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Centrifugal partition chromatographic reaction for the production of chiral amino acids

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Abstract

The use of a centrifugal partition chromatographic reactor is investigated for the production of chiral amino acids from racemic mixtures. Chirally selective enzymatic hydrolysis of *N*-acetyl-L-methionine into acetic acid and L-methionine was carried out in the chromatographic reactor to demonstrate the concept of integrated reaction and separation in centrifugal partition chromatography (CPC). The products L-methionine and acetic acid, as well as the unconverted substrate, *N*-acetyl-D-methionine are obtained separately. An aqueous two-phase system, consisting of PEG 600, potassium phosphate and water was successfully applied as liquid–liquid two-phase system in CPC. A model is presented, which describes the reaction chromatograms on the basis of the independently measured partition and mass transfer coefficients of the individual (reacting) components. The model appears to be a valuable tool for optimizing the reaction–separation process.

Keywords: Centrifugal partition chromatography; Aqueous two-phase systems; Chiral conversions; Amino acids; *N*-Acetyl-L-methionine

1. Introduction

1.1. Chromatographic reaction for the production of chiral amino acids

Chromatographic reaction, in which (bio)conversion is integrated with a chromatographic separation, is a promising process for increasing the conversion of equilibrium limited processes. The concept of chromatographic reaction has been studied intensively during the last three decades [1,2]. In this work, the possibility of shifting an equilibrium limited conversion beyond its thermodynamic equilibrium is considered. By simultaneous chromatographic separation of the products, the reverse reaction is virtually suppressed and conversions beyond the thermodynamic equilibrium are theoretically possible. Although the concept of chromatographic reaction is broadly applicable, this work is limited to the production of chiral amino acids. A number of industrial methods to produce chiral amino acids has been reviewed by Jansen [3]. An equilibrium limited reaction in which two products are being formed is the chirally selective enzymatic hydrolysis of *N*-acetyl-L-methionine into acetic acid and L-methionine (Fig. 1). The remaining *N*-acetyl-D-

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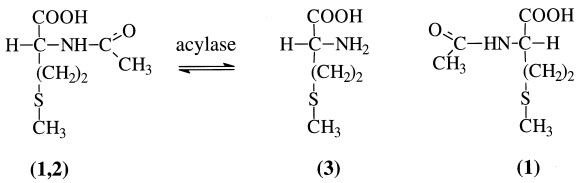

Fig. 1. Selective enzymatic hydrolysis of *N*-acetyl-D,L-methionine (**1**, **2**) into L-methionine (**3**) and acetic acid (not shown).

methionine can be separated from the desired product L-methionine. By chromatographic separation of the product from the unconverted substrate, it is possible to obtain chirally pure L-methionine from the racemic mixture. The equilibrium and kinetics of this reaction have been studied extensively by Wandrey and Flaschel [4]. The degree of conversion at equilibrium depends on the initial substrate concentration, as shown in Fig. 2. Fig. 2 is calculated for an initial concentration *N*-acetyl-L-methionine between 0 and 500 mol m^{-3}, equilibrium constant K_{eq} is 2750. This lower equilibrium conversion at higher substrate concentration leads to voluminous equipment or large recycle streams in conventional processes to produce chirally pure amino acids. Therefore, it is worthwhile to investigate alternative processes such as chromatographic reaction. The difference in migration velocities (elution times) of the two products in the chromatographic column (acetic acid and L-methionine) leads to separation of these products in the column and effectively suppresses the reverse reaction.

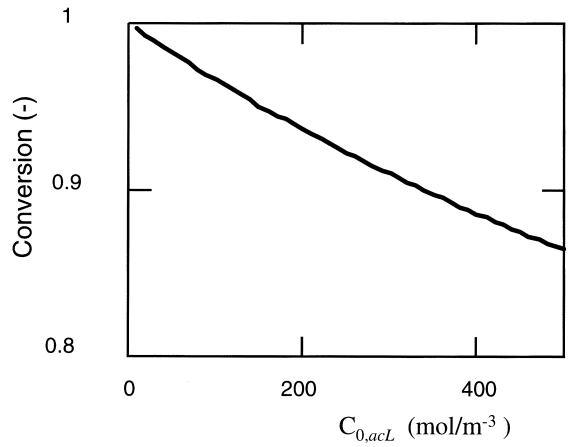

Fig. 2. Conversion as function of the initial substrate concentration according to Wandrey and Flaschel [4]. $C_{0,acL}$ is the initial concentration *N*-acetyl-L-methionine.

1.2. Chromatographic column

A relatively new type of chromatographic technique is used for the integrated reaction and separation: centrifugal partition chromatography (CPC). This chromatographic technique is based on the difference in distribution of two or more components over two liquid phases. The liquid stationary phase is kept in the column by a combination of channel geometry, centrifugal force and a density difference between the two immiscible liquid phases. This type of liquid–liquid chromatography has a number of advantages over conventional (liquid–sorbent) chromatography [5]: the stationary liquid phase has a large capacity, especially when compared to chromatographic columns with a solid (or supported liquid) stationary phase. In CPC, the capacity of the column is limited by solubility of the components and volume ratio of the two liquid phases, rather than by sorbent capacity.

