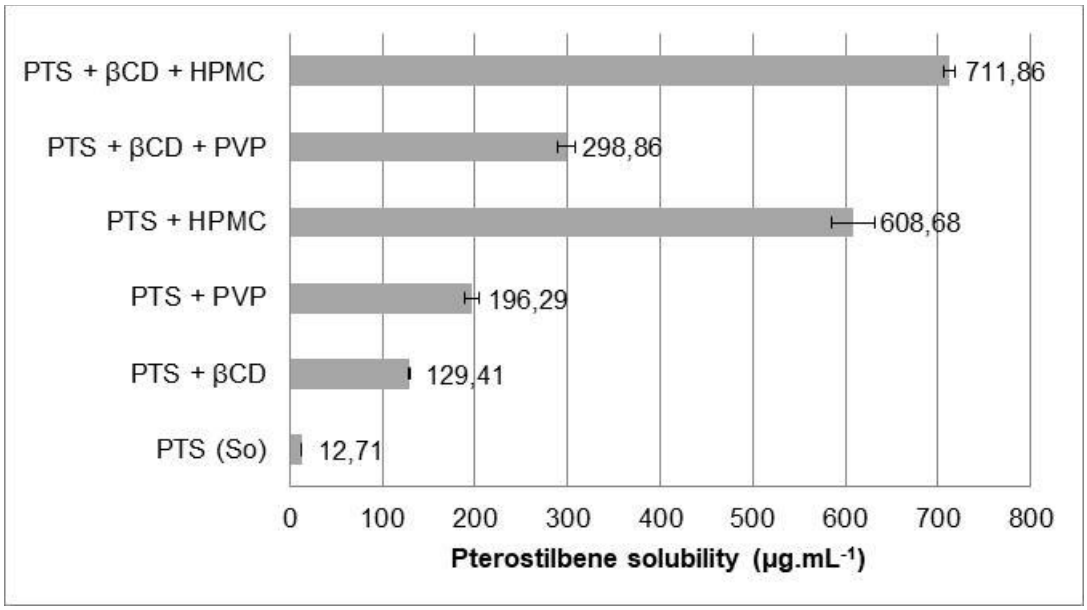

Figure 4: Aqueous solubility of PTS in the presence or absence of hydrophilic polymers (1% w/w) and βCD (4mM). Were, βCD = β-cyclodextrin; PVP = polyvinylpyrrolidone; HPMC = hydroxypropylmethylcellulose; S_0 = intrinsic solubility.

Table 3: Effect of β-cyclodextrin (4mM) or β-cyclodextrin combined to hydrophilic polymers (1.0% w/w) on the apparent stability constant (K_s), complexation efficiency (CE), molar ration (PTS:CD) and aqueous solubility of PTS.

Complex/ ternary system	K_s (M^{-1})	CE	Molar ratio (PTS:βCD)	Solubility increase (fold)*
PTS:βCD	2568.86	0.127	1:9	10
PTS:βCD:PVP	5148.68	0.255	1:5	24
PTS:βCD:HPMC	18479.54	0.916	1:2	56

* calculated based on the intrinsic solubility of PTS (12,71 µg.mL-1)
PVP = polyvinylpyrrolydone. HPMC = Hydroxypropylmethylcellulose
K_s = apparent stability constant CE = complexation efficiency

3.4 Characterization of PTS:βCD complexes and PTS:βCD:hydrophilic polymer ternary systems

3.4.1 Differential scanning calorimetry

The presence of interactions in the formation of complexes or associations can be observed in the profiles of thermograms, from the enthalpy changes and disappearance of endothermic and exothermic peaks, comparing the free and associated molecules (FIGUEIRAS et al., 2007). DSC thermograms obtained for pure PTS, βCD, HPMC, the combinations among these compounds binary and ternary systems as well as complexes and the corresponding physical mixtures are shown in **Figure 5.**

The thermogram obtained for PTS (**Figure 5a**) exhibited a unique sharp endothermic peak at 94.38°C, which corresponds to PTS melting point. In the βCD thermogram (**Figure 5b**) a broad endothermic band is observed at around 100°C, corresponding to its dehydration, without observing endothermic melting peaks, corroborating the amorphous structure of βCD. The thermogram observed for the HPMC polymer (**Figure 5c**), presents only a broad and smooth endothermic range before 100°C, which may correspond to its dehydratation.

For the PTS:βCD physical mixture (**Figure 5d**), a superposition of the thermograms of both components can be observed, but the intensity of the peak corresponding to the PTS melting point is drastically reduced, but well defined even the low amount of PTS in the physical mixture. The same is observed in the PTS:βCD:HPMC physical mixture (**Figure 5g**), but in a more discrete peaks. For the binary complexes obtained in aqueous media (**Figure 5e**) or using ethanol as cosolvent (hydroethanol solution) (**Figure 5f**), as well as for the ternary systems obtained in aqueous media (**Figure 5h**) or in hydroethanol solution (**Figure 5i**) the complete disappearance of the PTS melting peak is observed, indicating interaction among them. It is found that above 300°C occurs the decomposition of the materials.

