NOVEL AQUEOUS TWO-PHASE SYSTEMS FORMED BY BIOCOMPATIBLE AND BIODEGRADABLE POLYSACCHARIDES AND ACETONITRILE

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RESUMO –In this work, it is shown that aqueous two-phase systems can be formed by the combination of acetonitrile and polysaccharides, namely dextran biopolymers. The respective ternary phase diagrams were determined at 25 ºC for the systems composed of water + acetonitrile + dextran. The effect of the dextran molecular weight was also 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, namely 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.

1. INTRODUCTION

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 (Sidiqqui *et al*., 2014). The chemical structure of dextran is predominantly formed by 95% of linear α-(1 $\rightarrow$6) linkages as the main backbone and 5% of α-(1$\rightarrow$3) branch linkages (Vettori *et al* . , 2012). This homopolymer of glucose has been applied as a constituent in aqueous two-phase system - ATPS (Tubio *et al*., 2004).

ATPS are usually formed by mixing two polymers in aqueous media (PEG and Dextran - Karakatsanis and Liakopoulou-Kyriakides, 2007); or by one polymer and one salt (PEG and phosphate-based salts – Lima *et al*., 2002). However, some other pairs of phase-forming components can be used in the creation of alternative ATPS, such as alcohol + salt (Reis et al., 2012), ionic liquid + salt (Ventura et al., 2012).

In this work, dextran was used with acetonrile (ACN) to format the novel ATPS. Acetonitrile (CH$_3$CN) – ACN is an aprotic solvent, strongly polar, which is obtained as a by-product from the manufacture of acrylonitrile (Pollak *et al*., 2000). This solvent is miscible with water in all proportions (Mandal *et al*., 2011) and its molecules are unable to strongly associate with themselves leaving a hydrogen-bond network formed by water (Piekarski and Kubalczyk, 2005).

ATPS are widely applied in biotechnology for the isolation and purification of enzymes as lipase (Barbosa *et al*., 2011), antioxidants as rutin (Reis *et al.*, 2014), alkaloids as theobromine, theophyline, nicotine and caffeine (Passos *et al.*, 2013) and antibodies (Samatou *et al*., 2007). 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 (Gündüz, 2000).

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).

2. MATERIAL AND METHODS

2.1 Materials

The ATPS studied in this work were formed by dextran from *Leuconostoc* spp. (Mw = 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. Distilled and deionized water was used in all experiments.

2.2 Phase Diagrams

The ternary phase diagrams for water, acetonitrile and the different molecular weight dextran were determined at (25 ± 1) ^{o}C 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^{-5} 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 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. 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,

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

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 (RT) in the top phase was evaluated using equation 2,

$$R_{\mathrm{T}} = \frac{100}{1 + \dfrac{1}{K_{\mathrm{van}} \times R_{v}}} \tag{2}$$

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

3. RESULTS

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 25°C and atmospheric pressure. The binodal curves experimentally determined in molality units for an enhanced understanding on the impact of the distinct dextrans through the formation of ATPS, the diagrams are depicted in Figure 1.

The analysis of Figure 1 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 (Cardoso *et al.*, 2013) 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 Freire *et al.*, (2011), 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 (Cardoso *et al*., 2014) also demonstrated that polyols with more hydroxyl groups are more able to form ATPS with acetonitrile.

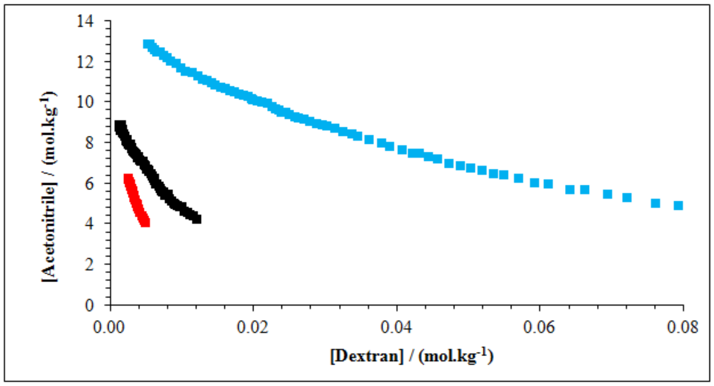

Figure 1. Binodal curves in molality units (a) and weight fraction (b) for the ternary systems composed of dextran + acetonitrile + water at 25 ºC and atmospheric pressure: 🟦- Dx-6; ⬛- Dx-40; and 🟥- Dx-100.

