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THE INFLUENCE OF pH AND SOLVENT POLARITY ON THE MECHANISM AND EFFICIENCY OF FOLIC ACID EXTRACTION WITH AMBERLITE LA-2

Studies on the reactive extraction of folic acid with Amberlite LA-2 in three solvents of different polarity (*n*-heptane, chloroform, dichloromethane), at various extractant concentrations and pH-values of the aqueous solutions, indicated that the pH-value controls the extraction mechanism through the dissociation of carboxylic groups of folic acid. Thus, the solute and the extractant participate at the interfacial reaction at a molar ratio of 1:2 for pH below pK_1 , and 1:1 for $pK_1 < pH < pK_2$.

The solvent polarity and pH-value exhibit a significant effect both on the extraction degree of folic acid (for an Amberlite LA-2 concentration of 80 g/l the extraction degree in dichloromethane was about 2–2.7 times greater than that corresponding to extraction in *n*-heptane, this difference increased with increasing pH of the aqueous solution) and on the extraction constant (by increasing the pH-value from 3 to 5.2, the extraction constants obtained for dichloromethane increased about 2.7 to 27.1 times compared with those obtained for extraction in *n*-heptane).

Key words: reactive extraction, folic acid, Amberlite LA-2, *n*-heptane, chloroform, dichloromethane, dielectric constant, extraction mechanism, extraction degree, extraction constant.

Folic acid, also called pteroylglutamic acid or vitamin B₉, is one of the important members of the vitamin B group (Figure 1).

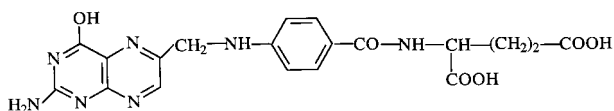


Figure 1. Chemical structure of folic acid

It is a growth factor, essential for making genetic material (DNA and RNA), red blood cells, for building muscle tissues, especially during periods of infancy, adolescence and pregnancy. It also intervenes in many metabolic functions, such as choline and amino acid biosynthesis. It has a direct effect on lessening depression, it helps regulate sleeps and appetite and prevents some birth defects [1]. Folic acid deficiency can cause poor growth, tongue inflammation, gingivitis, loss of appetite, irritability, mental sluggishness etc.

Folic acid could be obtained by extraction at acidic pH from vegetables (spinach leaves, cereals, lemons) or animal organs (liver) [2]. But, this method needs a high amount of raw materials and laborious stages for separation and purifications.

Its chemical synthesis is applied at an industrial scale and consists of the condensation of *p*-amino-(L)-glutamic acid with 2,3-dibromopro-pionaldehyde

and 2,4,5-triamino-6-hydroxy-pyridine/HCl in basic medium [1,3]. The overall yield of folic acid does not exceed 40–50%, the acid purity being 80%.

The condensation reactions can also be catalyzed by enzymes. Thus, folic acid can be obtained from precursors such as glutamic acid, *p*-aminobenzoic acid or 2-amino-4-hydroxypyridine-6-carboxyaldehyde using enzymes extracted from *Escherichia coli*, *Lactobacillus arabinosus* or *Mycobacterium avium*, with or without needing supplementary stages of the chemical synthesis type [1]. Due to economic limitations, these methods are only used on a laboratory scale.

Folic acid production by biosynthesis has been recently developed, the obtained product possessing a superior bioavailability compared to that obtained by chemical pathways [4]. This method is based on the high metabolic potential of mutant *Bacillus subtilis* to biosynthesize folic acid on a glucose substrate. The obtained conversion of glucose to folic acid was 0.16 moles folic acid/mole glucose, a value several times greater than that reached when wild strains of *Bacillus subtilis* were used.

The separation and purification of folic acid from fermentation broths implies biomass filtration and acid sorption on anionic exchangers, after preliminary purification of the filtrate by thermal or chemical treatments. These stages are not difficult, but the elution of anionites with basic solutions (sodium hydroxide) must be carried out gently, due to the low stability of folic acid at pH greater than 10, which is an important limitation of the separation technology.