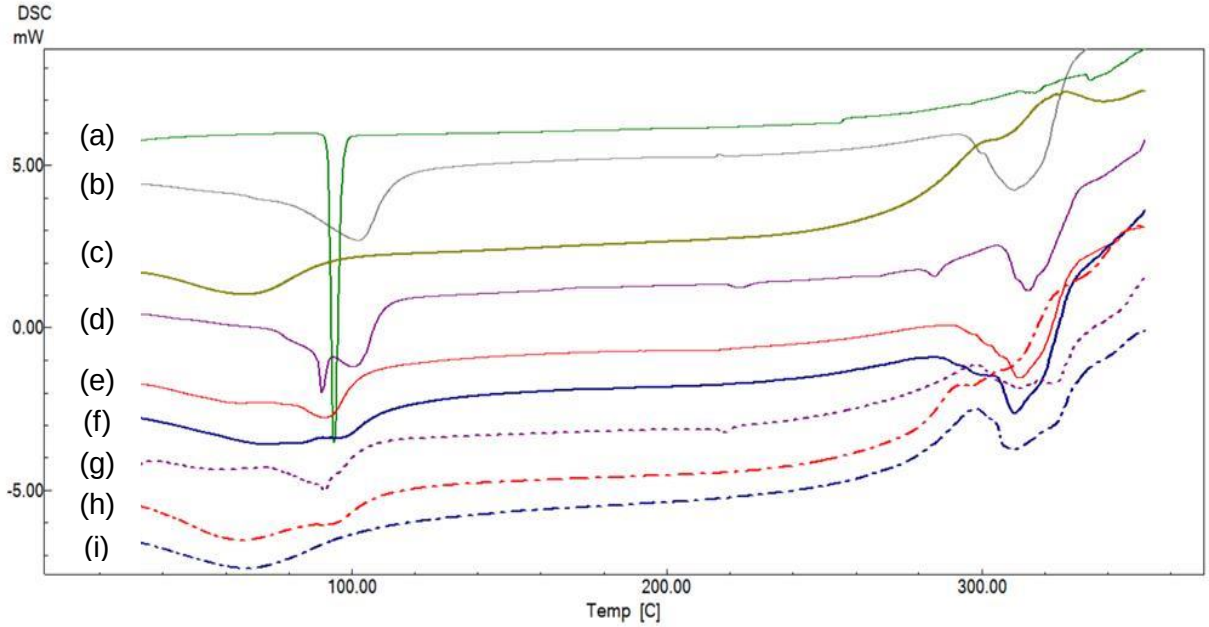

Figure 5: Differential scanning calorimetry analysis. Curves of (a) PTS, (b) βCD, (c) HPMC, (d) PTS:βCD physical mixture, (e) PTS:βCD complex obtained in aqueous solution, (f) PTS:βCD complex obtained in hydroethanol solution, (g) PTS:βCD:HPMC physical mixture, (h) PTS:βCD:HPMC complex obtained in aqueous solution and (i) PTS:βCD:HPMC complex obtained in hydroethanol solution.

3.4.2 Scanning electron microscopy

The photomicrographs obtained for raw material (PTS, βCD and HPMC) and the corresponding binary and ternary solid systems are reported in **Figure 6**. PTS particles **(Figure 6A)** are characterized by regular shaped crystals, presenting as columnar particles and of smooth surface. βCD **(Figure 6B)** is composed of particles with amorphous character with some irregularities in surface, whereas HPMC particles **(Figure 6C)** were larger long, with rough surface. In the physical mixtures **(Figure 6D and 6E)**, the particles of each component are not clearly distinguishable from each other, but it is possible to verify that the particles are adhered to each other on their surfaces; thus presenting an appearance of irregular amorphous agglomeration. However, the solid PTS:βCD associations **(Figure 6F and 6G)** were completely different from pure PTS or βCD. In these binary complexes there is a drastic change in the shape and appearance of the particles, presenting as a solid of rhombic shape, with smooth and non-porous surface. In the complex obtained using ethanol as cosolvent, more homogeneous crystalline structures are observed than those observed in the complex obtained in aqueous solution, demonstrating the

influence of the cosolvent for to increase the intrinsic solubility before complexation. The micrographs of the ternary complexes **(Figure 6H and 6I)** show amorphous and irregular structures, with filamentous appearance. In these complexes the individual components are no longer distinguishable each from other.

The SEM technique is not conclusive, however, the microphotographs obtained for both binary and ternary complexes show that there is interaction between the elements and support the idea of obtaining a new product (FIGUEIRAS et al., 2007).

