UNIVERSIDADE TIRADENTES – UNIT

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS - PEP

DESENVOLVIMENTO DE NOVOS SISTEMAS AQUOSOS BIFÁSICOS COMPOSTOS POR ACETONITRILA PARA A EXTRAÇÃO DE VANILINA

ARACAJU, SE – BRASIL
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Doutorando: Gustavo de Brito Cardoso
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Profª. Drª.Cleide Mara Faria Soares

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Gustavo de Brito Cardoso

TESE SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS DA UNIVERSIDADE TIRADENTES COMO PARTE DOS REQUISITOS NECESSÁRIOS PARA A OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA DE PROCESSOS

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Nunca se ouviu dizer que filho valente tivera nascido de mãe temerosa.

(Thomas Carlyle, adaptado)

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DESENVOLVIMENTO DE NOVOS SISTEMAS AQUOSOS BIFÁSICOS COMPOSTOS POR ACETONITRILA PARA A EXTRAÇÃO DE VANILINA

Sistemas aquosos bifásicos (SAB's) são amplamente empregados na purificação de biomoléculas, pois propiciam alto grau de purificação e recuperação. Os SAB's utilizam componentes menos agressivos ao meio ambiente, quando comparado a solventes tradicionais utilizados em extrações, possuem baixo custo de operação e evidenciam o seu potencial de aplicação em nível industrial, identificando-os como um dos sistemas mais adequados para separação de determinadas biomoléculas. Em meio aquoso, muitos pares de solutos podem ser utilizados para preparar SAB's, entretanto, poucos trabalhos têm sido realizados relatando o uso de carboidratos, polióis, polímeros naturais (dextrana) e álcool polivinílico. Neste sentido, o objetivo deste trabalho é desenvolver novos SAB's baseados em acetonitrila associada aos compostos já citados, e sua aplicação na partição da vanilina. Para isto, os diagramas de fase foram determinados e o efeito da estrutura dos carboidratos, polióis, dextrana e álcool polivinílico sobre a formação do sistema bifásico foi avaliada. Além de carboidratos de elevada pureza, açúcares comerciais de grau alimentar também foram testados e mostraram ser capazes de formar SAB's. Nos sistemas avaliados a vanilina migrou preferencialmente para a fase rica em acetonitrila. A recuperação de vanilina foi maior que 70% a temperatura de 15 ºC utilizando SAB's constituídos por acetonitrila/dextrana, cujo coeficiente de partição foi 7,66. Os SAB's formados por acetonitrila/carboidratos apresentaram coeficientes de partição superior a 3,0, e os SAB's utilizando acetonitrila/polióis apresentaram coeficientes de partição entre 7 e 67 e são dependentes da capacidade de *salting-out* de cada poliol. Em ambos os sistemas a recuperação da vanilina foi superior a 90%, enquanto que nos sistemas constituídos por acetonitrila/PVA o coeficiente de partição foi 2,24 e a recuperação foi de 78,84% para um pH de 4,7 e a temperatura de 5 ºC.

Palavras-chave: Sistema aquoso bifásico; carboidrato; polióis; álcool polivinílico, dextrana e vanilina.

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

DEVELOPMENT OF NEW BIPHASIC AQUEOUS SYSTEMS COMPOUNDED BY ACETONITRILE FOR THE EXTRACTION OF VANILLIN

Aqueous Two phase system (ATPS) are widely used in the purification of biomolecules, since they allow high degree of purification and recovery. ATPS use affordable components and are less harmful to the environment; they also have low operating costs and demonstrate their potential application in industrial level, identifying them as one of the most suitable systems for parting of certain biomolecules. In aqueous media, many pairs of solutes can be used to prepare ATPS, however, few studies have been conducted reporting the use of carbohydrates, polyols, natural polymers (dextran) and polyvinyl alcohol. In this sense, the objective of this work is to develop new ATPS based on acetonitrile associated with the aforementioned compounds, and its application in vanillin partition. To do so, the phase diagrams were determined and the effect of the structure of carbohydrates, polyols, dextran and polyvinyl alcohol to liquid-liquid separation was evaluated. Besides high-purity carbohydrates, commercial food-grade sugars have also been tested and showed to be capable of forming ATPS. In the evaluated systems, vanillin preferentially migrated to the acetonitrile rich phase. The lowest recovery of vanillin was of 70.65% at 15º C, using ATPS consisting of acetonitrile/dextran, whose partition coefficient was 7.66. The ATPS formed from acetonitrile/carbohydrate had partition coefficients greater than 3.0, and the ATPS using acetonitrile/polyols exhibited partition coefficients between 7 and 67 and are dependent on the salting-out ability of each polyol. In both systems the recovery of vanillin was higher than 90%. While the ATPS consisting of acetonitrile/PVA partition coefficient was 2.24 and the recovery was of 78.84% for a pH of 4.7 and a temperature of 5° C.

Key words: Aqueous Two phase system, carbohydrate, polyols, polyvinyl alcohol, dextran and vanillin.

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LISTA DE SIGLAS

ACN: Acetonitrila

C^0_p: Calor específico a pressão constante

$C_{T\ ou\ B}$: Concentração na fase de topo ou fundo (g/L e/ou mg/100g)

ELL: Extração líquido-líquido

G^0_m: Energia livre de Gibbs

H^0_m: Entalpia

IL: Líquidos Iônicos

K: Coeficiente de partição (admensional)

P: Pressão

PEG: Polietilenoglicol

PEO: Óxido de polietileno

PPO: Óxido de polipropileno

PVA: Álcool Polivinílico

R: Constante universal dos gases

RT ou B: Recuperação na fase de topo ou fundo (admensional)

S^0_m: Entropia

SAB (ATPS): Sistema Aquoso Bifásico (Aqueous two-phase System)

T: Temperatura

TL: *tie line* (linha de amarração)

TLL: *tie line length* (comprimento da linha de amarração)

STL: *slop tie line* (inclinação da linha de amarração)

V: Volume

VAN: Vanilina

RT ou B: Recuperação na fase de topo ou fundo (admensional)

INTRODUÇÃO

Atualmente, a indústria requer técnicas de biosseparação que sejam eficientes, efetivas e que permitam atingir elevado grau de pureza, rendimento e recuperação de moléculas com atividade biológica. Baseando-se nas regulamentações ambientais, diversos trabalhos são descritos na literatura com o objetivo de minimizar o descarte de resíduos nocivos ao ambiente e à saúde humana, assim o desenvolvimento de métodos menos poluentes, seguros e viáveis economicamente em larga escala são imprescindíveis.

Uma das alternativas aplicadas para obtenção de biomoléculas que possibilita a separação de vários analitos é a extração líquido-líquido utilizando solventes orgânicos, empregada para melhorar a solubilidade e eficiência do processo de transferência de massa. Uma opção neste processo é o uso de sistemas aquosos bifásicos (SAB's), para a extração e purificação de biocompostos. O SAB é constituídos majoritariamente por água outros componentes, em sua maioria não tóxicos ou inflamáveis, tornando-o um método de extração e purificação menos agressivo.

Os SAB's são geralmente formados por soluções aquosas de diversos compostos, como: polímeros, sais inorgânicos, líquidos iônicos e alcoóis; os quais, acima da concentração crítica promovem espontaneamente à separação de fases. Em cada fase predomina um dos componentes do sistema. A partição de biomoléculas ocorre em condições não-desnaturantes, devido à elevada concentração de água em ambas as fases, como anteriormente descrito. Outra característica dos SAB's é sua capacidade de promover a partição de solutos em um meio pouco agressivo, de forma que substâncias como macromoléculas, partículas virais, fragmentos de células ou mesmo organelas celulares possam ser particionadas e purificadas sem perda de atividade biológica (ALBERTSSON, 1986; SILVA & LOH, 2006).

Atualmente uma nova classe de constituintes de SAB's com resultados promissores para recuperação e purificação de biomoléculas, tem despertado a necessidade de estudos relacionados a este processo de separação. Em geral, esses novos constituintes dos SAB's são compostos não tóxicos, que possuem grande afinidade pela água e são utilizados para conferir estabilidade termodinâmica a macromoléculas biológicas, além de evitar a perda de atividade enzimática de alguma molécula alvo (BACK *et al*., 1979; BRADBURY & JAKOBY, 1972).

Neste sentido, este trabalho desenvolve sistemas utilizando acetonitrila como constituinte ainda pouco referenciado na literatura e restrito apenas a sua utilização com alguns

carboidratos, aqui se amplia a combinação deste constituintes com poliois, álcool polivinílico e dextrana.

A metodologia de biosseparação em SAB é aconselhável na purificação de biomoléculas em larga escala, pois permite separação seletiva, baixa tensão interfacial, boa biocompatibilidade e relação custo-benefício favorável. Quando comparada a outras técnicas de separação apresenta vantagens como: rápida operação, altos rendimentos e minimização da desnaturação de biomoléculas (MALPIENDI *et al*., 2009; CASCONE *et al*., 1991; LIMA *et al*., 2002).

O aumento da população mundial impulsiona o crescimento de mercados voltados à produção de alimentos, aumentando a demanda por aditivos alimentares, em especial a vanilina. Apesar dos produtos sintéticos terem se tornado importante para o sustento da população mundial existe uma preocupação da população em geral no consumo de produtos naturais (FURUKAWA *et al*., 1998). Dessa forma, a biotecnologia vem se mostrando como uma promissora solução na obtenção de produtos como a vanilina, por meio da extração e purificação em SAB's.

Diante deste contexto, a vanilina (4-hidroxi-3-metoxibenzaldeído), um dos aromatizantes mais apreciados no mundo por ser um importante flavorizante para alimentos, bebidas e produtos farmacêuticos (DAUGSCH & PASTORE, 2005), se mostra uma biomolécula de interesse. O aroma da vanilina, a baunilha, é obtido de forma tradicional de uma orquídea tropical a *Vanilla planifólia,* no entanto essa fonte natural fornece somente cerca de 0,2 % da baunilha consumida (BERGER& ONKEN, 1999). O potencial de mercado para o consumo de vanilina é alto, devido a esta necessidade, novas tecnologias são utilizadas como, por exemplo, a síntese de vanilina a partir da lignina contida no licor negro, um resíduo das indústrias de papel e celulose (KORTEKAAS *et al*., 1998). Atualmente, a maior parte da vanilina é produzida por meio de precursores derivados do petróleo, onde os mais utilizados são o guaiacol e o p-cresol devido ao maior rendimento (MUKHOPADHYAY, 2005).

A vanilina produzida biotecnologicamente pode ser obtida a partir de fontes renováveis, como extratos enzimáticos ou enzimas purificadas, micro-organismos e culturas de células de plantas, sendo as principais fontes utilizadas na sua produção biotecnológica o eugenol, o isogenol e o ácido ferúlico (RAMACHANDRA & RAVISHANKAR, 2002). Entretanto, a síntese da vanilina utilizando micro-organismos possui limitações como baixo rendimento e a toxicidade do substrato e do produto (BERGER, 1999). Por outro lado, a biotransformação de vanilina sintética a partir de células de plantas tem recebido bastante

atenção, por serem produzidas em valores de pH e temperatura brandos e também por serem estereosseletivas (QI & HEDGES, 1995).

Capítulo I

1. REVISÃO BIBLIOGRÁFICA

Nesta seção será apresentado o estado da arte referente à técnica de extração, concentração e purificação de biomoléculas por Sistemas Aquosos Bifásicos (SAB), sua formação e características, bem como sua aplicação na partição de vanilina e suas propriedades.

1.1. Métodos de Extração e Purificação de Biomoléculas

Um procedimento de purificação e de extração de biomoléculas a ser desenvolvido deve ser adequado e rigoroso dependendo da aplicação (HATTI-KAUL, 2001). O método ideal de extração deve ser quantitativo e não destrutivo para a biomolécula. Na literatura relatam-se métodos de extração, separação e identificação de biomoléculas a partir da biomassa utilizando solventes orgânicos (ZHANG *et al*., 2013), além de outras técnicas, como a extração supercrítica (DIMITRIESKA-STOJKOVIC & ZDRAVKOVSKI, 2003), extração assistida por micro-ondas (ZHANG *et al*., 2009) e a extração assistida por ultrassom (YANG & ZHANG, 2008).

A purificação de biomoléculas, geralmente é realizada em presença de solventes orgânicos, tendo com vantagens a obtenção de maior grau de pureza, seletividade adequada e baixo custo. Deve-se salientar que o método de extração por solventes propicia uma boa combinação entre os passos de recuperação e purificação (MARINOVA & YANICHLIEVA, 1997). Essa técnica geralmente depende do tipo de solvente utilizado, da agitação empregada para melhorar a solubilidade e eficiência do processo de transferência de massa (PEDERSEN-BJERGAARD *et al*., 2000).

O estado supercrítico de fluidos pode ser definido como o estado no qual o líquido e o gás são indistinguíveis entre si, apresentando assim características tanto de um gás como de um líquido. A extração realizada com fluido supercrítico utiliza um fluido submetido à pressão e temperatura acima de seu ponto crítico modificando propriedades como a densidade, tornando-a similar a dos líquidos; e a viscosidade, a qual passa a apresentar valores próximos

aos determinados para os gases (HERRERO *et al*., 2006). O dioxido de carbono (CO_2) é o fluido mais utilizado em meio pressurizado devido à sua baixa temperatura (31,3 ^oC) e pressão (72,9 atm) crítica. Entretanto, o CO_2 supercrítico possui um menor efeito nas extrações de biomoléculas de alta polaridade, por esta razão a adição de co-solventes como metanol e etanol nos processos pode ser uma alternativa de processo (RAVENTÓS *et al*., 2002).

O uso de processos de extração assistida por micro-ondas para biomoléculas é uma alternativa viável apesar do alto custo para determinação de contaminantes orgânicos em amostras ambientais (ZHANG *et al.*, 2013). Estudos recentes compararam extração assistida por micro-ondas a métodos tradicionais de extração sólido-líquido para a remoção de compostos fenólicos de cascas de amendoim, verificando-se que ete tipo de irradiação é altamente promissor devido aos rendimentos de extração (BALLARD *et al.*, 2010). O uso de líquidos iônicos associado a extração assistida por micro-ondas mostrou eficiência ao extrair compostos polifenólicos de plantas medicinais com maior rendimento e menor tempo (DU *et al.*, 2009).

A utilização do ultra-som em processos de extração tem apresentado um rápido desenvolvimento, com diversas aplicações para a extração de metabólitos de plantas (KNORR, 2003), polissacarídeos (YANG *et al*., 2008), dentre outros. Este método faz uso de fenômenos físicos e químicos que apresentam vantagens em termos de rendimento, produtividade e seletividade (BARBOZA & SERRA, 1992). As ondas ultra-sônicas de alta potência produzidas causam mudanças físicas e químicas devido à variação de pressão, produzindo cavitação e microfluxos nos líquidos, aquecimento e ruptura nos sólidos e instabilidade na superfície da interface de sistemas líquido-líquido e líquido-gás (CHEMAT *et al.*, 2011). A eficiência da extração está relacionada ao aumento da transferência de massa, que por sua vez, resultam do processo de cavitação (BREITBACH *et al.*, 2003).

As técnicas alternativas de extração e separação de biocompostos como a extração realizada com fluido supercrítico e a utilização do ultrassom, requerem geralmente condições mais drásticas, ou seja, temperaturas e pressões elevadas, e também dependem de um equipamento sofisticado transformando a extração em um processo com custos mais elevados e maior complexidade de manuseio. Contudo, a escolha da técnica de separação e purificação de biomoléculas a ser utilizada deve ser feita levando em consideração o grau de purificação necessário para sua aplicação em escala industrial. Na escolha da técnica se deve avaliar o custo-benefício das alternativas de extração e purificação, a fim de se obter processos simples, baratos e de alta eficiência.

Em comparação às técnicas citadas anteriormente, a partição em sistema aquoso bifásico (SAB) é uma alternativa bastante eficiente para a separação e purificação de biomoléculas. As vantagens oferecidas por este tipo de sistema são: ambiente biocompatível, baixa tensão interfacial, baixa energia, fácil aplicação em grande escala, operação contínua, alta sensibilidade no reconhecimento das interações das biomoléculas (SELVAKUMARA *et al.*, 2010) e muitas vezes ambientalmente corretas pois suas fases são predominantemente formadas por água (60-95%) (SILVA & LOH, 2006).

1.2. Sistema Aquoso Bifásico (SAB)

A formação dos sistemas aquosos bifásicos (SAB's) é conhecida desde o final do século XIX, e de forma cronológica a evolução da aplicação dos SAB's é apresentada a seguir (ALBERTSSON *et al*., 1990; ZAFARANI-MOATTAR & NASIRI, 2010):

- 1896: Beijerinck observou a formação de duas fases líquidas após misturar soluções aquosas de gelatina e ágar, ou gelatina e amido solúvel, notando que a fase inferior tornou-se rica em ágar e na fase superior rica em gelatina.

- 1929: Ostwald e Hertel, em estudos posteriores, verificaram que para variadas fontes de amido, diferentes concentrações eram necessárias para a separação das fases.

- 1947: Dobry e Boyer-Kawenoki estudaram a miscibilidade de pares de polímeros solúveis em água ou em solventes orgânicos, e a ocorrência ou não de separação de fases, constatando que dos 35 pares de macromoléculas estudados, apenas 4 não produziram a formação das duas fases, e concluiram que a incompatibilidade entre polímeros era um fenômeno geral

- 1958: Albertsson foi o primeiro a observar, que quando uma proteína é introduzida em um sistema aquoso bifásico, esta biomolécula é distribuída desigualmente entre essas duas fases. Isto propiciou analogias com processos de extração líquido–líquidos, que são completamente comuns em indústrias químicas. A partir dessas observações, ficou evidente para a comunidade científica a grande

potencialidade de aplicação destes sistemas à partição/purificação de materiais biológicos, desde proteínas até células (MERCHUK *et al*., 1998).

- 1971: Ryden & Albertsson, fizeram a primeira publicação relatando resultados de tensão interfacial de SAB's em sistemas formados por PEG e Dextrana. Neste trabalho já era relatado um comportamento característico de γ em função de duas variáveis termodinâmicas; o comprimento da linha de amarração (CLA) e a massa molar dos dois polímeros.

- 1984: Brooks e colaboradores, estudaram o mesmo sistema e propuseram uma relação exponencial entre a tensão interfacial e a diferença de composição entre as duas fases, expressa pelo comprimento da linha de amarração (CLA) (BAMBERG *et al.*, 1984).

- 1998: Silva e Loh, descobriram que a adição de CH_2Cl_2 a um sistema aquoso bifásico formado por Na_2SO_4/PEO/H_2O resultava em um sistema trifásico, onde, a fase mais densa era formada pela mistura H_2O/Na_2SO_4, a fase intermediária era composta por PEO/CH_2Cl_2, enquanto a fase superior era formada por PEO/H_2O. A adição de n-hexano, a este sistema trifásico, gerava um sistema tetrafásico em que a nova fase era a menos densa e formada por mistura de CH_2Cl_2/n-hexano (SILVA & LOH, 2006).

- 2003: Roger e colaboradores foram os primeiros a mostrar que as soluções aquosas de líquidos iônicos à base de imidazólio podem formar SAB em presença de soluções aquosas de certos sais inorgânicos, como por exemplo K_3PO_4 (GUTOWSKI *et al.*, 2003).

- 2007: Gu e Zhang estudaram a partição de biomoléculas em sistemas acetonitrila e água à baixa temperatura (GU & ZHANG, 2007).

- 2009: Coutinho e colaboradores utilizaram líquidos iônicos como adjuvantes na formação de sistemas bifásicos formados por PEG/sulfato e sua aplicação na partição de aminoácidos (PEREIRA *et al*, 2009).

De forma geral, a formação dos SAB's ocorre quando dois compostos hidrofílicos como polímeros (polietilenoglicol, dextrana, dentre outros) e sais (fosfatos, sulfatos, citratos,

etc.) são misturados acima de certa concentração crítica resultando em duas fases imiscíveis, onde o maior constituinte é água (GARZA-MADRID *et al.,* 2010). Acima deste ponto, as composições e temperaturas dividem-se em composições diferentes, porém em equilíbrio termodinâmico (VENTURA *et al*., 2011). A aplicação do SAB na separação de vários biocompostos tem sido estudada por diversos pesquisadores. A quantidade de reagentes químicos consumidos, como sais e polímeros, determina a competitividade entre a extração com SAB e as outras técnicas de bioseparação (COIMBRA *et al.*, 2003).

Os SAB's formados por PEG e dextrana ou PEG e sais são amplamente utilizados por não serem tóxicos, possuírem alta seletividade, possibilidade de reciclagem dos reagentes e por manterem a integridade das biomoléculas (FERREIRA *et al*., 2009). Todavia, o elevado custo da dextrana torna inviável a sua aplicação em escala industrial. Assim, o sistema PEG e sais foram empregados para a extração de enzimas em larga escala, pelo reduzido custo, baixa viscosidade e elevada seletividade (MALPIEDI *et al.*, 2009; ZHAO *et al.*, 2011).

Os sistemas formados por alcoóis e sais (sais de potássio) foram também utilizados na separação de biomoléculas por apresentarem baixa viscosidade, alta polaridade e também devido à fácil recuperação dos alcoóis e dos sais (OOI *et al.*, 2009). A substituição destes alcoóis por líquidos iônicos (usualmente referido como IL's do inglês *Ionic Liquids*) já foi descrita na literatura como uma alternativa na substituição dos solventes orgânicos (GUTOWSKI *et al.*, 2003; REIS *et al.*, 2012).

Os IL's são uma nova classe de sais com ponto de fusão abaixo de 100°C, cujas características principais incluem alta capacidade de solvatação, baixa inflamabilidade e alta estabilidade térmica e química (MONIRUZZAMAN *et al.*, 2010). Dentre os diferentes estudos recentes, Pereira *et al.* (2010) estudaram a influência de vários IL's no SAB constituído por polietilenoglicol e Na_2SO_4, e perceberam que a separação do L-triptofano na presença dos IL's foi bastante eficiente no sistema, induzindo diretamente a mudança do coeficiente de partição da biomolécula.

A partir das inúmeras vantagens do uso de SAB´s quando comparada as demais técnicas tradicionais (MALPIEDI *et al*., 2009; CLAUDIO *et al*., 2010), pode-se verificar na Tabela 1 estudos recentes em composições com diferentes constituintes evidenciando assim o potencial de aplicação destes sistemas no desenvolvimento de tecnologias para a inovação dos processos industriais.

Tabela 1: Exemplos de grupos de SAB e seus constituintes.

Grupos do SAB	Constituintes	Referência
Polímero – Polímero	PEG/Dextrana	Gunduz & Korkmaz, 2000
	PEG/PVA	Pessoa Junior & Kilikian, 2005
	PEG/Poli ácido acrílico	Saravanan *et al.*, 2008
Polímero – Sal Inorgânico	PEG/Fosfato de Potássio	Haraguchi *et al.*, 2004
	PEG/Sulfato de Magnésio	Oliveira *et al*., 2009
	PEG/Tartarato de Sódio	Mageste *et al.*, 2009
Solvente Orgânico – Sal Inorgânico	Álcool/Citrato de Sódio	Ooi *et al.*, 2009
	Álcool/K_2HPO_4...	Wang *et al.*, 2011
	Álcool/Sais de Potássio	Reis *et al.*, 2012
Líquido Iônico – Sal Inorgânico	[C_4min]Cl.../Fosfato de Potássio	Louros *et al.*, 2010
	[C_7mim]Cl.../Fosfato de Potássio	Ventura *et al.*, 2011
Líquido Iônico – Carboidratos	[C_4min]Cl.../D-Maltitol...	Freire *et al.*, 2011
Líquido Iônico – Aminoácidos	[C_4min]Cl.../L-Lisina...	Dominguez-Perez *et al*., 2009

1.3. Constituintes do Sistema Aquoso Bifásico

Uma diversidade de constituintes para SAB's vem sendo discritos na literatura, portanto serão descritos abaixo os compostos utilizados como formadores dos sistemas desenvolvidos neste trabalho.

1.3.1. Acetonitrila

A acetonitrila (CH_3CN) (Figura 1), também chamada de cianeto de metila é um composto orgânico translúcido, inflamável, com temperaturas de fusão e ebulição de -45 ºC e 81,6 ºC, respectivamente. A acetonitrila é produzida industrialmente pela desidratação da acetamida ou pela reação entre acetileno e amônia, entretanto sua principal rota de produção, dar-se pela amoxidação da acroleína (processo industrial utilizado na produção de nitrilas utilizando amônia e oxigênio), para a qual um precursor pode ser utilizado sob condições

específicas para que a quantidade de acetonitrila produzida seja pelo menos o dobro da quantidade de acrilonitrila (TAKAMUKU *et al*., 1998).

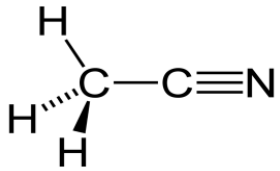


Figura 1. Estrutura química da acetonitrila.

A recuperação da acetonitrila é importante para a redução de custos, pois é amplamente utilizada como solvente para a extração de produtos de alto valor devido as suas propriedades físico-químicas (DHAMOLE *et al.*, 2010).

Uma das principais propriedades deste composto é que as suas moléculas não interagem fortemente entre si e tendem a formar ligações de hidrogênio com as moléculas da água (TAKAMUKU *et al*., 1998). OKANO *et al.* (2013) observaram que a acetonitrila é miscível em água em várias proporções a temperatura ambiente, além de verificarem que na presença de IL ocorre a separação das fases entre a água e a acetonitrila, formando assim um sistema bifásico. GU & ZHANG (2007) também estudaram os coeficientes de partição de algumas biomoléculas (peptídeos, aminoácidos e antibióticos) com sistemas formados por acetonitrila e água a baixas temperaturas. Em ambos os trabalhos a biomolécula migrou preferencialmente para a fase rica em acetonitrila.

A acetonitrila é aplicada como solvente polar aprótico na química sintética, sendo miscível em água, metanol, acetatos de metila e etila, acetona, éter etílico, hidrocarbonetos insaturados, clorofórmio, tetracloreto de carbono e cloreto de vinila a temperaturas próximas da temperatura ambiente (ZHANG *et al.*, 2011).

Apesar das nitrilas serem amplamente utilizadas na síntese de aminas, amidas, cetonas, aldeídos e uma variedade de outros compostos, a acetonitrila é usada principalmente como solvente em técnicas de cromatografia líquida de alta eficiência (CLAE), graças as suas características únicas, como: baixa viscosidade, baixa absorção de luz ultravioleta e alta miscibilidade com solventes apolares e água (SADEK, 2002).

A acetonitrila é um importante produto químico, largamente utilizado nas indústrias de cosméticos, borracha, pesticidas e produtos farmacêuticos. O uso deste solvente pode possibilitar rendimentos similares ou superiores aos solventes tradicionais, sendo que do ponto de vista ambiental e da saúde humana é o mais recomendado (SANTOS *et al.*, 2010). Na

literatura existem poucos relatos do uso de acetonitrila em SAB's, dentre eles, podemos citar os descritos na Tabela 2.

Tabela 2: Exemplos de SAB's formados por acetonitrila.

Constituintes	Referência
ACN/ Glucose	DHAMOLE *et al.*, 2010
ACN/NaCl	ZHANG *et al.*, 2011
ACN/Tampão	TAHA *et al.*, 2012

Na análise de carboidratos como sacarose, glicose e frutose, a ACN é o solvente de escolha, pois misturada à água em diferentes proporções, resulta em boa seletividade cromatográfica (NOLLET, 1992). Trabalhos recentes demonstraram a possibilidade da partição da vanilina em SAB's formados por acetonitrila e carboidratos, com elevada recuperação (DHAMOLE *et al.*, 2010; CARDOSO *et al.*, 2013).

1.3.2. Carboidratos

Os carboidratos ou hidratos de carbono possuem fórmula geral $(CH_2O)_n$, podendo conter também nitrogênio, enxofre ou fósforo. Portanto, apresentam uma diversidade de compostos orgânicos, incluindo açúcares, quintina, celulose e amido. São as biomoléculas mais abundantes na natureza, alguns carboidratos (como açúcar comum e amido) são à base da dieta na maior parte do mundo e a oxidação metabólica fornecedora de energia na maioria das células não-fotossintéticas. De acordo com a literatura, polímeros insolúveis de carboidratos funcionam tanto como elementos estruturais quanto de proteção nas paredes celulares (ALLINGER *et al.*, 1985; LEHNINGER *et al*., 2006).

Os carboidratos estão presentes na natureza e alguns existem praticamente puros, como: sacarose, glicose, frutose, amido e celulose. Em termos de volume de produção mundial, os carboidratos ficam apenas atrás dos óleos vegetais. Na utilização industrial, exceto pela indústria alimentícia, seu uso está restrito a alguns mono e dissacarídeos de baixo peso molecular, sendo extremamente atrativos como matéria-prima para experimentos laboratoriais, devido a sua disponibilidade em grandes quantidades, baixo custo e ecologicamente adequados (FERREIRA *et al.*, 2001).

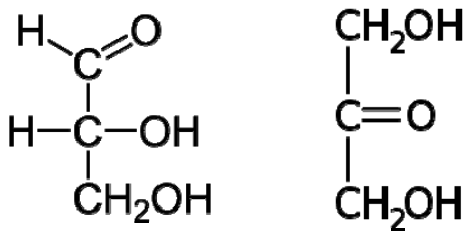
Os carboidratos são poliidroxialdeídos ou poliidroxicetonas, classificados como: monossacarídios que consistem de uma única unidade de poliidroxialdeído ou cetona,

oligossacarídios, formados por cadeias curtas de unidades monossacarídicas unidos por ligações glicosídicas, sendo os mais abundantes os dissacarídeos e polissacarídios, são polímeros formados por mais de vinte unidades de monossacarídeos (SOLOMONS & FRYHLE, 2002).

1.3.2.1. Monossacarídeos

Os monossacarídeos são os carboidratos mais simples, e quimicamente são aldeídos ou cetonas podendo conter um ou mais grupos hidroxila na molécula. Os monossacarídeos são compostos incolores, solúveis em água, porém insolúveis em solventes apolares. As moléculas dos monossacarídeos são constituídas por uma cadeia carbônica não-ramificada na qual todos os átomos de carbono estão unidos entre si por ligações covalentes simples. Na nomenclatura oficial, o sufixo "-ose" é anexado ao prefixo para denotar o número de átomos de carbono dos monossacarídeos: trioses (n=3), treoses (n=4), pentoses (n=5), hexoses (n=6) (LEHNINGER *et al.*, 2006; CONN & STUMPF, 1975).

Na cadeia aberta, um dos átomos de carbono está unido por uma ligação dupla a um átomo de oxigênio para formar um grupo carbonila, onde cada um dos outros átomos de carbono possuem ligação com um grupo hidroxila. Portanto, quando um grupo carbonila está em uma das extremidades da cadeia, o monossacarídeo é então denominado uma aldose, se o grupo carbonila está em qualquer outra posição na cadeia carbônica, o monossacarídeo é então chamado de cetose, como pode ser observado na Figura 2 (LEHNINGER *et al.*, 2006).



(a) (b)

Figura 2: Representação da fórmula molecular de: (a) aldose (gliceraldeído) e (b) Cetose (diidroxiacetona).

O gliceraldeído é uma aldotriose e a diidroxiacetona é uma cetotriose. As tetroses, as pentoses, as hexoses, as heptoses e as octoses, ocorrem em duas séries, isto é, aldotetroses e cetotetroses, aldopentoses e cetopentoses, aldoexoses e cetoexoses (LEHNINGER *et al.*, 2006).

