Solid–Liquid Equilibrium of Substrates and Products of the Enzymatic Synthesis of Ampicillin

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The solid–liquid equilibrium of precursors and products of the enzymatic synthesis of ampicillin (AMP) [6-aminopencillanic acid (6-APA) and D(−)phenylglycine (PG)] was investigated at different temperatures (283–298 K) and pHs (5.5–7.5). Solubility data were obtained using an analytical methodology. Equilibrium dissociation constants were experimentally measured at several temperatures for AMP, 6-APA, PG, and D(−)phenylglycine methyl ester. A model based on the simplified perturbed hard sphere theory proposed by Khoshkbarchi and Vera (Ind Eng Chem Res. 1996;35:4319-4327) was fitted against solubility data. The model could describe the water solubility behavior for AMP and PG as function of pH and temperature, but a bias was observed when fitting the model to the solubility of 6-APA. © 2009 American Institute of Chemical Engineers *AIChE J,* 56: 1578–1583, 2010

Keywords: β-lactam antibiotics, green chemistry, solubility, solid–liquid equilibrium, thermodynamic modeling

Introduction

Ampicillin (AMP) is one of the most important β-lactam antibiotics. It can be produced through chemical or enzymatic syntheses, as well as other semisynthetic antibiotics. Because of its high yields, the chemical route is the most common practice in industry. However, the chemical process comprises several steps (including protection–deprotection of side chains) at low temperatures (bellow 248 K), using toxic organochloride solvents and generating considerable amounts of nonrecyclable waste.[1] On the other hand, the enzymatic route is more environmental-friendly, within the "green chemistry" scope: it may be carried out in aqueous medium, at room temperature (298 K) and almost neutral pH.[2]

Two enzymatic approaches to synthesize β-lactam antibiotics may be used: the thermodynamically controlled synthesis (TCS) and the kinetically controlled synthesis (KCS). However, the latter one is the only feasible for semisynthetic penicillins (amoxicillin, AMP) without the aid of cosolvents, since the reaction equilibrium does not favor the synthesis.[3] KCS consists of a reacting activated precursor of the acyl moiety (an ester or amide) with β-lactam nucleus

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[6-aminopenicillanic acid (6-APA)]. Usually, immobilized penicillin G acylase from *Escherichia coli* is used as biocatalyst. This enzyme also catalyzes the hydrolysis of the acyl-donor derivative and the hydrolysis of the antibiotic itself (see Figure 1). Therefore, the yield of the antibiotic is driven by the reactions kinetics. AMP arises as an intermediate component, and vanishes at equilibrium conditions. Hence, the goal of achieving a high selectivity (ratio between concentrations of antibiotic and hydrolyzed acyl-donor) falls into a classic optimization problem, where the intermediate product has to be maximized within a set of series-parallel reactions.

The use of a semicontinuous integrated reactor, where substrates are fed and product is crystallized,[4–7] may overcome many obstacles of the KCS and is a promising alternative for large-scale production. Indeed, if the antibiotic migrates to the solid phase, it is subtracted from the action of the enzyme, and the process selectivity increases. Still, this process has to be very well tuned to compete with the chemical route.[3]

Of course, the solubility of the components involved in the KCS of AMP play an essential role in the performance of the integrated reactor. The aqueous phase of the reaction medium is a nonideal solution and consistent experimental data of the solid–liquid equilibrium of these components would be very important to optimize the semicontinuous integrated reactor. A number of data methodically collected can be used to fit theoretical or empirical models that would describe the solubility of the components involved in the synthesis as function of concentration, temperature, and pH. Those models would be particularly useful for the dynamic optimization of the reactor, when operating in fed-batch model.