The separation of biosynthetic products from fermentation broths by extraction constitutes an efficient method for many technologies. For compounds which dissociate in aqueous solutions, the performance of

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extraction process can be enhanced by reactive extraction with an extractant added to the organic phase [5–7]. Reactive extraction using extractants of organophosphorus compounds or height molecular mine derivatives types have been successfully applied to the separation of some carboxylic acids, such as: acetic acid, lactic acid, citric acid, succinic acid, maleic acid, ascorbic acid or beta-lactamic antibiotics [5–18].

Due to the insolubility of folic acid in organic solvents [1], its separation by physical extraction is impossible. But, its extraction could become possible by adding an extractant which reacts with folic acid to the solvent, leading to the formation of a hydrophobic compound, as in the case of the above mentioned carboxylic acid extraction. For this purpose, our studies were focused on the reactive extraction of folic acid with Amberlite LA-2 (lauryl-trialkyl-methylamine). Because the solvent polarity and the pH-value of aqueous solution are important factors that control the extraction, the cumulative influence of these factors on the mechanism and efficiency of the reactive extraction of folic acid in three different solvents (n-heptane, chloroform, dichloromethane) was analyzed in this paper.

MATERIALS AND METHOD

The experiments were carried out using an extraction column with vibratory mixing. The laboratory equipment was described in detail in previous papers [19]. Phase mixing was performed by means of a perforated disk of 45 mm diameter and 20% free section. The vibrations had a frequency of 50 s^{-1} and a 5 mm amplitude. The mixer position was maintained at the initial contact interface between the aqueous and organic phases. The extraction time was 1 minute at a constant temperature of 20°C . The resulting emulsion was evacuated at the base of the column and phase-separated in a centrifugal separator at 5000 rpm.

The folic acid initial concentration in aqueous solution was 0.08 g/l. The reactive extraction was performed with Amberlite LA-2 solved in three solvents with different dielectric constants (Table 1). The extractant concentration in the organic phase was varied between 10 and 160 g/l. The volumetric ratio of the aqueous and organic phase was 1 (20 ml of each phase).

Table 1. Dielectric constants of the used solvents at 20°C [20]

Solvent	n-Heptane	Chloroform	Dichloro-methane
Dielectric constant	1.90	4.806	9.08

The pH-value of the initial aqueous solution was varied between 3 and 10. The pH was adjusted with a solution of 3% sulfuric acid or 3% sodium hydroxide,

depending on the defined pH-value. The pH-values were determined using a digital pH-meter (Consort C836) and were recorded throughout each experiment. Any pH change was noted during the extraction experiments.

The extraction process was analyzed by means of the extraction degree and distribution coefficient. For calculating these parameters, the folic acid concentration in the initial solution was measured by high performance liquid chromatography (HPLC) with a Shim-pack CLC-ODS column (6 mm diameter and 150 mm length) and UV detector at 210 nm. The mobile phase was a mixture of 100 mM phosphate buffer (pH=6) and 0.8 mM n-octylsulphonate sodium salt/acetonitrile in a volume ratio of 9/1. A mass balance was used to calculate folic acid concentration in the organic phase.

RESULTS AND DISCUSSION

The reactive extraction of folic acid, $\text{R}(\text{COOH})_2$, with Amberlite LA-2, Q, occurs by means of an interfacial reaction with the formation of a strong hydrophobic compound. The interaction between the acid and the extractant could be realized by hydrogen bonding, with undissociated carboxylic groups, or by ion-pair formation, if the acid dissociates in the aqueous solution. Furthermore, compounds of the acidic or aminic adduct type could be formed at the interface, depending on the chemical characteristics of the extraction system components and solvent polarity [5,6].