Os monossacarídeos com cinco ou mais átomos de carbono na cadeia, quando estão em solução aquosa, ocorrem predominantemente como estruturas cíclicas nas quais o grupo carbonila forma uma ligação covalente com o oxigênio de um grupo hidroxila ao longo da cadeia. A formação destas estruturas em anel é o resultado da reação geral que ocorre entre aldeídos ou cetonas e alcoóis formando derivados denominados hemiacetais ou hemicetais. Os monossacarídeos são muitos reativos, podem ser oxidados por agentes oxidantes relativamente suaves, possuem capacidade de serem oxidados por íons cúpricos (Cu^{+2}) e férricos (Fe^{+3}), por isso são chamados de açúcares redutores. O grupo carbonila é oxidado a carboxila com a concomitante redução, por exemplo, do íon cúprico (Cu^{+2}) à cuproso (Cu^{+}), sendo tal princípio utilizado na análise de açúcares (LEHNINGER *et al.*, 2006).

Os carboidratos em geral possuem afinidade com a água uma vez que vários grupos -OH, com caráter doador/receptor, podem ser envolvidos em ligações de hidrogênio e, portanto, apresentam um caráter "*salting-out*" inerente, também conhecido como efeito "*sugaring-out*", sendo então os hidratos de carbono, potenciais substitutos para os sais convencionais utilizados na formação do SAB (MONTEIRO-FILHO, 2010). Os monossacarídeos utilizados neste trabalho no preparo de SAB's são: Glicose, manose, galactose, frutose, xilose e arabinose.

1.3.2.2. Oligossacarídeos

Os oligossacarídeos são polímeros pequenos de vários monossacarídeos unidos por ligações glicosídicas, a qual é formada quando um grupo hidroxila de uma molécula de açúcar reage com o átomo de carbono anomérico da outra molécula de açúcar. As ligações glicosídicas são facilmente hidrolisadas por ácido, mas resistem à clivagem por base. Assim os oligossacarídeos podem ser hidrolisados com ácido em solução. Os oligossacarídeos mais comuns são os dissacarídeos, dos quais se destacam a sacarose e a lactose, importantes dissacarídeos presentes no leite e na cana-de-açúcar, ambos representados na Figura 3 (HODGE & OSMAN, 1976).

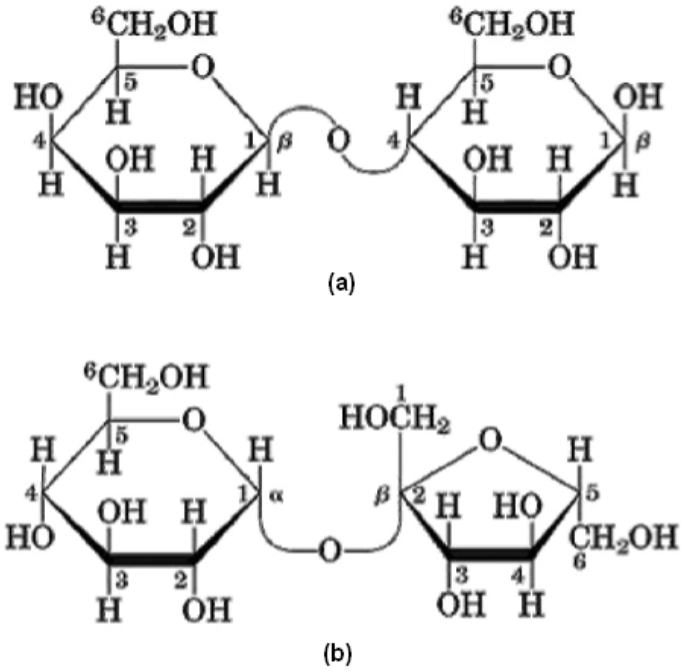


Figura 3: Fórmulas moleculares de lactose (a) e sacarose (b).

A sacarose é hoje no Brasil um dos mais importantes produtos devido à produção do álcool combustível, cuja obtenção se dá também por fermentação. Da hidrólise da sacarose se obtém uma mistura de glicose e frutose [O-β-D-frutofuranosil-(2→1)-α-D-glucopiranosideo], também conhecida por açúcar invertido. O termo invertido é empregado devido ao desvio da luz polarizada que sofre inversão de sentido, após a reação de hidrólise, inicialmente para a direita e, após a hidrólise para a esquerda. Em contraste com a maioria dos dissacarídeos e oligossacarídeos, a sacarose não possui átomos de carbono anomérico livre, pois, os átomos anoméricos de carbono das duas hexoses estão ligados entre si e por essa razão não age como açúcar redutor (LEHNINGER *et al.*, 2006).

O dissacarídeo utilizado neste trabalho em SAB é a maltose, formado pela junção de duas moléculas de D-glicose unidas por uma ligação glicosídica entre C-1 (carbono anomérico) de uma unidade de glicose e C-4 de outra unidade de glicose.

1.3.2.3. Polissacarídeos

Polissacarídeos são polímeros de alto peso molecular, resultantes da condensação de um grande número de moléculas de aldoses e cetoses. Também chamados de glicanos, diferem entre si na identidade das suas unidades monossacarídicas repetitivas, que podem ser de uma única espécie ou de espécies alternadas, nos tipos de ligação que as unem, no

comprimento das suas cadeias e no grau de ramificação das cadeias (LEHNINGER *et al.*, 2006; MURRAY *et al.*, 1994).

A maioria dos carboidratos encontrados na natureza compõe-se de polissacarídeos de alto peso molecular. Na hidrólise completa com ácido ou enzimas específicas, esses polissacarídeos produzem monossacarídeos e/ou derivados monossacarídicos simples. Nos polissacarídeos, a unidade monossacarídica predominante é a D-glicose, mas também são comuns os polissacarídeos de D-manose, D-frutose, D e L-galactose, D-xilose, D-arabinose, dextranas (LEHNINGER *et al.*, 2006).

Os polissacarídeos podem ser utilizados "*in natura*" na alimentação, na fabricação de produtos têxteis, papel, madeira para construção, revestimentos industriais, cosméticos etc. Também podem sofrer modificações nas cadeias poliméricas, de maneira que possam ser adaptados para usos específicos, por exemplo, o rayon e a quitosana, obtidos respectivamente da celulose e da quitina, mantêm as estruturas gerais dos polissacarídeos correspondentes (FERREIRA *et al.*, 2009).

A utilização de carboidratos como substitutos potenciais de sais inorgânicos e polímeros para a formação de SAB, são recentes. A utilização de açúcares em SAB foi já abordado por Wang *et al.* (2008), em combinação com acetonitrila, bem como por Wu *et al.* (2008), e Freire *et al.* (2011) que utilizaram os açúcares em combinação com os líquidos iônicos. Os carboidratos são utilizados em SAB's na separação de determinadas substâncias que não toleram a presença de sais (MONTEIRO-FILHO, 2010).

1.3.3. Polióis

Os alcoóis polihídricos, polióis ou açúcares alcoóis, diferenciam-se de outros sacarídeos devido à redução das funções cetona ou aldeído. Constituem uma classe especial de carboidratos, podendo ser monossacarídicos (sorbitol, manitol, xilitol, eritritol), dissacarídicos (maltitol, lactitol, isomalte) e mistura de sacarídeos e polissacarídeos hidrogenados (BILLAUX *et al*., 1991).

Os polióis são utilizados em diversas áreas, incluindo alimentos, produtos farmacêuticos, e cosméticos (VELEZMORO MEIRELLES, 1998). Os polióis também são considerados como uma nova geração da plataforma de energia verde. Com o esgotamento dos recursos de petróleo, a produção de polióis a partir de biomassa satisfaz as necessidades do desenvolvimento sustentável. Wang *et al*. (2012) analisaram uma das rotas mais atrativas para a

reação da utilização de celulose e sua conversão em polióis, e observaram que a celulose pode ser convertida em C_2 e C_6 polióis com o rendimento até 38% catalisada por Ru/nanotubos de carbono.

São obtidos por hidrogenação da maltose, lactose, palatinose, glicose e xilose, estando disponíveis em várias formas cristalinas ou como xaropes líquidos. A solubilidade varia com a temperatura e a higroscopicidade varia com a umidade relativa (ZUMBÉ, 2001). A conversão do grupo carbonílico (aldeído ou cetona) de açúcares em álcool, com conseqüente transformação de estruturas cíclicas a lineares, confere aos polióis importantes propriedades, como resistência ao escurecimento, diminuição da susceptibilidade à fermentação, maior resistência à cristalização e maior estabilidade química (NINNI *et al*., 1999).

Os polióis em solução possuem grande afinidade pela água, sendo largamente utilizados por conferir estabilidade termodinâmica a macromoléculas biológicas em solução. A atividade dos polióis com as moléculas de água está estreitamente relacionada com outras propriedades termodinâmicas, tais como pressão de vapor, coeficiente osmótico, coeficiente de atividade, entalpia de excesso, entre outros (SADEGHI & ZIAMAJIDI, 2006). É ainda importante na elucidação da natureza das interações entre grupos polares e não polares com água, na concepção de processos de separação e devido à sua relação com os sistemas biológicos. Contudo, o comportamento deste tipo de solutos na água não está bem compreendido devido à complexidade das interações que ocorrem em solução aquosa e à falta de dados experimentais das suas propriedades termodinâmicas (ROMERO & PÁEZ, 2006).

Os principais polióis utilizados neste trabalho foram glicerol, eritritol, xilitol, sorbitol e maltitol. O glicerol constituído por um tri-álcool com 3 carbonos (1,2,3-propanotriol), é um líquido incolor, com gosto adocicado, sem cheiro e muito viscoso. A presença de três grupos hidroxila na estrutura do glicerol é responsável pela solubilidade em água e sua natureza higroscópica. É uma molécula altamente flexível formando ligações de hidrogênio tanto intra como intermoleculares. De acordo com estudos teóricos, usando métodos de teoria de densidade funcional (DFT), existem 126 possíveis confôrmeros para o glicerol (CALLAM *et al*., 2001).

Eritritol é um poliól encontrado em frutas, cogumelos e alguns alimentos fermentados (SASAKI, 1989), podendo ser produzido a partir de sacarose por processos biotecnológicos (AOKI *et al*., 1993; KIM *et al*., 2000)

O xilitol, poliálcool cuja fórmula molecular é $C_5H_{12}O_5$ (1,2,3,4,5-pentaidroxipentano) de estrutura aberta, possui cinco grupos hidroxila, cada um deles ligado a um átomo de carbono, razão pela qual esse composto é conhecido como poliidroxiálcool

acíclico ou pentitol (MÄKINEN, 2000). Esse poliálcool é atóxico devido à ausência de grupos aldeídicos ou cetônicos em sua molécula (MANZ *et al*., 1973).

O sorbitol é o poliol mais conhecido da família dos alcoóis poli-hídricos. Sua aplicação na indústria de alimentos é amplamente difundida. Uma das principais características do sorbitol é a sua capacidade de reter umidade, e a sua utilização como um agente umectante, mantendo o alimento fresco por um período maior de tempo (DEIS, 1993). A sua extração é realizada naturalmente a partir de frutas (ameixa, cereja, maçã, pêssego). Entretanto, como as quantidades presentes na natureza não são suficientes para sua extração comercial, este poliol pode ser produzido industrialmente a partir da sacarose ou do amido, seguida de hidrogenação catalítica da D-glucose. Possui propriedade espessante, edulcorante, inibidor de cristalização, plastificante, anticongelante (reduz o ponto de congelamento) e crioprotetor. Apresenta estabilidade química e térmica, baixa higroscopicidade, não absorvendo umidade a temperaturas de até 25 °C e umidades relativas do ar de até 85%, permitindo o desenvolvimento de produtos altamente estáveis à estocagem (VIGGIANO, 2003).

O maltitol ou 4-O-α-D - glicopiranosil-D-sorbitol, é um poliol dissacarídico, não encontrado na natureza, é produzido industrialmente baseado na hidrogenação catalítica da maltose, produzida pela hidrólise do amido de milho ou de batata hidrolisada enzimaticarnente. A hidrogenação do xarope de maltose resulta no xarope de maltitol, o qual é purificado, desidratado e cristalizado. O xarope contém cerca de 80% dos sólidos representado por maltitol, 9,5% a 13,5% de maltotriol, 3% de sorbitol e 6,5% a 13% por polióis polissacarídeos e oligossacarídeos hidrogenados. O maltitol é solúvel em água, o aumento de solubilidade é devido à presença de maltotriol e a maior concentração de oligossacarídeos e polissacarídeos hidrogenados, maltitol possui alta higroscopicidade, e possui boa estabilidade química, térmica e enzimática (MARIE e PIGGOTT, 1991).

1.3.4. Dextrana

Dextrana é o nome dado a uma classe de polissacarídeos bacterianos extracelulares, são constituídos quase que exclusivamente da unidade monomérica α-D-glicose ligadas por ligações α-(1,6) na cadeia principal e α-(1,4), α-(1,3) e α-(1,2) nas ramificações (ALSOP, 1983). A massa molecular e o número de ramificações e outras propriedades das dextranas são propriedades que definem seu comportamento em soluções, o número de ramificações tende a diminuir simultaneamente com a redução da massa molar (AQUINO & FRANCO, 2008).

Dextranas que possuem maior número de ligações α-(1,6) são mais solúveis em água, possuindo elevada estabilidade sob condições ácidas e alcalinas (KIM *et al*., 2003).

A presença de sacarose e outros açúcares e sua concentração, as condições de temperatura e pH influenciam diretamente na massa molar da dextrana produzida (ROBYT *et al*., 1974). Outros açúcares promovem um crescimento vegetativo da bactéria, mas sem produção da enzima. Portanto, a dextrana não é um produto formado diretamente pela ação microbiana, mas provém da ação da enzima dextrana-sacarase, produzida através da ação da bactéria sobre a sacarose (KARTHIKEYAN *et al*., 1999).

Muitas bactérias sintetizam dextrana extracelularmente, sendo as principais produtoras pertencentes ao gênero *Leuconostoc mesenteroides*. A grande diversidade dentro da classe das dextranas ocorre devido a essa grande variedade de microorganismos que as produzem, sendo que a espécie determina sua propriedade e estrutura (JUNG & MAYER, 1981).

As dextranas e os seus derivados vêm adquirindo importância industrial devido às suas aplicações na indústria química, farmacêutica, alimentícia e petrolífera e seus usos depende de seu peso molecular, o qual é função das condições da síntese, como temperatura, concentração de sacarose e presença de outros açúcares (ROBYT &WALSETH, 1979). Quando é sintetizada somente com sacarose, denomina-se dextrana nativa, apresenta em média peso molecular entre 9 e 50 milhões de daltons e alta viscosidade (DE BELDER, 1987). Baixos pesos moleculares são obtidos na presença de outros açúcares, que são chamados de aceptores, como a maltose e isomaltose. A redução do peso molecular pode ser feita pela hidrólise da dextrana nativa (MONSAN *et al.*, 1986).

1.3.5. Álcool Polivinílico

O álcool polivinílico (PVA), é um polímero sintético, hidrofílico, biodegradável, não tóxico e biocompatível, com excelente capacidade de formação de filmes por deposição à temperatura ambiente, possuem baixo custo e alta disponibilidade. O PVA é um pó granular de cor inodoro e insípido, translúcido, branco ou creme, e estão disponíveis em várias formas cristalinas ou como xaropes líquidos (MORAES *et al*., 2008).

A primeira síntese do PVA foi realizada por Hermann e Haehnel em 1924 a partir da hidrólise de acetato de polivinila em etanol com KOH. O álcool polivinílico é produzido comercialmente a partir de acetato de polivinila, em geral por um processo contínuo. As

características físicas e suas específicas utilizações funcionais dependem do grau de polimerização e do grau de hidrólise. O álcool polivinílico é classificado em duas classes, nomeadamente: parcialmente hidrolisado e totalmente hidrolisado. Parcialmente hidrolisada PVA é usado nos alimentos (MARTEN, 1985; CHOU, 2010).

O PVA é utilizado em diversas aplicações nas indústrias de papel, têxtil, farmacêutica, química e automobilística (MARUSINCOVÁ *et al.*, 2013; RUMYANTSEV, 2013). As propriedades físicas e resistência química deste polímero sintético o caracterizam com boa resistência a solventes, óleos e resistente à passagem de oxigênio (KRUMOVA *et al.*, 2000). É um dos poucos polímeros semicristalinos solúveis em água, e apresenta boas características interfaciais e mecânicas. O PVA é frequentemente usado em processamento de papel e fibras (OKAYA *et al*., 1999; ISENBERG & WONG, 2006), na obtenção de membranas anfifílicas para imobilização de enzimas (ARANHA & LUCAS, 2001) e é conhecido por ser um dos polímeros mais amplamente utilizados para a separação de misturas contituídas de compostos orgânicos e água (MANDAL *et al.*, 2011).

As características biodegradáveis e a não toxidez do PVA permite sua utilização como filme protetor da umidade para comprimidos de suplementos alimentares e alimentos secos (ZUMBÉ, 2001; MARUSINCOVÁ *et al.*, 2013). O PVA também é utilizado como hidrogéis por exibir um alto nível de absorção de água ou líquidos biológicos. Por causa destas propriedades, o PVA é capaz de simular o tecido natural e encontra numerosas aplicações na engenharia (LI & NEOH, 2004).

A solubilidade do PVA varia com a temperatura, sendo associado à quebra das ligações de hidrogênio intra e intermoleculares, o aumento da temperatura pode quebrar também as ligações de hidrogênio com a água, diminuindo a solubilidade. A sua higroscopicidade varia com a umidade relativa (OLIVEIRA *et al*., 2002). Para o PVA 98% hidrolisado, a sua solubilidade aumenta com a diminuição do grau de polimerização. Para o PVA parcialmente hidrolisado (88%), a solubilidade é relativamente independente do grau de polimerização. Para o PVA 80% hidrolisado, a solubilidade a baixa temperatura é muito maior que para o 88% hidrolisado, mas decresce rapidamente a partir de 30°C (LIU *et al*., 1997; ISENBERG & WONG, 2006).

1.4. Termodinâmica em Sistema Aquoso Bifásico

Para o estudo termodinâmico de qualquer sistema, diversas propriedades devem ser avaliadas. Dentre elas, podemos destacar as mais comuns, como: a entalpia (H), a entropia (S) e a energia livre de Gibbs (G) (COOPER, 1999). Para estudos de equilíbrio de fases e desenvolvimento de diagrama de fases faz-se necessário a determinação das curvas binodais, linhas de amarração (usualmente referido como TL, do inglês *Tie Line*), comprimento dessas TL (usualmente referido como TLL, do inglês *Tie Line Length*), sua inclinação (usualmente referido como STL, do inglês *Slant Tie Line*) e o seu ponto crítico (P_c), e para a partição de biomoléculas aplicando estes sistemas, alguns outros parâmetros tornam-se indispensáveis como, por exemplo: o coeficiente de partição (K) e recuperação (R_T),.

Os parâmetros termodinâmicos envolvidos para a determinação da partição de uma biomolécula em SAB's são determinados pela teoria de Flory-Huggins, na qual descreve a energia necessária para a obtenção do ΔG_m^o (JOHANSSON *et al.*, 1998; COOPER, 1999).

A energia livre de Gibbs (G) é o parâmetro que expressa o equilíbrio molecular, ela indica a direção dos processos, bem como a quantidade de trabalho necessária para que ele ocorra (DREYER *et al.*, 2009; SOUSA *et al.*, 2009). Ela pode ser expressa em função da energia livre a pressão constante (equação 1) e a nível molecular (equação 2).

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

$$\Delta G_m^o = -RT\ln K \tag{2}$$

onde R é a constante dos gases e K é a constante de equilíbrio.

Geralmente, estes parâmetros citados anteriormente são obtidos pelo ajuste linear da equação obtida pelo correlacionamento do coeficiente de partiçãoe e o inverso da temperatura em graus Kelvin (JOHANSSON *et al.*, 1998).

O coeficiente de partição é definido como a razão entre a concentração de uma biomolécula nas fases de topo e de fundo (WANG *et al.*, 2011). As biomoléculas, aplicadas nos SAB's para extração e purificação distribuem-se entre as duas fases aquosas, podendo ser definido um coeficiente de partição desta biomolécula, pela utilização da equação 3:

$$K = \frac{C_T}{C_F} \quad (3)$$

onde, C_T e C_F são as concentrações do soluto nas fases de Topo e Fundo.

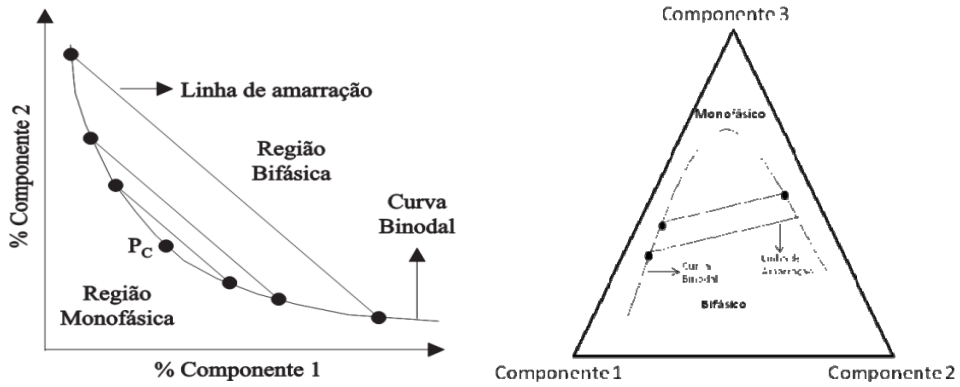
Essa distribuição depende tanto das características da biomolécula que se distribui (massa molar, carga, hidrofobicidade superficial, etc.), quanto da natureza do SAB utilizado (reagentes, massa molar, concentração, pH, força iônica, etc.). Embora a manipulação das propriedades do sistema venha a ser uma forma de controlar a partição das biomoléculas no sistema, controlá-la ainda é uma difícil tarefa (GIRALDO-ZUÑIGA, 2001; CHAIWUT *et al*., 2010).

Os mecanismos que interferem a partição de biomoléculas não são ainda entendidos completamente. Segundo Johansson *et al*. (2008) e Dreyer *et al.* (2009) a partição de biomoléculas em sistemas aquosos bifásicos pode ser descrita com base na repulsão entrópica (log K_S), interações eletrostáticas (log K_{el}), hidrofóbicas (log K_{hy}) e a força motriz eletroquímica proveniente do sal do sistema, onde o total da partição (K_{tot}) está representada na equação 4.

$$\log K_{Tot} = \log K_s + \log K_{el} + \log K_{hy} + \log k_{salt} \quad (4)$$

As propriedades das fases também contribuem na partição das biomoléculas, como a natureza química, composição do SAB, presença de ligantes ao longo da cadeia polimérica, pH, temperatura, densidade (CHAIWUT *et al*., 2010). As duas fases dos SAB estão em equilíbrio, nenhuma propriedade termodinâmica está variando em uma dimensão temporal. Estas fases estão separadas por uma interface que é a região na qual as propriedades termodinâmicas de cada fase transitam para valores diferentes, sempre tendendo para o valor daquela propriedade no seio da outra fase em equilíbrio (RÍOS, 2004).

Os sistemas termodinâmicos para SAB's podem ser representados por meio de diagramas retangulares e triangulares (Figura 4).



(a) (b)

Figura 4: Diagrama de fase expresso em coordenadas retangulares (a) e coordenadas triangulares (b).

Nestes diagramas pode ser observada a composição química das duas fases que se encontram em equilíbrio termodinâmico, os quais são expressos em diversas unidades, geralmente é utilizada a representação em percentuais mássicos (% m/m). A partir destes diagramas, podemos obter as composições globais do sistema, com a delimitação das regiões monofásica e bifásica por meio da curva binodal ou curva de solubilidade. Existem vários tipos de curvas binodais para um sistema ternário, que podem ser classificadas pelo número de pares parcialmente miscíveis, algumas dessas estão representadas na Figura 5 (HACKBART, 2007).

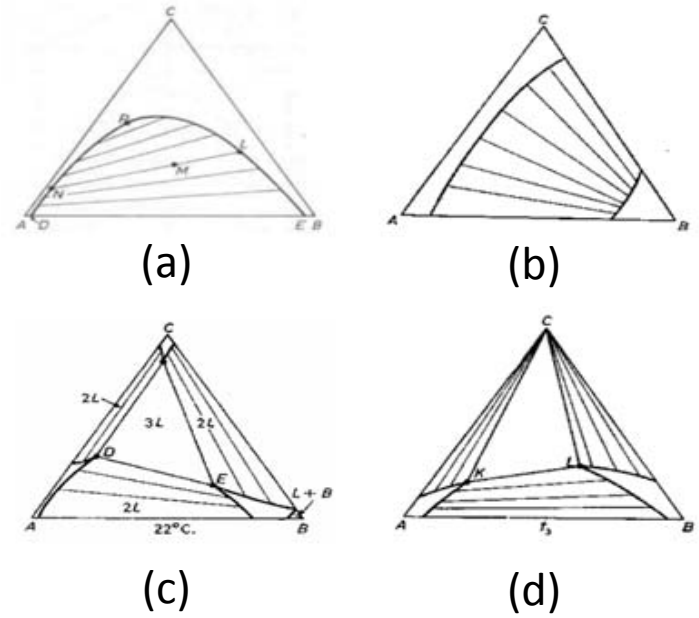


Figura 5: Equilíbrio líquido-líquido ternário: (a) formação de um par de líquidos parcialmente miscíveis, (b) formação de dois pares de líquidos parcialmente miscíveis, (c) formação de três pares de líquidos parcialmente miscíveis e (d) formação de fases sólidas.

Após a determinação gráfica das curvas binodais se faz necessário a construção das TL, onde quaisquer pontos sobre ela representam um sistema com a mesma composição que possuem propriedades termodinâmicas intensivas iguais (composição, densidade, volume molar, entalpia molar, etc.). Entretanto, deve-se considerar que as variáveis termodinâmicas extensivas são diferentes (massa, volume, etc.). Portanto, se aplica o mesmo raciocínio para as fases inferiores formadas a partir de composições globais localizadas sobre uma mesma TL (ZASLAVSKY, 1995; SILVA & LOH, 2006). A análise de uma grande parte de diagramas de fases reportadas na literatura revela que as TL são paralelas entre si, porém Zaslavsky (1994) têm mostrado que esse paralelismo entre elas, não é uma regra para os SAB's.

Outro parâmetro termodinâmico comumente utilizado é o comprimento da linha de amarração (TLL) (RÍOS, 2004), que é a medida das retas que ligam pontos de mistura e da composição das fases em equilíbrio no diagrama que representam (PEI *et al.*, 2012). Quaisquer pontos sobre essa mesma linha fornecerão fases de topo e fundo com a mesma composição final, porém com diferentes relações de volumes ou de massas entre as fases (SILVA & LOH, 2006).

Segundo Malpiedi *et al.* (2009), o aumento da TLL ocasiona uma diminuição do volume livre na fase de fundo do sistema. Portanto, este parâmetro termodinâmico importante representa o quão distintas são as propriedades termodinâmicas das fases do sistema. A TLL pode ser calculadaa partir das diferenças nas concentrações dos componentes em cada fase, conforme indicado na Equação 5.

$$\mathrm{TLL} = \left[\left(C_P - C_{Po} \right)^2 + \left(C_S - C_{So} \right)^2 \right]^{1/2} \tag{5}$$

onde, C_P e C_{Po} são as concentrações do componente 1; e C_S e C_{So} são as concentrações do componente 2 (% (m/m)) nas fases de topo e fundo, respectivamente.

À medida que o valor do TLL aumenta, torna-se maior a diferença de composição entre as fases, elevando, conseqüentemente, a eficiência na extração e/ou partição do soluto de interesse (PEI *et al.*, 2012).

A inclinação da linha de amarração, usualmente definido como STL, é uma medida de como a composição das fases pode variar com a alteração de uma propriedade físico-química, como a temperatura e a massa molar, por exemplo (ALBERTSSON, 1986). O valor da inclinação pode ser calculado de acordo com a Equação 6:

$$STL = (C_P - C_{Po}) / (C_S - C_{So}) \quad (6)$$

Este parâmetro apresenta-se atualmente como uma característica importante dos diagramas de fases uma vez que o seu valor é geralmente constante, quando está presente em um SAB de polímeros (ALBERTSSON, 1986) e a sua avaliação, comparação com uma média para um dado sistema e nova medição para valores afastados dessa média é provavelmente o melhor método para obter diagramas de fases confiáveis (ZASLAVSKY, 1995).

Outra particularidade de um diagrama de fases é o ponto crítico (P_c), neste ponto as propriedades físico-químicas (composição e volume, entre outras) das duas fases são teoricamente iguais. Desta forma, quanto mais a composição do sistema se aproxima do P_c, menor é a diferença entre as fases, ou seja, no P_c, as composições e os volumes entre as fases são teoricamente iguais. No entanto, nas proximidades do P_c existem pequenas alterações na composição dos sistemas, que provocam drásticas mudanças na qual o sistema varia de uma para duas fases e vice-versa (ALBERTSSON, 1986). Este ponto pode ser obtido pela intersecção de uma linha que passa pelo ponto médio de várias TL, com a binodal. O ponto limite é o ponto onde a binodal é tangente à linha que une os segmentos iguais nos eixos do diagrama. A posição relativa do ponto limite e do P_c define a simetria do diagrama de fases. Quando estes dois pontos coincidem o diagrama de fases considera-se simétrico (RODRIGUES *et al*, 2001).

Diferentes modelos termodinâmicos têm sido desenvolvidos para a modelagem de SAB. Dentre estes o modelo da equação virial NRTL (*Non Random Two Liquids*) é bastante utilizado, ele se baseia no conhecimento da pressão osmótica de um solvente. Geralmente, este modelos é frequentemente utilizado para correlação e predição do equilíbrio de SAB constituído por polímero e sal. Além deste, diversos modelos são utilizados para a determinação da TL conforme descritos na literatura (LADDHA & DEGALEESAN, 1976; MERCHUCK *et al*, 1998; PRAUSNITZ *et al*., 1999; RIOS, 2004; MARTINS *et al.*, 2010; ZAFARANI-MOATTAR & NASIRI, 2010). Dentre eles, podemos citar o Método de Merchuck, que é bastante utilizado para partição e recuperação de biomoléculas em SAB's, como por exemplo: as enzimas pectinolíticas (LIMA *et al.*, 2002), a lipase (SOUSA *et al.*, 2010; VENTURA *et al.*, 2011), a vanilina (CLAUDIO *et al.*, 2010; REIS *et al.*, 2012), dentre outros.

1.5. Vanilina

A vanilina ($CH_3O(OH)C_6H_3CHO$) apresentada na Figura 6 é um aldeído aromático, pertencente ao grupo de compostos fenólicos simples, onde sua estrutura possui três grupos funcionais incluindo um aldeído, um éter e um grupo fenol. A vanilina possui um aspecto de um pó branco cristalino com um odor intenso e agradável. É solúvel em clorofórmio, éter e água à temperatura ambiente (CONVERTI *et al*., 2010). As propriedades termofísicas estão relatadas na Tabela 3.

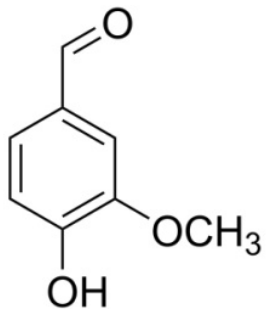


Figura 6. Estrutura molecular da vanilina.

Tabela 3: Propriedades Termofísicas da Vanilina a 298K.

Propriedade	Valor
Massa Molar ($g.mol^{-1}$)	152,15
Densidade ($g.cm^{-3}$)	2,056 a 298K
Ponto de Fusão (K)	353-354
Ponto de Ebulição (K)	558
Solubilidade em Água ($g.dm^{-3}$)	10 a 298K
pK_a	8,2 a 298K

Fonte: Tarabanko *et al.*, 2007.

