UNIVERSIDADE TIRADENTES – UNIT

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS - PEP

EXTRAÇÃO DE CAPSAICINA POR MÉTODOS CONVENCIONAIS E NÃO CONVENCIONAIS E PURIFICAÇÃO UTILIZANDO SISTEMAS AQUOSOS BIFÁSICOS

ARACAJU, SE - BRASIL

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Poliane Lima Santos

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"A única gula que não é pecado,é a do conhecimento"
(Autor desconhecido)

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EXTRAÇÃO DE CAPSAICINA POR MÉTODOS CONVENCIONAIS E NÃO CONVENCIONAIS E PURIFICAÇÃO UTILIZANDO SISTEMAS AQUOSOS BIFÁSICOS

Poliane Lima Santos

A capsaicina (CPS) é um alcaloide que está presente, exclusivamente, nas pimentas do gênero *Capsicum* e é o principal responsável pela pungência típica, com potencial terapêutico e industrial. Assim, é importante estudar e desenvolver técnicas de extração e purificação rápidas, com alta eficiência e simplicidade. Este trabalho teve o objetivo de extrair e purificar a CPS presente em três variedades de frutos *Capsicum* (*C. frutescens*, *C. chinense*, *C. annuum*), em dois estágios de maturação, utilizando métodos de extração convencionais e não convencionais, seguido de processo de purificação utilizando novos sistemas aquosos bifásicos (SAB). Foram realizadas extrações convencionais com solvente orgânico à frio e à quente, as condições de extração dos métodos não convencionais assistidos por ultrassom e por micro-ondas foram otimizadas e, para a etapa de purificação por SAB, foram construídos os diagramas de fases a 25°C e avaliados os efeitos do tipo e concentração dos componentes e da temperatura do sistema sobre a partição da CPS. O maior teor de CPS foi detectado na pimenta cumari-do-Pará madura (4,59 mg de CPS.g^{-1} pimenta). Os métodos não convencionais foram mais eficientes (≥ 600%) do que os convencionais, sendo método assistido por micro-ondas a melhor técnica para extrair a CPS. Os estudos com SAB mostraram que a capsaicina migrou, preferencialmente, para a fase de topo (rica em solvente orgânico) e que os sistemas propostos conseguiram separar e purificar a CPS a partir de sua fonte natural com sucesso (K_{CPS} > 40, EE_{CPS} > 89% e PF_{CPS} > 2), sendo os melhores resultados obtidos para o sistema composto por etanol e sal (NaH_2PO_4). Portanto, este estudo demonstrou que a combinação de um método de extração eficiente seguido da purificação por SAB é alternativa interessante e promissora para extrair e purificar a CPS a partir de sua fonte natural, as pimentas do gênero *Capsicum*.

Palavras-chave: capsaicina, solvente orgânico, extração, micro-ondas, purificação, sistema aquoso bifásico.

Abstract of the thesis presented to the Post-graduation Program in Process Engineering of Tiradentes University as part of the requirements for the Doctor degree in Engineering Processes

EXTRACTION OF CAPSAICINA FOR CONVENTIONAL AND UNCONVENTIONAL METHODS AND PURIFICATION USING AQUOUS TWO-PHASES SYSTEMS

Poliane Lima Santos

Capsaicin (CPS) is an alkaloid that is present, exclusively in *Capsicum* peppers and it is the main responsible for the typical pungency, therapeutic and industrial potential. Thereby, it is important to study and develop techniques of extraction and purification quick, with high efficiency and simplicity. This study aimed to extract and purify CPS in three varieties of *Capsicum* fruit (*C. frutescens*, *C. chinense*, *C. annuum*), in two ripening stages and using conventional extraction methods and unconventional, following by purification process using new aqueous two-phase systems (ATPS). Conventional extraction with organic solvent cold and hot were performed, the extraction conditions of unconventional methods assisted by ultrasound and microwave were optimized and, for the purification step by ATPS, the phase diagrams were constructed at 25 °C and evaluated the effects the type and concentration of components and temperature of the system partition on the CPS. The highest content of CPS was detected in pepper cumari-do-Pará mature (4.59 mg of CPS.g^{-1} pepper. Unconventional methods were more efficient (≥ 600%) than the conventional method, being the technique assisted by microwave the best for extracting CPS. ATPS showed that capsaicin migrated, preferentially, to the top phase (rich in organic solvent), the proposed systems were optimized and were able to separate and purify the CPS from its natural source with success (K_{CPS} > 40 EE_{CPS} > 89% and PF_{CPS} > 2), with the best results obtained for the system composed of ethanol and salt (NaH_2PO_4). Therefore, this study showed that the combination of an efficient extraction method followed by purification by ATPS is an interesting and promising alternative to extracting and purify CPS from its natural source, the peppers of the *Capsicum* genus.

Keywords capsaicin, organic solvent, extraction, microwave, purification, aqueous two-phase systems.

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ARTIGO II - SUPPORTING INFORMATION

LISTA DE SIGLAS

[Ch][Bit]: Bitartarato de colina

[Ch][Cl]: Cloreto de colina

[Ch][DHcit]: Dihidrogeno citrato de colina

ACN: acetonitrila

CAPS: capsaicinoides

CPS: capsaicina

$C_6H_5Na_3O_7$: citrato de sódio

$C_6H_5K_3O_7$: citrato de potássio

K_2CO_3: carbonato de potássio

K_2HPO_4: fosfato de potássio dibásico

K_3PO_4: fosfato de potássio tribásico

K_2HPO_4/KH_2PO_4: Tampão fosfato de potássio

$NaC_2H_3O_2$: acetato de sódio

NaCl: cloreto de sódio

NaClO: hipoclorito de sódio

$NaClO_3$: clorato de sódio

Na_2CO_3: carbonato de sódio

NaH_2PO_4: fosfato de sódio

$NaNO_3$: nitrato de sódio

Na_2SO_4: sulfato de sódio

$Na_2S_2O_3$: tiossulfato de sódio

A, B, C: Constante de correlação das equações empíricas

ABS/ATPS/SAB: Sistema aquoso bifásico

C_T ou $_F$: Concentração na fase de topo ou fundo (g/L)

EE: Eficiência de extração (%)

EHF: *extremely high frequency* – frequência extremamente alta

ε: constante dielétrica

$FP_{T\ ou\ F}$: fator de purificação na fase de topo ou fundo (adimensional)

FT: Fenóis Totais

G_m^0: Energia livre de Gibbs (kJ/mol)

H_m^0: Entalpia molar (kJ/mol)

K: Coeficiente de partição (admensional)

K_{ow}: Coeficiente de partição octanol-água

LI ou IL: Líquido iônico

Ln: Logarítmo neperiano

P: Pressão

pK_a: Constante de dissociação ácida

R: Constante universal dos gases (8,31434 J/K.mol)

R_v: razão volumétrica (adimensional)

SHF: *super high frequency* – frequência super alta

SHU: Unidade de Calor Scoville

T: Temperatura (ºC ou K)

ton/ha: tonelada/hectare

TL: Linha de amarração

TLL: Comprimento da linha de amarração

UHF: *ultra high frequency* – frequência ultra alta

V_T: Volume de topo (mL)

V_F: Volume de fundo (mL)

X: Porcentagem mássica no fundo

Y: Porcentagem mássica no topo

ΔG, ΔH, e ΔS: Variação da Energia de Gibbs, da Entalpia e da Entropia (KJ/mol)

Capítulo I

INTRODUÇÃO

A pimenta está presente no cardápio dos seres humanos há muitos anos, podendo ser considerada uma das especiarias mais consumidas e cultivadas no mundo. Estima-se que em 2011 os principais produtores mundiais (China, México, Turquia e Índia) produziram juntos mais de 30 milhões de toneladas de pimentas do gênero *Capsicum* (FAOSTAT, 2015).

No Brasil, a produção de pimenta é importante não somente por servir como fonte de renda para diversas famílias de agricultores, mas também porque possui potencial funcional e cultural, sendo utilizada em diversas preparações culinárias que fazem parte da tradição de diversas cidades. Minas Gerais, Goiás, São Paulo, Ceará e Rio Grande do Sul são os principais Estados produtores e cultivam juntos uma área estimada entre 10 a 30 ton/ha, cuja produtividade varia de acordo com o tipo de pimenta plantado (PAULA et al., 2011). Na região Centro-Sul do Estado de Sergipe, especificamente no município de Lagarto, o cultivo de pimentas do gênero *Capsicum* se intensificou nos últimos anos em função da instalação de uma indústria processadora de molho de pimenta, que incentiva o cultivo e adquire a matéria-prima desses pequenos produtores, gerando renda para a comunidade local (SILVA et al., 2010).

Os frutos do gênero *Capsicum* possuem mais de 20 espécies descritas e o conteúdo de capsaicina é um dos principais parâmetros de qualidade. A capsaicina (CPS) é um tipo de alcaloide produzido nas glândulas da placenta da pimenta, principal responsável pela pungência típica dos frutos deste gênero e, assim como outros tipos de alcaloides, é conhecida por sua atividade biológica, farmacológica e terapêutica, possuindo potencial cicatrizante, termogênico, analgésico, antioxidante, anticancerígeno, além de ser uma excelente fonte de vitaminas (LUO et al., 2011; SHARMA et al., 2013).

Apesar da importância econômica e funcional que a pimenta possui, o número de estudos realizados com relação aos métodos de extração e purificação da CPS, ainda é pequeno. Desta forma, a busca por tecnologias que consigam extrair e purificar esta biomolécula, a partir de sua fonte natural, de forma mais rápida e eficiente é necessária não somente para agregar maior valor à pimenta, mas também para que a CPS possa ser disponibilizada para as indústrias alimentícia e farmacêutica com maior grau de pureza.

Os métodos de extração de biomoléculas podem ser divididos em métodos convencionais (com solvente à frio ou à quente) e não convencionais (assistido por ultrassom ou micro-ondas, por exemplo). Normalmente, os métodos convencionais requerem maior quantidade de solvente e tempo de extração do que os não convencionais, que se destacam pela rapidez e eficiência de extração. Para obter um extrato natural com grau de pureza elevado, além do processo de extração, é necessário realizar uma etapa de purificação e, neste contexto, a extração líquido-líquido por meio de Sistemas Aquosos Bifásicos (SABs), proposta na década de 50 por Albertsson é uma alternativa (ALBERTSSON, 1958).

O SAB utiliza duas soluções aquosas imiscíveis que interagem entre si e, após atingir o equilíbrio termodinâmico, se separam em duas fases e desloca a biomolécula de interesse para a fase que esta possui maior afinidade. Pode ser formado por polímeros, sais, solvente orgânico, carboidratos, polióis e líquidos iônicos, por exemplo, e já foi aplicado com sucesso na partição de enzimas, vanilina, rutina, ácido gálico, antibióticos, alcaloides, dentre outros (REIS et al., 2015). Esta técnica vem se destacando pela simplicidade, eficiência e seletividade (WU et al., 2011).

A realização de um estudo prévio que inclui a construção de diagramas de fase e estabelecimento das condições iniciais de separação das fases é fundamental para partição de uma biomolécula por SAB. Apesar de já constar na literatura um trabalho sobre capsaicina e SAB (ZHAO et al., 2015), é importante ressaltar que, neste estudo, o sistema aquoso foi utilizado como método de extração e não de purificação, a qual foi feita por cromatografia de fase reversa. Além disso, os experimentos foram realizados utilizando uma oleoresina adquirida comercialmente e as condições de partição não foram completamente estudadas, o que reforça caráter inovador dos sistemas propostos, dos dados apresentados e a importância da realização desta tese, que comparou métodos convencionais com não convencionais para extrair capsaicina a partir de pimentas do gênero *Capsicum*, estudou a formação de SABs compostos por acetonitrila com líquidos iônicos à base de colina, sais de sódio e sais de potássio, etanol com sais de sódio, avaliou sua eficiência para separar e purificar a capsaicina a partir de sua fonte natural e assim, propôs um novo método para purificação desta biomolécula.

Capítulo II

OBJETIVOS

2.1 OBJETIVO *GERAL*

Extrair capsaicina de pimentas do gênero *Capsicum* utilizando métodos de extração convencional (com solvente a frio e a quente) e não convencional (assistida por ultrassom e por micro-ondas), seguida de purificação utilizando sistemas aquosos bifásicos.

2.2 OBJETIVOS ESPECÍFICOS

Este trabalho teve como objetivos específicos:

- Estudar a extração da capsaicina presente nas seguintes pimentas do gênero *Capsicum*: jalapeño (*C. annuum*), cumari-do-Pará (*C. chinense*) e malagueta (*C. frutescens*), utilizando métodos convencionais à frio e à quente;
- Estudar e otimizar os métodos de extração não convencionais assistidos por ultrassom e micro-ondas;
- Construir diagramas de fases para os sistemas formados por acetonitrila com líquidos iônicos à base de colina (cloreto, bitartarato e dihidrogeno citrato) e com sais ($Na_2S_2O_3$, $C_6H_5Na_3O_7$, $C_6H_5K_3O_7$, Na_2CO_3, K_2CO_3, K_2HPO_4, K_3PO_4), etanol com sais ($Na_2C_2H_3O_2$, Na_2CO_3, $NaClO_3$, Na_2NO_3, Na_2SO_4, $Na_2S_2O_3$ e NaH_2PO_4);
- Estudar e avaliar o efeito do tipo e concentração dos componentes e da temperatura sobre a partição da capsaicina nos sistemas modelos, utilizando capsaicina sintética;
- Determinar os parâmetros de extração (coeficiente de partição e eficiência de extração) e os parâmetros termodinâmicos (entalpia, entropia e energia livre de *Gibbs*), para cada sistema modelo;
- Purificar a capsaicina, a partir de um extrato natural de pimenta *Capsicum*, utilizando estes sistemas aquosos bifásicos.

Capítulo III

REVISÃO BIBLIOGRÁFICA

Neste capítulo será apresenta informações sobre a capsaicina, uma biomolécula de interesse comercial, no tocante a suas propriedades, seguido do estado da arte referente à extração e purificação de capsaicina utilizando métodos convencionais e não convencionais e sua purificação por diferentes sistemas aquosos bifásicos.

3.1 PIMENTAS DO GÊNERO *CAPSICUM*

O cultivo de pimentas é realizado em todos os continentes e, aproximadamente, 89 % da área total encontra-se no Continente Asiático (Índia, Coréia, Tailândia, China, Vietnã, Sirilanka e Indonésia), 7% nos Estados Unidos e México e 4% entre países da Europa, África e Oriente Médio (RUFINO e PENTEADO, 2006). Apesar do Brasil não se encontrar na lista dos principais produtores mundiais, o país tem aumentado sua produção em todas as regiões. Segundo dados do Censo Agropecuário realizado em 2006, o Brasil produziu 18.682 toneladas de pimenta, sendo a região Nordeste (6.417 ton) a maior produtora do país, seguida das regiões Sudeste (5.629 ton), Norte (4.231 ton), Centro-Oeste (1.660 ton) e Sul (746 ton). O Estado de Sergipe ocupou a posição de terceiro maior produtor do Nordeste (558 ton), perdendo para o Ceará (3354 ton) e Bahia (1097 ton) (IBGE, 2006).

Da família das Solanáceas assim como o tomate, a batata, a berinjela e o tabaco, as pimentas do gênero *Capsicum* possuem mais de 90 espécies descritas, entretanto, nem todas são conhecidas (ISMAIL e REVATHI, 2006). As variedades mais citadas na literatura são a *C. annuum* (jalapeño), *C. baccatum* (dedo-de-moça), *C. chinense* (cumari-do-Pará), *C. frutescens* (malagueta).

A *C. annuum* (Figura 3.1a) é originária do México, mas cultivada em todo o mundo. Apresenta grande variabilidade genética, tornando-se conhecida por diversas espécies, sendo umas menos picantes como, por exemplo, o pimentão e outras mais picantes, como a jalapeño (FERRÃO, 1993; REIFSCHNEIDER, 2000).

Dentre as pimentas mais populares e picantes, o gênero *C. chinense* (Figura 3.1b) é representado pelas variedades pimenta-de-cheiro, pimenta-de-bode, cumari-do-pará, habanero, é considerado o mais brasileiro de todos os gêneros, uma vez que foi domesticado pelos índios da Bacia Amazônica (CARVALHO e BIANCHETTI, 2008). Outro gênero

também popular e com pungência conhecida, são as pimentas *C. frutescens* (Figura 3.1C) são representadas, principalmente, pela pimenta malagueta. Amplamente cultivadas no Brasil, costumam ser usadas no preparo de peixes, acarajés, molhos e conservas. Seus frutos são pequenos, verdes ou amarelos quando imaturos e vermelhos quando em completo estágio de maturação (FERRÃO, 1993). A Tabela 3.1 apresenta a composição centesimal de algumas pimentas do gênero *Capsicum*.

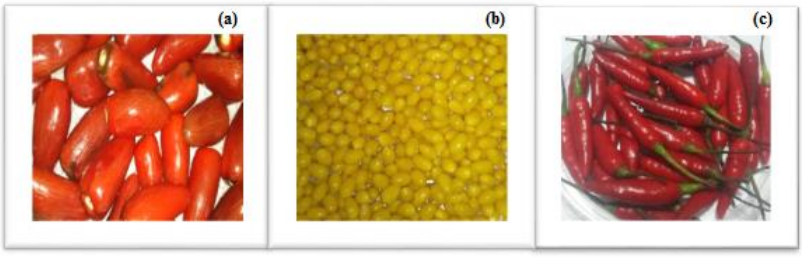

Figura 3.1: Aspecto da pimenta *Capsicum* nas variedades: **(a)** jalapeño (*C. annuum*); **(b)** cumari-do-pará (*C. chinense*); **(c)** malagueta (*C. frutescens*).

Tabela 3.1: Composição centesimal de pimentas do gênero *Capsicum*.

Parâmetros	***C. frutescens****	***C. baccatum*****	***C. chinense*****
Acidez	0,04 ± 0,04 %	4,00 ± 0,20 %	-
pH	5,48 ± 0,05	5,14 ± 0,05	-
Umidade	29,4 ± 0,10 %	89,60 ± 0,50 %	-
Lipídios	0,63 ± 0,02 %	0,14 ± 0,01 %	4,34 ± 0,06 g/kg
Proteínas	4,8 ± 0,10 %	0,95 ± 0,01 %	5,20 ± 0,02 g/kg
Cinzas	0,04 ± 0,03 %	0,14 ± 0,04 %	5,18 ± 0,02 g/kg
Vit. C	121,5 ± 0,30 mg/100g	-	232,33 ± 0,10 mg/kg

Fonte: * Rebouças et al., 2013; **Nascimento et al., 2012; *** Reis et al., 2013.

As pimentas do gênero *Capsicum* são comumente utilizadas como matéria-prima para indústrias alimentícias, farmacêuticas, cosméticas e como condimento para preparações culinárias. Apresentam-se como excelentes fontes de vitaminas (C, complexo B, A e E), que estão associadas ao conteúdo de compostos fenólicos, flavonoides, carotenoides e capsaicinoides (DAVIS et al., 2007; GHASEMNEZHAD et al., 2011; REIS et al., 2013).

3.2 CAPSAICINOIDES (CAPS)

Dentre a gama de compostos químicos que estão presentes nas pimentas do gênero *Capsicum*, os principais componentes são os capsaicinoides, um tipo de alcaloide do grupo das amidas da vanilamina (4-hidróxi-3-metóxi-benzilamina) (DAVIS et al., 2007). A estrutura química básica dos CAPS possui um anel aromático com um grupo hidroxila fenólico, um grupo metóxi e um grupo amido ácido (vanililamida), que é responsável pela pungência típica destes compostos (REILLY et al., 2001). A Figura 3.2 apresenta a estrutura química básica de um CAPS.

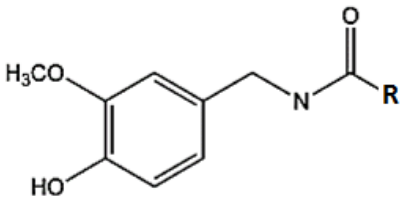

Figura 3.2: Fórmula estrutural básica dos CAPS, onde R representa as cadeias carbônicas alifáticas. Fonte: Adaptada de Chauhan et al. (2011).

Os capsaicinoides mais comuns e que estão presentes nas pimentas do gênero *Capsicum*, segundo Wesolowska et al. (2011), são a capsaicina, di-hidrocapsaicina, nordi-hidrocapsaicina, homo-capsaicina e homodi-hidrocapsaicina, sendo que a capsaicina é mais e a nordi-hidrocapsacina é menos pungente (PINTO et al., 2013). A Figura 3.3 apresenta a estrutura química de alguns CAPS.

A concentração de capsaicinoides começou a ser determinada em 1912 através do teste organoléptico de Scoville, feito extraindo a capsaicina em álcool e diluindo até a pungência ser detectada ao colocar apenas uma gota na língua, sendo o resultado expresso em Unidade de Calor Scoville (SHU), que corresponde ao número de vezes que o extrato de pimenta precisa ser diluído para perder a ardência (SCOVILLE, 1912; CHANCELLOR e GROAT, 1999). O teste de Scoville não possui tanta precisão porque depende da subjetividade de um avaliador e, além disso, através dele não é possível distinguir o tipo de capsaicinoide presente na amostra. A Tabela 3.2 apresenta o teor total de capsacinoides em algumas espécies de *Capsicum* e da capsaicina pura, medido em SHU.

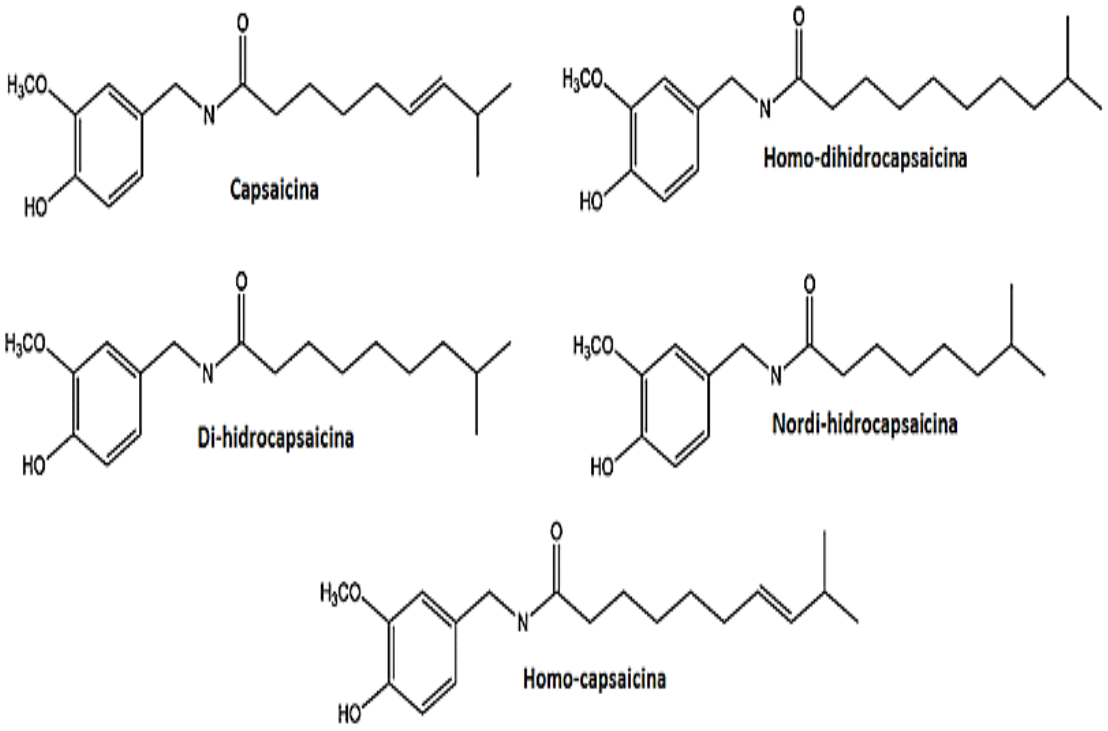

Figura 3.3: Estrutura química de alguns CAPS. Fonte: Adaptada de Davis et al. (2007).

Tabela 3.2: Teor de capsaicinoides total em de pimentas do gênero *Capsicum*.

Pimenta	SHU
Pimentão (*Capsicum annuum*)	0 – 1
Pepperocini (*Capsicum annuum*)	100 – 500
Jalapeño (*Capsicum annuum*)	1.000
Caiena (*Capsicum baccatum*)	30.000 – 50.000
Thai (*Capsicum frutescens*)	50.000 – 100.000
Jamaicana e Habanero (*Capsicum chinense*)	100.000 – 300.000
Capsaicina pura	16.000.000

Fonte: Adaptada de Chancellor e Groat (1999).

O conteúdo de capsaicinoides nas pimentas pode ser afetado pelo estágio de desenvolvimento, idade e tamanho do fruto. Além disso, fatores como temperatura, luz, composição do solo, nível de fertilizantes e estação do ano também são determinantes na formação destes compostos, pimentas cultivadas na primavera-verão tendem a ser mais pungentes do que as do outono-inverno (ESTRADA et al., 2000; KIRSCHBAUM-TITZE et al., 2002).

3.2.1 *Capsaicina (CPS)*

A capsaicina ($C_{18}H_{27}NO_3$), cuja fórmula estrutural está apresentada na Figura 3.4, é um dos tipos alcaloides, dentre o grupo dos CAPS, produzido exclusivamente pelos frutos do gênero *Capsicum*, é o principal composto responsável pela pungência, a ardência característica destes (WALSH e HOOT, 2001). Segundo Bosland (1996), a CPS é produzida somente nas glândulas da placenta do fruto e não nas sementes, que se desenvolvem na região próxima a placenta e, ocasionalmente, absorvem a biomolécula. A Figura 3.5 apresenta a anatomia da pimenta.

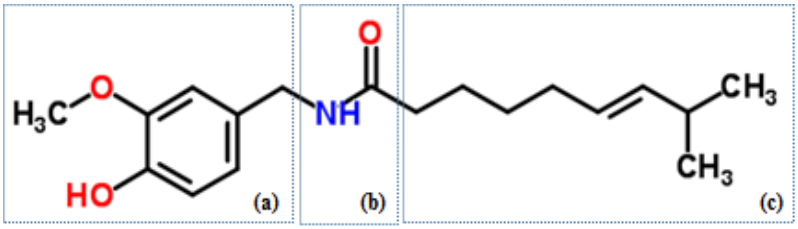

Figura 3.4: Fórmula estrutural da capsaicina. **(a)** Região do anel aromático - POLAR; **(b)** Região amida; **(c)** Cadeia lateral hidrofóbica - APOLAR. Fonte: Chauhan et al. (2011).

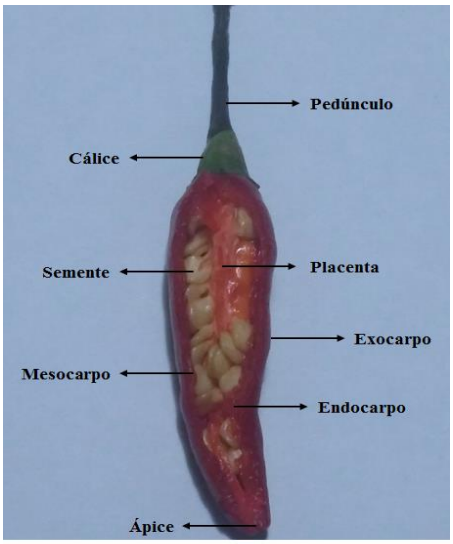

Figura 3.5: Corte longitudinal de uma pimenta do gênero *Capsicum*.

Denominada pela IUPAC como 8-metil-N-vanilil-trans-6-nonenamida, a capsaicina é um composto sem cor e sem cheiro. Possui peso molecular igual a 305,4 g/mol, ponto de fusão a 65°C, ebulição a 210-220°C e sublimação a 115°C. É insolúvel em água e solúvel em solventes organicos, é resistente a soluções ácidas e alcalinas a temperatura ambiente

(CHAHUAN et al., 2011). Sua degradação térmica pode ser dividida em dois estágios: o primeiro inicia a 200 °C e ocorre mais rápido a partir de 360 °C, sugerindo a destruição dos grupos amidas; o segundo estágio ocorre a 450 °C e indica a degradação de compostos orgânicos (WANG et al., 2013).

A biossíntese da capsaicina pode ser feita via fenilpropanóide ou via ácido graxo de cadeia ramificada (CHAUHAN et al., 2011). A CPS pode ser utilizada como precursor natural de vanilina, um composto que desperta interesse por seu amplo potencial industrial, cuja produção via conversão enzimática da capsaicina é feita pela ação das enzimas acilase e amino oxidase, a primeira hidrolisa capsaicina em vanililamina e a segunda produz a vanilina a partir da vanililamina (ROMANO et al., 2011; CHO et al., 2014). A Figura 3.6 ilustra as rotas de biossíntese e a conversão enzimática da capsaicina.

A capsaicina pode ser administrada tanto via oral quanto local e tem sido utilizada para o alívio de dor causada por artrite reumatoide, fibromioalgia, inflamação e hiperalgesia (FRAENKEL et al., 2004), como ingrediente ativo em analgésico pós-cirúrgico e no tratamento de osteoartrose (REMADEVI e SZALLISI, 2008). Com relação ao potencial anticancerígeno e quimiopreventivo, seu efeito está relacionado com sua capacidade para impedir a multiplicação das células e migração, além de induzir a apoptose celular (LUO et al., 2011). Também foi relatado o efeito protetor contra os altos níveis de colesterol e obesidade (JOO et al., 2010; LEUNG, 2008; KEMPAIAH et al., 2005), além de efeitos benéficos ao sistema cardiovascular e gastrointestinal (PENG e LI 2010).

Estudos indicam que os efeitos clínicos decorrente da ingestão de pimenta ou extrato de capsaicina correspondem a uma excitação dos nervos sensoriais que se refletem, fisiologicamente, na redução da vasodilatação, dor por calor e aumento da sensibilidade até ocorrer uma insensibilização (BARCELOUX et al., 2009). No entanto, o conhecimento sobre sua toxicidade ainda é limitado e Meghvanzi et al. (2010) relatou a dificuldade em estimar os valores da toxicidade devido a individualidade de cada ser em relação à resistência a capsaicina. Segundo Lewis (2000), a DL_{50} oral aguda dos extratos de capsaicina em seres humanos pode ser estimada em 0,5-5,0 g/kg. Baez et al. (2010) mostrou que o consumo excessivo de pimentas pode aumentar o risco de desenvolvimento de câncer gástrico em vez de trazer benefícios, visto que os metabólitos da capsaicina podem atacar o DNA e provocar conversão de tumores benignos em malignos.

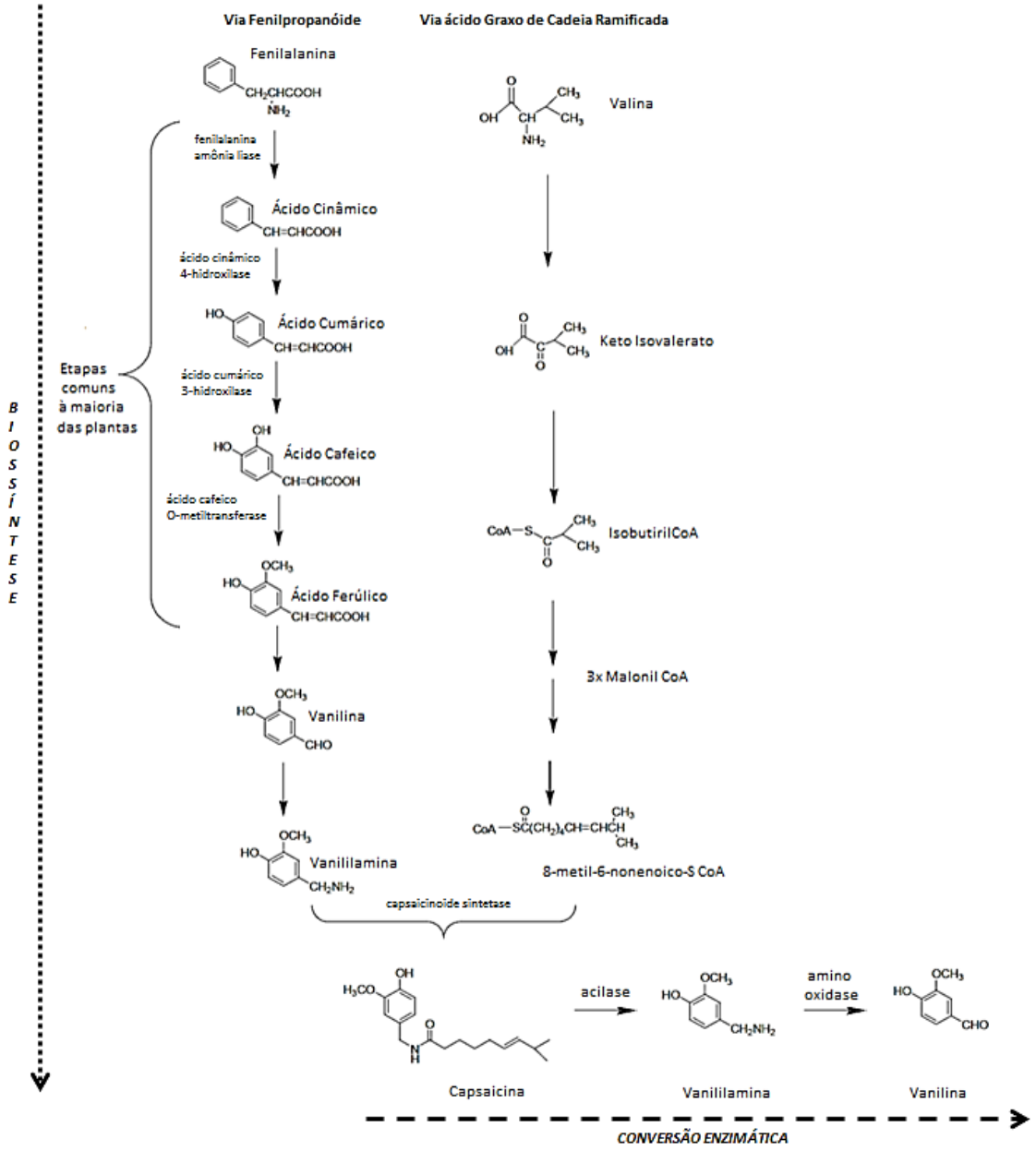

Figura 3.6: Ilustração das rotas de biossíntese e conversão enzimática da capsaicina. Fonte: Adaptada de Chauhan et al. (2011) e Romano et al. (2011).

Está evidente que a capsaicina possui um grande potencial industrial. No entanto, para que esta biomolécula possa ser utilizada, primeiramente é necessário extraí-la de sua matriz e realizar etapas de purificação que resultem num produto final com elevado grau de pureza. Assim, a realização de estudos visando o desenvolvimento e aprimoramento de técnicas para extração e purificação de biomoléculas tem se intensificado com o intuito de atender a crescente demanda do setor industrial por processos mais rápidos, eficientes e com custo reduzido.

3.3 EXTRAÇÃO E PURIFICAÇÃO DE BIOMOLÉCULAS

Biomoléculas ou moléculas bioativas são compostos sintetizados por organismos vivos, que possuem efeito farmacológico ou toxicológico e podem atuar no metabolismo, digestão, proteção e locomoção destes seres, a depender de sua necessidade (GREISLER, 2003). Por exemplo, as flores sintetizam aroma para atrair espécies que atuam em seu processo de polinização e fertilização, enquanto outros organismos sintetizam compostos químicos tóxicos para afastar predadores e patógenos (DUDAREVA e PICHERSKY, 2000).

De acordo com a sua origem, as biomoléculas podem ser do tipo endógena, quando o próprio organismo a sintetiza como é o caso dos lipídios, proteínas e ácidos nucleicos ou ser do tipo exógena que não são sintetizadas naturalmente pelo organismo e dependem de fator externo para serem produzidas, como, por exemplo, os antioxidantes, minerais, retinol e bioflavina (GREISLER, 2003). Entretanto, para obter esses compostos a partir de sua fonte natural, primeiramente é necessário realizar um processo de extração.

A extração é um tipo de operação unitária que tem o objetivo de separar substâncias a partir de diversas matrizes, tanto sólidas quanto líquidas, utilizando processos químicos que empregam solventes químicos e processos físicos como, por exemplo, a utilização de calor e radiação, ou ainda, da combinação destes dois processos. O processo de extração compreende, basicamente, em cinco etapas: 1- dessorção do composto (analito) do sítio ativo da matriz; 2- difusão para a própria matriz; 3- dissolução do analito pelo líquido extrator; 4- difusão do analito no líquido extrator; 5- coleta do extrato (STICHER, 2008).

Existe uma grande variedade de métodos de extração de biomoléculas, que podem ser divididos em convencionais, que são métodos mais tradicionais e já consolidados na literatura como, por exemplo, maceração e extração com refluxo de solvente à quente utilizando o aparato de Sohxlet, e os não-convencionais, que requerem o uso de técnicas mais sofisticadas como micro-ondas, ultrassom, fluido supercrítico, entre outros. No entanto, apesar das diferenças quanto aos princípios e procedimentos utilizados, todos os métodos possuem os mesmos objetivos que são: extrair biomoléculas a partir de amostras complexas, ser seletivo e sensível ao analito de interesse; ser rápido e eficiente (SMITH, 2002). A Tabela 3.3 apresenta alguns métodos para extrair capsaicina a partir de sua fonte natural disponíveis na literatura.