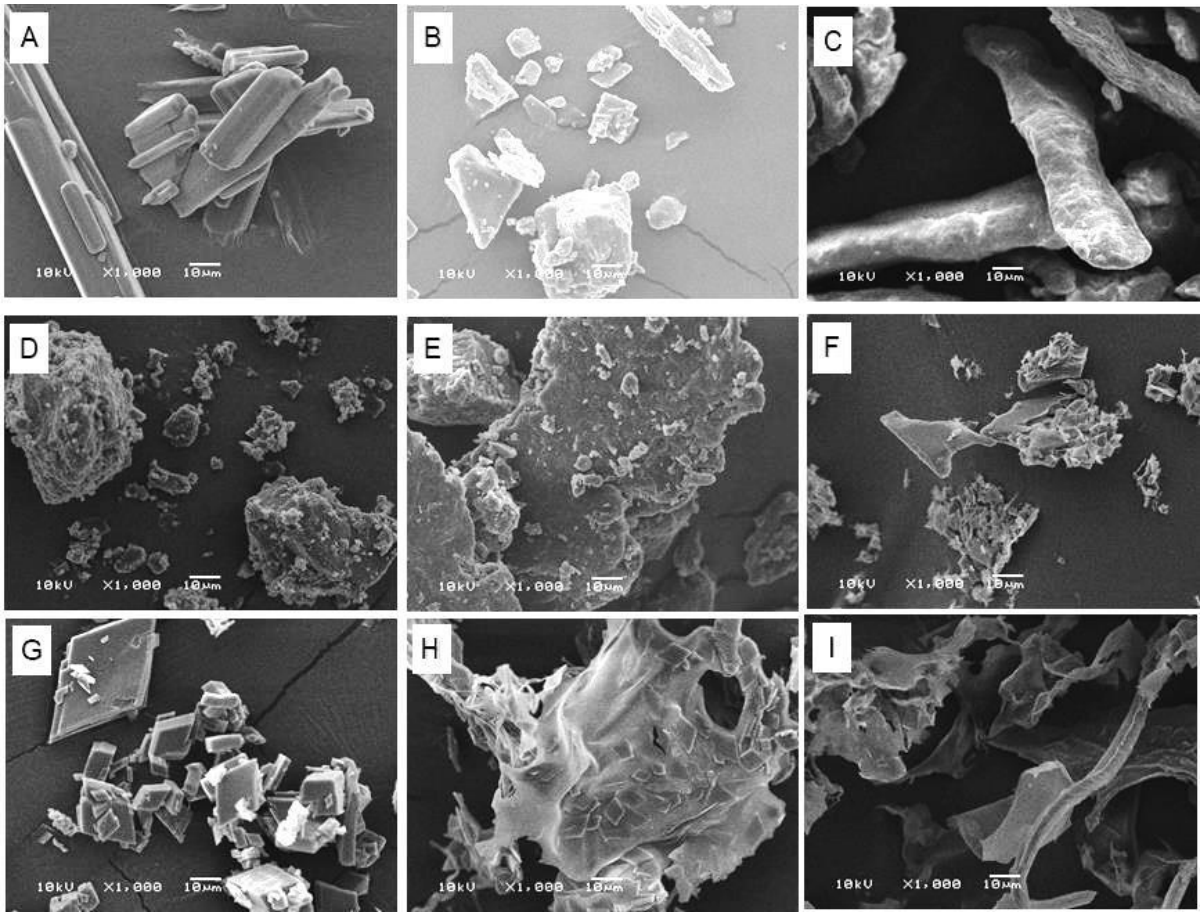

Figure 6: Photomicrographs obtained by scanning electron microscopy of (A) PTS, (B) βCD, (C) HPMC, (D) PTS/βCD physical mixture, (E) PTS/βCD/HPMC physical mixture (F) PTS/βCD solid complex obtained in aqueous solution, (G) PTS/βCD solid complex obtained in hydroethanol solution, (H) PTS/βCD/HPMC solid complex obtained in aqueous solution and (I) PTS/βCD/HPMC solid complex obtained in hydroethanol solution.

3.4.3 *Fourier* transformed-infrared (FTIR) spectroscopy

The FTIR analysis provides useful information regarding interactions between different functional groups of the molecules, allowing to investigate the functional groups that are involved in the complexation with cyclodextrins. **Figure 7** shows the FTIR spectra by the pure substances, physical mixtures and PTS:βCD and PTS:βCD:HPMC associations.

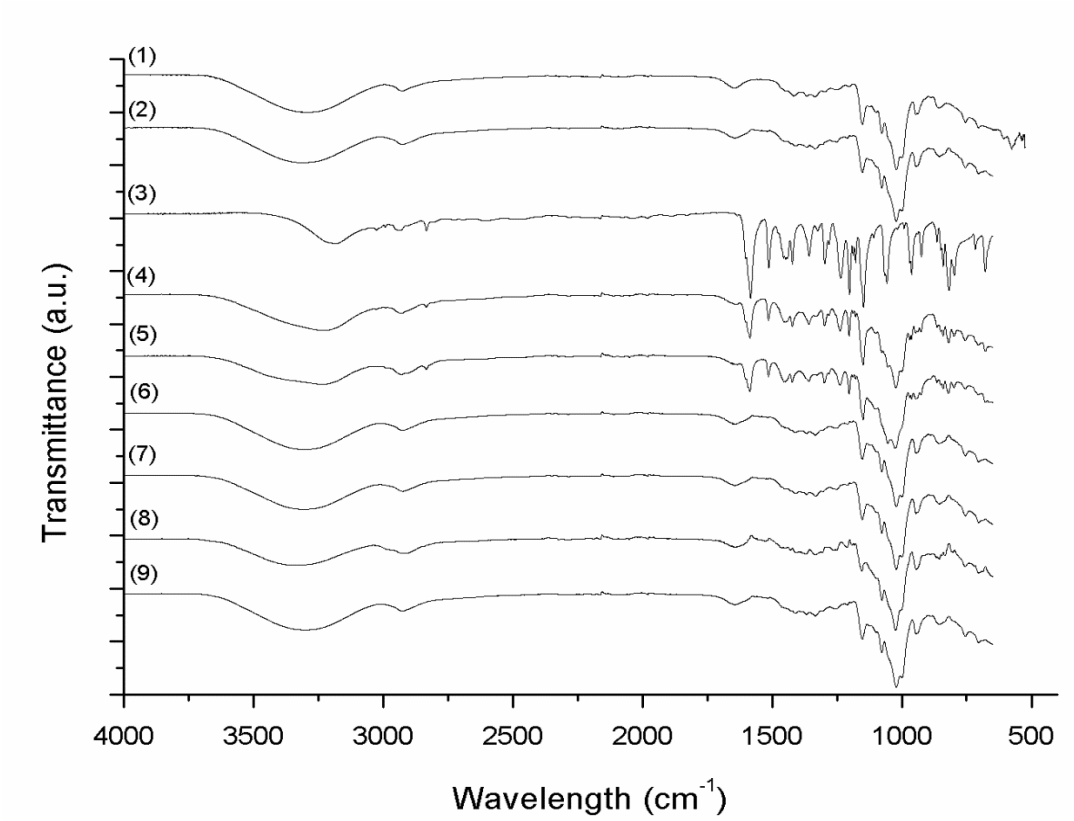

Figure 7: FTIR spectra of (1) βCD; (2) HPMC; (3) PTS; (4) PTS:βCD physical mixture; (5) PTS:βCD:HPMC physical mixture; (6) PTS:βCD association in aqueos solution; (7) PTS:βCD association in hydroethanol solution; (8) PTS:βCD:HPMC in aqueos solution and (9) PTS:βCD:HPMC in hydroethanol solution.

The FTIR spectrum obtained for PTS exhibited characteristic bands at 3200 cm^{-1} (O-H stretching), 3000–2830 cm^{-1} (C-H stretching of aromatic groups), 1585; 1510 and 1450 cm^{-1} (C-C aromatic double bond), and 817 cm^{-1} (C-H stretching) as observed by Silva et al. and Lacerda et al. (LACERDA et al., 2018; SILVA et al., 2014) A few more bands were observed, which may be related to the substitution position in the benzene rings (meta and para substituted), in addition to a band at 1296 cm^{-1} (C-O phenolic) and at 1240 cm^{-1} (C-O aromatic ether). FTIR spectra of both βCD and HPMC showed a large band in the around region 3300 cm^{-1} (O-H

stretching), a short band in 2925 cm^{-1} (C-H stretching), and a large band, which presents distinct peaks in the region of 900–1200 cm^{-1} (BORGHETTI et al., 2009; KOESTER et al., 2003).

