

Separation of 6-Aminopenicillanic Acid by Reactive Extraction

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Abstract

6-Aminopenicillanic acid exhibits an amphoteric character and can react both with acidic and basic extracting agents. The study on individual reactive extraction of 6-Aminopenicillanic acid with di-(2-ethylhexyl)phosphoric acid and Amberlite LA-2 indicated that the separation is more effective with Amberlite LA-2 (at pH=10, the extraction yield with Amberlite LA-2 is over 4.7 times greater compared with physical extraction with 1,2-dichloroethane or reactive extraction with di-(2-ethylhexyl)phosphoric acid). The separation occurs by means of an interfacial reaction between the components at equimolecular ratio, the reaction mechanism being controlled by the extracting agent type and aqueous phase pH-value.

By means of the influences of the aqueous phase pH-value and the Amberlite

LA-2 concentration in 1,2-dichloroethane on the reactive extraction of 6-Aminopenicillanic acid, a mathematical model that describes the extraction process was proposed.

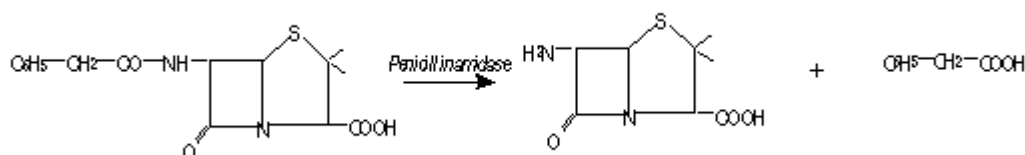
Keywords: Physical extraction, reactive extraction, 6-Aminopenicillanic acid, Amberlite LA-2, di-(2-ethylhexyl) phosphoric acid.

Introduction

6-Aminopenicillanic acid (6APA) is the main component of the semisynthetic Penicillins, antibiotics that are obtained by 6APA acylation and formation of amidic bonds differing from the natural ones. 6APA is the biosynthetic product of some Penicillin producing fungus grown on nutritive media without precursors. Thus, in absence of phenylacetic acid, at the end of *P. chrysogenum* fermentation cycle, the broths contains about 50%

6APA, 30% Penicillin K, 15% Penicillin DF and 5% Penicillin F [1]. 6APA could be obtained even if the precursors are added into the media (phenylacetic acid for Penicillin G biosynthesis, phenoxyacetic acid for Penicillin V), but at a very low concentration level (generally, 0.2 – 0.4%, rarely 2 – 3%). Because the biosynthesis of this compound is economically inefficient, some chemical or enzymatic methods for Penicillin G hydrolysis to 6APA were proposed and applied at industrial scale [2-5].

The chemical hydrolysis of Penicillin G requires a lot of stages and uses special chemical agents and low temperature (-60°C), these limiting the application of the method [2,3]. The enzymatic hydrolysis is catalyzed by *penicillinamidase* and can be described by the following reaction:



Penicillin G

6-Aminopenicillanic acid Phenylacetic acid

The most used *penicillinamidase* at industrial scale is produced by *E. coli*. This enzyme could be used after biomass separation, free or immobilized on different polymeric supports, or without biomass filtration, the Penicillin G hydrolysis occurring simultaneously with enzyme production. The best results were obtained for immobilized enzyme (the Penicillin G conversion exceeds 90%), the process being carried out at 35 – 40°C and pH=8.2 [2-4]. Recently have been isolated and purified *penicillinamidases* biosynthesized by *Bacillus megaterium*, *Streptomyces lavendulae*, *Achromobacter sp.*, *Proteus rettgeri*, *Actinoplanes sp.*, *Bovista plumbea*, *Kluyvera atrophila*, *Pseudomonas melanogenum*, *Fusarium sp.*, *Chainia*, for Penicillin G or V hydrolysis [5,6].

The solution obtained by Penicillin G enzymatic hydrolysis contains 6APA, phenylacetic acid (PAA) and unhydrolyzed Penicillin G (PG). The industrial separation of 6APA is achieved by acidification at pH=2.5, extraction of PG and PAA with butyl acetate, concentration and acidification with hydrochloric acid for 6APA precipitation [2,3]. This separation technology needs high materials and energy consumption, thus increasing the 6APA cost.