Tabela 3.3: Métodos de extração de capsaicina.

Método	Solvente	Tempo de extração	Temperatura	[capsaicina]	Referência
EAU*	Metanol	20 min	50 °C	573. 44-15220 µg/g de pimenta seca	(KEHAROM et al., 2015)
Extração acelerada com solvente associada à EFS**	Metanol	5 min estático + três ciclos de EFS	200 °C	2,307.0-9047.3 µg/g de pimenta seca	(CHANTHAI et al., 2012)
EAU*	Metanol	10 min	50 °C	275-448 µmol/kg de pimenta fresca	(BARBERO et al., 2008)
Extração supercrítica assistida por ultrassom	CO_2	60 min	40 °C	1,78-1-93 mg/g de pimenta fresca	(SANTOS et al., 2015)
Soxhlet	Etanol	140 min	140 °C	0,009626 g/g de pimenta	(XU et al., 2009)
EAU*	Etanol	3 h	45 °C	85 % de recuperação	(BOONKIRD et al., 2008)
Extração com água pressurizada	Água	30 min	200 °C	285-46451 µg/kg de pimenta seca	(BAJER et al., 2015)
MEFS + ultrassom	Metanol	20 min	-	77 % de recuperação	(SPICER e ALMIRALL, 2005)
Extração em shaker	Acetona	20 min	50 °C	8-16,5 mg/g de pimenta seca	(CHINN et al., 2011)
EAM****	Etanol	5 min	125 °C	265,2-451,6 µmol/kg de pimenta fresca	(BARBERO et al., 2006)

* EAU: Extração assistida por ultrassom;**EFS: Extração em fase sólida; ***MEFS: Micro extração em fase sólida; ****EAM: Extração assistida por micro-ondas.

3.3.1 Métodos de Extração Convencionais

Tradicionalmente, a extração de biomoléculas é feita utilizando os métodos convencionais com solventes à frio ou à quente, uma vez que estes já estão estabelecidos e, normalmente, são de fácil execução, pouco sofisticado e de baixo custo. Entretanto, fatores como a utilização de grande quantidade de solvente, a necessidade de longo tempo de contato e o baixo rendimento, contribuem para a redução da viabilidade destes métodos (YANG et al., 2008).

Os métodos convencionais consistem basicamente em submeter o material ao contato de um solvente, por certo tempo e temperatura, com ou sem agitação como, por exemplo, a extração utilizando o aparelho de Soxhlet, maceração, lixiviação, imersão, entre outros (AZMIR et al., 2013; CHAN et al., 2011). A extração de capsaicina já foi realizada por meio dos seguintes métodos convencionais: maceração (KIRSCHBAUM-TITZE et al., 2002), agitação (CONTRERAS-PADILLA e YAHIA, 1998; CISNEROS-PINEDA et al., 2007), agitação em banho-maria (CHINN et al., 2011) e empregando Sohxlet, que costuma ser utilizado como modelo para comparação com novos métodos de extração (BOONKIRD et al., 2008; CHUICHULCHERM et al., 2013; BAJER et al., 2015). Segundo Cowan (1999), a eficiência de qualquer método de extração convencional depende, principalmente, da escolha do solvente que, por sua vez, deve considerar a polaridade do analito de interesse.

Chinn et al. (2011) avaliaram o efeito do método de preparação das amostras de *C. chinense*, fresca ou seca, e o tipo de solvente orgânico para a extração da capsaicina. As amostras frescas foram armazenadas em freezer e secas, foram desidratadas a 65°C até adquirir o peso constante. As amostras foram extraídas pelo método de agitação em banho a 50°C por 1h, utilizando etanol, acetona e acetonitrila como solventes e a quantificação feita por CLAE. Os três solventes utilizados apresentaram bons resultados para a extração da capsaicina. Entretanto, os maiores rendimentos foram obtidos a partir da extração utilizando acetona como solvente (16,5 mg/g de pimenta seca).

Já Chuichulcherm et al. (2013) compararam o método tradicional por Soxhlet com duas técnicas diferentes, a extração assistida por micro-ondas (EAM) e por ultrassom (EAU), para extrair capsaicinoides de pimentas secas (*Capsicum frutescens* Linn.). Os autores avaliaram o volume de solvente, 200-250-300 mL de etanol, utilizado na extração e observaram que não houve diferença significativa quanto ao conteúdo de capsaicinoides, encontrando 5,24 mg de capsaicinoides/g de pimenta seca utilizando 200 mL de etanol, após 5 h de extração. Para os métodos assistidos por micro-ondas e ultrassom, os resultados obtidos

após 20 min de extração foram iguais a 5,28 e 4,0 mg de capsaicinoides/g de pimenta seca, respectivamente. Apesar de melhor resultado ter sido encontrado utilizando EAM, o método de EAU foi escolhido em função do tempo de análise reduzido e do menor gasto com energia durante o processo.

3.3.2 Métodos de Extração Não-Convencionais

Segundo Luque-Garcia e Castro (2003), alguns desafios da extração convencional correspondem aos longos tempo de extração, a grande quantia de solvente requerida, grande perda de solvente por evaporação, baixa seletividade e degradação de compostos mais sensíveis à temperatura. Assim, em função dos problemas inerentes à utilização dos métodos convencionais para a extração de biomoléculas, existe a necessidade do desenvolvimento de técnicas que preserve a estrutura do composto, utilize menor quantidade de solvente e reduza o tempo de processo. Neste contexto, métodos de extração não convencionais como a extração por micro-ondas, fluido supercrítico, ultrassom e ultrafiltração, por exemplo, vêm despontando pela eficiência e menor custo de processo (CHEMAT et al., 2011).

3.3.2.1 Extração Assistida por Ultrassom (EAU)

A Extração Assistido por Ultrassom (EAU) é uma das alternativas descritas na literatura como técnica promissora para introduzir a inovação em processos de extração de biomoléculas (ESCLAPEZ et al., 2011). Entre as potenciais vantagens na utilização desta técnica, quando em comparação com métodos de extração convencionais, pode-se citar o menor tempo de extração, alto rendimento e a facilidade de controlar os parâmetros do processo. Estas vantagens são decorrentes, principalmente, dos fenômenos de cavitação (RATHOD e RATHOD, 2014). Além disso, as ondas ultrassônicas reduzem a temperatura necessária ao processo e favorece a solubilização do analito de interesse no solvente (ESCLAPEZ et al., 2011)

O método consiste na aplicação de ondas ultrassonônicas de alta potência (acima de 20 kHz), produzidas num banho de ultrassom ou por ultrassom de sonda, que irão provocar efeitos físicos (aumentam as taxas de transferência de massa e, portanto, também aumentam o rendimento da extração), efeitos térmicos (causam a ruptura das paredes das células, redução no tamanho da partícula e aumentam a transferência de massa através das membranas celulares) e efeitos mecânicos (podem aumentar a área de superfície de contato entre as fases

sólida e líquida, devido à possibilidade de redução do tamanho da matriz sólida) (VETAL et al., 2013).

As ondas ultrassônicas de alta frequência são capazes de provocar cavitação devido aos ciclos de expansão e compressão que o material passa quando submetido a ultrassons (SANTOS et al., 2015). A expansão pode criar bolhas e resultar numa pressão negativa, já na contração ocorre o colapso das bolhas que podem resultar na cavitação e o consequente rompimento da estrutura celular (ESCLAPEZ et al., 2011). Estes ciclos que perturbam as paredes das células da matriz vegetal favorecem a penetração do solvente ao aumentar a permeabilidade da parede celular, facilitando a dilatação e hidratação da amostra ao promover o estresse mecânico das células e, consequentemente, favorece a transferência de massa através do aumento do tamanho dos poros da parece celular, aumentando assim a taxa de extração e o rendimento do processo (TOMA et al., 2001; LUQUE-GARCIA e CASTRO, 2003). Por meio da Figura 3.7 é possível visualizar o efeito da EAU sobre uma matriz vegetal.

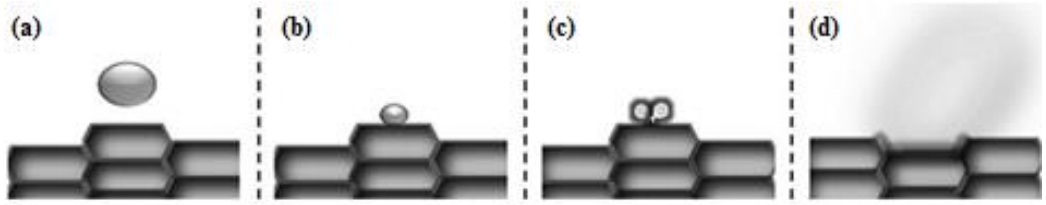

Figura 3.7: Representação esquemática dos efeitos do processo de extração assistida por ultrassom sobre uma matriz vegetal Fonte: Esclapez et al. (2011).

Na Figura 3.7, pode ser observada a formação das bolhas, que ao sofrer o processo de expansão (Figura 3.7a) e compressão (Figura 3.7b), sofrerão colapso (Figura 3.7c) ou implosão (Figura 3.7d). Quando o colapso ocorre perto da interface da matriz, pode ocorrer a formação de ondas de choque que perturbarão a parede da matriz e, consequentemente, liberação do material intracelular no solvente (Figura 3.7d) (ESCLAPEZ et al., 2011).

Dentre as variáveis que influenciam o processo de extração assistido por ultrassom, podem ser citadas a razão massa/solvente, temperatura, tipo de solvente, frequência e configuração do extrator, sendo as duas últimas as mais importantes (ESCLAPEZ et al., 2011). A frequência, a depender da estrutura da matriz e a biomolécula a ser extraída, costuma influenciar o rendimento e a cinética do processo. Segundo Takeuchi et al. (2009),

frequências mais baixas evitam a degradação de compostos. Os autores afirmam ainda que a morfologia da partícula também influencia o rendimento da extração.

Os equipamentos extratores por ultrassom podem ter o transdutor acoplado ao vaso de extração (Figura 3.8a), que possui a desvantagem da falta de uniformidade de distribuição de ondas, ou ter uma sonda ultrassônica imersa no meio (Figura 3.8b e 3.8c), que distribui melhor as ondas no meio de extração porque a sonda fica imersa diretamente na mistura da matriz com o solvente (ESCLAPEZ et al., 2011).

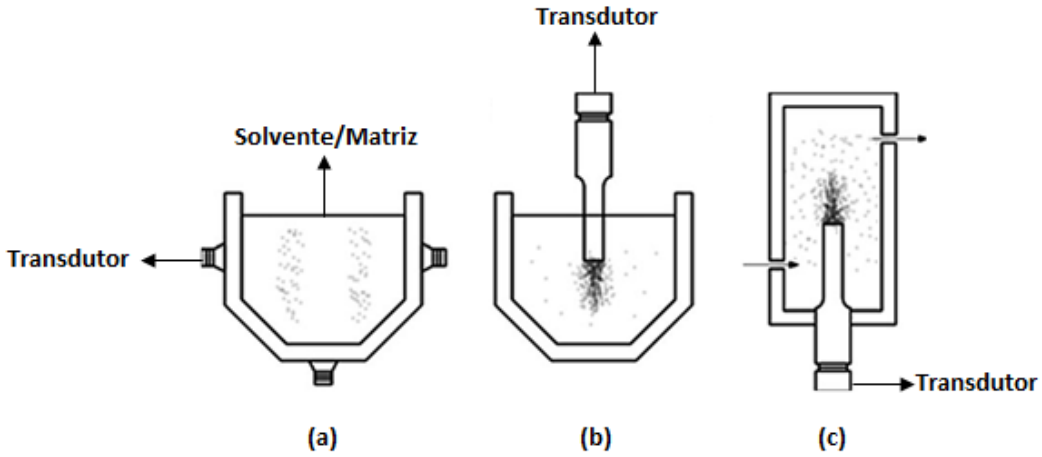

Figura 3.8: Configurações típicas de extratores: **(a)** transdutor acoplado a um vaso; transdutor (sonda) imerso no meio solvente/matriz vegetal, em batelada **(b)** e em modo contínuo **(c)**.
Fonte: Esclapez et al. (2011).

Barbero et al. (2008) otimizaram as condições de extração de capsaicinoides por ultrassom, avaliando o tipo de solvente (acetonitrila, metanol, etanol e água), temperatura de extração (10 – 60 °C), tempo de extração (2 – 25 min), quantidade de amostra (0,2 – 2 g) e volume de solvente (15 – 50 mL). Concluíram que as melhores condições de extração por ultrassom devem ser feitas a 50 °C, durante 10 min., utilizando 1 g de amostra e 25 mL de metanol. Assim, as amostras de pimenta caiena (*C. frutescens*) apresentaram 448 µmol de capsaicina/kg de pimenta fresca.

Boonkird et al. (2008) avaliaram a influência do tipo de solvente, razão entre o solvente e a amostra, o efeito da quantidade de água na mistura com etanol, tempo e temperatura de extração, para extração de capsaicinoides a partir de pimentas *Capsicum frutescens* desidratadas. A condição otimizada encontrada utilizando 1 g de amostra, 5 mL de

solução de etanol 95%, a 45 °C durante 3 h conseguiu extrair 85% dos capsicinoides presentes na matriz.

Sganzerla et al. (2014) avaliaram a influência do tipo de solvente, proporção metanol:acetona e tempo de extração assistida por ultrassom de acordo com um delineamento experimental de 11 ensaios. Além disso, desenvolveram e validaram um método por cromatografia líquida de ultra performance para analisar capsaicinoides em pimenta cumari-do-pará (*Capsicum chinense*). Os melhores resultados foram obtidos utilizando 100% de metanol, durante 10min de extração assistida por ultrassom, com conteúdo de capsaicina variando de 156 a 1.442 g de capsaicina/g de pimenta fresca. O método analítico foi otimizado e permitiu a separação de oito capsaicinoides num tempo de corrida igual a 2 min e gastando 2 mL de fase móvel.

Santos et al. (2015) avaliaram os efeitos das ondas ultrassônicas na extração com fluido supercrítico (SFE) de pimenta malagueta (*C. frutescens* L.) quanto ao conteúdo de capsaicinoides total nos extratos. Os resultados mostraram que o uso do ultrassom (360 W durante 60min) aumentou até 77% do rendimento global do processo com fluido supercrítico (CO_2 como solvente a 15 MPa e 40 °C), sem alterar o perfil cromatográfico dos capsaicinoides.

3.3.2.2 Extração Assistida por Micro-ondas (EAM)

As micro-ondas são radiações eletromagnéticas não ionizantes, que consistem em campos elétricos e magnéticos que oscila perpendicularmente uns aos outros nas bandas de frequência entre 0,3 e 300 GHz, variando entre 0,001 e 1 metro (CHAN et al., 2011; ROUSSY e PEARCE, 1995). Fortuny et al. (2008) dividiram a faixa de frequência eletromagnética em três bandas típicas: a frequência ultra alta (UHF – *ultra high frequency*, de 300MHz a 3GHz), frequência super alta (SHF – *super high frequency*, de 3 a 30GHz) e a frequência extremamente alta (EHF – *extremely high frequency*, de 30 a 300GHz). Figura 3.9 ilustra a faixa das micro-ondas no espectro eletromagnético.

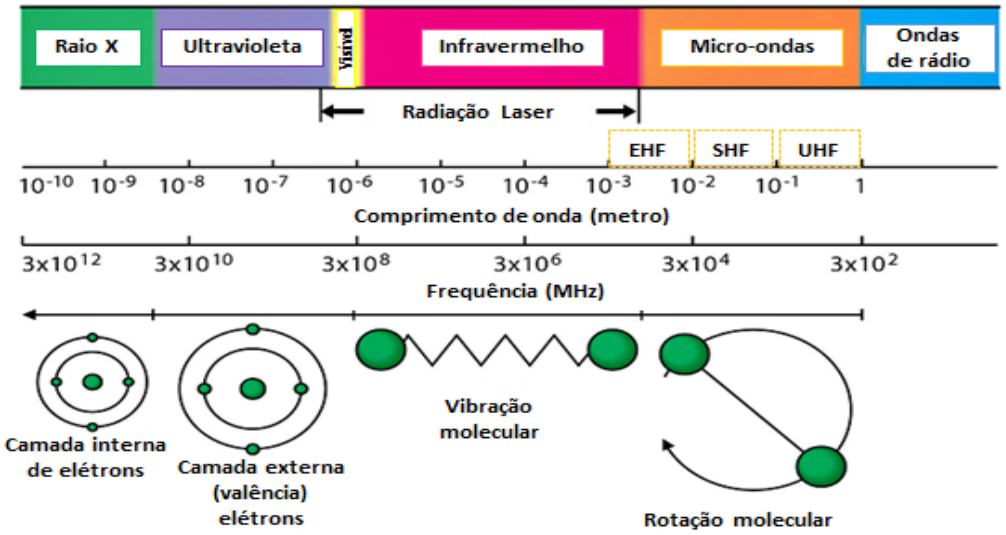

Figura 3.9: Espectro eletromagnético. Fonte: Adaptada de Fortuny et al. (2008) e Hayes (2002).

A Figura 3.10 mostra o esquema de aquecimento no método convencional por condução e no método por ondas eletromagnéticas. Nos métodos de extração convencionais que utilizam aquecimento, normalmente, a energia é transferida para o material por meio da condução, utilizando uma fonte externa que conduz o calor para o meio reacional através das paredes do recipiente, um processo que demanda longo tempo até atingir o equilíbrio térmico. Já no processo de aquecimento por micro-ondas, a energia é transferida diretamente ao material através de interações entre as moléculas e o campo eletromagnético e, desta forma, ao invés de transferência de calor, ocorre conversão de energia eletromagnética em térmica (STICHER, 2008; HAYES, 2002).

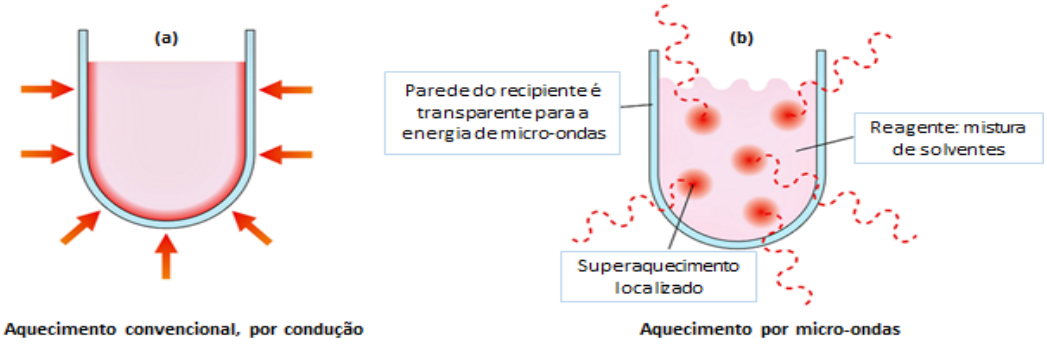

Figura 3.10: Esquema de aquecimento de amostra através do método convencional por condução **(a)** e do não-convencional por micro-ondas **(b)**. Fonte: Adaptada de Hayes (2002).

Como o aquecimento por micro-ondas não depende da condutibilidade térmica dos materiais do equipamento, o aquecimento ocorre de modo mais rápido e forma regiões de superaquecimento localizado, onde ocorrem as reações (HAYES, 2002). Esse aquecimento seletivo da matriz, resulta no aumento do volume celular até ocorrer a explosão das células, liberação do analito para a fase líquida e absorção das micro-ondas na fase líquida, que promove o aumento da velocidade de difusão e uniformidade do calor em todo o material (ALUPULUI et al., 2009). Desta forma, a utilização de energia micro-ondas para a extração de biomoléculas resulta em sistemas de aquecimento mais eficaz, transferência de energia mais rápida, redução de gradientes térmicos, aquecimento seletivo, melhor controle do processo de aquecimento, menor tempo de extração, alta eficiência, boa reprodutibilidade, e, além destes fatores, a utilização de reduzida quantidade de solvente orgânico, torna esse método favorável ao meio ambiente (CRAVOTTO et al., 2008; SPIGNO e FAVERI, 2009; ALUPULUI et al., 2009; ALUPULUI et al., 2012).

O aquecimento de materiais por meio de micro-ondas pode ser explicado por dois mecanismos: rotação dipolar e condução iônica, sendo que a água é o dipolo responsável pela maioria dos processos de aquecimento ao absorver a energia e, por superaquecimento, provoca a ruptura da estrutura celular permitindo a penetração do solvente na matriz (FORTUNY et al., 2008; ROUTRAY e ORSAT, 2012). No processo de aquecimento por rotação de dipolo, a energia é transformada em calor devido ao atrito dos dipolos com as moléculas vizinhas, que geram calor por fricção (VENKATESH e RAGHAVAN, 2004). No aquecimento por condução iônica, a amostra se comporta como um semicondutor elétrico, que ao sofrer com as mudanças no campo elétrico, movimentam os íons através da matriz em direções opostas, que provocarão o aquecimento da amostra ao transformar energia cinética em calor por meio da aceleração das cargas (FORTUNY et al., 2008; VENKATESH e RAGHAVAN, 2004).

De acordo com Spigno e Faveri (2009), os fatores que influenciam no processo de extração assistido por micro-ondas são o tempo de irradiação, razão matriz/solvente, temperatura e tipo de solvente utilizado. Os autores destacam também outro fator importante e que interfere bastante na eficiência do processo é a taxa de aquecimento, que é influenciada pela constante dielétrica dos solventes utilizados no processo. Solvente com constante dielétrica (descreve a polarização de uma molécula num campo elétrico) elevada, aquece o meio submetido à radiação mais rapidamente e, por conta do valor dessa constante, a água é o

melhor solvente para EAM e a sua adição em misturas de solventes costuma ser feita para aumentar o índice de polaridade da solução e assim melhorar a eficiência do processo.

Barbero et al. (2006) desenvolveram um método para a extração de capsaicinoides em pimentas (*C. frutescens*) empregando extração assistida por micro-ondas. Os autores estudaram o tipo de solvente (metanol, etanol, acetona, acetato de etilo e água), a temperatura (50-200 °C), a quantidade da amostra (0,1-1 g), volume de solvente (15-50 mL) e o tempo de extração (5-20 min). Os resultados obtidos mostraram que as condições ótimas são a uma temperatura de 125 °C, utilizando 25 mL de solvente, 0,5 g de amostra, etanol 100% como solvente num tempo de extração de 5min. Com o método otimizado, foi extraído 451,6 µmol de capsaicina/kg de pimenta fresca.

3.3.3 Sistema Aquoso Bifásico

A formação dos sistemas aquosos bifásicos (SAB) é conhecida desde o final do século XIX. Em 1896, o pesquisador Beijerinck, notou que soluções aquosas de gelatina e agar ou gelatina e amido, quando misturadas numa determinada faixa de temperatura e concentração, formavam misturas turvas que após ficarem em repouso se separavam de forma espontânea em duas fases líquidas límpidas, além disso, detectou que a fase inferior era mais densa e enriquecida em agar ou amido e que a fase superior era rica em gelatina, sendo a água o componente majoritário nas duas fases (SILVA e LOH, 2006).

Posteriormente, Ostwald e Hertel verificaram que amidos provenientes de diferentes matrizes produziram diferentes diagramas de fases. Já Dobry e Boyer-Kawenoki detectaram que existe uma incompatibilidade entre os polímeros. Entretanto, somente em meados da década de 50, com os trabalhos desenvolvidos por Per-Âke Albertsson, é que a comunidade científica pode conhecer melhor o grande potencial do SAB para a extração/reconcentração/partição/purificação de biomoléculas (ALBERTSSON et al., 1958; ZAFARANI-MOATTAR e NASIRI, 2010). Nas décadas seguintes, uma grande quantidade de trabalho foi desenvolvida utilizando o SAB. Até que em 2003, Gutowski e seus colaboradores apresentaram uma pesquisa pioneira sobre a possível criação de SAB utilizando sais inorgânicos às soluções de líquidos iônicos (GUTOWSKI et al., 2003).

O SAB pode ser definido como sendo o sistema de extração/purificação de compostos, baseado no princípio da extração líquido-líquido, que utiliza líquidos imiscíveis que, quando atingem o equilíbrio termodinâmico, se separam em duas fases (uma fase de topo e outra de fundo) transportando o analito de interesse para a fase que este possui maior afinidade e os

compostos considerados como contaminante, tendem a ir para a fase oposta. Podem ser formados por mistura de dois polímeros diferentes, por mistura de polímero com solução salina, mistura de álcool e sal, ou ainda utilizando líquidos iônicos, que são sais de baixo ponto de fusão que se encontram no estado líquido, quando em temperatura ambiente (WU et al., 2011). A Tabela 3.4 apresenta exemplos de tipos e composição para sistemas aquosos bifásicos do SAB que foi proposta por Pessoa Júnior e Kilikian (2005), com ampliação de exemplos.

Os primeiros trabalhos publicados com SAB utilizavam sistema composto por polímero e sal (ALBERTSSON, 1958), no entanto, em função da viscosidade do polímero, ao longo dos anos diversos sistemas alternativos foram sendo desenvolvidos como, por exemplo, álcool – K_3PO_4, K_2HPO_4 e K_2HPO_4/KH_2PO_4 (REIS et al., 2012; REIS et al., 2014; REIS et al., 2015), álcool – $(NH_4)SO_4$, NaH_2PO_4, K_2CO_3, K_3PO_4 (TAN et al., 2014), líquido iônico – $C_6H_5K_3O_4.5H_2O$ (PASSOS et al., 2014), surfactante – $Na_3C_6H_5O_7.5H_2O$, $MgSO_4.7H_20$, Na_2SO_4 (TAGHVIVAND et al., 2014).

Tabela 3.4: Diferentes composições de sistemas aquosos bifásicos.

Tipo de SAB	Exemplo de composição
Dois polímeros não iônicos	- Polietilenoglicol (PEG) + polissacarídeo, Dextrana, Polivinil Álcool - Polipropilenoglicol (PPG) + Dextrana
Polieletrólito e polímero não iônico	- Sulfato Dextrana de Sódio + PPG - Carboximetilcelulose de Sódio + Metil Celulose
Dois polieletrólitos	- Carboximetildextrana de Sódio + Carboximetilcelulose de Sódio, Sulfato Dextrana de Sódio
Polímero não iônico e composto de baixa massa molecular (Sal)	- PPG + Fosfato de Potássio, Glicose - PEG + Fosfato de Potássio, Glicose, Sulfato de magnésio - Metoxipolietilenoglicol + Fosfato de Potássio
Líquido iônico e Sal	- Líquido Iônico + Fosfato de Potássio
Solventes orgânicos e Sal	- Álcoois + Fosfato de Potássio
Líquido iônico e composto de alta massa molecular	- Líquido Iônico + Açúcares

A escolha do sal a ser utilizado no sistema, normalmente, é baseada na Série de Hofmeister, também conhecida como Série Liotrópica (Figura 3.11), que ordena um conjunto de cátions e ânions de acordo com sua capacidade de induzir o efeito *salting in* ou *salting out*

e, através desta sequência, é possível compreender a capacidade que os íons possuem para formar sistema bifásico (HOFMEISTER et al., 1888; SILVÉRIO et al., 2013).

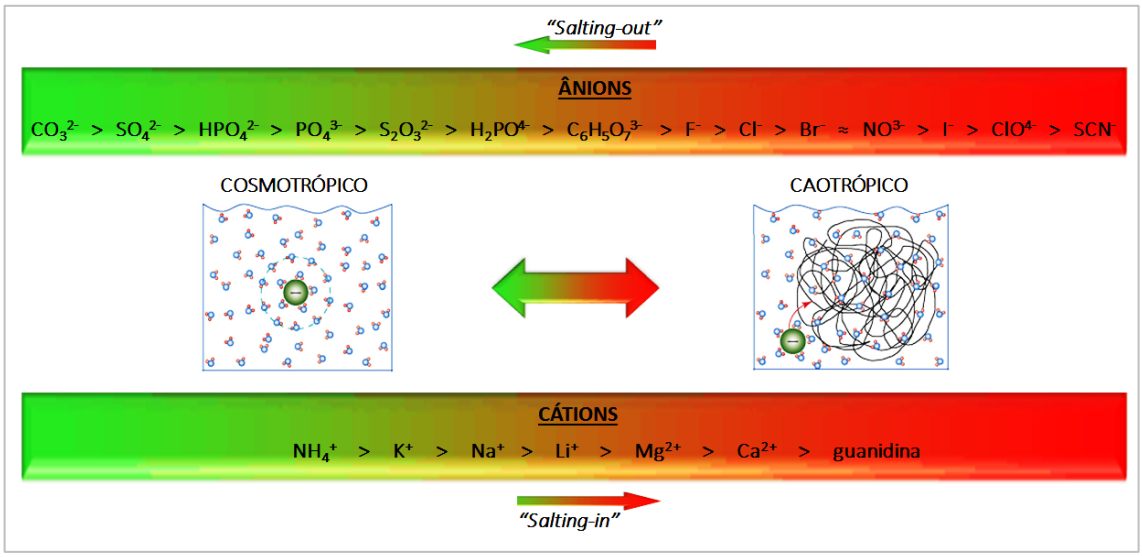

Figura 3.11: Série de Hofmeister. Fonte: Adaptada de Zhang et al. (2006), Hofmeister et al. (1988) e http://tinyurl.com/ed5gj.

Na Figura 3.11 também é possível analisar a capacidade que os íons possuem de interagir com a molécula de água. Partindo do Cl^- em direção à esquerda, os íons são classificados como cosmotrópicos, são íons formadores da estrutura da água por meio de pontes de hidrogênio, fortemente hidratado e estável, conduzem ao efeito *salting out* de macromoléculas. Já em direção à direita estão os chamados caotrópicos, que são tidos como desestabilizadores de estruturas da água que promovem o efeito *salting in* (ZHANG et al., 2006). Ainda de acordo com Zhang et al. (2006), as interações diretas entre íons e macromoléculas são, em grande parte dos casos, responsáveis pela maioria dos aspectos da capacidade que os íons têm de alterar a ligação de hidrogênio da água.

Nos últimos anos, a busca por SAB com nova e diferentes composições se intensificou. A acetonitrila (ACN, cianeto de metila, CH_3CN) é um solvente da classe dos apróticos, possui polaridade média, miscível em água a temperatura ambiente, amplamente utilizado em síntese orgânica, além de ser o solvente orgânico mais utilizado como fase móvel para separação de compostos, especialmente por cromatografia líquida, em função de suas propriedades físico-químicas favoráveis como a baixa viscosidade, alta resolução e baixo ponto de ebulição (NEMATI e SHEKAARI, 2013). Neste contexto, diversos SABs compostos por acetonitrila foram desenvolvidos em combinação com matéria-prima de custo

reduzido, quando comparado aos polímeros como, por exemplo, carboidratos (CARDOSO et al., 2013), polissacarídeos (CARDOSO et al., 2014b), polióis (CARDOSO et al., 2014a), polivinil ácool (CARDOSO et al., 2015) e líquido iônico [MBClm]SO_3Cl e [MBAIm]HSO_4 (OKANO et al., 2013).

Atualmente, os estudos com os líquidos iônicos foram intensificados em consequência de suas excelentes caraterísticas. Líquidos iônicos são sais que possuem baixa temperatura de fusão (< 100 °C) e que podem ser projetados para fins específicos por pré-seleção de combinações de cátions e ânions. A possibilidade de adaptar suas polaridades e afinidades para conseguir extrair biomoléculas específicas, apresenta-se como a principal vantagem de sua utilização. Sua ampla versatilidade permite a síntese de novos fluidos com um impacto ambiental aceitável e melhor biocompatibilidade (SHAHRIARI et al., 2013; PASSOS et al., 2014). Dentre a gama de líquidos iônicos que podem ser desenvolvidos, por diversas combinações, os líquidos iônicos baseados em íons colina tem sido sugerido para a preparação de SAB em combinação com sal (PEREIRA et al., 2013; PEREIRA et al., 2014; TAHA et al., 2015), polímero (SHAHRIARI et al., 2013) e tetrahidrofurano (SOUZA et al., 2015).

A colina foi oficialmente reconhecida como nutriente essencial para o funcionamento do corpo em 1998 pelo Instituto de Medicina dos Estados Unidos (Institute of Medicine, 1998). O Instituto destaca que a colina é necessária para a síntese de neurotransmissor, sinalização de membrana celular, transporte de lipídios, metabolismo metil-grupo e, além disso, desempenha papel importante no cérebro e desenvolvimento da memória ainda na fase fetal (ZEIZEL e COSTA, 2009). O cloreto de colina (cloreto de 2-hidroxietiltrimetilamônio ou vitamina B4) é a base para esta família de líquido iônico e destaca-se por ser uma matéria-prima barata, com alto ponto de fusão (298 – 304°C), biodegradável e de baixa toxicidade, o que permite que ela seja utilizada como aditivo alimentar (PETKOVIC et al., 2010; KHAN et al., 2014; SILVA et al., 2014). VENTURA et al., (2014) avaliaram a ecotoxicidade das colinas e concluíram que sua toxicidade depende do tipo de ânion e do cátion e que, em geral, apresentam baixa toxicidade para o organismo testado, a bactéria marinha *Vibrio ficheri*. As colinas também podem ser combinadas com ânions como o bicarbonato, etil malato, dihidrogeno citrato, bitartarato, entre outros (PEREIRA et al., 2013). A Figura 3.12 apresenta algumas estruturas químicas de líquidos iônicos baseados em colina.

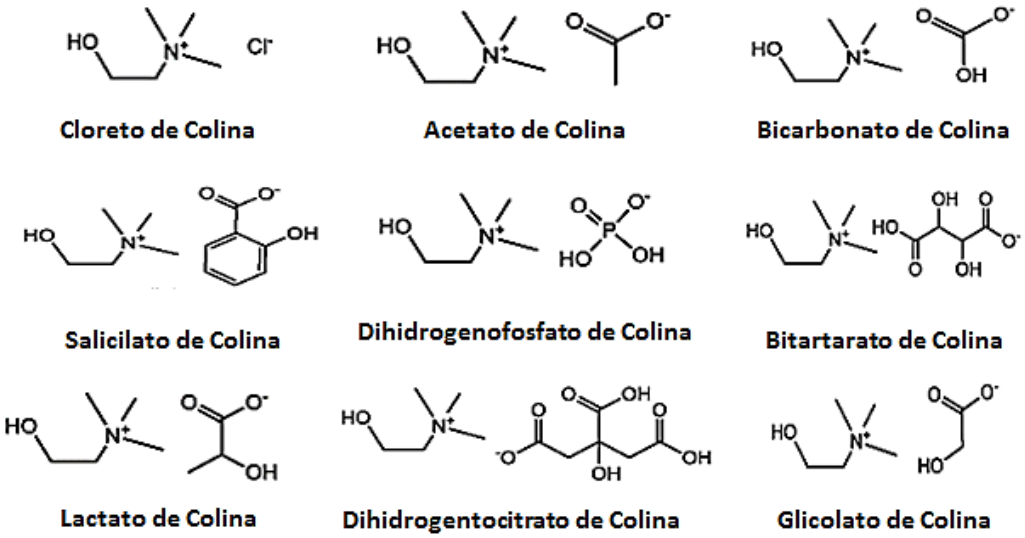

Figura 3.12: Estrutura química de líquidos iônicos baseados em colina. Fonte: Khan et al. (2014).

A ferramenta básica para o desenvolvimento dos estudos com SAB é o diagrama de equilíbrio de fases, que representa graficamente, em temperatura e pressão definidas, a composição em que as duas fases líquidas estão em equilíbrio termodinâmico. A Figura 3.13 apresenta o esquema representativo do diagrama de equilíbrio.

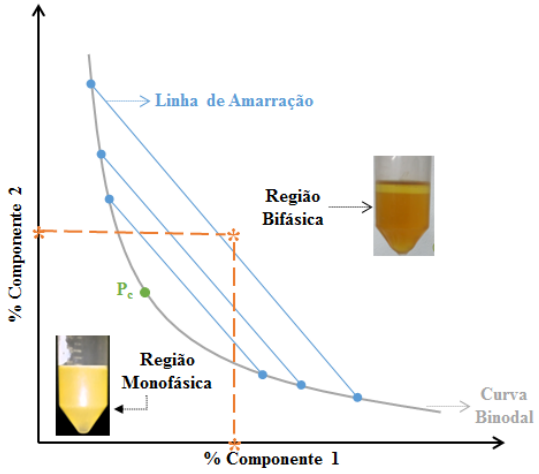

Figura 3.13: Diagrama de fases para sistema aquoso bifásico, expresso em coordenada retangular. Fonte: Adaptada de Silva e Loh (2006).