As observed for the reactive extraction of others compounds having voluminous molecules with Amberlite LA-2 and due to the initial concentration of folic acid, which was much lower than that of Amberlite LA-2, it could be assumed that formation of the acidic adduct is sterically hindered [5,6,17,19]. Therefore, the interfacial compounds could be of the $\text{R}(\text{COOH})_2\text{Q}$ or $\text{R}(\text{COOH})_2\text{Q}_2$ type, formed by neutralization of one or both carboxylic groups with extractant molecules, or of the aminic adduct type, $\text{R}(\text{COOH})_2\text{Q}_n$ with $n > 2$. The formation of molecular associations is more pronounced in low-polar solvents and increases the hydrophobicity of the interfacial compound [5].

The pH-value of the aqueous phase exhibits a significant influence on the reactive extraction efficiency. As could be observed from Figure 2, the reactive extraction degree of folic acid strongly decreases with pH increase.

Folic acid contains in its structure three groups that could dissociate depending on the aqueous solution pH-value, their acid values being: $k_1 = 2 \cdot 10^{-5}$, $k_2 = 1.58 \cdot 10^{-7}$, $k_3 = 1 \cdot 10^{-9}$ at 25°C [23]. In this context, the variation of extraction efficiency with pH increase, cumulated with the insolubility of folic acid in the used solvents, suggests that folic acid can be extracted with Amberlite LA-2 if it exists in the aqueous phase in undissociated or partially dissociated from.

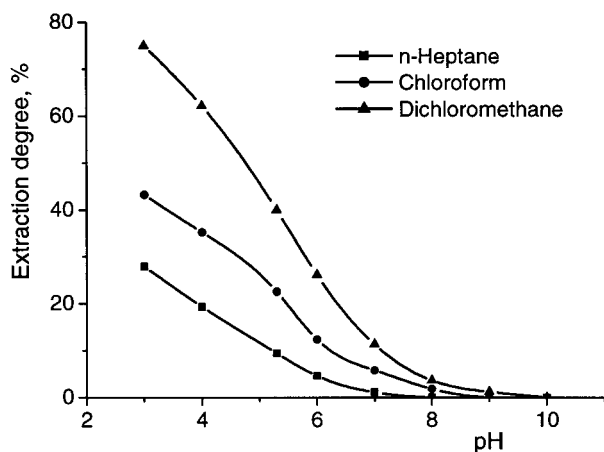
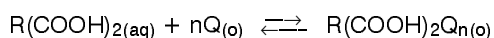


Figure 2. Influence of the pH of the aqueous phase on the reactive extraction degree of folic acid into different solvents

For studying the cumulated influence of pH-value and solvent polarity on the reactive extraction mechanism, in the next step the experiments were carried out at pH = 3, which corresponds to the undissociated form of folic acid ($pK_1 = 4.69$), and at pH = 5.2, this value corresponding to its partially dissociated form ($pK_2 = 6.80$).

For the case of aminic adduct formation, the interfacial reaction can be expressed as follows:



Therefore, the distribution coefficient, D , is calculated using the relationship:

$$D = \frac{[R(\text{COOH})_2Q_{n(o)}]}{[R(\text{COOH})_2(\text{aq})]} \quad (1)$$

where $[R(\text{COOH})_2(\text{aq})]$ and $[R(\text{COOH})_2Q_{n(o)}]$ represent the overall concentrations of folic acid and extracted compound at the equilibrium state.

According to the interfacial equilibrium, the extraction constant, K_E , can be calculated by means of the following expression:

$$K_E = \frac{[R(\text{COOH})_2Q_{n(o)}]}{[R(\text{COOH})_2(\text{aq})] \cdot [Q_{(o)}]^n} \quad (2)$$

$$\Rightarrow [R(\text{COOH})_2Q_{n(o)}] = K_E \cdot [R(\text{COOH})_2(\text{aq})] \cdot [Q_{(o)}]^n \quad (3)$$