Albertsson [6] pointed out the challenging potentials of aqueous two-phase systems (ATPS) for the separation of biochemical products. ATPS are formed when two polymers or one polymer and a salt are added to water. Above certain concentrations of the polymers and the salt, phase separation occurs and two liquid phases are formed. Both phases consist of more than 50 wt. % water. The advantage of ATPS over aqueous–organic phase systems is mostly based on the mild properties of the aqueous phases towards biochemical compounds. Furthermore, the solubility of amino acids in organic solvents (including alcohols) is far too low to apply organic solvents successfully in bi-phasic systems for the integrated reaction separation process. Although substantial decrease of enzyme activity might occur, it is possible, because of the mild conditions, to perform enzymatic reaction in the aqueous phases. The use of an aqueous two-phase system for the enzymatic hydrolysis of *N*-acetyl-D,L-methionine has been described by Kuhlmann et al. [7].

1.3. Goal of this research

In this work, the applicability of ATPS for integrated reaction and separation in CPC is investigated. The important subsystems of the integrated reaction and separation process, such as partition and mass transfer coefficients of the components and the kinetic parameters of the enzymatic reaction, are determined separately. A model is developed to predict the reaction chromatograms, using the selective enzymatic hydrolysis of *N*-acetyl-L-methionine as a model system.

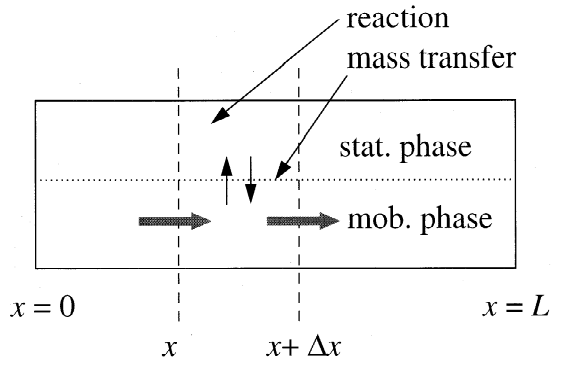

Fig. 3. Scheme for modeling the integrated reaction separation in CPC.

2. Model

2.1. Modeling integrated reaction and separation in CPC

The influence of partitioning behaviour and mass transfer effects on effluent profiles in CPC has been described by Van Buel et al. [8]. Their model included partitioning of the components over mobile and stationary phases, where the mobile phase was described as plug flow with axial dispersion and the stationary phase was considered to be unmixed. Mass transfer between the mobile and the stationary phase was described with the bifilm model by Lewis and Whitman [9]. Effluent profiles were accurately predicted by this model. In this work, a similar approach has been used to describe the chromatographic reaction. Because of the limited effect of dispersion in the mobile phase on peak broadening [8], this term is neglected. The model structure is shown schematically in Fig. 3. The partial differential equations describing the mobile phase concentration of species i are as follows:

$$\frac{\partial c_i}{\partial t} = -v\frac{\partial c_i}{\partial x} - \frac{k_o a}{1-\varepsilon_s}(K_i c_i - q_i) \tag{1}$$

where c is the concentration in the mobile phase, t is time, v is the linear velocity of a component in the mobile phase, $k_o a$ is the overall volumetric mass transfer coefficient, x is the position in the column, ε_s is the stationary phase hold-up, q is the concentration in the stationary phase and K is the partition coefficient. Subscript i stands for component i. A reaction term describing the enzymatic conversion (r_s) is added to the model. It is assumed that the enzymatic reaction occurs in the stationary phase only. The partial differential equations for the stationary phase are as follows:

$$\frac{\partial q_i}{\partial t} = \frac{k_o a}{\varepsilon_s}(K_i c_i - q_i) + \mu_i r_s \tag{2}$$

where μ_i is the stoichiometric coefficient of component i which is negative for reactants, positive for products and zero for inerts. r_s is the reaction rate per volume of stationary phase. In Eqs. (1) and (2), the molarity based partition coefficient K is defined as:

$$K_i = \frac{q_i}{c_i} \tag{3}$$

The enzymatic reaction has been described extensively by Wandrey and Flaschel [4]. They derived an expression for the enzymatic conversion rate, including substrate and product inhibition. For the enzymatic reaction in the stationary phase, including equilibrium limitation, this expression is given by Eq. (4).

$$r_s = \frac{EV_{max}}{1+\frac{q_{amL}+q_{amD}}{k_{in}}} \frac{q_{amL} - \left(\frac{q_m q_a}{k_{eq}}\right)}{K_m\left(1+\frac{q_m}{k_{ic,m}}\frac{q_a}{k_{ic,a}}\right)+q_{amL}} \tag{4}$$

in which E is the enzyme concentration (g l^{-1}), V_{max} is the specific maximum conversion rate (mol g^{-1} s^{-1}), the subscripts amL and amD indicate the L- and D-substrate, a indicates acetate and m methionine. K_{eq} is the equilibrium constant, k_{in} stands for the substrate inhibition coefficient and k_{ic} is the product inhibition constant. All constants in the kinetic rate equation depend on composition and nature of the phase system (solvent) in which the enzymatic reaction takes place. The concentration-based kinetic and equilibrium parameters depend on temperature and phase composition. Wandrey and Flaschel [4] studied the kinetics of the hydrolysis reaction in aqueous environment at different pH values. The optimum pH was found to be 7.0. In ATPS the kinetic rate constants can deviate significantly from those in aqueous environment.