A Figura 3.13 apresenta o esboço de um diagrama de fases para um SAB. É possível visualizar a curva binodal, as linhas de amarração, o ponto crítico (Pc), além das regiões bifásica e monofásica. Também é possível obter informações sobre em quais composições

globais o sistema encontra-se na Região Monofásica ou Bifásica, estas regiões são delimitadas pela Curva Binodal. Segundo Martins et al. (2009), a posição da curva no diagrama pode variar de acordo com a hidrofilicidade/hidrofobicidade, peso molecular e natureza química dos compostos, temperatura e pH do meio.

Experimentalmente, a curva binodal pode ser determinada por cromatografia líquida de alta eficiência (CLAE) (ALBERTSSON, 1986), no entanto, o método do ponto de nuvem ou de turvação é o mais utilizado. Para correlacionar os pontos experimentais, Merchuck et al. (1998) propuseram um modelo matemático (Equação 3.1) que é amplamente utilizado até os dias de hoje, porém, outros modelos como o de Othmer-Tobias (Equação 3.2) e Bancroft (Equação 3.3) também podem ser aplicados (TAGHAVIVAND e PAZUKI, 2014).

$$[\mathrm{Y}] = \mathrm{A}\,x\,\exp\,\{(\mathrm{B}\,x\,[\mathrm{X}]^{0,5}) - \,(\mathrm{C}\,x\,[\mathrm{X}]^{3})\} \tag{3.1}$$

$$ln\left(\frac{100-Y_T}{Y_T}\right) = a + b\;x\;ln\left(\frac{100-X_F}{X_F}\right) \tag{3.2}$$

$$ln\left(\frac{100-X_F-Y_F}{X_F}\right) = c + d\;x\;ln\left(\frac{100-X_T-Y_T}{X_T}\right) \tag{3.3}$$

onde X e Y são as porcentagens em fração mássica do componente 1 e 2, respectivamente, os subscritos T e F referem-se as fases de topo e fundo, e os parâmetros de ajuste a, b, c e d são obtidos pela regressão dos mínimos quadrados.

As retas que ligam pontos na curva representam as composições de equilíbrio e são denominadas Linhas de Amarração ("*tie lines*" - TL), qualquer conjunto de pontos que pertençam à região bifásica e que estejam sobre a mesma linha de amarração fornecerá fases de topo e fundo com mesma composição, mas com diferente relação de volume entre as fases (SILVA e LOH, 2006). A determinação das TL pode ser elaborada segundo Merchuck et al. (1998). Ainda na curva binodal, está demarcado o Ponto Crítico (PC), neste as propriedades físico-químicas das duas fases são teoricamente iguais e a composição e volume das fases são iguais (ALBERTSSON, 1986).

O cálculo do comprimento da linha de amarração (frequentemente referido como TLL do inglês "*Tie Line Length*") é um importante parâmetro termodinâmico e, geralmente, é

usado como variável determinante do processo de partição. Segundo Silva e Loh (2006), o TLL pode ser calculado por meio da equação (3.4):

$$\mathrm{TLL} = \sqrt{([\mathrm{X}]_\mathrm{T} - [\mathrm{X}]_\mathrm{F})^2 + ([\mathrm{Y}]_\mathrm{T} - [\mathrm{Y}]_\mathrm{F})^2} \quad (3.4)$$

Normalmente, o SAB pode ser avaliado calculando dois parâmetros, a eficiência da extração (EE), que informa o quanto o sistema foi eficiente na transferência da biomolécula para a fase de topo, ou por meio do cálculo do coeficiente de partição (K), que relaciona a concentração da biomolécula que migrou para a fase de topo com a concentração que migrou para a fase de fundo, através da equação (3.5).

$$\mathrm{k} = \frac{\mathrm{C_T}}{\mathrm{C_F}} \quad (3.5)$$

onde, C_T é a concentração da biomolécula na fase de topo e C_F na fase de fundo.

Vale ressaltar que:

- Se k = 1, a biomolécula está presente na fase de topo e de fundo em concentrações iguais.
- Se k >> 1, a biomolécula está mais concentrada na fase de topo.
- Se k << 1, a biomolécula está mais concentrada na fase de fundo.

Ao desenvolver um estudo com SAB, é importante avaliar alguns efeitos que podem ocorrer durante a formação e estabilização do sistema (CHAIWUT et a., 2010). Segundo Wang et al. (2010), esses efeitos influenciam não somente a formação do sistema, mas também a partição da biomolécula. Os principais efeitos relatados pelos autores estão destacados abaixo:

- Massa molar e concentração dos componentes: ao alterar a composição das fases de topo e fundo, pode ocorrer mudança na solubilidade da biomolécula que, a depender de seu caráter, sofrerá maior efeito *salting-out* ou *salting-in* e particionará, preferencialmente, para a fase mais hidrofóbica ou hidrofílica, respectivamente (BABU et al., 2008). Ao aumentar a concentração de um componente, pode ocorrer o efeito do volume de exclusão por ocupar os espaços intersticiais de uma fase, podendo resultar na redução da solubilidade e aumento da viscosidade na fase oposta (RAWDKUEN et al., 2011).

- pH: pode alterar a carga elétrica (positiva/neutra/negativa) e induzir processos de associação ou dissociação, estas alterações podem provocar mudanças na forma como a biomolécula interage com o sistema e modificar o comportamento da partição (WANG et al., 2010; SILVA e LOH, 2006).
- Temperatura: seu efeito varia de acordo com a composição do sistema e o tipo de biomolécula a ser particionada. Segundo Coimbra et al. (2003), sistemas cuja composição se aproxima do ponto crítico são mais afetados por mudanças de temperatura, devido à instabilidade inerente à região do ponto crítico. Alguns trabalhos relatam aumento do coeficiente de partição com o aumento da temperatura (REIS et al., 2014; PESSOA-JUNIOR e KILIKIAN, 2005). O efeito da temperatura sobre o equilíbrio de fases pode ser melhor estudado analisando as propriedades termodinâmicas.

Segundo Cooper (1999), as propriedades termodinâmicas que melhor descrevem um sistema em equilíbrio é a entalpia (H), entropia (S) e a energia livre de Gibbs (G). Considerando a partição de uma biomolécula por SAB, os parâmetros termodinâmicos devem ser determinados de acordo com a teoria de Flory-Huggins, que descreve a energia necessária para a obtenção da energia livre de Gibbs na mistura ($\Delta G_m°$) (COOPER, 1999; JOHANSSON et al., 2011). Assim, a função da energia livre à pressão constante e a nível molecular estão apresentadas nas equações seguintes.

$$\Delta G^0{}_m = \Delta H^0{}_m - T\ x\ \Delta S^0{}_m \tag{3.6}$$

$$\ln(K) = -\frac{\Delta H^0{}_m}{R}\ x\ \frac{1}{T} + \frac{\Delta S^0{}_m}{R} \tag{3.7}$$

onde R é a constante universal dos gases (8,314 J/mol.K), T é a temperatura (Kelvin) e K é o coeficiente de partição da biomolécula.

Considerando a Equação 3.6, observa-se que $\Delta G_m°$ possui contribuição de natureza entálpica ($\Delta H_m°$) e entrópica. ($\Delta S_m°$). No SAB, o valor de $\Delta G_m°$ nunca poderá ser zero porque sempre haverá contribuição e, desta forma, a $\Delta G_m°$ será função das interações resultantes presentes em solução, bem como das diferentes distribuições das moléculas na solução. Termodinamicamente, quando $\Delta G_m° < 0$, a partição da biomolécula no sistema ocorre de forma espontânea, se $\Delta H_m° < 0$ o processo é exotérmico e se $\Delta H_m° > 0$ é endotérmico. Para saber qual tipo de força governa a migração da biomolécula, pode ser feita uma análise

baseada na Equação 3.7 e, desta forma, se T x $\Delta S_m°$ > $\Delta H_m°$, o processo é governado por forças entrópicas, que tende a deslocar a biomolécula para a fase de topo do sistema, do contrário, as forças entálpicas governaram a partição e direcionam o analito para a fase de fundo (SILVA e LOH, 2006).

Cardoso et al. (2014) estudaram a partição da vanilina utilizando SAB com composição inédita (polivinil álcool com acetonitrila) e obtiveram os melhores resultados (K = 2,24 e R_T = 79%) utilizando um sistema composto por 49% em peso de ACN + 9% em peso de PVA a 5°C. A vanilina migrou para a fase superior (rica em acetonitrila) de forma espontânea e num processo exotérmico.

Souza et al. (2015) desenvolveram um SAB composto por líquido iônico a base de colina e tetra-hidrofurano (THF) para purificar lipase. As condições ótimas para partição utilizaram 40% em peso de THF e 30% em peso de bitartarato de colina a 25ºC, que resultaram num fator de purificação de 130,1 ± 11,7 vezes, rendimento de 90,0 ± 0,7% e um coeficiente de partição da enzima para a fase de fundo rica em colina igual a 0,11 ± 0,01 e proteína contaminantes para a fase rica em THF (K = 1,16 ± 0,1). Termodinamicamente, o processo acontecer de forma espontânea, endotérmica e governada por forças entrópicas.

Reis et al. (2014) extraíram rutina a partir de resíduos de acerola por SAB utilizando um sistema composto por álcool e sais de fosfato de potássio e observaram que os SABs estudados são versáteis, pois permitiram que a biomolécula seja recuperada tanto na fase de topo quanto na fase de fundo, ricas em álcool e sal, respectivamente. O maior coeficiente de partição (51,47) e eficiência de extração (98,64%) foram obtidos em SAB consistindo de 1-propanol (40% em peso) + K_2HPO_4 / KH_2PO_4 (20% em peso) + água (40% em peso) e 2,5 (% em peso) de NaCl à 298,15 K. A migração da rutina aconteceu de forma, termodinamicamente, espontânea, de forma endotérmica e governada por forças entrópicas. Os autores obtiveram sucesso ao extrair a rutina de amostras reais e os resultados concordaram e validaram o sistema modelo proposto.

Tan et al. (2014) separaram e purificaram ácido clorogênico (CGA), a partir da folha de rami (*Boehmeria nivea)*, utilizando SAB composto por etanol e NaH_2PO_4. Os resultados mostraram que o CGA migrou, preferencialmente, para a fase rica em álcool com um rendimento de 95,76% a 25 °C e pH 3,29 em um SAB composto por 15,2 % de etanol, 28,1 % de sal e 56,7 % de água. Os autores também destacaram a importância do estudo da termodinâmica junto com a cinética e propuseram um sistema de reciclagem das fases de forma fácil, ecológica, mais barata e viável para produção em larga escala.

Capítulo IV

INTRODUÇÃO AO CAPÍTULO IV

O capítulo IV será apresentado dividido em forma de artigos científicos (ARTIGO I, II, III e IV) os quais correspondem aos resultados referentes aos estudos da extração e purificação da capsaicina a partir de sua fonte natural, as pimentas do gênero *Capsicum*. Os artigos foram organizados de acordo com as normas propostas pelo periódico de publicação.

ARTIGO I – Extraction of capsaicin from *Capsicum* fruits: a comparison study between conventional methods and unconventional assisted by ultrasound and microwave extraction. Artigo submetido ao periódico *Food Chemistry*.

ARTIGO II – Recovery of capsaicin from *Capsicum frutescens* by applying aqueous two-phase systems based on acetonitrile and cholinium-based ionic liquid. Artigo aprovado no periódico *Chemical Engineering Research and Design*.

ARTIGO III – Aqueous two-phase systems based on acetonitrile and salts of Na^+ and K^+ to purification of capsaicin from *Capsicum frutescens*. Artigo a ser submetido ao periódico *Separation and Purification Technology*.

ARTIGO IV – Partitioning of capsaicin using aqueous two-phase systems based on ethanol + sodium salts. Artigo submetido ao *periódico Process Biochemistry*.

ARTIGO I

Extraction of capsaicin from *Capsicum* fruits: a comparison study between conventional methods and unconventional assisted by ultrasound and microwave extraction

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Abstract

Capsaicin is the main component responsible for characteristic pungency and therapeutic potential of Capsicum peppers; notwithstanding the pharmaceutical application, it is necessary to develop a fast and efficient method to extract it from biomass. This paper aims to extract capsaicin from Capsicum peppers using conventional methods and unconventional methods assisted by ultrasound and microwave. Three different varieties of peppers were used (cumari-do-Pará, malagueta and jalapeño) in two stages of maturation. The highest capsaicin content was found in mature pepper cumari-do-Pará (411.73 μg of capsaicin.g^{-1} pepper). The unconventional methods were more efficient (≥ 600%) than the conventional methods; furthermore, the technique assisted by microwave was 55% better than ultrasound, extracting 4.59 and 2.96 mg of capsaicin.g^{-1} pepper, respectively. The optimised conditions for the method assisted by microwave comprised aqueous solution with 70% ethanol, 0.2 of the ratio of solid/liquid at 125°C, 250 W, and without stirring for 1 min.

Keywords: capsaicin; extraction; ultrasound-assisted extraction; microwave-assisted extraction.

1. Introduction

The peppers are widely used in the culinary industry due to the flavour and aroma that confer to food; furthermore, it is a rich source of metabolites such as vitamins (C, B complex, A and E) and antioxidants (flavonoids, phenolic acids, carotenoids and capsaicinoids) with a high potential to promote health (Topuz & Ozdemir, 2007). The genus *Capsicum* comprises a large number of wild and cultivated pepper species (Wahyuni et al., 2013), which are cultivated all over the world, especially species such as *C. annuum*, *C. chinense* and *C. frutescens*.

The species *C. chinense* is originated from the Amazon region; however, it is commercially grown throughout southern and northern Brazil, due to its adaptability to different soils and local climates, and its popular citrus-like aroma. Fruits from this species show an enormous variability in size and shape and in the different intensities of yellow, orange or red when ripe (Lannes et al., 2007). Among the species, the variety cumari-do-pará has a special highlight due to yellow colouring when mature, flavour, aroma and pungency.

The pungency of the *Capsicum* fruits is a characteristic unique to peppers of this genus, due to the accumulation of capsaicinoids in its placenta, and the concentration of these compounds is one of the most important quality parameters for peppers (Keyhaninejad et al., 2014). The group of capsaicinoids is composed of nordihydrocapsaicin, dihydrocapsaicin, homodihydrocapsaicin, homocapsaicin and capsaicin, which is the main component responsible for pungency of the peppers (Davis et al., 2007).

The interest in the capsaicinoids, primarily capsaicin, extends beyond their sensory and nutritional properties. Capsaicin has therapeutic and pharmacological activity, with potential analgesic, antioxidant, cancer prevention, weight reduction, cardiovascular and gastrointestinal benefits (Sharma et al., 2013). However, to take advantage of the functional

potential of this biomolecule, firstly, it is necessary to extract it from its natural source, the pepper.

Traditionally, the biomolecule extraction is performed using conventional methods already established and which generally are easy to use and do not require sophisticated equipment and using cold or hot solvent, e.g. stirring in water bath (Chinn et al., 2011) and Soxhlet (Bajer et al., 2015). However, factors such as the use of large amounts of solvent, the need for long contact time, and the low yield contribute to reducing the viability of these methods (Yang et al., 2008). In this context, non-conventional methods of extraction are emerging as processes which are fast and efficient, such as the extraction by supercritical fluid (Aguiar et al., 2013), assisted by ultrasound (Barbero et al., 2008; Boonkird et al., 2008; Santos et al., 2015) and assisted by microwave (Barbero et al., 2008).

The use of ultrasound may be a promising way to introduce innovation in extraction processes (Esclapez et al., 2011). This technique is based on the formation of high-frequency ultrasonic waves, which are capable of causing cavitation due to the expansion and contraction cycles through which the material goes when submitted to ultrasound (Santos et al., 2015). These cycles disrupt the cell walls of the vegetable matrix, favouring the penetration of the solvent and the mass transfer, thus increasing the extraction rate and yield (Toma et al., 2001). Among the potential advantages in the use of extraction assisted by ultrasound when compared to conventional extraction methods, the shorter extraction time, the high yield, and the facility to control parameters of the process are cited, which are due, principally, to the phenomenon of cavitation (Rathod & Rathod, 2014). Ultrasound-assisted extraction (UAE) has been used in the extraction of pigments (Maran et al., 2015), alkaloids (Hossain et al., 2014), and piperine (Rathod & Rathod, 2014), among other biomolecules.

A microwave is defined as an electromagnetic wave which consists of electric and magnetic fields oscillating perpendicularly to each other in a frequency ranging from 0.3 to

300 GHz (Chan et al., 2011). During absorption, the microwaves' energy is converted into kinetic energy, enabling the selective heating of the microwave-absorbent parts of the plant material. The volume increased in this way makes cells explode, releasing their content into the liquid phase. When the liquid phase absorbs the microwaves, the kinetic energy of its molecules increases and, consequently, the diffusion rate increases (Alupului et al., 2009). The use of microwave energy for the extraction of biomolecules results in more effective heating, faster energy transfer, reduced thermal gradients, selective heating, faster response to process heating control, faster start-up, and increased production. In addition to these factors, the use of reduced content of organic solvent also contributes to this technique and can be considered environment-friendly (Alupului et al., 2009). Microwave-assisted extraction (MAE) is found as a promising technique for sample preparation because of the rapidity, simplicity, high extraction efficiency, and low solvent and sample consumption (Chen et al., 2008; Xie et al., 2014). Recent studies have shown that MAE has been applied successfully in the extraction of polyphenols (Dahmoune et al., 2015), polysaccharide (Maran et al., 2015), and pectin (Maran et al., 2013), among others.

Considering that the capsaicin content can vary according to the species and that the improvement of extraction techniques is a continuous need, this study aims to compare conventional extraction techniques, using cold and hot organic solvent with unconventional techniques assisted by ultrasound and microwave, to extract the capsaicin from *Capsicum* fruit in varieties jalapeno, malagueta and cumari-do-pará, a variety rarely studied.

2. Materials and Methods

2.1 Materials

Ethanol, 1-propanol and 2-propanol were obtained from Vetec (Rio de Janeiro, Brazil), all with purities of ≥ 98 wt.%. The acetonitrile, an HPLC grade with purity of 99.9 wt.%, was purchased from Sigma. Ultrapure and distilled water was used in all experiments.

Capsicum peppers used in this study were acquired from a local producer from Lagarto, Sergipe (Brazil), in two maturation stages (green and mature) and in the varieties jalapeño (*C. annuum*), malagueta (*C. frutescens*) and cumari-do-pará (*C. chinense*), which are presented in Figure 1. The peppers were selected, sanitised with sodium hypochlorite solution (10 mg.L^{-1}), dried in an oven at 65 ± 1 °C until a constant weight (which means free of water), triturated in a mill, packed in polypropylene bags, and stored at room temperature in a dry and sheltered place of light until the next experiment.

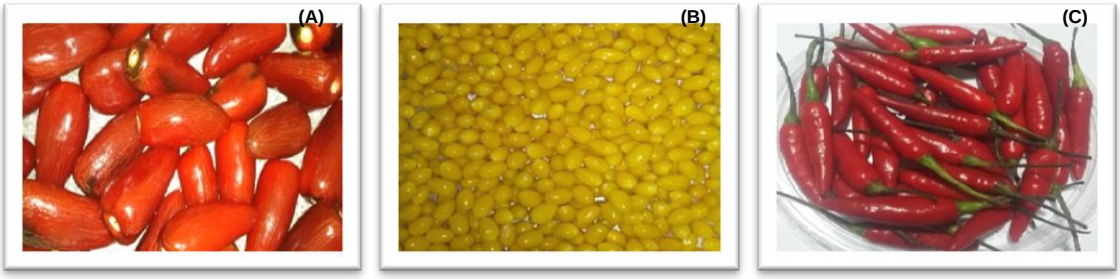

Figure 1: Capsicum peppers aspect of the varieties studied, in mature stage: **(A)** jalapeño (*C. annuum*); **(B)** cumari-do-pará (*C. chinense*); **(C)** malagueta (*C. frutescens*).

2.2 Conventional methods

2.2.1 Stirring in water bath

The extraction of capsaicin was carried out following methodology described by Chinn et al. (2011), with modifications. Briefly, 1 g of pepper (previously prepared as

described above) was weighed and 25 mL of solvents (ethanol, 1-propanol, 2-propanol, methanol, acetonitrile and water) was used. Then the samples were placed in a bath at 25 ± 1 ^{o}C, under constant stirring, for 4 hours, using a Marconi MA-095 shaker. The pepper extracts were filtered through a Millipore filter (0.45 µm) and analysed by HPLC.

2.2.2 Soxhlet extraction

The extraction was performed using a 7.2g sample in a flat-bottomed flask; 180 mL of ethanol was added and the system was placed under reflux for 2 h (Bajer et al., 2015). After this period, the flask was cooled down to room temperature, concentrated until 10 ml, and the extract filtered through a Millipore filter (0.45 µm) and analysed by HPLC.

2.3 Ultrasound-assisted extraction (UAE)

The extraction of capsaicin from peppers using ultrasound was carried out while employing different extraction conditions: temperature: 25–70°C; solvents: ethanol, 1-propanol, 2-propanol, methanol, acetonitrile and water; ratio of mass/volume: 0.01–0.4; extraction time: 5–30 min; solvent concentration: 0–100%; and percentage of power: 0–100%. The pepper extracts were filtered through a Millipore filter (0.45 µm) and analysed by HPLC. The UAE process was performed in an ultrasonic cleaner, model USC-2850A.

2.4 Microwave-assisted extraction (MAE)

The extraction of capsaicin from peppers using microwaves was carried out while employing different extraction conditions: temperature: 25–150°C; solvents: ethanol, 1-propanol, 2-propanol, methanol, acetonitrile and water; power: 50–300 W; ratio of mass/volume: 0.01–0.2; extraction time: 1–30 min; solvent concentration: 0–100%; magnetic stirring: off–high. The pepper extracts were filtered through a Millipore filter (0.45µm) and

analysed by HPLC. The MAE process was performed in a Discover SP ActiVent microwave extractor (CEM, Matthews, North Carolina, USA).

2.5 Analytical method

The extract of pepper was analysed by a high-performance liquid chromatography (HPLC) (model Prominence, brand Shimadzu) system with a UV-VIS detector, wavelength 280 nm, C18 column type, mobile phase consisting of acetonitrile:water (60:40) at a flow rate of 1.0 mL/min, isocratic mode, column temperature 30°C, and injection volume equal to 20 µL. The conditions were adapted from Chinn et al. (2011). For identification and quantification of compounds, we first constructed a calibration curve using standard capsaicin solution. A known concentration was injected and the curve thus constructed.

2.6 Statistical analysis

The experimental data were analysed using the SAS 9.0 statistical program. Treatment means were compared by a Tukey test at 5% and their significance was established by ANOVA. Differences of $p > 0.05$ were considered statistically significant. For each extraction condition tested, analyses were performed in triplicate and results of capsaicin concentration presented as the mean ± SD.

3. Results and Discussion

3.1 Conventional methods

According to Wahyuni et al. (2011), the concentration of capsaicin in the pepper is variable and depends on genetic and environmental factors, crop management, and harvesting age. Based on the results presented in Tables 1 and 2, it is possible to evaluate the influence of

the variety and maturation stage on the content of capsaicin and, besides, the effect of the type of solvents used to extract capsaicin from *Capsicum* peppers.

Evaluating the content of capsaicin in relation to varieties studied, the results showed that there is a statistically significant difference between the three varieties, with as much the green stage (Table 1) as the mature stage (Table 2). It is noted that the pepper cumari-do-pará has higher capsaicin content (green: 11.23–200.89 $\mu g.g^{-1}$ of pepper; mature: 11.46–284.73 $\mu g.g^{-1}$ of pepper) than the malagueta pepper (green: 25.14–106.59 $\mu g.g^{-1}$ of pepper; mature pepper: 8.85–117.61 μg.g-1 of pepper) or jalapeño pepper (green: 3.79–14.32 $\mu g.g^{-1}$ of pepper; mature pepper: 1.25–7.43 $\mu g.g^{-1}$ of pepper). Considering the extraction time and temperature used, the results are in accordance with the values found by Othman et al. (2011) that varied between 0.99 and 4249 $\mu g.g^{-1}$ of pepper.

Comparing the results shown in Table 1 with Table 2 in relation to the maturation stage, it is observed that the cumari-do-pará and malagueta peppers in the mature stage have a higher content of capsaicin than the green stage. According to Gnayfeed et al. (2001), capsaicin content tends to be higher in mature fruit than green fruit. However, this tendency was not observed for the jalapeño pepper and the results showed that the green pepper has higher capsaicin content (3.79–14.32 $\mu g.g^{-1}$ of dry pepper) than the mature pepper (1.25–7.43 $\mu g.g^{-1}$ of dry pepper), which can be related to the climate and soil of the cultivation region, crop management or peroxidase activity, and the type of enzyme which participates in the biosynthesis of capsaicin in the cells of fruit placenta. Contreras-Padilla and Yahia (1998) studied the evolution of capsaicinoid content during development, maturation and senescence in *C. annuum* peppers and they observed that the capsaicin content can be related to the peroxidase activity, since the capsaicin concentration decreased with the increase of maturation and the peroxidase activity, indicating that this enzyme is also involved in the degradation of capsaicinoids. Nevertheless, the capsaicin content found in this study agreed

with the values reported in the literature by Kozukue et al. (2005), which found capsaicin values for Jalapeño peppers ranging from 2.6 to 8.2 μg of capsaicin.g^{-1} of pepper, and Harvell and Bosland (1997) explained that the capsaicinoid content may differ by up to 90 mg per fruit depending on environmental factors.

The selection of a solvent to extract a bioactive compound must consider the molecular affinity between the solvent and solute to mass transfer, environmental security, toxicity, and financial viability (Azmir et al., 2013). Analysing Tables 1 and 2, it is also possible to evaluate the potential of different solvents used to extract capsaicin from *Capsicum* peppers. It is observed that, among the solvents tested, methanol was the best and water was the worst solvent to extract the biomolecule. According to Sharma et al. (2013), capsaicin is a compound that presents more nonpolar character and, due to high polarity of water, this result was already expected.

We considered the polarity of solvents used: 2-propanol – 3.9; 1-propanol – 4.0; methanol – 5.1; ethanol – 5.2; acetonitrile – 5.8; water – 9.0 (Byers, 2009). Methanol and ethanol are solvents with intermediate polarity and their potential extractive was reported in literature by Aguiar et al. (2014). It is noted in Tables 1 and 2 that there was no statistically significant difference ($p > 0.05$) between the capsaicin content extracted using methanol (the best solvent extractor, in absolute value) and ethanol from both malagueta and jalapeño peppers. However, in the case of the cumari-do-pará pepper, it is observed that there was a statistically significant difference ($p > 0.05$) between methanol and ethanol values in the two maturation stages (Table 1). The genetic characteristics and content of capsaicin of each pepper may have contributed to this different behaviour observed between the varieties of peppers and, in the case of the cumari-do-pará pepper, the difference in polarity between methanol and ethanol influenced the extraction of capsaicin.

Table 1: Capsaicin concentration (μg.g^{-1} of dry pepper) in green pepper, using conventional method by stirring in bath with different solvents.

Green Pepper	Solvents (μg.g^{-1} of dry pepper)					
	2-propanol	**1-propanol**	**methanol**	**ethanol**	**acetonitrile**	**water**
Cumari-do-pará	171.78 ± 1.23 Ac	188.48 ± 1.63 Ab	200.89 ± 0.83 Aa	166.83 ± 3.34 Ad	127.79 ± 0.82 Ae	11.23 ± 0.14 Af
Malagueta	99.40 ± 1.06 Bc	102.34 ± 0.38 Bbc	106.59 ± 0.20 Ba	104.29 ± 0.02 Bab	84.68 ± 0.22 Bd	25.14 ± 0.17 Be
Jalapeño	8.99 ± 0.11 Ccd	9.99 ± 0.41 Cbcd	14.32 ± 0.18 Ca	13.09 ± 0.16 Cab	11.62 ± 0.21 Cac	3.79 ± 0.11 Ce

* Means followed by the same capital letter in the column and lower case on the line do not differ statistically by Tukey test ($p > 0.05$).

Table 2: Capsaicin concentration (μg.g^{-1} of dry pepper) in mature pepper, using conventional method by stirring in bath with different solvents.

Mature Pepper	Solvents (μg.g^{-1} of dry pepper)					
	2-propanol	**1-propanol**	**methanol**	**ethanol**	**acetonitrile**	**water**
Cumari-do-pará	183.87 ± 1.21 Ad	195.66 ± 4.02 Abc	284.73 ± 5.26 Aa	199.05 ± 5.05 Ab	193.47 ± 8.30 Abc	11.46 ± 0,43 Ae
Malagueta	110.53 ± 0.62 Ba	113.23 ± 0.33 Ba	117.61 ± 0.40 Ba	115.99 ± 0.34 Ba	106.38 ± 0.07 Ba	8.85 ± 0.31 Bb
Jalapeño	5.49 ± 0.04 Ca	6.11 ± 0.20 Ca	7.43 ± 0.23 Ca	6.59 ± 0.10 Ca	5.50 ± 0.26 Ca	1.25 ± 0.07 Ca

* Means followed by the same capital letter in the column and lower case on the line do not differ statistically by Tukey test ($p > 0.05$).

The cumari-do-pará pepper, in the mature stage, showed the best results in relation to malagueta and jalapeño peppers using methanol (284.73 μg.g^{-1} of dry pepper). However, methanol is a solvent which presents toxicity, which limits its application in foods and pharmaceutical products (FDA, 2012). Therefore, as the second-best result was obtained using ethanol (199.05 μg.g^{-1} of dry pepper), it was chosen as the best solvent to extract capsaicin from *Capsicum* peppers.

Soxhlet extraction is an established technique and widely used for extraction of bioactive compounds from their natural sources (Azmir et al., 2013). In general, the extraction yields obtained by Soxhlet are higher than by maceration and agitation, since it uses the solvent in boiling temperature, which facilitates the action of the solvent within the solid matrix (Aguiar et al., 2014). The results were presented in Table 3.

Table 3: Capsaicin concentration (μg.g^{-1} of dry pepper), by Soxhlet extraction using ethanol.

Mature Pepper	**Maturation Stage (μg.g^{-1} of dry pepper)**	
	Green	**Mature**
Cumari-do-pará	340.68 ± 2.99 Ab	411.73 ± 16.85 Aa
Malagueta	231.14 ± 0.90 Ba	239.55 ± 5.88 Ba
Jalapeño	33.31 ± 2.32 Ca	21.05 ± 0.63 Ca

* Means followed by the same capital letter in the column and lower case on the line do not differ statistically by Tukey test ($p > 0.05$).

The results showed that there was a statistically significant difference ($p > 0.05$) between the varieties studied. The cumari-do-pará pepper has a higher content of capsaicin (green: 340.68 and mature: 411.72 μg.g^{-1} of dry pepper) than the malagueta (green: 231.14 and mature: 239.55 μg.g^{-1} of dry pepper) and jalapeño (green: 21.06 and mature: 33.31 μg.g^{-1}

of dry pepper). However, only the cumari-do-pará pepper presented a difference between the maturation stage, and the best results were found in the mature pepper. The results agree with the values found by Bajer et al. (2015), who analysed with Soxhlet the same species studied in this work, but with different varieties, using methanol as solvent for 2 h and capsaicin detected in a range between 285 and 3,191 $\mu g.g^{-1}$ of pepper. The method applied in this study used ethanol as solvent for 2 hours and found values within the range observed by Peña-Alvarez et al. (2009) (0.15–5.93 $mg.g^{-1}$ of pepper), using ethanol as solvent for 5 h.

3.2 Unconventional methods

3.2.1 Ultrasound Assisted Extraction (UAE)

Six parameters (type of solvent, extraction time, ratio of mass/volume, solvent concentration, power, and temperature) were investigated for the best extraction of capsaicin, as shown in Figure 2. Initial extractions were performed based on the conditions optimised by Barbero et al. (2008), using a 0.5g sample and 12.5 mL of solvent extraction for 10 minutes at room temperature.

It is noted in Figure 2A that methanol was the best solvent to extract capsaicin from the cumari-do-pará pepper, followed by ethanol. Again, the polarity influenced the yield of extraction. However, due to the reasons presented in section 3.1.1, ethanol was selected to continue the experiment. Figure 2B depicts that 10 minutes was enough to get good results in the extraction of capsaicin; from that, there was a slight decrease in yield, possibly caused by a saturation in the extraction system, since after a certain period the solute concentration in the solid matrix and in the solution tends to attain an equilibrium or due to degradation of biomolecules in function to the contact time between the sample and the solvent on the ultrasonic waves (Maran et al., 2015).

The reduction in the amount of solvent and, therefore, the increase in the ratio of mass/volume have improved the mass transfer and the extraction efficiency because the capsaicin was concentrated in a lesser amount of extract (Figure 2C). This fact can be justified by the collapsing in the cell wall cavities caused by the ultrasound energy absorbed; this action can result in enlargement of the pores or cause disruption of the cell wall, increasing the adsorption of solvent, improving the mass transfer and, consequently, increasing the extraction of capsaicin (Esclapez et al., 2011). Despite the ratio of mass/volume being equal to 0.4 having presented the best result, this ratio was not chosen to continue the experiment because of the small volume of final extract and consequent difficulty in performing the membrane filtration, which is a necessary step for the realisation of HPLC analysis. Then the ratio chosen for realisation of the next study was equal to 0.2.

Boonkird et al. (2008) studied the influence of some operational parameters for capsaicinoid extraction in a laboratory and pilot plant scale, and obtained better results using 75% ethanol, although they chose to work with 95% solvent due to high cost and energy consumption for recovery of the solvent in the final extract. The results presented in Figure 2D showed that the addition of water in ethanol did not influence the extraction of capsaicin by use of ultrasonic waves. It was observed that the higher the amount of water in the extract, the less efficient the extraction. This occurred, possibly, due to the polarity difference between the aqueous solution and the capsaicin, which is considered a biomolecule practically insoluble in water (Sharma et al., 2013). Even though there was a little difference in performance between the extraction using 90% ethanol and 100% (2.3 and 2.5 $mg.g^{-1}$ of dry pepper, respectively), the experiment followed using 100% ethanol, considering the explanation by Boonkird et al. (2008) and the statistical difference between the two values.

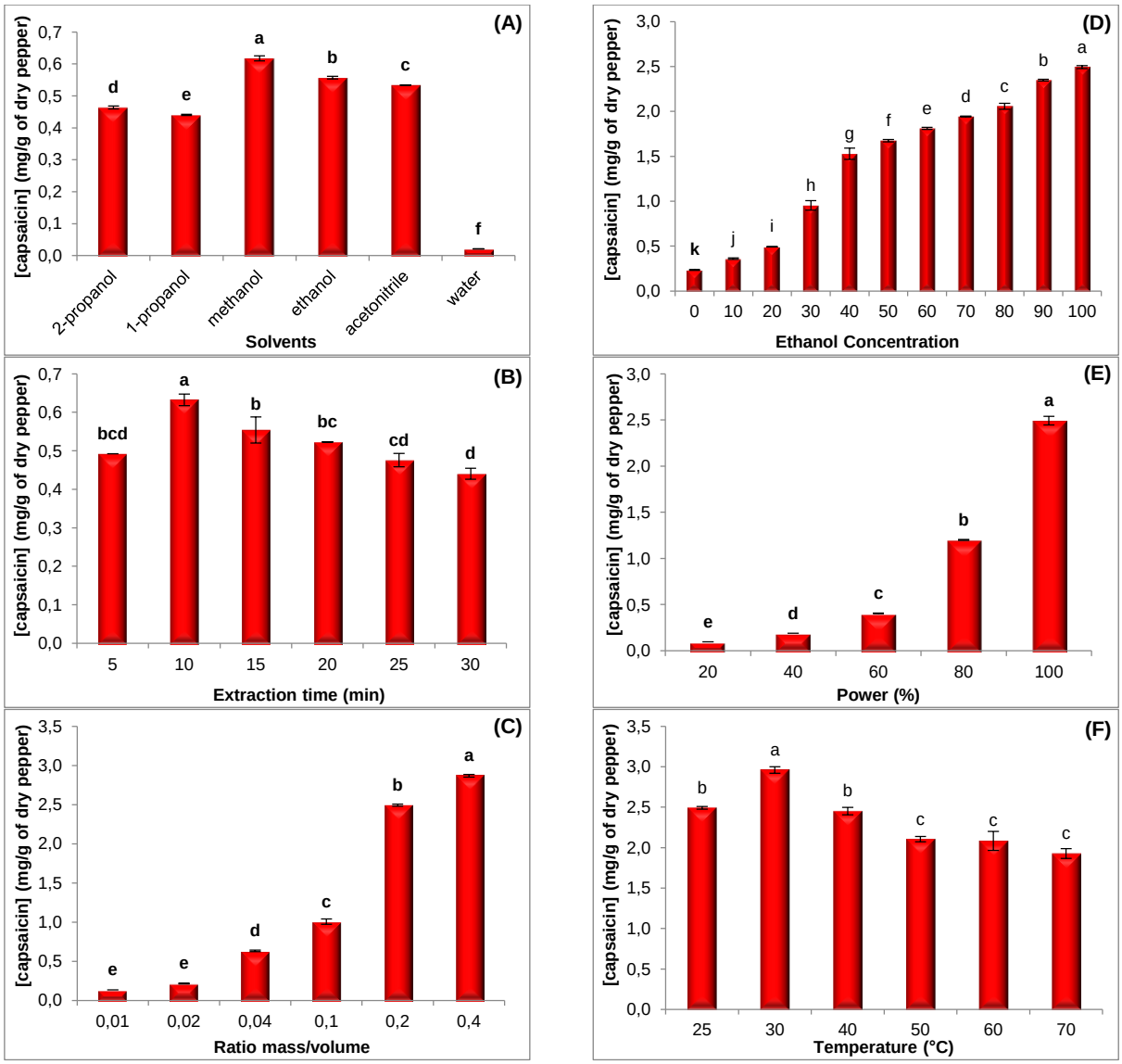

Figure 2: Extraction of capsaicin from *Capsicum chinense* var. cumari-do-pará using UAE. **A)** effect of solvents (0.04 ratio mass/volume of solvents at 100%, 10 min, room temperature, 100% power); **B)** effect of extraction time (0.04 ratio mass/volume of ethanol at 100%, room temperature, 100% power); **C)** effect of ratio mass/volume (ethanol 100%, 10 min, room temperature, 100% power); **D)** effect of ethanol concentration (0.2 ratio mass/volume, 10 min, room temperature, 100% power); **E)** effect of power (0.2 ratio mass/volume of ethanol at 100%, 10 min, room temperature); **F)** effect of temperature (0.2 ratio mass/volume of ethanol at 100%, 10 min, 100% power). * Means followed by the same letter do not differ statistically by Tukey test ($p > 0.05$).