There are very few data of solid–liquid equilibrium of β-lactam antibiotics. Rudolph et al.[8] presented data of solubility at different temperatures (at the isoelectric point of each component) and pH (for a constant temperature, 298.15K) for AMP, amoxicillin and their precursors [D(−)phenylglycine (PG), *p*-hydroxy D(−)phenylglycine, and 6-APA) in pure water. Additionally, the authors fitted the data using a model initially developed by Khoshkbarchi and Vera[9] to describe the solubility of natural amino acids in water (see the Methods section) including the dissociation

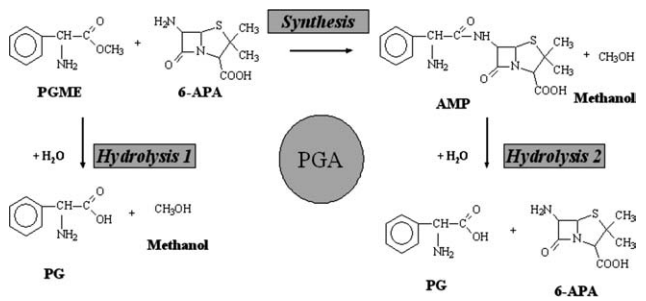

Figure 1. An example of the kinetically controlled synthesis (KCS) of ampicillin.

AMP, ampicillin; PGME, D(−)phenylglycine methyl ester (acyl-donor derivative); 6-APA, 6-aminopenicillanic acid (β-lactam nucleus); PG, D(−)phenylglycine (hydrolyzed acyl-donor); PGA, penicillin G acylase (enzyme).

Table 1. Dipole Moments Used to Fit the Solubility of Ampicillin, Phenylglycine, and 6-Aminopenicillanic Acid

Component	D (Debye)
Ampicillin	32.4693
D(−)phenylglycine	5.8307
6-APA	17.7621

effects. Three parameters were estimated for each component: enthalpy, entropy, and potential-well depth. The hard-core diameter of each component was estimated using van der Waals volumes and surfaces and dipole moments were computed using a quantum mechanics model. The model represented the qualitative behavior of the system. The values of the resulting parameter agreed in magnitude with those obtained by Khoshkbarchi and Vera[9] for natural amino acids. Rudolph et al.[8,10] also presented experimental data of solubility and partition coefficient for the same components in a water + 1-butanol mixture. The data were fitted using a model based on the solubility excess, suggested by Gude et al.[11] to describe the partition coefficient of amino acid mixtures in water + butanol.

Youshko et al.[5] studying AMP synthesis in an aqueous-precipitated system, presented data of solubility at 298.15K of substrates and products in water, pure and in mixtures. The authors used thermostatic cells and a pH-stat to control temperature and pH respectively. Samples of the supernatant were diluted and analyzed in HPLC. Results showed a substantial variation in the solubility of AMP, PG, and 6-APA when D(−)phenylglycine methyl ester (PGME) concentrations were changed. These results make obvious the nonideality of the medium. The authors also noticed a supersaturation effect during synthesis of AMP in a semicontinuous integrated reactor.

Vieira[12] reported data of solubility for the components involved in the KCS of AMP: PGME, AMP, 6-APA and PG, as a function of pH at 298.15K.

The objective of the current work is to present reliable data and confidence limits of solubility at different

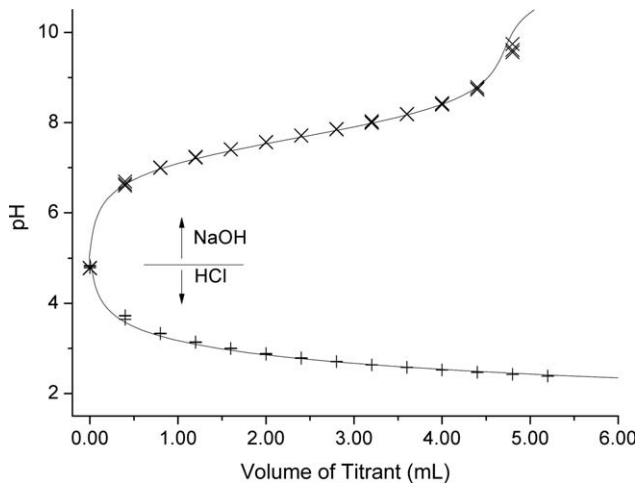

Figure 2. Titration curve of ampicillin at 283.15 K (in triplicate).