Poly(ethylene glycol) (PEG) [7] and trace amounts of metal ions [4] can influence the reaction rate significantly as well. Kuhlmann et al. [7] found a decrease in initial activity in a PEG 300–potassium phosphate–water phase system of approximately 90% at an initial substrate concentration of 112 mol m^{-3}. It is also known from literature that PEG can have a positive effect on enzyme stability and influences both the position of the thermodynamic equilibrium and the reaction kinetics [11]. Depending on the concentration of the phosphate salts, present in the stationary phase, the buffering capacity of these salts may be sufficient to control the pH of the phase system. Possible changes in pH of the phase system influences both reaction kinetics and the partition coefficients of the components. The effect of the salts present in the stationary phase on the reaction rate has not been investigated in detail. The reaction rate in the top-phase of the ATPS is measured because these effects (PEG content, salt content and pH) influence the specific reaction rate of the enzyme. Deactivation of the enzyme through wash-out of metal ions can be avoided by adding cobalt chloride to the phase system via the mobile phase feed. Co^{2+} was found to have the most powerful effect among some other metal ions in enhancing the reaction rate [4].

2.2. Partitioning behaviour of the components

The degree of separation of the components in CPC is mainly determined by the partition coefficients in the two-phase system. In recent years, work has been done by a number of research groups to describe the partition behaviour of components in an ATPS. The composition of the two phases has a direct influence on the partition coefficient of a component [10] according to the following relation (Eq. (5)).

$$\ln K = k \cdot \Delta w \tag{5}$$

where, Δw is the composition difference of one of the phase forming components between the two phases and k is a constant, which depends on the properties of both the solute and the phase system. The partition coefficient can be fine-tuned by varying the composition of an ATPS, thereby making it possible to optimize the CPC separation. By varying the composition of the phase system, both the partition behaviour of the components and the hydrodynamic properties of the phase system are influenced. The change of the partition coefficients depends on the molecular properties of the species (hydrophobicity, charge, surface properties, etc.) and therefore the partition coefficients of each of the species can not be set independently. The hydrodynamic character of the phase system depends on the phase system composition. Because some properties of the ATPS (such as viscosity, surface tension, density difference and settling time) have to be within a certain range for application in CPC [5], the range in which the phase system composition can be varied is limited. The partition coefficients of the components have a large influence on peak broadening as well [8]. The following relation for the overall mass transfer coefficient was found to be valid:

$$\frac{1}{k_o a} = \frac{K}{k_m a} + \frac{1}{k_s a} \tag{6}$$

Eq. (6) shows the increase in mass transfer resistance (the main cause of peak broadening) at increasing partition coefficient. $k_m a$ and $k_s a$ stand for the volumetric mass transfer coefficients in mobile and stationary phase, respectively. These values are of the same order of magnitude for components with a similar size in the same phase system [8].

The partition coefficients are determined in shake flask experiments and by fitting them to an experimental chromatogram as described by Van Buel et al. [8]. This last method is only useful if the

components do not influence the composition of the individual phases (in general this method is only valid at lower concentrations). In this work both techniques were used to determine the partition coefficients.

3. Experimental

3.1. Chemicals

Table 1 shows the supplier and the purity of the chemicals. The enzyme, *N*-acylamino acid amidohydrolase, EC 3.5.1.14, Grade I, is used for the experiments, it contains 75 wt. % protein and has a specific activity of 0.56–0.83 mol (kg protein s)$^{-1}$ at 25°C.

3.2. Phase system and shake flask experiments

The ATPS were prepared by weighing appropriate amounts of water, dipotassium hydrogenphosphate, monopotassium dihydrogenphosphate and PEG 600 under vigorous shaking. Before addition, PEG 600 was slightly heated (20 min in a water bath at 40°C) to decrease the viscosity of PEG. After vigorous shaking and (overnight) equilibration at 25°C, the two phases were separated in a separation funnel.

The phase diagram of the PEG 600–H_2PO_4–K_2HPO_4–water system is obtained by constructing a two-phase system with known composition. Subsequently, under vigorous mixing, a weighted amount of water was added until the phase separation disappeared (the system turned clear). Subsequently, PEG 600 was added until the solution became turbid again. Water was added and this cycle of events was repeated. Every composition that showed a ''turbidity change'' added one point to the phase diagram. This method is called the titration method and is described by Albertsson [6]. Four tie-lines were obtained by preparing a two-phase system with known composition and analyzing both upper and lower phases. The amount of PEG 600 in the phase system was determined thermally by a High-Temperature Total Organic Carbon measurement (Dohrmann® DC-190, Rosemount Analytical, Santa Clara, CA, USA, 680°C). The amount of salt followed from the previously obtained binodal curve.