Figure 2E depicts that the extraction of capsaicin gradually improved with the increase in power used during the process. The increased ultrasound energy favoured the extraction because, possibly, to increase the amplitude of ultrasonic waves through the liquid medium, the cavitation bubbles start to collide more violently, favouring the penetration of solvent into the matrix and mass transfer in the system (Toma et al., 2001).

Temperature is an important factor to be considered in a biomolecule extraction process, since it can affect the solubility, mass transfer rate, and cavitation (Rathod & Rathod, 2014). It is observed in Figure 2F that the best value was obtained when the extraction was realised at 30°C and, from this temperature, a reduction in the amount of extracted capsaicin was detected at 40°C, followed by constancy in the range of 50 to 70°C, which does not present a statistically significant difference between its values. This gradual reduction in the yield of extraction may have been caused by high power combined with temperature, which, possibly, reduced the cavitation effect and resulted in a lower yield of extraction. According to Yang et al. (2013), the thermal effect alone is not capable of contributing to the performance of large extraction. Barbero et al. (2008) selected 50°C as the working temperature and reported that the range of 30–60°C can be considered optimal for extraction of capsaicin because, in this range of values, capsaicinoids do not degrade.

Then, after evaluating the extraction parameters using UAE, it was found that the best results for the extraction of capsaicin through this method were obtained using the following conditions: 100% ethanol in a ratio of mass/volume equal to 0.2, for 10 min at 30°C, 100% power of the equipment. Using these conditions, 2.96 $mg.g^{-1}$ of dry pepper was extracted from the cumari-do-pará pepper.

3.2.2 Microwave Assisted Extraction (MAE)

To extract capsaicin using the extraction method assisted by microwave we evaluated seven parameters (type of solvent, ratio of mass/volume, extraction time, temperature,

percentage of solvent, power, and stirring). The initial condition of extraction used in this work was based on Barbero et al. (2006), who obtained the best results using the following: 0.5g sample, 25 mL of ethanol, extraction time equal to 5 minutes, temperature at 125°C, and power at 500 W. The results were shown in Figure 3.

Choosing the best solvent for extraction of biomolecules is a fundamental step towards the development of extraction methods. In the case of MAE, the efficiency of extraction mainly depends on the solvent capacity to absorb and transmit electromagnetic waves (Barbero et al., 2006). This study evaluated the extraction capacity of 2-propanol, 1-propanol, methanol, ethanol, acetonitrile and water. The results presented in Figure 3A showed the same situation as those reported in sections 3.1 and 3.2.1: methanol as the best solvent, followed by ethanol. Again, ethanol was chosen to continue the experiment. In general, ethanol is the most used because it is a good microwave absorber and, besides, its toxicity is lower than that of methanol, favouring the future application of this method in food and pharmaceutical industries (Zhou & Liu, 2006; FDA, 2012).

The ratio between mass and volume is a parameter very important in MAE because it ensures a homogeneous heating system, which can result in increasing process efficiency, minimisation of solvent consumption and, consequently, reduction of operating costs in industry, since the heating acts directly on the molecules (Chan et al., 2011). The use of an inadequate amount of solvent can result in excessive swelling of the material, which can cause irregular heating of the system and a reduction in the absorption of microwave in the material, limiting the distribution of compounds in the middle and reducing extraction efficiency (Maran et al., 2013). In Figure 3B, it is observed that there was a gradual increase in capsaicin extraction as the ratio of mass/volume increased and the best values were found using 0.2 (2.72 $mg.g^{-1}$ of pepper).

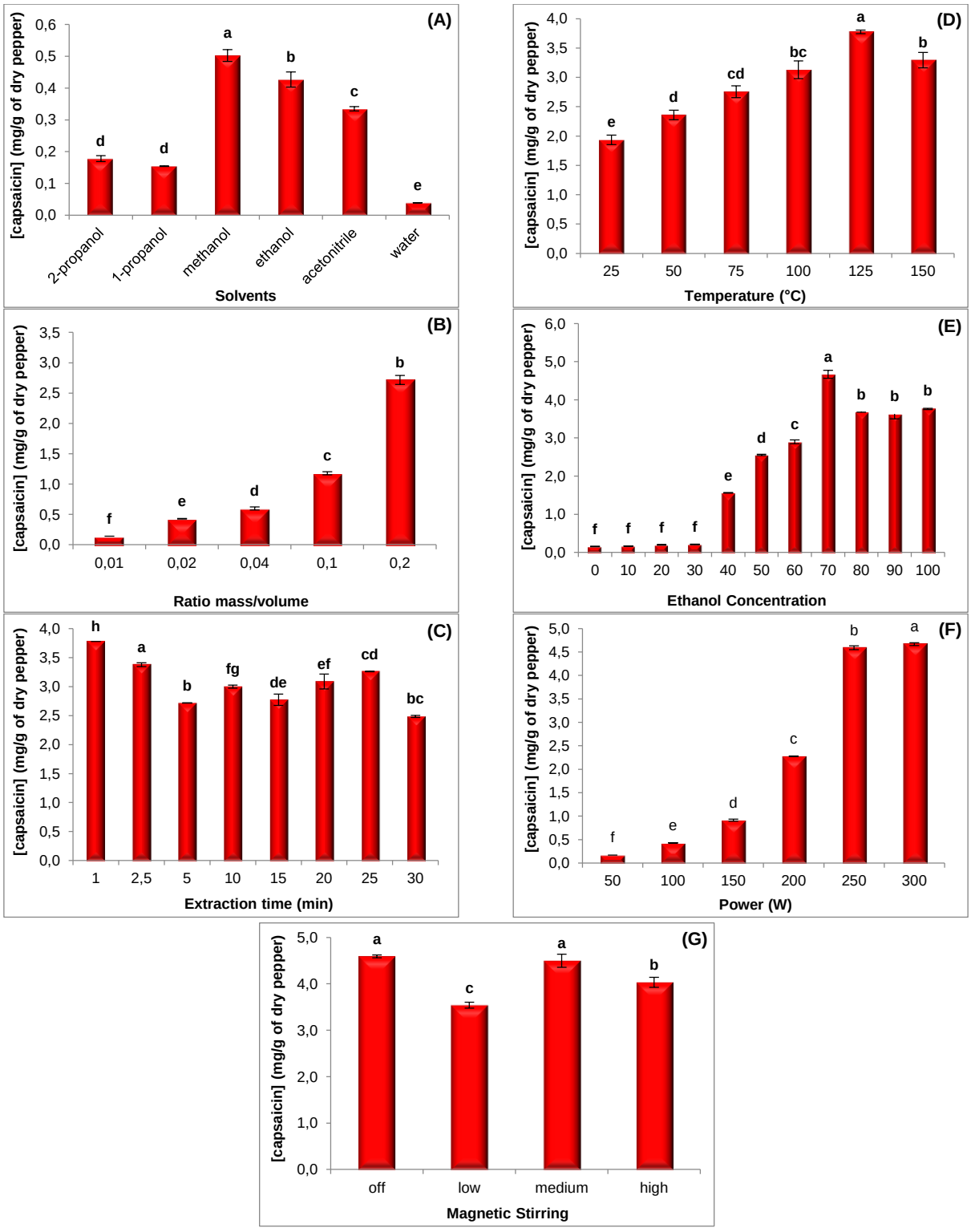

Figure 3: Extraction of capsaicin from *Capsicum chinense* var. cumari-do-pará using MAE. **A)** effect of solvents (0.04 ratio mass/volume of solvent at 100%, 5 min, 125°C, 300W, without stirring); **B)** effect of ratio mass/volume (ethanol at 100%, 5 min, 125°C, 300W, without stirring); **C)** effect of extraction time (0.2 ratio mass/volume of ethanol at 100%,

125°C, 300W, without stirring); **D)** effect of temperature (0.2 ratio mass/volume of ethanol at 100%, 1 min, 300W, without stirring); **E)** effect of ethanol concentration (0.2 ratio mass/volume of ethanol, 1 min, 125°C, 300W, without stirring); **F)** effect of power (0.2 ratio mass/volume of ethanol at 70%, 1 min, 125°C, without stirring); **G)** effect of magnetic stirring (0.2 ratio mass/volume of ethanol at 70%, 1 min, 125°C, 250W). * Means followed by the same letter do not differ statistically by Tukey test ($p > 0.05$).

A major advantage of the use of MAE is the short time required to obtain the maximum extraction of the compound, usually minutes, compared with conventional methods, which typically require extraction hours. Generally, the increase in extraction time also increases the amount of extracted analyte, although there is the risk of degradation of the compound of interest (Alupului et al., 2009). Barbero et al. (2006) did not obtain the best results with the increase in extraction time, and then they chose 5 min as sufficient time to extract the capsaicinoids for their samples. From the results shown in Figure 3C, it is noted that the best performance was obtained with only one minute of extraction; after this time, there was a reduction in efficiency, possibly due to the degradation of capsaicin in the function of drastic conditions of extraction.

In Figure 3D, the effect of temperature on the extraction of capsaicin using MAE is observed. The selection and control of the temperature of extraction depend on the stability and performance of the analyte of interest (Chan et al., 2011). The results showed a gradual increase in the capsaicin extraction when the temperature increased from 25 to 125°C, and a decrease in the extraction at 150°C. This behaviour suggests that, possibly, if one increases the temperature above 150°C, the yield of the process tends to decrease. Thus, based on the results presented in Figure 3D, in order to ensure that degradation of the compound of interest does not occur, the selected working temperature was 125°C.

Because of the nature of capsaicin being more nonpolar, water is not considered a good solvent for the extraction of this compound (Sharma et al., 2013). However, according to Spigno and De Faveri (2009), the water strongly absorbs microwave energy, promoting an internal heating which results in cell disruption and the ease of extraction of active compounds from a vegetable matrix. The results presented in Figure 3E showed that the use of a mixture composed of 30% water and 70% ethanol extracted more capsaicin than using 100% ethanol in the extract. Figure 3E also showed that when the water content in the system was less than 30%, there was a reduction in the extraction of the analyte and, according to Dahmoune et al. (2015), this reduction in extraction may be attributed to a difference in dielectric properties of the solvent ($\varepsilon_{Ethanol} = 30$ e $\varepsilon_{Água} = 80$) due to a reduction in the quantity of water, which promotes greater internal heating. This study stage proved again that the method proposed in this work is promising because, in addition to being fast (extraction time equal to one minute), it is proposed to reduce the amount of organic solvent in the process, without compromising its effectiveness.

The power application in the MAE method aims to provide localised heating in the sample and a driving force in which the microwave destroys the matrix and releases the analyte of interest so that it dissolves in the solvent, thereby improving the yield in less extraction time (Chan et al., 2011). However, the power tends to decrease the yield, possibly because of the intense irradiation that drastically disrupts the system and results in degradation of the compounds. Through Figure 3F, it is noted that there was a gradual increase in the capsaicin extraction with the power increase (50–200 W). From 250 to 300 W (maximum power of equipment used), there was not a significant difference in extraction. In order to ensure operational safety and maintenance of equipment, 250 W was selected as the most efficient power.

Stirring tends to accelerate the extraction speed, desorption and dissolution of active compounds present in the sample (Ruan & Li, 2007). However, the literature hardly has research about assessing the effect of agitation on the extraction of biomolecules by MAE. In this study, we performed extractions without agitation and with low, medium and high agitation, according to the specifications of the equipment used. The results presented in Figure 3G show that the stirring did not significantly influence the extraction of capsaicin because the best extraction was achieved without a system of stirring. Finally, according to the results shown in Figure 3, the optimal conditions for extraction of capsaicin from the *Capsicum chinense* pepper *var*. cumari-do-pará (4.59 $mg.g^{-1}$ of dry pepper) comprised 0.5 g of pepper, 2.5 mL of a ratio of mass/volume equal to 0.2, aqueous solution with 70% ethanol for 1 min at 125°C, 250W power system, and without stirring.

3.4 Comparison between methods

The use of conventional methods to extract biomolecules consists, basically, of submitting the material to the contact with a solvent, during a determined time and temperature, with or without stirring (Azmir et al., 2013). Typically, these methods are easy to execute; however, they require higher amounts of solvent, long analysis time, and present a low yield (Yang et al., 2008). The results presented in Table 3 showed that the increase in temperature in the process (Method 2) contributed to improving the extraction yield above 100%, when compared to Method 1, which was performed at room temperature. Comparing the conventional methods (1 and 2) with the unconventional methods (3 and 4), it was observed that the yield was >600% and >1000% higher compared to UAE and MAE, respectively. In addition, it is noted that there was a significant reduction in the amount of sample, solvent and time required to realise the analysis, confirming the superiority of unconventional in relation to conventional methods.

Comparing the methods (3 and 4), it is noted that the extraction yield by MAE was 55.16% higher than UAE. This result can be explained by a mechanism of action on the biomolecule. The UAE method consists of the application of ultrasonic waves, which promotes the occurrence of cavitation phenomena due to expansion and compression cycles. These cycles increase the extraction rate and yield of the process to promote the mechanical stress in cells, which results in rupture of the cellular structure, increasing cell wall permeability and favouring the penetration of the solvent and, consequently, the mass transfer (TOMA et al., 2001). In the case of MAE, the energy is transferred directly to the material through interactions between the molecules and the electromagnetic field; thus, instead of transference, conversion of electromagnetic energy occurs in heat (Alupului et al., 2009). As heating by microwave is not dependent on thermal conductivity of the equipment materials, the heating occurs rapidly and selectively, resulting in the increase of cell volume until the explosion of cells. The analyte is released into the liquid phase and absorption of microwaves in this phase increases the diffusion rate and uniformity of heat throughout the material (Alupului et al., 2009). Because of this rapid and selective heating, the transfer of energy and the release of analyte in the middle occur more quickly, efficiently and with less chance of degrading the biomolecule during the process. That is why, while even using high temperature to extract the capsaicin (125°C), the method by MAE was 55.16% better than UAE.

Proper and efficient use of energy is essential to the success of an industrial process because of reduced costs, increased productivity, and lucre of the company. Therefore, the amount of consumed energy (kJ) was calculated for each method and the results (Table 4) showed that MAE showed exceptional performance, since it extracted a greater amount of capsaicin (4,590.54 $\mu g.g^{-1}$ of dry pepper) in less time (1 min) and it consumed about 88% less energy than other methods.

Table 4: Energy Consumption (kJ) and Extraction Yield (%) in each method.

Method	Conditions					[capsaicin] (μg.g-1 of dry pepper)**	Extraction Yield (%)			Energy Consumption (kJ)
	sample (g)	Vol. (mL)*	T (°C)	Time (min)	Power Equipment (W)		(2) *x* (1)	(3) *x* (1), (2)	(4) *x* (1), (2), (3)	
Stirring in water bath (1)	1	25	25	240	1520	199.05 ± 5.05d	-	1386.39	2,206.23	21,888
Soxhlet (2)	7.2	180	78	120	3000	411.72 ± 16.85c	106.84	618.61	1,014.96	21,600
UAE (3)	0.5	2.5	30	10	220	2,958.66 ± 39.87b	-	-	55.16	134.64
MAE (4)	0.5	2.5	125	1	250	4,590.54 ± 29.80a	-	-	-	15.3

* In method (4), the ethanol solution was used at 70% and the other 100% ethanol.

** Means followed by the same letter do not differ statistically by Tukey test ($p > 0.05$).

4. Conclusions

The capsaicin content in *Capsicum* peppers was influenced by the fruit maturity stage and both choices of solvent as the extraction method were fundamental to extract the capsaicin from its natural source with maximum efficiency. Among the peppers studied, the cumari-do-pará pepper has the major content of capsaicin, followed by malagueta and jalapeño peppers, principally when in the mature stage. Comparing the conventional methods, the results proved that the temperature influenced positively in the process and the Soxhlet technique was more efficient than stirring at room temperature. However, the Soxhlet technique was not sufficiently better than the unconventional methods assisted by ultrasound (UAE) and microwave (MAE) that presented efficiency ≥ 600%.

The optimised conditions by the MAE method were a ratio of mass/volume equal to 0.2 with an aqueous solution comprising 70% ethanol for 1 min at 125°C, 250 W, and without stirring — the results were 55% superior to UAE. Thus, this study proved that unconventional methods were better than conventional methods; beyond that, it was possible to develop a method to extract capsaicin from *Capsicum* fruits quickly and efficiently, requiring less organic solvent and energetic consumption.

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ARTIGO II

Recovery of capsaicin from *Capsicum frutescens* by applying aqueous two-phase systems based on acetonitrile and cholinium-based ionic liquids

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Abstract

Peppers are the principal source of natural capsaicinoids, a class of bioactive compounds with different therapeutic properties (anticancer, antioxidant, anti-obesity) which make it interesting to the development of extraction and purification processes. This work aims at developing an integrated process of extraction and purification to recover capsaicin from pepper *Capsicum frutescens*. Thus, ATPS consisting of acetonitrile and some cholinium-based ionic liquids were investigated, characterized and the partition of capsaicin on these systems properly optimized. The main results showed that capsaicin is preferentially migrating for the acetonitrile (top) phase. With a simple technology, mild conditions and less solvents, the success of the capsaicin purification from the pepper biomass was achieved (K_{CPS} = 60.95 ± 1.29; EE_{CPS} = 90.57 ± 0.48 %, and PF_{CPS} = 3.26 ± 0.08) using ATPS. Summing up, this work allowed the development of an integrated process of extraction and purification of capsaicin from the chili pepper biomass.

Keywords: capsaicin, aqueous two-phase systems, acetonitrile, cholinium-based ionic liquids, integrated process.

1. Introduction

The peppers of genus *Capsicum* belong to the *Solanaceae* family, as tomatoes, potatoes, eggplant and tobacco, and have more than 90 species already described (Ismail and Revathi, 2006). China, Mexico, Turkey and India are the main world producers of *Capsicum*, being responsible together, for the production of more than 30 million tons of *Capsicum* (fresh and dried products) in 2011 (FAOSTAT, 2015). This spice is widely consumed due to its flavor, color, content on vitamins C and E, provitamin A, carotenoids, and alkaloids (responsible for the spice pungency), those in particular designed as capsaicinoids, for example nordihydrocapsaicin, dihydrocapsaicin, homodihydrocapsaicin, homocapsaicin and, specially, capsaicin (Davis et al., 2007). They are a group of chemicals with important pharmacological effects, with capsaicin being one of the most popular capsaicinoids widely recognized by its biological and pharmacological activities. The ingestion of capsaicin, either through direct consumption of pepper or *via* oral or local drug administration, has shown a positive effect on the human health, as a pain relief chemical (Fraenkel et al., 2004), principally against the rheumatoid arthritis or fibromyalgia (Fraenkel et al., 2004). Moreover, this natural compound is also identified by its anti-inflammatory (Desai et al., 2013), anticancer (Luo et al., 2011), and antioxidant (Lee et al., 2011) properties, as well as by its benefits against cardiovascular and gastrointestinal diseases (Peng and Li, 2010).

Capsaicin (Figure A1 in Supporting Information) is a phenolic compound whose biosynthesis can be done by condensation of fatty acids and vanillyllamine (Thiele et al., 2008). It is a volatile, pungent, colorless and odorless chemical, considered as practically insoluble in water and easily soluble in various organic solvents, in particular, alcohols, ethers, benzene and chloroform (Chauhan et al. 2011; Sharma et al., 2013). Capsaicin can be extracted from its natural sources using conventional techniques such as the maceration (Kirschbaum-Titze et al., 2002, Contreras-Padilla and Yahia, 1998) or through unconventional techniques, in particular by supercritical fluid extraction (Barbero et al., 2006a; Aguiar et al., 2013), microwave-assisted extraction (Barbero et al., 2006b), micro-solid phase extraction (Peña-Alvarez et al., 2009; Chanthai et al., 2012) and ultrasonic-assisted extraction (Barbero et al., 2008; Boonkird et al., 2008). Regarding the maceration, solvents such as water, methanol, ethanol, ethyl acetate, hexane and acetonitrile were investigated (Barbero et al., 2006a,b; Barbero et al., 2008; Chanthai et al., 2012; Aguiar et al., 2013). However, both maceration and other less conventional techniques have led to crude extracts of low purity. To enhance

the purity of the final product, a purification step must complement the extraction, namely aqueous two-phase systems (ATPS), well described and deeply studied in different processes in the last decades. This particular case of liquid-liquid extraction systems are normally used as purification technologies due to their capacity to allow the fractionation of several compounds from the simplest to the most complex matrices. They are normally considered as biocompatible and more sustainable techniques of extraction and purification, due to the higher content of water constituting them, which makes these systems a favorable environment for the biomolecules, capable to keep their chemical structure and main activities (Wu et al., 2011; Reis et al., 2012; Cardoso et al., 2014a).

The application of ATPS as extraction/(re)concentration/purification techniques was firstly recognized in the 1950's (Albertsson, 1958) and, since then, these methods have been developed and recurrently applied in the fractionation of various bioactive compounds (Albertsson, 1958; Freire et al., 2012). However, there are still some constraints in the use of polymeric-based ATPS, namely their high viscosities and cost, which have been minimized by the use of alternative phase formers and, consequently ATPS, such as alcohol-salt (Reis et al., 2012; Reis et al., 2014), acetonitrile-carbohydrates (Cardoso et al., 2013), acetonitrile-polyols (Cardoso et al., 2014a), and ionic liquid-acetonitrile (Okano et al., 2013) systems.

Ionic liquids (ILs) are low temperature melting salts that, due to their "tunable" nature, have been established as "designer solvents", thus letting the possibility of to change their properties through specific anion/cation combinations, allowing them to be designed to meet the requirements of a particular process. They have some physico-chemical properties advantageous over conventional molecular organic solvents such as their high solvation ability, high chemical and thermal stability, high selectivity, excellent microwave-absorbing ability, broad liquid temperature range, and lower environmental impact (at least in the air compartment) that makes them good choices for the extraction and purification of biomolecules (Wasserscheid and Keim, 2000; Sheldon et al., 2002; Passos et al., 2014).

Among the various IL families, the cholinium based have recently been the focus of attention as ATPS phase formers, since they can form biphasic systems with both salts (Pereira et al., 2013; Pereira et al., 2014; Taha et al., 2015) and polymers (Shahriari et al., 2013). Cholinium chloride (2-hydroxyethyltrimethylammonium chloride) in one of the most recent families of ILs being utilized in the extraction and purification of biomolecules, due to its claimed biocompatibility, which was firstly attributed due to the fact that this cation derives from an important nutrient for the structural integrity of cell membranes, methyl metabolism,

cholinergic neurotransmission, transmembrane signaling, and lipid and cholesterol transport and metabolism (Institute of Medicine, 1998; Zeizel and Costa, 2009). Associated with its biocompatibility, it is a cheap raw material, widely used as food additive, and thus a safer and more environmentally friendly salt when compared with some of the most common ILs' cations (Petkovic et al., 2010; Santos et al., 2015; Silva et al., 2014; Sintra et al., 2015; Ventura et al., 2014).

Acetonitrile (ACN) or methyl cyanide is well known as a medium-polarity solvent, miscible with water at ambient temperature and widely used in organic synthesis. This solvent belongs to the class of dipolar aprotic solvents and it is also one of the most preferred organic solvents or mobile phase in various separations techniques, due to its physicochemical properties such as low viscosity, high resolution and low boiling point (Nemati-Kande and Shekaari, 2013). The extraction of biomolecules using acetonitrile–water systems was focused on the partition of antibiotics, peptides and amino-acids under the effect of negative temperatures (Gu and Zhang, 2007), but they were also used for the extraction of metals at room temperature using ATPS based in acetonitrile (Zhang, et al., 2012). More recently, the use of acetonitrile + carbohydrates-, acetonitrile + poly(vinyl alcohol)- and acetonitrile + polyols-based ATPS was proposed for the extraction of vanillin (Cardoso et al., 2015; Cardoso et al., 2014a; Cardoso et al., 2013; Cardoso et al., 2014b). As discussed, the number of works dealing with aqueous solutions of acetonitrile as the main solvent for the extraction of biomolecules is very limited, and not previously attempted for the extraction of capsaicin. In fact, the use of common or alternative solvents to extract or purify capsaicin from its natural source are scarce or practically inexistent, as proved by checking the last complete revisions made about the development of techniques/processes with ILs aqueous solutions or with ILs-based ATPS, respectively to extract and to purify capsaicin (Freire et al., 2012; Passos et al., 2014).

Considering the industrial potential of capsaicin and the continuous need for the development of more efficient and sustainable purification techniques, this work proposes the development of new ATPS by combining cholinium compounds and acetonitrile to purify capsaicin from its natural source, the pepper *Capsicum frutescens var*. malagueta. The binodal curves of the ternary systems composed of cholinium + acetonitrile + water were established at (298 ± 1) K and atmospheric pressure. Then, these systems were applied in the optimization of the ATPS regarding their partition and purification performances experimentally determined with a commercial standard of capsaicin. In the optimization step, the capsaicin partition between both aqueous phases was assessed considering the effect of several processing conditions,

namely the cholinium structure by assessing the effect of different anions, the mixture point selected (allowing the study of several cholinium and acetonitrile concentrations), and the temperature of the partition process. Through the optimization study the best conditions were identified and these systems used in the purification of capsaicin from an acetonitrile crude extract, obtained from the solid-liquid extraction of capsaicin from the pepper *Capsicum frutescens var.* malagueta, by applying the best ATPS selected.

2. Materials and Methods

2.1 Materials

The acetonitrile, HPLC grade with purity of 99.9 wt% was purchased from Tedia. The cholinium ionic structures were acquired at Sigma-Aldrich: cholinium chloride [Ch]Cl, cholinium bitartrate [Ch][Bit], and cholinium dihydrogen citrate [Ch][DHCit]. The purity of each cholinium-based structure is ≥ 98 wt%. The water used in all the experiments is ultrapure and distilled. A commercial sample of capsaicin (CPS) was acquired at Sigma-Aldrich with high purity (≥ 97%). All the chemical structures are represented in Figure A1 in the Supporting Information.

The peppers *Capsicum frutescens var.* malagueta (*C. frutescens*) used in this work were locally acquired in the city of Lagarto, Sergipe - Brazil, in their ripe stage. The peppers were selected, sanitized with a sodium hypochlorite solution (10 $mg.L^{-1}$), dried in an oven at (338 ± 1) K until constant weight (or free of water), macerated in a blender, packed in polypropylene bags and stored for next tasks.

2.2 Phase diagrams and tie-lines

The ATPS were formed using aqueous solutions of acetonitrile at 80 wt% and aqueous solutions of three cholinium-based ILs, in particular, the [Ch][Bit], [Ch][DHCit], [Ch]Cl, at 50, 60 and 65 wt% of maximum concentration, respectively. The phase diagrams were determined at 298 ± 1 K and at atmospheric pressure, by the cloud point titration method (Sintra et al., 2014) and the tie-lines (TLs) were determined according to the gravimetric method well reported in literature (Merchuk et al., 1998). Briefly, the mixing points located in the biphasic region of the diagram were chosen and the solutions prepared, vigorously stirred and centrifuged at 3000 g for 10 min. After the equilibrium time reached (at 298 K for at least 18 h), the top and bottom phases were separated and weighed. Each experimental binodal curve was correlated using Equation (1) (Merchuk et al., 1998).

$$[\mathrm{ACN}] = \mathrm{A} \times \exp\ \{(\mathrm{B} \times [\mathrm{Ch}]X^{0,5}) -\ (\mathrm{C} \times [\mathrm{Ch}]X^{3})\} \quad (1)$$

where [ACN] and [[Ch]X] refer, respectively, to the acetonitrile and cholinium-based ILs weight fraction percentages (X represents the anion species which could be one of the three anions tested, Cl, [DHCit] or [Bit])and A, B and C are constants parameters obtained by the regression.

The TLs were determined using Equations (2) to (5) for unknown values of $[\mathrm{ACN}]_{\mathrm{T}}$, $[\mathrm{ACN}]_{\mathrm{B}}$, $[\mathrm{IL}]_{\mathrm{T}}$ and $[\mathrm{IL}]_{\mathrm{B}}$.

$$[\mathrm{ACN}]_{\mathrm{T}} = \mathrm{A} \times \exp\left\{\left(\mathrm{B} \times [\mathrm{Ch}]X_{\mathrm{T}}^{0,5}\right) - (\mathrm{C} \times [\mathrm{Ch}]X_{\mathrm{T}}^{3})\right\} \quad (2)$$

$$[\mathrm{ACN}]_{\mathrm{B}} = \mathrm{A}\ \times \exp\left\{\left(\mathrm{B} \times [\mathrm{Ch}]X_{\mathrm{B}}^{0,5}\right) - (\mathrm{C} \times [\mathrm{Ch}]X_{\mathrm{B}}^{3})\right\} \quad (3)$$

$$[\mathrm{ACN}]_{\mathrm{T}} = ([\mathrm{ACN}]_{M}/\alpha) - ((1-\alpha)/\alpha)\ \times [\mathrm{ACN}]_{\mathrm{B}} \quad (4)$$

$$[\mathrm{Ch}]_{\mathrm{T}} = \left([\mathrm{Ch}]_{X_M}/\alpha\right) - ((1-\alpha)/\alpha) \times [\mathrm{Ch}]X_{\mathrm{B}} \quad (5)$$

where the subscripts M, T and B refer, respectively, to the initial mixture, top and bottom phase. The value of α corresponds to the ratio between the mass of the top phase and the total mass of the mixture.

The length of each tie-line (TLL) was calculated from Equation (6).

$$\mathrm{TLL} = \sqrt{([\mathrm{Ch}]X_{\mathrm{T}} - [\mathrm{Ch}]X_{\mathrm{B}})^2 + ([\mathrm{ACN}]_{\mathrm{T}} -\ [\mathrm{ACN}]_{\mathrm{B}})^2} \quad (6)$$

2.3 Optimization study - Capsaicin partition in the ATPS

The partition systems were prepared using graduated centrifuge tubes (50 mL) by weighting the appropriate amount of acetonitrile, [Ch]X and a capsaicin aqueous solution (60 $\mathrm{mg.L^{-1}}$) in a total mass of 15 g. The mixtures were then gently stirred and centrifuged at 3000 rpm for 10 minutes. The graduated tubes were placed at different temperatures, ranging from 278 to 318 K and at atmospheric pressure, for at least 18 hours, using a thermostatic bath MARCONI MA-127, to reach the equilibrium and to promote the complete capsaicin migration. The two phases were then carefully collected for the determination of their volume and weight, and the capsaicin was properly quantified in triplicate in both phases, using a Varian Cary-50 Bio UV-visible Spectrophotometer, at 280 nm. In this task, to evaluate the capsaicin partition,

different parameters were calculated, namely the partition coefficient (K_{CPS}), the extraction efficiency (EE_{CPS}) and the volume ratio (R_v) for each ATPS under study - Equations (7) to (9).

$$K_{CPS} = [CPS]_T/[CPS]_B \quad (7)$$

$$R_V = V_T/V_B \quad (8)$$

$$EE_{CPS} = (K_{CPS} \times R_V)/(1 + (K_{CPS} \times R_V)) \times 100 \quad (9)$$

It should be remarked that for all ATPS studied, the top phase was the acetonitrile-rich phase while the bottom phase corresponds to the cholinium-rich phase.

2.4 Thermodynamic functions

The thermodynamic parameters of phase transfer, such as the standard molar Gibbs energy of transfer ($\Delta_{tr}G^o_m$ - J.mol^{-1}), the standard molar enthalpy of transfer ($\Delta_{tr}H^o_m$ - J.mol^{-1}) and the standard molar entropy of transfer ($\Delta_{tr}S^o_m$ - J.mol^{-1}.K^{-1}) were determined through the van't Hoff methodology and calculated according to Equations (10) and (11):

$$ln(K_{CPS}) = -\left(\left(\Delta_{tr}H^0_m/R\right) x \left(1/T_{ref}\right)\right) + \left(\Delta_{tr}S^0_m/R\right) \quad (10)$$

$$\Delta_{tr}G^0_m = \Delta_{tr}H^0_m - T_{ref}\, x\, \Delta_{tr}S^0_m \quad (11)$$

where T_{ref} represents the temperature (Kelvin), K_{CPS} is the partition coefficient of capsaicin, and R is the universal gas constant (8.314 J.mol^{-1}.K^{-1}).

2.5 Extraction and purification of CPS from pepper Capsicum frutescens var. malagueta

The extraction of capsaicin was carried following the steps of the conventional methodology described by Chinn and co-workers (2011). The first step was the solid-liquid extraction of capsaicin from the pepper biomass through the use of aqueous solutions of acetonitrile, obtaining thus a crude acetonitrile aqueous extracts rich in capsaicin. Briefly, 1 g of pepper previously prepared as described in Section 2.1 was weighed and 25 mL of an acetonitrile aqueous solution were added. To evaluate the best acetonitrile:water mixture to carry this extraction, acetonitrile aqueous solutions with concentrations ranging from 0-100% were tested. Then, the samples were placed in a bath at (298 ± 1) K, under constant stirring, for 5

hours, using a Marconi MA-095 shaker. The water solutions and acetonitrile aqueous extracts were filtered through a Millipore filter 0.45 μm and analyzed by High Performance Liquid Chromatography (HPLC). To the crudde acetonitrile extracts rich in capsaicin obtained, distinct amounts of the cholinium-based ILs and water were added to prepare the ATPS up to a final weight of 15 g. The selected ATPS were prepared considering the conditions previously selected in the optimization section regarding the systems and conditions of maximum partition and extraction performances. The mixtures were stirred, centrifuged at 3000 x g for 10 minutes, placed in equilibrium in the optimum temperature condition, for at least 18 hours. Then, both phases were separated, collected and their volume and weight measured as well as the CPS content quantified by HPLC (model Prominence, brand Shimadzu system with UV-VIS detector, at 280 nm, C18 column type, mobile phase consisted of acetonitrile:water (60:40) at a flow rate of 1.0 mL.min^{-1}, isocratic mode, column temperature 30 °C, 20 μL of injection volume). The phenolic content (PC) in the crude extracts obtained in the solid-liquid (or solvent) extraction step and in both aqueous phases was assessed by colorimetric spectrophotometry using the Folin-Ciocalteau method, using the gallic acid as standard (Swain and Hillis, 1959). The purification factor of capsaicin (PF_{CPS}) was determined by the ratio between the specific concentration of capsaicin (SC_{CPS}) present in the acetonitrile crude extract rich in capsaicin (obtained from the solid-liquid extraction) and in each phase of the ATPS according to Equations (12) and (13), respectively.

$$SC_{CPS} = C_{CPS}/C_{PC} \quad (12)$$

$$PF_{CPS} = (SC_{CPS})_T/(SC_{CPS})_E \quad (13)$$

where, C_{CPS} is the concentration of capsaicin, C_{PC} represents the concentration of phenolic compounds simultaneously extracted in the solid-liquid step and the subscripts T and E are indicative of the top and acetonitrile crude extract rich in capsaicin, respectively.

3. Results

3.1 PHASE DIAGRAMS AND TIE-LINES

The formation of ATPS of acetonitrile with three cholinium-based ILs was assessed, through the experimental determination of the respective phase diagrams at 298 (± 1) K, depicted in Figure 1. The composition values were expressed in molality units to avoid discrepancies in the evaluation of the ILs potential to induce the liquid-liquid demixing related with the

different molecular weights of the ILs involved and the acetonitrile as well. The experimental weight fraction data of the binodal curves are provided in the Supporting Information (Table A1). By analyzing Figure 1, it is observed that the potential of the cholinium compounds to form ATPS increases as follows: [Ch][DHCit] ≈ [Ch][Bit] > [Ch]Cl. This sequence is related to the lipophilic/hydrophilic nature of the cholinium salts, as derived from their octanol–water partition coefficients (log K_{ow}) (Chemspider, 2015). The tendency observed for the ATPS formation is related to the decrease in their log K_{ow}, namely log K_{ow} ([Ch][DHCit]) = -1.32, log K_{ow} ([Ch][Bit]) = -1.43 and log K_{ow} ([Ch]Cl) = -3.70.

