Sugaring out for separation of acetonitrile and extraction of proteins and antibiotics
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ABSTRACT
In this study we report a novel method for separation of acetonitrile (ACN) and extraction of proteins and antibiotics from ACN-water mixtures. It was observed that addition of a monomeric saccharide or disaccharide into an ACN-water mixture results in phase separation with the upper phase rich in ACN. The extraction of selected proteins (bovine serum albumin, trypsin, and pepsin) and antibiotics (erythromycin, streptomycin, and nalidixic acid) was studied. High glucose concentration (16.5 %) and low temperature (6 °C) resulted in better separation and improved ACN recovery (up to 62 % w/w). The distribution of proteins and antibiotics in the upper phase and lower phase was determined by their hydrophobic/hydrophilic nature. After a sugaring-out phase partition, 95-100 % (w/w) of the hydrophilic proteins (bovine serum albumin, trypsin, and pepsin) were retained in the lower aqueous phase. Hydrophobic erythromycin was extracted in the ACN-rich phase with a concentration of up to 65 % (w/w). High concentrations of streptomycin (80–90 %, w/w) and nalidixic acid (91–94 %, w/w) were retained in the aqueous phase. The effective separation of proteins and antibiotics from ACN-water mixtures at a temperature close to room temperature and with potentially minimal damage to the products makes this method promising for large number of applications in the pharmaceutical and biotechnology industries.

Keywords: sugaring out; two phase separation; acetonitrile; liquid-liquid extraction

INTRODUCTION
Separation and purification of biological products involves a sequence of separation processes. Liquid-liquid extraction, salting out and reversed phase-high pressure liquid chromatography are typical processes in the separation of biomolecules especially proteins and biomolecules. Amongst these methods acetonitrile is preferred as a solvent or mobile phase because of its physicochemical properties like low viscosity, high resolution, and low boiling point, etc [1]. However, acetonitrile is miscible in all proportions with water and separating it from aqueous phase is a major problem. Its recovery is accomplished traditionally by distillation [2-3], cooling below subzero temperatures (-20 °C) [4-5], or salting out [6-8]. Distillation under vacuum is an energy intensive process [3] while the high temperatures encountered in atmospheric pressure distillations (>100 °C) pose problems to temperature sensitive biomolecules. In an effort to remove ACN from the effluent fraction of RP-HPLC, Gu et al [4] observed a phase separation at a subzero temperature of -17 °C where the top phase was rich in ACN (88 % v/v), with most of proteins (99 %) remained in the lower phase. Pence and Gu [9] studied the liquid-liquid equilibrium of ACN-water systems and obtained an ACN-rich upper phase containing 0.738 mass fraction of ACN at -18.6 °C. Other researchers have utilized salting out for the separation of ACN from a water mixture at a higher temperature. Le et al., [7] added a salt (NaCl, Na2SO4, or NH4Cl) to separate ACN and erythromycin from an aqueous solution at room temperature and the solvent-rich phase had an ACN concentration of 90 % (v/v) at NaCl of 10 %. Gu and Shih [8] tested the use of different salts (K2HPO4, KH2PO4, NaCl, NH4HCO3, and KCl) for the separation of ACN from an aqueous phase containing proteins (bovine serum albumin and myoglobin) at 4 °C and found that the best separation was achieved with K2HPO4. The limitations of salting out are high concentrations of salt, unwanted reactions [10], and destructive effect to some biological products [11]. Another major problem in application of salting out is the corrosion of equipment [12]. In addition, some salts (e.g., K2HPO4) may alter the pH and damage the product [8].

Sugaring out is a new phase separation method which uses sugar as a mass separating agent. It has been observed that the addition of a monomeric sugar or a disaccharide to an acetonitrile (ACN)-water mixture
created two phases, one solvent-rich and the other aqueous [13-14]. This method does not require a subzero temperature for phase partition. Moreover, the use of sugar as a phase separating agent does not alter environment conditions (e.g., pH). In this study we have explored this method for the separation of ACN from an aqueous phase and for the extraction and recovery of biomolecules (proteins: BSA, trypsin, pepsin and antibiotics: erythromycin, streptomycin, nalidixic acid). Our earlier experiments showed a phase separation at 1 °C for relatively low sugar concentrations (15-50 g/l) [13-14] and best phase separation results were obtained with glucose as a phase separating agent. In this study we have studied the effect of temperature and glucose concentration on sugaring out.