The partition coefficients of the components (*N*-acetyl-D,L-methionine, L-methionine and acetate) were determined from shake flask experiments. Four ATPS with different compositions were used in the experiments. The overall compositions of these phase systems and the compositions of the upper and lower phases is given in Table 2. The densities of the top and bottom phases were measured separately (AP PAAR, DMA 48). The viscosities of the upper and lower phase of the phase systems was measured with a Haake VT 550 viscometer. The pH of the upper and lower phases were measured (Mettler Toledo, pH-electrode) as well.

To determine the partition coefficients with shake flask experiments, solutions with different concentrations of *N*-acetyl-methionine, methionine and acetate (25, 50 and 67 mol m^{-3} phase system) were prepared. A flask (Chrompack, 50-ml bottle with

Table 1
Supplier and purity of the chemicals

Chemical	Formula	Supplier	Purity (%)
N-Acetyl-L-methionine	$C_{17}H_{13}NO_3S$	Sigma	>99
N-Acetyl-L,D-methionine	$C_{17}H_{13}NO_3S$	Sigma	>99
Acylase I	–	Sigma	Unknown
D,L-Methionine	$C_5H_{11}NO_2S$	Sigma	99.5
Potassium dihydrogenphosphate	KH_2PO_4	Lamers and Pleuger	98
Dipotassium monohydrogenphosphate	K_2HPO_4	Merck	98
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	Sigma	98.8
Sodium azide	NaN_3	Merck	>99
Sodium acetate	CH_3COONa	Baker	>99
PEG 600[a]	$HO\text{-}(CH_2CH_2O)_n\text{-}H$	Merck	Unknown
1,10-Phenanthroline	$Cl_2H_8N_2 \cdot H_2O$	Sigma	Unknown
Water	H_2O	Milli-Q	Unknown

[a] M=570–630 g mol^{-1} according to supplier, the molecular mass distribution is not determined.

Table 2
Composition of the PEG 600–potassium phosphate–water phase systems, before and after phase split at 25°C

Phase system	Composition PEG	(*w*, %) Salt	Phase	Comp. PEG (*w*, %)	Δw_{PEG}	Salt (*w*, %)	ρ (g ml^{-1})	η (mPa s)	pH
I	16.0	16.0	Upper	29.4	28.0	6.4	1.0982	6.67	7.25
			Lower	1.4		26.8	1.2274	2.74	7.09
II	18.0	18.0	Upper	35.3	34.6	4.6	1.0394	8.86	7.36
			Lower	0.7		31.8	1.2760	3.16	7.10
III	21.2	20.9	Upper	45.1	45.0	2.5	1.0970	13.77	7.40
			Lower	0.16		37.9	1.3394	3.97	7.14
IV	24.0	24.0	Upper	51.6	51.0	1.8	1.1018	20.23	7.78
			Lower	0.07		43.6	1.4013	5.62	7.25

PTFE cap) was filled with a weighed amount of both phases, and a weighed amount of the component was added. The phase system was stirred vigorously overnight in a water bath at 25°C. After equilibration, a sample of the upper phase and a sample of the lower phase were taken from the shake flask. The methionine and *N*-acetyl-D,L-methionine concentration of the upper and lower phase were determined by UV absorbance measurement (Ultrospec III spectrophotometer, Uppsala, Sweden), acetate concentration was determined by high-performance liquid chromatography (HPLC) on a Bio-Rad HPX-87H column of 300×7.8 mm I.D., operating at 60°C. The eluent was a 10 m*M* phosphoric acid solution of pH 2.0 at a flow-rate of 0.6 ml min^{-1}. Acetic acid was detected by UV absorbance at 210 nm.

3.3. Batch kinetic measurements

The kinetic parameters were determined from batch conversion experiments. Because in the integrated CPC experiments the reaction is assumed to occur in the stationary phase only, the kinetic experiments were carried out in the top-phase of the two-phase system. Cobalt chloride was added to a final concentration of 0.5 mol m^{-3} (the same concentration was used in the integrated experiments) to 100 ml top-phase to increase (or stabilize) the enzyme activity. Initial concentrations of 100 mol m^{-3}, *N*-acetyl-D,L-methionine were used in the batch experiments. The reaction was started by adding 2 ml of a 10 g l^{-1} enzyme solution in demineralised water. The reaction vessel was placed in a water bath at 25°C. Off-line colorimetric measurement of the L-methionine concentration were performed to follow the conversion. The reaction was stopped by adding 500 μl of the sample to 1 ml, 15 m*M* 1,10-phenanthroline solution in water.