The binodal curve data were fitted using Equation 1, firstly applied by Asenjo and collaborators (Merchuk et al., 1998). The parameters A, B and C (estimated by least-squares regression), and the corresponding standard deviations (std) and regression coefficients (R^2) are reported in Table A2 (Supporting Information). At the same time, Figure A2 presents the complete phase diagrams with the TLs and respective TLLs calculated (the numerical results are reported in Table A3 of Supporting Information).

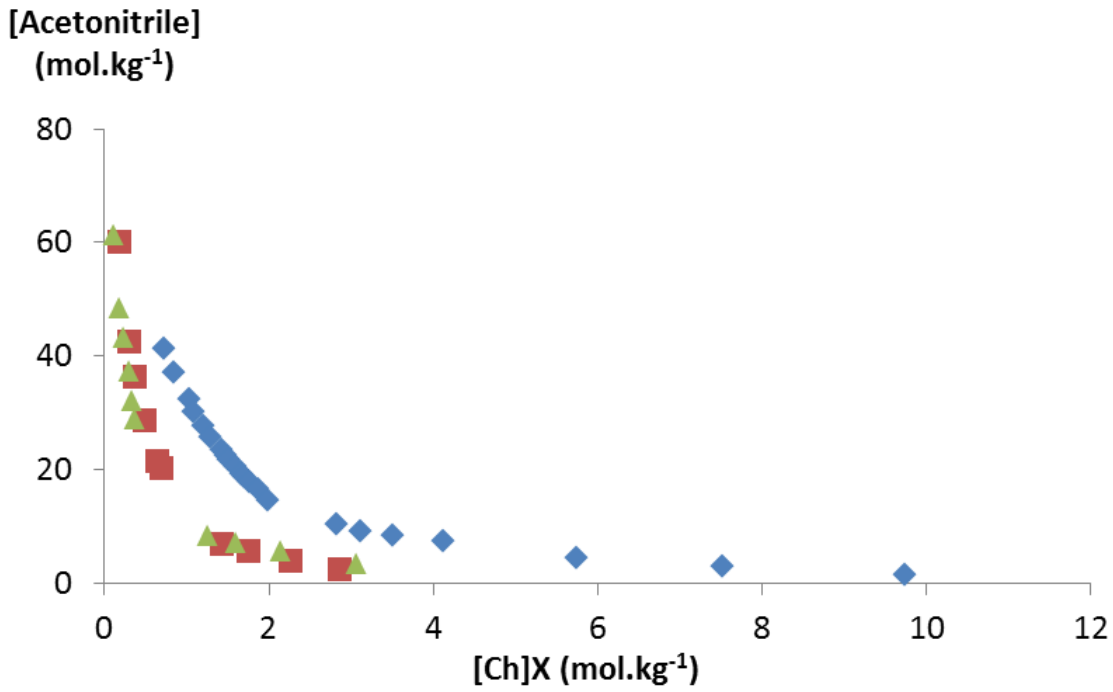

Figure 1: Phase diagrams for the ternary systems composed of acetonitrile + [Ch]X + water at 25°C: (◆) [Ch]Cl, (■) [Ch][Bit], and (▲) [Ch][DHCit].

Finally, the ATPS here developed were also defined in terms of the pH of each aqueous phase formed, as shown in Table A4 (Supporting Information). In general terms, the pH is in its

acidic form, in the range of 3.58 to 6.22. According to the speciation curves shown in the Supporting Information (Figure A3) and the results of Table A4, it is observed that the effect of pH on the capsaicin partition can be explained by its effect on the electrical charge, which influenced its solubility in the top phase. The system composed of [Ch]Cl presented pH values for the top phase in the range of 6.22-5.66,and in this range of pH, the capsaicin is more negatively charged, thus favoring the occurrence of electrostatic interactions favorable to the migration of capsaicin for this phase. Regarding the systems composed of [Ch][Bit] and [Ch][DHCit], the top phase presented pH values between 4.62-5.21, which was not much influenced by electrostatic forces because in this range, the capsaicin is mostly in its neutral form, which explains the poorest affinity of capsaicin for the top-phase, and the smaller value of K_{CPS}. The pH values found in the bottom phase are included in the same range found elsewhere (Souza et al., 2015). In this work, ATPS composed of tetrahydrofuran and cholinium-based compounds were applied on the purification of a lipase and observed that the pH also influenced the partition of the enzyme by the electrostatic interactions formed.

3.2 Optimization of CPS Partitioning on the ATPS

In this work, different ATPS based in acetonitrile and three cholinium salts were used to investigate the partition of capsaicin, as an alternative method to purify it from the pepper *Capsicum frutescens var.* malagueta. After the adequate definition of the phase diagrams, and consequently, of the biphasic region for each system, the optimization of the partition of capsaicin using a commercial sample with high purity (≥ 97% of purity) as a model compound was investigated. Different parameters were evaluated to optimize and identify the best extraction/partition conditions, not only regarding the most appropriate ATPS, but also the most adequate processing conditions. Examples of those conditions are the cholinium anion, the composition of the system (regarding the mixture point used to prepare each ATPS) and the temperature of extraction, whose results are depicted in Figure 2.

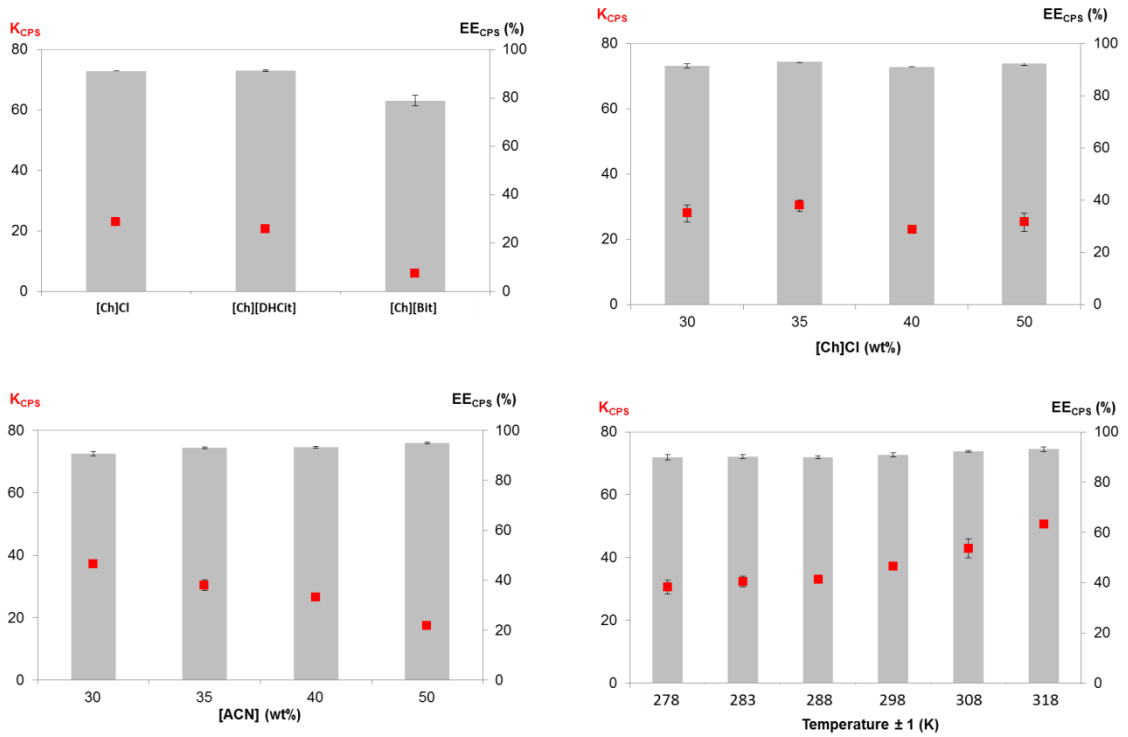

Figure 2: Partition Coefficient, K_{CPS} (■) and Extraction Efficiency, EE_{CPS} (grey bars) of capsaicin determined for different conditions: **A)** ATPS composed by 40 wt% [Ch]X + 35 wt% acetonitrile, at 298 (± 1) K; **B)** effect of the initial concentration of [Ch]Cl: ATPS composed by 30 to 50 wt% [Ch]Cl + 35 wt% acetonitrile, at 298 (± 1) K; **C)** effect of the acetonitrile initial concentration: ATPS composed by 35 wt% [Ch]Cl + 30 to 50 wt% of acetonitrile, at 298 (± 1) K; **D)** effect of temperature of extraction: ATPS composed by 35 wt% [Ch]Cl + 30 wt% of acetonitrile, at 278 to 318 (± 1) K.

3.2.1. Effect of the cholinium anion

The results presented in Figure 2(A) show the partition coefficients (squares) and the extraction efficiency results (grey bars) obtained for capsaicin (numerical data is reported in Supporting Information - Table A5) when applied the three ATPS, namely 40 wt% of [Ch]X (X is representing Cl, [DHCit] or [Bit]) + 35 wt% of acetonitrile + 25 wt% of water. The experiments show that the capsaicin partitions preferentially for the top acetonitrile-rich phase, with values of K_{CPS} of 22.92, 20.55 and 5.79, for [Ch]Cl, [Ch][Bit] and [Ch][DHCit], respectively. Moreover, this trend is in close agreement with the extraction efficiencies (EE_{CPS}) results ranging from 78.85 to 91.29 %. These results demonstrated the higher affinity of capsaicin for the acetonitrile phase [log K_{ow} = -0.17 (Chemspider, 2015)], due to its hydrophobic nature, as demonstrated by the octanol-water partition coefficient (log K_{ow} = 3.75), being the biomolecule affinity decreased when the cholinium-based ILs with more

hydrophobic anions. In this sense, [Ch]Cl is the most efficient regarding the concentration of capsaicin in the acetonitrile-rich phase. In addition to the most favorable results found for [Ch]Cl regarding the concentration of capsaicin in the top phase, [Ch]Cl is much cheaper, widely available in industrial scale and a harmless compound (that is actually an essential nutrient for human nutrition and widely used as supplement in animal feed), and thus, the ATPS based on this cholinium compound will be used in the next optimization steps.

3.2.2. Composition of the system

Considering the ATPS based in [Ch]Cl + acetonitrile + water, identified as the best system to partition capsaicin into the acetonitrile-rich phase, the effect of different mixture points was assessed. The results regarding the various extraction points are represented in Figure 2(B) (numerical data in Supporting Information, Table A6). In this case, the ATPS was composed of 35 wt% of acetonitrile and various concentrations of [Ch]Cl ranging from 30 to 50 wt%. The results indicate that the effect of different mixture points is not very relevant for the partition of capsaicin, since the data of K_{CPS} (ranging from 22.93 - 30.35) and EE_{CPS} (included in the range of 91.14 - 93.06%) are similar between the various ILs compositions. The effect of different concentrations of acetonitrile was also tested, as depicted in Figure 2(C), for systems composed of 35 wt% of [Ch]Cl and the concentration of acetonitrile varying from 30 to 50 wt% (numerical results reported in Table A7 of Supporting Information). In this case, the migration of capsaicin was influenced by the variations in the acetonitrile concentration, *i.e* the partition coefficient decreases with the increase in the acetonitrile content (ranging from 17.58 – 37.29). However, due to the change in the relative phase volumes, the extraction efficiencies change in the opposite way, but only marginally, between 90.74 and 94.93%.

3.2.3. Effect of temperature

In order to study the effect of temperature in the partition of capsaicin, the range between 278 and 318 (± 1) K was evaluated and the experimental results depicted in Figure 2(D) (detailed data at Table A8 in Supporting Information). The selected system was based on the best conditions found to maximise the capsaicin migration to the top phase rich in acetonitrile, namely composed of 30 wt% of acetonitrile + 35 wt% of [Ch]Cl + 35 wt% water. From the results presented in Figure 2(D), it can be seen that the K_{CPS} increases with temperature, until the maximum of 50.68, achieved at 318 (± 1) K. This behavior is justified by the increase in the solubility of capsaicin into the top phase, promoted by entropic factors. Meanwhile, the

EE_{CPS} is not significantly affected by temperature, ranging from 89.75 to 93.07%. Following the study of the temperature effect on the capsaicin migration, the thermodynamic parameters were also evaluated, aiming at achieving a better understanding of the partition process of capsaicin between the aqueous phases. The thermodynamic parameters of transfer, namely the standard molar Gibbs energy ($\Delta_{tr}G^{o}_{m}$), enthalpy ($\Delta_{tr}H^{o}_{m}$) and entropy ($\Delta_{tr}S^{o}_{m}$) were calculated considering Equations (10) and (11) and the results presented in Figure 3 and Table A9. It is possible to conclude that the capsaicin migration for the acetonitrile-rich phase using these specific ATPS is a spontaneous process ($\Delta_{tr}G^{o}_{m}$ is negative, equal to -18.17 kJ.mol^{-1}). Moreover, the $\Delta_{tr}H^{o}_{m}$ is positive (9.13 kJ.mol^{-1}), suggesting that the transference of capsaicin from the [Ch]Cl to the acetonitrile-rich phase is an endothermic process ($\Delta_{tr}H^{o}_{m} > 0$ kJ.mol^{-1}). In this case, it is possible to recognize the important role of the entropic effects, since the absolute value of $T\Delta_{tr}S^{o}_{m}$ (18.18 kJ.mol^{-1}) is higher than $\Delta_{tr}H^{o}_{m}$ (9.13 kJ.mol^{-1}).

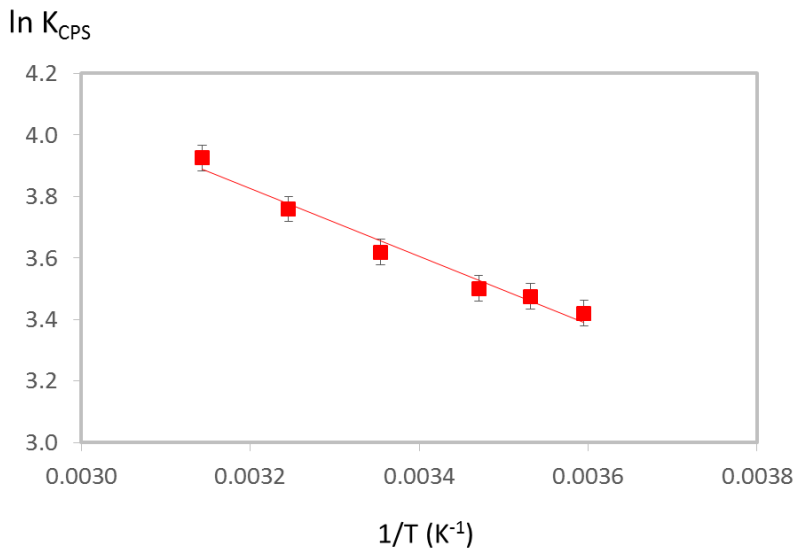

Figure 3: Correlation between the logarithmic function of K_{CPS} - ln(K_{CPS}) - versus T^{-1} (K^{-1}) for the CPS partitioning considering the system 30 wt% of acetonitrile + 35 wt% of [Ch]Cl + water at different temperatures, namely 318, 308, 298, 288, 283, 278 (± 1) K.

3.3 Purification of Capsaicin from Pepper Capsicum Frutescens var. Malagueta

Having evaluated and optimized the partition coefficients of capsaicin on the ATPS using the commercial standard of capsaicin (high level of purity), they were further applied in the purification process considering the removal of capsaicin from the pepper *Capsicum frutescens* var. malagueta. The partition of the target capsaicinoid and the main contaminants (normally phenolic compounds) present in the pepper biomass were considered in the

analysis. The integrated process proposed is depicted in Figure 4, which represents the process diagram for the extraction of capsaicin from the *Capsicum frutescens.*

The samples of *Capsicum frutescens* are subjected to a solid-liquid extraction starting with a mechanical maceration of the pepper biomass, assisted by solvent extraction with aqueous solutions of acetonitrile (different concentrations of the organic solvent were tested), aiming at the complete extraction of capsaicin from the biomass. The acetonitrile aqueous extract rich in capsaicin obtained was then used in the implementation of the final step of purification. In this step, the acetonitrile aqueous solution rich in the capsaicinoid was used to prepare the ATPS with the best performance composed of 35 wt% of [Ch]Cl + 30 wt% of acetonitrile + 35 wt% of acetonitrile (conditions previously optimized, Section 3.2), at temperatures of 298 and 318 (±1) K.

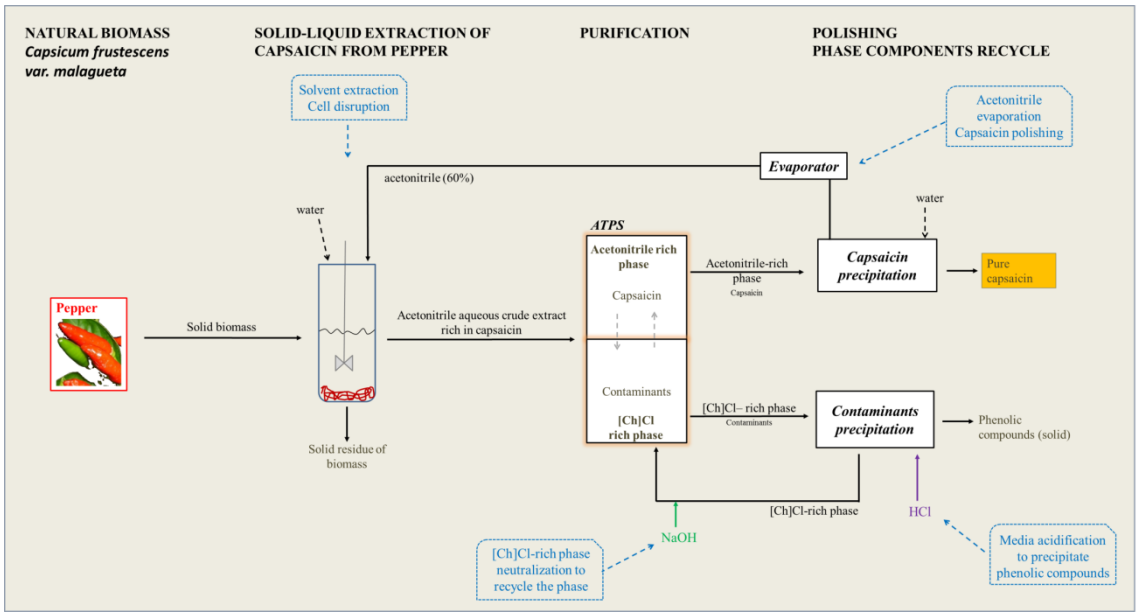

Figure 4: Flowchart of the integrated process for the extraction and purification of capsaicin from *Capsicum frustescens var.* malagueta. The process starts with the solvent extraction of capsaicin from the biomass through the use of water or aqueous solutions of acetonitrile, followed by the purification of capsaicin from the acetonitrile aqueous extract rich in capsaicin by applying the ATPS based in the [Ch]Cl under the optimized conditions of composition and temperature (the phenolic compounds are the main contaminants considered). The polishing of capsaicin and the recycle of the phase components are steps also included in the purification process.

3.3.1. Solid-liquid extraction of capsaicin from Capsicum frutescens var. malagueta

To recover any bioactive compound from the natural biomass, the first step to be conducted must be a solid-liquid extraction. The simplest and industrially most used approach is the maceration in which the solvent contacts directly with the grounded biomass at temperatures not far removed from the ambient. To carry this extraction, it is necessary to start with the selection of the adequate solvent or a combination of solvents, taking into account the affinity of the target bioactive chemical to be recovered from this solvent or mixture of solvents. In this work, the extraction of capsaicin from the solid biomass was evaluated in aqueous solutions of acetonitrile with concentrations ranging from 0 to 100% (Figure 5). These results seem to indicate that the pure solvents are not the best choices to extract capsaicin from the biomass, actually the data demonstrated that the mixture of water/acetonitrile improves the extraction of capsaicin from the pepper biomass, which is agreement with other studies (Barbero et al., 2006b). The best solvent mixture to extract capsaicin from the pepper was found to be the mixture of 40% of water and 60% of acetonitrile, represented by a concentration of capsaicin extracted of 0.146 ± 0.001 mg of capsaicin.g^{-1} of pepper (Figure 5), conditions used in the preparation and implementation of ATPS as purification systems to capsaicin.

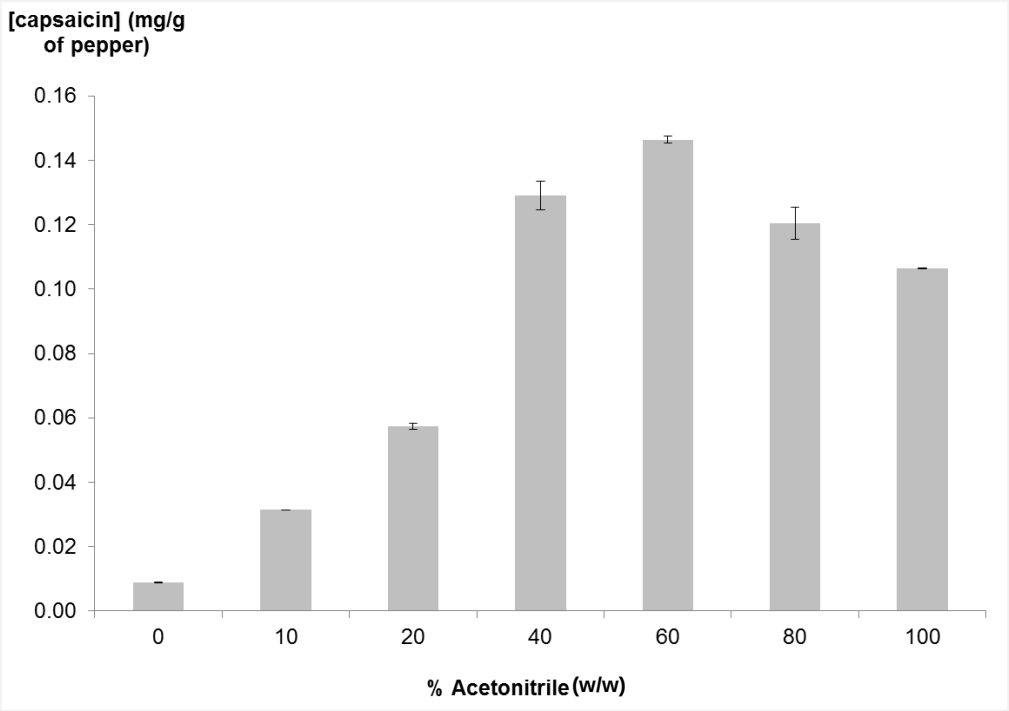

Figure 5: Effect of the acetonitrile concentration on the extraction of capsaicin from the *Capsicum frutescens* biomass.

3.3.2. Purification of capsaicin from Capsicum frutescens var. malagueta using ATPS

After the optimization carried out using the capsaicin commercial standard, the ATPS presenting the higher K_{CPS} parameters were considered in the purification of the natural capsaicin from the acetonitrile aqueous crude extract. This extract obtained from the maceration was used to prepare the ATPS composed of 30 wt% of acetonitrile + 35 wt% of [Ch]Cl + 35 wt% of water selected in the Optimization Study (Section 3.2.). The purification was conducted at 298 and 318 (±1) K, to evaluate the effect of temperature when the capsaicin is purified from a natural source. The partition coefficient and extraction efficiency data were determined to conclude about the purification performance achieved by the selected ATPS when a much more complex matrix is investigated. In this case, the purification factor (Table 1), described by Equation (13) is also relevant to measure the ATPS performance, since it is directly measuring the separation of capsaicin from the phenolic compounds, the main contaminants present in the extract.

Table 1: Partition Coefficient (K_{CPS}), Extraction Efficiency (EE_{CPS}) and Purification Factor (PF_{CPS}) of capsaicin from the pepper *Capsicum frutescens var.* malagueta obtained using the optimized ATPS: 35 wt% of [Ch]Cl + 30 wt% of acetonitrile at 298 or 318 (±1) K.

Temperature ±1 (K)	**K_{CPS}**	**EE_{CPS} (%)**	**PF_{CPS}**
298	60.95 ± 1.29	90.57 ± 0.48	3.26 ± 0.08
318	67.71 ± 0.96	90.93 ± 0.12	3.20 ± 0.10

Regarding the partition behavior of the natural capsaicin, the results for the best ATPS, at 318 (±1) K, evidenced the preferential partition of the biomolecule for the acetonitrile (top)-rich phase ($K_{CPS} >> 1$), and the EE_{CPS} values remained constant at circa 90%. By the application of these ATPS, it was found a good purification factor (PF_{CPS} = 3.20 or 3.26), meaning that the capsaicin concentrated in the acetonitrile phase is free of some of the contaminants. The standard and samples chromatograms [commercial capsaicin, the natural pepper extract obtained in Section 3.3.1 and the top phases obtained after the purification of capsaicin at 298 and 318 (±1) K], obtained by HPLC analysis were reported in Supporting Information (Figure A4). The chromatograms confirm that the use of ATPS was a successfully step on the development of the capsaicin' purification process, due to the significant increase in the

specific capsaicin concentration in the top phase (Figure A3). Regarding the temperature effect, it was observed an increase in the value of K_{CPS} (from 60.9 to 67.7) however, its impact on the extraction efficiency and purification factor was not very significant. In this case, the lower temperature was selected as the most adequate, since it allows the minimization of the energy costs enhancing the process economic viability while maintaining the system purification capacity. Furthermore, the recovery of capsaicin from the solid biomass is practically the same obtained from a liquid chromatography - electrospray ionization (86% of capsaicin recovery) by Álvarez-Fernández and co-workers (Garcés-Claver et al., 2006), but in our case, using a simpler and low-cost methodology.

For the process proposed to be of industrial relevance, the isolation of capsaicin from the acetonitrile-rich phase (also known as a polishing step) and the recycling of the phase components must be addressed. It is here proposed the recycling of both phases by the evaporation of acetonitrile and its reuse in the extraction process (more details in the flowchart of the integrated process proposed, Figure 4), the [Ch]Cl is then washed out with water and capsaicin is precipitated due to its lower solubility in water. The precipitation of capsaicin will be promoted by the addition of high amounts of water as anti-solvent (at low temperature, if needed), due to the limited solubility of capsaicin in water (Turgut et al., 2004). The acidification of the [Ch]Cl-rich phase is here proposed aiming at the removal of the phenolic compounds by precipitation at very low pH values (Li et al., 2014). After the removal of the contaminants and capsaicin from both aqueous phases, the acetonitrile phase will be directly reintroduced in the ATPS preparation and the IL-rich phase will be neutralized with a base, and then reintroduced in the purification system (in the step of ATPS preparation).

4. Conclusions

Cholinium and acetonitrile based ATPS were successfully developed and applied in the purification of capsaicin from crude extracts obtained from the pepper *Capsicum frutescens* var. malagueta. From the optimization study carried out to previously select the best ATPS and processing conditions, high partition coefficients and extraction efficiencies at the acetonitrile-rich phase were achieved, and the best ATPS, regarding its capacity to concentrate capsaicin in the acetonitrile-rich phase was selected: 30 wt% of acetonitrile, 35 wt% of [Ch]Cl and 35 wt% of water, at 318 (±1) K to further perform the purification of the natural capsaicin from the pepper. With a simple technology like ATPS, with mild conditions

and requiring less solvents, the success of the capsaicin purification from pepper was achieved (K_{CPS} = 60.95 ± 1.29; EE_{CPS} = 90.57 ± 0.48 %, and PF_{CPS} = 3.26 ± 0.08), with lower environmental impacts and costs when compared with the conventional methodologies already applied. Summing up, with the integrated purification process here developed, it would be possible to purify capsaicin from the chili pepper using aqueous solutions of acetonitrile, by means of an effective and simple purification process integrating ATPS.

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APPENDIX A. Supporting Information

Supporting data associated with this article can be found, in the online version.

Supporting Information

Recovery of capsaicin from *Capsicum frutescens* by applying aqueous two-phase systems based on acetonitrile and cholinium-based ionic liquids

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Table A1. Experimental weight fraction data for the ternary systems composed of acetonitrile (1) + [Ch]X (2) + water (3) at (298 ± 1) K.

[Ch]Cl		[Ch][Bit]		[Ch][DHCit]	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
62.8563	9.2202	71.1468	4.2531	71.6187	3.1138
60.3061	10.4731	63.5342	7.1232	66.6140	5.2122
57.0784	12.5774	59.9395	8.3918	64.0474	6.2177
55.4299	13.1141	54.0500	11.0281	60.5983	8.2424
53.2495	14.3531	46.7848	13.9427	56.8075	8.7756
51.3750	15.3293	45.5615	14.9713	54.3477	9.7349
49.1066	16.6477	10.0870	41.9574	14.1471	47.5421
48.0790	17.0682	16.6201	36.4885	23.0770	38.7327
47.0576	17.4849	23.1781	30.8163	29.2911	32.0344
45.6110	18.2478	28.3087	26.5800	33.9811	27.1721
44.3091	18.9260				
43.4637	19.1301				
42.2643	19.8005				
40.4854	20.6708				
37.4062	21.7243				
5.8013	57.6442				
10.4410	51.2479				
15.0991	44.5098				
23.1628	36.5326				
25.6168	32.9059				
27.3557	30.3087				
29.7829	28.2401				

Table A2: Adjusted parameters and respective standard deviations (std) obtained from the application of the Merchuk equation (Eq. 1) for the ternary systems composed of [Ch]X + acetonitrile + water, at (298 ± 1) K.

[Ch]X	Regression Parameters			
	A ± std	**B ± std**	**C ± std**	**R^2**
Cl	144.9 ± 6.7	-0.265 ± 0.012	$5.9 \times 10^{-6} \pm 6.6 \times 10^{-7}$	0.9980
[Bit]	114.4 ± 3.1	-0.224 ± 0.009	$1.3 \times 10^{-5} \pm 9.7 \times 10^{-7}$	0.9994
[DHCit]	99.9 ± 3.2	-0.183 ± 0.012	$6.1 \times 10^{-6} \pm 9.5 \times 10^{-7}$	0.9987

Table A3: Mass fraction compositions and respective standard deviations (std) for the TLs and respective tie-line lengths (TLLs), at the Top (T) and Bottom (B) phases, and at the initial biphasic composition of the mixture (M), composed of acetonitrile (Y) and [Ch]X (X), at (298 ± 1) K and atmospheric pressure.

[Ch]X	100 x weight fraction (wt%)						
	Y_M ± std	**X_M ± std**	**Y_T ± std**	**X_T ± std**	**Y_B ± std**	**X_B ± std**	**TLL**
Cl	39.99 ± 0.02	30.00± 0.01	90.37 ± 0.07	2.89 ± 0.05	16.78 ± 0.08	42.02 ± 0.06	85.11
	34.99 ± 0.02	39.98± 0.01	95.91 ± 0.05	2.41 ± 0.04	6.51 ± 0.03	58.53 ± 0.03	99.78
[Bit]	39.97 ± 0.01	30.02± 0.03	80.96 ± 0.08	2.22 ± 0.01	3.39 ± 0.05	54.57 ± 0.04	92.89
	34.96 ± 0.02	39.96 ± 0.03	93.33 ± 0.03	0.90 ± 0.01	1.23 ± 0.02	61.95 ± 0.01	95.50
[DHCit]	39.99 ± 0.05	29.99 ± 0.04	87.13 ± 0.01	0.56 ± 0.04	17.84 ± 0.01	43.82 ± 0.03	81.68
	34.91 ± 0.06	40.01 ± 0.02	95.97 ± 0.02	0.06 ± 0.03	6.99 ± 0.02	58.84 ± 0.01	98.06

Table A4: pH values of both top and bottom phases for each system under study; System A: 30 wt% of [Ch]Cl + 40 wt% of acetonitrile + 30 wt% of water; System B: 40 wt% of [Ch]Cl + 35 wt% of acetonitrile + 25 wt% of water, at (298 ± 1) K.

[Ch]X	System A		System B	
	Top phase	Bottom phase	Top phase	Bottom phase
Cl	6.22	4.34	5.66	4.24
[Bit]	4.64	3.61	4.75	3.58
[DHCit]	4.62	4.44	5.21	4.41

Table A5. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at (298 ± 1) K, obtained in the study of the effect of different cholinium-ILs structures.

[Ch]X	100 x Mass fraction composition (wt%)			K_{CPS}	EE_{CPS} (%)
	acetonitrile	[Ch]Cl	water		
Cl	39.97	30.02	30.01	16.13 ± 0.80	90.04 ± 0.26
[Bit]	39.99	30.01	30.00	7.15 ± 0.60	84.85 ± 1.12
[DHCit]	40.01	29.98	30.01	11.66 ± 0.97	87.99 ± 1.45

Table A6. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at (298 ± 1) K obtained in the study of the effect of different [Ch]Cl concentrations.

[Ch]Cl wt%	100 x Mass fraction composition (wt%)			K_{CPS}	EE_{CPS} (%)
	Acetonitrile	[Ch]Cl	water		
30	35.01	29.99	35.00	27.95 ± 2.68	91.45 ± 0.80
35	34.99	34.99	30.02	30.35 ± 1.72	93.06 ± 0.40
40	35.00	40.00	25.00	22.93 ± 0.36	91.14 ± 0.02
50	35.00	49.99	15.01	25.19 ± 2.85	92.47 ± 0.79

Table A7. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at (298 ± 1) K obtained in the study of the effect of different acetonitrile concentrations.

Acetonitrile wt%	100 x Mass fraction composition (wt%)			K_{CPS}	EE_{CPS} (%)
	acetonitrile	[Ch]Cl	water		
30	30.00	35.00	35.00	37.29 ± 1.19	90.74 ± 0.80
35	34.99	34.99	30.02	30.35 ± 1.72	93.06 ± 0.40
40	40.00	35.00	25.00	26.53 ± 1.30	93.31 ± 0.42
50	49.97	35.00	15.03	17.58 ± 0.93	94.93 ± 0.34

Table A8. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at (298 ± 1) K obtained in the study of the effect of different temperatures.

Temperature (± 1 K)	**100 x Mass fraction composition (wt%)**			**K_{CPS}**	**EE_{CPS} (%)**
	acetonitrile	**[Ch]Cl**	**water**		
278	30.01	35.00	34.99	30.57 ± 2.14	89.75 ± 1.05
283	30.00	35.00	35.00	32.29 ± 1.70	90.07 ± 0.91
288	30.02	35.00	34.98	33.15 ± 0.01	89.88 ± 0.50
298	30.00	35.00	35.00	37.29 ± 1.19	90.74 ± 0.80
308	30.00	35.00	35.00	42.90 ± 3.02	92.28 ± 0.35
318	30.00	35.00	35.00	50.68 ± 1.07	93.07 ± 0.85

Table A9. Standard molar thermodynamic functions of transfer for capsaicin from 278 to 318 (± 1) K, using the ternary system composed of 30.0 wt% of acetonitrile + 35 wt% of [Ch]Cl + 35 wt% of water.

$\Delta_{tr}H^{o}_{m}$ (kJ.mol^{-1})	$\Delta_{tr}S^{o}_{m}$ (J.mol^{-1}K^{-1})	$\Delta_{tr}G^{o}_{m}$ (kJ.mol^{-1})	$T\Delta_{tr}S^{o}_{m}$ (kJ.mol^{-1})
9.13	61.01	-18.17	18.18

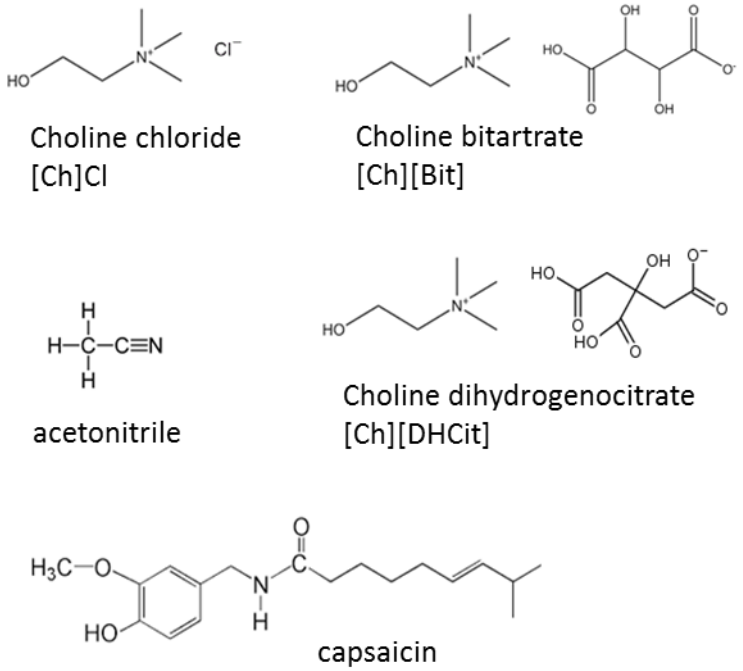

Figure A1: Chemical structure of acetonitrile, capsaicin and the three cholinium-based ILs and respective abbreviation names.

