



## Short communication

**Liquid-liquid equilibrium of the acetonitrile-water system for protein purification**

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**Abstract**

Liquid-Liquid Equilibria (LLE) were measured at atmospheric pressure for the binary acetonitrile-water system at temperatures ranging from  $-1.3$  to  $-18.6^{\circ}\text{C}$ . Data points were taken using two methods, the cloud point method and the analysis of phase compositions using gas chromatography. Both methods show excellent agreement in their representation of the phase envelope. Temperature dependent NRTL (non-random two liquid) parameters were calculated from the binary data. The parameters calculated were  $\tau_{12} = 355.7 - 31.49T(^{\circ}\text{C})$  and  $\tau_{21} = 967.2 - 26.59T(^{\circ}\text{C})$  with the  $\alpha$  value held constant at 0.3.

*Keywords:* Equilibrium; Purification; Acetonitrile; Water; Protein

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**1. Introduction**

The acetonitrile-water system is one of the most widely used mobile phases in reversed phase high performance liquid chromatography (RP-HPLC). RP-HPLC is frequently used as a final step in protein purification. The protein fraction is typically a protein solution containing acetonitrile (ACN), water, and 0.1% (v/v) trifluoroacetic acid (TFA). Recently, it was noticed that when a protein fraction containing the human growth hormone protein was placed in a freezer at around  $-15^{\circ}\text{C}$ , two liquid phases formed [1]. More than 99% of the protein stayed in the water-rich bottom phase. The majority of ACN was in the ACN-rich top phase. This seems to be a convenient method to remove the majority of ACN.

Surprisingly, few data are available in the literature for the acetonitrile-water system at sub-zero temperatures. In this work, data were taken on this system

using two methods: the cloud point method [2] and the analysis of phase compositions using gas chromatography (GC). Temperature-dependent NRTL parameters were correlated from the data to allow reconstruction of the phase envelope for liquid-liquid equilibrium (LLE). The phase envelope can then be used to predict the compositions of the two phases and the lever rule can be used to calculate the phase volumes for a given temperature.

**2. Experimental****2.1. Materials**

The water used was purified from a standard in-house ultrafiltration cartridge system. The acetonitrile used in phase separation was purchased from Fisher Scientific (Pittsburgh, PA). It was HPLC grade and had a minimum stated purity from the manufacturer of 99.9%. The histological grade ethylene glycol used in the temperature bath was also purchased from Fisher Scientific.

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## 2.2. Apparatus and procedure

A Neslab RTE-100LP (Neslab Inc., Portsmouth, NH) low temperature bath ( $\pm 0.1^\circ\text{C}$  accuracy) filled with 60% (v/v) ethylene glycol and 40% (v/v) water was used for temperature control. All temperature measurements were performed with an ASTM 62C thermometer with subdivisions of  $0.1^\circ\text{C}$ .

Cloud point measurements were performed in  $16 \times 100$ -mm screw cap glass test tubes. For each data point ten test tubes were prepared each increasing by approximately a tenth of a mole fraction ACN. They were then submerged in the temperature bath and allowed to equilibrate overnight. If in one of the test tubes, there were two liquid phases and one phase had only a tiny drop in volume, the compositions of the two phases became a data point for LLE. In this fashion, a point on the phase envelope could be determined within  $\pm 0.1$  mol% acetonitrile.

GC measurements were performed on a Hewlett Packard (Palo Alto, CA) 5790A gas chromatography system with a thermal conductivity detector. For each measurement a mixture of approximately 40 mol% acetonitrile and 60 mol% water was placed in a  $16 \times 100$  mm screw cap glass tube. The test tubes were then submerged in the temperature bath and allowed to equilibrate overnight. Samples were taken from each phase with glass pipettes. They were then analyzed with GC for ACN compositions.

## 3. Results and discussion

The LLE data obtained from the cloud point method and GC method are shown in Tables 1 and 2, respectively. The data were plotted in Fig. 1. Fig. 1 shows that the two methods have an excellent agreement in their representation of the phase envelope. The critical solution temperature (i.e. the maximum temperature at which a phase separation can occur) in this work was found to be  $-1.32^\circ\text{C}$ . This is in close agreement to a critical solution temperature of  $-1.29^\circ\text{C}$  reported by Hurth and Woerman [3].