The aim of experiments is to establish the possibilities of 6APA separation by reactive extraction. For selecting the most efficient extraction system, the individual extraction of

6APA with extracting agents of organophosphoric acids (di-(2-ethylhexyl) phosphoric acid, D2EHPA) and high molecular weight amines type (lauryl-trialkyl-methylamine, Amberlite LA-2) was studied.

By means of the experimental data, a correlation between 6APA extraction efficiency and main parameters that influence the reactive extraction (pH-value, extracting agent concentration) was established.

Materials and Methods

The experiments have been carried out in an extraction column with vibratory mixing has been used, this laboratory equipment being described in detail in previous papers [7,8]. The phase mixing was made by mean of a perforated disk with 45 mm diameter and 20% free section. The vibrations had a frequency of 50 s⁻¹ and 4 mm amplitude. The mixer position was maintained at the initial contact interface between the aqueous and organic phases. The extraction time was of 1 minute. The resulted emulsion was evacuated at the base of the column and broken in a centrifugal separator at 5000 rpm.

The solvent was 1,2-dichloroethane, being individually used or as a solution of two extracting agents types: D2EHPA and Amberlite LA-2. The extractants concentrations in organic phase varied between 0 and 80 g.l⁻¹. The volume ratio between the aqueous solution and organic solvent was of 1, each phase volume being of 50 ml.

The initial aqueous solution pH adjustment has been made with a solution of 0.1N hydrochloric acid or 0.1N sodium hydroxide, depending on the desired pH value (pH values were determined using a digital pH-meter of Consort C832 type). The pH values have been recorded throughout each experiment and any pH change was noted.

The extraction degree has been calculated by means of 6APA concentration in the initial solution and in the raffinate:

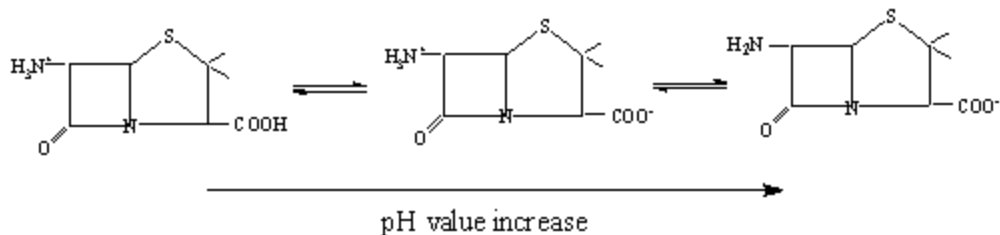
$$Y = \left(1 - \frac{C_R}{C_0} \right) \cdot 100, \%$$

6APA concentration has been measured using high performance liquid chromatography technique (HPLC) with a Lichrospher 100 RP - 18 column (5 μm) and an UV detector at 225 nm. The mobile phase was a mixture of phosphate buffer (pH = 6) and acetonitrile in a volume ratio of 4 : 1.

Results and Discussion

1. Reactive extraction of 6-Aminopenicillanic acid

Owing to the presence of amine and carboxylic groups in 6APA chemical structure, this compound exhibits an amphoteric character, the increase of the aqueous solution pH-value inducing the following dissociation equilibriums:



The corresponding pK-values were determined by potentiometric titration with solutions of 10^{-3} N hydrochloric acid and 10^{-3} N sodium hydroxide. The obtained values were closed to those given in literature [4,9]:

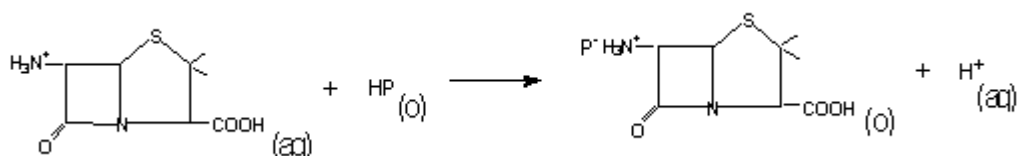
$$pK_{\text{COOH}} = 2.3 \quad pK_1 = 3.8 \quad pK_{\text{NH}_3^+} = 8$$

These ionic species formation considerably reduces the 6APA solubility in nonpolar solvents. But, due to its acidic and basic properties, 6APA can react with acidic or basic extractants, this allowing its solubilization in an organic phase. For this reason, the reactive extraction with D2EHPA and Amberlite LA-2 was studied. Thus, the extraction mechanism was comparatively analyzed by means of the pH-value of aqueous phase and extracting agent concentration in organic phase influences on extraction degree.