In the FTIR spectra obtained for the binary and ternary associations by physical mixing, characteristic bands of the PTS overlapped with the bands of βCD, and HPMC in ternary associations, suggesting no interaction between the components. The FTIR spectra of the binary and ternary complexes were compared to the physical mixtures and pure raw materials. In the spectra of the PTS:βCD and PTS:βCD:HPMC associations, the bands corresponding to the cyclodextrin or polymer are predominantly observed, indicating substantial masking of the absorption bands of PTS. In fact, when the formation of inclusion complexes occurs, the guest molecule becomes protected by the host molecule, and the final structure is almost completely rearranged with a configuration in macrocycle (PINHO et al., 2014). As there is the presence of excess βCD there is a need of using of other techniques for more conclusive results regarding the PTS:βCD and PTS:βCD:HPMC interactions in the complex.

3.4.4 Nuclear magnetic resonance spectroscopy

1H-NMR technique has been one of the most important tool for enlighten the interactions between host and guests in cyclodextrin complexation. One-dimensional ^{1}H NMR spectra obtained for the PTS, βCD and PTS:βCD solid complex are shown in **Figure 8**. The βCD spectrum presented a characteristic profile, with signals corresponding to the hydrogens of the cavity, H3 and H5, respectively, at 3.87 to 3.76 ppm. In the spectrum of PTS, its characteristic signals are also present, hydrogens H5'/3' (6,79 ppm), H6'/2' (7.39 ppm) and methoxyl (3.80 ppm). 1HNMR spectrum obtained for PTS:βCD complex (**Figure 8C**) presented hydrogen signals of PTS and βCD. It has been observed some changes in chemical shift of some peaks, mainly of PTS, this may be related to both the use of different solvents used in the analysis and the formation of complexes. The data are in agreement with the reported in the literature, for both βCD and PTS (LACERDA et al., 2018; MEDEIROS, 2015)

The **Figure 9** shows ^{1}H homonuclear 2D-ROESY contour maps of the PTS:βCD complex. The expansion of the interaction region in the spectrum of PTS:βCD complex reveals intermolecular cross-peaks between the hydrogen H5'/3' (6.67 ppm)

of PTS and the hydrogen H5 (3.80 ppm) and H2 (ppm) of βCD. Considering that the hydrogens 3 and 5 of βCD are present within their cavity, this interaction between the phenol moiety of PTS and the hydrogen H5 located inside of the cyclodextrin cavity demonstrates that the PTS was included in the βCD cavity.

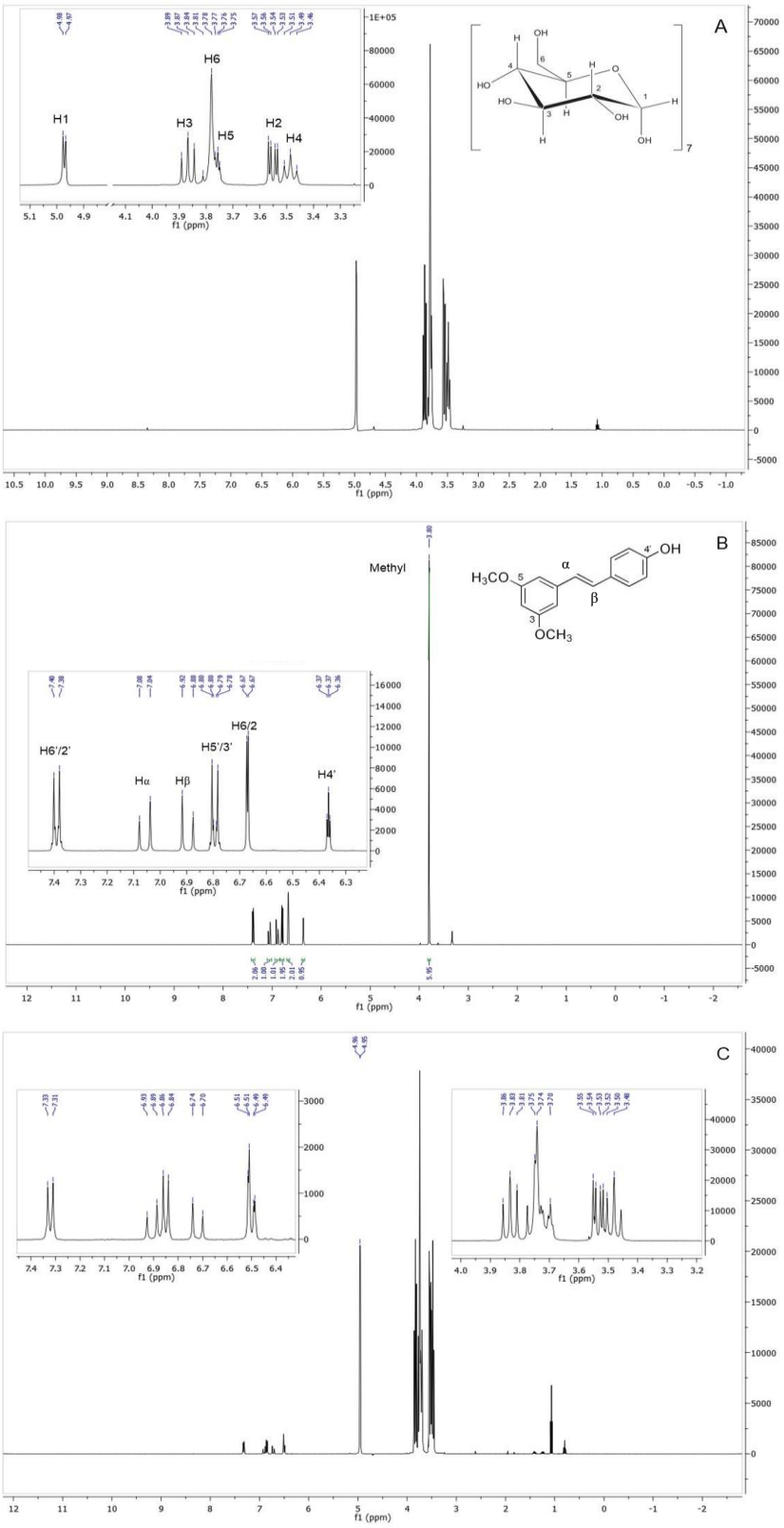

Figure 8: One-dimensional ^{1}H NMR spectra of the (A) βCD (B) PTS and (C) PTS:βCD complex.