The thermodynamic criterion for the existence of liquid-liquid equilibrium is such that the second-order derivative of the Gibbs free energy with respect to composition is less than or equal to zero [4], i.e.

$$\frac{\partial^2(G^E/RT)}{\partial x^2} \leq 0 \quad (1)$$

In the NRTL equation, the Gibbs free energy can be represented by:

$$G^E/RT = -x_1 x_2 \left[ \frac{\tau_{21} \exp(-\alpha\tau_{21})}{x_1 + x_2 \exp(-\alpha\tau_{21})} \right.$$

Table 1

Binary LLE data from the cloud point method

$T(^{\circ}\text{C})$	$x_{\text{ACN}}$
-11.17	0.666
-4.81	0.567
-1.53	0.325
-5.01	0.214
-1.62	0.412
-8.63	0.184

Table 2

Binary LLE data from gas chromatography

$T(^{\circ}\text{C})$	Water-rich phase $x_{\text{ACN}}$	ACN-rich phase $x_{\text{ACN}}$
-1.32	0.365	0.414
-1.82	0.289	0.452
-2.83	0.235	0.529
-3.52	0.232	0.540
-5.18	0.213	0.579
-5.88	0.201	0.593
-6.41	0.201	0.606
-7.43	0.187	0.622
-7.98	0.178	0.630
-9.13	0.158	0.643
-10.13	0.149	0.660
-11.16	0.154	0.682
-13.73	0.143	0.690
-14.49	0.149	0.707
-17.53	0.141	0.717
-18.60	0.134	0.738

$$\left. + \frac{\tau_{12} \exp(-\alpha\tau_{12})}{x_2 + x_1 \exp(-\alpha\tau_{12})} \right] \quad (2)$$

where

$$\tau_{12} = (g_{12} - g_{22})/RT = a_{12} + b_{12} \cdot T(^{\circ}\text{C}) \quad (3)$$

$$\tau_{21} = (g_{21} - g_{11})/RT = a_{21} + b_{21} \cdot T(^{\circ}\text{C}) \quad (4)$$

$$\alpha = 0.3 \quad (5)$$

In this model  $g_{11}$  and  $g_{22}$  represent the Gibbs free energies of the pure components, while  $g_{12}$  and  $g_{21}$  represent Gibbs interaction energies between the two components. It is assumed  $g_{12} = g_{21}$ . These values are represented by the constants  $a_{12}$  and  $a_{21}$  while their temperature dependence is represented by  $b_{12}$  and  $b_{21}$ . Alpha is tied to the non-randomness of the mixture, although it is often viewed as a strictly empirical constant. It frequently has a value in the range of 0.1–0.5, although a value of  $-1$  is also sometimes employed [4]. In this work  $\alpha$  was artificially held constant at a value of 0.3. This was to allow comparison of calculated NRTL parameters for LLE to published [5] NRTL parameters for vapor-liquid equilib-

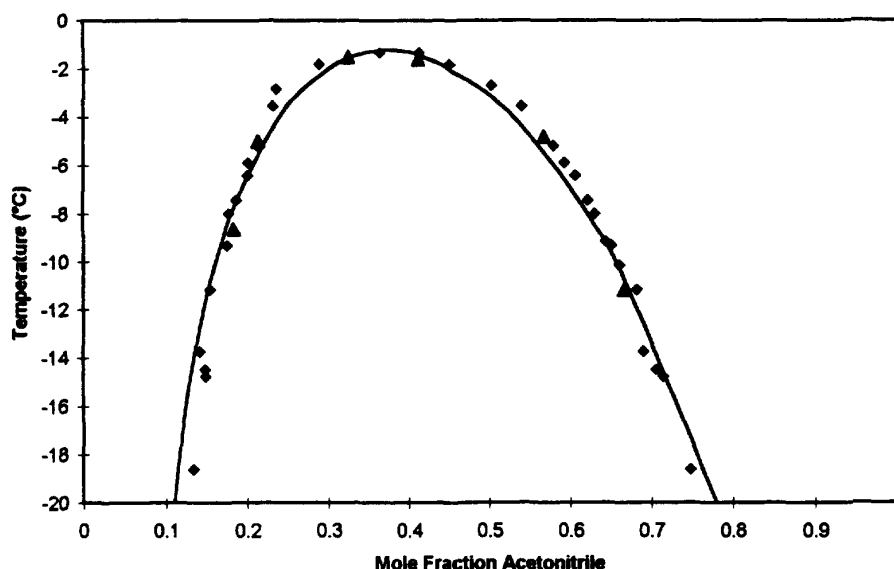


Fig. 1. Comparison of experimental data and data predicted by the NRTL model. (—) NRTL Prediction; (▲) cloud point method; (◆) gas chromatography.

rium (VLE). The published VLE parameters had been used to predict LLE data for the ACN-water system, but the results were not satisfactory. That is why experimental LLE data were used to obtain the NRTL parameters for LLE.