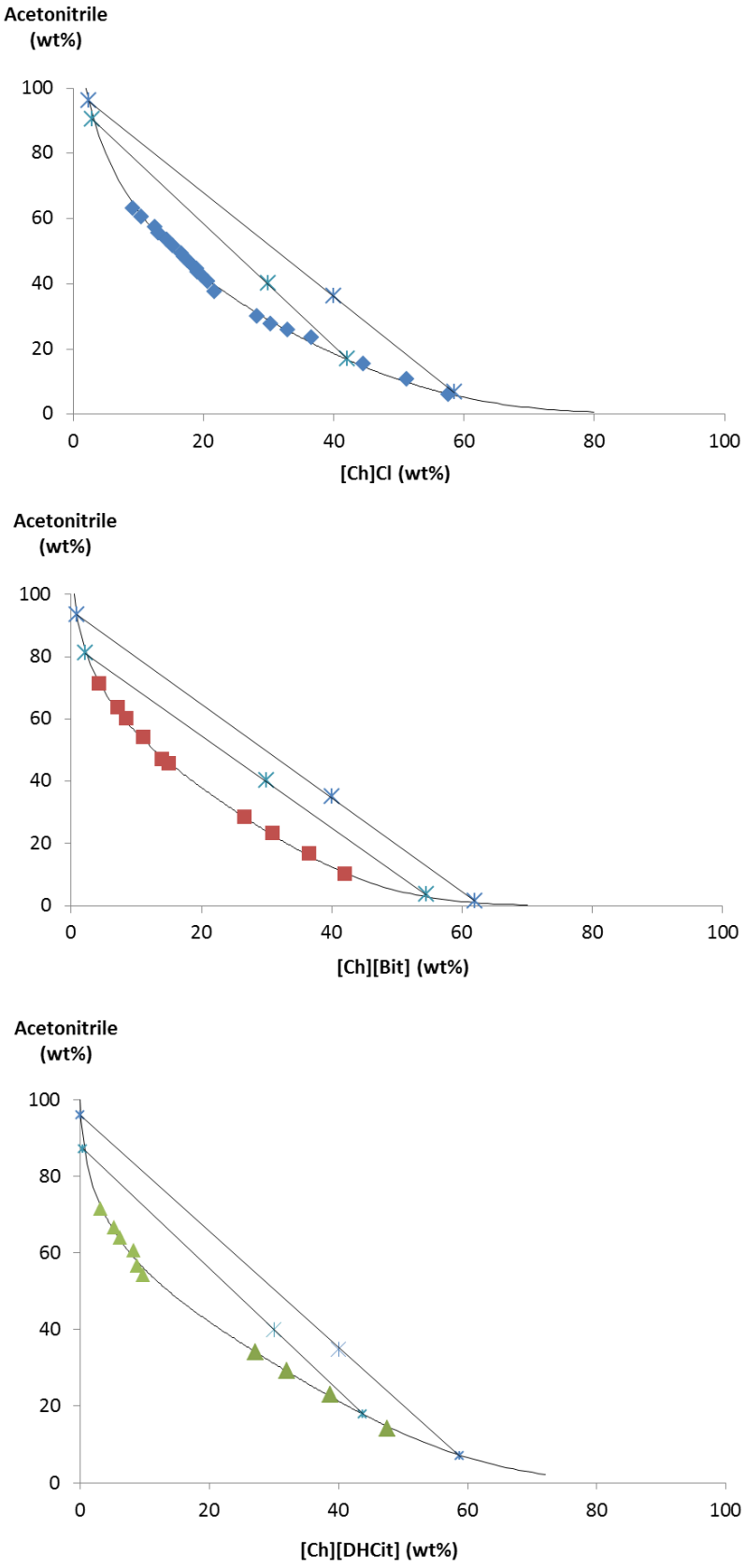

Figure A2: Phase diagrams for the ternary systems composed of acetonitrile + [Ch]X + water at 25°C. (◆) [Ch]Cl, (■) [Ch][Bit], (▲) [Ch][DHCit], (✳) tie-line data, (—) binodal curve adjusted data through Eq. (1).

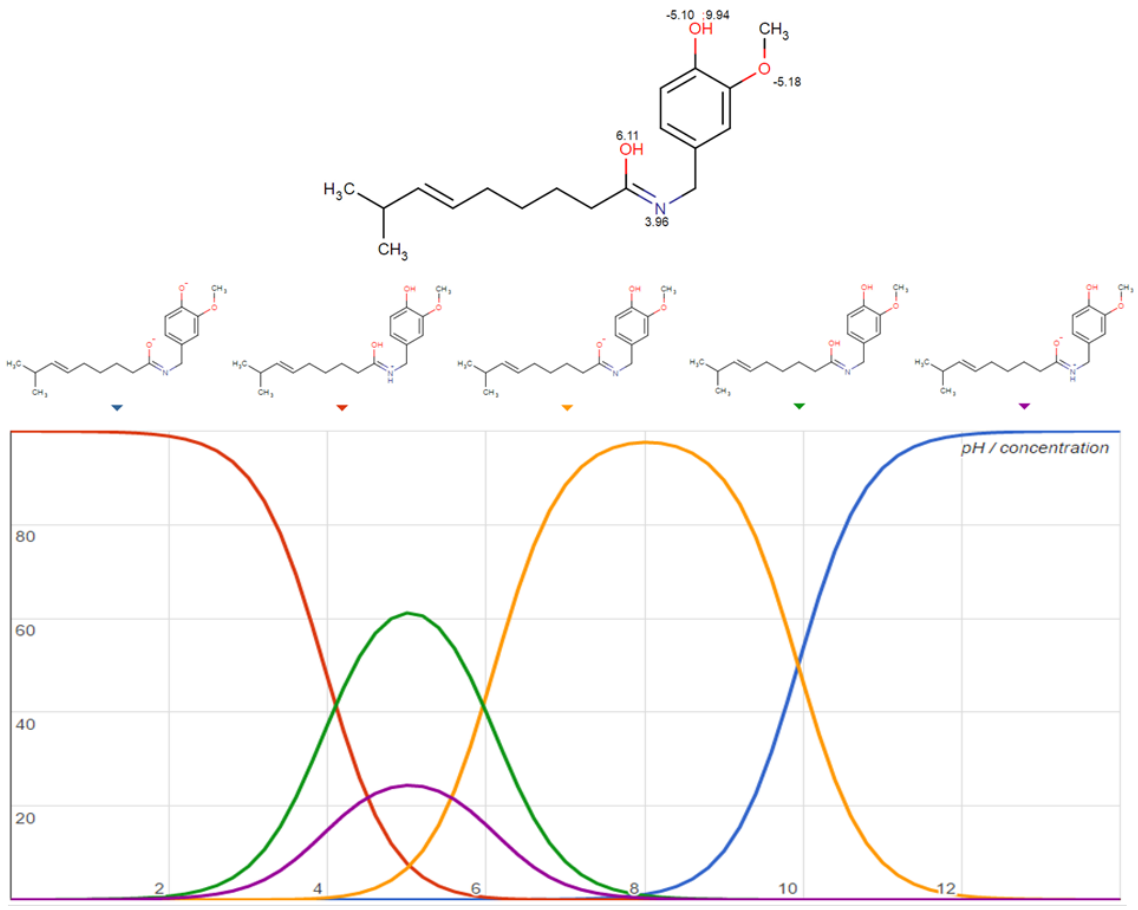

Figure A3: Speciation curves for capsaicin, obtained for different pH values. This content was adapted from the Chemspider chemical database (http://www.chemspider.com/).

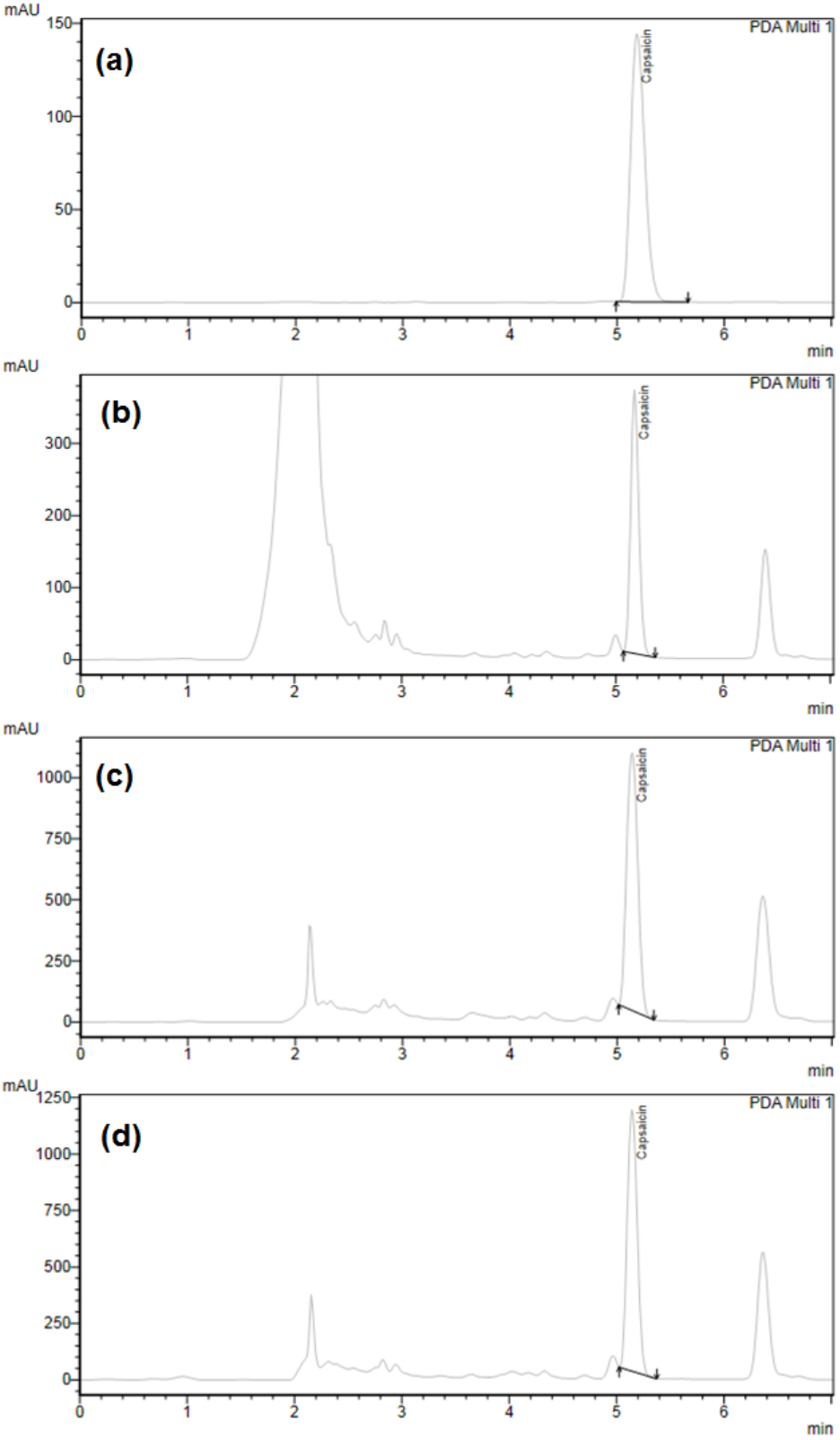

Figure A4: Standard and samples chromatograms: (a) commercial standard CPS, (b) natural pepper extract obtained in section 3.3.1, (c) top phases obtained after the purification of CPS at 298 (±1) K, (d) top phases obtained after the purification of CPS at 318 (±1) K).

ARTIGO III

Aqueous two-phase systems based on acetonitrile and salts of Na^+ and K^+ to purification of capsaicin from *Capsicum frutescens*

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Abstract

Capsaicin is a compound of interest to the pharmaceutical and nutritional industry due to its functional and therapeutic potential and therefore, it is necessary to develop efficient techniques for its recovery from a natural source. This study evaluates the combination of acetonitrile with salts of Na^+ and K^+ to form Aqueous Two-phase System (ATPS) and its application to the purification of capsaicin from *Capsicum* peppers. The results showed that capsaicin migrated, preferentially, to the top phase (acetonitrile-rich) and the optimum conditions for its purification were determined to be 20 wt.% of acetonitrile, 15 wt.% of K_2CO_3 and 65 wt.% of water at 45 °C. The model system was applied successfully in real sample (K_{CPS} = 43.95 ± 1.52; EE_{CPS} = 89.06 ± 1.17 %; and PF_{CPS} = 2.65 ± 0.01).

Keywords: capsaicin, aqueous two-phase systems, acetonitrile, salts.

1. Introduction

The liquid-liquid extraction is a technique commonly used in many industrial processes with the aim of extracting and separating compounds from a matrix, however, this method can cause irreversible damage to the structure of biomolecules [1]. In this context, the Aqueous Two-Phase Systems (ATPS) was proposed in the 50s by Albertsson as alternative method to minimize the occurring this disadvantage and, since then, the ATPS has been detached for simplicity, selectivity and efficiency in partition and purification of biomolecules [2, 3].

The ATPS can be defined as the system of extraction/purification of compounds, based on the principle of liquid-liquid extraction, that uses two immiscible aqueous solutions, which after reaching thermodynamic equilibrium, separate into two phases and displaces the biomolecule for the phase highest affinity [3]. This system can be formed by mixing polymer – polymer [4], polymer – salt [5], polymer - ionic liquids [6], organic solvent – salt [7], ionic liquids- salt [8], among others. The main difference and advantage of the use of ATPS in relation to the liquid-liquid conventional method is that the ATPS requires use of large amounts of water to form the system, a versatile solvent, cheap, environmentally safe and it contributes to reducing the occurrence of structural changes and loss of activity of the biomolecules [9, 10].

After the initial studies, it become necessary to develop alternative systems to improve the characteristic such as cost and viscosity of the components then new systems began to be studied as, for example, alcohols – salt, ionic liquid – salt, among others [11, 12, 13]. The choice of salt to be used in the system is usually made based on the Hofmeister series, also known as lyotropic series, which ordering a set of cations and anions according to its ability to induce *salting in* or *salting out*, and through this sequence, it is possible to explain the ability of the ions have to form two-phase system [14, 15].

Recently, the search for ATPS with new compositions has intensified and, in this context, the acetonitrile (ACN, methyl cyanide, C_2H_3N), which is an aprotic solvent, medium polarity, miscible in water at room temperature, widely used in synthesis and separation of organic compounds [16]. ACN is a solvent that presents low viscosity, high resolution, low boiling point and, due to these properties, it has already been used successfully in ATPS composed by carbohydrates [16], polysaccharides [17], polyols [18], polyvinyl alcohol [19] and ionic liquid [20].

Various biomolecules has already been partitioned using ATPS as, for example, enzymes [7, 21], vanillin [16, 17], rutin [22], gallic acid [11, 23], ascorbic acid [24], antibiotics [25, 26], alkaloids [27, 28], dyes [29], and recently capsaicin [30].

Capsaicin (CPS, 8-methyl-N-vanillyl-trans-6-nonenamide, C18H27NO3), chemically, is a phenolic compound and its biosynthesis can be made phenylpropanoid or fatty acid branched pathway, presents colorless and odorless, molecular weight 305.4 g.mol-1, melting point at 65 °C, boiling point at 210-220 °C, the CPS is insoluble in water and soluble in alcohol, ether, benzene and chloroform, and resistant the acidic and alkaline solutions at room temperature [31]. Organically, the CPS is an alkaloid type produced exclusively in the glands of the placenta from the Capsicum fruits and that, among the group of capsaicinoids, it is the main component responsible for the pungency characteristic of peppers of this kind and also it is known for its biological, pharmacological and therapeutic activities, which it confers potential thermogenic, analgesic, antioxidant, anticancer and, besides this, it is an excellent source of vitamins (C, B complex, A and E) [32, 33, 34, 35].

The pepper is present on the menu of humans for many years and can be considered one of the most consumed spices and grown spices in the world. In 2011, the world production of pepper was superior to 30 million tons [36]. The pepper cultivation in Brazil is important not only to serve as a source of income for many farm families but also due to its functional potential, which is closely related to capsaicin content present in different varieties. However, despite the social, cultural and economic importance that capsaicin confers to the peppers, the amount of available studies about the extraction and purification methods of this biomolecule, it is still limited. Therefore, the search for technologies that are capable to extract and purify capsaicin from its natural source, quickly and efficiently, it became necessary to attend the demand of the food and pharmaceutical industries. In this context, considering the pharmacological and industrial potential of capsaicin, this work proposes the development of ATPS composed of acetonitrile and salts of Na^+ and K^+ to separate, concentrate and purify this biomolecule from pepper *Capsicum frutescens var.* malagueta. The Corresponding phase diagrams, tie lines and tie line lengths were determined at 25°C. Before applying the ATPS for the purify of capsaicin from its natural source, the model system conditions were optimized through evaluation of the effects of the type and concentration of salt, acetonitrile concentration and temperature on the partition synthetic capsaicin.

2. Materials and Methods

2.1 Materials

The acetonitrile, HPLC grade with purity of 99.9 % was purchased from Tedia. The salts were acquired at Sigma-Aldrich® with purity ≥98 %: potassium citrate ($C_6H_5K_3O_7$), sodium citrate ($C_6H_5Na_3O_7$), sodium thiosulfate ($Na_2S_2O_3$), tripotassium phosphate (K_3PO_4), dipotassium phosphate (K_2HPO_4), potassium carbonate (K_2CO_3) and sodium carbonate (Na_2CO_3). The water used during all the experiments is ultrapure, treated with a Milli-Q plus 185 water purification apparatus. Synthetic capsaicin was acquired at Sigma-Aldrich® with high purity (≥ 97%). All the chemical structures and molecular formulas are represented in Supporting Information (Table A1).

The peppers *Capsicum frutescens var.* malagueta (*C. frutescens*) used in this work were acquired at the producer located in the city of Lagarto, Sergipe - Brazil, in the ripe stage. The peppers were selected, sanitized with a sodium hypochlorite solution (10 $mg.L^{-1}$), dried in an oven at 35 ± 1°C until constant weight, macerated in a blender, packed in polypropylene bags and stored for next tasks.

2.2 Phase diagrams and tie-lines

The ATPS were formed using aqueous solutions of acetonitrile at 80 wt.% and seven aqueous solutions of salts ($C_6H_5K_3O_7$, $C_6H_5Na_3O_7$, $Na_2S_2O_3$, K_3PO_4, K_2HPO_4, K_2CO_3 e Na_2CO_3) standardized at 20 wt.%. The phase diagrams were determined at 25 °C and at atmospheric pressure, by the cloud point titration method [7, 19] and the tie-lines (TLs) were determined according to the gravimetric method well reported in the literature [37]. Briefly, the mixing points located in the biphasic region of the diagram were chosen and the solutions prepared, vigorously stirred and centrifuged at 3000 g for 10 min. After the system reached the thermodynamic equilibrium (at 25 °C for at least 18 h), the top and bottom phases were separated and weighed. Each experimental binodal curve was correlated using Equation (1) [37].

$$[\mathrm{ACN}] = \mathrm{A}\, x \exp\{(\mathrm{B}\, x\, [\mathrm{Salt}] X^{0,5}) - (\mathrm{C}\, x\, [\mathrm{Salt}] X^{3})\} \quad (1)$$

where [ACN] and [Salt] were expressed in weight fraction percentages and A, B and C are constants parameters obtained by regression.

The TLs were determined using Equations (2) to (5) for unknown values of $[ACN]_T$, $[ACN]_B$, $[Salt]X_T$ and $[Salt]X_B$.

$$[ACN]_T = A\ x\ \exp\left\{\left(B\ x\ [Salt]X_T^{0,5}\right) - (C\ x\ [Salt]X_T^3)\right\} \quad (2)$$

$$[ACN]_B = A\ x\ \exp\left\{\left(B\ x\ [Salt]X_B^{0,5}\right) - (C\ x\ [Salt]X_B^3)\right\} \quad (3)$$

$$[ACN]_T = ([ACN]_M/\alpha) - ((1-\alpha)/\alpha)\ x\ [ACN]_B \quad (4)$$

$$[Salt]_T = \left([Salt]_{X_M}/\alpha\right) - ((1-\alpha)/\alpha)\ x\ [Salt]X_B \quad (5)$$

where the subscripts M, T and B refer, respectively, to the initial mixture, top and bottom phase. The value of α corresponds to the ratio between the mass of the top phase and the total mass of the mixture.

The length of each tie-line (TLL) was calculated through Equation (6).

$$TLL = \sqrt{([Salt]X_T - [Salt]X_B)^2 + ([ACN]_T - [ACN]_B)^2} \quad (6)$$

2.3 Capsaicin partitioning in the ATPS

The partition systems were prepared in graduated centrifuge tubes (50 mL) and an appropriate amount of salt, water and capsaicin in acetonitrile solution (60 $mg.L^{-1}$) were weighted making a total mass equal to 15 g. The mixtures were then gently stirred and centrifuged at 3000 rpm for 10 minutes. The graduated tubes were placed at the respective temperature, from 5 to 45 °C and atmospheric pressure, for at least 18 hours, using a thermostatic bath MARCONI MA-127, to reach the equilibrium. The two phases were then carefully collected for the determination of their volume and weight, and the capsaicin was properly quantified in both phases in triplicate, using a Varian Cary-50 Bio UV-visible Spectrophotometer, at 280 nm. In this task, to evaluate the capsaicin partition different parameters were calculated namely the partition coefficients (K_{CPS}), the volume ratio (R_v) and the extraction efficiencies (EE_{CPS}) for each ATPS (Equations 7 to 9).

$$K_{CPS} = C_T/C_B \quad (7)$$

$$R_V = V_T/V_B \quad (8)$$

$$EE_{CPS} = (K_{CPS}\ x\ R_V)/(1 + (K_{CPS}\ x\ R_V))\ x\ 100 \quad (9)$$

It should be remarked that for all ATPS studied, the top phase was the acetonitrile-rich phase while the bottom phase corresponds to the salt-rich phase.

2.4 Thermodynamic functions

The thermodynamic parameters of phase transfer, such as the standard molar Gibbs energy of transfer ($\Delta_{tr}G^{o}_{m}$ - KJ.mol^{-1}), the standard molar enthalpy of transfer ($\Delta_{tr}H^{o}_{m}$ - KJ.mol^{-1}) and the standard molar entropy of transfer ($\Delta_{tr}S^{o}_{m}$ - J.mol^{-1}.K^{-1}) were determined through the van't Hoff methodology and calculated according to Equations (10) and (11):

$$ln(K_{CPS}) = -\left(\left(\Delta_{tr}H^{0}_{m}/_{R}\right) x \left(1/_{T_{ref}}\right)\right) + \left(\Delta_{tr}S^{0}_{m}/_{R}\right) \quad (10)$$

$$\Delta_{tr}G^{0}_{m} = \Delta_{tr}H^{0}_{m} - T_{ref}\, x\, \Delta_{tr}S^{0}_{m} \quad (11)$$

where T_{ref} represents the temperature (Kelvin) and R is the universal gas constant (8.314 J.mol^{-1}.K^{-1}).

2.5 Extraction and purification of CPS from pepper Capsicum frutescens var. malagueta

The extract of capsaicin from *Capsicum frutescens var*. malagueta was prepared according methodology described by Santos and co-workers [38], using acetonitrile aqueous solution at 60%. The selected ATPS, considering maximum partition and extraction parameters, were prepared in according to procedure described in partitioning section (item 2.3) and using natural extract of pepper in acetonitrile solution. After the equilibration period, the two phases were separated, collected and the volume, weight and the CPS content determined by HPLC. Then, the PF_{CPS} was determined by the ratio between the specific concentration of capsaicin (SC_{CPS}) present in the acetonitrile crude extract rich in capsaicin and in each phase according to Equations 12 and 13, respectively.

$$SC_{CPS} = C_{CPS}/C_{PC} \quad (12)$$

$$PF_{CPS} = (SC_{CPS})_T/(SC_{CPS})_E \quad (13)$$

where, C_{CPS} is the concentration of capsaicin C_{PC} represents the concentration of phenolic compounds and the subscripts T and E are indicative of the top and acetonitrile crude extract rich in capsaicin, respectively.

The concentration determination of capsaicin by HPLC was performed in equipment model Prominence, brand Shimadzu system with UV-VIS detector, at 280 nm, C18 column type, mobile phase consisted of acetonitrile:water (60:40) at a flow rate of 1.0 mL/min, isocratic mode, column temperature 30 °C, 20 μL of injection volume.

The phenolic content of pepper (PC) in the extract prepared in the solvent extraction step and in both aqueous phases was assessed by colorimetric spectrophotometry using the Folin-Ciocalteau method, using gallic acid as standard [39].

3. Results

3.1 Phase diagrams and Tie-lines

The experimental determination of phase diagrams relative to the formation of ATPS composed by acetonitrile and seven salts was carried out at 25 °C and the binodal curves presented in Figure 1. The values were expressed in molality units to avoid disparities in the evaluation of salts potential to induce the liquid-liquid demixing, that could be a direct consequence of the different molecular weights of the salts involved. Mixture composition above the binodal curve there is the formation of two phases and below this curve, only one phase is formed. The experimental weight fraction data were provided in the Supporting Information (Table A2).

The capacity of salts to form ATPS with acetonitrile (Figure 1) occurred in the following order: $C_6H_5K_3O_7$ > $C_6H_5Na_3O_7$ > $Na_2S_2O_3$ > K_3PO_4 > K_2HPO_4 > K_2CO_3 > Na_2CO_3. It is noted that the ATPS formation following the tendency described by Hofmeister Series in relation to cations (K^+ > Na^+) and anions ($C_6H_5O_7^{3-}$ < $S_2O_3^{2-}$ < PO_4^{3-} > HPO_4^{2-} < CO_3^{2-}) [14]. These results also agreed with the tendency presented by Shahriari and co-workers [40], except to the anion PO_4^{3-}, which formed ATPS easier that $C_6H_5O_7^{3-}$.

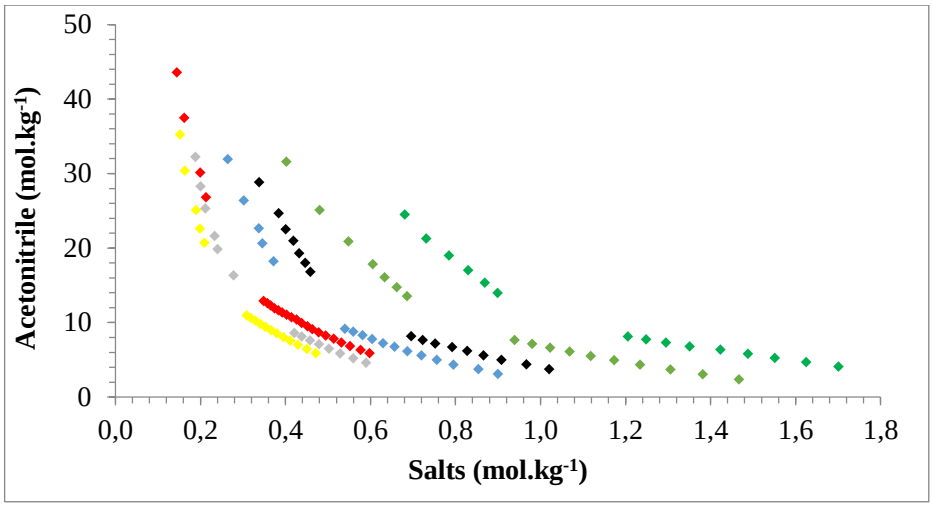

Figure 1: Phase diagrams to the ternary system composed of acetonitrile + salt + water at 25°C. (◆) $C_6H_5K_3O_7$, (◆) $C_6H_5Na_3O_7$, (◆) $Na_2S_2O_3$, (◆) K_3PO_4, (◆) K_2HPO_4, (◆) K_2CO_3, (◆) Na_2CO_3.

The experimental binodal curves data were fitted using the empirical model described by Equation 1, according to Merchuck et al. [37]. The parameters A, B and C (estimated by least-squares regression), and the corresponding standard deviations (σ) and regression coefficients (R^2) are reported in Supporting Information (Table A3). The values shown provide an accurate description of the experimental binodal curves. In Supporting Information are presents the complete phase diagrams with TLs and respective TLLs calculated (Figure A1) and its values are reported (Table A4).

3.2. Capsaicin Partitioning in ATPS

In this work, the use of acetonitrile and seven types of salts for ATPS was investigated as an alternative method for the extraction and purification of capsaicin from pepper *Capsicum frutescens var*. malagueta. In the previous section (section 3.1), the phase diagrams were studied and determined the biphasic region for the system proposed. Then, to determine the best partition conditions of the biomolecule, a model system was developed using synthetic capsaicin and evaluated according to the salt type used, composition of the system and effect of temperature. Figure 2 depicts the results of CPS partitioning.

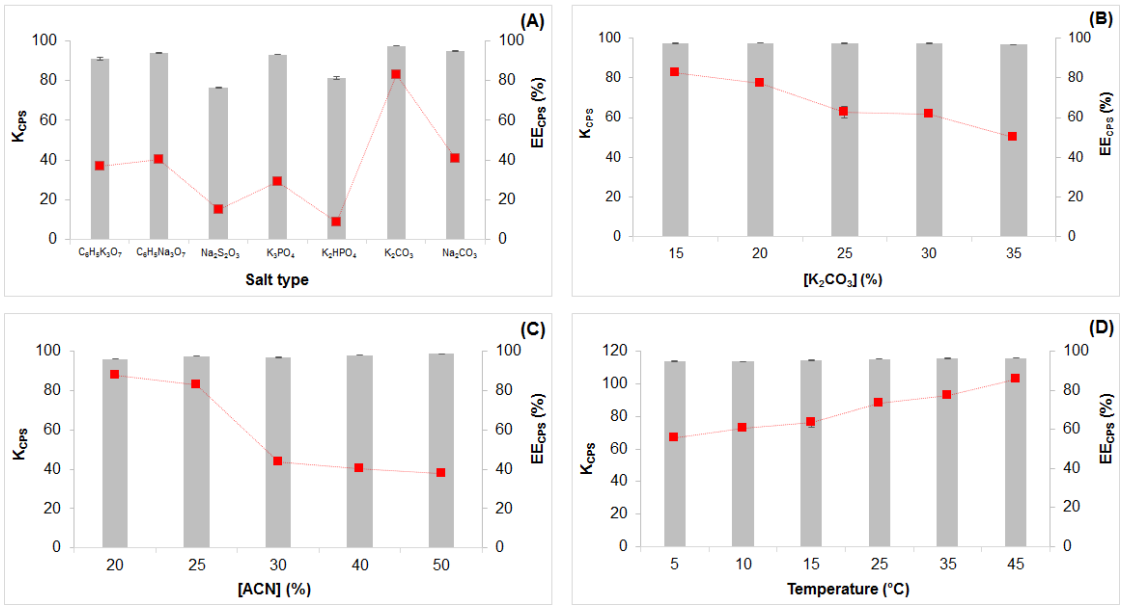

Figure 2: Partition Coefficient, K_{CPS} (■) and Extraction Efficiency, EE_{CPS} (grey bars) of capsaicin determined for different conditions: **A)** Effect of salt type in ATPS composed by 15 wt% salt + 25 wt% acetonitrile + 60 wt% water, at 25 °C; **B)** Effect of the K_2CO_3 concentration in ATPS composed by 15 to 35 wt% K_2CO_3 + 25 wt% acetonitrile, at 25 °C; **C)** Effect of the acetonitrile concentration in ATPS composed by 15 wt% K_2CO_3 + 20 to 50 wt% of acetonitrile, at 25 °C; **D)** Effect of temperature in ATPS composed by 15 wt% K_2CO_3 + 20 wt% of acetonitrile + 65 wt% water, at 5 to 45°C.

3.2.1. Effect of salt type

Analyzing the molecular structure of capsaicin (Table A1, in Supporting Information), it is possible to divide it into three portions: aromatic, amide and hydrocarbon. Barbero and co-workers [41] reported that the length of the side chain of capsaicinoids is an important factor for its bioactivity, since the maximum activity is in the portion with 8 and 9 carbon atoms in the lateral chain, that it presents a lower number of hydrogen-bond acceptors, confers the capsaicin a more hydrophobic character, and consequently higher aptitude for the hydrophobic phase, in this case, the phase rich in acetonitrile.

The results presented in Figure 2A proved that the capsaicin migrated, preferentially, to the top phase rich in acetonitrile ($K_{CPS} >> 1$), with values of K_{CPS} ranging from 8.82 to 82.82, to the system composed of 25 wt.% of acetonitrile + 15 wt.% of salt + 60 wt.% of water. This trend agrees with the extraction efficiencies (EE_{CPS}) results which range from 76.60 to 97.41 %. The results also showed that the capsaicin partition following the tendency of Hofmeister Series in relation to cations ($K^+ > Na^+$), except to the citrate salts. The data of

weight fraction compositions, partition coefficients (K_{CPS}) and extraction efficiency (EE_{CPS}) of capsaicin, determined at 25 °C are shown in Supporting Information (Table A5).

The lipophilicity/hydrophilicity nature of the compounds is related with the octanol–water partition coefficients values (log K_{ow}) and can be also used to evaluate to the ATPS formation and the partition of biomolecules. The values of log K_{ow} of the system components are: CPS = 3.75, ACN = -0.17, $C_6H_5K_3O_7$ = -1.32, $C_6H_5Na_3O_7$ = -1.32, $Na_2S_2O_3$ = 0.05, K_3PO_4 = -1.02, K_2HPO_4 = -1.02, K_2CO_3 = 0.25 and Na_2CO_3 = 0.25 [42]. Based on the log K_{ow} value, it is confirmed the more hydrophobic nature of the capsaicin and, consequently, justify its higher affinity for top phase ($K_{CPS} >> 1$), rich in acetonitrile. Moreover, observing the results of the Figure 2A, it is noted that the K_2CO_3, the second salt more hydrophobic, presented the highest K_{CPS} value (K_{CPS} = 82.82), thereby, it is possible to infer that the combination of acetonitrile with this salt favored the capsaicin partition to the top phase.

The change of the electrical charge form can affect the hydrophobicity/hydrophilicity and influence the solubility of biomolecule in the top phase [43]. The ATPS studied can be also evaluated in relation to pH of each phase, the Table A6 (Supporting Information) presents the pH values. It is observed that the pH of the phases showed more basic characteristic, with a range between 8.20 and 12.85, except the $Na_2S_2O_3$, which showed character slightly acid (pH range between 6.47 and 7.27). However, considering the speciation curves shown in the support information (Figure A2), it was observed that this pH range favored the occurrence of electrostatic interactions by changing the electrical charge of the capsaicin, becoming it more negative and, consequently, influencing its solubility in the top phase. The higher pH values were presented by K_3PO_4 (top: 12.67, bottom: 12.71) and K_2CO_3 (top: 12.59, bottom: 12.28). Then, considering the hydrophobic characteristics and the influence of pH promoted by the presence of K_2CO_3 in the system, which resulted in the higher K_{CPS} and EE_{CPS}, this salt was chosen to the following experiments.

3.2.2. Composition of the system

After choosing the K_2CO_3 as the best kind of salt to partition capsaicin with acetonitrile, the effect of system composition was evaluated. Firstly, it was tested the influence of salt concentration (Figure 2B) that ranged from 15 to 35 wt.% and the acetonitrile composition was fixed in 25 wt.% (numerical data are presents in Table A7, Supporting Information). It was expected that the K_{CPS} value increase with the increasing salt concentration due the *salting out* effect that it should move the capsaicin to the top phase. However, the results indicated that the K_{CPS} value decreased from 82.82 to 50.07. As the

acetonitrile concentration in the system did not change, it is possible that the effect of the R_V became higher than *salting out*, since increasing the salt concentration, increased the value of R_V and also the hydrophobicity in the system, consequently, the CPS developed more affinity for the bottom phase rich in salt, which resulted in reduction of K_{CPS} value. Besides that the biomolecule also can suffer the pH action, becoming more negative and available to interact with ions K^+ and OH^- formed after the interaction between K_2CO_3 and water or yet, the capsaicin can be migrated to the bottom phase due its solubility in alkaline solutions [44]. The similar situation was obtained by Wang and co-workers [45], in their study using ATPS composed by polymer and salt, the increase of the salt concentration increased the ‘‘*salting out* effect” and ‘‘solubilization effect”, reducing the solubility of protein in bottom phase, which in this case, was the phase of interest. The EE_{CPS} value has remained fairly constant in 97%, approximately.

The effect of the acetonitrile concentration on the partition of capsaicin at 25 °C is presented in Figure 2C (Table A8 of Supporting Information). The system was composed of 15 wt.% of K_2CO_3 and the concentration of acetonitrile varied from 20 to 50 wt.%. In this case, the EE_{CPS} value was little influenced by variations in acetonitrile concentration and its value was in the range of 97%. However, in relation to K_{CPS} value, it is observed that occurred a sharp drop when the acetonitrile concentration varied from 20 to 50 wt.%. In general, the lower value of acetonitrile concentration resulted in higher K_{CPS} value due to a reduction in the volume of top phase. According BABU and co-workers [46], the biomolecule tends to concentrate more in phase with lower volume. Then, the optimized composition to the system suggests the use of 15 wt.% of K_2CO_3 + 20 wt.% of de acetonitrile + 65 wt.% of water.