A vanilina é obtida tradicionalmente da vagem de uma orquídea tropical (*Vanilla planifólia*) (Figura 7). O maior produtor mundial de vanilina natural é Madagascar, seguida por outros países como México, Tahiti e Indonésia (BYTHROW, 2005).

Figura 7: *Vanilla planifólia.*

O interesse sobre a vanilina é principalmente apreciado por criar sabores artificiais em uma ampla gama de produtos comerciais, é atualmente utilizada em alimentos, bebidas, na indústria de cosméticos devido à sua fragrância e em produtos químicos especiais para aplicações técnicas, tais como a análise por cromatografia em camada fina (HOCKING, 1997; NOUBIGH *et al*., 2010). Além destas aplicações, aproximadamente 50% da produção de vanilina sintética é usada como um produto intermediário na produção de produtos farmacêuticos, tais como: papaverina, L-dopa, L-metildopa e agentes antimicrobianos (NOUBIGH *et al*., 2010). Devido ao seu caráter fenólico, a vanilina possui propriedades antimicrobianas e antioxidantes, que são devidas à interação com espécies radicalares, evitando, dessa forma, os processos oxidativos (ANTONIOLLI *et al*., 2004). Além disso, algumas pesquisas ilustram o seu potencial antimutagênico (MAURYA *et al*., 2007).

Os custos elevados, somados aos pequenos rendimentos da produção de vanilina natural aproximadamente 0,2%, estimularam a pesquisa por rotas de produção de vanilina sintética, então em 1875 a vanilina foi sintetizada a partir do eugenol (Figura 8), preparado a partir do óleo de cravo (HOCKING, 1997), e no século XX a vanilina passou a ser sintetizada a partir da lignina contida no licor negro, um resíduo das indústrias de papel e celulose. Este componente representa 30% de sua constituição, sendo que o extrato sintético de vanilina fornece apenas a nota sensorial principal do "flavour" de baunilha (KORTEKAAS *et al*., 1998).

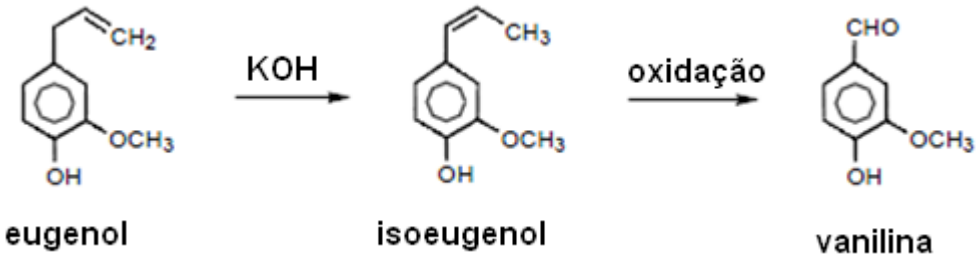


Figura 8. Produção de vanilina a partir do eugenol.

Para a indústria, tem-se tornado cada vez mais importante o uso de compostos aromatizantes que possam ser denominados "naturais", a fim de obter a aceitação do consumidor. A vanilina produzida por meio de processos biotecnológicos é oriunda de compostos de fontes renováveis e com custos relativamente baixos se comparados ao processo de extração da vanilina natural. A produção biotecnológica da vanilina ocorre mediante o uso de extratos enzimáticos brutos ou enzimas purificadas produzidas principalmente por microrganismos ou plantas ou ainda por culturas de células (DAUGSCH & PASTORE, 2005).

O aumento da população mundial impulsionou o crescimento de mercados especialmente voltados à produção de alimentos. Concomitante ao aumento do consumo de alimentos encontra-se o aumento da demanda por aditivos alimentares, tais como a vanilina (FURUKAWA *et al*., 1998). Sendo então de grande importância estudos envolvendo sua produção, separação e purificação, mantendo suas características funcionais inalteradas, o sistema aquoso bifásico se encaixa neste perfil.

Cláudio *et al*. (2010) extraíram a vanilina utilizando sistemas de duas fases aquosas formado por líquidos iônicos, onde foram determinados os coeficientes de partição da vanilina. Três parâmetros principais foram avaliados através do processo de separação da vanilina: o líquido iônico (IL), estrutura cátion e ânion, a temperatura de equilíbrio e a concentração de vanilina disponível no sistema global. Além disso, a viscosidade e densidade de ambas as fases aquosas foram experimentalmente medidas nas composições de fração de massa para os quais os coeficientes de partição foram determinados. Em todos os sistemas e condições testadas, a vanilina migra preferencialmente para a fase rica em IL. Os resultados obtidos neste trabalho indicam que SAB contendo IL pode ser adicionalmente utilizada na extração e purificação de vanilina a partir de diferentes matrizes, como confirmado pelos grandes coeficientes de partição obtidos e melhoria dos sistemas de baixa viscosidade.

1.6. Aplicações dos SAB's

Os SAB's vêm sendo empregados com muito sucesso na separação e purificação de diversas biomoléculas. Encontram-se na literatura diversos trabalhos que evidenciam o seu potencial de aplicação a nível industrial e que auxiliam a identificar os sistemas mais adequados na separação e partição de algumas biomoléculas, como: as enzimas (PORTO *et al*., 2011), os aminoácidos (SALABAT *et al*., 2011), antibióticos como o ciprofloxacino

(MOKHTARANI *et al*., 2008) e antocianinas (WU *et al*., 2011), compostos de aroma como a vanilina (CLAUDIO *et al*., 2010), antioxidantes (REIS *et al*., 2012) , e alcalóides (PASSOS *et al*., 2013).

Durante algum tempo os SAB's foram utilizados apenas em laboratórios, mas devido sua simplicidade, biocompatibilidade e facilidade de operação em grande escala, o seu uso foi expandido para as indústrias (HATTI-KAUL, 2001; MONTEIRO FILHO, 2010). De acordo com a Tabela 4, podemos observar a possibilidade de separar e recuperar uma grande variedade de biomoléculas utilizando SAB's. Além disso, esta técnica de separação vem sendo aplicada em outros segmentos como no tratamento de efluentes (WAZIRI *et al.*, 2003), na remoção de íons metálicos (GRABER *et al.*, 2000) e na remoção de poluentes orgânicos do meio ambiente (ROGERS *et al.*, 1998).

Tabela 4: Aplicações de Sistemas Aquosos Bifásicos.

Biomolécula	**SAB**
Glutenina, Lisina, glisina, β-galactosidade, lisozima, Pululanase, BSA e ovalbumina	PEG – Dextrana
β-lg, BSA, caseína	Goma guar – Dextrana ou PEG
BSA	Ficoll – Dextrana
Ácido lático	EOPO – Dextrana
Endopoligalacturonase	PEG – PVA
α –amilase	PEG – $MgSO_4$
Lisozima e conalbumina	PEG – $C_6H_5Na_3O_7$ ou Na_2SO_4
Insulina	PEO/PPO/PEO – Tampão de fosfato
β-lg, antitripsina, caseína, BSA e amiloglucosidade	PEO – K_3PO_4 ou $MgSO_4$ ou K_2HPO_4 ou Na_2SO_4
B-caroteno	IL – Carboidratos ou K_3PO_4

Rabelo *et al.* (2004) utilizaram o SAB para a purificação de bromelina, uma enzima presente nos frutos do abacaxi. Depois de triturados os frutos, o suco obtido foi filtrado e alíquotas foram adicionadas a sistemas bifásicos óxido de polietileno (PEO) - Óxido de polipropileno (PPO) - óxido de polietileno (PEO). Medidas laboratoriais indicaram que houve, sob condições adequadas, uma purificação adequada da enzima diretamente a partir do seu meio de obtenção, mantendo-se boa atividade e a possibilidade de aplicação tecnológica.

Alves *et al.* (2000) estudaram a purificação de insulina obtida de suínos, bastante semelhante à humana, simulando sua obtenção em um meio fermentado. A proteína foi satisfatoriamente purificada, mantendo sua atividade após o processo, o que indica o potencial de utilização desta metodologia na indústria farmacêutica.

Machado (1999) estudou a purificação de células inteiras (*Lactobacillus acidophilus* H2B20) por meio de sistemas aquosos bifásicos formados por poli (etileno glicol) e maltodextrina. Empregou-se como meio de fermentação o soro de queijo ultrafiltrado e o objetivo foi obter um concentrado de células possível de ser adicionado a um alimento não fermentado, tendo-se em vista eliminar o problema da baixa aceitação de produtos fermentados com o microrganismo, que por sua vez possui características benéficas. Obteve-se como resultado final um método de purificação de *L. acidophilus* H2B20 que não apenas foi capaz de preservar a atividade microbiana, mas também de suportar seu crescimento.

O maior uso dos sistemas aquosos bifásicos tem sido na etapa de concentração e no processo de purificação de proteínas. De acordo com Cláudio *et al.* (2010), várias novas matrizes para produção de vanilina estão sendo estudadas, onde os SAB's na presença de IL se mostraram eficazes para extração e purificação.

Capítulo II

2. OBJETIVOS

2.1. Objetivo Geral

Este trabalho teve como objetivo geral o desenvolvimento de novos sistemas aquosos bifásicos baseados em acetonitrila e sua aplicação na partição de vanilina.

2.2. Objetivos Específicos

Os objetivos específicos deste trabalho foram:

- Construir diagramas de fases para sistemas formados por acetonitrila e carboidratos, e sua aplicação na partição de vanilina.
- Construir diagramas de fase para sistemas formados por acetonitrila e polióis, e sua aplicação na partição da vanilina.
- Construir diagramas de fases para sistemas formados por acetonitrila e dextrana, e sua aplicação na partição da vanilina.
- Construir diagramas de fases para sistema formado por acetonitrila e álcool polivinílico, e sua aplicação na partição da vanilina.

INTRODUÇÃO AOS CAPÍTULOS III, IV, V e VI

Os capítulos III, IV, V e VI são apresentados em forma de artigos científicos, e estão organizados conforme as normas propostas pelo periódico de cada publicação. Estes capítulos trazem uma pequena introdução, o material e métodos utilizados no desenvolvimento de cada artigo, os resultados obtidos e sua discussão, além das conclusões de cada etapa.

No primeiro artigo (Capítulo III – "Aqueous two-phase systems based on acetonitrile and cabohydrates and their application to the extraction of vanillin"), foram estudados a formação e o uso de sistemas aquosos bifásicos formados por acetonitrila/carboidratos na extração da vanilina. Este artigo está publicado no periódico "Separation and Purification Technology", p. 106-113, 2013. DOI: 10.1016/j.seppur.2012.11.001.

O segundo artigo (Capítulo IV – "Novel Aqueous Two-Phase Systems Composed of Acetonitrile and Polyols: Phase Diagrams and Extractive Performance"), foram estudados a formação e o uso de sistemas aquosos bifásicos formados por acetonitrila/polióis na extração da vanilina. Este artigo foi aceito no periódico "Separation and Purification Technology".

O terceiro artigo (Capítulo V – "Aqueous Two-Phase Systems formed by Biocompatible and Biodegradable Polysaccharides and Acetonitrile") foi estudado a formação e o uso de sistemas aquosos bifásicos formados por acetonitrila/dextrana na extração da vanilina. Este artigo foi submetido ao periódico "Journal of Chromotography B".

O quarto artigo (Capítulo VI – "Poly (vinyl alcohol) as a Novel Constituent to form Aqueous Two-Phase Systems with Acetonitrile: Phase Diagram and Partitioning Experiments"), foi estudado a formação e o uso de sistemas aquosos bifásicos formados por acetonitrila/PVA na extração da vanilina. Este artigo foi submetido ao periódico "Chemical Engineering Research and Design".

Capítulo III

Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin

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Abstract

Aqueous two-phase systems (ATPS) are an important technique for the extraction and purification of biomolecules. In aqueous media, many pairs of solutes can be used to prepare ATPS and, in spite of their interest, scarce attention has been given to the use of mono- and disaccharides. In this context, this work addresses the use of acetonitrile and carbohydrates to prepare aqueous two-phase systems and applies them in the partition of vanillin. The phase diagrams were determined at 298 K and the impact of the carbohydrate structure on the liquid-liquid demixing was evaluated. Besides high purity carbohydrates, commercial food grade sugars were also tested and are shown to be able to form ATPS. Their impurities affect, however, the phase separation and tend to reduce the two-phase region. The studied ATPS were investigated for the extraction of vanillin that favorably partitions towards the acetonitrile-rich phase with partition coefficients higher than 3.0 and recoveries up to 90 % attained in a single step.

Key-words: aqueous two-phase system, acetonitrile, carbohydrate, vanillin, extraction.

1. Introduction

Aqueous two-phase systems (ATPS) are formed when a pair of solutes leads to the formation of two macroscopic liquid phases when dissolved in water above certain concentrations. This phenomenon was first observed by Beijerinck in 1896; however, it was not until 1956 that the potential use of these systems as a separation technique in biotechnology was realized [1]. Nowadays, the scientific literature is prodigal in studies concerning ATPS for the extraction and purification of biomolecules such as antibiotics (e.g. ciprofloxacin [2] and anthocyanins [3]), amino acids (e.g. L-methionine [4]), proteins (e.g. lectin [5]) and enzymes (e.g. lipase [6,7]).

Their versatility, high efficiency, high yield, improved purification factor, selectivity, low-cost and fast mass transfer rates are the main advantages of ATPS [8,9]. Originally, these systems were based on aqueous mixtures of two incompatible polymers such as polyethylene glycol (PEG), dextran and maltodextrin [10-12]. Nevertheless, the high viscosity of the coexisting phases led to the development of systems formed by polymers and inorganic salts such as potassium phosphate, sodium citrate and calcium chloride [13-15]. Other components like organic solvents have also been used, *e.g.* alcohols [16-17], but the application of this type of systems is limited due to the interference of alcohols in the biological activity of several biomolecules. Recently, a new kind of ATPS was reported using ionic liquids (ILs) and inorganic salts [18-20] or saccharides [21,22]. ILs have also been proposed as potential adjuvants in conventional polymer-salt ATPS aiming at tailoring their extraction efficiency for particular added-value compounds [23].

Scarce attention has been devoted to the use of carbohydrates as potential substitutes of inorganic salts and polymers for the formation of ATPS. The use of sugars in ATPS was already addressed by Wang and co-workers [24] in combination with acetonitrile, as well as by Wu and co-workers [21] and Freire and co-workers [22] in combination with ionic liquids.

Acetonitrile, CH_3CN, also known as cyanomethane or methyl cyanide, is a colorless aprotic solvent which is fully miscible in water at temperatures close to room temperature. The acetonitrile molecules do not strongly interact with themselves and tend to form a hydrogen bond network with water molecules [25]. Acetonitrile is an important chemical widely used in industry in the production of perfumes, rubber products, pesticides or pharmaceuticals. It is also usually applied as a mobile phase in high-performance liquid chromatographic or as solvent to extract fatty acids from animal and vegetable oils [26]. The extraction of biomolecules using acetonitrile-water systems was attempted at temperatures below 0°C [27] whereas extractions of Pt(IV), Pd(II) and Rh(III) at room temperature were performed using ATPS composed of

acetonitrile and carbohydrates [28]. No reports were found of extracting vanillin in these systems.

Carbohydrates, with the general formula $(CH_2O)_x$, are a large and diverse group of organic compounds including sugars, cellulose and starch. These molecules are non-charged, biodegradable, nontoxic and a renewable feedstock. They are classified in monosaccharides, oligosaccharides (2-10 linked monosaccharides) and polysaccharides (> 10 linked monosaccharides) [29]. Carbohydrates are polyhydroxy aldehydes or ketones with high affinity for water since several –OH groups, with a dual donor/acceptor character, can be involved in hydrogen bonding, and thus, present an inherent salting-out character (also known as *sugaring-out* effect). Therefore, carbohydrates are potential substitutes to conventional salts used in the formation of ATPS with the advantage of creating more friendly environments to the biomolecules.

This work is focused on the development of novel carbohydrate-acetonitrile-based ATPS and on the evaluation of the carbohydrate structure through the phase separation ability. For that purpose, various carbohydrates were investigated, namely monosaccharides (glucose, mannose, galactose, xylose, arabinose and fructose) and disaccharides (sucrose and mannose). Moreover, commercial sucrose, fructose and glucose commonly used in the food industry were also tested. The ternary phase diagrams were determined at 298 K, and the respective binodal curves, tie-lines and tie-line lengths are reported. Finally, to appraise the extractive potential of the proposed ATPS, the partitioning of a common antioxidant, vanillin was additionally investigated.

2. Materials and methods

2.1. Materials

The ATPS studied in this work were formed by several carbohydrates and acetonitrile. The carbohydrates used were sucrose (> 99.5 wt% pure from Himedia), D-(+)-maltose ($\geq$ 98.0

wt % pure from Sigma), D-(+)-glucose (> 99.5 wt% pure from Scharlau), D-(+)-mannose (> 99.0 wt% pure from Aldrich), D-(+)-galactose (> 98.0 wt% pure from GPR Rectapur), D-(+)-xylose (≥99.0 wt% pure from Carlo Erba), L-(+)-arabinose (> 99.0 wt% pure from BHD Biochemicals) and D-(-)-fructose (> 98.0 wt% pure from Panreac). The acetonitrile, HPLC grade with a purity of 99.9 wt%, was purchased from Sigma. The vanillin (> 99 wt% pure) was supplied by Aldrich. Commercial fructose, sucrose and glucose are of food grade and were obtained in a local supermarket at Aracaju, Sergipe, Brazil. Distilled and deionized water was used in all experiments.

2.2. Phase diagrams and tie-lines

The studied systems comprise acetonitrile and different carbohydrates, and which can be divided into monosaccharides (D-(+)-glucose, D-(+)-mannose, D-(+)-galactose, D-(+)-xylose, L-(+)-arabinose and D-(-)-fructose) and disaccharides (sucrose and D-(+)-maltose). The ternary phase diagrams were determined at 298 (± 1) K and at atmospheric pressure by the cloud point titration method. Stock solutions of the carbohydrates (≈ 40-70 wt%, depending on the carbohydrate solubility saturation in water) and acetonitrile (≈ 80 wt%) were previously prepared and used for the determination of the phase diagrams. Repetitive drop-wise addition of the carbohydrate solution to the aqueous solution of acetonitrile was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultra-pure water until the detection of a monophasic region (clear and limpid solution). All these additions were carried out under continuous stirring.

The tie-lines (TLs) were obtained through a gravimetric method originally described by Merchuck and co-workers [30]. For the calculation of TLs, a mixture at the biphasic region of each ternary system was prepared, vigorously stirred and allowed to reach equilibrium, and phase separation, for a minimum of 18 h, at 298 (± 1) K. After the equilibration step, both top

and bottom phases were separated and weighted using a Metller Toledo AL-204 balance (± 0.0001 g). Each individual TL was determined by the application of the lever arm rule, which describes the relationship between the weight of the top phase and the overall system weight and composition. For that purpose, the binodal curves were correlated using equation 1,

$$Y = A \times \exp\left(BX^{0.5} - CX^{3}\right) \tag{1}$$

where Y and X are the acetonitrile and carbohydrate weight percentages, respectively, and A, B and C are constants parameters obtained by the regression.

The determination of the TLs was then accomplished by solving the following system of four equations (equations 2 to 5) for the four unknown values of Y_T, Y_B, X_T and X_B,

$$Y_T = A \exp\left(BX_T^{0.5} - CX_T^{3}\right) \tag{2}$$

$$Y_B = A \exp\left(BX_B^{0.5} - CX_B^{3}\right) \tag{3}$$

$$Y_T = \left(Y_M / \alpha\right) - \left(\left(1-\alpha\right)/\alpha\right)Y_B \tag{4}$$

$$X_T = \left(X_M / \alpha\right) - \left(\left(1-\alpha\right)/\alpha\right)X_B \tag{5}$$

where the subscripts M, T and B denote, respectively, the initial mixture, and the top and bottom phases. The value of α is the ratio between the mass of the top phase and the total mass of the mixture. The system solution results in the acetonitrile and carbohydrate concentration in the top and bottom phases, and thus, TLs can be simply represented.

The tie-line length (TLL) was determined through the application of equation 6,

$$\text{TLL} = \sqrt{(X_T - X_B)^2 - (Y_T - Y_B)^2} \tag{6}$$

2.3. Partitioning of vanillin

The partitioning systems for vanillin were prepared in graduated glass centrifuge tubes weighing the appropriate amounts of carbohydrate, acetonitrile and an aqueous solution containing vanillin. Vanillin was at a concentration of 0.4 $g.dm^{-3}$ in the initial aqueous solution. After the complete mixing of all components, each system was centrifuged at 3,000 x *g* for 10 minutes to favor the phase separation, and then each tube was placed in a thermostatic bath at 298.15 (± 0.01) K for at least 18 h. The volume of each phase was initially measured. After, both phases were carefully separated for the quantification of vanillin and for the determination of their density, viscosity and pH values.

The density and viscosity of the bottom phase (carbohydrate-rich) were determined in the temperature range from (298.15 to 323.15) K, and at atmospheric pressure, using an automated SVM 300 Anton Paar rotational Stabinger viscosimeter-densimeter. The pH values (± 0.02) of the top and bottom phases were measured at 298 K using a HI 9321 Microprocessor pH meter (HANNA Instruments).

The concentration of vanillin at each aqueous phase was quantified through UV-spectroscopy, using a Varian Cary 50 Bio UV-Vis spectrophotometer, and at a wavelength of 280 nm making use of a calibration curve previously established.

The partition coefficient of vanillin was determined taking into account the concentration of the antioxidant in each phase and according to equation 7,

$$K_{van} = \frac{C_T}{C_B} \tag{7}$$

whereK_{van} is the partition coefficient of vanillin, *C* represents the vanillin concentration, and the subscripts *T* and *B*denote the top (acetonitrile-rich) and bottom (carbohydrate-rich) phases, respectively. The recoveries of vanillin (R_T) for the top phase was evaluated using equation 8,

$$R_T = \frac{C_T}{(C_T + C_B)} \times 100 \tag{8}$$

where *C* and the subscripts *T* and *B*are described above.

3. Results and Discussion

3.1. Phase diagrams and tie-lines

The systems investigated in this work are formed by acetonitrile and a large array of carbohydrates. The molecular structures of the studied carbohydrates are depicted in Figure 1. The experimental phase diagrams for each monosaccharide (D-(+)-glucose, D-(+)-mannose, D-(+)-galactose, D-(+)-xylose, L-(+)-arabinose and D-(-)-fructose), disaccharide (sucrose and D-(+)-maltose) and commercial carbohydrates (glucose, fructose and sucrose), were determined at 298 K and atmospheric pressure. The corresponding phase diagrams are presented in Figures 2 to 4 and allow the analysis of the carbohydrate potential to induce an ATPS. All binodal curves are represented in molality units to avoid disparities in the evaluation of the carbohydrate potential in inducing the liquid-liquid demixing and which could simple result from their distinct molecular weights. The experimental weight fraction data are provided in the Supplementary Information (Tables S1-S4).

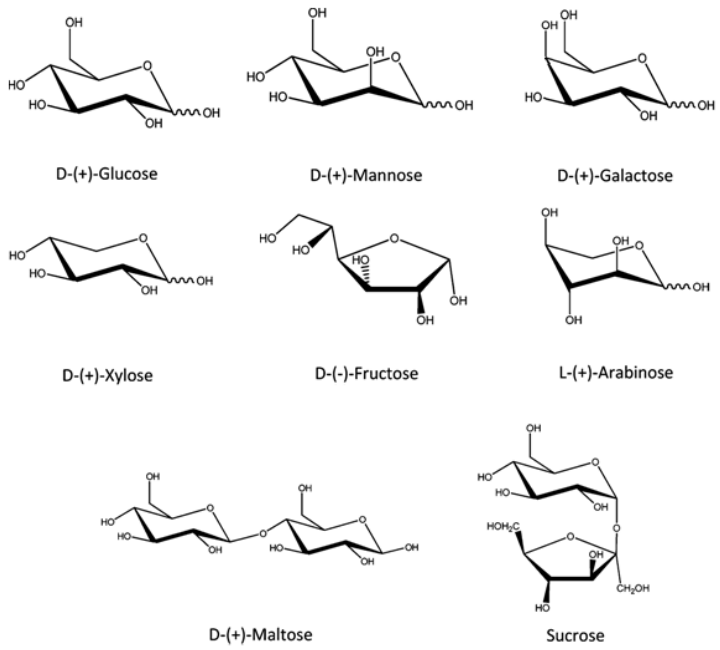

Figure 1.Chemical structure of the monosaccharides and disaccharides studied.

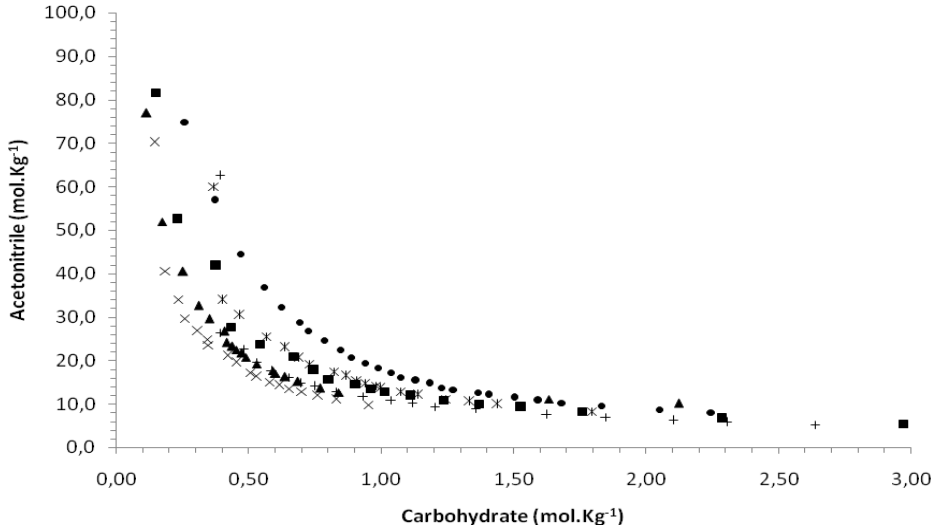

Figure 2. Phase diagrams for the ternary systems composed of acetonitrile + carbohydrate + water at 298 K. ■- D-(-)-Fructose, ▲- D-(+)-Glucose, ●- D-(+)-Xylose, ×- D-(+)-Galactose, ✳- L-(+)-Arabinose, ┼- D-(+)-Mannose.

The addition of a concentrated carbohydrate aqueous solution to acetonitrile leads to phase separation: a top acetonitrile-rich phase and a bottom carbohydrate-rich phase. According to Galema and co-workers [31] the hydration of carbohydrates depends on the ratio between the axial and equatorial hydroxyl groups. Thus, the carbohydrates can be classified into three groups of decreasing hydration: (a) both OH(2) and OH(4) are axial (D-(+)-talose); (b) OH(4) is equatorial and OH(2) is either axial (D-(+)-mannose) or equatorial (D-(+)-glucose); (c) OH(4) is axial and OH(2) is equatorial (D-(+)-galactose). The binodal curves for the systems with acetonitrile and the various monosaccharides, and depicted in Figure 2, show indeed an increasing tendency of phase separation proportional to their hydration ability: D-(+)-xylose < L-(+)-arabinose ≈ D-(-)-fructose < D-(+)-glucose < D-(+)-mannose < D-(+)-galactose.

Aldoses with 5 carbon atoms such as L-(+)-arabinose and D-(+)-xylose are less effective in promoting the ATPS formation due to a lower number of hydroxyl groups and, consequently, a lower hydration ability and less favorable conformation for hydrogen bounding with water.

The comparison between the isomers D-(+)-glucose (an aldose with a 6-sided ring) and D-(-)-fructose (a ketose with a 5-sided ring) suggests that ketoses are less effective in inducing the formation of two aqueous phases. Epimers of aldoses with 6 carbon atoms, which are distinguished by the position of the hydroxyl group at carbon 2, epimers D-(+)-glucose and D-(+)-mannose, have similar abilities to induce ATPS formation. However, the position of the hydroxyl group at carbon 4, as for the epimers D-(+)-glucose and D-(+)-galactose, is relevant and facilitates the phase formation with D-(+)-galactose. Therefore, the orientation of the hydroxyl at carbon 4 plays an important role in the ATPS formation ability.

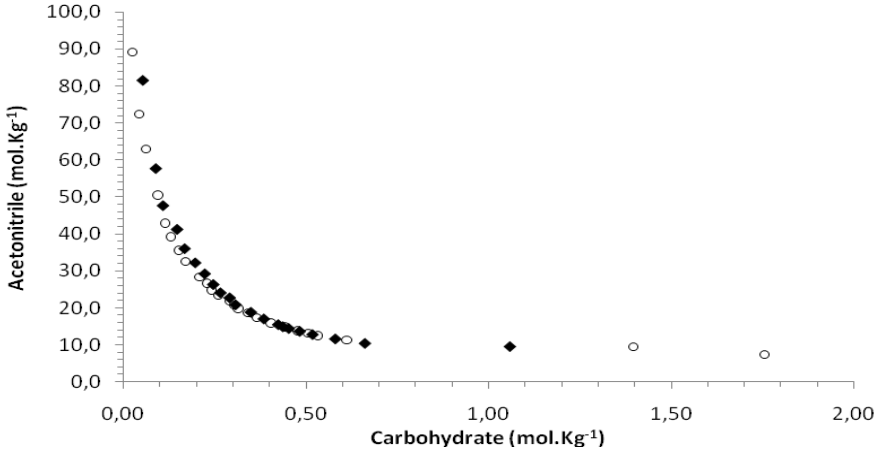

Figure 3. Phase diagrams for the ternary systems composed of acetonitrile + carbohydrate + water at 298 K. ◆- Sucrose; ○- D-(+)-Maltose.

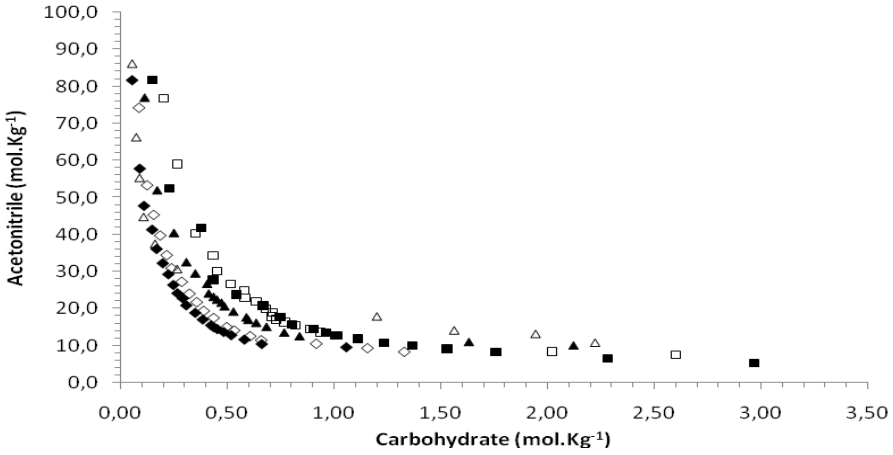

Figure 4. Phase diagrams for the ternary systems composed of acetonitrile + carbohydrate + water at 298 K. ◆- Sucrose; ◇- Commercial sucrose, ■- D-(-)-Fructose; □- Commercial fructose, ▲- D-(+)-Glucose, △- Commercial glucose.