As it could be observed from **Figure 1**, the increase of pH initially leads to the reduce of physical or reactive extraction efficiency to a minimum value that corresponds to pH=6-7, followed by an extraction yield increase. This phenomena is more pronounced for reactive extraction with Amberlite LA-2, the extraction degree being significantly higher compared to those obtained for physical or reactive extraction with D2EHPA.

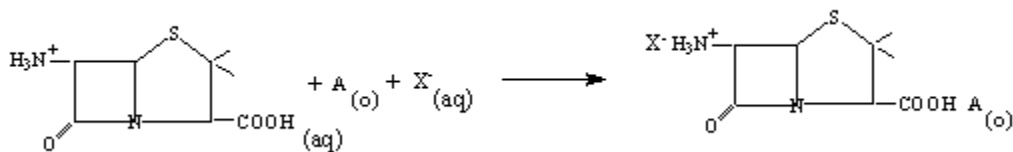
The variation of separation efficiency for the considered extraction systems can offer informations about the extraction mechanism. Therefore, the physical extraction, which is based only on the physical solubilization of 6APA in 1,2-dichloroethane, is strongly limited by this compound ionization in aqueous solution. The physical extraction yield is very low, reaching the maximum values only for the pH domain corresponding to the mono-ionized species formation (Y=7.7% for pH=2, respectively 14% for pH=12). For the domain of pH favorable to the zwitterions appearance, the extraction efficiency is supplementary reduced, becoming 0 at pH=6.

The reactive extraction with D2EHPA is possible only in acidic domain and occurs by means of the following interfacial ionic exchange reaction:



This mechanism is similar to the amino acids extraction with D2EHPA and requires the 6APA existence as cation in aqueous solution, as found at pH-values below the isoelectric point [10-12]. Compared with the amino acids extraction, the efficiency of 6APA extraction is considerably lower, the maximum value of extraction degree being of 28% for pH=2 and 80 g.l⁻¹ D2EHPA in 1,2-dichloroethane. The increase of pH induces the formation in an extended amount of zwitterions and consequently the decrease of extraction degree. For pH-value over the isoelectric point, the efficiency of reactive extraction with D2EHPA becomes equal to that of physical extraction, suggesting that reactive extraction with D2EHPA doesn't occur in this pH interval.

The best results were obtained for reactive extraction of 6APA with Amberlite LA-2. Although the variation of extraction degree with pH-value is similar to those for the former studied systems, it indicates two possible mechanisms for reactive extraction. Therefore, for the pH domain below isoelectric point, the separation of 6APA is the result of the interfacial reaction:



with ion-pair formation in organic phase (X⁻ is the contraion existed in aqueous solution, respectively the anion of the acid used for pH correction). This mechanism is similar to the extraction of amino acids with Amberlite LA-2 [8].

The increase of pH-value leads to the reduce of extraction yield, due to the zwitterions appearance in aqueous solution. At greater pH-values than $pK_{NH_3^+}$ the extraction efficiency strongly increases and reaches a maximum level at pH=10. For this pH domain, the extraction mechanism is changed, the interfacial reaction between 6APA and Amberlite LA-2 becoming similar to that for Penicillins extraction [12]:

The further decrease of extraction degree, for pH>10, could be the result both of the rapid chemical inactivation of 6APA in strong alkaline media, and of its sodium salt formation at an important concentration level, process which induces the 6APA reextraction from solvent to aqueous phase.

The proposed mechanisms were verified by analyzing the influence of extracting agent concentration in 1,2-dichloroethane on extraction degree. As it can be seen from **Figure 2**, the separation efficiency continuously increases with extractants concentration. But, for both extracting agents, the increase of extraction degree is initially pronounced, diminishing afterwards. The modification of extraction yield evolution is produced at an extractant concentration value corresponding to the equimolecular ratio between 6APA and Amberlite LA-2, respectively D2EHPA. This result confirms the formation of 6APA

- extractant complex in organic phase by chemical reaction between one molecule of each compound.