Experimental data: (×) NaOH as titrant; (+) HCl as titrant. Line: fitted model.

Table 2. Dissociation Constants Obtained Experimentally at Temperatures Ranged From 283.06 to 298.03 K

	Ampicillin				D(−)phenylglycine				6-APA				PGME	
Temperature (K)	pKa_1	s.d.	pKa_2	s.d.	pKa_1	s.d.	pKa_2	s.d.	pKa_1	s.d.	pKa_2	s.d.	pKa	s.d.
283.1	2.27	0.096	7.66	0.160	1.60	0.233	9.51	0.050	1.98	0.106	5.34	0.043	6.84	0.041
288.1	2.24	0.252	7.61	0.122	1.70	0.248	9.35	0.064	2.04	0.112	5.25	0.053	7.00	0.095
293.1	2.14	0.122	7.45	0.252	1.82	0.297	9.36	0.095	2.10	0.325	5.18	0.165	7.17	0.065
298.1	2.14	0.096	7.31	0.104	2.08	0.230	9.14	0.087	2.20	0.815	4.83	0.456	7.24	0.063

Standard deviation of the estimated parameter was evaluated during fitting procedure.

temperatures and pHs, for some of the components involved in the enzymatic synthesis of AMP, including phenylglycine ethyl ester, a nonconventional precursor of AMP. A wider range of pH and temperature was spanned here, when comparing to the data presented in the literature. The experimental data were used to fit the model developed by Khoshkbarchi and Vera.[9]

Materials and Methods

Materials

Reactants were supplied by Sigma-Aldrich: PGME 97%; 6-APA 96%; AMP 96%; and PG 99%. All reactants were used without any further purification. Ultra-pure water, obtained by reverse osmosis, was used in all procedures.

Methods

Solubility of the Components

Solubility of AMP, 6-APA and PG was determined experimentally using a saturation approach suggested by Gude et al.[11] and adapted by Vieira.[12] The method consists in preparing saturated solutions of the components at determined pH's, then controlling the temperature of the solution until apparent equilibrium is reached. The saturated solutions were prepared and then transferred to a shaker with controlled temperature (resolution of ± 0.1 K), where they were stirred at 150 rpm for 6 h. The resulting suspensions were left in rest to sediment for 1 h. Small aliquots were withdrawn from supernatant using a 0.22 μm filter attached to a syringe. For 6-APA and PG, the supernatant was diluted (1:200) and analyzed in a VARIAN ultraviolet spectrophotometer at $\lambda = 240$ nm. For AMP, the supernatant was diluted (1:1000) and analyzed in a Shimadzu HPLC. To evaluate possible degradation, samples of the AMP solution were analyzed in HPLC before and after the assay. The assays were carried out in triplicates. Temperatures ranged from 283 to 298 K and pH from 5.5 to 7.5. No buffer was added. Instead, pH was corrected only at the beginning of each assay by addition of NaOH or HCl and further monitored along the assay. No pH variation was noticed during solubility assays. Masses of the samples were measured using a KERN analytical balance, model 410, then diluted with water.

HPLC analyses were carried out at 25°C and $\lambda = 225$ nm using a C18 column (Waters Nova-Pack, USA, 60E, 4 μm, 3.9×150 mm^2) and 1 mL/min of eluent (mobile phase). The mobile phase was composed of 35% acetonitrile, 5 mM SDS (lauryl sodium sulfate), and 5 mM phosphate buffer.

Determination of Dissociation Constants. Dissociation constants ($Ka = 10^{-pKa}$) are very important properties of amino acids and other molecules with dissociable hydrogen. They make possible the evaluation of the ratio of charged forms as a function of pH. The charged form of the molecules (including enzyme residues) involved in the enzymatic synthesis of β-lactam antibiotics plays an important role in every aspect of the synthesis, such as reaction rates, selectivity (reaction mechanism), and solubility of the substrates and products. Dissociation constants of the components involved in the enzymatic synthesis of AMP are presented in the literature but in a scattered way. They are also rarely encountered at temperatures different of 298.15 K.