Figure 2 shows a general comparison between the strongest sugaring-out agents previously reported (a dissacharide and a polyol) (Cardoso *et al*., 2014; Cardoso *et al*., 2013), 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.

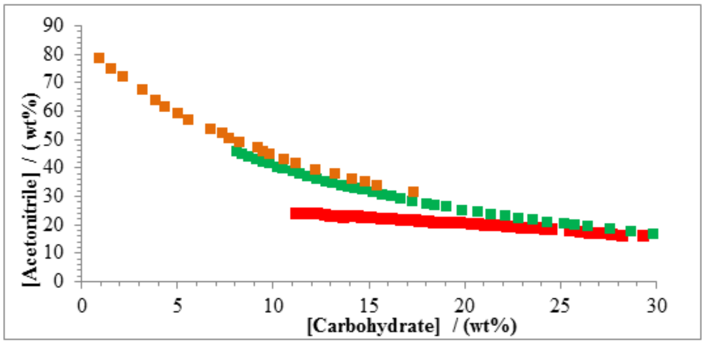

Figure 2. Binodal curves for the ternary systems composed of acetonitrile + (dextran -🟥, maltitol - 🟩 (Cardoso et al., 2014) or maltose - 🟧 (Cardoso et al., 2013) + water at 25 ºC and atmospheric pressure.

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. The results are depicted in Figure 3.

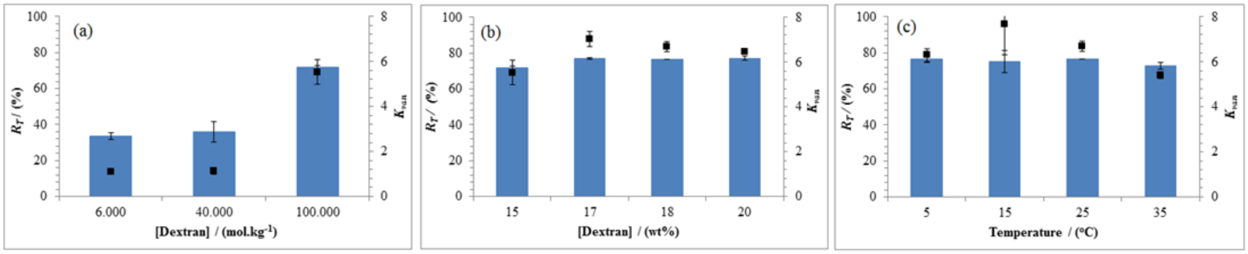

Figure 3. Effect of the dextran molecular weight (a), dextran concentration (b) and temperature (b) on the vanillin partitioning at 25 ^{o}C for the system composed of dextran-acetonitrile (15-30 wt%). Bars – recovery of vanillin (R_T) in the top phase; symbols – partition coefficient (K_{van}) of vanilin in the acetonitrile-rich phase.

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 25^{o}C. The partition coefficient of vanillin (K_{van}) and recovery of vanillin (R_T) in the top phase are shown in Figure 3 (a), which allows the inspection through the influence of the dextran molecular weight on the vanillin partitioning among the two phases.

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 (Albertsson, 1986), 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 (Bassani *et al.*, 2007) and protease (Yavari *et al.*, 2013). However, and as previously shown by us (Cardoso et al., 2014; Cardoso *et al.*, 2013), 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.

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 3(b). 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 Tubio *et al*., (2004) 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 (Reis *et al*., 2012).

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 35^{o}C, for the common mixture composition of 30 wt% of ACN + 18 wt% of Dx-100. The results obtained are depicted in Figure 3(c).

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 (Cláudio *et al*., 2010), and of acetonitrile + polyols ($7.16 < K_{van} < 66.58$) (Cardoso *et al*., 2014). However, the partition coefficients obtained in this work are similar to those reported by Cardoso *et al*., (2013) using ATPS formed by acetonitrile and carbohydrates ($3.06 < Kvan < 9.67$). The recovery of vanillin for all system ranges between 72.70 and 76.58 %.

4. CONCLUSION

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 vanillin partition. 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 15^{o}C, with a respective recovery in top phase of 70.65%.

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