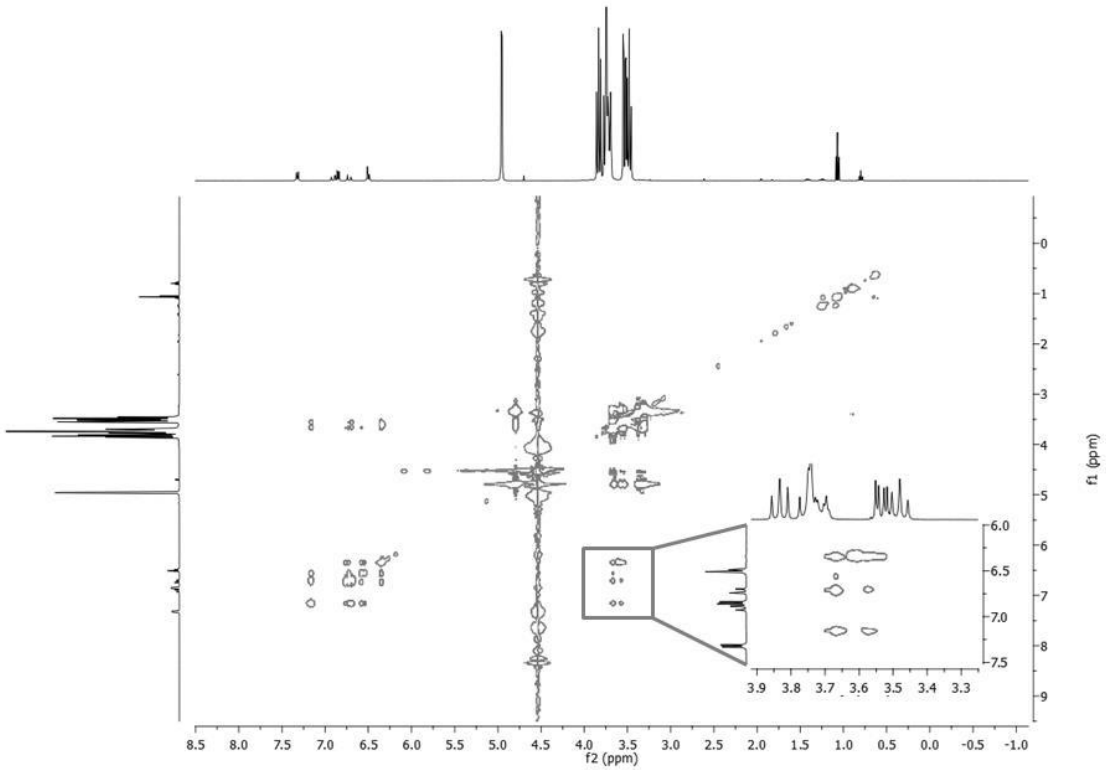

Figure 9: ^{1}H homonuclear 2D-ROESY contour map containing PTS:βCD complex (400 MHz, D2O).

3.4.5 Mass Spectrometry

The 1:1 stoichiometric ratio for PTS:βCD what was preliminarly observed in the the phase-solubility study was confirmed by ESI–MS. The signal at m/z 718.2308 **(Figure 10)** correspond to the 1:1 complex of PTS with βCD. The MS spectrum of the complex shows a signal presenting the highest intensity for a 1:1 molar ratio, followed by a 1:2 molar ratio signal **(Table 4)**. These results corroborate that complexes with different stoichiometries can be formed and coexist in solution, predominating those with lower energy formation and higher stability (DE SOUSA et al., 2008; FRANCO et al., 2009).

Table 4: Spectral data of ESI-MS obtained for PTS:βCD complex.

Complex stoichiometry	Detected *m/z* (u)	Molecular formula	Calculated mass (u)	Experimental error (ppm)
PTS:βCD 1:1	718.2308	$[M+2Na]^{+2}$	718.2290	2.50
	718.7347		718.7307	5.56
	719.2342		719.2321	2.91
	719.7332		719.7335	-0,41
	720.2382		720.2348	4.72

PTS:βCD 1:2	864.6100	$[M+3Na]^{+3}$	864.6057	4.97
	864.9496		864.9401	10.98
	865.2799		865.2744	6.35
	865.6166		865.6087	9.12
	865.9518		865.9430	10.16

βCD = β-cyclodextrin; PTS = pterostilbene; M = Molecular formula (βCD = $C_{42}H_{70}O_{35}$; PTS = $C_{16}H_{16}O_{3)}$