The phase diagrams shown in Figure 3 show the effect of the two disaccharides on the formation of ATPS. Sucrose consists of glucose and fructose linked by a glycosidic bond while maltose is formed by two glucose units. These disaccharides have similar capabilities for ATPS formation in systems formed with acetonitrile at 298 K.

Figure 4 shows the comparison between the high purity and commercial forms of sucrose, glucose and fructose towards the ATPS formation. The binodal curves show a decreasing order in inducing ATPS according to: sucrose > commercial sucrose > glucose > fructose ≈ commercial fructose > commercial glucose. The use of commercial carbohydrates leads to a decrease of the biphasic region envelope which may be a result of a low purity level and to the presence of impurities. The difference was more pronounced when using commercial glucose (corn syrup, glucose) due to the presence of other sugars such as isomaltose, maltose and maltotriose and as already pointed out by Pontoh and Low [32].

All the binodal curves were fitted using equation 1. The regression coefficients (R^2) and the fitted parameters *A*, *B* and *C*, estimated by least-squares regression, are reported in Table 1. Figure 5 presents three examples of the correlation of the data for the systems composed of acetonitrile + carbohydrate (D-(-)-fructose, sucrose or D-(+)-galactose) + water.

Table 1. Adjusted parameters obtained from the regression of equation 1 for the ternary systems composed of acetonitrile + carbohydrate + water at 298 K and atmospheric pressure.

Carbohydrate	Regression Parameters			
	A	*B*	*C*	R^2
Sucrose	114.5 ± 2.2	-0.280 ± 0.008	$2.8 \times 10^{-5} \pm 0.5 \times 10^{-5}$	0.9964
D-(+)-Maltose	102.0 ± 1.3	-0.245 ± 0.006	$3.8 \times 10^{-5} \pm 0.6 \times 10^{-5}$	0.9962
D-(+)-Glucose	122.6 ± 2.7	-0.332 ± 0.011	$4.4 \times 10^{-5} \pm 1.3 \times 10^{-5}$	0.9962
D-(+)-Mannose	127.6 ± 5.8	-0.356 ± 0.014	$2.8 \times 10^{-16} \pm 1.7 \times 10^{-6}$	0.9954
D-(+)-Galactose	123.3 ± 3.0	-0.375 ± 0.011	$1.1 \times 10^{-5} \pm 9.0 \times 10^{-6}$	0.9978
D-(-)-Fructose	134.6 ± 2.2	-0.342 ± 0.006	$7.1 \times 10^{-16} \pm 1.1 \times 10^{-6}$	0.9978
D-(+)-Xylose	177.7 ± 6.2	-0.394 ± 0.012	$3.4 \times 10^{-6} \pm 3.2 \times 10^{-6}$	0.9960
L-(+)-Arabinose	151.6 ± 5.6	-0.393 ± 0.006	$4.1 \times 10^{-7} \pm 4.1 \times 10^{-6}$	0.9965

The results for the remaining systems are presented in Supplementary Information (Figure S1-S4). To complete the phase diagrams, several TLs and respective TLLs were further

calculated and their values are reported in Table 2. Some examples of the TLs representation are shown in Figure 5.

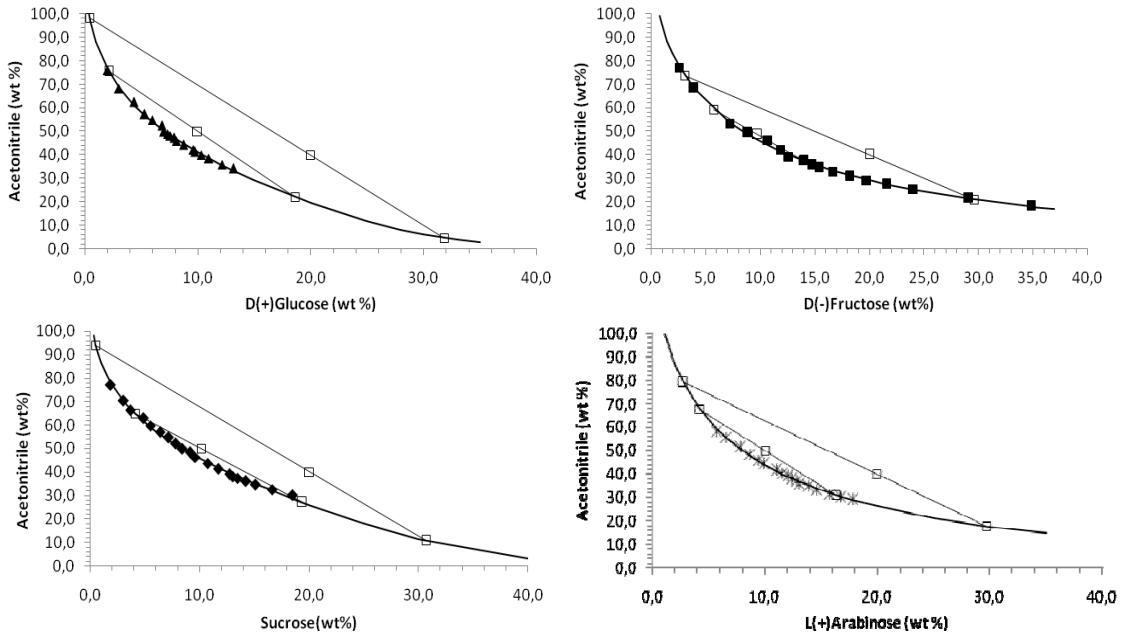

Figure 5. Phase diagrams for the ternary systems composed of acetonitrile + carbohydrate at 298 K (■- D-(+)-Fructose, ▲- D-(+)-Glucose, ∗- D-(+)-Arabinose and ◆- Sucrose), □- TL data, (—) binodal adjusted data through equation 1.

Table 2. Weight fraction compositions (TLs) at the top (*T*) and bottom (*B*) phases, initial mixture composition (*M*), and respective TLLs for the several systems composed of acetonitrile (*Y*) and carbohydrate (*X*) at 298 K and atmospheric pressure.

Carbohydrate	100 x weight fraction / wt %						
	Y_M	X_M	Y_T	X_T	Y_B	X_B	TLL
Sucrose	39.97±0.03	20.07±0.05	93.91±0.01	0.50±1.99	10.71±0.09	30.68±0.03	88.51
	49.94±0.02	10.14±0.10	64.86±0.02	4.09±0.24	27.15±0.04	19.38±0.05	40.69
D-(+)-Maltose	40.05±0.03	19.96±0.05	92.15±0.01	0.17±5.84	6.59±0.15	32.66±0.03	91.52
	49.95±0.02	10.02±0.10	65.24±0.02	3.31±0.30	18.13±0.06	23.97±0.04	51.44
D-(+)-Glucose	39.98±0.03	19.99±0.05	98.37±0.01	0.44±2.26	4.56±0.03	31.86±0.22	58.98
	49.98±0.02	10.00±0.10	75.42±0.02	2.14±0.47	22.09±0.05	18.61±0.05	55.81
D-(+)-Mannose	40.04±0.03	20.00±0.05	77.14±0.02	1.99±0.50	17.62±0.06	30.88±0.03	66.15
	49.92±0.02	9.98±0.10	69.29±0.02	2.93±0.34	28.34±0.04	17.84±0.06	43.58
D-(+)-Galactose	49.99±0.02	10.01±0.10	73.92±0.02	1.87±0.54	21.46±0.01	19.73±0.05	55.42
	45.00±0.02	8.01±0.12	32.02±0.03	12.54±0.08	54.21±0.02	4.80±0.21	23.50
D-(-)-Fructose	40.03±0.03	20.03±0.05	73.66±0.02	3.11±0.32	20.96±0.03	29.62±0.05	58.98
	48.65±0.02	9.76±0.10	59.28±0.02	5.76±0.17	37.61±0.03	13.92±0.07	23.15
D-(+)-Xylose	39.95±0.03	20.05±0.05	79.31±0.01	4.20±0.24	20.77±0.01	27.78±0.04	63.11
	49.99±0.02	10.02±0.10	50.06±0.02	10.26±0.10	50.06±0.02	10.26±0.10	0.00
L-(+)-Arabinose	39.97±0.03	19.91±0.05	79.15±0.02	2.73±0.37	17.59±0.01	29.72±0.03	67.21
	50.01±0.02	10.00±0.02	67.51±0.02	4.24±0.24	30.97±0.03	16.28±0.06	38.47

The application of ATPS in industrial processes for biomolecules extraction and purification also depends on their physical properties. Particularly, large differences in the densities of both phases favor the phase separation whereas low viscosities increase the mass transfer coefficients. Hence, the characterization of the densities and viscosities of the phases are important issues when envisaging the process scale-up. In this sense, the densities and viscosities for the sugar-rich phase were here determined. It should be remarked that acetonitrile, at 298.15 K, presents a density of 0.7766 g.cm^{-3} and a viscosity of 0.3369 mPa.s [33]. These values are below the values of pure water at the same temperature (0.9991 g.cm^{-3} and 1.0 mPa.s) [34] and thus the properties of the acetonitrile-rich phase were not determined due to a lack of a proper equipment to measure densities and viscosities within this range. Furthermore, the sugar composition (the more dense and viscous compound) in the acetonitrile-rich phase is always below 7 wt% (Supplementary Information - Tables S1-S4).

Table 3. Experimental value of densities (ρ) and viscosities (η) of the acarbohydrate-rich phase at 298.15 K and 323.15 K.

		ρ / (g.cm^{-3})		η/ (mPa.s^{-1})	
Carbohydrate	System	298.15 K	323.15 K	298.15 K	323.15 K
Sucrose	A	1.0984	1.0808	3.5606	1.8145
	B	1.0535	1.0343	2.2117	1.1956
D-(+)-Maltose	A	1.0968	1.0793	3.5977	1.8345
	B	1.0678	1.0495	2.5711	1.3809
D-(+)-Glucose	A	1.0991	1.0825	3.2582	1.6831
	B	1.0358	1.0173	1.8355	1.0510
D-(+)-Mannose	A	1.0990	1.0813	3.0513	1.6009
	B	1.0314	1.0121	1.7401	0.9794
D-(+)-Galactose	B	1.0429	1.0243	1.8812	1.0643
	C	1.0004	0,9797	1.3995	0.8233
D-(+)-Xylose	A	1.0738	1.0550	2.5039	1.3760
	B	1.0091	0.9872	1.4637	0.8606
L-(+)-Arabinose	A	1.0919	1.0729	2.6091	1.4102
	B	1.0288	1.0083	1.6412	0.9372
D-(-)-Frutose	A	1.0977	1.0782	2.8406	1.4917
	B	1.0228	1.0021	1.6096	0,9216

A: 40 wt% acetonitrile + 20 wt% carbohydrate; B: 50 wt% acetonitrile + 10 wt% carbohydrate); C (45 wt% acetonitrile + 8 wt% carbohydrate).

For the carbohydrate-rich phase the densities range from 1.0004 $g.cm^{-3}$ (galactose) to 1.0991 $g.cm^{-3}$ (glucose) whereas the viscosities are between 1.3995 mPa.s (galactose) and 3.5977 mPa.s (maltose). The densities and viscosities at 298.15 K and 323.15 K for the carbohydrate-rich phase of different systems are presented in Table 3. These values are significantly lower than the viscosities obtained for ATPS constituted by polymers such as polypropylene glycol (polymer-rich phase: 18.1 – 64.7 mPa.s and Na_2SO_4-rich phase: 1.91 – 3.73 mPa.s [35]) or ionic liquids (ionic-liquid-rich phase: 8.0 – 1.03 mPa.s [8]). The low viscosity of acetonitrile-carbohydrate ATPS favor the mass transfer on extraction processes as well as the phases handling at industrial scale.

3.2. Partitioning of vanillin

The application of the investigated systems as alternative extractive techniques was studied using the partitioning of vanillin, a widely used flavoring agent, [36] as model system. For each system, two different compositions were investigated: 20 wt% carbohydrate + 40 wt% acetonitrile and 10 wt% carbohydrate + 50 wt% acetonitrile. The pH values of both phases of each ATPS are presented in Table 4. These values range between 5.48 and 7.06. Vanillin is present as a neutral molecule at these conditions [37]. The influence of the pH in the chemical structure of vanillin is shown in Figure S5 in Supporting Information.

Table 4.pHvalues of the acetonitrile (top) and carbohydrate (bottom) rich phases at 298 K.

	System A		System B	
Carbohydrate	Top phase	Bottom Phase	Top phase	Bottom Phase
Sucrose	7.06	6.35	6.97	6.76
D-(+)-Maltose	6.55	5.97	6.92	6.64
D-(+)-Glucose	6.84	5.69	6.98	6.28
D-(+)-Mannose	7.00	6.28	6.89	6.41
D-(+)-Galactose	-	-	6.81	6.09
D-(+)-Xylose	6.64	5.83	5.96	5.95
L-(+)-Arabinose	6.78	5.73	6.34	6.14
D-(-)-Fructose	6.36	5.80	6.34	5.48

A: 40 wt% acetonitrile + 20 wt% carbohydrate; B: 50 wt% acetonitrile + 10 wt% carbohydrate.

For all systems the partition coefficients of vanillin are higher than 1 and demonstrate the preferential affinity of vanillin towards the acetonitrile-rich phase (Figure 6). This preferential migration is in good agreement with the octanol-water partition coefficient of vanillin (logK_{ow} = 1.19 [38]) which indicates the preferential affinity of vanillin for more hydrophobic phases. Acetonitrile (logK_{ow} = -0.17) is indeed more hydrophobic than carbohydrates (-2.30 < logK_{ow}< -4.70) and support the trend observed (http://chemicalspider.com).

The effect of system composition, namely the TLL, on the extraction ability was studied by changing the point of the initial mixture, acetonitrile-carbohydrate, from 40-20 wt% to 50-10 wt%. The composition of each phase is described in Table 2. A large decrease in the partition coefficient was observed with the system composed of mannose (K_{van} = (9.67 ± 0.04) and (3.66 ± 0.01)) with the decrease of the TLL. An opposite pattern was verified with the system constituted by xylose and for which the partition coefficient increases from (6.95 ± 0.01) to (8.74 ± 0.03) with a decrease in the TLL. It should be remarked that Gu and Zhang [27] studied the partitioning of various biomolecules in the system composed of acetonitrile and water at sub-zero temperatures (-10ºC). Most compounds preferentially partitioned for the water-rich phase [27] contrarily to what was observed here.

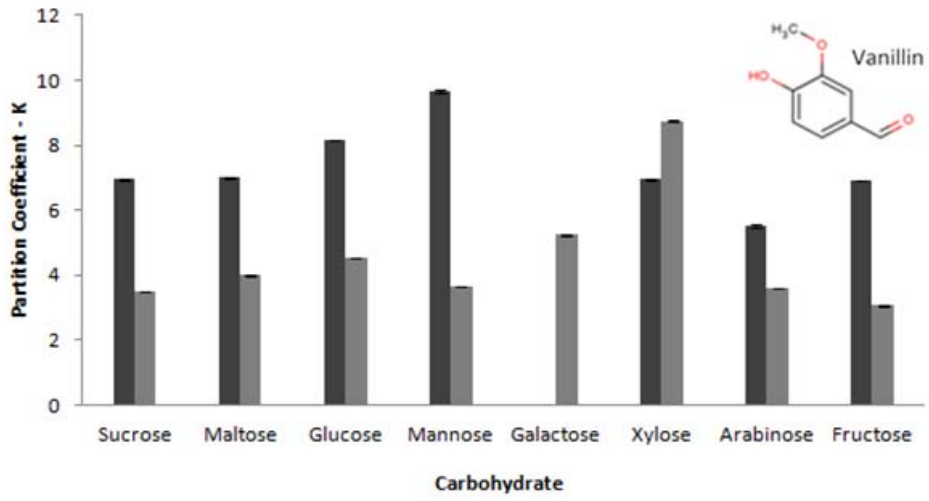

Figure 6. Partition coefficient of vanillin between the acetonitrile- and the carbohydrate-rich phase at 298 K. ■- 40-20 wt% acetonitrile-carbohydrate and ■50-10 wt% acetonitrile-carbohydrate.

The K_{van} rank at different mixtures compositions is similar to the order of formation of ATPS previously noted. For instance, for the mixture composition constituted by 20 wt% of carbohydrate and 40 wt% of acetonitrile, the order of partition coefficients is,

Aldoses with 6C: D-(+)-glucose < D-(+)-mannose

Aldoses with 5C: L-(+)-arabinose < D-(+)-xylose

Monossacharides: Aldoses with 5C ≈ D-(-)-fructose (Ketose) < Aldose with 6C.

Dissacharides: sucrose ≈ D-(+)-maltose

In addition, for the mixture point composed of 10 wt% of carbohydrate and 50 wt% of acetonitrile, the partition coefficient values increase according to,

Aldoses with 6C: D-(+)-mannose < D-(+)-glucose < D-(+)-galactose

Aldoses with 5C: L-(+)-arabinose < D-(+)-xylose

Monossacharides: Aldoses with 5C ≈ D-(-)-fructose (Ketose) < Aldose with 6C

Dissacharides: sucrose < D-(+)-maltose

All the results indicate that the hydration capacity of the carbohydrate leads to an exclusion effect of the biomolecule towards the acetonitrile-rich phase and confirms the *sugaring-out* effect reported by other authors [24, 39]. In addition, for aldoses with 6 carbon atoms, the order is inversely proportional to the dielectric constant of each carbohydrate: D-(+)-mannose (4.25) ≈ D-(+)-glucose (4.27) > D-(+)-galactose (3.28) [40].

Based on the quantification of vanillin and on the volume of each phase, the recoveries of vanillin at the acetonitrile-rich phase were also determined and are presented in Figure 7. As observed with the partition coefficients, the recoveries indicate a preferential migration of vanillin to the acetonitrile-rich phase. The recovery of vanillin ranges between (73.01 ± 0.06)%, with the system formed by acetonitrile and galactose, and (93.42 ± 0.06)%, with the

system constituted by acetonitrile and glucose. In general, high recovery efficiencies are attained in a single step.

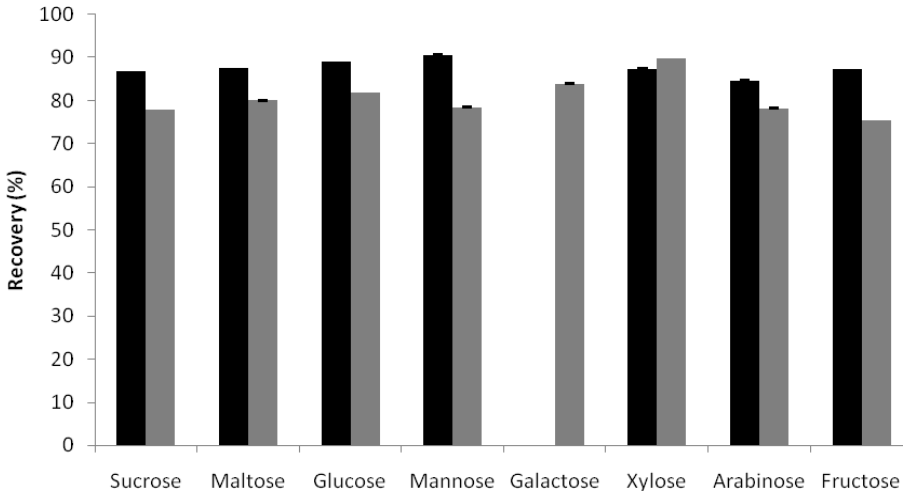

Figure 7. Recovery of vanillin in the top phase (acetonitrile-rich phase) at 298 K. ■- 40-20 wt% acetonitrile-carbohydrate and ■50-10 wt% acetonitrile-carbohydrate.

4. Conclusions

This study reports novel ATPS formed by acetonitrile and a large array of carbohydrates (monosaccharides and disaccharides). The ternary phase diagrams, tie-lines and tie-line lengths were determined at 298 K and at atmospheric pressure. Based on the phase diagrams behavior it was shown that the ATPS formation is a result of the hydration capacity of each sugar. Besides high purity carbohydrates, commercial food grade sugars were also investigated and shown to be less able to form ATPS.

To explore the applicability of the investigated systems, the partitioning of vanillin was studied in several ATPS at two different mixture compositions. In all the extraction essays vanillin preferentially migrates for the acetonitrile-rich phase. The trend on the partition coefficients is dependent on the hydration capacity of each carbohydrate. The recovery of vanillin in the acetonitrile-rich phase ranged between 73 and 95% in a single step.

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Supporting Information

Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin

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Table S1. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Commercial Sugar (2) + Water (3) at 298 K

Glucose		Fructose		Sucrose	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
77.955	0.992	75.929	3.565	75.312	2.930
73.117	1.332	70.796	4.587	68.674	4.123
69.413	1.601	62.311	5.985	65.089	5.089
64.753	1.927	58.516	7.274	62.059	6.017
60.610	2.852	55.238	7.578	58.653	6.925
55.732	4.584	52.229	8.510	56.018	7.599
42.308	17.803	50.546	9.507	52.900	8.960
35.050	25.957	48.522	9.519	49.785	9.972
30.650	28.612	47.248	10.300	47.305	10.908
36.676	21.988	45.014	10.963	44.484	11.790
		43.599	11.431	41.952	12.999
		42.194	11.313	38.318	14.561
		41.174	11.636	36.867	15.419
		40.065	12.199	34.267	17.212
		38.777	12.870	32.261	18.409
		37.135	13.868	30.423	23.871
		35.726	14.458	27.963	28.338
		34.102	15.392	25.781	31.236
		25.484	26.725		
		23.501	31.916		

Table S2. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Aldose with 6 carbon atoms (2) + Water (3) at 298 K

D-(+)-Glucose		D-(+)-Mannose		D-(+)-Galactose	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
75.952	2.037	51.956	6.583	62.487	3.269
68.083	3.043	48.270	7.973	58.263	4.061
62.418	4.352	44.567	8.729	54.868	4.461
57.228	5.301	41.834	9.598	52.307	5.282
54.799	5.973	39.811	10.516	50.454	5.816
52.322	6.851	37.683	11.150	49.167	5.938
49.768	6.963	36.625	11.888	46.324	7.030
48.810	7.322	34.530	13.026	44.471	7.564
47.971	7.567	32.636	14.398	41.386	8.350
47.079	7.885	30.837	15.761	40.031	8.749
45.882	8.105	29.327	16.774	38.243	9.449
44.101	8.732	27.856	17.821	36.978	9.987
42.067	9.635	26.561	19.642	35.764	10.520
41.117	9.763	23.620	22.664	34.582	11.242
39.985	10.301	22.110	24.985	32.843	12.081
38.321	10.997	20.200	27.473	31.061	13.009
35.813	12.171	19.129	29.368	28.840	14.641
34.133	13.153	17.564	32.218		

Table S3. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Aldose with 5 carbon atoms (2) + Water (3) at 298 K.

L-(+)-Arabinose		**D-(+)-Xylose**	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**
58.229	5.681	69.999	5.309
55.546	6.552	64.610	6.597
51.164	7.855	60.156	7.746
48.547	8.696	56.955	8.573
45.838	9.363	52.365	9.804
44.123	9.857	50.217	10.544
41.656	10.996	47.913	11.279
40.337	11.509	45.866	11.742
38.932	12.008	44.228	12.376
37.758	12.366	42.857	12.935
36.755	12.812	41.296	13.485
35.885	13.001	39.737	13.895
34.467	13.873	38.693	14.526
33.191	14.591	36.147	15.548
31.389	15.737	35.281	16.056
30.270	16.695	33.211	17.442
28.908	17.774	31.921	18.413
		30.689	19.317
		29.593	20.171
		26.277	23.548

Table S4. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Ketose – D-(-)-Fructose or Disaccharides (2) + Water (3) at 298 K

D-(-)-Fructose		D-(+)-Maltose		Sucrose	
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
77.014	2.614	78.541	0.935	77.006	1.872
68.306	3.962	74.816	1.553	70.338	3.028
53.262	7.224	72.090	2.141	66.216	3.663
49.396	8.900	67.466	3.193	62.917	4.866
46.157	10.736	63.792	3.824	59.724	5.505
42.142	11.853	61.734	4.336	57.000	6.383
39.186	12.645	59.387	5.006	54.619	7.155
37.405	14.025	57.168	5.570	52.039	7.837
35.601	14.756	53.783	6.752	49.833	8.404
34.451	15.428	52.310	7.338	48.374	9.123
32.813	16.674	50.501	7.708	46.237	9.576
30.814	18.200	49.086	8.214	43.735	10.733
29.103	19.775	47.287	9.155	41.303	11.693
27.463	21.597	46.097	9.455	39.031	12.733
25.323	24.062	44.889	9.776	38.148	13.060
21.494	29.154	43.329	10.525	37.394	13.473
18.159	34.844	41.764	11.187	36.138	14.234
		39.588	12.195	34.652	15.108
		37.891	13.228	32.513	16.617
		35.132	14.818	30.174	18.503
		34.018	15.436		

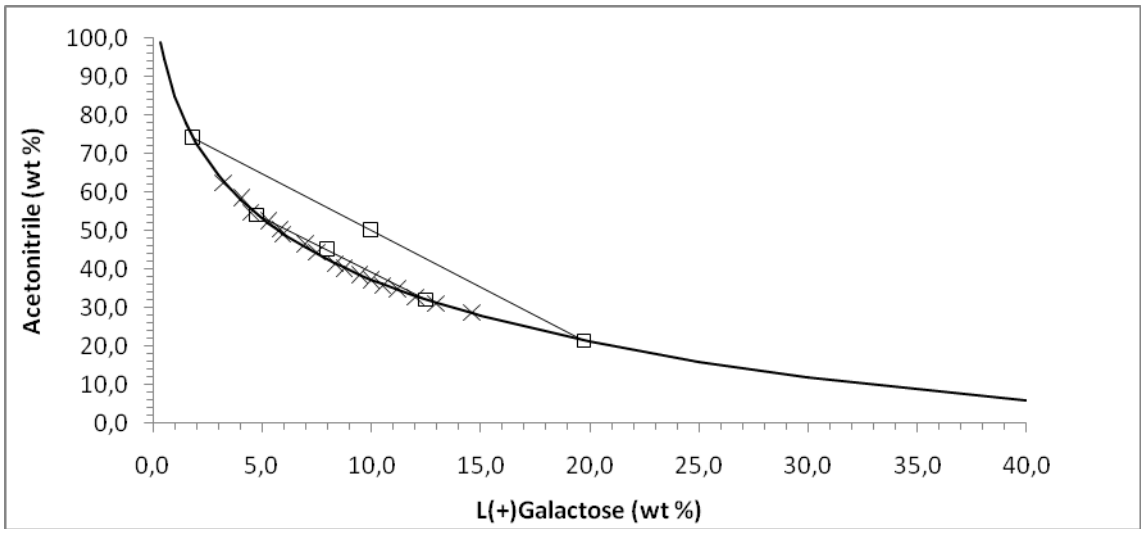

Figure S1. Phase diagram for the ternary system composed of acetonitrile + L(+)-Galactose at 298 K (×), □- TL data, (—) binodal adjusted data through equation 1.

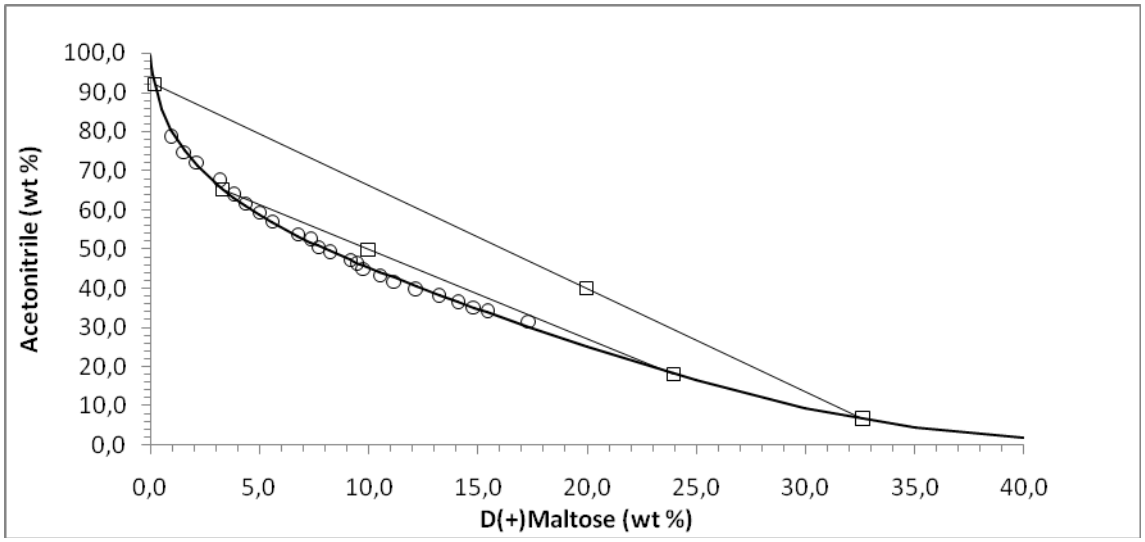

Figure S2. Phase diagram for the ternary system composed of acetonitrile + D(+)-Maltose at 298 K (○), □- TL data, (—) binodal adjusted data through equation 1.

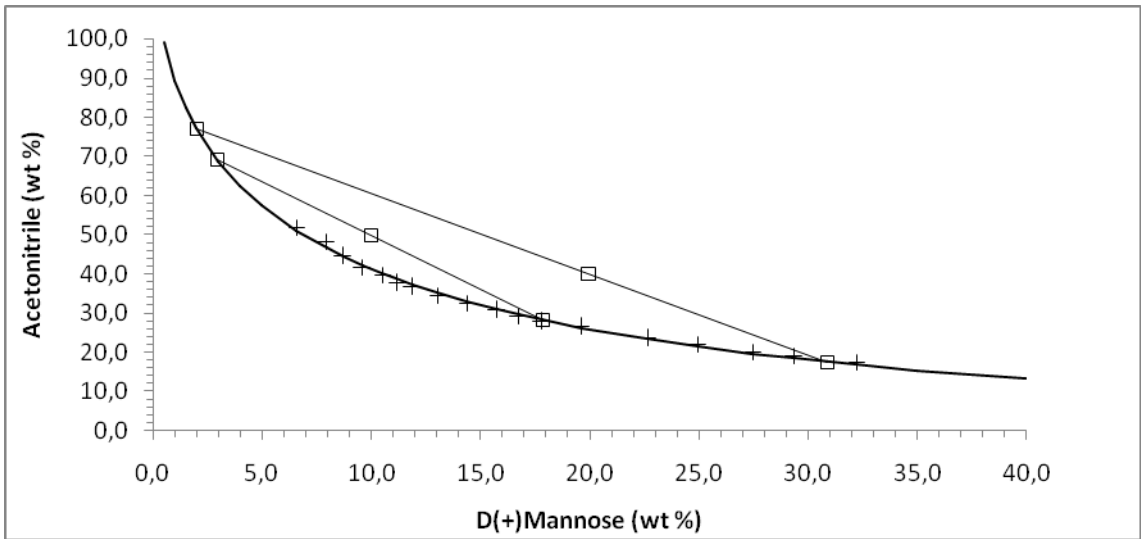

Figure S3. Phase diagram for the ternary system composed of acetonitrile + D(+)-Mannose at 298 K (+), □- TL data, (—) binodal adjusted data through equation 1.