Measurements of pKa were done with 40 mL of 5 mM solutions of AMP, PG, 6-APA, and PGME placed into an equilibrium cell. The assays were carried out at different temperatures between 283.0 and 298.0 K. Values of pKa were determined using potentiometric titration.[13] Titrants NaOH (42.4 mM) and HCl (50.0 mM) were added to the solutions by a burette (25.0 mL). Stirring was done by a slow stream of nitrogen bubbles which was stopped before each measurement. Determination of the apparent pKa values was carried out by fitting the theoretical model to the titration curve. The error of the added titrant volume (0.05 mL) could not be neglected in comparison with the resolution of the pH (0.01). Hence, a maximum likelihood method was used, where the resolutions of the burette and of the pH-meter were taken into account as weights.[14] Correcting activity coefficient by Debye–Hückel formula to take ionic strength into account did not improved the estimated dissociation constants beyond their estimates uncertainties, and the results are shown here.

Thermodynamic Model. The simplified perturbed hard-sphere model, proposed by Khoshkbarchi and Vera[9] to describe the solubility of amino acids in water (Eqs. 1–3) was fitted to the experimental data. It is the only method cited in the literature for these compounds which considers the pH influence on the solubility, for the best of our knowledge. In this model, the solvent is assumed a dielectric

Table 3. Comparison Among Dissociation Constant Obtained in this Work in Water and Data from the Literature, at 298.15 K

	6-APA		PG		PGME	AMP	
Source	pKa_1	pKa_2	pKa_1	pKa_2	pKa	pKa_1	pKa_2
This work	2.20	4.83	2.08	9.14	7.24	2.14	7.31
Ref. 15	2.50	4.90	–	–	6.89	–	–
Ref. 9	2.60	5.40	–	–	–	–	–
Ref. 16	–	4.60	2.20	9.30	–	–	–
Ref. 12	2.47	4.93	1.96	9.02	–	2.66	7.24

Table 4. Solubility (±s.d. of Triplicates) in Water of Ampicillin, D(−)Phenylglycine and 6-Aminopenicillanic Acid for Different Temperatures and pHs

	Ampicillin		D(−)Phenylglycine		6-Aminopenicillanic acid	
pH	Solubility (mmol/L)	s.d. (mmol/L)	Solubility (mmol/L)	s.d. (mmol/L)	Solubility (mmol/L)	s.d. (mmol/L)
283.06 K						
5.5	13.45	0.0965	11.11	0.671	15.69	0.00964
6.0	14.07	0.437	11.77	0.154	50.58	0.368
6.5	15.96	0.257	12.16	0.279	99.07	0.0366
7.0	18.73	0.110	13.15	0.767	275.3	0.275
7.5	39.59	0.0502	13.99	0.120	436.3	0.379
288.01 K						
5.5	14.75	0.0201	19.12	0.811	20.49	0.0899
6.0	16.35	0.190	20.77	0.385	60.39	0.125
6.5	17.19	0.378	21.57	0.396	115.3	0.249
7.0	19.29	0.366	22.69	0.277	302.6	0.315
7.5	47.00	0.296	22.98	0.0297	498.7	0.236
292.95 K						
5.5	16.88	0.134	25.46	0.441	30.68	0.105
6.0	17.17	0.0973	25.96	0.322	70.11	0.0199
6.5	18.50	0.0705	27.56	0.498	139.7	0.508
7.0	27.48	0.0387	29.12	0.979	319.9	0.335
7.5	49.80	0.0640	30.57	0.498	511.1	0.124
298.03 K						
5.5	20.89	0.912	33.92	0.660	41.45	0.416
6.0	21.61	0.161	36.78	0.367	85.53	0.294
6.5	22.27	0.0142	37.65	0.278	170.6	0.402
7.0	28.39	0.00114	38.51	0.215	353.9	0.377
7.5	51.37	0.140	39.38	0.213	587.3	0.266

continuum, which reduces the solute/water solution to a single-component system. The second factor of the right hand size of Eq. 1 arises from the assumption that only the zwitterionic (dipolar) form of the amino acid crystallizes. Equation 2 shows the resulting expression for the activity coefficient, defined in the unsymmetrical convention.