If the pH is 3, the concentration of undissociated folic acid in aqueous solution, $[R(\text{COOH})_2(\text{aq})]$, is equal to its overall concentration in aqueous solution, $[R(\text{COOH})_2(\text{aq})]$. Thus, the distribution coefficient can be calculated as a function of the extraction constant using the correlation:

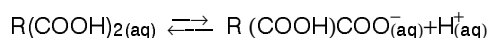
$$D = K_E \cdot [Q_{(o)}]^n \quad (4)$$

Correlation (4) in the logarithmic form is linear:

$$\ln D = \ln K_E + n \cdot \ln[Q_{(o)}] \quad (5)$$

Because the initial concentration of extractant is far higher than the initial concentration of folic acid, $[Q_{(o)}]$ could be assumed to be the initial concentration of Amberlite LA-2 in the organic phase. Consequently, from the slope of the straight line it is possible to determine the number of extractant molecules, n , which participate in the formation of the interfacial compound, and from the intercept the value of the extraction constant, K_E .

For pH = 5.2, the concentration of undissociated folic acid can be calculated from its overall concentration in the aqueous phase and the dissociated acid concentration. The dissociation constant, k_1 , corresponds to the following dissociation equilibrium:



and is determined by the relationship:

$$k_1 = \frac{[R(\text{COOH})\text{COO}^-(\text{aq})] \cdot [\text{H}^+]}{[R(\text{COOH})_2(\text{aq})]} \quad (6)$$

Thus, the concentration of undissociated folic acid becomes:

$$[R(\text{COOH})_2(\text{aq})] = [R(\text{COOH})_2(\text{aq})] - [R(\text{COOH})\text{COO}^-(\text{aq})] \quad (7)$$

$$[R(\text{COOH})\text{COO}^-(\text{aq})] = k_1 \frac{[R(\text{COOH})_2(\text{aq})]}{[\text{H}^+]} \quad (8)$$

$$[R(\text{COOH})\text{COO}^-(\text{aq})] = k_1 \frac{[R(\text{COOH})_2(\text{aq})]}{1 + \frac{k_1}{[\text{H}^+]}} \quad (9)$$

In this case, the distribution coefficient can be obtained by combining equations (1), (3) and (9):

$$D = K_E \cdot \frac{[Q_{(o)}]^n}{1 + \frac{k_1}{[\text{H}^+]}} \quad (10)$$

The logarithmic form of equation (10) is linear:

$$\ln D + \ln \left(1 + \frac{k_1}{[\text{H}^+]} \right) = \ln K_E + n \cdot \ln [Q_{(o)}] \quad (11)$$

which may be used to determine n and K_E graphically, similarly to the calculation for pH = 3.

In order to determine the number of extractant molecules which react with folic acid, the influence of extractant concentration on the extraction efficiency was analyzed. The experimental results, plotted in Figure 3 for the two pH-values and the three solvents, indicated that the extraction degree increased with Amberlite LA-2 concentration in the organic phase.

At pH=3, the extraction degree strongly increases for Amberlite LA-2 concentrations lower than 80 g/l, which is not the case at higher concentrations. The effect is more pronounced for solvents with higher