2. Modelling of reactive extraction of 6-Aminopenicillanic acid with Amberlite LA-2

The experimental data on reactive extraction of 6APA showed that the highest extraction degrees are obtained using Amberlite LA-2 as extracting agent (at pH=10, the extraction yield with Amberlite LA-2 is over 4.7 times greater compared to the physical extraction or reactive extraction with D2EHPA). By means of the effects of the pH-value and Amberlite LA-2 concentration on 6APA reactive extraction correlation between 6APA extraction degree and these parameters level was established.

As it was suggested by the variations plotted in **Figure 3**, the proposed mathematical model is of polynomial regression equation type of six orders:

$$Y = a_0 + a_1 \cdot x + a_2 \cdot x^2 + a_3 \cdot x^3 + a_4 \cdot x^4 + a_5 \cdot x^5 + a_6 \cdot x^6$$

where:

x - pH-value of aqueous phase

a_0, \dots, a_6 - regression coefficients, which are logarithmic functions of extracting agent concentration.

The values of regression coefficients were calculated using Microcal Origin 6.0 software, the following correlation for reactive extraction of 6APA being obtained:

$$Y = 24.10 + 43.96 \ln C_A - (18.02 + 33.59 \ln C_A) \cdot \text{pH} + (0.56 + 27.88 \ln C_A) \cdot \text{pH}^2 - (3.7 + 9.51 \ln C_A) \cdot \text{pH}^3 + (0.56 + 1.32 \ln C_A) \cdot \text{pH}^4 - (5 \cdot 10^{-2} + 2 \cdot 10^{-2} \cdot \ln C_A) \cdot \text{pH}^5 + (8 \cdot 10^{-4} + 7 \cdot 10^{-4} \ln C_A) \cdot \text{pH}^6$$

The surface describing the cumulated influence of the considered factors on 6APA reactive extraction yield is given in **Figure 4**. Compared with the experimental results, the average deviation of the calculated values is of $\pm 10.5\%$.

Conclusions

Owing to its amphoteric character, 6APA can react both with acidic extracting agents and basic ones. The study on individual reactive extraction of 6APA with D2EHPA and Amberlite LA-2 indicated that the separation is more effective with Amberlite LA-2 (at pH=10, the extraction yield with Amberlite LA-2 is over 4.7 times greater compared with physical extraction or reactive extraction with D2EHPA). The separation occurs by means of an interfacial reaction between the components at equimolecular ratio, the reaction mechanism being controlled by the extracting agent type and aqueous phase pH-

value.

Using the influences of the aqueous phase pH-value and Amberlite LA-2 concentration in 1,2-dichloroethane on reactive extraction of 6APA, a mathematical model that describes the extraction process was proposed.

Notations

A - Amberlite LA-2

C6APA0 - initial concentration of 6APA in aqueous phase, g.l-1

CA - Amberlite LA-2 concentration in organic phase, g.l-1

CD2EHPA - D2EHPA concentration in organic phase, g.l-1

HP - D2EHPA

pKi - pK-value corresponding to the isoelectric point

Y - extraction degree, %

(aq) - aqueous phase

(o) - organic phase.