$$x_i = \exp\left(\frac{\Delta S_{i\pm}}{R} - \frac{\Delta h_{i\pm}}{RT}\right) \times \left(1 + \frac{a_{H^+}}{Ka_i^{(1)}} + \frac{Ka_i^{(2)}}{a_{H^+}}\right) \frac{1}{\gamma_{i\pm}} \quad (1)$$

$$\ln \gamma_i = \frac{8\xi - 9\xi^2 + 3\xi^3}{(1-\xi)^3} - \frac{4\pi\rho}{kT}\left\{\frac{8}{9}\in \sigma^3 + \frac{D^4}{9(4\pi \in_0 \in_r)^2 kT\sigma^3}\right\} \quad (2)$$

with

$$\xi = \frac{\pi}{6}\rho\sigma^3 \quad (3)$$

Equations 1–3 were used to fit experimental data of solubility of AMP, PG, and 6-APA. Adjustable parameters for each component were: the entropic and enthalpic change of solution of the solid crystalline solute into an infinitely dilute solution ($\Delta s_{i\pm}$, $\Delta s_{i\pm}$); the hard core diameter (σ); and the potential-well depth ($\in$). For a better fitting, the temperature dependence of the parameter $\in$ was considered as follows:

$$\in = a(1 + bT) \quad (4)$$

Dipole moments have been computed through *ab-initio* quantum calculations using Gaussian98. During the computations, the initial geometry of the molecule in the vacuum was stepwise-optimized, gradually increasing the complexity

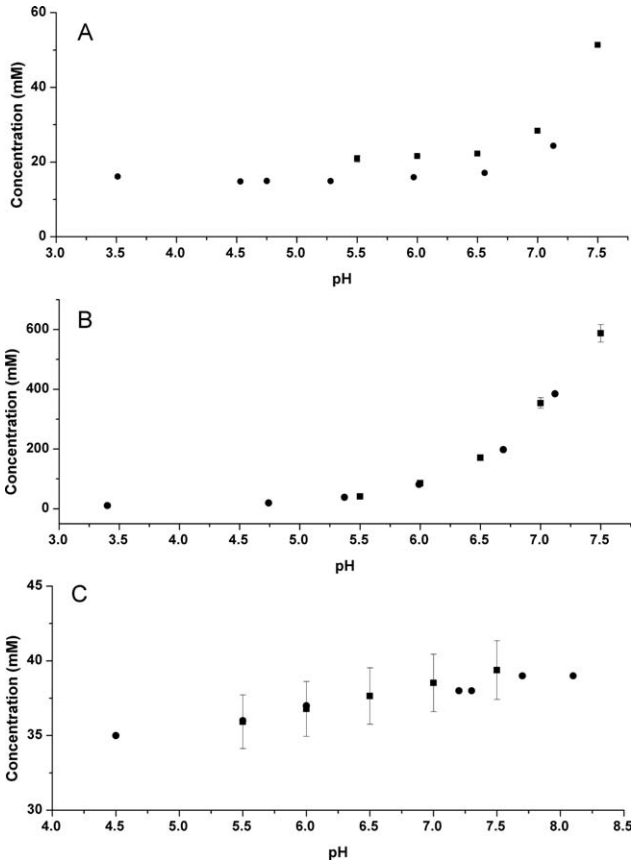

Figure 3. Solubility in water at 298 K as function of pH.