MATERIALS & METHODS

Chemicals
Glucose (certified ACS), HPLC-grade ACN (purity 99.9 %), and 1-butanol (99.9 %) were purchased from Fisher Scientific Co. (Pittsburgh, PA). Erythromycin (98 %) was purchased from Acros organics Pvt LTD. (Geel, Belgium) and biomolecules BSA (>96 %), trypsin, pepsin, streptomycin, and nalidixic acids of analytical grade were procured from Sigma Aldrich Chemicals (St. Louis, MO).

Sugaring-out phase separation and extraction of biomolecules
Glucose at a concentration of 105-165 g/l with a 15 g/l increment was dissolved in DI water at room temperature. The hydrophilic biomolecules (BSA, trypsin, pepsin, streptomycin, and nalidixic acid) were dissolved in the above sugar solutions while the erythromycin molecules were dissolved in ACN at a concentration of 10 mg/ml before adding sugar solution. Sugar solution (5 ml) containing the biomolecules (hydrophilic molecules) were mixed with 5 ml of ACN at a ratio of 1:1 (v/v) in 15 mL scale test tubes. For hydrophobic compound, 5 ml ACN containing erythromycin was mixed with 5 mL sugar solution. After gentle mixing of the components, tubes were placed in a water bath filled with DI water (Poly Science Digital Temperature Controller). Temperature of the water bath was controlled to ± 0.2 °C. Tubes were incubated at a fixed temperature (ranging from 6 °C to 18 °C with a 3 °C increment) for 24 h to reach the equilibrium. The upper phase and lower phase volumes were recorded after phase separation. Samples were collected using a disposable syringe and analyzed for solute (protein/antibiotic) concentration.

The results were expressed in terms of Phase ratio (PR) defined as the ratio of the upper phase volume to the lower phase volume and extraction (%) defined as the ratio of the amount of solute in the phase to the amount of solute added.

Analysis
Acetonitrile concentration in the upper phase was determined by a gas chromatograph unit (GC Hewlett Packard 5890 Series II, Avondale, PA) using DB-WAX (30 m × 0.250 mm × 0.25 µm) fused silica capillary column (J & W scientific, Agilent Technologies, Germany). The GC was equipped with a flame ionization detector (FID) and an auto sample injector (HP 7673A Automatic Injector). The oven temperature was programmed from 40 °C to 190 °C at 20°C min⁻¹. The injector and detector temperature was set to 220 °C and 250 °C, respectively. n-butanol was chosen as an internal standard. The carrier gas was He at a flow rate of 0.72 ml min⁻¹.

Protein concentrations in the ACN-rich phase were determined by a standard protein assay following the manufacturer’s protocol (Bio-Rad, CA, USA). The concentration of the antibiotics was estimated by taking the absorbance of ACN-rich phase at different wavelengths for each antibiotic. For erythromycin, streptomycin, and nalidixic acid, the λmax was 288 nm, 328 nm, and 326 nm, respectively. Standard solutions with concentrations of 2-10 mg/ml were prepared for each biomolecule for calibration in the UV detection.

RESULTS & DISCUSSION

Effect of temperature and glucose concentration on phase separation and acetonitrile recovery
Increasing temperature from 6 °C to 18 °C was accompanied by a gradual reduction in the upper phase volume and hence a reduction in the phase ratio (Fig. 1a). When the temperature was fixed, the upper phase volume and phase ratio increased with an increase in the glucose concentration. The effect of temperature on phase separation was more pronounced at low glucose concentration (105 g/l) than at high glucose concentration (165 g/l). The glucose concentration had little effect on phase separation at low temperature (6
No phase separation was observed for a glucose concentration of 105 g/l above 15 °C. Under the experimental conditions used in this study, the highest phase ratio (0.59 ± 0.017) was obtained at the highest glucose concentration (165 g/l) and lowest temperature (6 °C).