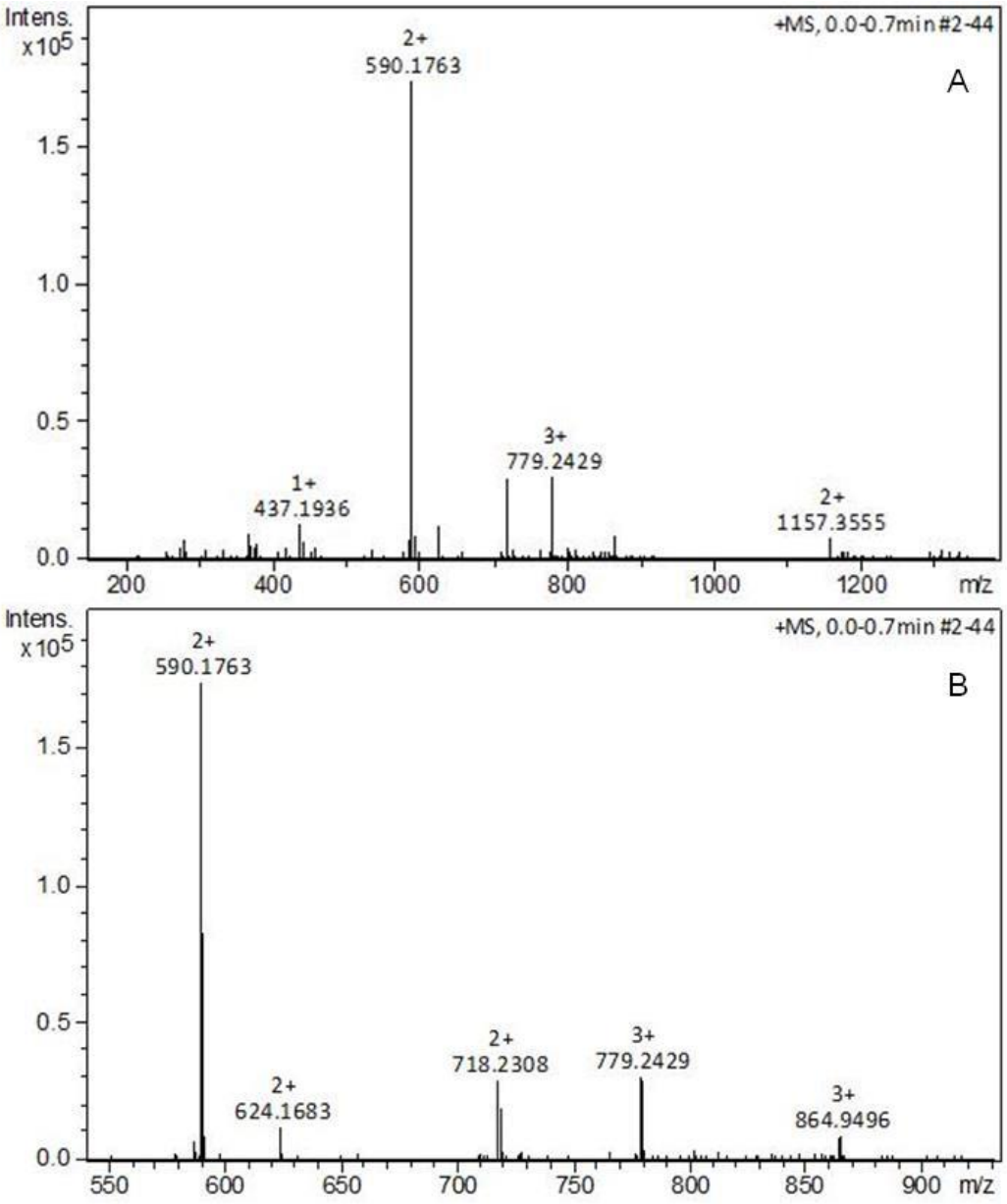

Figure 10: Mass spectrum obtained by ESI-MS for (A) PTS: βCD complex; (B) Zoom spectrum mass.

3.4.6 Content of pterostilbene in the solid complex or ternary system

Once the solid complexes presented strong evidences about the formation of inclusion complexes, the content of PTS in the binary and ternary solid complexes was evaluated **(Table 5).**

Table 5: Content of PTS in the solid complexes**.**

Solid complex (freeze dried powder)	PTS content (mg/g) ± RSD (%)	
	Complexation medium	
	Aqueous	Hydroethanol
PTS:βCD	36.20 ± 1.90	40.60 ± 3.17
PTS:βCD:HPMC	34.78 ± 4.18	116.65 ± 1.40

PTS = pterostilbene; βCD = β-cyclodextrin; HPMC = hydroxypropylmethylcellulose; RSD = relative standard deviation (%)

The ternary system PTS:βCD:HPMC obtained using ethanol as cosolvent presented a statistically significant difference ($p < 0.05$) in relation to the PTS content in all the other systems presented in **Table 5**. The other complexes showed similar PTS content. The higher PTS content in this system can be explained by the combined use of two methods that increase the CE of the complex formed (KURKOV; LOFTSSON, 2013): the use of ethanol as cosolvent and HPMC as hydrophilic polymer which promote the solubilization of the components for complexes formation. Ethanol favors the solubilization of both CD and non-water soluble drugs in water, so more soluble molecules are in the medium, more molecules are available for complexation (DEL VALLE, 2004). As PTS showed an aqueous solubility of only 12.71 µg/mL and βCD also has limited aqueous solubility (18.5 mg/mL) (JANSOOK; OGAWA; LOFTSSON, 2018), the use of an organic solvent as cosolvent was used to increase its S_0 . The hydrophilic polymer HPMC showed its effect of increasing K_s and S_0. Consequently both, HPMC and ethanol were able to significantly improve CE and reduce the CD amount in the PTS solubilization.

4. Conclusions

This study demonstrated for the first time the influence of operating conditions on PTS:βCD complexation using a Box-Behnken design. The best complexation

conditions were found to be 37°C temperature, 2 hours stirring and 4mM PTS excess. The the phase-solubility diagram can be classified as A_L type, indicating the predominant formation of a 1:1 PTS:βCD complex, what was corroborate by MS analysis. The characterization of solid complexes and the corresponding physical mixture suggest that PTS interacts with βCD by the formation of inclusion complexes. Moreover, the conjunction of the two strategies to improve the complexation efficiency, addition of hydrophilic polymer and the use of a cosolvent resulted in an excellent solubilizing effect of the PTS. Using HPMC and ethanol to obtain the ternary system, both, K_s and S_0 were improved and consequently the complexation efficiency. In summary, this first report on ternary system containing PTS successfully demonstrated that the combination of PTS:βCD:HPMC it is able to drastically improve the PTS water solubility what allow to reduce the amount of cyclodextrin used in the complexation media. The freeze dried βCD: PTS: HPMC system can be regarded as a promising intermediate product for new pharmaceutical or food products with improved PTS bioavailability.