(■) Present work, (●) data from literature: (A) ampicillin (●) Rudolph et al.[10]; (B) 6-APA, (●) Rudolph et al.[10]; and (C) PG, (●) Youshko et al.[5]

Table 5. Parameter Values Resulting from the Fitting Procedure of the Model Given by Equations 2–5

Solute	σ, 10^{10} (m)	a, 10^{21} (J)	b, 10^{3} (K^{-1})	Δh, 10^{-4} $(J/mol)^{-1}$	Δs $(J/mol/K)^{-1}$	s.d. (M)
AMP	2.98510	−1628.26	1.35800	0.95523	−14.0312	0.0018
6-APA	7.43292	5.61829	7.16481	1.26876	−45.3188	0.0719
PG	4.00970	203.343	22.1415	6.11467	106.847	0.0017

of the theory and the bases of the molecular orbital computation. The final optimization was accomplished via the method of three hybrid parameters (B3LYP) using base 6-311, including polarization of heavy atoms. Optimization of the geometry for the different charged species (positive, negative, and zwitterionic) was also accomplished. The values of dipole moment used in the fitting procedure are presented in Table 1.

Results and Discussions

Dissociation constants

The variation of p*K*a with temperature was measured for the molecules that are most relevant to the enzymatic synthesis of AMP. Figure 2 shows an example of the titration curves that were used to estimate p*K*a values.

Table 2 presents the obtained dissociation constants for AMP, 6-APA, PG, and PGME. Table 3 compares these results with data from the literature.

Solubility assays

Table 4 presents the mean value and standard deviation of the solubility of AMP, PG, and 6-APA, at different temperatures and pHs. The low-standard deviations (of triplicates) indicate the measurements accuracy.

The values of solubility obtained at 298.03 K were compared with results of the literature at 298.15 K as function of pH, as depicted in Figure 3. Results obtained for 6-APA and AMP closely agree with the data published by Rudolph et al.[10] The solubility of PG is also close to those obtained by Youshko et al.[5] U-shaped curves are usually expected for molecules with amino and carboxylic moieties. The low solubility of the zwitterionic form in water makes the component to have lower solubility near its isoelectric point. Since the range of pH used was greater than the pKa_1 of the considered components, only half of the expected U-shaped curve can be seen. It is worth to notice that Youshko et al.[5] did not report a table of solubility data, the authors provide only graphics, but for the sake of comparison, we have approximated their data. Still, the results shown in Figure 3C agreed within the precision of the obtained data.

Model fitting

Table 5 shows the estimated parameters and the global standard deviation obtained after the fitting procedure. The fitting procedure showed very distinct values comparing to those estimated by Rudolph et al.[8] The parameters however are highly correlated and the estimative of their uncertainties (not shown here) tend to their absolute values. Thus, the estimated values greatly depend on the initial guesses used in the fitting procedure.

The qualitative behavior of the model predictions can be assessed in Figures 4–6. Although a bias deviation is observed between the fitted model and the data for AMP and phenylglycine, the model closely followed the data trend (Figures 4 and 6). On the other hand, a noticeably higher deviation between data and model was observed for 6-APA (Figure 5). This deviation could also be seen in the figure presented in Rudolph et al.[8] work. In this work, the model was also quantitatively compared to Khoshbarchi and Vera[9] model through mean and global deviations as shown in Table 6. The results show clearly that this model could not be used to describe quantitatively this system.

The focus in this work was to obtain new experimental data, and the Khoshbarchi and Vera[9] model is the only

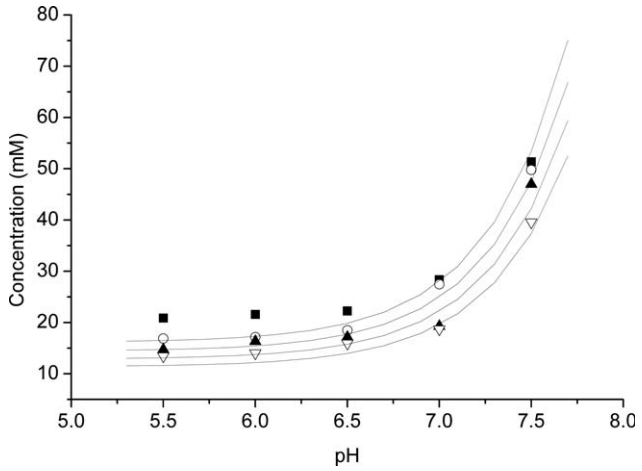

Figure 4. Solubility of ampicillin in water as function of pH at different temperatures (K): 283.1 (▽); 288.1 (▲); 293.1 (○); 298.1 (■). Lines: model.