Although the phase ratio is an indicator of separation efficiency, it is not a quantitative measure to estimate the ACN recovery in the upper phase. Hence, the upper phase samples were analyzed for ACN concentration. A trend similar to the phase ratio was observed for ACN extraction. Increasing temperature lowered the ACN extraction efficiency (Fig. 1b). As the sugar concentration was increased, the amount of ACN in the upper phase increased. The maximum ACN recovery in the upper phase was 62 % which was achieved at the conditions for the highest phase ratio. At high sugar concentrations (16.5 %), the effect of temperature (6-18 °C) on phase separation and ACN recovery was less evident (53 -62 %). However, the effect of temperature at low sugar concentrations (10.5 %) is significant on ACN recovery (0 – 48 %). High sugar concentration and low temperature are desirable for maximum ACN recovery and better separation. Similar results were reported for salting out [7-8].

To study the relation between the phase ratio and ACN recovery, the phase ratio was plotted against the ACN recovered (Fig. 1c). As can be seen from Fig. 1c, the ACN recovery increases linearly (R² = 0.99) with phase ratio. Thus one can estimate the amount of ACN extraction from the phase ratio.

Phenomena of sugaring-out can be explained on the basis of interaction between glucose, acetonitrile and water molecules. Takamuku et al [15] reported that ACN molecules form three dimensional clusters and these clusters are surrounded by water molecule through hydrogen bonding and dipole-dipole interactions. Hydrogen bond network between ACN and water is enhanced when the mass fraction of ACN is less than 0.6. In the present study the mass fraction was always less than 0.6. It is thus proposed that sugar molecules may have replaced the hydrogen bonds between ACN and water molecules. This process may force ACN molecules out of the mixture resulting into a two phase formation. As a result, when sugar concentration is increased it results in an increase in upper phase (ACN-rich) volume as more ACN molecules are forced out.

At low temperatures, the ACN extraction increased for a fixed glucose concentration (Fig. 1b). This could be attributed to water structure at low temperatures. The degree of hydrogen bonding depends on temperature. As the temperature is lowered, the distance between nearest neighboring water molecules is decreased [16]. This means that the solute (ACN) will have a less chance to replace a water molecule at a lower temperature than at a higher temperature. Further, in an ACN-water-glucose mixture, glucose may replace the hydrogen bonds between ACN and water molecule. An outcome of this may be translated to a better phase separation in an ACN-water system when sugar is added at a low temperature.

Effect of temperature and glucose concentration on extraction of proteins and antibiotics

BSA, trypsin and pepsin were selected as model proteins in this study. All these proteins are hydrophilic in nature. It can be seen from Table 1 that more than 95 % of proteins are retained in the aqueous phase. An increase in the glucose concentration and temperature did not affect the protein extraction. No phase separation was observed at 18 °C and 105 g/l glucose. This shows that almost all the protein is retained in aqueous phase and very little protein (less than 2 %) is lost during the ACN separation process. Thus sugaring-out can be used for an effective recovery or purification of proteins from the ACN-containing effluent of a RP-HPLC process.

In the case of antibiotics, streptomycin, erythromycin and nalidixic acid were selected as model antibiotics for the study. The distribution of antibiotics was decided by its hydrophobic/hydrophilic nature. Erythromycin is hydrophobic whereas streptomycin and nalidixic acid are hydrophilic molecules. Table 2 shows the extraction of erythromycin in the ACN-rich phase and that of streptomycin and nalidixic acid in aqueous phase. No phase separation was observed at 18 °C and 105 g/l glucose. For erythromycin, a phase separation did not occur even at 120 g/l glucose at 18 °C. 80-90 % of the streptomycin was retained in the aqueous phase. Similarly, 91-94 % nalidixic acid was retained in the water-rich phase (Table 2). The glucose concentration and temperature had insignificant effect on the extraction of streptomycin and nalidixic acid (less than 10 %). On the other hand, 48-65 % of erythromycin was extracted in the ACN-rich phase. The erythromycin extraction increased with an increase in the glucose concentration at both temperatures. It
increased from 48% to 65% when the glucose concentration was increased from 105 g/l to 165 g/l at 6°C. Similarly, extraction increased from 0% to 59% when the glucose concentration was increased from 105 g/l to 165 g/l at 18°C.