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DISCUSSÃO

O PTS pertence a classe dos estilbenos (PENG et al., 2018) e apresenta diversas atividades farmacológicas descritas na literatura, o que vem atraindo a atenção de pesquisadores e aumentado o consumo deste constituinte pela população, especialmente na forma de alimentos funcionais, visando a exploração de seus benefícios à saúde (SILVA et al., 2014). Porém, sua baixa hidrossolubilidade, bem como sua instabilidade química, representa uma limitação para o desenvolvimento de produtos e realização de estudos nos meios fisiológicos, que são de natureza aquosa (BETHUNE; SCHULTHEISS; HENCK, 2011; YEO; HO; LIN, 2013). Assim, o objetivo geral do trabalho foi obter e caracterizar complexos e sistemas ternários contendo PTS, βCD e polímeros hidrossolúveis, visando sobrepujar suas limitações farmacêuticas decorrentes das características químicas e físico-químicas da molécula. Com o objetivo de subsidiar as etapas centrais do presente trabalho, a primeira etapa, foi dedicada ao desenvolvimento de método indicativo de estabilidade química do PTS, ante diversas condições de estresse. O ponto central do trabalho foi melhorar a hidrossolubilidade do PTS com o uso de uma ciclodextrina de baixo custo e elevada aplicabilidade, a βCD. A fim de aumentar a eficiência de complexação entre PTS e βCD foram utilizadas duas estratégias, o uso de cossolvente e uso de polímeros hidrofílicos (JANSOOK; OGAWA; LOFTSSON, 2018). Assim, a dissertação foi organizada em três capítulos.

O primeiro capítulo deste trabalho foi dedicado à revisão bibliográfica do tema em questão. Foram abordadas as atividades biológicas, as características estruturais e físico-químicas do PTS, sua instabilidade química e as estratégias tecnológicas já relatadas para melhorar os problemas relacionados a esta molécula. Foi discutida a utilização de ciclodextrinas, polímeros solúveis em água e etanol como cossolvente com a finalidade de aumentar a hidrossolubilidade de fármacos pouco solúveis e a eficiência de complexação fármaco:ciclodextrina. Em seguida, foi discutido o uso de desenho experimental como ferramenta para otimização e verificação de robustez de um método analítico. Adicionalmente, foram abordados aspectos relativos à validação de metodologia analítica.

O referido capítulo forneceu subsídios que demonstraram a importância do desenvolvimento de um método indicativo de estabilidade, assim como conhecer

mais sobre a estabilidade química do PTS. Ademais, este capítulo evidenciou dois aspectos inéditos: a utilização de βCD e polímeros hidrofílicos para associação com PTS e o uso concomitante de cossolvente e polímeros com vistas na melhoria na hidrossolubilidade e no aumento do conteúdo de PTS nos complexos sólidos contendo este estilbeno.

No segundo capítulo, foi desenvolvida e validada uma nova metodologia analítica para quantificação do PTS. A conhecida instabilidade de estilbenos diante de luz, oxidação e elevadas temperaturas também motivou um estudo indicativo de estabilidade por CLAE (exposição ao calor, luz ultravioleta e meios ácido, básico e oxidativo), objetivando identificar a ocorrência de formação de produtos de degradação e excluir sua eventual interferência na quantificação do analito.

O método analítico desenvolvido mostrou-se específico e indicativo de estabilidade para PTS ante exposição as condições de estresse. O PTS apresentou instabilidade quando exposto à luz ultravioleta, ao meio oxidativo e à elevadas temperaturas, e os produtos de degradação apresentaram picos com tempos de retenção diferentes do pico correspondente ao PTS, não interferindo em sua quantificação. Os resultados indicaram a importância da proteção do PTS perante exposição à luz ultravioleta, bem como, o cuidado que se deve ter em relação à exposição ao meio oxidante e a elevadas temperaturas. O perfil de degradação observado neste estudo está de acordo com relatos da literatura, sendo que já é bem caracterizada a instabilidade de estilbenos diante de temperatura (KWASNIEWSKI et al., 2003), e principalmente diante de luz ultravioleta (HENDRICKSON; CRAM; HAMMOND, 1970; MALLORY; MALLORY, 1984) e meios oxidativos (OGATA; TOMIZAWA; IKEDA, 1979).

O método foi validado segundo compêndio oficial (ICH, 2005), mostrando-se linear, exato, preciso e robusto na faixa de 1,0 a 20 µg/mL. O Box-Behnken design aplicado à robustez permitiu verificar como os parâmetros de análise interferem na resposta analítica, e denota a importância de manter as condições preestabelecidas na validação do método. Em conclusão, o capítulo descreve um método por CLAE que mostrou-se adequado para a quantificação de PTS, aplicado a extrato de mirtilo, complexo PTS:βCD, e sistemas ternários PTS:βCD:polímeros hidrofílicos (PVP ou HPMC).

O capítulo III refere-se ao desenvolvimento tecnológico de complexos e sistemas ternários contendo PTS, visto que esta molécula apresenta reduzida hidrossolubilidade, o que representa um fator limitante ao seu emprego em formulações farmacêuticas. Baseado em dados da literatura, optou-se pela complexação do PTS com βCD. Na otimização das condições de complexação, entre PTS e βCD, obtendo-se como resultado que banho a 37°C, 2 horas de agitação e 4mM de PTS no meio são as condições ideias para realizar-se a interação entre estas moléculas, considerando a viabilidade prática e econômica. Na proporção molar de 1:1 (PTS:βCD), a βCD aumentou a solubilidade do PTS em 10 vezes, com uma adequada eficiência de complexação.

Numa etapa subsequente, um componente novo foi explorado, o estudo de sistemas ternários PTS:βCD com polímeros hidrofílicos (hidroxipropilmetilcelulose, HPMC ou polivinilpirrolidona, PVP) com vistas a melhorar a hidrossolubilidade do fármaco reduzindo a quantidade de ciclodextrina necessária para melhorar a hidrossolubilidade do PTS, ou seja a razão molar PTS:βCD no meio de complexação.