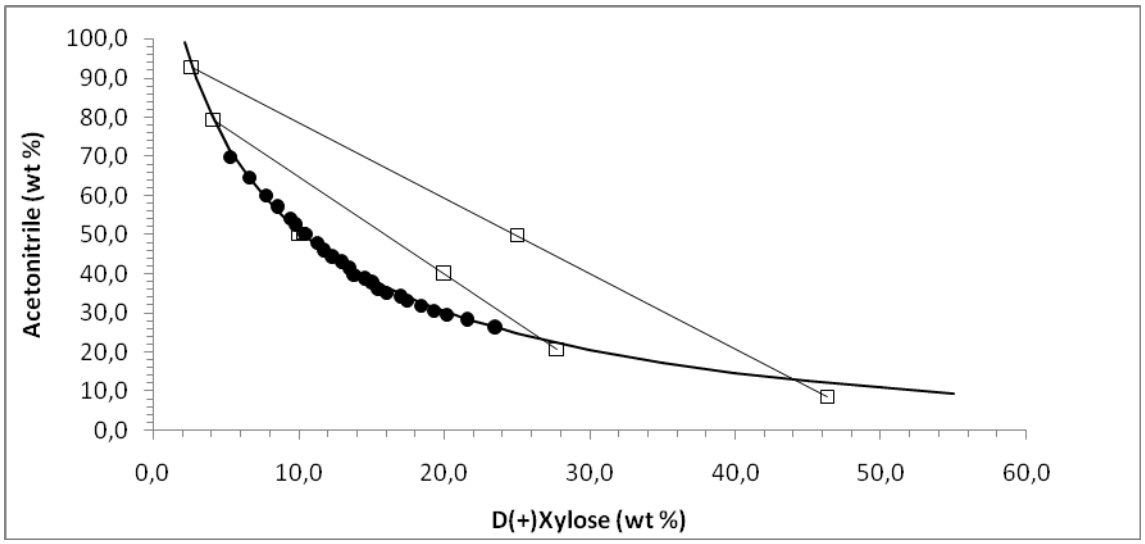

Figure S4. Phase diagram for the ternary systems composed of acetonitrile + D(+)-Xylose at 298 K (●), □- TL data, (—) binodal adjusted data through equation 1.

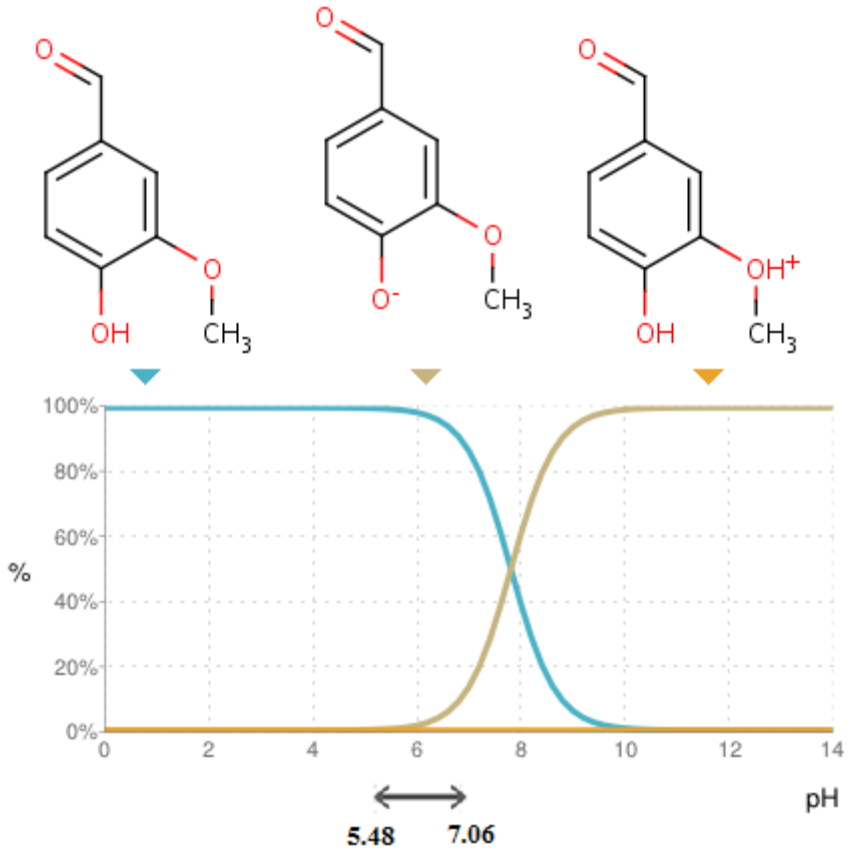

Figure S5. Chemical structure of vanillin at different pH values. This content was adapted from the Chemspider chemical database (http://www.chemspider.com/).

Capítulo IV

Novel Aqueous Two-Phase Systems Composed of Acetonitrile and Polyols: Phase Diagrams and Extractive Performance

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Abstract

A large number of works has been devoted to the study of alternative constituents to form aqueous two-phase systems (ATPS); however, scarce attention has been given to polyols as two-phase forming components. This work addresses the potential use of polyols (glycerol, erythritol, xylitol, sorbitol and maltitol) to create ATPS in presence of acetonitrile. Novel ternary phase diagrams were determined at 298 K and the impact of the polyol chemical structure through the liquid-liquid demixing was evaluated. It is shown that the ability for phase separation largely depends on the number of hydroxyl groups present in each polyol. Polyols with a higher number of hydroxyl groups are better phase separating agents increasing thus the ability for two-phase formation. The partitioning of a model biomolecule, vanillin, was also assessed to ascertain on these systems applicability as alternative extractive techniques. In all systems, vanillin preferentially migrates to the acetonitrile-rich phase (more hydrophobic layer) with recoveries higher than 8%, except to glycerol. This pattern was confirmed by solid-liquid solubility studies of vanillin in aqueous solutions containing diverse polyols supporting thus their phase separating ability. These novel systems can be used as alternative ATPS for the extraction and recovery of added-value biomolecules.

Keywords: aqueous two-phase system, acetonitrile, polyol, recovery, vanillin.

1. Introduction

The extraction of biomolecules usually requires the use of several and combined processes, such as solvent and ultrasound assisted extraction [1], microwave assisted extraction [2] and supercritical fluid extraction [3], followed by purification steps involving precipitation, centrifugation, filtration, dialysis or chromatography [4]. This two-step process makes the downstream processing responsible for 50-80% of the final

cost of biotechnological-based products [5]. In this sense, aqueous two-phase systems (ATPS) can be foreseen as a possible alternative that is easy to scale up, presents low cost and leads to a high product purity as well as to a high yield, while maintaining the biological activity of the molecules due to their water-rich environment [6,7].

ATPS have been studied in the recovery and purification of diverse biomolecules, namely proteins [8], enzymes [9,10], nucleic acids [11], flavor compounds (vanillin [12]; 6-pentyl-α-pyrone [7]), antioxidants (ascorbic acid [13]), alkaloids [14], and antibiotics (tetracycline [15-17]).

Since the first observation (by Beijerinck in 1886) demonstrating that ATPS can be formed by mixtures of agar and starch or gelatin in aqueous media many other pairs of phase-forming constituents have been explored [18]. In the past decades, ATPS have shown capable to be created by two polymers (dextran/polyethylene glycol [19]) or by a polymer-salt combination (polypropylene glycol/$(NH_4)_2SO_4$, $MgSO_4$, KCl or KCH_3CO_2 [20]), and which can be labeled as "traditional systems". In recent times, other compounds have been successfully used in the replacement of the traditional constituents, such as the pairs alcohol - salt [13], ionic liquid - salt [21-23], ionic liquid - polymer [24,25], and ionic liquid - carbohydrate [26]. Recently, pioneering ATPS based on acetonitrile and sugars have also been reported [27-29].

Acetonitrile is an organic solvent widely used by industry in the production of perfumes, rubber products, pesticides or pharmaceuticals [30] or as a mobile phase in reverse phase high performance liquid chromatography (HPLC) in separation and purification processes [31,32]. Acetonitrile is also a by-product from the manufacture of acrylonitrile [33]. Acetonitrile, CH_3CN, also known as cyanomethane or methyl cyanide, is one aprotic solvent miscible with water in the whole composition range,

similar to the dimethyl sulfoxide or acetone behavior, and its molecules do not strongly interact with themselves leaving a hydrogen bond network formed by water [34].

Polyols, usually known as sugar alcohols, are a hydrogenated form of carbohydrates and whose carbonyl group has been reduced to a primary or secondary hydroxyl group [35]. Polyols may mimic the structure of water and maintain an artificial sphere of hydration around macromolecules [36]. Due to their properties, polyols are widely used in pharmaceuticals, confectionery products, chewing gums, mixed juice [37] and as substituent of sucrose in foodstuffs [38].

Taking into account the continuous investigation on novel phase-forming components to create ATPS, this work addresses innovative ATPS formed by polyols of different chemical structure and acetonitrile. The corresponding phase diagrams, tie-lines and tie-line lengths were determined at 298 K. Moreover, to investigate the extractive performance of these novel systems, they were used in the partitioning of vanillin (used here as a standard biomolecule). Vanillin (3-methoxy-4-hydroxybenzaldehyde) is the major component of natural vanilla and it is widely used as a flavoring material in confectionery, food products, beverages, perfumes and in pharmaceutical preparations [39]. Currently, vanillin is naturally produced via a multistep curing process of the green vanilla pods of the orchid plant (10%). However, the majority of vanillin (90%) is actually synthetically produced [40].

2. Material and Methods

2.1. Materials

The ATPS studied in this work were formed by polyols and acetonitrile. All reagents were purchased from Sigma-Aldrich: glycerol (> 99.5 wt% pure), erythritol (≥ 99 wt % pure), xylitol (> 99 wt% pure), sorbitol (> 98 wt% pure), maltitol (> 98 wt%

pure), acetonitrile (HPLC grade with a purity of 99.9 wt%) and vanillin (> 99 wt% pure). Distilled and deionized water was used in all experiments.

2.2. Phase diagrams and tie-lines

The ternary phase diagrams were determined for each polyol and acetonitrile at 298 (± 1) K and atmospheric pressure by the cloud point titration method. Stock solutions of each polyol (≈30-80 wt%, depending on the polyol solubility saturation in water) and acetonitrile (≈80-100 wt%) were previously prepared and used for the determination of the phase diagrams. Repetitive drop-wise addition of the polyols solution to the aqueous solution of acetonitrile was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultra-pure water until the detection of a monophasic region (clear and limpid solution). These additions were carried out under continuous stirring and the saturation curves were determined gravimetrically within ± 10^{-4} g.

The tie-lines (TLs) were obtained through a gravimetric method originally described by Merchuck and co-workers [41]. A mixture at the biphasic region of each ternary system was prepared, vigorously stirred, and allowed to reach equilibrium and phase separation, for a minimum of 18 h at 298 (± 1) K. After the equilibration step, the top and bottom phases were carefully separated and weighted within ± 10^{-4} g. Each individual TL was determined by the application of the lever-arm rule, which describes the relationship between the weight of the top phase and the overall system weight and composition. For that purpose, the binodal curves were correlated using equation 1,

$$[\mathrm{ACN}] = A\exp\{(B\times[\mathrm{Polyol}]^{0.5} - (C\times[\mathrm{Polyol}]^{3})\} \quad (1)$$

where [ACN] and [Polyol] are the acetonitrile and polyol weight fraction percentages, respectively, and *A*, *B* and *C* are constants parameters obtained by the regression of the experimental binodal data.

The determination of the TLs was then accomplished by solving the following system of four equations (equations 2 to 5) for the four unknown values of $[ACN]_T$, $[ACN]_B$, $[Polyol]_T$ and $[Polyol]_B$,

$$[ACN]_T = A\exp\{(B\times[Polyol]_T^{0.5} - (C\times[Polyol]_T^3)\} \quad (2)$$

$$[ACN]_B = A\exp\{(B\times[Polyol]_B^{0.5} - (C\times[Polyol]_B^3)\} \quad (3)$$

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

$$[Polyol]_T = ([Polyol]_M/\alpha) - ((1-\alpha)/\alpha)[Polyol]_B \quad (5)$$

where the subscripts M, T and B denote, respectively, the initial mixture, and the top and bottom phases. The value of α is the ratio between the mass of the top phase and the total weight of the mixture. The system solution results in the acetonitrile and polyol concentration in the top and bottom phases, and thus, TLs can be simply represented.

The respective tie-line lengths (TLLs) were determined through the application of equation 6,

$$TLL = \sqrt{([Polyol]_T - [Polyol]_B)^2 - ([ACN]_T - [ACN]_B)^2} \quad (6)$$

2.3. Partitioning of vanillin

The partitioning liquid-liquid systems for vanillin were prepared in graduated glass centrifuge tubes weighing the appropriate amounts of each polyol, acetonitrile and an aqueous solution containing vanillin. Vanillin was at 0.4 $g.dm^{-3}$ in the initial aqueous solution. After the complete mixing of all components for a given mixture composition, each system was centrifuged at 2,000 x *g* for 10 min, and then each tube was placed in a thermostatic bath at 298.15 (± 0.01) K for at least 18 h. After the two phases become

clear and transparent, and the interface was well defined, the bottom phase was carefully withdrawn using a long needle syringe and a pipette for removing the top phase [42]. The volume of each phase was initially measured and both phases were further separated for the quantification of vanillin and for the determination of their pH values. At least three independent replicates were made and the average partition coefficients and associated standard deviations were therefore determined.

The pH values (± 0.02) of the top and bottom phases were measured at 298 K using a DIGIMED DM-20 pH meter.

The concentration of vanillin at each aqueous phase was quantified through UV-spectroscopy, using a Varian Cary 50 Bio UV-Vis spectrophotometer, and at a wavelength of 280 nm using a calibration curve previously established [12].

The partition coefficient of vanillin was determined taking into account the concentration of the antioxidant in each phase and according to,

$$K_{\text{van}} = \frac{C_{\text{T}}}{C_{\text{B}}} \tag{7}$$

where K_{van} is the partition coefficient of vanillin, C represents the vanillin concentration, and the subscripts T and B denote the top (acetonitrile-rich) and bottom (polyol-rich) phases, respectively.

The recovery of vanillin (R_T) in the top phase was evaluated using equation 8,

$$R_{\text{T}} = \frac{100}{1 + \frac{1}{K_{\text{van}} \times R_v}} \tag{8}$$

where R_v is the ratio between the volumes of the top (V_T) and bottom (V_B) phase.

2.4. Determination of vanillin solubility

Vanillin was added in excess amounts to aqueous solutions of xylitol and sorbitol (20, 15, 10 and 5 wt %) and equilibrated in an air oven under constant agitation using an Eppendorf Thermomixer Comfort equipment. The equilibrium temperature was 303 (± 0.5) K. Previously optimized equilibration conditions were established: stirring velocity of 750 rpm and at least for 72 h. After the saturation conditions all samples were centrifuged in a Hettich Mikro 120 centrifuge to properly separate the macroscopic phases during 20 min at 4500 rpm. After centrifugation, samples of the liquid phase were carefully collected and the amount of vanillin was quantified through UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 280 nm. A proper calibration curve was previously established. At least three individual samples of each aqueous solution, and at each concentration of polyol, were quantified in order to determine the average solubility of vanillin and the respective standard deviation.

3. Results and Discussion

3.1. Phase diagrams and tie-lines

Although there are many reports in literature describing ATPS, this work is the first evidence that systems based on acetonitrile and polyols also undergo phase separation in aqueous media. The molecular structures of the constituents of these novel systems are depicted in Figure 1.

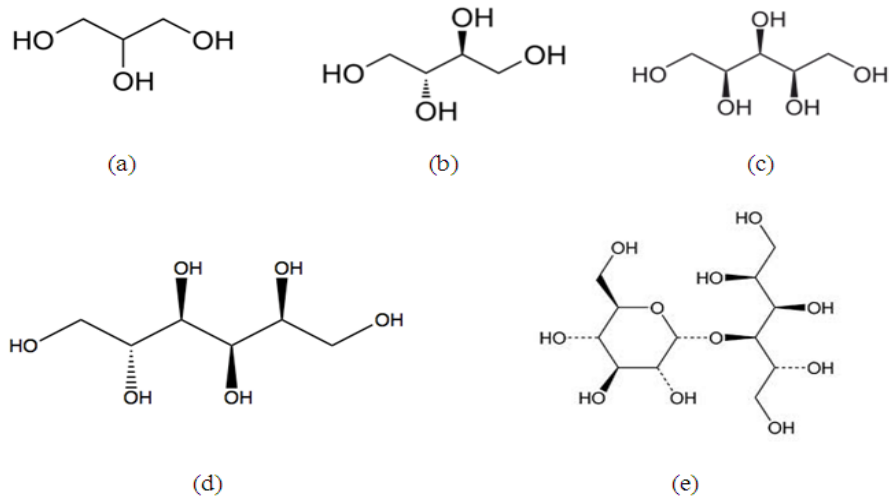

Figure 1. Chemical structure of the studied polyols: (a) glycerol; (b) erythritol, (c) xylitol, (d) sorbitol and (e) maltitol.

The solubility of a given solute in water is affected by the presence of other species that can act as phase separating agent. Polyols are non-ionic compounds with an enhanced ability to be hydrated due to their large number of –OH groups [43]. Therefore, polyols tend to act as Phase separating agent [26,44,45].

The experimental phase diagrams for each polyol (glycerol, erythritol, xylitol, sorbitol and maltitol) and acetonitrile were determined at 298 K and atmospheric pressure. The experimental weight fraction data are provided in the Supporting Information (Tables S1-S2). The corresponding phase diagrams are depicted in Figure 2 and allow the analysis of the polyol potential to induce the liquid-liquid demixing. All solubility curves are represented in molality units to avoid disparities in the evaluation of the polyol capability to form ATPS and which could result from their different molecular weights. It should be remarked that for the studied systems based on acetonitrile and each polyol, the bottom phase corresponds to the polyol-rich phase whereas the top phase is the acetonitrile-rich phase.

According to Figure 2, the formation of ATPS is favoured in the following order: glycerol < erythritol < xylitol < sorbitol < maltitol. In general, the capability of alditols for ATPS formation is directly proportional to the increasing carbon number, and thus, of hydroxyl groups from 3 (glycerol) to 9 (maltitol) - *cf.* Figure 1. According to Freire and co-workers [26] the number of hydroxyl groups present in each polyol is directly associated with its capability to hydrogen-bond with water and, therefore, to act as a phase separating species. In this context, polyols with more hydroxyl groups are those that are more able to form ATPS with acetonitrile that is excluded towards a second aqueous liquid phase.

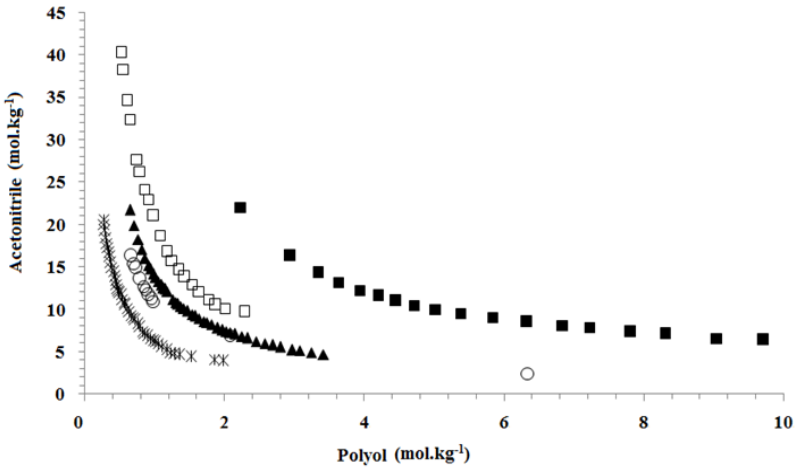

Figure 2. Binodal curves for ternary systems composed of acetonitrile + polyol + water at 298 K and atmospheric pressure. ■, glycerol; □, erithrytol; ▲, xylitol; ○, sorbitol; ∗, maltitol.

All experimental binodal data were fitted using the empirical relationship described by equation 1. The fitted parameters A, B and C (estimated by least-squares regression), and the corresponding standard deviations (σ) and regression coefficients (R^2) are reported in Table 1.

Table 1. Parameters *A*, *B* and *C* (and corresponding standard deviation, σ), obtained from the regression of the experimental binodal data by equation 1, and correlation coefficients (R^2), for the ternary systems composed of acetonitrile + polyol + water at 298 K and atmospheric pressure.

Polyol	$A \pm \sigma$	$B \pm \sigma$	$C \pm \sigma$	R^2
Glycerol	155.3 ± 6.2	-0.295 ± 0.008	$4.0\text{x}10^{-17} \pm 1.4\text{x}10^{-7}$	0.997
Erythritol	160.9 ± 5.4	-0.384 ± 0.012	$3.6\text{x}10^{-14} \pm 3.5\text{x}10^{-6}$	0.999
Xylitol	142.2 ± 2.1	-0.375 ± 0.004	$1.1\text{x}10^{-14} \pm 4.0\text{x}10^{-7}$	0.999
Sorbitol	115.1 ± 14.3	-0.329 ± 0.035	$7.4\text{x}10^{-7} \pm 1.2\text{x}10^{-6}$	0.989
Maltitol	132.3 ± 2.1	-0.371 ± 0.005	$6.3\text{x}10^{-15} \pm 4.3\text{x}10^{-7}$	0.999

. As can be seen by the correlation coefficients obtained, equation 1 provides an accurate description of the experimental binodal saturation curves. Figure 3 presents the correlation of the data by equation 1 for the several systems investigated and composed of acetonitrile + polyol + water. To complete the phase diagrams, several TLs and respective TLLs were further calculated and their values are reported in Table 2. The TLs are also represented in Figure 3.

Table 2. Mass fraction composition for the TLs and respective TLLs, at the top (T) and bottom (B) phase, and initial biphasic composition of the mixture (M), composed of acetonitrile ([ACN]) and polyol ([Polyol]) at 298 K and atmospheric pressure.

Polyol	Weigth Fraction / (wt%)						
	$[ACN]_M$	$[Polyol]_M$	$[ACN]_T$	$[Polyol]_T$	$[ACN]_B$	$[Polyol]_B$	TLL
Glycerol	40.15±0.03	30.04±0.03	15.62±0.02	5.94±0.17	23.14±0.04	41.59±0.02	63.18
	29.92±0.03	34.99±0.03	59.69±0.02	10.49±0.10	24.34±0.04	39.41±0.03	45.66
Erythritol	39.71±0.03	30.42±0.03	88.12±0.01	2.45±0.41	11.62±0.09	46.64±0.02	88.34
Xylitol	39.94±0.03	29.98±0.03	88.13±0.01	1.63±0.61	10.87±0.01	47.08±0.02	89.64
	29.52±0.03	34.40±0.03	83.70±0.01	2.00±0.50	11.44±0.09	45.21±0.02	84.19
Sorbitol	40.00±0.03	30.05±0.03	84.84±0.01	0.86±1.16	10.47±0.01	49.26±0.02	88.73
	29.99±0.03	35.04±0.03	79.58±0.01	1.26±0.79	10.85±0.09	48.04±0.02	83.15
Maltitol	39.78±0.03	30.34±0.03	82.99±0.01	1.58±0.63	9.49±0.11	50.50±0.02	88.29
	30.59±0.03	34.68±0.03	80.54±0.01	1.79±0.56	9.92±0.10	48.81±0.02	84.84

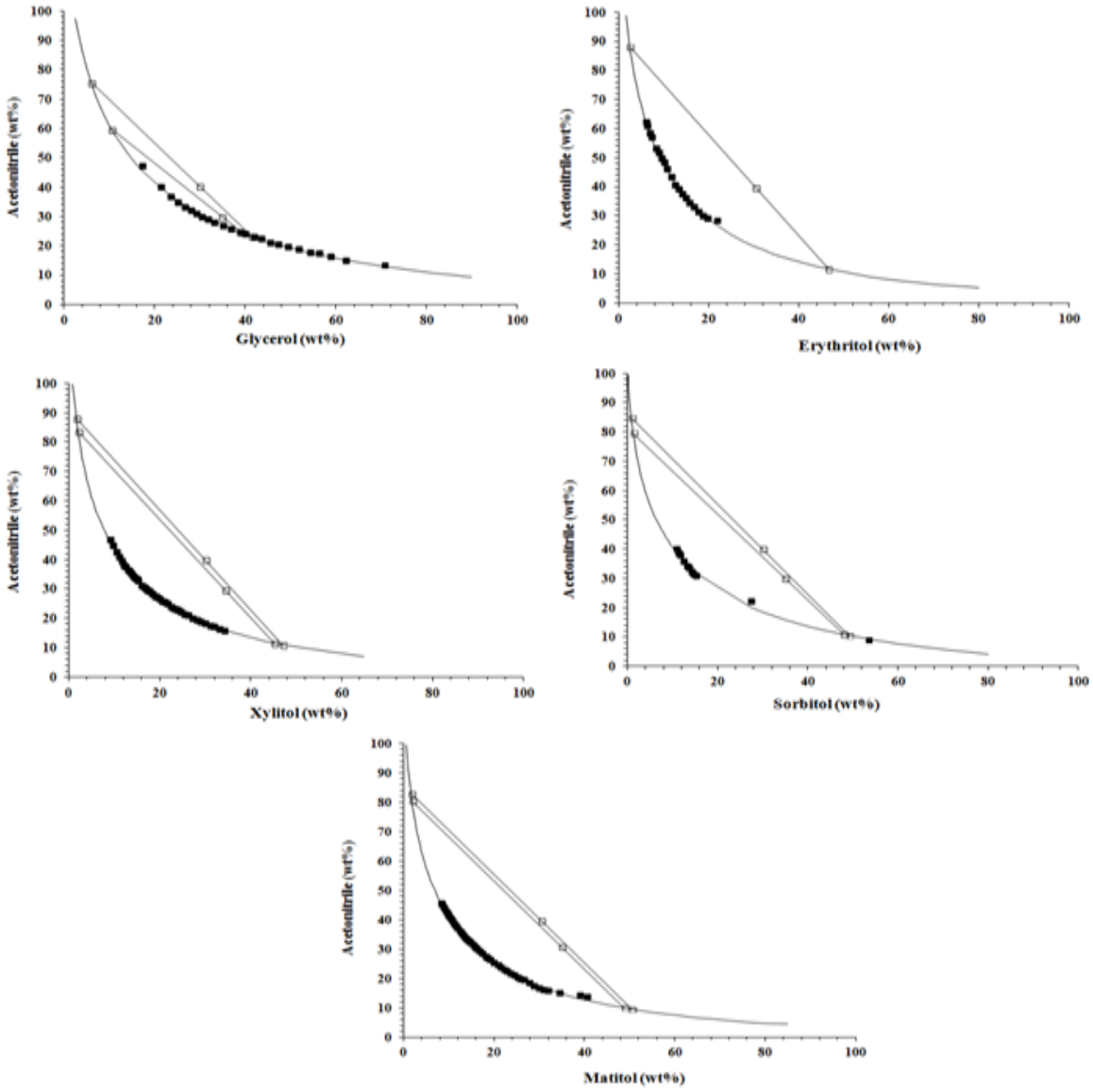

Figure 3. Phase diagrams for ternary system composed of acetonitrile + polyol + water at 298 K and atmospheric pressure. ■, experimental solubility data; □, TL data; —, fitting by equation 1.

3.2. Partitioning of vanillin

In order to evaluate the polyol structure and respective concentration in the extraction of vanillin, two different mixtures compositions (30 wt% polyol + 30 wt% acetonitrile and 35 wt% polyol + 30 wt% acetonitrile) were investigated.

In all systems, the top phase (acetonitrile-rich) is more acid (pH ranging between 4.7 and 5.7) than the bottom (pH varying between 6.5 and 6.8). The pH values of the coexisting phases used in the partitioning experiments are reported in Table 3. In all

systems, vanillin is mainly present as a neutral molecule (pKa = 8.2) [46,47]. The influence of the pH in the chemical structure of vanillin is shown in Supporting Information (Figure S1).

The partition coefficient of a given biomolecule depends on the main interactions and solute/solvent properties which favour an one-side migration. These can include dispersive-type interactions, hydrogen-bonding and eletrostatic forces, as well as the biomolecule size, solubility and affinity for a given phase. Moreover, the associated magnitude futher depends on the composition of the system and biomolecules nature [5].

Table 3.pH values of the top (acetonitrile-rich) and bottom (polyol-rich) phases at 298 K and atmospheric pressure.

Polyol	System A		System B	
	Top	Bottom	Top	Bottom
Glycerol	4.74	5.62	5.40	6.80
Erithrytol	4.96	5.93	5.05	6.52
Xylitol	5.34	6.10	5.46	6.76
Sorbitol	4.70	5.83	5.54	6.79
Maltitol	5.68	6.57	5.46	6.82

System A: 40 wt% acetonitrile + 30 wt% polyol; System B: 30 wt% acetonitrile + 35 wt% polyol.

The partition coefficients of vanillin at two different mixture compositions, at 298.15 K, are shown in Figure 4 and Table S.3 of the Supporting Information. The compositions of the coexisting phases, *i.e.*, the respective TLs are reported in Table 2. In all systems, vanillin preferentially migrates for the acetonitrile-rich phase with partition coefficient values higher than 7.16 ± 0.43. Vanillin has preference for more hydrophobic and organic phases as reflected by its octanol-water partition (K_{ow}) coefficient value: log(K_{ow}) = 1.19 [48]. In fact, acetonitrile is more hydrophobic (log(K_{ow}) = -0.17) than all the studied polyols, namely glycerol (log(K_{ow}) = -1.84), erythritol (log(K_{ow}) = -2.47), xylitol (log(K_{ow}) = -3.10), sorbitol (log(K_{ow}) = -3.73) and maltitol (log(K_{ow}) = -5.50) [49].

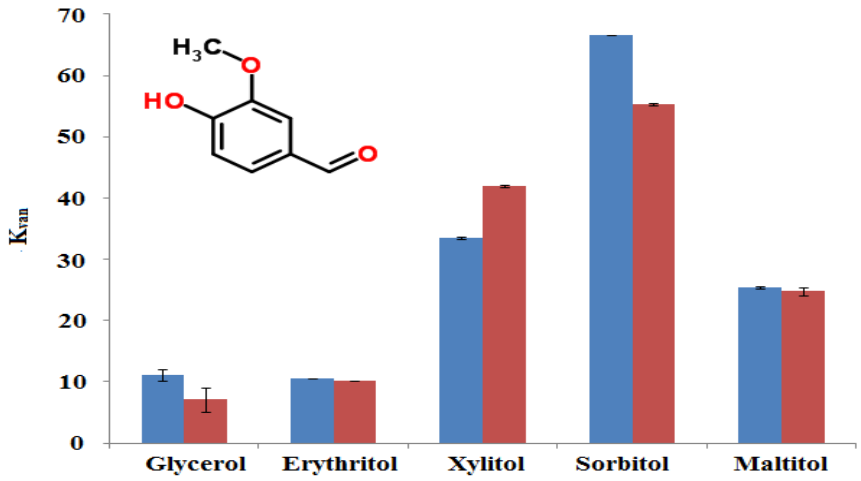

Figure 4. Partition coefficients of vanillin (K_{van}) in ATPS composed of: -30 wt% polyol + 40 wt% acetonitrile and 30 wt% water; - 35 wt% polyol + 30 wt% acetonitrile and 35 wt% water. The chemical structure of vanillin is also shown as an insert.

Moreover, the partition coefficient of vanillin for the acetonitrile-rich increases with the hydrophilicity of the polyol, with the exception of maltitol. This trend also follows the ability for two-phase formation previously described indicating that stronger phase separating species improves the migration of the biomolecule for the opposite phase. This pattern of the phase separating capacity of the different polyols was further confirmed by the determination of the solubility of vanillin in aqueous solutions containing polyols at different concentrations. The results obtained are depicted in Figure 5 and Table S.4 of the Supporting Information, and it show a decrease on the vanillin solubility with the increase on the polyols concentration supporting thus their phase separating effect over vanillin.