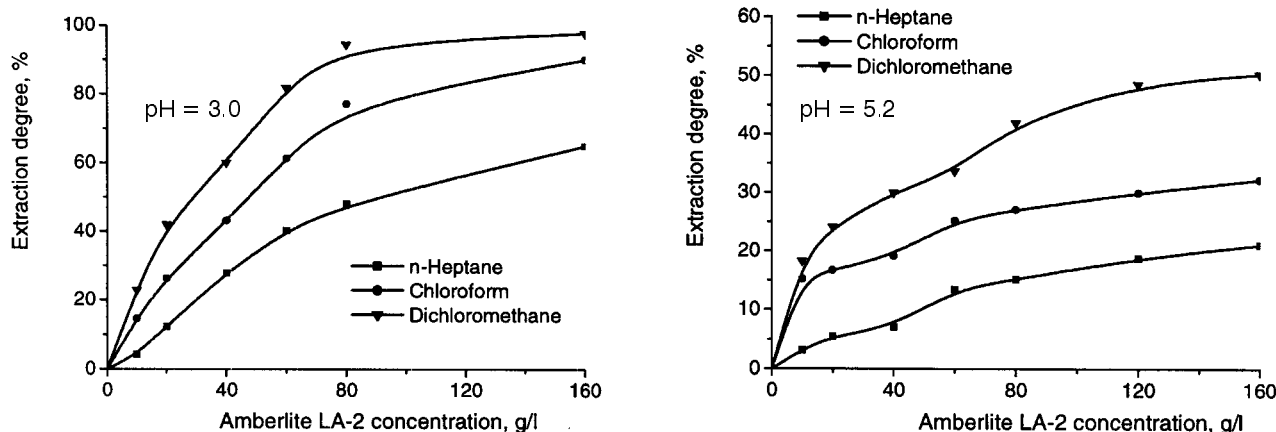


Figure 3. Influence of Amberlite LA-2 concentration on the reactive extraction degree of folic acid into different solvents

polarity (chloroform and dichloromethane). On the other hand, for pH = 5.2, the extraction degree continuously increases for the entire domain of extractant concentration increase. The increase of extraction efficiency with increasing solvent polarity was recorded for both pH-values of the aqueous phase.

Using the experimental data from Figure 3 and by plotting equations (5) and (11), the straight lines in Figure 4 were obtained.

It could be observed that the slope of the straight line depends on the pH of the aqueous solution:

- for pH = 3 $n = 1.91 - 2.05$
- for pH = 5.2 $n = 0.89 - 1.10$

thus indicating modification of the interfacial compound structure as a function of pH. In the case of undissociated folic acid, reactive extraction occurs by the interfacial formation of $R(COOH)_2Q_2$, by coupling the extractant with both carboxylic groups of the solute by means of hydrogen bonds. The dissociation of folic acid molecules limits their capacity to interact with extractant molecules. Consequently, at pH=5.2 only one molecule of Amberlite LA-2 interacts with the folic acid molecule,

by bonding the undissociated carboxylic group. The solvent polarity does not exhibit any influence on the structure of the interfacial compound formed at a certain pH.

But, Figure 3 suggests that for reaching high levels of extraction efficiency, high extractant concentrations is required, higher than the stoichiometric values needed for reacting with folic acid. This behavior of the extraction system is the result of one of the following reasons:

- a. Due to the low acidity of folic acid, the interfacial reaction occurs slowly, the accumulation of one reactant at the interface (Amberlite LA-2) increasing the rate of chemical reaction, consequently increasing the amount of extracted solute for a certain duration of the extraction.
- b. High molecular weight amines, such as Amberlite LA-2, can form molecular aggregates (20-40 monomers) of the micellar type, especially in low-polar solvents [5]. The formation of these aminic aggregates could induce an increase of the interfacial product solubility in the organic phase by entrapping it into the micelles. On the other hand, at high concentration, the