3.4. The CPC column

The CPC experiments were performed with a C.P. Chromatograph LLB-M, manufactured by Sanki Engineering (Tokyo, Japan). The column consisted of a single Sanki Engineering HPCPC 1000 cartridge. This cartridge contained two identical parts with 1068 channels each. All experiments were performed in the so-called ''descending'' mode (in which the lighter phase is retained in the column). The column volume was 101.2 ml.

A more detailed description of the operating procedure of the CPC column is presented by Van Buel et al. [8]. The experimental set-up is shown in Fig. 4. The mobile phase was pumped (Shimadzu LC-8A) through an injection loop (Rheodyne, 2 ml). A switch valve (Rheodyne) included or excluded the CPC cartridge from the loop. A small flow of 0.15 ml min^{-1} water was added to avoid formation of a biphasic system by the formed product acetate. The latter effect was found to disturb the spectrophotometric measurement of methionine. The temperature of the column was kept approximately constant by thermostatting the mobile phase with a water bath at 25°C. The components were detected at different wavelengths. The wavelengths at which the components were detected are given in Table 3.

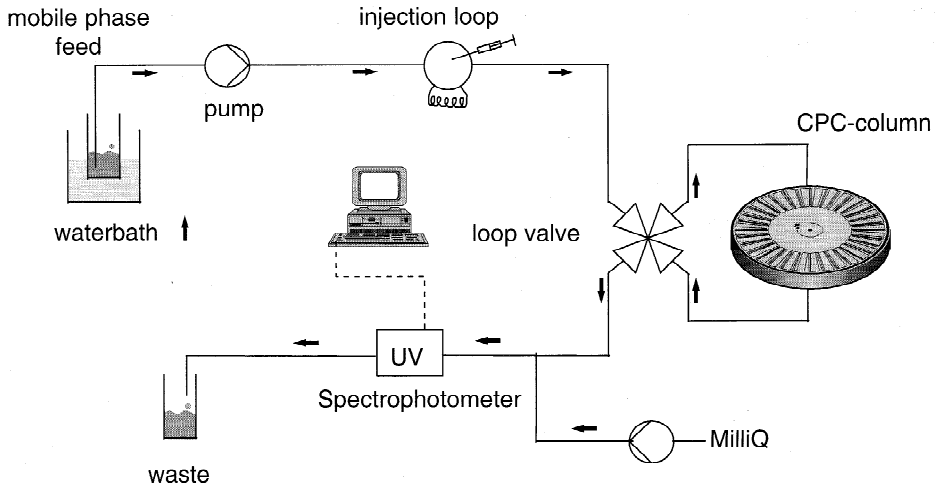

Fig. 4. Equipment.

Firstly, the tubes and the CPC cartridge were rinsed with demineralised water (40°C) to remove the remainder of previous experiments. To avoid air bubbles in the column during CPC operation, the mobile phase was deaerated by pouring it over a G4 glass filter while applying vacuum. The column was filled with the lighter stationary phase while the rotor rotated at 200 rpm, and the column was switched in the ascending mode. In this manner gas bubbles present in the (the lighter phase) were removed. In all experiments at least 200 ml of stationary phase were used to fill the column. The column was disconnected by switching the loop valve and all tubing was rinsed with demineralised water. Subsequently, all tubing was filled with the mobile phase. The column was rotated at the desired rotational frequency in the descending mode (1000 rpm) and the flow-rate was set to 5 ml min^{-1}. The mobile phase was pumped through the column in combination with the column volume. The stationary phase hold-up was determined by measuring the amount of stationary phase that flowed out of the column before the injection of the substrate [8]. During the experiment the stationary phase hold-up was not monitored.

Table 3
Wavelength for UV detection

Components	Optimal wavelength (nm)
N-Acetyl-L,D-methionine	230
Acetic acid	214
D,L-Methionine	225
Acylase I	220

Single component injection experiments to determine K and $k_o a$ were performed for methionine and acetyl methionine. Because of the experimentally observed limiting solubility of methionine in the biphasic system (approximately 80 mol m^{-3}) and the stoichiometry of the reaction, these experiments were performed at concentrations acetyl-L-methionine up to 36 mol m^{-3}. The components were dissolved in the bottom (mobile) phase. Because of problems with the spectrophotometric detection of acetate, no CPC experiments could be performed to determine K and $k_o a$ for this component.

3.5. Experimental reaction chromatograms

To carry out the enzymatic reaction in the biphasic system, 100 mg l^{-1} enzyme was added to the phase system. Because of the low solubility of the enzyme in the mobile phase [7], it was assumed that the enzyme was present in the stationary (top) phase only. Because the overall composition of the phase system and the position of the tie-line, the enzyme concentration in the stationary phase was assumed to be around 200 mg l^{-1}.