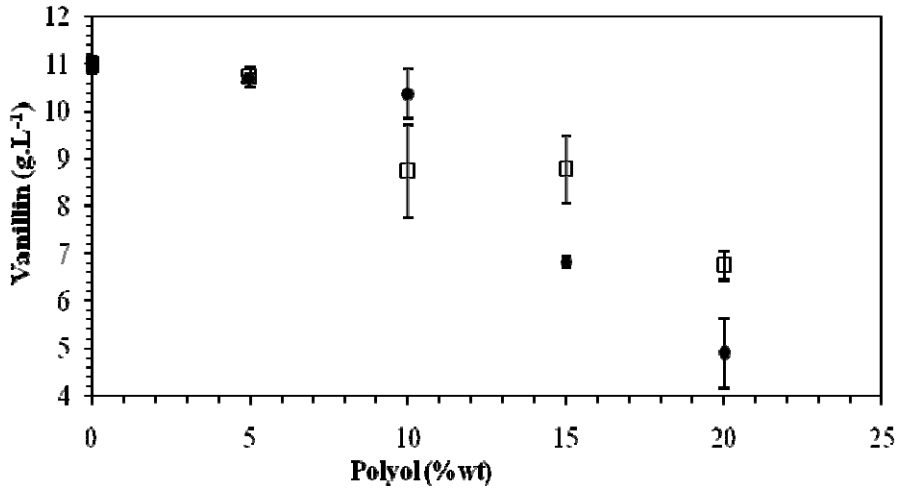

Figure 5. Solubility of vanillin at 303 K in ■ H_2O, and aqueous solutions of, ● sorbitol and □ xylitol.

The maximum partition coefficient was observed with sorbitol whereas the lowest value was observed with maltitol. With an increase in the hydroxyl groups number in the polyol structure there is an increase in interactions by hydrogen-bonding (glycerol – 3 and sorbitol – 6) with vanillin, which did not overcome the affinity of vanillin with the top phase and the increase of *K*. However, maltitol has almost the double of the -OH groups (11) compared to sorbitol, and probably it occurs a stronger interaction of this polyol with vanillin leading to a subsequent decrease of the partition coefficient of vanillin when compared with other polyols.

The partition coefficients of vanillin observed in this work in acetonitrile-polyol ATPS are higher than those observed in other systems composed of acetonitrile + mannose (K_{van} = 9.67) [28] or ionic liquid + K_3PO_4 (K_{van} = 49.5) [12], although lower than those observed in ethanol + K_2HPO_4 (K_{van} = 430) [13]) systems. The gathered results support the idea that non-ionic ATPS formed by acetonitrile and polyols are therefore an alternative extractive route for added-value biomolecules, such as vanillin.

The influence of the polyol chemical structure was also evaluated through the vanillin recovery parameter - Figure 6 and Table S.3 of the Supporting Information. In

general, the recovery values are above 89%, except for glycerol, and reflect the high preference of vanillin for the acetonitrile-rich phase. In terms of recovery, the values tend to increase with the increase in the number of hydroxyl groups at the polyol and from glycerol to maltitol.

Polyols with less than 4 hydroxyl groups present an average of R_T below 0f 82% (glycerol), and similar to the value reported by Cardoso and co-workers [28] using acetonitrile + sugar ATPS. Polyols with more than 4 hydroxyl groups lead to an average R_T above 89% (xylitol, sorbitol and maltitol), and similar to the value described by Reis and co-workers [13] using systems constituted by alcohols + potassium phosphate salts.

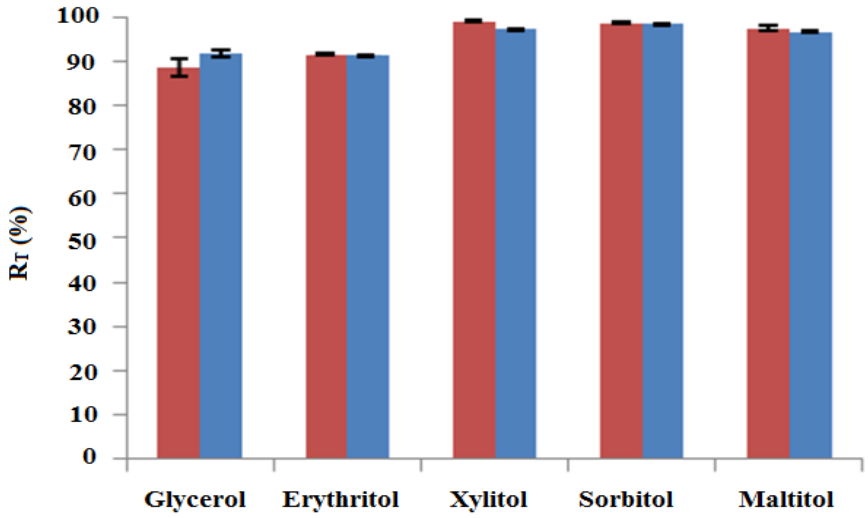

Figure 6. Recovery of vanillin in the acetonitrile-rich phase (R_T) in ATPS composed of: -30 wt% polyol + 40 wt% acetonitrile and 30 wt% water; - 35 wt% polyol + 30 wt% acetonitrile and 35 wt% water.

The enhanced recovery of vanillin to the acetonitrile-rich phase suggests that the systems investigated in this work represent a viable alternative for extractive purposes while avoiding the use of charged species or high-charge density salts. Dhamole and co-workers reported recovery of proteins in CAN-glucose aqueous two-phase system above 97.16 % at 18ºC [50].

4. Conclusion

This work shows, for the first time, that ATPS can be formed by acetonitrile and a wide variety of polyols at specific concentrations in aqueous media. The ternary phase diagrams, tie-lines and tie-line lengths were determined at 298 K and atmospheric pressure. The results obtained indicate that polyols act as phase separating species leading to the exclusion of a second acetonitrile-rich phase. Moreover, the higher the number of hydroxyl groups at the polyol the higher it is the salting-out effect observed. The two-phase separation is favored in the following order: glycerol < erithrytol < xylitol < sorbitol < maltitol. A proof of principle showing that these novel ATPS can be used as to extract biomolecules was also demonstrated using vanillin as a model compound. Vanillin preferentially partitions for the acetonitrile-rich phase with partition coefficients ranging from 7 to 67 and which are dependent on the phase separating ability of each polyol. Moreover, the recovery of vanillin at the acetonitrile-rich phase showed to be higher than 89%, except to glycerol, supporting the huge potential of these novel systems to be explored in the extraction of the most diverse added-value compounds.

Acknowledgments

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Supporting Information

Novel Aqueous Two-Phase Systems Composed of Acetonitrile and Polyols: Phase Diagrams and Extractive Performance

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Table S1. Experimental binodal mass fraction data for the system composed of acetonitrile (1) + polyol (2) + water (3) at 298 K.

Glycerol		Erythritol		Xylitol	
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
13.4707	70.6650	28.3688	21.7702	15.9459	34.1446
15.1554	62.0598	29.3176	19.6508	16.5776	32.9716
16.5415	58.8050	30.1316	18.6177	17.3540	31.8141
17.6039	56.0647	31.4279	17.7353	17.5530	31.0832
17.8784	54.1234	33.1807	16.6372	18.5229	29.8283
18.8147	51.7095	34.6368	15.7465	19.0826	28.9499
19.9110	49.3151	36.1316	14.8358	19.5743	28.1329
20.6244	47.1989	37.5378	14.0212	20.2177	27.1230
21.2153	45.4320	39.2257	13.2128	21.3020	26.0750
22.4357	43.3144	40.7459	12.4853	21.6810	25.4467
23.0865	41.8207	43.4371	11.5886	22.4526	24.5857
24.2442	39.9221	46.3024	10.7213	22.8184	24.0051
24.7994	38.6336	48.4718	9.9889	23.2547	23.5465
25.7890	36.8074	49.7324	9.4348	23.6178	23.0432
26.9825	34.9378	51.8732	8.8098	24.1450	22.3930
28.0834	33.0875	53.1640	8.3486	25.0943	21.5627
29.0064	31.6224	57.0877	7.2974	25.6667	20.9645
30.0345	30.2231	58.6589	6.8675	25.9430	20.5126
31.0551	29.0647	61.1347	6.2969	26.7362	19.8588
32.1884	27.8757	62.2992	5.9704	27.3620	19.2885
33.3033	26.5890			27.7507	18.8577
35.0559	25.0372			28.6141	18.2010
36.9285	23.4725			29.2736	17.7065

Table S1 (Cont.). Experimental binodal mass fraction data for the system composed of acetonitrile (1) + polyol (2) + water (3) at 298 K.

Glycerol		Erythritol		Xylitol	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
40.0409	21.2913			29.6974	17.2858
				30.3325	16.8286
				30.7409	16.4624
				31.4073	15.9957
				33.0914	15.2055
				33.6847	14.8510
				33.9335	14.5498
				34.5348	14.1579
				35.2608	13.7260
				35.9919	13.3193
				36.7727	12.9245
				37.6971	12.4338
				38.3159	12.0845
				39.6327	11.5443
				41.1851	10.9482
				42.8309	10.3420

Table S2. Experimental binodal mass fraction data for the system composed of acetonitrile (1) + polyol (2) + water (3) at 298 K.

Sorbitol		Maltitol	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**
8.8906	53.5915	13.7184	40.4609
22.1038	27.3615	14.2575	38.8956
30.8835	15.1962	15.1868	34.2960
31.5178	14.7079	15.8585	31.6896
32.3839	14.2284	16.2014	30.7489
33.5869	13.6393	16.6561	29.8190
34.2509	13.2132	17.4807	28.6785
35.7251	12.5854	18.5447	27.5972
37.9299	11.7505	19.6439	26.4053
38.6926	11.3560	20.0024	25.6942
40.0359	10.8295	20.3501	25.1633
		21.1668	24.2791
		21.8214	23.5211
		22.5443	22.7770
		23.1494	22.0825
		23.7613	21.3644
		24.4871	20.6791
		25.2750	19.8621
		26.3338	19.0204
		27.0228	18.4350
		27.4838	17.9957
		28.5445	17.2435

Table S2 (Cont.). Experimental binodal mass fraction data for the system composed of acetonitrile (1) + polyol (2) + water (3) at 298 K.

Sorbitol		Maltitol	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**
		29.3127	16.6680
		30.1169	16.1373
		30.6401	15.6931
		31.3547	15.2188
		32.3562	14.6199
		32.8435	14.2339
		33.3326	13.8850
		33.8105	13.5436
		34.6718	13.0922
		35.3134	12.7106
		36.1577	12.2691
		37.1069	11.8009
		37.9438	11.3872
		38.7737	11.0223
		39.8771	10.5081
		40.5355	10.1888
		41.5304	9.7704
		42.1578	9.4885
		43.1244	9.1148
		44.0964	8.7356
		45.7885	8.0859

Table S.3. Partition coefficients (K_{van}) and recovery of vanillin in top phase (R_t) in ATPS composed of: 30 wt% polyol + 40 wt% acetonitrile and 30 wt% water (System A); and 35 wt% polyol + 30 wt% acetonitrile and 35 wt% water (System B).

Polyol	K_{VAN}		R_T (%)	
	System A	SystemB	System A	SystemB
Glycerol	7.16 ± 0.43	11.11 ± 1.32	81.85 ± 3.13	64.69 ± 2.92
Erythritol	10.25 ± 0.01	10.53 ± 0.01	89.43 ± 0.01	89.43 ± 0.01
Xylitol	41.90 ± 7.89	33.49 ± 4.48	98.32 ± 0.60	94.33 ± 0.14
Sorbitol	55.33 ± 2.85	66.58 ± 3.01	98.39 ± 0.31	96.78 ± 0.35
Maltitol	24.83 ± 0.55	25.44 ± 1.19	96.95 ± 0.92	93.63 ± 0.54

Tabela S.4. Solubility of vanillin at 303 K in aqueous solutions of xylitol and sorbitol.

Polyol (wt%)	Solubility in Xilytol ($g.L^{-1}$)	Solubility in Sorbitol ($g.L^{-1}$)
0	10.98 ± 0.20	10.98 ± 0.20
5	10.74 ± 0.21	10.69 ± 0.10
10	8.72 ± 0.98	10.38 ± 0.52
15	8.77 ± 0.7	6.83 ± 0.11
20	6.75 ± 0.30	4.89 ± 0.73

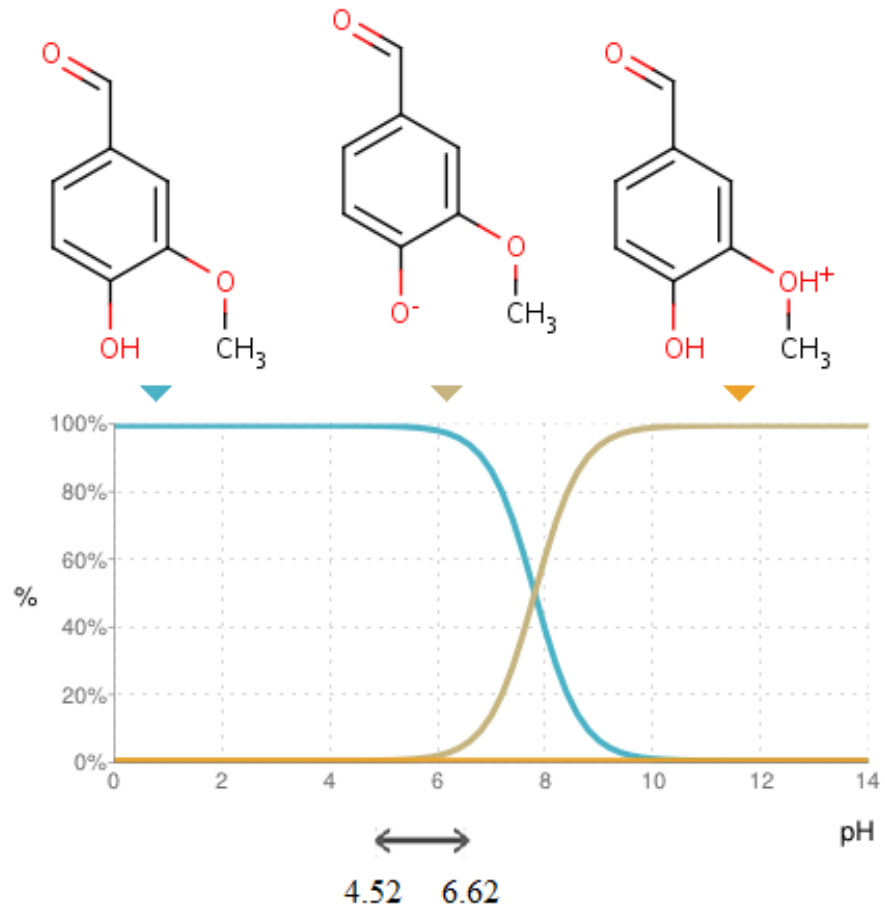

Figure S1. Chemical structure of vanillin at different pH values. This content was adapted from the Chemspider chemical database (http://www.chemspider.com/).

Capítulo V

Aqueous Two-Phase Systems formed by Biocompatible and Biodegradable Polysaccharides and Acetonitrile

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Abstract

In this work, it is shown that novel aqueous two-phase systems can be formed by the combination of acetonitrile and polysaccharides, namely dextran. Several ternary phase diagrams were determined at 25 ºC for the systems composed of water + acetonitrile + dextran. The effect of the dextran molecular weight (6,000, 40,000 and 100,000 $g.mol^{-1}$) was ascertained toward their ability to undergo liquid-liquid demixing. An increase in the dextran molecular weight favors the phase separation. In general, acetonitrile is enriched in the top phase while dextran is majorly concentrated in the bottom phase. The applicability of this new type of two-phase systems as liquid-liquid extraction approaches was also evaluated by the study of the partition behavior of a well-known antioxidant – vanillin - and used here as a model biomolecule. The optimized conditions led to a recovery of vanillin of 70.7% at the acetonitrile-rich phase. Based on different partition coefficients obtained at diverse temperatures, it can be concluded that the vanillin partitioning process for the acetonitrile-rich phase is spontaneous, and either an exothermic or endothermic process depending on the temperature range evaluated.

Keywords: aqueous two-phase system, acetonitrile, dextran, vanillin, extraction.

1. Introduction

Aqueous two-phase system (ATPS) are widely applied in biotechnology for the isolation and purification of enzymes as lipase [1-3], antioxidants as rutin [4] and gallic acid [5], alkaloids as theobromine, theophyline, nicotine and caffeine [6], antibiotics as tetracycline [7,8]; and antibodies [9,10]. The main advantages of ATPS rely on their

scale-up possibility, on the rapid mass transfer and phase equilibrium, possibility of a continuous processing, low energy requirements, among others [11].

ATPS are usually formed by mixing two polymers in aqueous media (PEG and Dextran [12,13]; PEG and Maltodextrin [14]), or by one polymer and one salt (PEG and phosphate-based salts [15-17]; PEG-citrate-based salts [18,19]). However, some other pairs of phase-forming components can be used in the creation of alternative ATPS, such as alcohol + salt [20], ionic liquid + salt [21-23], ionic liquid + PEG [24,25] and ionic liquid + carbohydrate [26] mixtures.

Continuing the search on novel ATPS, it has been demonstrated that biphasic aqueous systems can be formed either by dextran and polyethylene glycol (ATPS) [27] or by ethanol/2-propanol and ammonium sulfate [28]. Previously we have also demonstrated that alternative aqueous biphasic systems can be created by combining acetonitrile and carbohydrates (monosaccharides and disaccharides) [29] as well as with polyols [30]. In this context, we attempted now the formation of novel ATPS formed by acetonitrile and polysaccharides, namely dextran.

Dextran is a water soluble biopolymer produced by a variety of lactic acid bacteria such as *Leuconostoc* sp., and which presents two valuable properties: biodegradability and biocompatibility [31]. The chemical structure of dextran is predominantly formed by 95% of linear α-(1 →6) linkages as the main backbone and 5% of α-(1→3) branch linkages [32]. This homopolymer of glucose has several targeted industrial applications, varying from food, cosmetic, pharmaceutical to oil drilling industries [33].

Acetonitrile (CH_3CN) – ACN is an interesting solvent due to its properties; it is aprotic and strongly polar and is obtained as a by-product from the manufacture of acrylonitrile [34]. ACN is widely used by industry in the production of perfumes, rubber

products, pesticides or pharmaceuticals [35], and in chromatography process as mobile phase in high performance liquid chromatography – HPLC [36,37]. This solvent is miscible with water in all proportions [38] and its molecules are unable to strongly associate with themselves leaving a hydrogen-bond network formed by water [39,40].

The aim of the present work is to study novel aqueous two-phase systems based on acetonitrile and several dextrans of different molecular weights. The ternary phase diagrams were determined at 25 ºC and the effect of the polysaccharide molecular weight was evaluated. These systems were also ascertained on what regards their applicability on extraction routes, and in particular on the extraction of a well-known antioxidant, vanillin (4-hydroxy-3-methoxybenzaldehyde). Vanillin is the major flavor constituent of natural vanilla - *Vanilla plantifolia*, extracted at a rate of 12,000 ton/year [41]. Vanillin is widely used as a flavoring material in confectionery, food products, beverages, perfumes and in pharmaceutical preparations [42]. Natural vanillin costs between 1,200 and 4,000 USD/kg, while synthetic vanillin costs around 15 USD/kg [43]. However, the chemical process usually leads to a low quality vanillin that further requires a sensitive extraction and purification procedure [44], and for which ATPS can be foreseen as an alternative approach.

2. Material and Methods

2.1. Materials

The ATPS studied in this work were formed by dextran from *Leuconostoc* spp. (M_w = 100,000 g.mol^{-1} – Dx-100; 40,000 g.mol^{-1} – Dx-40; and 6,000 g.mol^{-1} – Dx-6) and acetonitrile. Dextran and acetonitrile (purity of 99.9 wt%), as well as vanillin (> 99 wt% pure) were purchased from Sigma-Aldrich. The chemical structures of the phase-forming components of the ATPS studied and of the target biomolecule used in the

partitioning experiments are shown in Figure 1. Distilled and deionized water was used in all experiments.

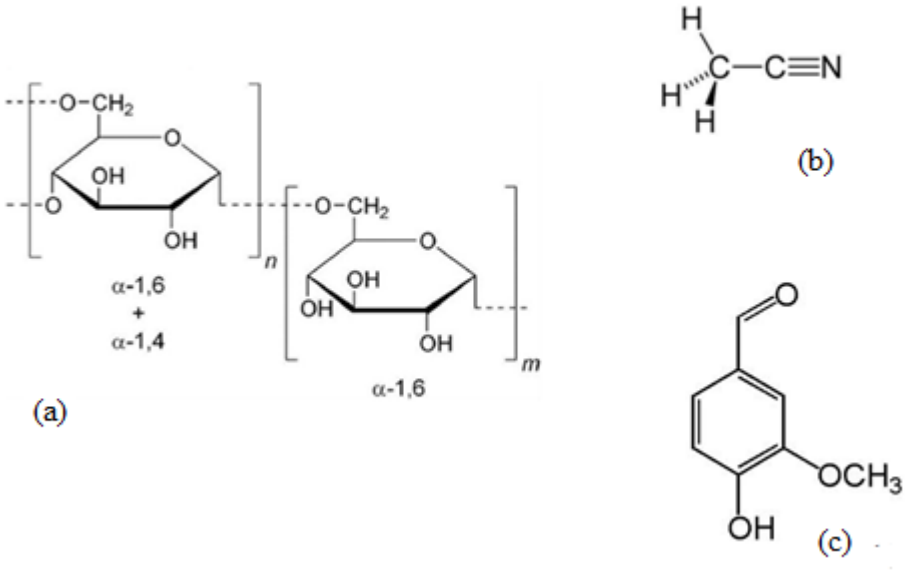

Figure 1. Chemical structures of the phase-forming components used in the ATPS formation and of the biomolecule used as a partitioning solute:(a) dextran; (b) acetonitrile; (c) vanillin.

2.2 Ternary Phase Diagrams

The ternary phase diagrams for water, acetonitrile and the different molecular weight dextran were determined at (25 ± 1) ºC and atmospheric pressure by the cloud point titration method. Stock solutions of dextran (40 wt%) and acetonitrile (80 wt%) were previously prepared and used for the determination of the binodal curves. Repetitive drop-wise addition of the acetonitrile solution to the aqueous solution of dextran was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultra-pure water until the inspection of a monophasic region (clear and

limpid solution). These additions were carried out under continuous stirring and the saturation curves were determined gravimetrically within ± 10^{-4} g.

2.3. Partitioning of vanillin

Dextran, acetonitrile and water were successively added into graduated glass centrifuge tubes with vanillin at a final concentration of 0.4 $g.L^{-1}$. After the complete mixing of all components, for a given mixture composition, each system was centrifuged at 2,000 x *g* for 10 min to favour the phase separation, and then each tube was placed in a thermostatic bath at (5 to 35ºC) for at least 18 h. The volume of each phase was measured and both phases were further separated for the quantification of vanillin. At least three independent replicates were made and the average partition coefficients and associated standard deviations were therefore determined.

The concentration of vanillin at each aqueous phase was quantified through UV-spectroscopy, using a Varian Cary 50 Bio UV-Vis spectrophotometer, and at a wavelength of 280 nm using a calibration curve previously established.

The partition coefficient of vanillin was determined taking into account the concentration of the antioxidant in each phase and according to,

$$K_{\text{van}} = \frac{C_{\text{T}}}{C_{\text{B}}} \tag{1}$$

where K_{van} is the partition coefficient of vanillin, C represents the vanillin concentration, and the subscripts T and B symbolize the top (acetonitrile-rich) and bottom (dextran-rich) phases, respectively.

The recovery of vanillin (R_T) in the top phase was evaluated using equation 2,

$$R_{\text{T}} = \frac{100}{1 + \frac{1}{K_{\text{van}} \times R_v}} \tag{2}$$

where R_v is the ratio between the volumes of the top (V_T) and bottom (V_B) phase.

The standard molar Gibbs free energy of transfer ($\triangle_{tr}G_o^m$), the standard molar enthalpy of transfer ($\triangle_{tr}H_o^m$) and standard molar entropy of transfer ($\triangle_{tr}S_o^m$) associated with the vanillin partition coefficient were determined by the van't Hoff approach at different temperatures (5 to 35ºC). The following isochors were used to determine the molar thermodynamic functions of transfer (equations 3-5):

$$\ln K_{van} = -\Delta_{tr}H_m^o \times \frac{1}{T} + \frac{\Delta_{tr}S_m^o}{R} \quad (3)$$

$$\Delta_{tr}G_m^o = \Delta_{tr}H_m^o - T\Delta_{tr}S_m^o \quad (4)$$

$$\Delta_{tr}G_m^o = -RT\ln(K_{van}) \quad (5)$$

where T is the temperature (Kelvin) and R is the ideal gas constant. The enthalpy and entropy contributions can be directly deduced from the linear approximation of ln(K_{van}) *versus* T^{-1}.

3. Results and Discussion

The effect of different molecular weights of dextran towards the phase separation in acetonitrile-aqueous solutions was firstly addressed. Further, the potentiality of these systems for the extraction of biomolecules was assessed. Moreover, the effects of the dextran concentration and temperature were also evaluated in what concerns the vanillin partitioning for the acetonitrile-rich phase.

3.1. Ternary Phase Diagrams

Three high molecular weight dextrans were used in combination with acetonitrile in aqueous media, and the respective phase diagrams were determined at 25ºC and atmospheric pressure. The binodal curves experimentally determined in

molality units and weight fraction are depicted in Figure 2. The phase diagrams are also reported in molality units for an enhanced understanding on the impact of the distinct dextrans through the formation of ATPS.

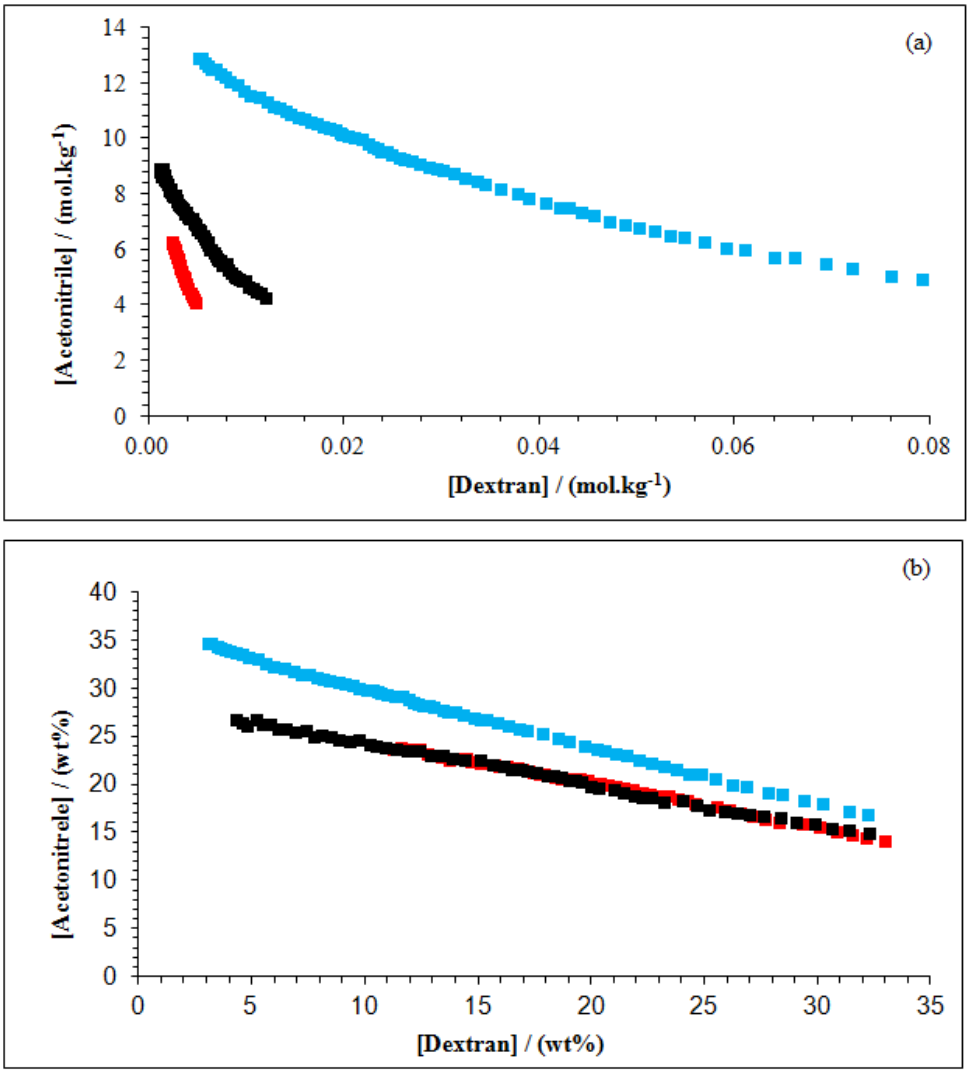

Figure 2. Binodal curves in molality units (a) and weight fraction (b) for the ternary systems composed of dextran + acetonitrile + water at 25 ºC and atmospheric pressure: ■- Dx-6; ■- Dx-40; and ■- Dx-100.

The experimental weight fraction data are provided in the Supporting Information. The analysis of Figure 2 reveals a strong displacement of the binodal

curves towards the origin in the following order: Dx-6 > Dx-40 > Dx-100. In general, the higher the molecular weight of the polysaccharide the more able it is to form two aqueous rich phases with acetonitrile. These results corroborate those found by Lima and co-workers [29] where higher molecular weight carbohydrates, namely monosaccharides and disaccharides, result in a higher ability for phase separation. The increase of the molecular weight of dextran leads to an increase on the number of hydroxyl groups *per* mole of biopolymer. Indeed, and as previously discussed by Coutinho and co-workers [26], the number of hydroxyl groups present in carbohydrates as phase-forming components of ATPS is directly associated with its capability to hydrogen-bond with water and, therefore, to act as salting-out/sugaring-out species. In the same context, Lima and co-workers [30] also demonstrated that polyols with more hydroxyl groups are more able to form ATPS with acetonitrile.

Figure 3 shows a general comparison between the strongest sugaring-out agents previously reported (a dissacharide and a polyol) [29,30], and the dextran with the highest molecular weight determined here, on their ability to form ATPS with acetonitrile. In fact, it is clearly seen that polysaccharides perform as better sugaring-out agents when compared with carbohydrates of lower molecular weight and polyols. In summary, lower amounts of dextran are required to induce the liquid-liquid demixing when compared to disaccharides and polyols and which can be foreseen as a major advantage towards the sustainability concept.

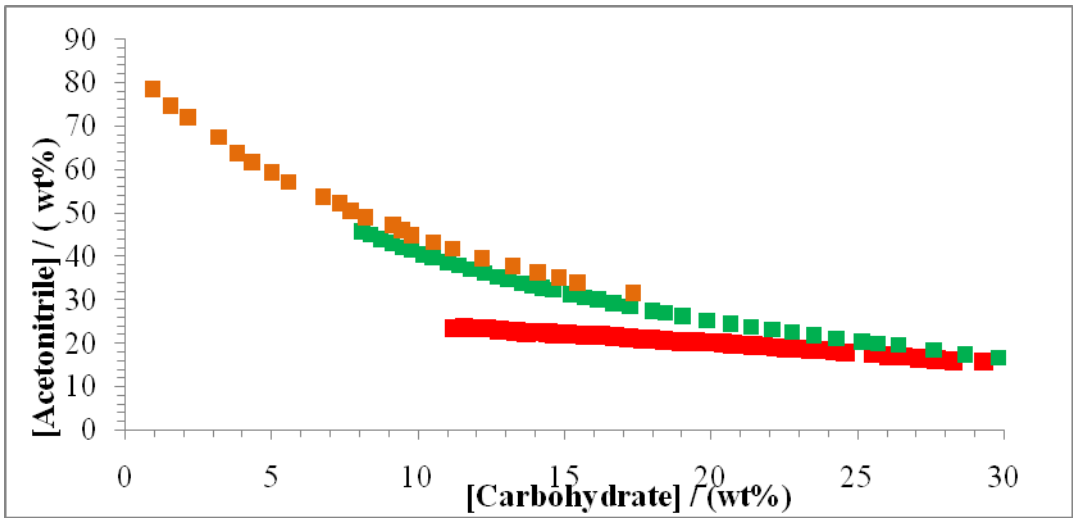

Figure 3. Binodal curves for the ternary systems composed of acetonitrile + (dextran - ■, maltitol - ■ [30] or maltose - ■ [29]) + water at 25 ºC and atmospheric pressure.