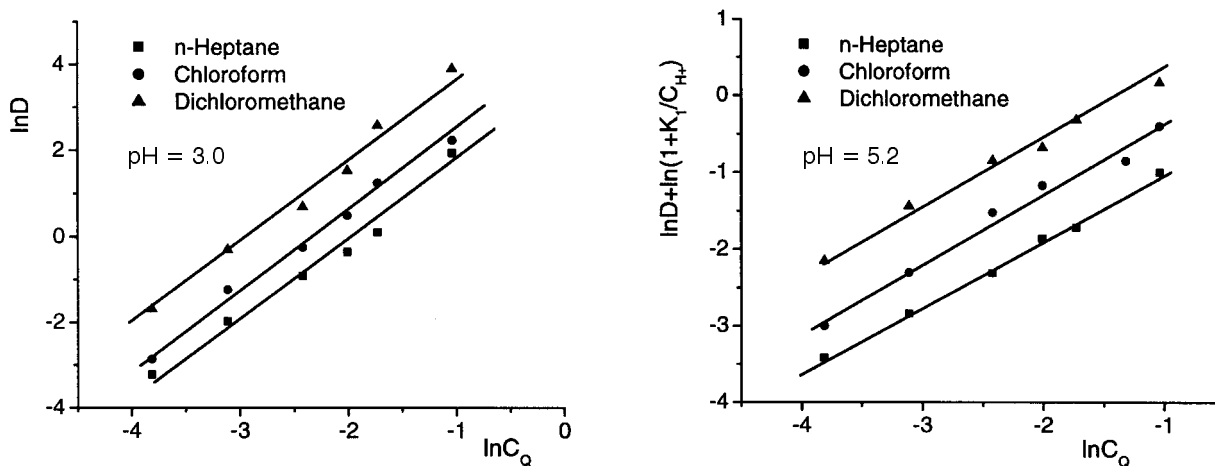


Figure 4. Graphical representation of the straight lines defined by equations (5) and (11)

extractant can solvate the interfacial compound, thus also increasing the solute solubility in solvent, this process being promoted by the increase of the solvent dielectric constant.

In order to establish the mechanism responsible for the influence of extractant concentration on extraction efficiency, the effect of extraction duration was analyzed. The experimental data indicated that the duration did not relevantly influence the extraction yield, a significant increase of the extraction yield being recording only for times up to 10 s. This variation suggests that the rate of the interfacial reaction is rather high and that the limiting step of the extraction is not of the kinetic type.

As presented above, solvent polarity represents an important parameter that controls the extraction of ionizable solutes. The dielectric constant is considered to be a characteristic of the solvent – solute local interactions, by inducing the limitation of solute solvation by solvent or extractant, due to the presence of ionizable groups in the solute chemical structure [21,22,24,25]. Modification of the dielectric constant has a smaller effect on the solubility and extraction of non-electrolytes or weak electrolytes, but it becomes an important factor for the extraction of solutes that dissociate.

Solvent polarity exhibits a significant influence on the reactive extraction degree of folic acid, its increase inducing the enhancement of separation process efficiency (Figures 2 and 3). Thus, for an Amberlite LA-2 concentration of 80 g/l the extraction degree in dichloromethane was about 2–2.7 times greater than that corresponding to the extraction in n-heptane, this difference being increased by increasing the pH of the aqueous solution.

The enhancement of extraction efficiency due to the increase in solvent polarity cumulated with the high rate of the interfacial reaction underlines that the solubilization and diffusion of the interfacial compound into the organic phase bulk represents the limiting step of the separation process. Moreover, the cumulated favorable effects of the increase of both extractant concentration and solvent polarity indicate that solubilization of the interfacial compound is achieved by its solvation by extractant molecules and not by its entrapment into aminic micelles.

The solvent polarity also significantly influences the extraction constant, K_E . It can be seen from Figure 5 that an increase of the solvent dielectric constant from 1.9 to 9.08 leads to a significant increase of the extraction constant, regardless of the pH of the folic acid solution.

Because the effect of solvent polarity becomes more important in the extraction of dissociated molecules, the influence of dielectric constant increase is more pronounced in the case of folic acid extraction at

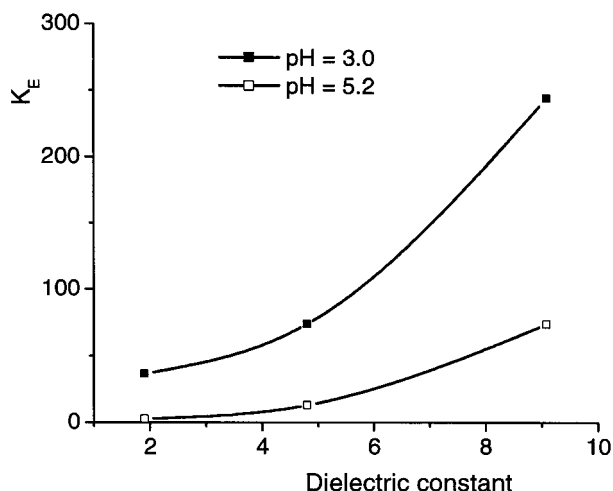


Figure 5. Influence of the dielectric constant of the solvent on extraction constant

pH = 5.2 (at pH = 5.2 the extraction constant for dichloromethane was about 27.1 times greater than that for n-heptane, respectively, about 2.7 times greater at pH = 3).