A utilização de polímeros solúveis em água melhorou a constante de estabilidade aparente dos complexos e, por consequência, a eficiência de complexação e a hidrossolubilidade do PTS. Essa melhora foi mais pronunciada com o uso de HPMC, onde verificou-se um aumento de hidrossolubilidade do PTS de 56 vezes em relação a sua solubilidade aquosa intrínseca, e por isso este sistema ternário PTS:βCD:HPMC foi selecionado para a sequência do estudo. Esta estratégia resultou numa menor razão molar entre PTS:βCD (1:2), significando que é necessário apenas 2 moléculas de βCD no meio de complexação para resultar na formação de uma molécula de complexo PTS:βCD, no sistema ternário PTS:βCD:HPMC, diferentemente do que ocorre na ausência de polímero (razão PTS:βCD de 1:9).

O uso de etanol como cossolvente na formação do sistema ternário PTS:βCD:HPMC resultou num conteúdo de PTS 3 vezes maior, no produto final liofilizado, do que no mesmo produto obtido em meio aquoso e nos complexos PTS:βCD (obtido tanto em meio aquoso quanto usando cossolvente).

Os complexos e sistema ternário foram caracterizados utilizando-se microscopia eletrônica de varredura, calorimetria exploratória diferencial, espectroscopia no infravermelho, ressonância magnética nuclear, espectroscopia de massas e cromatografia líquida de alta eficiência. As análises revelaram interação entre o PTS e cavidade da βCD, demonstrando ocorrer a formação de complexos de inclusão. A razão estequiométrica de 1:1 entre PTS e βCD constatada no estudo de solubilidade de fase foi confirmada pela técnica de espectroscopia de massas, que permitiu afirmar a ocorrência de formação de complexos de inclusão, principalmente na razão 1:1, mas também na razão 1:2 PTS:βCD. Este é o primeiro relato do uso de espectroscopia de massas para identificação da estequiometria dos complexos formados por PTS e βCD.

Esse conjunto de resultados conduz às conclusões que são detalhadas a seguir e motivam diversas perspectivas de continuidade da investigação científica na área de formulação.

CONCLUSÕES

- A metodologia analítica, indicativa de estabilidade, para quantificação do PTS por CLAE mostrou-se linear, precisa, exata, seletiva e robusta nas condições testadas.

- O PTS demonstrou ser estável diante da exposição ao meio ácido (HCl 1M, 30h) e alcalino (NaOH 1M, 30h), mas instável ante exposição térmica (80°C, 30h) e oxidação (H_2O_2 1,5%, 10h); e muito instável ante luz UV (UVA 10 min).

- Como resultado da otimização por Box-Behnken dos parâmetros críticos para formação de complexo entre PTS e βCD, observou-se que dentre as condições testadas as melhores foram: banho de 37°C, 2 horas de agitação com 4mM de PTS no meio.

- O diagrama de solubilidade de fases demonstrou um incremento de hidrosolubilidade do PTS em 10 vezes, quando associado à βCD, na proporção molar 1:1.

- A avaliação das combinações do PTS com βCD e HPMC por microscopia eletrônica de varredura, calorimetria diferencial exploratória, espectrofotometria no infravermelho e ressonância magnética nuclear demonstram haver interação entre o PTS e βCD, indicando a formação de complexo de inclusão do PTS com a cavidade da ciclodextrina.

- A razão estequiométrica de 1:1 entre PTS e βCD constatada no estudo de solubilidade de fase foi confirmada por espectroscopia de massas. Esta técnica permite afirmar que ocorre a formação de complexos de inclusão principalmente na razão 1:1, mas também na razão 1:2 (PTS:βCD).

- O estudo de solubilidade revelou que as misturas binárias entre PTS:βCD e PTS:polímero hidrofílico promoveram a hidrossolubilidade do PTS. Porém, o sistema ternário PTS:βCD:HPMC apresentou resultados mais significativos, aumentando em 56 vezes a solubilidade aquosa do PTS em relação a sua solubilidade intrínseca.

- A constante de estabilidade aparente, que indica a força de ligação entre molécula hóspede e hospedeira, e a eficiência de complexação do complexo PTS:βCD aumentaram significativamente com o uso dos polímeros hidrofílicos. Por

consequência, a razão molar necessária para formar complexo entre PTS e βCD foi drasticamente reduzida. Na ausência de polímero hidrofílico a razão molar era de 1:9 e, na presença de HPMC, foi reduzida a 1:2 (PTS:βCD), denotando que a quantidade de ciclodextrina necessária para obter o incremento de hidrossolubilidade do PTS de 56 vezes foi reduzida.

- A associação de ciclodextrina a um polímero solúvel em água permite a redução na quantidade de ciclodextrina utilizada, sem prejudicar o efeito solubilizante do PTS. Os polímeros hidrofílicos PVP e HPMC são capazes de melhorar as propriedades farmacêuticas e, possivelmente, biológicas dos complexos PTS:βCD.

- O emprego de etanol, como cossolvente no meio de complexação determinou um aumento de 3 vezes no conteúdo de PTS no sistema ternário PTS:βCD:HPMC sólido, quando comparado com a sua obtenção em meio aquoso, denotando a influência positiva deste cossolvente.

Em conclusão, os resultados inéditos obtidos na presente dissertação demonstraram o sucesso da proposta formulada, em que o uso dos dois métodos para melhorar a eficiência de complexação, uso de cossolvente e uso de polímeros hidrofílicos, representa uma estratégia tecnológica relevante e pertinente, possibilitando o uso de menor quantidade de ciclodextrina e alcançando incrementos significativos da solubilidade aparente do PTS em água. Entre os produtos desenvolvidos, o produto ternário PTS:βCD:HPMC, obtido com o uso de etanol como cossolvente apresenta-se como o produto intermediário mais promissor para formulações farmacêuticas, e até mesmo, cosméticas e alimentícias.

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