3.2. Partitioning of vanillin

In order to evaluate the potential of the studied ATPS to be applied as extractive systems, vanillin was thorough used. The partition coefficients and the recovery of vanillin at the acetonitrile-rich phase were determined at different compositions and at several temperatures.

3.2.1. Influence of Dextran molecular weight

Two-phase systems composed of 15 wt% of dextran and 30 wt% of ACN, and for the 3 different molecular weights dextran (Dx-6; Dx-40 and Dx-100), were used to study the partitioning behavior of vanillin. All these results were obtained at 25ºC. The partition coefficient of vanillin (K_{van}) and recovery of vanillin (R_T) in the top phase are shown in Figure 4 and the corresponding numeric values are reported in the Supporting Information. Figure 4 allows the inspection through the influence of the dextran molecular weight on the vanillin partitioning among the two phases.

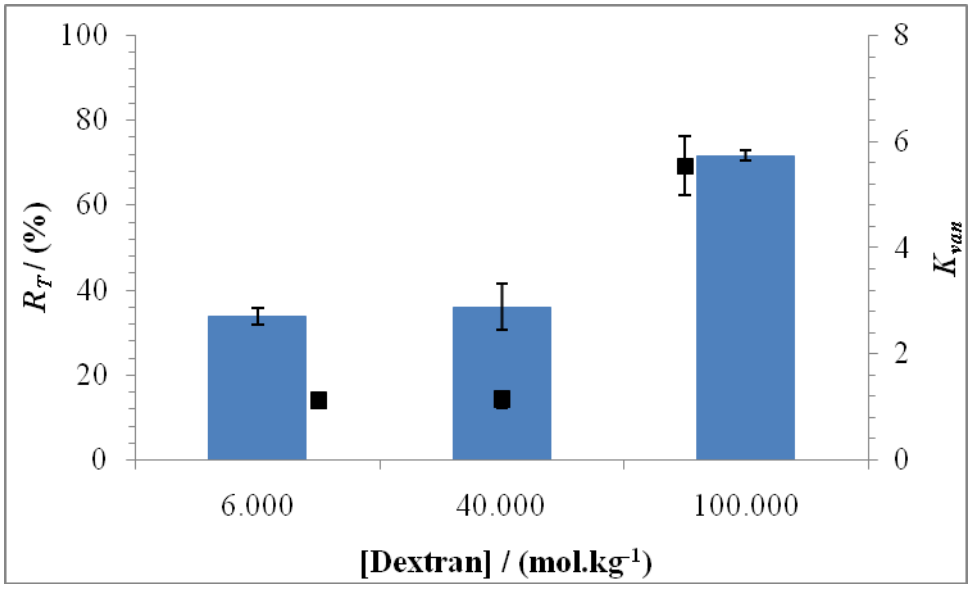

Figure 4. Effect of the dextran molecular weight on the vanillin partitioning at 25 ºC for the system composed of dextran-acetonitrile (15-30 wt%). Bars – recovery of vanillin (R_T) in the top phase; symbols – partition coefficient (K_{van}) of vanilin in the acetonitrile-rich phase.

For Dx-6 and Dx-40, vanillin displays a similar partitioning behavior between the coexisting phases ($K_{van} \approx 1.0$). Nevertheless, the use of Dx-100 leads to a preferentially partitioning of vanillin for the ACN-rich phase ($K_{van} > 1.0$). In general, an increase on the molecular weight of dextran induces an increase on the partition coefficient of vanillin, particularly seen with Dx-100. This effect can be explained by a volume exclusion model [45], where the intermolecular spaces in the dextran-rich phase are reduced with the increase on the molecular weight, and consequently, vanillin migrates for the top phase (ACN-rich phase). Other authors also observed the volume exclusion effect using polymers as polyethylene glycol for the partition behaviour of bromelin [46] and protease [47]. However, and as previously shown by us [29,30], this

increase on the partition coefficients for the ACN-rich phase can be also a result of a sugaring-out phenomenon of the carbohydrate over the target biomolecule.

The recovery of vanillin in the ACN-rich phase ranges between 33.69 and 71.61%. In addition, the partition coefficients are within 1.09 and 5.53. In summary, the highest partition coefficient and recovery of vanillin in the ACN-rich phase was observed with the system composed of Dx-100, the higher molecular weight biopolymer investigated.

3.2.2. Influence of the Dextran concentration

To evaluate the effect of the dextran concentration, a series of ATPS containing 30 wt% of ACN and 15-20 wt% of Dx-100 at 25ºC were assessed. The results obtained are depicted in Figure 5 and in the Supporting Information.

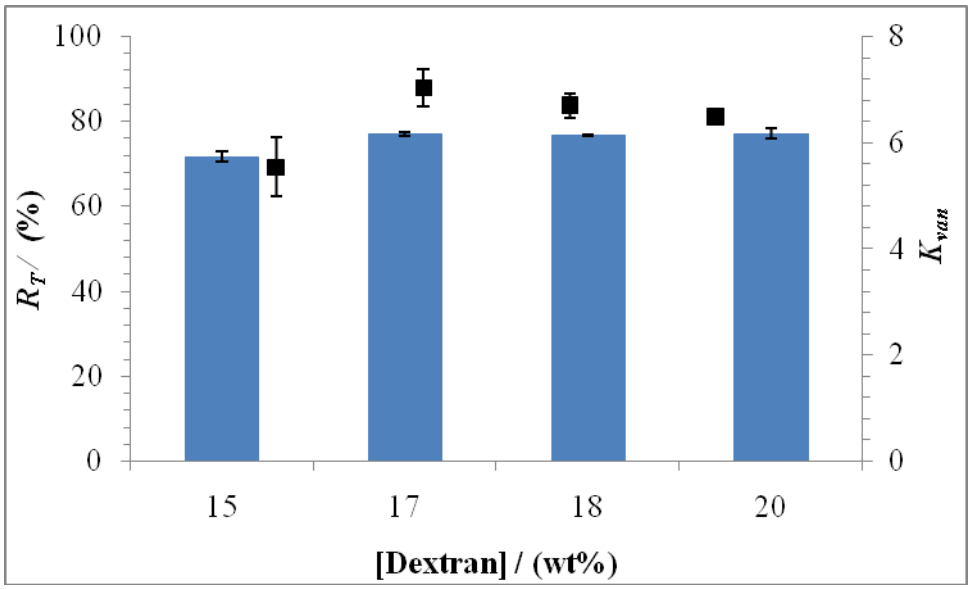

Figure 5. Effect of the dextran concentration on the vanillin partitioning at 25 ºC for the system composed of Dx-100 (15-20 wt%) + acetonitrile (30 wt%). Bars – recovery of vanillin (R_T) in the top phase; symbols – partition coefficient (K_{van}) of vanilin in the acetonitrile-rich phase.

In the current study, an increase in the partition coefficient was observed with the increase on the Dx-100 concentrations ranging between 15 and 17 wt%. However, for dextran concentrations above 17 wt% the K_{van} values are almost constant, considering the associated standard. This trend suggests the presence of a saturation process linked to the dextran concentration, as reported by Picó and co-workers [27] in the albumin partitioning behavior using a PEG-3350/dextran ATPS. The recovery values of vanillin at the ACN-rich phase range between 71.61 and 77.07 %, values lower than that those found by us (98.37 and 99.94 %) using ATPS constituted by alcohols and potassium phosphate salts [20].

3.2.3. Influence of temperature

In the previous sections it was demonstrated that by choosing a high molecular weight dextran (Dx-100) at a concentration ranging between 15-20 wt%, vanillin can be effectively separated and concentrated into the top phase. Therefore, the effect of temperature was also evaluated at temperatures varying between 5 and 35ºC, for the common mixture composition of 30 wt% of ACN + 18 wt% of Dx-100. The results obtained are depicted in Figure 6 whereas the respective values are presented in the Supporting Information.

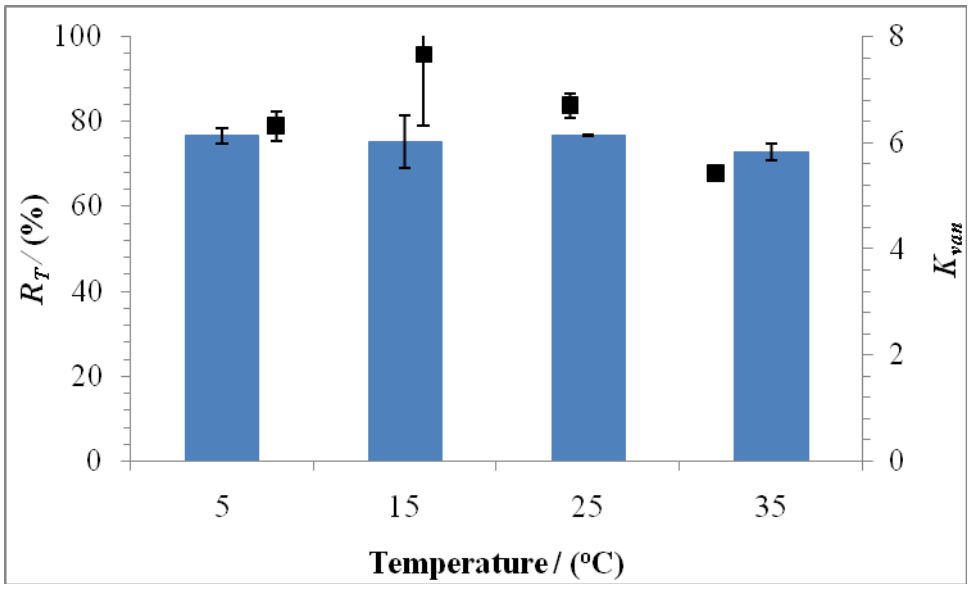

Figure 6. Effect of temperature on the vanillin partitioning for the system Dx-100-acetonitrile (18-30 wt%). Bars – recovery of vanillin (R_T) in the top phase; symbols – partition coefficient (K) of vanilin in the acetonitrile-rich phase.

At all temperatures, vanillin preferentially migrates for the top phase ($5.39 < K_{van} < 7.66$). However, these values are somewhat lower than those observed with ATPS composed of ionic liquids + potassium phosphate ($6.69 < K_{van} < 49.98$) previously [48], and of acetonitrile + polyols ($7.16 < K_{van} < 66.58$) [30]. However, the partition coefficients obtained in this work are similar to those reported by Lima and co-workers [29] using ATPS formed by acetonitrile and carbohydrates ($3.06 < K_{van} < 9.67$).

The results obtained indicate that temperature influences the vanillin partitioning. This effect can be divided into two parts: (i) between 5 and 15 ºC in which the K_{van} reached the maximal value (7.7 ± 1.4); (ii) and between 15 and 35 ºC, in which the partition coefficient of vanillin decreases with an increase in temperature. Similarly to the results on the vanillin partitioning in ionic-liquid-salt ATPS [48], it was also observed here a maximum value in the partition coefficient as a function of temperature. This maximum as a function of temperature suggests that the partition phenomenon is

driven by opposite effects resulting from enthalpic and entropic contributions. The recovery of vanillin for all system ranges between 72.70 and 76.58 %.

The thermodynamic parameters involved in the partitioning of vanillin where obtained from the application of Equations 3 to 5 for two temperature ranges (5-15ºC and 15-35ºC). The thermodynamic functions of transfer values are presented in Table 1. The obtained regression coefficient of $\ln(K_{van})$ *versus* T^{-1} is higher than 0.97 and which supports a reasonable determination of $\Delta_{tr}H_m^o$ and $\Delta_{tr}S_m^o$.

Table 1. Standard molar thermodynamic functions of transfer of vanillin in the ATPS composed of acetonitrile – Dx-100 (30 wt% – 18 wt%) at 25 ºC.

Temperature (ºC)	$\Delta_{tr}G_m^o$ / (kJ.mol^{-1})	$\Delta_{tr}H_m^o$ / (kJ.mol^{-1})	$\Delta_{tr}S_m^o$ / (J.mol^{-1}.K^{-1})
5 – 15	-54.96	+15.72	62.27
15 - 35	-46.30	-12.87	-27.66

The calculated $\Delta_{tr}G_m^o$ values are negative for both temperature intervals (from 5 to 15ºC and from 15 to 35ºC), reflecting thus the spontaneous and preferential partition of vanillin for the acetonitrile-rich phase ($K_{van} > 1.0$). $\Delta_{tr}H_m^o$ values are negative for the temperatures ranging between 15 and 35ºC, revealing that the transference of vanillin from the dextran-rich phase to acetonitrile-rich phase is an exothermic process. This observation is according to the findings by Ni and co-workers [49] in partitioning studies of chloramphenicol in aqueous two-phase systems based on ionic liquids and potassium tartrate. On the contrary, from 5 to 15ºC, the transference of vanillin for the acetonitrile-rich phase is an endothermic process. This last trend was also verified in the bovine serum albumin and trypsin partitioning using an ATPS constituted by maltodextrin and a propylene oxide copolymer [50].

4. Conclusion

In this work, novel aqueous two-phase systems based on acetonitrile and dextran were proposed. The binodal curves, which define the mono- and biphasic regimes, were determined at 25ºC and at atmospheric pressure. Three dextran polysaccharides of different molecular weight were investigated, namely 6,000, 10,000 and 40,000 $mol.kg^{-1}$. In general, the higher the molecular weight of dextran, the higher is the ability of the system to undergo phase separation.

The novel ATPS were also evaluated in what concerns their potential for extraction purposes. Vanillin was used as a model antioxidant or bioactive substance. In all situations, vanillin preferentially migrates for the acetonitrile-rich phase. The higher partition coefficients and recoveries of vanillin at the acetonitrile-rich phase were observed with the dextran with the higher molecular weight (Dx-100). For a fixed composition, the highest value obtained for the partition coefficient of vanillin is 5.53 whereas the improved recovery in the top phase is 71.61 %, and obtained with Dx-100. Moreover, the Dx-100 concentration and temperature were also optimized towards the improvement on the partition process. The highest value of K_{van} (7.66) was found using an ATPS composed of 18 wt% of Dx-100 and 30 wt% of acetonitrile at 15ºC, with a respective recovery in top phase of 70.65%. The vanillin partitioning process for the ACN-rich phase is spontaneous for all temperatures and an exothermic or endothermic process depending on the temperature range.

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Supporting Information

Aqueous Two-Phase Systems formed by Biocompatible and Biodegradable Polysaccharides and Acetonitrile

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Table S1. Experimental binodal mass fraction data for the system composed of dextran 6,000 mol.kg^{-1} (1) + acetonitrile (2) + water (3) at 25ºC.

100 w_1	100 w_2	100 w_3
32.2040	16.8375	50.9585
31.3175	17.1719	51.5106
30.2099	17.9849	51.8052
29.3740	18.3616	52.2644
28.4140	18.9891	52.5969
27.7757	19.0488	53.1755
26.8395	19.7613	53.3992
26.1852	19.9462	53.8687
25.4496	20.4954	54.0550
24.7855	20.9885	54.2260
24.2795	21.0818	54.6386
23.7207	21.5690	54.7103
23.1949	21.8157	54.9894
22.6280	22.1246	55.2474
22.0555	22.4245	55.5200
21.4990	22.9416	55.5595
21.0266	23.2169	55.7564
20.5571	23.5258	55.9171
20.1986	23.5553	56.2461
19.6343	24.0264	56.3394
18.9435	24.3827	56.6738
18.5131	24.7763	56.7107
17.8044	25.2232	56.9724
17.1434	25.5229	57.3337
16.7973	25.7924	57.4103
16.2746	26.0609	57.6645
15.8154	26.3754	57.8092
15.3049	26.6437	58.0514
15.0319	26.7661	58.2020
14.7502	26.8956	58.3542
14.3067	27.1258	58.5675
13.9284	27.4384	58.6332
13.5929	27.5354	58.8717
13.3815	27.7077	58.9108
12.9822	27.9134	59.1044
12.8221	28.1030	59.0749
12.5140	28.1516	59.3344
12.3301	28.3786	59.2913
12.1171	28.5311	59.3518
11.9128	28.7742	59.3131
11.5896	29.0422	59.3682
11.2822	29.1854	59.5324
10.9563	29.2556	59.7881
10.6784	29.3972	59.9244
10.5339	29.5432	59.9229

10.3253	29.7247	59.9500
10.0122	29.8379	60.1500
9.6888	29.9883	60.3229
9.3981	30.1980	60.4038
9.0788	30.3454	60.5758
8.7727	30.5252	60.7021
8.4144	30.6962	60.8894
8.0883	30.8870	61.0247
7.8136	31.0529	61.1335
7.5200	31.3453	61.1347
7.1570	31.4479	61.3951
6.7840	31.7105	61.5055
6.4164	32.0613	61.5223
5.9411	32.1852	61.8737
5.5866	32.4464	61.9670
5.2423	32.9482	61.8094
4.8324	33.1558	62.0118
4.5201	33.4165	62.0634
4.2500	33.6442	62.1058
4.0084	33.8916	62.1000
3.7605	33.9021	62.3374
3.5886	34.1002	62.3112
3.4078	34.3070	62.2852
3.1976	34.6081	62.1944
3.0260	34.6223	62.3517

Table S2. Experimental binodal mass fraction data for the system composed of dextran 40,000 mol.kg^{-1} (1) + acetonitrile (2) + water (3) at 25ºC.

100 w_1	100 w_2	100 w_3
32.2988	14.8878	52.8134
31.3878	15.2256	53.3865
30.6761	15.4548	53.8691
29.8601	15.8532	54.2867
29.0710	16.0237	54.9053
28.3490	16.5715	55.0795
27.6416	16.6607	55.6978
27.0097	16.8238	56.1665
26.4573	16.9708	56.5718
25.8736	17.1412	56.9852
25.2441	17.4049	57.3510
24.6611	17.7855	57.5534
24.0607	18.3556	57.5837
23.2411	18.1706	58.5883
22.6665	18.6058	58.7277
22.2294	18.6933	59.0772
21.8899	18.8050	59.3051
21.4648	19.0678	59.4674
20.9943	19.4219	59.5838
20.3499	19.6413	60.0087
20.0202	19.7222	60.2576
19.5951	20.1914	60.2135
19.3231	20.3506	60.3263
19.0256	20.4591	60.5153
18.7066	20.7536	60.5398
18.3750	20.9504	60.6746
18.0629	20.9814	60.9557
17.6202	21.3064	61.0734
17.2008	21.3761	61.4231
16.9274	21.5179	61.5547
16.4819	21.6009	61.9172
16.1504	21.9366	61.9131
15.6448	22.0906	62.2645
15.1263	22.5945	62.2792
14.4411	22.5488	63.0101
13.8667	22.7500	63.3833
13.4656	23.0569	63.4776
12.9244	22.9977	64.0780
12.4616	23.4149	64.1234
11.9077	23.5117	64.5805
11.4225	23.7030	64.8746
10.9117	23.8221	65.2662
10.5294	24.0501	65.4205
10.2270	24.1675	65.6055
9.7797	24.6152	65.6051

9.3397	24.4245	66.2359
8.8777	24.5945	66.5278
8.4839	24.8763	66.6398
8.0907	25.0684	66.8409
7.7583	25.0109	67.2308
7.4054	25.5183	67.0763
6.9214	25.4461	67.6326
6.5283	25.7534	67.7183
6.1497	25.8059	68.0444
5.8631	26.2031	67.9338
5.5067	26.2405	68.2528
5.2028	26.6719	68.1253
4.7871	26.0882	69.1247
4.5604	26.4775	68.9622
4.3197	26.7255	68.9548

Table S3. Experimental binodal mass fraction data for the system composed of dextran 100,000 mol.kg^{-1} (1) + acetonitrile (2) + water (3) at 25ºC.

100 w_1	100 w_2	100 w_3
32.9719	13.9675	53.0606
32.1178	14.3772	53.5050
31.5054	14.6154	53.8792
30.8137	15.0742	54.1121
30.0700	15.4096	54.5204
28.2887	15.9149	55.7965
27.6788	16.2606	56.0606
27.1088	16.5914	56.2998
26.5606	16.9515	56.4879
26.0678	17.1978	56.7344
25.5378	17.5412	56.9210
24.5902	17.9474	57.4624
24.2457	18.2817	57.4726
23.7962	18.4711	57.7327
23.3842	18.6904	57.9254
22.9586	18.7721	58.2693
22.5756	18.9201	58.5043
22.2057	19.0821	58.7122
21.7943	19.3907	58.8150
21.4237	19.5413	59.0350
21.0558	19.6603	59.2839
20.7298	19.8662	59.4040
20.3990	20.0266	59.5744
19.7847	20.3022	59.9131
19.4618	20.4656	60.0726
18.9618	20.4398	60.5984
18.6823	20.5600	60.7577
18.3990	20.7011	60.8999
18.1545	20.8064	61.0391
17.9236	20.9780	61.0984
17.6806	21.0617	61.2577
17.4446	21.1948	61.3606
17.1977	21.3225	61.4798
16.9561	21.4346	61.6093
16.7057	21.5757	61.7186
16.2425	21.8046	61.9529
15.8869	21.7700	62.3432
15.6943	21.8852	62.4206
15.1020	22.1848	62.7132
14.4243	22.5569	63.0188
13.3307	22.8001	63.8692
13.0390	22.9242	64.0369
12.7718	23.0367	64.1915
11.9397	23.5187	64.5417
11.2252	23.5369	65.2379

10.8612	23.8648	65.2740
10.6601	24.0040	65.3358
10.3944	24.1338	65.4718
10.0691	24.1834	65.7475
9.7113	24.4219	65.8669
9.5316	24.5060	65.9624
9.3085	24.5451	66.1463
8.9961	24.7003	66.3036
8.6664	24.6949	66.6387
8.3792	24.7602	66.8606
8.1271	24.6738	67.1991
7.9324	24.8401	67.2274
6.6760	25.1077	68.2163
6.4954	25.1035	68.4011
6.3247	25.1782	68.4971
6.1326	25.3715	68.4958
6.0011	25.3516	68.6473
5.8867	25.3966	68.7168
5.7562	25.3153	68.9284
5.6592	25.3743	68.9665
5.5753	25.4513	68.9734
5.4773	25.6025	68.9202
5.3781	25.5703	69.0516
5.2860	25.5703	69.1437
5.1839	25.5787	69.2374
5.1104	25.6165	69.2731
4.9309	25.6716	69.3976
4.8057	25.6343	69.5600
4.6840	25.9440	69.3720
4.2287	25.9539	69.8173
4.1020	25.8852	70.0128
3.9912	25.9078	70.1010
3.9005	26.0956	70.0039
3.7419	26.1159	70.1422
3.5739	26.3764	70.0497
3.4180	26.3001	70.2819
3.1839	26.5008	70.3153
2.6938	26.5714	70.7348

Table S4. Effect of the dextran molecular weight on K_{van} and R_T for the system composed of dextran-acetonitrile (15-30 wt%) at 25 ºC.

DEXTRAN / (MOL.KG^{-1})	K_{VAN}	R_T / (%)
6,000	1.09 ± 0.10	33.69 ± 1.85
40,000	1.12 ± 0.15	35.97 ± 5.48
100,000	5.53 ± 0.55	71.61 ± 1.16

Table S5. Effect of the dextran concentration on K_{van} and R_T for the system composed of Dx-100 (15-20 wt%) + acetonitrile (30 wt%) at 25 ºC.

DEXTRAN / (WT%)	K_{VAN}	R_T / (%)
15	5.53 ± 0.55	71.61 ± 1.16
17	7.03 ± 0.35	76.95 ± 0.37
18	6.68 ± 0.23	76.58 ± 0.18
20	6.46 ± 0.04	77.07 ± 1.14

Table S6. Effect of temperature on K_{van} and R_T for the system composed of Dx-100-acetonitrile (18-30% wt%).

DEXTRAN / (WT%)	K_{VAN}	R_T / (%)
5	6.30 ± 0.28	76.56 ± 1.79
15	7.66 ± 1.35	75.02 ± 6.17
25	6.68 ± 0.23	76.58 ± 0.18
35	5.40 ± 0.13	72.70 ± 1.91

Capítulo VI

Poly (vinyl alcohol) as a Novel Constituent to form Aqueous Two-Phase Systems with Acetonitrile: Phase Diagram and Partitioning Experiments

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Abstract

In this work it is shown, for the first time, that aqueous solutions of poly(vinyl alcohol) (PVA) and acetonitrile (ACN) undergo liquid-liquid demixing and form an aqueous two-phase system (ATPS). Ternary phase diagram (PVA, acetonitrile and water) and respective tie-lines was determined at 25ºC. ACN is enriched in the top phase while PVA is found in the bottom phase. To explore the potentiality of this ATPS for extraction strategies, the partitioning of vanillin among the coexisting phases was further evaluated. The effects of ACN and PVA concentrations, pH and temperature toward the vanillin partitioning were also investigated. An increased vanillin partitioning to the top phase was verified with the increase of the ACN concentration; however, the partition coefficient of vanillin for the ACN-rich phase decreases with the increase on both the PVA concentration and temperature. The pH value, at least in the range considered, is not relevant through the vanillin preferential migration. The highest partition coefficient of vanillin for the ACN-rich phase (2.24) was found with the system composed of 49 wt% of ACN + 9 wt% of PVA at 4.7 pH and 5ºC, with a recovery of 79%. The vanillin migration to the top phase is spontaneous and an exothermic process.

Keywords: aqueous two-phase system, acetonitrile, poly(vinyl alcohol), vanillin, partition.

1. Introduction

Poly(vinyl alcohol) (PVA) is a non-toxic, amorphous and biodegradable synthetic high-κ-polymer presenting, in addition, a randomly coiled and highly flexible chain when in solution (Sengwa and Sankhla, 2007). This polymer is obtained by hydrolysis of polyvinyl acetate and its properties depend on the polymerization and hydrolysis conditions, as well as on the drying and grinding (Krumova et al., 2000). PVA is a water soluble polymer with interesting properties, such as a low cost, good surface alignment effects, and an excellent film forming, emulsifying and adhesive properties (Chou et al., 2010). Due to these features, PVA is broadly used as a thickening, emulsifying or film-forming agent or as an adhesive in many household and industrial applications, especially in the paper, textile and chemical industries (Marusincova et al., 2013). In addition, PVA is usually employed as a phase forming-component in aqueous two-phase systems (ATPS) when combined with inorganic salts (Wu et al., 2001).

Acetonitrile, CH_3CN, also known as cyanomethane or methyl cyanide, is an aprotic and polar organic solvent miscible with water in all proportions (Mandal et al., 2011). The ACN molecules do not strongly interact with themselves and leave a hydrogen-bond network formed by water (Takamuku et al., 1998). ACN is also a by-product from the manufacture of acrylonitrile (Pollak et al., 2000), which is widely used by industry in the production of perfumes, rubber products, pesticides or pharmaceuticals (Zhang et al., 2011), or as a mobile phase in reverse phase high performance liquid chromatography (HPLC) (Taha et al., 2012; Gu and Shih, 2004).

ATPS have been divided into two categories: those which contain two polymers, such as polyethylene glycol (PEG) + dextran (Karakatsanis and Liakopoulou-Kyriakides, 2007; Chen and Lee, 1995) and PEG + maltodextrin (Silva and Meirelles.,

2000) systems, and those formed with a polymer and a salt, such as PEG-phosphate-based (Silva et al., 2013; Lima et al., 2002, Köhler et al., 1991) and PEG-citrate-based salts (Neves et al., 2012; Porto et al., 2008). However, many other pairs of compounds can be used to form ATPS, especially alcohol + salt (Reis et al., 2012), ionic liquid + salt (Ventura et al., 2012b; Neves et al., 2009; Gutowiski et al., 2003), ionic liquid + PEG (Pereira et al., 2013a; Freire et al., 2012) and ionic liquid + carbohydrate (Freire et al., 2011) mixtures. Recently, it was demonstrated that acetonitrile (ACN) can also form ATPS when combined with carbohydrates (Cardoso et al., 2013) or polyols (Cardoso et al., 2014).

ATPS can be an alternative option for extraction, separation and purification purposes over conventional systems which usually require organic solvents (Martínez-Aragón et al., 2009). Moreover, ATPS are easy to scale up, present low cost and typically lead to a high product purity as well as to a high yield, while maintaining the biological activity of the molecules due to their water-rich environment (Lu et al., 2013; Rito-Palomares et al., 2000). These systems have been used in the recovery and purification of many biomolecules, namely enzymes (lipase - Ventura et al., 2012a; Barbosa et al., 2011; Souza et al., 2010), antioxidants (rutin – Reis et al., 2014; gallic acid – Cláudio et al., 2012), alkaloids (caffeine – Cláudio et al, 2013; theobromine, theophyline, nicotine and caffeine – Passos et al., 2013), antibiotics (tetracycline – Pereira et al., 2013b; Wang et al., 2010) and antibodies (Azevedo et al., 2009; Samatou et al., 2007).

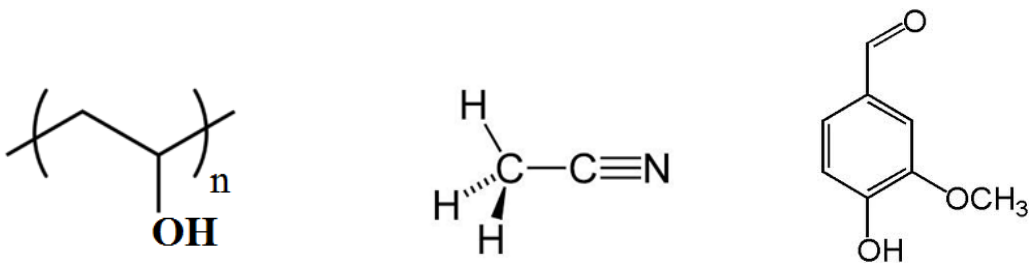
This work addresses novel ATPS based on acetonitrile and poly(vinyl alcohol). The corresponding ternary phase diagram, tie-lines and tie-line lengths at 25ºC were firstly determined. Further, its potential application to extract or separate a model antioxidant – vanillin (3-methoxy-4-hydroxybenzaldehyde) – was evaluated. This

biomolecule is used here as a standard biomolecule and representative of the phenolic compounds with antioxidant properties. Vanillin is the major component of natural vanilla and it is widely used as a flavoring material in confectionery, food products, beverages, perfumes and in pharmaceutical preparations (Walton et al., 2003).

2. Material and Methods

2.1. Materials

The ATPS studied in this work was formed by poly(vinyl alcohol) (M_w = 9,000-10,000 g.mol^{-1}) and acetonitrile. All reagents were purchased from Sigma-Aldrich: poly(vinyl alcohol) 80% hydrolysed (> 99.5 wt% pure), acetonitrile (HPLC grade with a purity of 99.9 wt%) and vanillin (> 99 wt% pure). The chemical structures of the phase-forming components of the ATPS and of the target biomolecule used in the partitioning experiments are shown in Figure 1. Distilled and deionized water was used in all experiments.