References

1. J.M. FERNANDEZ-CANON, A. REGLERO, H. MARTINEZ-BLANCO, J.M. LUENGO, J. *Antibiotics* 42, 1394 (1989).
2. C. ONISCU, *Chimia si tehnologia medicamentelor*, Technical Ed., Bucharest, pp. 108 (1988).
3. C. DAESCU, *Chimia si tehnologia medicamentelor*, E.D.P., Bucharest, pp. 245 (1994).
4. E. DIACONU, M. NECHIFOR, *Antibiotice beta-lactamice*, C.I.M.C., Bucharest, pp.131 (1988).
5. A. PARMAR, H. KUMAR, S.S. MARWAHA, J.F. KENNEDY, *Biotechnol. Adv.* 18, 289 (2000).
6. J. TORRES-BACETE, M. ARROYO, R. TORRES-GUZMAN, I. DE LA MATA, M.P. CASTILLON, C. ACEBAL, *Biotechnol. Appl. Biochem.* 32, 173 (2000).
7. D. CASCAVAL, R.Z. TUDOSE, C. ONISCU, *Hung. J. Ind. Chem.* 25, 245 (1997).
8. C. ONISCU, D. CASCAVAL, *Roum. Biotechnol. Lett.* 1, 23 (1996).
9. R.F. PRATT, M. DRYJANSKI, E.S. WUN, V.M. MARATHIAS, *J. Am. Chem. Soc.* 118, 8207 (1996).
10. M. ITOH, M.P. THIEN, T.A. HATTON, D.I.C. WANG, *Biotechnol. Bioeng.* 35, 853 (1990).
11. D. CASCAVAL, C. ONISCU, A.I. GALACTION, *Biochem. Eng. J.* 7, 171 (2001).
12. D. CASCAVAL, C. ONISCU, I.F. DUMITRU, A.I. GALACTION, *Roum. Biotechnol. Lett.* 6, 207 (2001).

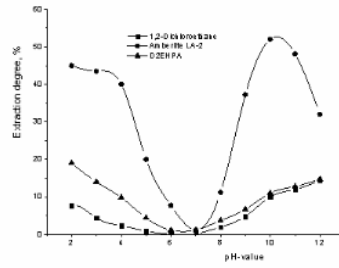
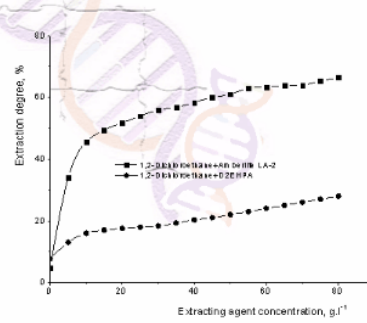


Figure 1. Influence of aqueous phase pH-value on physical and reactive extraction degree of 6APA ($C_{6APA0} = 3.30 \text{ g.l}^{-1}$, $C_A = 20 \text{ g.l}^{-1}$, $C_{D2EHPA} = 20 \text{ g.l}^{-1}$).



13. Figure 2. Influence of extracting agent concentration in 1,2-dichloroethane on extraction degree of 6APA ($C_{6APA0} = 6.67 \text{ g.l}^{-1}$, $\text{pH} = 10$ for extraction with Amberlite LA-2, $\text{pH} = 2$ for extraction with D2EHPA).

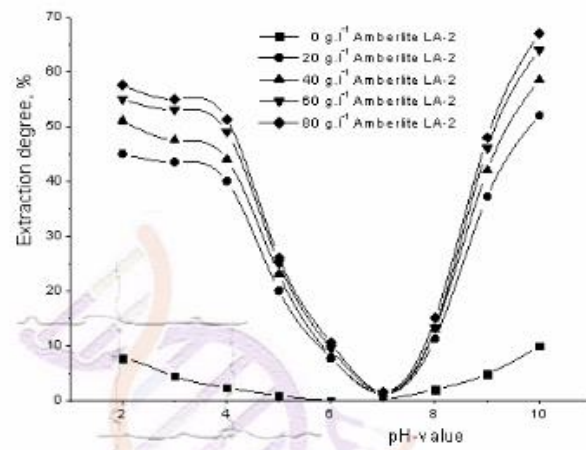


Figure 3. Cumulated influence of aqueous phase pH-value and Amberlite LA-2 concentration on extraction degree of 6APA ($C_{6APA0} = 5.04 \text{ g.l}^{-2}$).

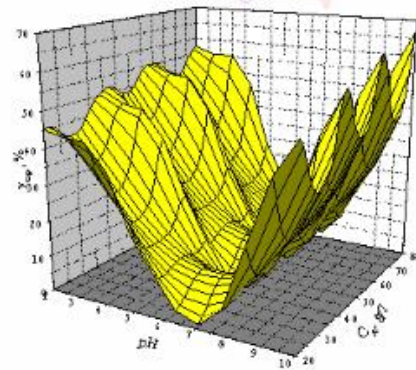


Figure 4. Surface plotted for cumulated influence of aqueous phase pH-value and Amberlite LA-2 concentration on extraction degree of 6APA.