3.2.3. Effect of temperature

After evaluating the effect of the composition of the system on the partition of capsaicin at 25 °C and determining that the best K_{CPS} and EE_{CPS} values were obtained using 20 wt.% acetonitrile +15 wt.% K_2CO_3 + 65 wt.% water, the effect of temperature on the partition was evaluated in the range from 5 to 45 °C and the experimental results are presented in Figure 2D (Table A9 of Supporting Information). It is observed that the increasing the temperature promoted the partition of capsaicin to the top phase, to improving its solubility in the system, resulting in K_{CPS} values between 66.73 and 103.13, in the range of temperature from 5 to 45 °C. The EE_{CPS} value changed from 94.77 to 96.48 % and therefore, it did not significantly influence the partition.

Then, the thermodynamic functions (standard molar Gibbs energy ($\Delta_{tr}G^{o}_{m}$), enthalpy ($\Delta_{tr}H^{o}_{m}$) and entropy ($\Delta_{tr}S^{o}_{m}$) were calculated based on Equations 10-11 in order to better understanding the migration process of capsaicin to the top phase, acetonitrile-rich. The results presented in Figure A3 (Supporting Information) indicate that the migration occurred by spontaneous and endothermic processes, because of negative ($\Delta_{tr}G^{o}_{m}$ = -10.86 kJ.mol^{-1}) and positive (($\Delta_{tr}H^{o}_{m}$ = 7.82 kJ.mol^{-1}) values. Analyzing the relation between temperature and entropy ($T\ x\ \Delta_{tr}S^{o}_{m}$) with the enthalpy ($\Delta_{tr}H^{o}_{m}$), it was noted that entropic effects governed the migration, since the absolute value of $T\ x\ \Delta_{tr}S^{o}_{m}$ (18.68 kJ.mol^{-1}) was superior to $\Delta_{tr}H^{o}_{m}$ (7.82 kJ.mol^{-1}). Thereby, it is possible to conclude that the migration process of capsaicin to the top phase (acetonitrile-rich) and the influence of temperature, it can be justified by the entropic effects which acted increasing the solubility of capsaicin in top phase and, consequently, increasing the K_{CPS} value.

3.3 Extraction and purification of CPS from pepper Capsicum frutescens var. malagueta

Firstly, it was realized a preliminary extraction to obtain the extract of capsaicin from pepper (*Capsicum frutescens var.* malagueta). Then, it was realized the separation and purification of using the model system developed to the ATPS composed of acetonitrile and salt. Figure 3 presents the schematic representative of the extraction and purification process of capsaicin from the pepper.

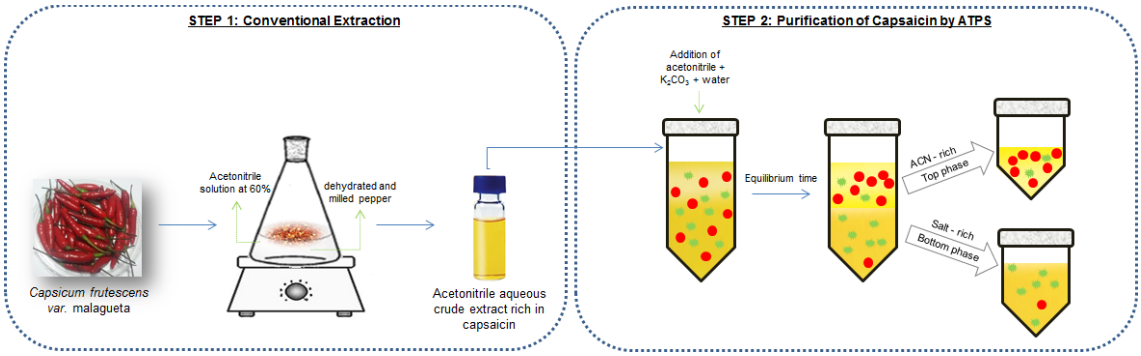

Figure 3: Schematic representative of the extraction and purification process of capsaicin from the pepper.

The validation step of model system was realized using pepper extract prepared with acetonitrile solution at 60% and the ATPS was formed using a system composed by 20 wt.% of acetonitrile, 15 wt.% of K_2CO_3 and 65 wt.% of water and monitored at 25 and 45°C, to

evaluate whether the beneficial effect of temperature remains when a real sample was used. The results of K_{CPS}, EE_{CPS} and PF_{CPS} were presented in Table 1.

The results presented in Table 1 shown that even using a real sample, the capsaicin partitioning to the top phase rich in acetonitrile with K_{CPS} value equal to 43.95 and 42.89 at 25 and 45 °C, respectively, and the EE_{CPS} values were similar to the two temperature studied at 89%. The values of PF_{CPS} in top phase, calculated using equation 13, were equal to 2.65 and 2.57 to 25 and 45 °C, respectively and can be considered a good value. The standard and samples chromatograms (synthetic CPS, the natural pepper extract obtained in the preliminary study, the top and bottom phases obtained after the purification of CPS at 25 and 45 °C, analyzed by liquid chromatography (HPLC) was reported in Figure 4.

The chromatograms confirmed that purification process was successful since the capsaicin was detected only in the top phase, in the bottom phase were detected the peaks of the contaminants with higher area and besides, there was a significant increase in specific CPS concentration on the top phase. Zhao and co-workers [30] combined the aqueous two-phase extraction (ATPE) with two sequence steps of chromatography (macrospores resin and reverse-phase resin, respectively) to extract and purify capsaicin from capsicum oleoresin and they have managed to recover at the end of the three steps between 80 and 93% of capsaicin. In case of this work, the capsaicin was separated and purified from its source with efficiency equal to 89%, in two steps (extraction by conventional method and purification by ATPS), proving that it is possible can say that the proposed system in this work it attained the objective to purify the capsaicin from its natural source with successfully and high efficiency.

The addition of acid in the system tends to reduce the pH effect on the capsaicin structure and, consequently, the partition by ATPS. Besides, the phenolic compounds (contaminants) precipitate in acid system. Then, with the increased stability of biomolecule, it is possible decreasing the phenolic compounds concentration in top phase and improving the PF_{CPS} value.

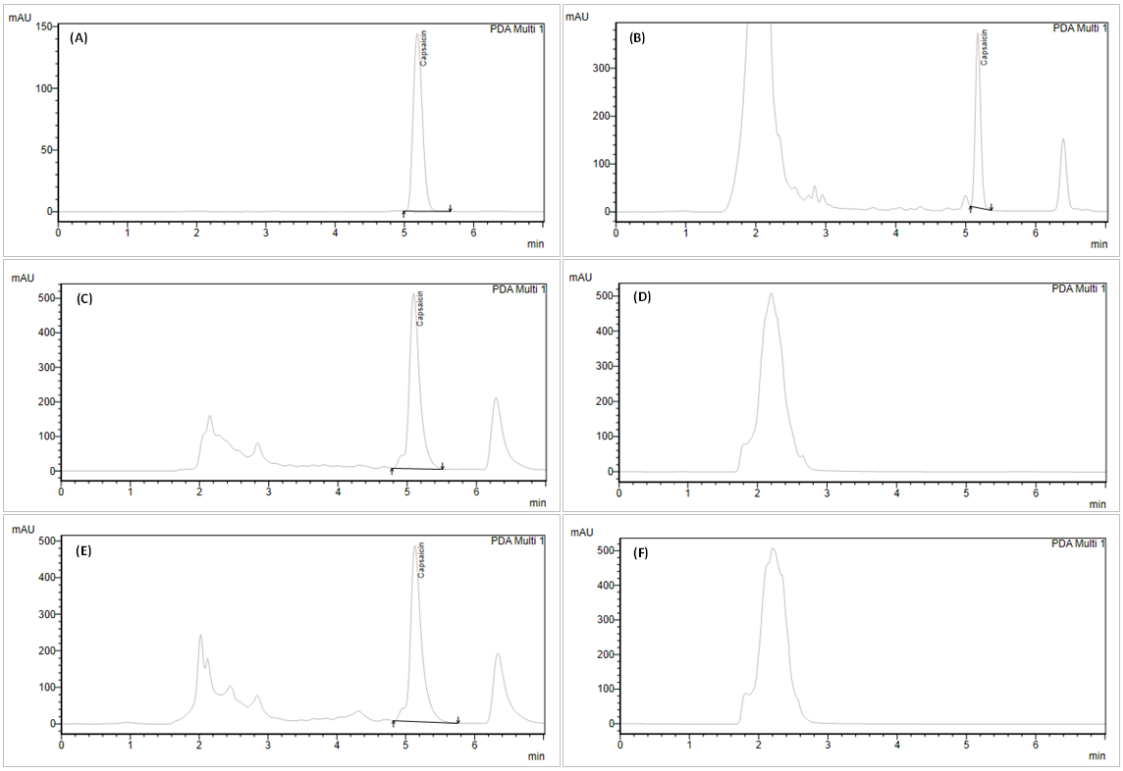

Figure 4: Standard and samples chromatograms: **(A)** synthetic CPS, **(B)** natural pepper extract obtained in section 3.3, **(C)** top phase obtained after the purification of CPS at 25 °C, **(D)** bottom phase obtained after the purification of CPS at 25 °C, **(E)** top phases obtained after the purification of CPS at 45 °C, **(F)** bottom phases obtained after the purification of CPS at 45 °C.

Table 1: Partition Coefficient (K_{CPS}), Extraction Efficiency (EE_{CPS}) and Purification Factor (PF_{CPS}) of capsaicin from the pepper *Capsicum frutescens var.* malagueta obtained using the optimized ATPS: 15 wt.% of K_2CO_3 + 20 wt.% of acetonitrile + 65 wt.% of water at 25 and 45 °C.

Temperature (°C)	K_{CPS}	EE_{CPS} (%)	PF_{CPS}
25	43.95 ± 1.52	89.06 ± 1.17	2.65 ± 0.01
45	42.89 ± 0.59	89.16 ± 1.11	2.57 ± 0.00

4. Conclusions

The ATPS composed of acetonitrile and salts were developed and applied to the purification of capsaicin from the natural extract of pepper *Capsicum frutescens var.*

malagueta with success and efficiency. The mixture of acetonitrile and salts formed ATPS easily and following the trend of Hofmeister Series, in relation to cations and anions. The best condition of the model system was conducted using synthetic capsaicin and the best K_{CPS} (103.13 ± 1.41) and EE_{CPS} (96.48 ± 0.07) values were found to the system composed of 20 wt.% of acetonitrile + 15 wt.% of K_2CO_3 + 65 wt.% of water at 45 °C. Then, to the apply the model system to purify the capsaicin from its natural extract, the successful was achieved (K_{CPS} = 43.95 ± 1.52; EE_{CPS} = 89.06 ± 1.17 %; PF_{CPS} = 2.65 ± 0.01). When compared with the method which combined aqueous two-phase extraction and chromatography in a sequence of three steps, the results obtained in this work (in two steps) were betters because required the use of lower quantity of acetonitrile and K_2CO_3 in its composition and the use of high water content contributed to reducing the environmental impacts and cost, besides become the process more rapid and simple.

Acknowledgments

The authors thank CNPq, CAPES and Fundação de Amparo à Pesquisa e Inovação Tecnológica do Estado de Sergipe – FAPITEC for financial support and scholarship of P. L. Santos and Programa de Bolsas de Iniciação Científica of Tiradentes University - PROBIC/UNIT scholarship of L. N. S. Santos.

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APPENDICE A. Supporting Information

Supporting data associated with this article can be found, in the online version.

Supporting Information

Aqueous two-phase systems based on acetonitrile and salts of Na^+ and K^+ to purification of capsaicin from *Capsicum frutescens*

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Table A1: Name, molecular formula, and chemical structure of the system components proposed.

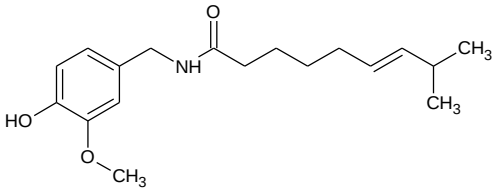
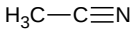
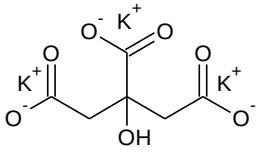
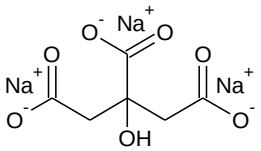
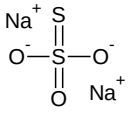
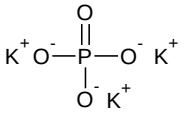
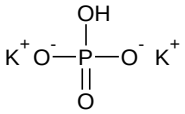
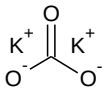
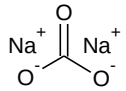
Name	Molecular Formula	Chemical Structure
Capsaicin	$C_{18}H_{27}NO_3$	
Acetonitrile	C_2H_3N	
Potassium Citrate	$C_6H_5K_3O_7$	
Sodium Citrate	$C_6H_5Na_3O_7$	
Sodium Thiosulfate	$Na_2S_2O_3$	
Tripotassium Phosphate	K_3PO_4	
Potassium Phosphate Dibasic	K_2HPO_4	
Potassium Carbonate	K_2CO_3	
Sodium Carbonate	Na_2CO_3	

Table A2. Experimental weight fraction data for the ternary systems composed of acetonitrile (1) + salt (2) + water (3) at 25 °C.

$C_6H_5K_3O_7$		$C_6H_5Na_3O_7$		$Na_2S_2O_3$		K_3PO_4		K_2HPO_4		K_2CO_3		Na_2CO_3	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
59.1052	4.7157	56.9344	5.2614	64.1270	3.4735	51.9655	6.0318	54.2101	5.5641	56.4609	5.2718	50.1307	6.7319
55.4893	5.0543	53.7237	5.5783	60.5913	3.8667	48.1562	6.6921	50.3160	6.2803	50.7089	6.2298	46.5970	7.1934
50.7221	5.8074	50.9398	5.8748	55.2728	4.7348	45.8380	6.8493	48.0155	6.5297	46.1639	7.0475	43.7856	7.6817
48.1197	6.0726	47.0055	6.4384	52.3751	5.0404	42.7863	7.3226	46.2643	6.8052	42.2168	7.7286	41.0740	8.0855
45.9157	6.3632	44.8805	6.6028	19.3952	12.9299	40.8566	7.4626	44.2029	7.0102	39.7314	8.0569	38.6022	8.4352
19.4076	13.2787	40.1014	7.5647	20.5317	12.5305	15.1006	14.4501	42.5070	7.2279	37.6707	8.3901	36.4409	8.7032
20.9309	12.7527	15.7878	14.7832	21.8586	12.0541	16.9215	13.8334	40.7873	7.4054	35.6584	8.6645	14.3427	15.2806
22.3441	12.2408	17.5183	14.1336	23.0819	11.6586	18.5770	13.2592	13.2675	15.0988	8.7935	16.8598	16.0310	14.6939
23.6527	11.7869	19.3581	13.4712	24.2491	11.3067	20.1513	12.7341	15.2462	14.4218	11.0693	16.0371	17.6299	14.1246
24.7950	11.3713	21.0275	12.8773	25.2702	10.9436	21.6285	12.2370	16.9726	13.6654	13.0680	15.2932	19.1391	13.6228
25.9137	10.9738	22.4778	12.3502	26.2594	10.6229	22.8693	11.7973	18.6237	13.1109	15.0569	14.5738	20.6934	13.1132
26.8707	10.6254	23.7745	11.8780	27.2145	10.3240	24.1772	11.3662	20.1738	12.6013	16.8217	13.9599	21.7185	12.5306
27.7962	10.2891	24.9414	11.4353	28.0474	10.0871	25.3465	10.9871	21.5956	12.1284	18.3936	13.3902	23.0313	12.0712
28.6663	9.9670	26.0419	11.0281	28.9290	9.8245	26.3940	10.6241	22.6687	11.5950	19.9836	12.8701	24.0379	11.6919
29.5095	9.6705	27.1258	10.6370	29.8781	9.5757	27.3122	10.2948	23.8543	11.1934	21.3444	12.3881	25.0121	11.3324
30.2807	9.3850	28.0557	10.2902	30.5296	9.3218			25.0220	10.8116	22.6009	11.9403		
31.0040	9.1159	28.9679	9.9533	31.1368	9.1012					23.8461	11.4939		
				31.7757	8.8877								
				32.3073	8.6938								
				32.8340	8.5031								
				33.4838	8.3254								
				34.0569	8.1679								
				34.6134	7.9761								

Table A3: Adjusted parameters and respective standard deviations (std) obtained from the application of the Merchuk equation (Eq. 1) for the ternary systems composed of salt + acetonitrile + water at 25 °C.

Salt	Regression Parameters			
	A ± std	**B ± std**	**C ± std**	**R^2**
$C_6H_5K_3O_7$	284.25 ± 6.7	-0.722 ± 0.024	$1.4 \times 10^{-5} \pm 1.9 \times 10^{-5}$	0.9989
$C_6H_5Na_3O_7$	295.81 ± 18.0	-0.821 ± 0.026	$3.4 \times 10^{-5} \pm 1.6 \times 10^{-5}$	0.9989
$Na_2S_2O_3$	193.58 ± 6.9	-0.585 ± 0.017	$8.5 \times 10^{-5} \pm 1.8 \times 10^{-5}$	0.9980
K_3PO_4	347.15 ± 37.0	-0.765 ± 0.044	$6.4 \times 10^{-5} \pm 2.5 \times 10^{-5}$	0.9985
K_2HPO_4	304.40 ± 30.9	-0.717 ± 0.042	$9.4 \times 10^{-5} \pm 2.4 \times 10^{-5}$	0.9985
K_2CO_3	205.77 ±13.54	-0.547 ± 0.027	$2.0 \times 10^{-4} \pm 1.4 \times 10^{-5}$	0.9988
Na_2CO_3	419.12 ±43.64	-0.812 ± 0.041	$4.6 \times 10^{-5} \pm 1.9 \times 10^{-5}$	0.9989

Table A4: Mass fraction compositions and respective standard deviations (std) for the TLs and respective tie-line lengths (TLLs), at the Top (T) and Bottom (B) phases, and at the initial biphasic composition of the mixture (M), composed of acetonitrile (Y) and salt (X), at 25 °C and atmospheric pressure.

Salt	100 x weight fraction (wt%)						
	Y_M ± std	X_M ± std	Y_T ± std	X_T ± std	Y_B ± std	X_B ± std	TLL
$C_6H_5K_3O_7$	30.00 ± 0.00	20.00± 0.00	87.54 ± 0.67	2.66 ± 0.03	4.77 ± 0.04	27.60 ± 0.08	86.45
	25.08 ± 0.05	15.12± 0.08	78.50 ± 0.53	3.18 ± 0.03	12.29 ± 0.15	17.98 ± 0.12	67.85
$C_6H_5Na_3O_7$	30.01 ± 0.01	20.00± 0.03	88.27 ± 0.40	2.81 ± 0.07	3.12 ± 0.10	27.93 ± 0.23	88.78
	25.02 ± 0.01	15.04 ± 0.03	72.76 ± 0.99	3.77 ± 0.07	10.89 ± 0.16	18.37 ± 0.12	63.57
$Na_2S_2O_3$	30.00 ± 0.00	20.01 ± 0.01	99.56 ± 0.42	1.38 ± 0.07	1.50 ± 0.13	27.64 ± 0.35	99.93
	25.02 ± 0.01	14.99 ± 0.01	96.20 ± 0.40	1.43 ± 0.20	10.17 ± 0.35	17.82 ± 0.22	87.59
K_3PO_4	30.00 ± 0.00	20.00 ± 0.00	87.25 ± 0.21	3.25 ± 0.01	1.38 ± 0.02	28.38 ± 0.08	89.47
	25.00 ± 0.02	15.01 ± 0.06	75.46 ± 0.40	3.96 ± 0.04	8.47 ± 0.21	18.63 ± 0.16	68.58
K_2HPO_4	30.00 ± 0.00	20.01 ± 0.00	85.89 ± 0.20	3.10 ± 0.01	0.67 ± 0.00	28.87 ± 0.02	89.03
	25.03 ± 0.02	15.06 ± 0.04	76.80 ± 0.29	3.67 ± 0.02	6.96 ± 0.14	19.05 ± 0.11	71.52
K_2CO_3	30.00 ± 0.00	20.01 ± 0.01	92.62 ± 0.07	2.12 ± 0.00	0.11 ± 0.00	28.55 ± 0.00	96.22
	25.00 ± 0.00	15.01 ± 0.00	86.93 ± 0.21	2.46 ± 0.03	4.63 ± 0.03	19.14 ± 0.02	83.97
Na_2CO_3	20.07 ± 0.04	20.08 ± 0.06	77.04 ± 0.28	4.27 ± 0.06	3.86 ± 0.02	24.50 ± 0.03	76.89
	25.00 ± 0.00	15.00 ± 0.01	68.20 ± 0.04	5.20 ± 0.02	9.09 ± 0.01	18.86 ± 0.01	57.95

Table A5. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at 25 °C, obtained in the study of the effect of different salts.

Salts	100 x Mass fraction composition (wt%)			K_{CPS}	EE_{CPS} (%)
	ACN	Salt	water		
$C_6H_5K_3O_7$	25.00	15.01	59.99	36.69±1.94	91.13±0.64
$C_6H_5Na_3O_7$	25.00	15.00	60.00	40.27±0.38	93.79±0.16
$Na_2S_2O_3$	25.01	15.00	59.99	15.11±0.25	76.60±0.30
K_3PO_4	25.01	15.01	59.98	29.33±0.45	93.05±0.04
K_2HPO_4	25.00	15.00	59.99	8.84±0.05	81.46±0.65
K_2CO_3	25.00	15.01	59.99	82.82±0.28	97.41±0.05
Na_2CO_3	25.00	15.00	59.99	40.97±0.98	94.74±0.20

Table A6: pH values of both top and bottom phases for the system composed of 25 wt% of acetonitrile + 15 wt% of salt + 60 wt% of water, at 25 °C.

Salt	Top phase	Bottom phase
$C_6H_5K_3O_7$	8.92	8.20
$C_6H_5Na_3O_7$	12.14	11.63
$Na_2S_2O_3$	7.18	6.47
K_3PO_4	12.67	12.71
K_2HPO_4	9.56	9.44
K_2CO_3	12.59	12.28
Na_2CO_3	12.14	11.63

Table A7. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at 25 °C obtained in the study of the effect of different K_2CO_3 concentrations.

K_2CO_3 wt%	100 x Mass fraction composition (wt%)			R_v	K_{CPS}	EE_{CPS} (%)
	ACN	K_2CO_3	water			
15	25.01	15.01	59.99	0.45 ± 0.01	82.82 ± 0.82	97.41 ± 0.05
20	25.00	20.01	54.99	0.52 ± 0.02	77.29 ± 0.31	97.58 ± 0.08
25	25.01	25.00	49.99	0.58 ± 0.01	62.58 ± 2.83	97.31 ± 0.16
30	25.00	30.00	45.00	0.59 ± 0.01	61.80 ± 0.41	97.32 ± 0.10
35	25.01	35.00	40.01	0.61 ± 0.01	50.07 ± 0.30	96.82 ± 0.04

Table A8. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at 25 °C obtained in the study of the effect of different acetonitrile concentrations.

ACN (wt%)	100 x Mass fraction composition (wt%)			R_v	K_{CPS}	EE_{CPS} (%)
	ACN	K_2CO_3	water			
20	20.00	15.00	64.99	0.28 ± 0.010	87.99 ± 0.09	96.09 ± 0.02
25	25.00	15.01	59.99	0.45 ± 0.01	82.82 ± 0.28	97.41 ± 0.05
30	30.00	15.00	54.99	0.71 ± 0.01	43.76 ± 1.15	96.86 ± 0.13
40	40.00	15.00	45.00	1.24 ± 0.05	40.14 ± 1.12	98.03 ± 0.03
50	49.99	15.00	35.00	2.06 ± 0.03	37.75 ± 0.15	98.73 ± 0.02

Table A9. Experimental weight fraction compositions, partition coefficient (K_{CPS}) data and extraction efficiency (EE_{CPS}) results determined at 25 °C obtained in the study of the effect of different temperatures.

Temperature (°C)	100 x Mass fraction composition (wt%)			K_{CPS}	EE_{CPS} (%)
	ACN	K_2CO_3	water		
5	20.00	15.00	64.99	66.73 ± 0.65	94.87 ± 0.16
10	20.01	15.00	64.99	72.45 ± 0.74	94.77 ± 0.05
15	20.00	15.01	64.99	76.13 ± 2.64	95.50 ± 0.29
25	20.00	15.00	64.99	87.99 ± 0.09	96.07 ± 0.00
35	20.00	15.01	64.99	92.76 ± 0.44	96.22 ± 0.24
45	20.00	15.00	64.99	103.13 ± 1.41	96.48 ± 0.07

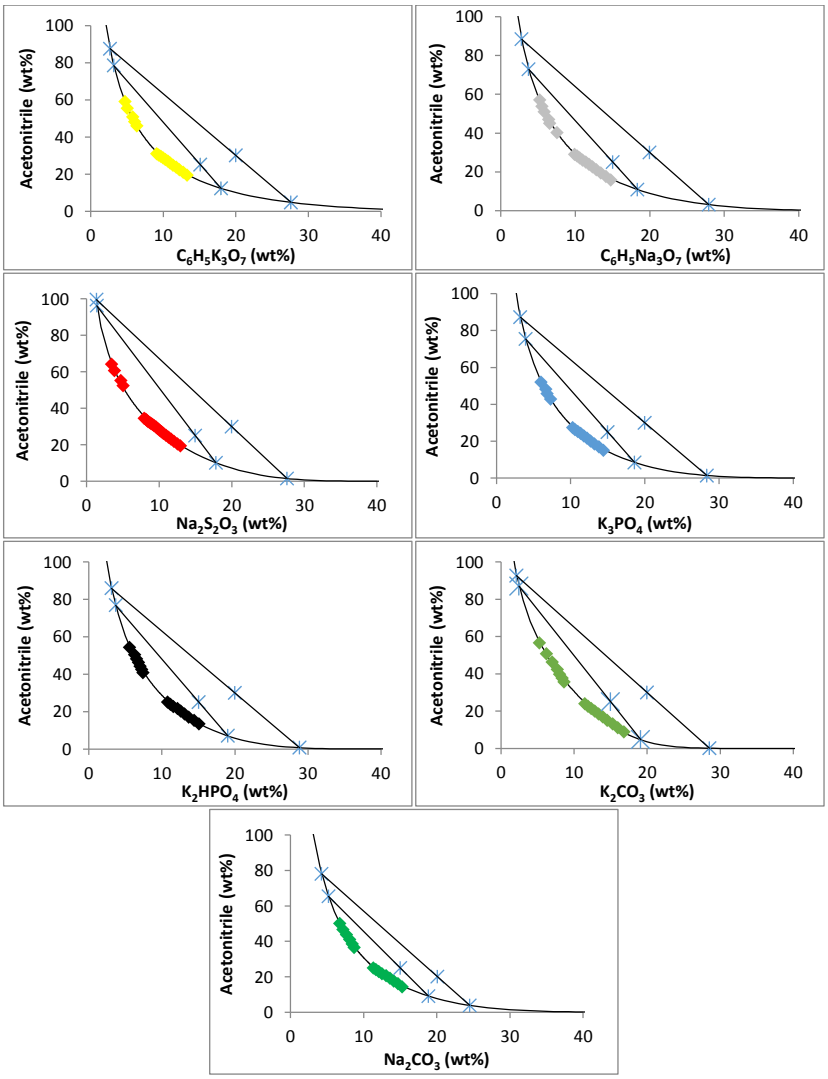


Figure A1: Phase diagrams for the ternary systems composed of ACN + salts + water at 25°C. (◇) experimental data, (✳) tie-line, (—) binodal curve adjusted data through Eq. (1).

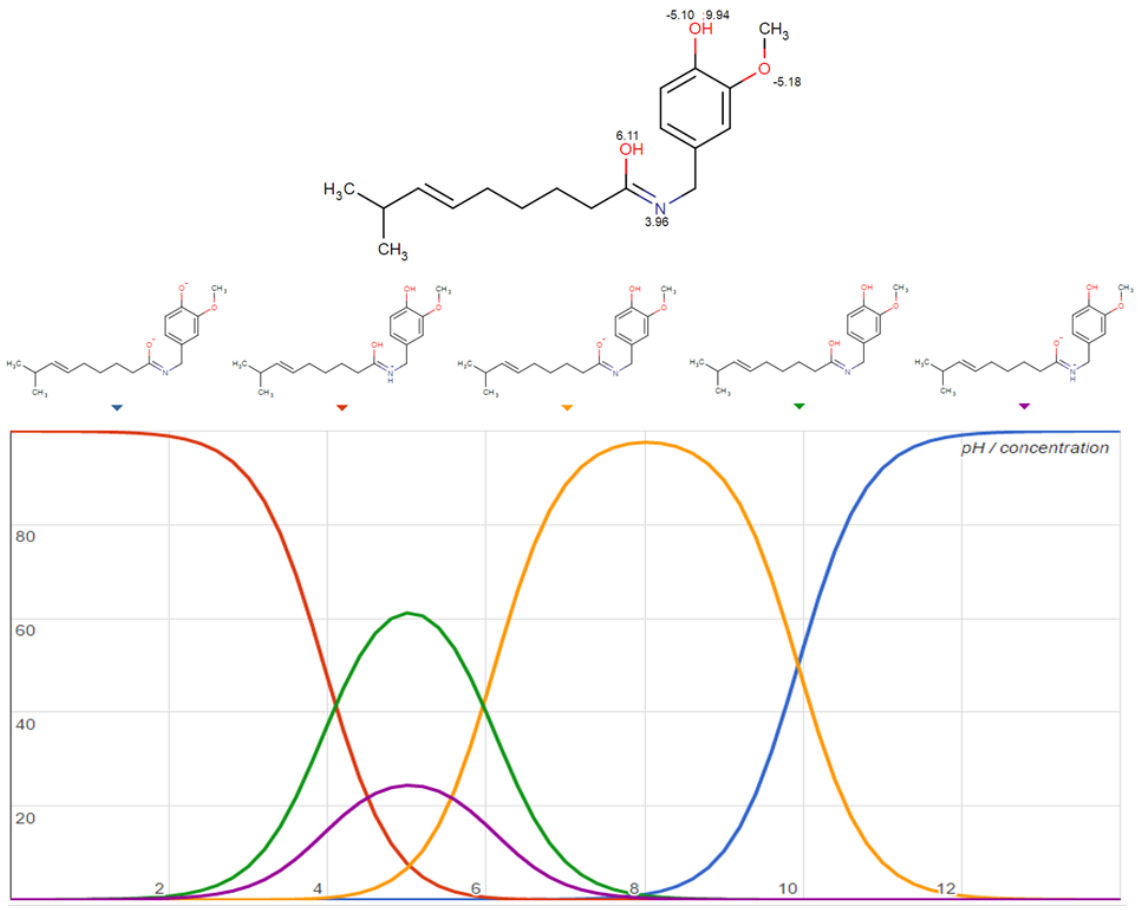


Figure A2: Speciation curves for capsaicin, obtained for different pH values. This content was adapted from the Chemspider chemical database (http://www.chemspider.com/).

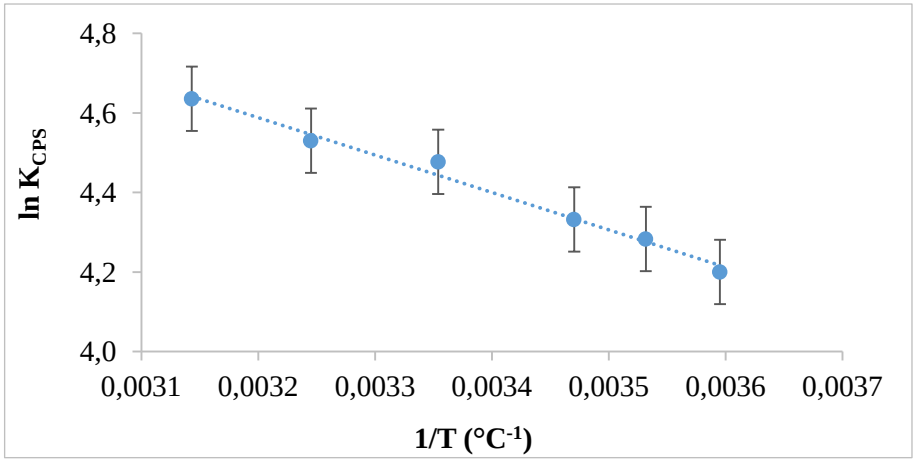


Figure A3: Relationship between K_{CPS} - $\ln(K_{CPS})$ - versus T^{-1} ($°C^{-1}$) for the CPS partitioning considering the system 20 wt% of acetonitrile + 15 wt.% of K_2CO_3 + 65 wt.% water at different temperatures.

ARTIGO IV

Partitioning of capsaicin using aqueous two-phase systems based on ethanol + sodium salts

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ABSTRACT: Capsaicin is a very powerful alkaloid with important pharmacological effects, namely as pain relief additives, and in the cancer prevention, weight reduction, cardiovascular benefits, among others. Liquid-liquid extraction technique applied to this capsaicinoids class is promising, in view of the development separation processes more biocompatible. In this sense, aqueous two-phase systems (ATPS) based on ethanol + sodium salts was applied for separation of capsaicin. The ability of the salts to promote the formation of ATPS with ethanol follows this trend: [$Na_2S_2O_3$] < [NaH_2PO_4] < [Na_2CO_3] ≈ [Na_2SO_4]. The optimum conditions for this separation were determined to be 20 wt.% of ethanol and 25 wt.% of NaH_2PO_4. The best extraction efficiency of 82.65 % ± 0.53 and partition coefficient of 5.23 ± 0.19 were obtained in ATPS. A purification factor of 4.38 ± 0.09 to the top phase, a capsaicin yield of 99.79 ± 0.01% and a partition coefficient for ethanol-rich phase of 1066.02 ± 65.17 were achieved. This result confirms the potential of this new ethanol-based ATPS for capsaicin separation from its natural source.

Keywords: capsaicin, separation, aqueous two-phase systems, ethanol.

Introduction

Chili brings the special flavors and colors needed in the food as an integrated between culture and customs of various countries of the world, especially in temperate regions of Central, South America and European countries, as well as tropical and subtropical regions of the Asian continent. The hot and spicy taste of chili pepper is produced by a chemical capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide) [1]. This is a very powerful alkaloid found in the placental tissue that surrounds the seeds in these fruits [2].

The concentration of capsaicinoids (which include capsaicin and its analogs as, for example, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin [3]) is depending on the type of chili. The chili that is slightly spicy has capsaicinoids concentration ranging from 0.003% to 0.01% dry weight of pepper, and strongly spicy in chili varieties exceeds 0.3% reaching about 1% dry weight of total capsaicinoids [4]. Other studies have shown that capsaicinoids content is genetically controlled, but also affected by environmental variables such as temperature, light, soil moisture levels or fertilizing [5-7]. Moreover, chili peppers are a great source of antioxidants capable of being used in the diet such as flavonoids, phenolic acids, carotenoids, vitamin A, ascorbic acid, and tocopherols [8]. Thus, the capsaicin has been used therapeutically as a topical analgesic treatment such as rheumatoid arthritis, post herpetic neuralgia [9], diabetic neuropathy [10], osteoarthritis and anticancer agent [11-13]. Extracts of capsaicinoids also has shown that contain antibacterial properties against *Salmonella typhimurium*, *Pseudomona aeruginosa* [14] and against *Helicobacter pylori* [15], among others [16, 17].

Conventional methods for capsaicin purification uses, commonly, chromatography [18, 19], but it have limitations to scale-up due of the high cost and requiring the use of sophisticated equipment. For this reason, the investigation turned their interest to the extraction of capsaicin. Aqueous two-phase systems (ATPS), originally proposed by Albertsson in 1958 [20], was used for the separation and purification of a great number of biological products as biological compounds [21], enzymes [22-24], alkaloids [25], antibiotics [26] and antioxidants [27], dyes [28], aroma compounds [29] and other organic compounds [30, 31]. ATPS consist of two immiscible aqueous-rich phases promoted by the addition of two water soluble solutes that can be polymer-polymer, or polymer-salt combinations [32]. The polymer-polymer and polymer-salt ATPSs have several disadvantages, due to the high viscosity and low polarity of the polymers, the slow segregation of the two phases and the complications associated with the recycling of phase components [22, 33]. The partitioning between both phases is

dependent on the surface properties of the molecule and in the composition of the two-phase system. And in this case, the use of ATPS composed of an organic solvent and a salt solution, have advantages that include its low viscosity, high polarity, and the alcohol can be recovered by evaporation [34]. Furthermore, organic solvent + salt can be a good alternative in the formations of ATPS. It is noteworthy that, the number of data in the literature on the extraction/purification of capsaicin using ATPS is quite limited, with only one published works until this moment [35].