(a) (b) (c)

Figure 1. Chemical structures of the phase-forming components used in the ATPS formation and of the biomolecule used as a partitioning solute:(a) poly(vinyl alcohol); (b) acetonitrile; (c) vanillin.

2.2. Phase diagram and tie-lines

The ternary phase diagram for poly(vinyl alcohol) and acetonitrile was determined at (25 ± 1) ºC and atmospheric pressure by the cloud point titration method. Stock solutions of poly(vinyl alcohol) (15 wt%) and acetonitrile (80 wt%) were previously prepared and used for the determination of the phase diagram. Repetitive drop-wise addition of the PVA solution to the aqueous solution of acetonitrile was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultra-pure water until the detection of a monophasic region (clear and limpid solution). These additions were carried out under continuous stirring and the saturation curves were determined gravimetrically within ± 10^{-5} g.

The tie-lines (TLs) were obtained through a gravimetric method originally described by Merchuck et al. (1998). Several mixtures at the biphasic region of the ternary system were prepared, vigorously stirred, and allowed to reach equilibrium and phase separation, for a minimum of 18 h at (25 ± 1) ºC. After the equilibration step, the top and bottom phases were carefully separated and weighted within ± 10^{-5} g. Each individual TL was determined by the application of the lever-arm rule, which describes the relationship between the weight of the top phase and the overall system weight and composition. For that purpose, the binodal curves were correlated using equation 1,

$$Y = A\exp\{(B \times X^{0.5} - (C \times X^{3})\} \quad (1)$$

where Y and X are the acetonitrile and poly(vinyl alcohol) weight fraction percentages, respectively, and A, B and C are constants parameters obtained by the regression of the experimental binodal data.

The determination of the TLs was then accomplished by solving the following system of four equations (equations 2 to 5) for the four unknown values of Y_T, Y_B, X_T and X_B,

$$Y_{\mathrm{T}} = A\exp\{(B \times X_{\mathrm{T}}^{0.5} - (C \times X_{\mathrm{T}}^{3})\} \quad (2)$$

$$Y_{\mathrm{B}} = A\exp\{(B \times X_{\mathrm{B}}^{0.5} - (C \times X_{\mathrm{B}}^{3})\} \quad (3)$$

$$Y_T = \left(Y_M / \alpha\right) - \left(\left(1-\alpha\right)/\alpha\right)Y_B \quad (4)$$

$$X_T = \left(X_M / \alpha\right) - \left(\left(1-\alpha\right)/\alpha\right)X_B \quad (5)$$

where the subscripts *M*, *T* and *B* denote, respectively, the initial mixture, and the top and bottom phases. The value of α is the ratio between the weight of the top phase and the total weight of the mixture. The system solution results in the acetonitrile and poly(vinyl alcohol) concentration in the top and bottom phases, and thus, TLs can be directly represented.

The respective tie-line lengths (TLLs) were determined through the application of equation 6,

$$\mathrm{TLL} = \sqrt{(X_T - X_B)^2 + (Y_T - Y_B)^2} \quad (6)$$

2.3. Partitioning of vanillin

The liquid-liquid systems for the partitioning of vanillin were prepared in graduated glass centrifuge tubes weighing the appropriate amounts of poly(vinyl alcohol), acetonitrile and an aqueous solution containing vanillin (with an initial pH value adjusted with HCl 0.1 M or NaOH 0.1 M). Vanillin was at 0.4 $g.L^{-1}$ in the initial aqueous solution. After the complete mixing of all components for a given mixture composition, each system was centrifuged at 2,000 x *g* for 10 min to favour the phase separation, and then each tube was placed in a thermostatic bath at (5 to 35^{o}C) for at least 18 h. The volume of each phase was measured and both phases were further separated for the quantification of vanillin and for the determination of their pH values.

At least three independent replicates were made and the average partition coefficients and associated standard deviations were therefore determined.

The pH values (± 0.02) of the top and bottom phases were measured at 25 ºC using a DIGIMED DM-20 pH meter.

The concentration of vanillin at each aqueous phase was quantified through UV-spectroscopy, using a Varian Cary 50 Bio UV-Vis spectrophotometer, and at a wavelength of 280 nm using a calibration curve previously established.

The partition coefficient of vanillin was determined taking into account the concentration of the antioxidant in each phase and according to,

$$K_{\text{van}} = \frac{C_{\text{T}}}{C_{\text{B}}} \tag{7}$$

where K_{van} is the partition coefficient of vanillin, C represents the vanillin concentration, and the subscripts T and B denote the top (acetonitrile-rich) and bottom (poly(vinyl alcohol)-rich) phases, respectively.

The recovery of vanillin (R_T) in the top phase was evaluated using equation 8,

$$R_{\text{T}} = \frac{100}{1 + \frac{1}{K_{\text{van}} \times R_v}} \tag{8}$$

where R_v is the ratio between the volumes of the top (V_T) and bottom (V_B) phase.

The standard molar Gibbs free energy of transfer ($\triangle_{tr}G_o^m$), the standard molar enthalpy of transfer ($\triangle_{tr}H_o^m$) and standard molar entropy of transfer ($\triangle_{tr}S_o^m$) associated with the vanillin partition coefficient were determined by the van't Hoff approach at different temperatures (5 to 35ºC). The following isochors were used to determine the molar thermodynamic functions of transfer (equations 9-11):

$$\ln K_{van} = -\Delta_{tr}H_m^o \times \frac{1}{T} + \frac{\Delta_{tr}S_m^o}{R} \tag{9}$$

$$\Delta_{tr}G_m^o = \Delta_{tr}H_m^o - T\Delta_{tr}S_m^o \quad (10)$$

$$\Delta_{tr}G_m^o = -RT\ln(K_{van}) \quad (11)$$

where T is temperature (Kelvin) and R is the ideal gas constant. The enthalpy and entropy contributions can be directly deduced from the linear approximation of $\ln(K_{van})$ *versus* T^{-1}.

3. Results and Discussion

3.1. Phase diagram and tie-lines

A remarkable number of phase diagrams can be found in literature for ATPS; however, this is the first evidence for an ATPS based on acetonitrile and PVA and its further application in the extraction of vanillin.

The solubility of a given solute in water is affected by the presence of other species that can act either as salting-in or salting-out agents. The high solubility of PVA in water is mainly due to hydrogen-bonding between water and the -OH groups of the polymeric chain (Sengwa and Sankhla, 2007).

The experimental phase diagram for acetonitrile and PVA was determined at 25ºC and atmospheric pressure. The experimental weight fraction data are provided in the Supporting Information (Table S1). The corresponding phase diagram is depicted in Figure 2 and allows the analysis of the PVA potential to induce the liquid-liquid demixing. In general, for any mixture within the biphasic envelope it is visible that high amounts of water are present, supporting thus the high potential of the solutes pair for phase separation in aqueous media. It should be remarked that for the studied system, the PVA is enriched at the bottom phase while acetonitrile is enriched at the top phase.

The experimental binodal data were fitted using the empirical relationship described by equation 1. The fitted parameters *A*, *B* and *C* (estimated by least-squares regression) and the corresponding standard deviations (σ), as well as the regression coefficient (R^2), are presented in Figure 2. As can be seen by the correlation coefficient obtained, equation 1 provides an accurate description of the experimental binodal saturation curve.

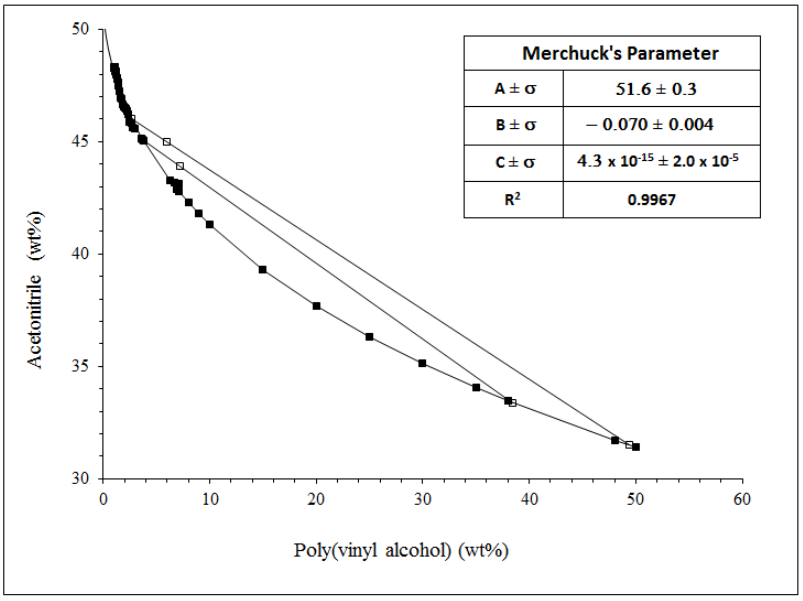


Figure 2. Phase diagram the for ternary system composed of acetonitrile + poly(vinyl alcohol) + water at 25 ºC and atmospheric pressure. ■, experimental solubility data; □, TL data; —, fitting by equation 1.

To complete the phase diagram, several TLs and the respective TLLs were further calculated and their values are given in Table 1. The TLs are also represented in Figure 2. The TL data allow the knowledge of the coexisting phases' compositions for any mixture point along the same TL.

Table 1. Mass fraction composition for the TLs and respective TLLs, at the top (T) and bottom (B) phases, and initial biphasic composition of the mixture (M), for the systems composed of acetonitrile (*Y*) and poly(vinyl alcohol) (*X*) at and pH 4.7.

WeigthFraction /(wt%)						
Y_M	X_M	Y_T	X_T	Y_B	X_B	TLL
44.99±0.02	6.01±0.17	46.04±0.02	2.63±0.38	31.50±0.03	49.41±0.02	48.99
43.92±0.02	7.18±0.14	45.11±0.02	3.66±0.27	33.38±0.03	38.45±0.03	36.72

3.2. Partitioning of vanillin

In order to evaluate the potential of the studied ATPS to be applied as an extractive system, it was further determined the partition coefficient and the recovery of vanillin at the acetonitrile-rich phase. The effects of poly(vinyl alcohol) and acetonitrile concentrations, the initial pH value of the aqueous solution of vanillin and temperature were also ascertained.

3.2.1. Influence of the PVA concentration

The effect of the PVA initial concentration (6 to 9 wt%) on the partitioning of vanillin (K_{van}) was studied and the results are depicted in Figure 3.

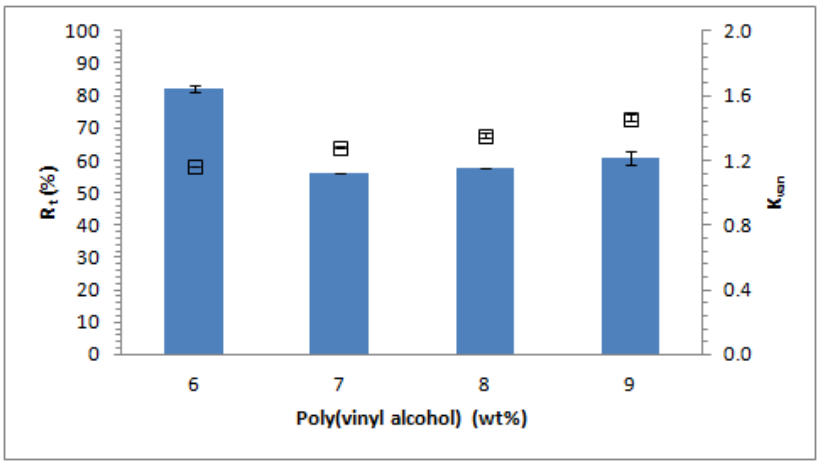


Figure 3. Influence of the poly(vinyl alcohol) concentration in the partition coefficient of vanillin (K_{van}) (symbols) and in the recovery of vanillin (R_t) (bars) in the ATPS composed of45 wt% of acetonitrile at pH 6.7 and 25 ºC.

For this study, the concentration of ACN was maintained constant at 45 wt% as well as the temperature, 25ºC, and initial pH (4.7). It can be observed that in all the conditions vanillin preferentially partitions for the ACN-rich phase (K_{van} > 1.0). The partition coefficient for vanillin increases with an increase in the PVA concentration maybe due to a salting-out effect exerted by PVA. This trend is in close agreement with our previous findings in ATPS composed of ACN and carbohydrates or polyols and where an increase on the –OH groups or their concentration favours the partitioning of vanillin for the opposite phase (Cardoso et al, 2014; Cardoso et al., 2013). According to Babu et al. (2008) the volume occupied by the polymer increases with an increase in the polymer concentration and which results in a reduced space to accommodate biomolecules - volume exclusion effect. Bassani et al. (2007) observed the volume exclusion effect when partitioning lipase using an ATPS formed by PEG 10,000 $g.mol^{-1}$ and potassium phosphate at pH 7.0 and 8 ºC. This phenomenon can also support the behaviour observed with the partitioning of vanillin as result of the polymer concentration. The recovery of vanillin in the ACN-rich phase ranges between 56.27 and 82.17%. Since the recovery values are dependent on the volume of the phases, the partition coefficients trend doesn't directly translate on the recoveries. In fact, the highest recovery of vanillin in the ACN-rich phase was observed with the system composed of 6 wt% of PVA.

3.2.2. Influence of the acetonitrile concentration

To analyze the effect of the ACN concentration, a series of ATPS containing 9 wt% of poly(vinyl alcohol) and 45-51 wt% of ACN, at 25ºC and pH 4.7, were assessed. The results obtained are depicted in Figure 4.

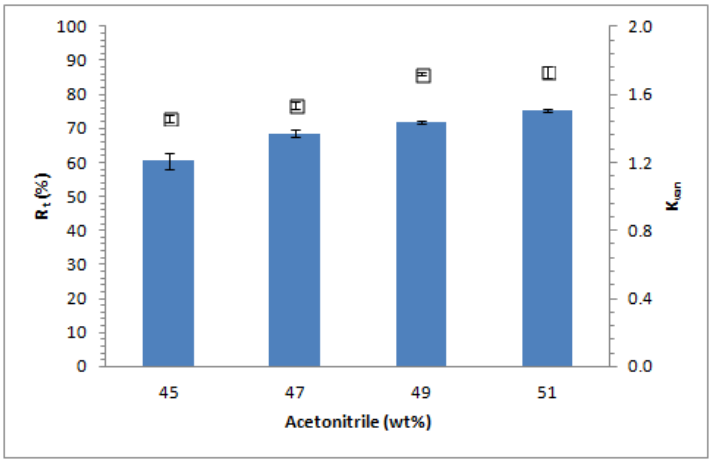


Figure 4. Influence of the acetonitrile concentration on the partition coefficient of vanillin (K_{van})(symbols) and in the recovery of vanillin (R_t) (bars) in the ATPS composed of9 wt% of poly(vinyl alcohol) at pH 6.7 and 25 ºC.

Acetonitrile presents hydrophobic characteristics and when its concentration increase in the mixture point the partition coefficient of vanillin also increases. Vanillin displays a higher affinity for more organic and hydrophobic phases as revealed by its octanol-water partition coefficient (log K_{ow} = 1.19 – Noubigh et al., 2010). The K_{van} reaches a value of 1.5 when using 51 wt% of ACN (Figure 4), a value very close to that found using 49 wt% of ACN. The recoveries of vanillin in the top phase also increase with the increase on the amount of ACN and reach a maximum value of about 80%. Cardoso et al. (2013) previously observed that vanillin preferentially migrates for the more hydrophobic phase (acetonitrile-rich phase over the carbohydrate-rich phase) with K_{van} > 3.0 and recoveries of vanillin ranging between 73 and 95%, agreeing with the results obtained here.

3.2.3. Influence of the initial pH

In order to study the influence of the pH of the aqueous medium (from 2.7 to 6.7) on the vanillin partitioning, the system constituted by 49 wt% of ACN and 9 wt%

PVA was used. The experiments were performed at 25ºC and the results are presented in Figure 5.

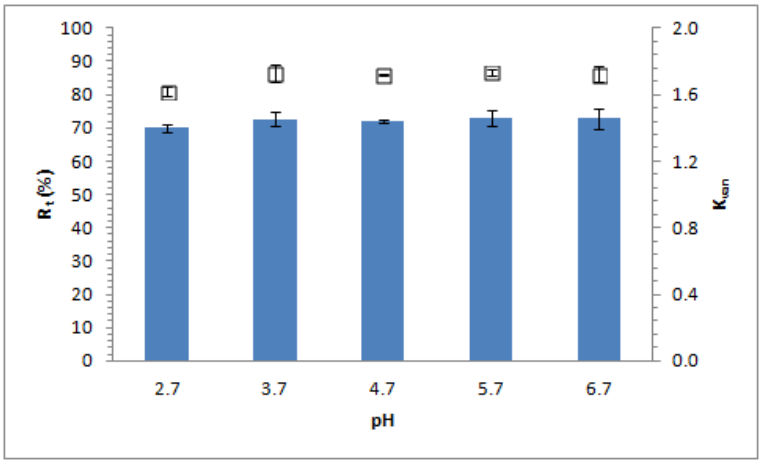


Figure 5. Influence of the initial pH on the partition coefficient of vanillin (K_{van}) symbols) and in the recovery of vanillin (R_t) (bars) in the ATPS composed of 9 wt% of poly(vinyl alcohol) and 49 wt% of acetonitrile at 25 ºC.

According to Albertsson (1986) the partition coefficient of a charged species depends on an electrostatic term ($z_pF\Delta\varphi/RT$), as described by equation 12,

$$\ln K_e = \ln K_o + \frac{z_p F \Delta\varphi}{RT} \qquad (12)$$

where K_e is the partition coefficient of a target biomolecule which is associated with its surface charge, z_p, and with the electrostatic potential difference between the two phases, $\Delta\varphi$. K_o includes all the other factors affecting the biomolecules partitioning.

Although vanillin can suffer speciation as a function of pH, the pH range considered in this work was chosen to maintain vanillin as a neutral molecule - *cf.* Supporting Information (Figure S1) with the speciation diagram of vanillin. Therefore, the K_{van} values are almost constant and do not depend on initial pH value as revealed in Figure 5. The recoveries into the top phase are also similar in all the ATPS investigated ($70.0\% \leq R_t \leq 73.16\%$).

3.2.4. Influence of temperature

Based on the previous results, a system composed of 49 wt% of ACN + 9 wt% PVA and at pH 4.7 was selected for the study of the vanillin partitioning at different temperatures (5 to 35ºC). The results obtained are presented in Figure 6.

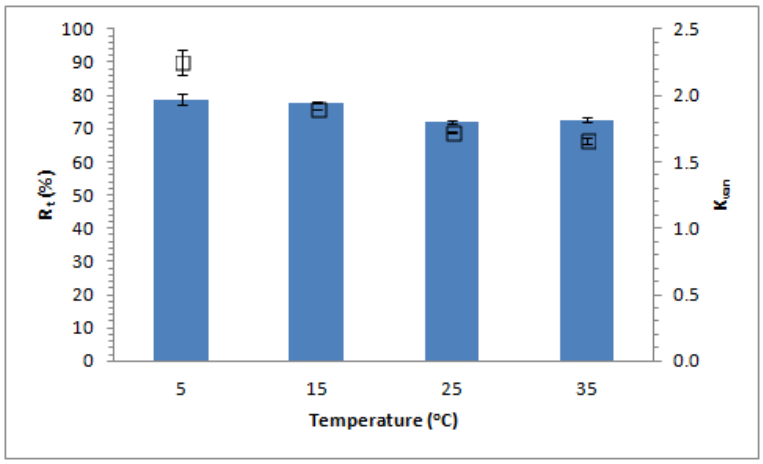


Figure 6. Influence of temperature on the partition coefficients of vanillin (K_{van})(symbols) and in the recovery of vanillin (R_t) (bars) in ATPS composed of 9 wt% of poly(alcohol) and 45 wt% of acetonitrile at pH 4.7.

The K_{van} and R_t decrease from 2.24 to 1.66 and from 78.84% to 71.93%, respectively, with the temperature increase from 5 ºC to 35 ºC. In general, an increase in temperature is not favorable for the partitioning of vanillin for the ACN-rich phase. This trend is in agreement with the data provided by Cláudio et al. (2010) where an increase in temperature also reduces the partitioning of vanillin for an ionic-liquid-rich phase. Saravanan et al. (2008) reported that the partition coefficients of proteins, such as myoglobin and ovalbumin, also decrease with an increase in the temperature of the system. However, temperature has a small influence on the distribution behavior of mulberry anthocyanins ($2.0 \leq K \leq 2.3$) in the temperature range from 25 ºC to 50 ºC.

Therefore, depending on the solute being partitioned, the increase on temperature can favor or not its preferential migration for a given phase.

The thermodynamic parameters involved in the partitioning of vanillin where obtained from the linear equation fitted for the experimental data. The obtained regression coefficient of $\ln(K_{van})$ *versus* T^{-1} is 0.937 and can thus support a reasonable determination of $\Delta_{tr}H_m^o$ and $\Delta_{tr}S_m^o$.

The $\Delta_{tr}G_m^o$ at 25ºC representative of the vanillin partitioning is negative ($\Delta_{tr}G_m^o$ = -1.41 KJ.mol^{-1}) meaning that the transfer process of vanillin from the PVA-rich to the ACN-rich phase is spontaneous. In fact, in all situations $K_{van} > 1$. In addition, the transference of vanillin is an exothermic process ($\Delta_{tr}H_m^o$ = -7.22 KJ.mol^{-1}). However, the enthalpic contribution is also relevant to the partitioning process ($\Delta_{tr}S_m^o$ = -19.50 J.mol^{-1}.K^{-1}).

4. Conclusion

In this work it was shown that novel ATPS can be formed by combining acetonitrile and PVA, at specific concentrations, in aqueous media. The ternary phase diagram, and respective tie-lines and tie-line lengths were determined at 25ºC and atmospheric pressure. Different system compositions of this novel ATPS were then used to study the partitioning behavior of vanillin. In all situations, vanillin preferentially partitions for the acetonitrile-rich phase (top phase). An increase in the PVA concentration leads to higher partition coefficients of vanillin where an increase in the ACN concentration results in the opposite behavior. The pH of the aqueous medium has no significant influence on the partition coefficients since the range of pH values considered guaranteed that vanillin was majorly present as a non-charged species. Furthermore, an increase in temperature leads to a decrease on K_{van}. The highest K_{van}

and R_t values occurred in the ATPS composed of 49 wt% of ACN + 9 wt% of PVA, at 4.7 pH and at 5 ºC (K_{van} = 2.24 ± 0.10 and R_t = 78.84 ± 1.52 %). The vanillin migration to top phase is a spontaneous and exothermic process.

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Supporting Information

Poly(vinyl alcohol) as a Novel Constituent to form Aqueous Two-Phase Systems with Acetonitrile: Phase Diagram and Partitioning Experiments

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Table S1. Experimental binodal mass fraction data for the system composed of acetonitrile (1) + PVA(2) + water (3) at 25ºC.

100 w_1	100 w_2	100 w_3
50.0003	31.4037	18.5960
48.0026	31.7199	20.2775
38.5561	33.3621	28.0818
35.6598	33.9236	30.4166
30.7432	34.9557	34.3011
25.2169	36.2630	38.5201
20.1254	37.6521	42.2225
15.4763	39.1402	45.3835
10.3598	41.1558	48.4844
9.6892	41.4630	48.8478
8.2735	42.1568	49.5697
7.1453	42.7627	50.0920
6.9270	42.8864	50.1866
6.7000	43.0175	50.2824
6.2788	43.2875	50.4336
3.7471	45.0592	51.1937
3.5817	45.1110	51.3073
2.9477	45.5536	51.4988
2.7878	45.6332	51.5790
2.6492	45.8455	51.5053
2.4816	45.8769	51.6415
2.3436	46.2112	51.4452

Table S1 (Cont.). Experimental binodal mass fraction data for the system composed of acetonitrile (1) + PVA (2) + water (3) at 25 ºC.

100 w_1	**100 w_2**	**100 w_3**
2.2144	46.3600	51.4256
2.1053	46.4726	51.4221
1.9846	46.4968	51.5187
1.8854	46.5353	51.5793
1.7942	46.6507	51.5551
1.7041	46.9151	51.3808
1.6042	46.9268	51.4689
1.5221	47.2432	51.2346
1.4582	47.4753	51.0665
1.3942	47.6330	50.9728
1.3287	47.7702	50.9011
1.2686	47.8410	50.8904
1.2173	47.9728	50.8100
1.1619	48.1065	50.7316
1.1104	48.1020	50.7876
1.0591	48.3011	50.6398
1.0161	48.3290	50.6549
0.9761	48.2702	50.7536

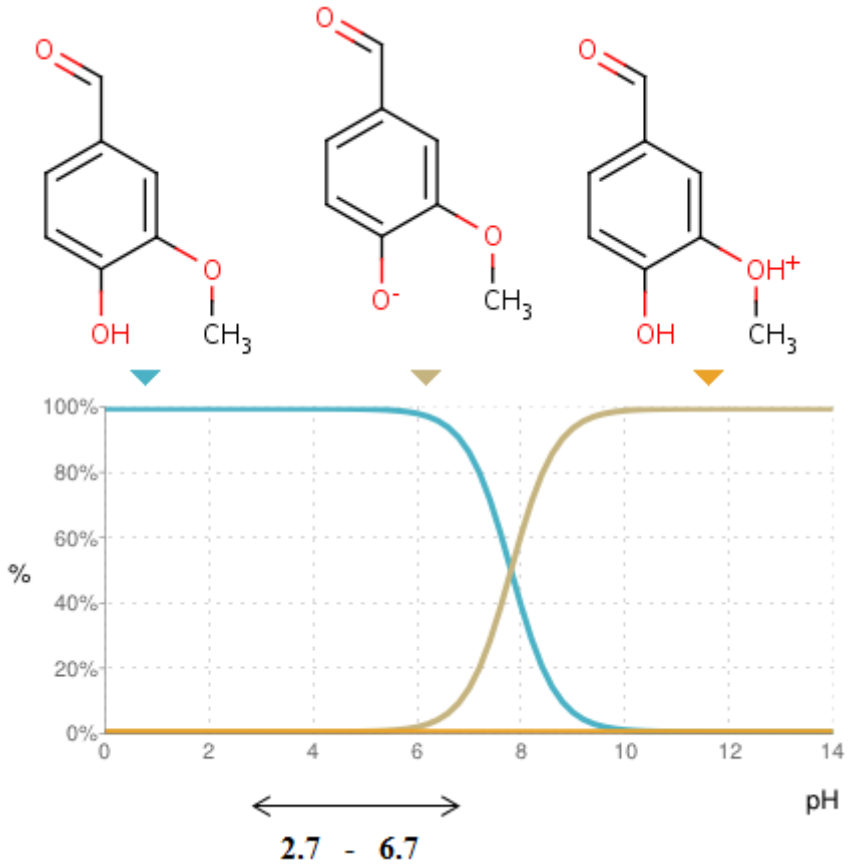

Figure S1. Chemical structure of vanillin at different pH values. This content was adapted from the Chemspider chemical database (http://www.chemspider.com/).

Capítulo VII

3. CONCLUSÕES

Os SABs formados por acetonitrila/carboidratos é um resultado da capacidade de hidratação de cada açúcar.

Carboidratos de elevada pureza se mostraram mais eficientes que açúcares comerciais de grau alimentar.

Este trabalho demonstra, pela primeira vez, que SABs podem ser formados por acetonitrila/polióis, acetonitrila/álcool polivinílico e acetonitrila e dextrana.

Os resultados obtidos indicam que na maioria dos casos quanto maior for o número de grupos hidroxila no poliol maior é o efeito "*salting-out*" observado.

A separação de duas fases é favorecida na seguinte ordem: glicerol < erithrytol < xilitol < sorbitol < maltitol.

O sistema composto por acetonitrila/dextrana revelou que quanto maior o seu peso molecular, melhor é a separação de fases, e os maiores são os valores para o coeficiente de partição e recuperação.

No sistema que continha acetonitrila/PVA o pH não mostrou influência significativa sobre o coeficiente de partição, enquanto que com o aumento da temperatura ocorreu a diminuição do coeficiente de partição.

A vanilina migrou preferencialmente para a fase rica em acetonitrila em todos os SABs (acetonitrila/carboidrato, acetonitrila/poliol, acetonitrila/dextrana, acetonitrila/PVA).

Nos sistemas formados por acetonitrila/carboidratos os coeficientes de partição foram superiores a 3,0, o sistema formado por acetonitrila/polióis apresentou coeficientes de partição entre 7 e 67 e que são dependentes da capacidade de "*salting-out*" de cada poliol.

O sistema formado por acetonitrila/dextrana, depois de otimizado (18 wt% Dx-100, 15ºC) apresentou um coeficiente de partição de 7,66 e recuperação de 70,65%. Com isso, podemos dizer que acima de 15ºC o processo é regido por contribuições entrópicas, enquanto que abaixo de 15ºC favorece a entalpia.

No sistema formado por acetonitrila/PVA o coeficiente de partição foi 2,24 e a recuperação foi de 78,84% para um pH de 4,7 e temperatura de 5 ºC.

A recuperação da vanilina na fase rica em acetonitrila mostrou-se superior a 90% em ambos os sistemas (acetonitrila/carboidrato, acetonitrila/poliol), apoiando o enorme potencial destes novos sistemas a serem explorados na extração dos mais diversos compostos de valor acrescentado.

Capítulo VIII

4. APRESENTAÇÃO DE TRABALHOS

Trabalhos em Congressos:

- Cardoso, G.B.; Mourão, T.; Pereira, F.M.; Freire, M.G.; Fricks, A.T.; Soares, C.M.F.; Coutinho, J.A.P.; Lima, A.S.. Aqueous biphasic systems composed of acetonitrile and sugar for the extraction of vanillin. In: International Congress of Chemical Engineering, 2012, Sevilla. Book of Abstract of International Congress of Chemical Engineering, 2012. p. T13-59-T13-59.
- Cardoso, G.B.; Mourão, T.; Pereira, F.M.; Freire, M.G.; Fricks, A.T.; Soares, C.M.F.; Coutinho, J.A.P.; Lima, A.S. Sugaring out effect in aqueous two-phase systems composed of acetonitrile and carbohydrate. In: 20th International Congress of Chemical and Process Engineering, 2012, Praga. CHISA Press 2012, 2012. p. 1-1.
- Cardoso, G.B.; Souza, I.N.; Pereira, F.M.; Soares, C.M.F.; Lima, A.S. Sistema aquoso bifásico composto por acetonitrila e polióis para a extração de vanilina. In: XIX SINAFERM, 2013, Foz do Iguaçu. Anais do XIX SINAFERM, 2013. v. 1. p. 1-4.

Artigo completo:

- Cardoso, G.B.; Mourão, T.; Pereira, F.M.; Fricks, A.T.; Freire, M.G.; Soares, C.M.F.; Lima, A.S. Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanilin. Separation and Purification Technology, v.104, p. 106-113, 2013.
- Cardoso, G.B.; Souza, I.N.; Freire, M.G.; Soares, C.M.F.; Lima, A.S. Novel Aqueous Two-Phase Systems Composed of Acetonitrile and Polyols: Phase Diagrams and Extractive Performance. Separation and Purification Technology, p. 54-60, 2014.

Capítulo IX

5. TRABALHOS FUTUROS

- Avaliar sistemas constituídos de acetonitrila/maltodextrina.

- Estudar a partição de outras biomoléculas dos sistemas propostos.

- Investigar outros modelos de correlação termodinâmica para os SAB's estudados.

- Explorar efeito da temperatura.

- Estudar modelos de reatores.

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