At both pH-values, the obtained variation of the extraction constant is the result of the increase of amount of extracted folic acid with increasing solvent polarity and of the corresponding decrease the folic acid concentration in the raffinate at equilibrium.

CONCLUSIONS

Studies of the reactive extraction of folic acid with Amberlite LA-2 in three solvents with different polarities (n-heptane, chloroform, dichloromethane), at various extractant concentrations and pH-values of the aqueous solutions indicated that reactive extraction occurs at the interface by means of hydrogen bonds established between the extractant molecules and non-dissociated carboxylic groups of the solute. A decisive role is played by the pH-value, which controls the molar ratio between the folic acid and the extractant through the dissociation of carboxylic groups (the folic acid:extractant molar ratio is 1:2 for pH below pK_1 , respectively 1:1 for $pK_1 < pH < pK_2$). For both pH domains, solubilization of the interfacial compound into the organic phase is achieved by solvation with extractant molecules, a process that is promoted at higher solvent dielectric constant.

The cumulated increase of solvent polarity and pH exhibits a significant effect both on the extraction degree of folic acid (for an Amberlite LA-2 concentration of 80 g/l the extraction degree in dichloromethane was about 2–2.7 times greater than that corresponding to extraction in n-heptane, this difference being enhanced by increasing the pH of the aqueous solution) and on the extraction constant (by increasing the pH from 3 to 5.2, the extraction constants obtained for dichloromethane

were greater about 2.7 to 27.1 times than those obtained for extraction into n-heptane).

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IZVOD

UTICAJ pH I POLARNOSTI RASTVARAČA NA MEHANIZAM I EFIKASNOST EKSTRAKCIJE FOLNE KISELINE SA AMBERLITOM LA-2

(rad)

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Proučavanje reaktivne ekstrakcije folne kiseline sa amberlitom LA-2 korišćenjem tri rastvarača različite polarnosti (n-heptan, hloroform, dihlormetan), pri različitim koncentracijama ekstragensa i pH-vrednostima vodenih rastvora, pokazala su da pH rastvora kontroliše mehanizam ekstrakcije putem disocijacije karboksilne grupe folne kiseline. Pokazano je da rastvorak i ekstrakciono sredstvo učestvuju u reakciji na međufaznoj površini pri molarnom odnosu 1:2 kada je pH vrednost manja od pK_1 , odnosno pri odnosu 1:1 kada je $pK_1 < pH < pK_2$.

Polarnost rastvarača i pH rastvora pokazuju značajan uticaj na stepen ekstrakcije folne kiseline. U slučaju korišćenog Amberlit LA-2 koncentracije 80 g/l i dihlormetana kao ekstragensa, stepen ekstrakcije je 2 – 2.7 puta veći od postignutog sa n-heptanom kao ekstragensom, pri čemu je utvrđeno da se razlika u ostvarenim stepenima ekstrakcije uvećava sa povećanjem pH vodenog rastvora. Takođe se povećava konstanta ekstrakcije povećanjem pH od 3 do 5,2, pa je u slučaju dihlormetana kao ekstragensa ona veća za 2,7 do 27,1 puta u odnosu na konstantu ekstrakcije folne kiseline u n-heptanu.

Ključne reči: reaktivna ekstrakcija, folna kiselina, Amberlit LA-2, n-heptan, hloroform, dihlormetan, dielektrična konstanta, mehanizam ekstrakcije, stepen ekstrakcije, konstanta ekstrakcije.