This work focuses in the design of several ternary systems, based in ethanol and seven sodium salts (sodium acetate [$NaC_2H_3O_2$], sodium carbonate [Na_2CO_3], sodium chlorate [$NaClO_3$], sodium nitrate [$NaNO_3$], sodium sulfate [Na_2SO_4], sodium thiosulfate [$Na_2S_2O_3$], sodium phosphate [NaH_2PO_4]). These ATPS are compared in terms of phase separation ability, and aiming at exploring the applicability of those ATPS is shown the ability of systems for the separation and purification of capsaicin. Subsequently, to establish the optimum conditions for the separation of capsaicin an analysis of response surface by central composite rotational design (CCRD) was used.

Materials and Methods

Material

The salts used were sodium acetate ($NaC_2H_3O_2$, purity ≥ 99 wt%), sodium carbonate (Na_2CO_3, purity ≥ 99 wt%), sodium chlorate ($NaClO_3$, purity ≥ 99 wt%), sodium nitrate ($NaNO_3$, purity ≥ 99 wt%), sodium sulfate (Na_2SO_4, purity ≥ 99 wt%), sodium thiosulfate ($Na_2S_2O_3$, purity ≥ 98 wt%), sodium phosphate (NaH_2PO_4, purity ≥ 99 wt%). The salts were purchased from Sigma-Aldrich® and used as received. Ethanol was purchased from Sigma-Aldrich® with a purity greater than 99% by weight. The water used in all the experiments was ultrapure and distilled. A commercial sample of capsaicin (N-vanillylnonanamide) was acquired from Sigma-Aldrich® with high purity ≥ 98%.

Experimental phase diagrams

Aqueous solutions of each salt with variable mass fractions (close to the saturation solubility in water at room temperature) and ethanol solution, were prepared for the determination of ternary phase diagrams, which were established at 298 (± 1) K and at atmospheric pressure, by the cloud point titration method [36]. The systems compositions were determined by weight quantification of all components added considering an uncertainty of ± 10^{-4} g. The

experimental solubility curves were correlated and evaluated using three equations: Equation 1 [37]; Equation 2 [38] and; Equation 3 [39].

$$w_1 = A\ exp[(Bw_2^{0,5}) - (Cw_2^3)] \quad (1)$$

$$w_1 = A + Bw_2^{0,5} + Cw_2 \quad (2)$$

$$w_1 = A + Bw_2^{0,5} + Cw_2 + Dw_2^2 \quad (3)$$

Where w_1 and w_2 are the mass fraction percentages of the ethanol and the salt, respectively. A, B, C and D are fitting parameters obtained by least squares regression.

The tie-lines (TLs) which describe the concentrations of ethanol and salts in the two phases, were calculated by the application of the lever-arm rule, measured with the procedure outlined in our previous work [36], being their lengths (*i.e.* TLL) described as the Euclidean distance between the top and bottom phase compositions as denoted in Equation 4 [40].

$$TLL = [(\Delta w_1)^2 + (\Delta w_2)^2]^{0,5} \quad (4)$$

The location of the critical point of the ternary systems was estimated by extrapolation from the TLs compositions applying the Eq. (5) [41].

$$[\mathrm{Et}] = f + g[\mathrm{IL}] \quad (5)$$

where *f* and *g* are fitting parameters.

Preparation of the ATPS

The biphasic systems were prepared in graduated centrifuge tubes (20 ml) by weighing the appropriate amounts of ethanol (20-25 wt%) and salts (20-35 wt%). All systems contained approximately 60 mg.L^{-1} (considering the total mixture) of CPS. Each mixture was prepared gravimetrically within ±10^{-4} g, vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) and at 25 ºC (±1 ºC). After this treatment, the two phases became clear and transparent and the interface was well defined. The phases were carefully separated using a pipette for the top phase and a syringe with a long needle for the bottom phase. The weight (uncertainty of ±10^{-4} g) and volume (uncertainty of ± 0.1 mL) were determined in graduated test tubes (the total mass of the extraction systems prepared is 15.0 g). Optimization of ATPS conditions for the partition coefficient of CPS was performed using a 2^2 central composite rotational design (CCRD). The CCRD was used with four axial points and three center points, resulting in 11 experiments.

The partition coefficient was defined as the capsaicin concentration (*K*), divided by the corresponding value in the bottom phase, as describe by Eq. (6)

$$K = \frac{C_T}{C_B} \quad (6)$$

where C_T and C_B are, respectively, the total capsaicin concentration ($mg.mL^{-1}$) in the top and bottom phases, respectively.

In order to evaluate the capsaicin extraction efficiencies (*EE*, %) and the volume ratio (R_v), in each ATPS, the following equations were used:

$$R_V = \frac{V_T}{V_B} \quad (7)$$

$$EE, \% = \left(\frac{K\,R_V}{1+K\,R_V}\right) \times 100 \quad (8)$$

where V is the phase volume, T, and B correspond to the top and bottom phases, respectively.

In optimization step, the capsaicin was quantified in the top and bottom phase by UV-spectroscopy, with Varian Cary-50 spectrophotometer at 280 nm based on the calibration curve of ethanol to know the real concentration of CPS in both phases of the ATPS. The quantification of CPS was performed in triplicate and, at least, three different assays for each system were carried out, being further reported the average values and the respective standard deviations associated.

Purification of capsaicin

The first step is the preparation of crude ethanol aqueous extracts rich in capsaicin from cumari-do-Pará peppers (*Capsicum chinense*), using optimized conditions for the method assisted by microwave, studied by Santos and co-workers [42]. Then, the ATPS was prepared in according to optimized conditions and using pepper ethanolic extracts prepared, salt and water up to a final weight of 15 g. The mixtures were stirred, centrifuged at 3000 x g for 10 minutes, placed in equilibrium in the optimum temperature condition, for at least 18 hours. Then, the two phases were separated, collected and the volume, weight, and the CPS content determined.

The pepper extracts and the phases (top and bottom) were analyzed by High Performance Liquid Chromatography - HPLC (model Prominence, brand Shimadzu system with UV-VIS detector, at 280 nm, C18 column type, mobile phase consisted of acetonitrile:water (60:40) at a flow rate of 1.0 mL/min, isocratic mode, column temperature 30 °C, 20 μL of injection volume).

The phenolic content of pepper (PC) was assessed by colorimetric spectrophotometry using the Folin-Ciocalteau method, using gallic acid as standard (SWAIN and HILLIS, 1959) in the extract prepared by extraction assisted by microwave and in both aqueous phases. The PF_{CPS} was determined by the ratio between the specific concentration of capsaicin (SC_{CPS}) present in the crude ethanolic extract rich in capsaicin and in each phase according to Equations 9 and 10, respectively.

$$SC_{CPS} = C_{CPS}/C_{PC} \quad (9)$$

$$PF_{CPS} = (SC_{CPS})_T/(SC_{CPS})_E \quad (10)$$

where, C_{CPS} is the concentration of capsaicin C_{PC} represents the concentration of phenolic compounds and the subscripts T and E are indicative of the top and crude ethanolic extract rich in capsaicin, respectively.

Results and Discussion

In this study, the use of alcohol-salt-based ATPS was investigated as an alternative for the extraction of CPS. It´s important to mention that the extraction of CPS studied and optimized here using the model system was performed using a commercial capsaicin. Different parameters like type of salts used and the composition of the system were evaluated regarding the partition coefficients and extraction efficiencies obtained.

Phase diagrams

The ethanol was used to evaluate the effects of different sodium salts, and their ability to form ATPS. Aqueous solutions of each salt (from 20 to 60 wt%) and of ethanol (60 wt% to pure ethanol) were initially prepared and used for the determination of the phase diagrams (or binodal curves) at 25 ºC (±1 ºC) and atmospheric pressure, through the cloud point titration method [36]. These phase diagrams provide information about (*i*) the concentration of phase-forming components required to form two phases (total mixture compositions above the binodal curve fall into the biphasic regime, whereas mixture compositions below the solubility curve are homogeneous); (*ii*) the concentration of phase components in the top and bottom phases; and (*iii*) the ratio of phase volumes [43].

In general, the ability of the salts to promote the formation of ATPS with ethanol follows this trend: $[Na_2S_2O_3] < [NaH_2PO_4] < [Na_2CO_3] \approx [Na_2SO_4]$ (Figure 1). Considering the fact that

these salts have the same cation (Na^+) but different anions, the phase-forming ability of these salts would be determined by the nature of their anions, *i.e.* by the hydration capacity of the anion. Anions with higher charge densities have a strong hydration capacity than those with a lower charge density [44]. Moreover, the kosmotropic ions (Hofmeister series), such as CO_3^{2}, SO_4^{2-}, $S_2O_3^{2-}$, $H_2PO_4^{-}$, which exhibit stronger interaction with water molecule [44, 45], favoring the salting-out effect and consequently ATPS [46].

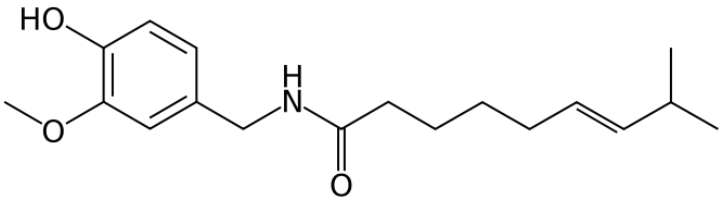

Figure 1. Molecular structure of capsaicin (CPS).

However, the ability to form two phases is a direct consequence the formation of hydration complexes between the water and salt, and the reducing the ability to hydrogen bond between the salting water and ethanol. Thus, three of the seven salts evaluated - *i.e.* sodium acetate ($NaC_2H_3O_2$), sodium chlorate ($NaClO_3$) and sodium nitrate ($NaNO_3$), do not have the ability to form ATPS with ethanol. This is because these salts possess anions located, more to right of known Hofmeister series ($[C_2H_3O_2]^- > [NO_3]^- > [ClO_3]^-$) [45].

The Figure 2 with various ternary systems investigated, result from different molecular weights of the salts, which are in molality units to obtain a diagram more accurate of the behavior of the components. It shows that the amount of water complexed to the salts was removed in the calculations of the molality of salts and added to the water composition of each phase diagram.

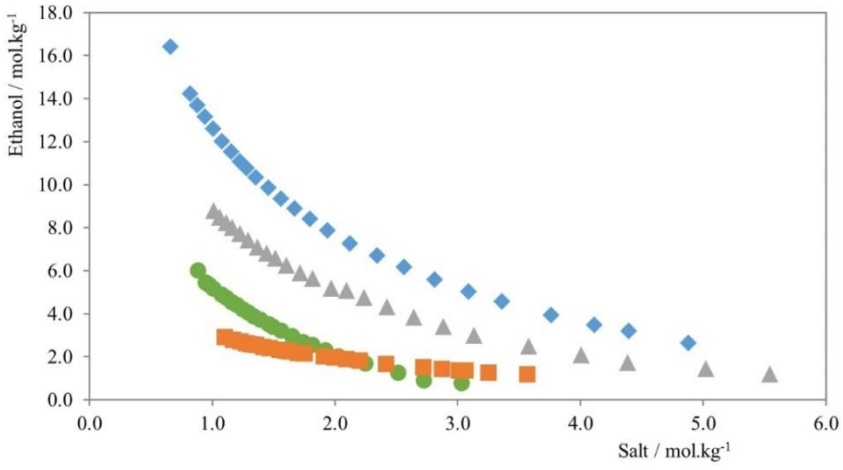

Figure 2. Binodal curves for ternary systems composed of ethanol + salt + water at 298 K and atmospheric pressure. (◆) $[Na_2S_2O_3]$; (▲) $[NaH_2PO_4]$; (●) $[Na_2CO_3]$, (■) $[Na_2SO_4]$.

In order to get a more accurate fitting, we used non-liner empirical expressions to correlate the binodal data. The regression parameters were estimated by least-squares regression using Eqs. 1, 2 and 3 and their values with the respective standard deviations (*std*) and correlation factors (R^2) along with the weight fraction experimental data (*w*) for the system. These parameters are given in Table 1, 2 and 3, respectively. On the basis of the obtained R^2 and std, in general, good correlation was obtained for the three equations used, indicating that these fittings can be used to predict data in a given region of the phase diagram where no experimental results are available. Additionally, as shown in Figure 3, the critical points for the studied systems were also estimated by intersection of a line passing through the midpoint of various TLs with the binodal. The midpoint is represented by point limit, which is the point where the binodal is tangent to the line joining the equal segments in the diagram axes. The relative position of the endpoint and the *critical point* defines the symmetry of the phase diagram [47].

Table 1: Correlation parameters and respective standard deviations (std) used in Equation 1 to describe the binodal at 298 K.

Ethanol + Salt system	***a* ± std**	***b* ± std**	***c* ± std**	**R^2**
NaH_2PO_4	71.6816 ± 1.9104	-0.2668 ± 0.0074	1.2E-5 ± 5.4E-7	0.9993
$Na_2S_2O_3$	84.6040 ± 0.5728	-0.2312 ± 0.0019	8.5E-6 ± 1.4E-7	0.9999
Na_2SO_4	70.1466 ± 1.2568	-0.3991 ± 0.0076	3.9E-5 ± 5.3E-6	0.9994
Na_2CO_3	69.2833 ± 3.6784	-0.3860 ± 0.0177	8.2E-5 ± 4.1E-6	0.9995

Table 2: Correlation parameters and respective standard deviations (std) used in Equation 2 to describe the binodal at 298 K.

Ethanol + Salt system	***a* ± std**	***b* ± std**	***c* ± std**	**R^2**
NaH_2PO_4	59.8986 ± 1.3710	-10.1190 ± 0.5915	0.2513 ± 0.0619	0.9992
$Na_2S_2O_3$	71.4654 ± 0.7253	-10.2357 ± 0.3191	0.1249 ± 0.0338	0.9997
Na_2SO_4	60.2559 ± 1.1038	-16.9415 ± 0.7619	1.2378 ± 0.1287	0.9994
Na_2CO_3	61.6059 ± 1.9499	-16.4466 ± 1.0212	0.9246 ± 0.1315	0.9986

Table 3: Correlation parameters and respective standard deviations (std) used in Equation 3 to describe the binodal at 298 K.

Ethanol + Salt system	***a* ± std**	***b*± std**	***c* ± std**	***d* ± std**	**R^2**
NaH_2PO_4	5.1629 ± 0.3437	-0.8719 ± 0.2065	0.1181 ± 0.0343	-0.0016 ± 0.0003	0.9994
$Na_2S_2O_3$	4.9610 ± 0.0454	-0.6005 ± 0.0285	0.0742 ± 0.0049	-0.0010 ± 4.0E-5	0.9999
Na_2SO_4	4.4583 ± 0.2666	-0.6178 ± 0.2513	0.0662 ± 0.0658	-0.0022 ± 0.0013	0.9995
Na_2CO_3	6.3797 ± 0.8281	-2.1130 ± 0.6054	0.4009 ± 0.1233	-0.0077 ± 0.0015	0.9991

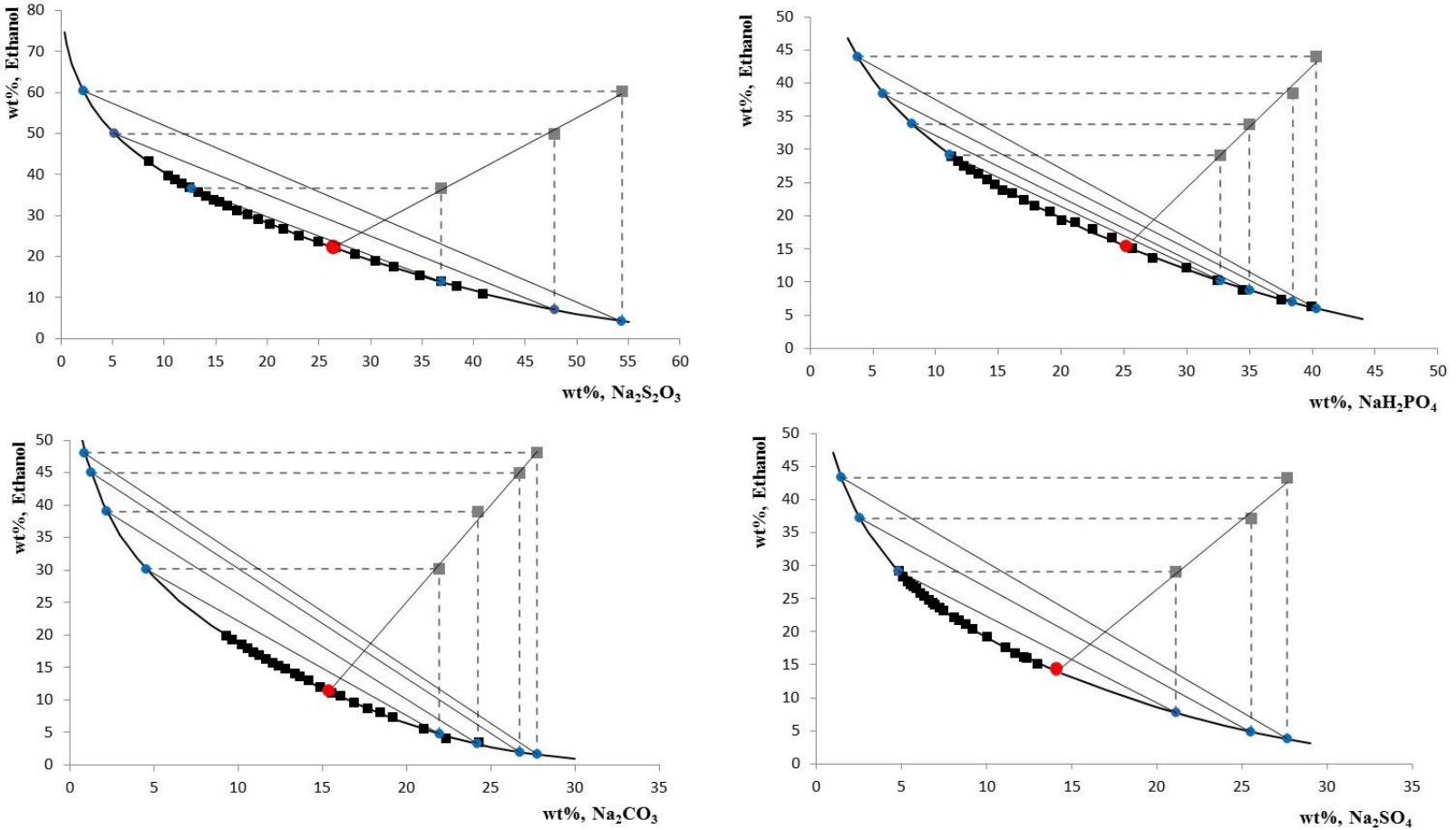

Figure 3. Phase diagrams for ternary systems composed of ethanol + sodium salts + water, at 298 K and atmospheric pressure. Experimental solubility data (■); TL data (●); fitting (—) by Eq.(1); auxiliary curve data (■); critical point (●) by Eq. (3).

Partition capsaicin

Although it is possible to form APTS with salts [$Na_2S_2O_3$], [NaH_2PO_4], [Na_2CO_3] and [Na_2SO_4] + ethanol (Figure 2), the actual experiments with the biomolecule in the case of sodium sulfate [Na_2SO_4] and sodium carbonate [Na_2CO_3] components, they do not form two phases due to salt precipitation, even by changing the concentrations of the components near the binodal curve. Thus, based on the results obtained, to assess the ability of ATPS for the extraction of commercial capsaicin, two different salts [$Na_2S_2O_3$] and [NaH_2PO_4] were investigated.

The tie-lines (TL) of the ternary systems are essential for extraction process due to the compositions in equilibrium of top and bottom phase in one ATPS. For each diagram, one TL was determined with the same length of tie-line (TLL ≈ 33), in order to perform out an effective comparison between the two systems for the partition of CPS, since the free space ratio of the system components and biomolecules are maintained (equal TLL). The results showed that the extraction efficiency (%, *EE*) was lower using ATPS based on $Na_2S_2O_3$ salt (*EE* = 68.16 % ± 0.03) compared with ATPS based on NaH_2PO_4 salt (*EE* = 73.62 % ± 1.07), due the capacity of ethanol/NaH_2PO_4 ATPS in partition of CPS preferentially for ethanol rich-phase (*K* = 2.33 ± 0.13). The coefficient partition can be described based on the entropic repulsion, electrostatic interactions and hydrophobic interactions in the system. Capsaicin is a bioactive molecule with a hydrophobic nature, as demonstrated by the octanol-water partition coefficient (log K_{ow} = 3.75). Its affinity for the ethanol phase is higher due to the most hydrophobic nature of this solvent (log K_{ow} = -0.16) [48], being this affinity increased when on the ATPS ethanol is conjugated with [NaH_2PO_4] (log K_{ow} = -1.02) [48] more hydrophobic salt. Therefore, the following optimization step, the system used was based on ethanol/NaH_2PO_4 to evaluate the best migration of molecules of capsaicin.

A surface response analysis by central composite rotational design (CCRD) was carried to evaluate the optimal partition conditions (Figure 4). The experimental design for the partitioning of capsaicin in ATPS is showed in Table 4. Table 5 shows the analysis of variance (ANOVA) including the significant factors for capsaicin separation by ATPS ethanol/salt. Concentrations of ethanol (X_1) haves significant effects on the coefficient partition ($p < 0.05$). Beside, the ANOVA revealed that the coefficient of determination (R^2 = 0.9611) and adjusted coefficient of determination (adjusted R^2 = 0.9222) were high. The model fit is also verified by the value obtained by the adjusted R^2. Values above 75% indicate that the factors in the experiment explain large percentage of the observed variation. In this case, the adjusted R^2 was equal to 92.22%.

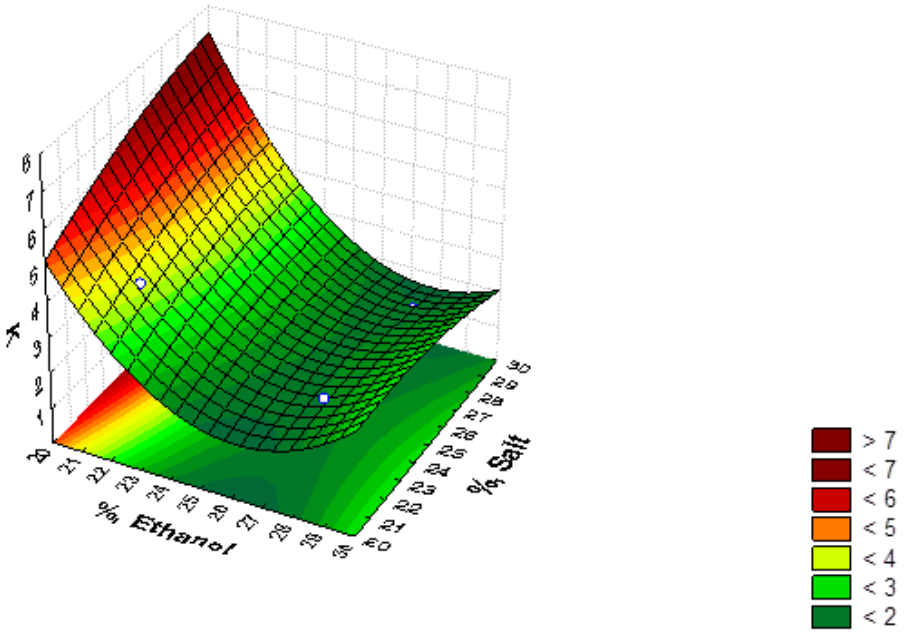

Figure 4. Response surface plot showing the effect of Ethanol concentration (wt%) and [NaH_2PO_4] concentration (wt%) in partitioning of CPS.

Table 4: Experimental design of the central composite rotational of the partitioning and extraction efficiency of capsaicin in ATPS.

Test set	Extraction conditions		Parameter of extraction
	X_1, Ethanol (wt%)	X_2, Salt (wt%)	*K*
1	-1 (22)	-1 (22)	4.09 ± 0.10
2	-1 (22)	1 (28)	4.83 ± 0.08
3	1 (28)	-1 (22)	2.34 ± 0.02
4	1 (28)	1 (28)	2.00 ± 0.03
5	-1.41 (20.77)	0 (25)	5.23 ± 0.12
6	1.41 (29.23)	0 (25)	2.28 ± 0.09
7	0 (25)	-1.41 (20.77)	1.45 ± 0.04
8	0 (25)	1.41 (29.23)	2.22 ± 0.09
9	0 (25)	0 (25)	2.33 ± 0.10
10	0 (25)	0 (25)	2.34 ± 0.08
11	0 (25)	0 (25)	2.31 ± 0.05

Table 5: Analysis of variance (ANOVA) for capsaicin partition in ATPS as response variable. The model of second order including as independent variables concentration (wt%) of ethanol (1) and concentration (wt%) of NaH_2PO_4 (2).

Source	SS	DF	*MS*	*F*-value	*p*-value
(1) Ethanol (L)	9.59696	1	9.596956	79.02275	0.000300*
Ethanol (Q)	4.02658	1	4.026585	33.15549	0.002218*
(2) Salt (L)	0.27846	1	0.278461	2.29289	0.190391
Salt (Q)	0.07403	1	0.074031	0.60958	0.470277
1L by L2	0.29589	1	0.295892	2.43642	0.179298
Pure error	0.60723	5	0.121445		
Total SS	15.60726	10			

SS = sum of square; DF = degree of freedom; MS = mean of square; $R^2 = 0.9611$. * Significative at level of 95%

Table 6 describes the coefficients of the regression model, from the coded matrix, linear terms are associated with the letter L and the quadratic terms with the letter Q. The regression coefficients were used to evaluate the weight of contribution of each factor to the response and the possible cross-effect among these variables, considering a 95% confidence interval.

Table 6: Effects estimated by the regression model for the variable partitioning of capsaicin.

Independent variables	Estimates Effects	Error	t-value	p-value
Interceptação	2.3261	0.2028	11.4677	0.00009
(1) Ethanol (L)	-2.1904	0.2484	-8.8170	0.00031
Ethanol (Q)	1.6849	0.2957	5.6983	0.00232
(2) Salt (L)	0.3734	0.2484	1.5030	0.19315
Salt (Q)	-0.2292	0.2957	-0.7751	0.47333
1L by 2L	-0.5440	0.35120	-1.5483	0.18223

Therefore, the factors having a significant effect on the response is only of concentration of ethanol solution (wt%) in linear and quadratic terms. The mathematical model to represent the process of capsaicin partition using ATPS ethanol/NaH_2PO_4, considering the significant variables is described by the Eq.(11).

$$Y_K = 2.32 - 2.19X_1 + 1.70{X_1}^2 \quad (11)$$

where Y_K represents the predicted value for coefficient partition (K) of CPS, X_1 represents concentration (wt%) of ethanol and X_2 represents concentration (wt%) of NaH_2PO_4 in ATPS. This equation shows the best result is found at lower concentration of ethanol and higher concentration of salt. Thus, future applications should be tested in conditions close to the indicated in this work.

Purification of capsaicin

After the optimization step, the best conditions found were applied to the purification of capsaicin pepper extract from *Capsicum chinense* var. cumari-do-Pará. The results showed that by using a natural extract, the value of the CPS partition coefficient (K = 1066.02 ± 65.17) for the top phase rich in ethanol was considerably higher than the value found in the optimization experiments (K = 5.23 ± 0.19) and this difference can be justified due to initial capsaicin concentration used to prepare the system. The natural capsaicin extract has a concentration almost 20 times higher than the used during optimization step (60 $mg.mL^{-1}$) and, the extraction efficiency was also superior (99.79 % ± 0.09).

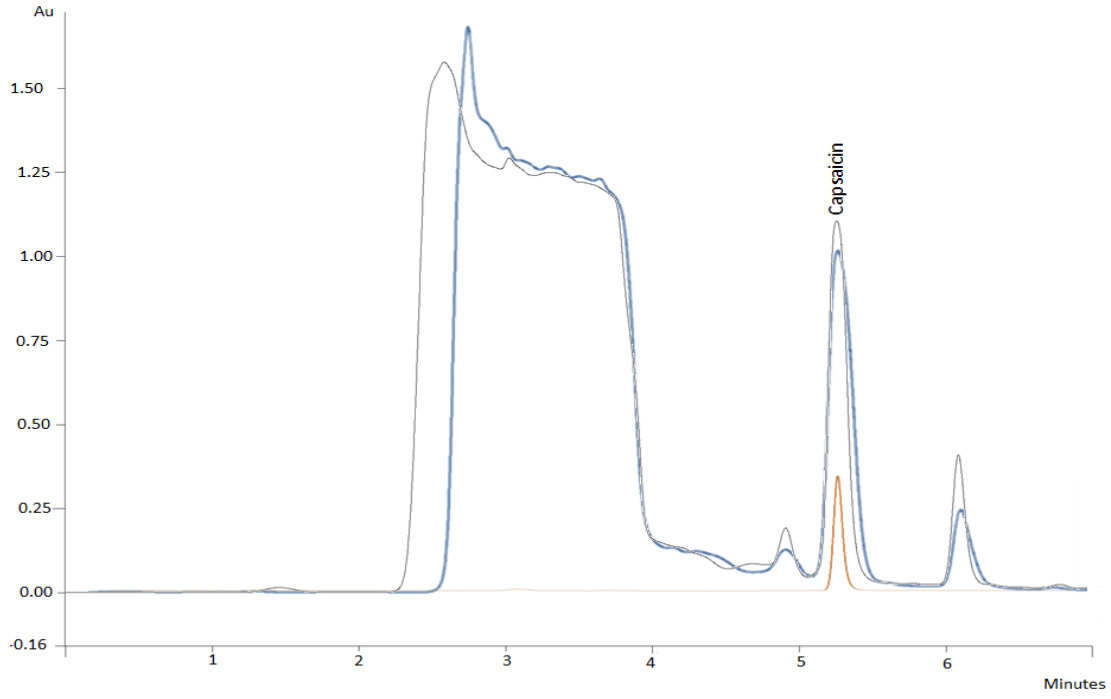

Figure 5. Standard and samples chromatograms: **(** —**)** synthetic CPS, **(** —**)** natural pepper extract obtained in the optimization study, **( )** top phase obtained after the purification of CPS.

The values of PF_{CPS} in top phase, calculated using equation 10, was equal to 4.38 ± 0.09 and can be considered a good value since the chromatograms presented in Figure 5 confirmed the higher area and besides, there was a significant increase in specific CPS concentration in the top phase.

Conclusions

Due to the compatibility between the components, only those salts [$Na_2S_2O_3$], [NaH_2PO_4], [Na_2CO_3] and [Na_2SO_4] were able to form two aqueous phase systems (ATPS) when mixed with a concentration above critical with ethanol. The ability of the salts to promote the formation of ATPS with ethanol follows this trend: [$Na_2S_2O_3$] < [NaH_2PO_4] < [Na_2CO_3] ≈ [Na_2SO_4].

The liquid-liquid extraction using aqueous two-phase systems is a useful tool for the isolation of biologically important molecules. That alcohol-salt system is attractive for purification of capsaicin because of the comparative advantages with other methods due to the low costs and feasibility of carrying to macro scale and environment friendly. Capsaicin preferentially migrates to the alcohol-rich-phase (K > 1) where the extraction efficiencies were higher. The optimum conditions for this separation were determined to be 20 wt% of ethanol and 25 wt% of NaH_2PO_4. The best extraction efficiency of 82.65 % ± 0.53 and partition coefficient of 5.23 ± 0.19 were obtained in ATPS. Thus, the capsaicin was separated and purified from its source with purification factor value satisfactory and efficiency superior to 99%, in two steps (extraction assisted by microwave and purification by ATPS). This confirms the potential of this new ethanol-based ATPS for separation capsaicin.

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Capítulo VI

CONSIDERAÇÕES FINAIS

As pimentas se destacam por seu potencial terapêutico, industrial e sensorial devido, principalmente, à presença da CPS e, é por isso, que é necessário que técnicas de extração e purificação sejam aperfeiçoadas. Neste trabalho foram estudados métodos de extração convencionais, utilizando solvente à frio e à quente, otimizados os métodos não convencionais assistidos por ultrassom e micro-ondas e foram desenvolvidos novos SABs para purificação desta biomolécula.

Ao comparar os métodos de extração convencionais com os não convencionais, os resultados mostraram que a variedade e estágio de maturação influenciam no conteúdo de CPS e os melhores resultados foram obtidos utilizando a pimenta cumari-do-pará madura. Quanto aos métodos testados, a utilização de temperatura no convencional aumentou em mais de 100% a eficiência do processo. Entretanto, o aquecimento convencional foi superado pelos métodos assistidos por ultrassom e micro-ondas, que apresentaram eficiência $\geq$ 600% superior. Com a realização deste estudo, foi possível desenvolver um método de extração rápido e eficiente assistido por micro-ondas, que extraiu 4,59 mg de CPS.g^{-1} de pimenta seca utilizando razão massa/volume igual a 0,2 com solução aquosa de etanol a 70% durante 1 minuto a 125 °C, 250 W e sem agitação.

Os estudos com SAB composto por ACN + cloreto de colina, ACN + K_2CO_3 e etanol + NaH_2PO_4, mostram que a CPS possui maior afinidade pela fase de topo, rica em solvente orgânico. Os sistemas modelo foram otimizados e os melhores resultados atingidos (K_{CPS} > 5, EE_{CPS} > 82%) utilizando as seguintes composições: 30 % (m/m) de ACN, 35 % (m/m) de cloreto de colina e 35 % (m/m) de água a 45 °C; 20 % (m/m) de ACN, 15 % (m/m) de K_2CO_3 e 65 % (m/m) de água a 45 °C; 20 % (m/m) de etanol, 25 % (m/m) de NaH_2PO_4 e 55 % (m/m) de água a 25 °C. Quando o sistema modelo foi aplicado para purificar a capsaicina a partir de sua fonte natural, os resultados mostraram que os sistemas propostos foram reprodutivos e eficientes (K_{CPS} > 40, EE_{CPS} > 89% e FP_{CPS} > 2), sendo que os melhores resultados foram atingidos utilizando o sistema composto por etanol e NaH_2PO_4, com FP > 4.

Diante do exposto, é possível concluir que a associação entre método de extração com o SAB foi fundamental para o sucesso na purificação da capsaicina e, desta forma, o sistema desenvolvido apresenta-se como excelente alternativa purificar a capsaicina a partir de sua fonte natural.

TRABALHOS APRESENTADOS

Até o presente momento, os resultados obtidos com o desenvolvimento desta tese foram publicados e aceitos em eventos nacionais e internacionais, conforme descritos abaixo:

1. SANTOS, P. L.; SANTOS, L. N. S.; SOUZA, R. L.; SOARES, C. M. F.; LIMA, A. S. Aplicação de novos Sistemas Aquosos Bifásicos baseados em Acetonitrila e Sais de Na^+ e K^+ na partição de capsaicina. In: XX Simpósio Nacional de Bioprocessos, 2015, Fortaleza. XX Simpósio Nacional de Bioprocessos, 2015.

2. SANTOS, P. L.; SANTOS, L. N. S.; SOUZA, R. L.; SOARES, C. M. F.; LIMA, A. S. Application of aqueous two-phase systems composed of acetonitrile and salts to partition capsaicin. In: Biopartitioning & Purification Conference 2015, 2015, Viena. Biopartitioning & Purification Conference 2015, 2015.

3. SANTOS, P. L.; SANTOS, L. N. S.; VENTURA, S. P. M.; COUTINHO, J. A. P.; SOARES, C. M. F.; LIMA, A. S. Partitioning of capsaicin using acetonitrile-choline ionic liquid aqueous two-phase system. In: 2nd International Conference on Ionic Liquids i Separation and Purification Technology, 2014, Toronto. Book of Abstract of 2nd International Conference on Ionic Liquids. Separation and Purification Technology, 2014.

4. SANTOS, P. L.; SANTOS, L. N. S.; SOARES, C. M. F.; LIMA, A. S. Extração de capsaicina presentes em *Capsicum Chinense* utilizando o método assistido por ultrassom. In: XXIV Congresso Brasileiro de Ciência e Tecnologia de Alimentos, 2014, Aracaju. XXIV Congresso Brasileiro de Ciência e Tecnologia de Alimentos, 2014.

5. SANTOS, P. L.; LIMA, A. S.; SOARES, C. M. F. Extração e Purificação de Capsaicina em Pimentas do Gênero *Capsicum*: um estudo utilizando sistemas aquosos bifásicos. In: 16ª SEMPESq - Semana de Pesquisa 'Ciência e Tecnologia para um Brasil sem Fronteiras', 2014, Aracaju. 16ª SEMPESq, 2014.

TRABALHOS FUTUROS

- Estudar os compostos fenólicos presentes nas pimentas deste gênero, que foram considerados contaminantes para o SAB.

- Estudar método cromatográfico de análise de capsaicina em sistema de cromatografia de ultra performance.

- Avaliar a eficiência dos SABs para separar e purificar os carotenoides presentes na pimenta.

- Estudar a utilização de equipamentos modernos para auxiliar na etapa de separação das fases e assim tentar reduzir o tempo de equilíbrio do SAB.

- Realizar os experimentos de reutilização das fases que foi proposto no artigo II.

- Avaliar a viabilidade destes sistemas em uma planta industrial.

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