The experiments including chromatographic reaction were performed with phase system I (Table 2)

Table 4
Experimental conditions of the integrated experiments

	All experiments		
Phase composition			
NaN_3	0.020 *w*, %		
CPC mode	Descending		
Rotational frequency	1000 rpm		
Flow-rate	$4.0 \cdot 10^{-6}$ m^3 min^{-1}		
Temperature	25°C		
CPC column volume	$101.3 \cdot 10^{-6}$ m^3		
Injection volume	$2 \cdot 10^{-6}$ m^3		
Phase system (Table 2)	I		
Acylase I concentration	0.10 g l^{-1}		
$CoCl_2$	0.50 mol m^{-3}		
	Experiment 1	Experiment 2	Experiment 3
Stationary phase hold-up	0.61	0.80	0.80
Injection sample	L	D,L	D,L
Sample concentration	35.06 mol m^{-3}	70.4 mol m^{-3}	70.4 mol m^{-3}

only. The experimental conditions are listed in Table 4. In the first integrated experiment (1), chirally pure *N*-acetyl-L-methionine was injected. From this experiment, it can be seen whether or not the substrate is converted in the chromatographic column. A second integrated experiment (2) was performed to study the effect of *N*-acetyl-D-methionine on the conversion of the L-substrate. Racemic substrate (*N*-acetyl-D,L-methionine) was injected. The experiments were performed with a biphasic system that was in equilibrium with cobalt chloride (0.50 mol m^{-3}) and acylase I (100 mg l^{-1}). *N*-Acetyl-methionine was dissolved in the bottom phase of the phase system. The column was filled and operated as described above. A volume of 2 ml was injected in the mobile phase at $t=t_0$. Analysis of the products was done by collecting samples of 1500 ml from the effluent. The enzymatic reaction was stopped instantaneously by adding the samples to 3 ml, 1,10-phenanthroline (15 mol m^{-3}) solution in water. On-line analysis of the effluent was performed spectrophotometrically. To compare the effluent profiles with the presented model, model calculations with the previously described model are given as well. To check the stability of the phase system, a third integrated experiment (3) was performed, with the same phase system and the same enzyme as used in experiment 2. Loss in stationary phase hold-up was indicated by different retention times of the products (or unconverted substrate). Deactivation of the enzyme could be observed as well with this experiment. Deactivation would result in a lower product over unconverted substrate ratio.

4. Results and discussion

4.1. Phase system

The phase diagram for the system (PEG 600–potassium phosphate–water) as determined in this work is given in Fig. 5. The compositions of the upper and lower phases of systems I–IV are given in Table 2. Viscosities, densities and pH values are presented as well. A small systematic pH difference between the two phases is observed. This pH difference increases with increasing difference in composition between the phases. Similar pH differences have been observed previously [10].

4.2. Shake flask experiments

Measured partition coefficients of the components are presented in Fig. 6a and Fig. 6b for phase systems I and II, respectively. The partition coefficient of *N*-acetyl-methionine is slightly dependent on concentration and decreases with increasing concentration *N*-acetyl-methionine in both phase sys-

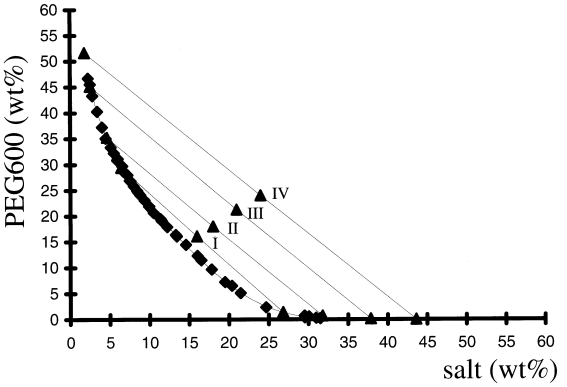

Fig. 5. Binodal of the phase system PEG 600–potassium phosphate ($K_2HPO_4/KH_2PO_4 = 1.82$ on mass basis)–water at 25°C (♦ represent binodal points, ▲ is the composition before and after phase split). Roman numbers refer to the phase systems in Table 2.

tems. The partition coefficient varies from 4.3 (50 mol m^{-3}) to 3.4 (200 mol m^{-3}) in phase system I and from 8.0 (50 mol m^{-3}) to 6.4 (200 mol m^{-3}) in phase system II. The partition coefficient of methionine does not seem to depend on concentration in the range from 25–67 mol m^{-3}. The average value for phase system I is 0.92, for phase system II it is 0.95. The partition coefficient of acetate decreases with increasing concentration. Especially at lower concentrations, the partition coefficient appears to be a strong function of the acetate concentration. This is probably due to the influence of charged acetate on the composition of the phase system.