Except at the critical temperature for LLE, there are two LLE points at the phase envelope for each temperature. A point where the phase envelope begins can be calculated from the NRTL equation by varying the composition of a component by a small increment and calculating the second-order derivative of the Gibbs free energy at this point. When this value just turns zero or starts to become negative, the system forms two phases and the first point on the phase envelope has been determined. Likewise, when the second-order derivative switches from a negative or zero value to a positive non-zero value the two phases no longer exist, and the second point on the phase envelope has been determined.

Each experimental data point gives a set of observed composition and temperature where the phase envelope begins or ends.  $x_o$  denotes the ACN mole fraction for an experimental data point. Likewise, for any given value of  $a_{12}$ ,  $b_{12}$ ,  $a_{21}$  and  $b_{21}$  and a specific temperature, there is a predicted ACN mole fraction at which the phase envelope begins and ends. This mole fraction is denoted  $x_p$ . The constants  $a_{12}$ ,  $b_{12}$ ,  $a_{21}$  and  $b_{21}$  were calculated by minimizing the objective function the  $\Sigma(x_p - x_o)^2$ . The summation sign indicates that the  $(x_p - x_o)^2$  is added up for all data points. The minimization of the objective function was performed with the Nelder-Mead Simplex

Method [6], and a FORTRAN 77 code was written to perform the calculations on an IBM-compatible Personal Computer (PC) with 75 MHz Pentium chip.

The NRTL parameters calculated in this manner for LLE have the same order of magnitude as those reported for VLE for the ACN-water system [1], but with a stronger temperature dependence. A comparison of the VLE and LLE parameters is shown in Table 3. The NRTL model with the temperature-dependent parameters listed in Table 3 correlates the experimental data well and should be sufficient for reconstruction of the phase envelope. The experimental data and data predicted by the NRTL model are presented in Fig. 1.

It is possible for impurities to have some effect on LLE data. In practical applications, the ACN-water system undergoes a phase separation in the presence of protein and TFA; GC measurements were performed on systems that contained protein and TFA. Proteins used were albumin,  $\alpha$ -chymotrypsin, lipase, and myoglobin. TFA was added at a concentration of 0.1% (v/v). The samples were centrifuged before GC analyses. For all studies the ACN composition at

Table 3  
Comparison of LLE and VLE NRTL parameters for the ACN-water system

	$a_{12}$	$b_{12}$	$a_{21}$	$b_{21}$	$\alpha$
LLE	355.7	-31.49	967.2	-26.59	0.3
VLE	604.1	-2.42	797.8	6.92	0.3

phase equilibrium varied by no more than 3 mol% from the composition predicted by the NRTL model.

#### 4. Conclusions

The two experimental methods for LLE employed in this work showed excellent agreement in their representation of LLE for the ACN-water system at sub-zero temperatures. The NRTL model with temperature-dependent parameters correlated from experimental data gives a good representation of this system. Using these parameters one can determine the phase composition and phase volume for a given temperature. The presence of proteins and 0.1% (v/v) TFA did not appear to have a significant effect on LLE in the ACN-water system.

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#### Notations

$a_{12}$	Correlation parameter for $\tau_{12}$
$a_{21}$	Correlation parameter for $\tau_{21}$
$b_{12}$	Correlation parameter for $\tau_{12}$
$b_{21}$	Correlation parameter for $\tau_{21}$
$G^E$	Gibbs free energy
$g_{11}$	Gibbs energy of component 1 (ACN)
$g_{12}$	Gibbs energy of interaction between components 1 and 2
$g_{21}$	Gibbs energy of interaction between components 1 and 2

$g_{22}$	Gibbs energy of component 2 (Water)
$R$	Gas constant
$T$	Equilibrium Temperature
$x_1$	mole fraction of ACN
$x_2$	mole fraction water, $1 - x_1$
$x_{ACN}$	mole fraction of ACN
$x_p$	Predicted ACN mole fraction at LLE
$x_o$	Experimental ACN mole fraction at LLE
$\alpha$	Parameter representing the non-randomness of the mixture
$\tau_{12}$	Temperature dependent NRTL parameter, $(g_{12} - g_{22})/RT$
$\tau_{21}$	Temperature dependent NRTL parameter, $(g_{21} - g_{11})/RT$

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