**Figure 1.** The effects of temperature and glucose concentration on sugaring out (1a); The effects of temperature and glucose concentration on acetonitrile recovery (1b and 1c).

Table 1. Effect of temperature and glucose concentration on extraction of proteins (BSA, Pepsin, and Trypsin) (ACN: water =1:1).

<table>
<thead>
<tr>
<th>Protein Recovery in Aqueous Phase (% w/w)</th>
<th>Pepsin</th>
<th>Trypsin</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose Conc. g/l</strong></td>
<td><strong>6 °C</strong></td>
<td><strong>18 °C</strong></td>
<td><strong>6 °C</strong></td>
</tr>
<tr>
<td>105</td>
<td>98.69 ± 1.03</td>
<td>98.13 ± 0.5</td>
<td>98.13 ± 0.5</td>
</tr>
<tr>
<td>120</td>
<td>98.88 ± 0.2</td>
<td>97.98 ± 0.98</td>
<td>97.98 ± 0.98</td>
</tr>
<tr>
<td>135</td>
<td>99.08 ± 0.27</td>
<td>98.42 ± 0.03</td>
<td>98.42 ± 0.03</td>
</tr>
<tr>
<td>150</td>
<td>98.91 ± 0.19</td>
<td>98.38 ± 0.41</td>
<td>98.38 ± 0.41</td>
</tr>
<tr>
<td>165</td>
<td>99.56 ± 0.23</td>
<td>98.59 ± 0.63</td>
<td>98.59 ± 0.63</td>
</tr>
</tbody>
</table>

* - No phase separation

Table 2. Effect of temperature and glucose concentration on extraction of antibiotics (Streptomycin, erythromycin, and nalidixic acid) (ACN: water =1:1).

<table>
<thead>
<tr>
<th>Average Extraction (% w/w)</th>
<th>Streptomycin (in aqueous phase)</th>
<th>Erythromycin (in ACN-rich phase)</th>
<th>Nalidixic Acid (in aqueous phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotic Glucose, g/l</strong></td>
<td><strong>6 °C</strong></td>
<td><strong>18 °C</strong></td>
<td><strong>6 °C</strong></td>
</tr>
<tr>
<td>105</td>
<td>80.64 ± 0.27</td>
<td>*</td>
<td>48.71 ± 3.5</td>
</tr>
<tr>
<td>120</td>
<td>77.80 ± 0.28</td>
<td>89.97 ± 0.46</td>
<td>58.52 ± 1.54</td>
</tr>
<tr>
<td>135</td>
<td>77.98 ± 0.0</td>
<td>89.17 ± 2.4</td>
<td>57.76 ± 3.59</td>
</tr>
<tr>
<td>150</td>
<td>80.12 ± 0.31</td>
<td>89.05 ± 0.04</td>
<td>66.62 ± 3.61</td>
</tr>
<tr>
<td>165</td>
<td>78.96 ± 0.31</td>
<td>89.59 ± 1.16</td>
<td>64.45 ± 2.9</td>
</tr>
</tbody>
</table>

* - No phase separation
CONCLUSIONS

Addition of glucose into an ACN-water mixture resulted into phase separation at relatively low temperature (6-18 °C).

1. Phase ratio and ACN extraction increased with an increase in glucose concentration (105-165 g/l) and with a decrease in temperature (from 18 °C to 6 °C).
2. More than 95 % (w/w) proteins were retained in the aqueous phase after sugaring-out separation of ACN.
3. Hydrophobic erythromycin (65 % w/w) was extracted in the ACN–rich phase and 80-90 % (w/w) streptomycin and 91-94 % (w/w) nalidixic acid were retained in the aqueous phase.

REFERENCES