4.3. Estimation of partition and mass transfer coefficients

The partition coefficients of *N*-acetyl-methionine and methionine were determined from pulse–response measurements in the CPC apparatus as well. The same method to fit the parameters as described by Van Buel et al. [8] was used. They found a good agreement between partition coefficients from shake flask experiments and partition coefficients fitted with the model. The results of the measurements (K and $k_o a$) are shown in Table 5. The fitted partition coefficients deviate significantly from the values found in the shake flask experiments. The difficulty

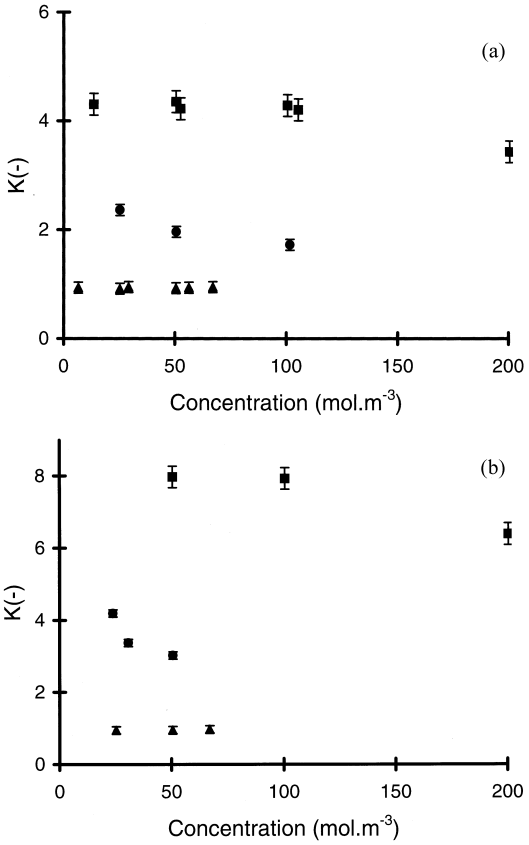

Fig. 6. (a) Partition coefficients (K) as function of the concentration for phase system I of Table 2, phase system I, $\Delta w_{PEG} = 28\%$, (■) acetyl-methionine, (●) acetic acid and (▲) methionine. Error bars are included. (b) Partition coefficients (K) as function of the concentration for phase system II of Table 2, phase system II, $\Delta w_{PEG} = 35\%$, (■) acetyl-methionine, (●) acetic acid and (▲) methionine. Error bars are included.

in determining the stationary phase hold-up could be the cause for this deviation. It has been shown that there is always some leakage of stationary phase [5]. This effect could explain the differences between the partition coefficients from batch experiments and from the pulse–response measurements. Therefore the fitted partition coefficients from the pulse–response measurements are probably not reliable and can not be used for the simulations.

4.4. Reaction kinetics of the hydrolysis

A limited number of kinetic data was collected in phase systems I, II and III. Fig. 7 shows the

Table 5
Partition coefficients from pulse–response experiments (K_p) and from shake flask experiments (K_{sf})

Component	Injection concentration (mol min^{-1})	Phase system I ($\varepsilon_s = 0.61$)			Phase system II ($\varepsilon_s = 0.65$)		
		K_p	K_{sf}	$k_o a$ (s^{-1})	K_p	K_{sf}	$k_o a$ (s^{-1})
N-Acetyl-L,D-methionine	70.6	5.9	4.3	0.0045	11.9	8.0	0.0019
D,L-Methionine	34.9	1.0	0.9	0.013	1.6	0.9	0.021

concentration of L-methionine as a function of time. From the limited number of batch kinetic experiments, it was not possible to determine all kinetic parameters in the expanded kinetic rate equation (Eq. (4)). Because of the separation of the products (methionine and acetic acid) from the substrate in the chromatographic column, product inhibition is neglected. Because of the low concentrations, the kinetic data are fitted with first-order kinetics with equilibrium limitation (Eq. (8)).

$$r_s = E\frac{V_{max}}{K_m}\left(q_{amL} - \frac{q_m q_a}{K_{eq}}\right) \quad (8)$$

The values of the fitted parameter (V_{max}/K_m) in the three phase systems are given in Table 6.

The reaction rate decreases with increasing PEG concentration in the phase system. The constant water concentration that is assumed in the equilibrium relation (appearing in Eq. (8)) does not hold for these ATPS. The decrease in enzymatic conversion rate at increasing PEG concentration is previously observed by Kuhlmann et al. [7].

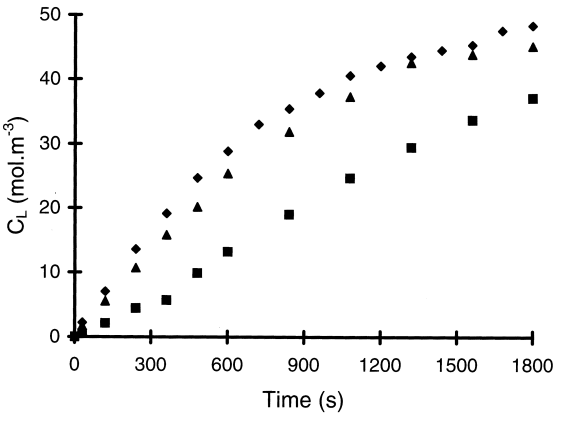

Fig. 7. Concentration of L-methionine, (C_L) as function of time. Concentration of acylase I is 0.20 g/l. The increase was measured in the top phase, (◆) phase system I, (▲) phase system II, (■) phase system III.