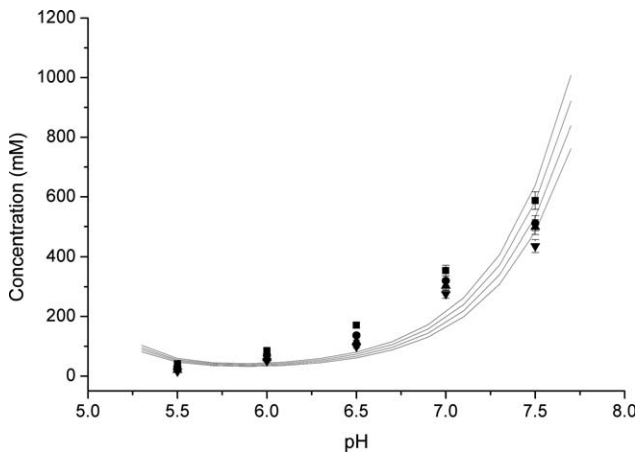

Figure 5. Solubility of 6-APA in water as function of pH at different temperatures (K): 283.06 (▼); 288.01 (▲); 292.95 (◆); 298.03 (■). Lines: model.

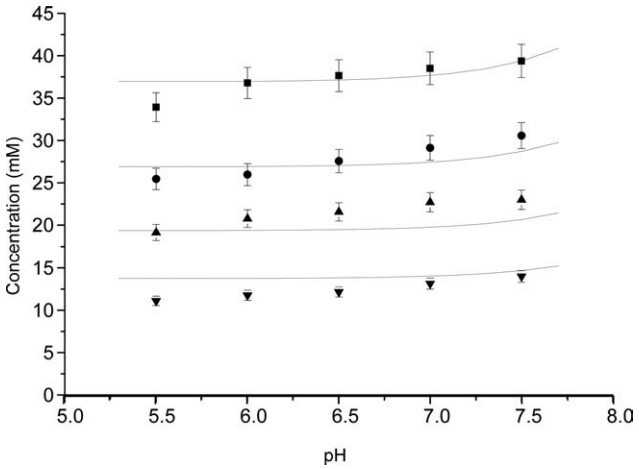

Figure 6. Solubility of PG in water as function of pH at different temperatures (K): 283.06 (▼); 288.01 (▲); 292.95 (◆); 298.03 (■). Lines: model.

Table 6. Average and Global Deviation Between Experimental and Calculated Values

T (K)	6-APA (%)	FG (%)	*T* (K)	AMPI (%)
298.03	54.0	2.6	298.1	8.1
292.95	52.6	5.6	293.1	5.9
288.01	48.6	7.2	288.1	10.6
283.06	47.2	26.1	283.1	6.6
Global	50.6	10.4	Global	7.8

available in literature for these compounds which includes directly the pH influence. So it was used here, but a better insight is clearly needed to achieve a quantitative model for the solubility of the compounds present in AMP synthesis. This will be the focus of future works.

Conclusions

In this work, new solubility experimental data for species present in the enzymatic synthesis of AMP (the antibiotic, 6-APA and PG) are presented. The saturation method proposed by Gude et al.[11] and modified by Vieira[12] was used. Data were obtained with a standard deviation of less than 1 mmol/L for all compounds. The obtained data at 298.03 K were satisfactorily compared with previously published data at 298.15 K. Dissociation constants (including PGME and PGEE) were also experimentally determined at several temperatures, and results at 298 K were satisfactorily compared with other literature data. Following the literature, the Khoshkbarchi and Vera[9] model was used to fit experimental data. The model was able to qualitatively describe the solubility data for AMP and PG. However, it was not able to describe 6-APA solubility. Hence, a more accurate model of the solubility of the compounds present in AMP synthesis is necessary, and will be pursued in future works.

Acknowledgments

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