4.5. Experimental reaction chromatograms

With the pulse–response model described by Van Buel et al [8], it is possible to fit the stationary phase hold-up, at ''fixed'' partition coefficient, flow-rate and column volume for single injection pulses. This method is mainly useful when the solute selectively partitions to one of the two phases (e.g., the partition coefficient deviates significant from unity). In this case the retention time of a solute is more influenced by the phase ratio. The stationary phase hold-up was fitted for experiments 1, 2 and 3. The partition coefficient of *N*-acetyl-methionine, determined in the batch partition measurements ($K = 4.1$) was used as an input for the model. The fitted hold-ups were 0.67, 0.80 and 0.80 for experiments 1, 2 and 3, respectively. Obviously no loss in hold-up is observed between experiments 2 and 3. The fitted hold-up is high compared to the hold-up that was determined by measuring the amount of stationary phase that leaves the column in the pulse-response measurements. For the simulation of the integrated experiments, the partition coefficients that were obtained from the batch experiments, in combination with the fitted hold-up were used as an input for the model. The volumetric mass transfer coefficients are

Table 6
Fitted kinetic parameters with first-order kinetics ($E = 0.20$ g l^{-1})

Phase system	PEG content (*w*, %)	V_{max}/K_m (l g^{-1} s^{-1})
I	29.4	0.0086
II	35.3	0.0059
III	45.1	0.0027

determined for *N*-acetyl methionine and methionine. The volumetric mass transfer coefficient of acetate is estimated from Eq. (5) with $K_{ac}=1.85$. From the linear interpolation using the values of K and $k_o a$ for methionine and *N*-acetyl-methionine, $k_o a$ for acetate in phase system I is estimated to be 0.016 s^{-1}.

The effluent profiles of the experiments (1, 2) and the model predictions are given in Fig. 8a and Fig. 8b. The time axis is replaced by the dimensionless time, by dividing the real time t by τ, (defined as the column volume, divided by the flow-rate, $\tau=1500$ s which equals the residence time of a component with $K=1$). The concentration profiles were obtained by converting the light absorption profiles into concentration profiles. In this method, it is assumed that all substrate is converted (same peak area as model predictions). Fig. 8a clearly shows the possibility to perform a chromatographic enzymatic reaction in an ATPS in CPC. *N*-Acetyl-L-methionine is converted in the CPC. Only a small amount of unconverted substrate appears to elute from the column. The elution time of the lower peak in Fig. 8a (retention time 4600 s) suggests that this component is unconverted *N*-acetyl-methionine. No analyses were performed to confirm the identity of the species. The parameters that are used to calculate the chromatograms with the model are given in Table 7.

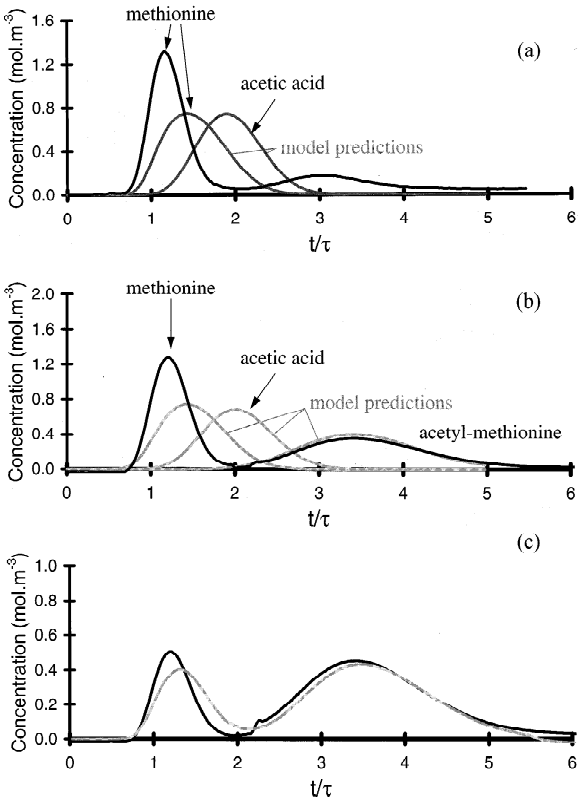

Fig. 8. (a) Experimental chromatogram of the enzymatic hydrolysis of *N*-acetyl-L-methionine by acylase I (experiment 1, see Table 4 for experimental conditions). Black lines are experimental values, gray lines are model predictions. The input parameters for the model are given in Table 7. (b) Experimental chromatogram of the enzymatic hydrolysis of a racemic mixture of *N*-acetyl-L,D-methionine by acylase I (experiment 2, see Table 4 for experimental conditions). Black lines are experimental values, gray lines are model predictions. The input parameters for the model are given in Table 7. (c) Chromatogram of the enzymatic hydrolysis of *N*-acetyl-L,D-methionine by acylase I. See Table 4 for experimental conditions. The black line is experiment 2 (day 1), the gray line is experiment 3 (day 2).

The calculations were carried out using a commercial numerical solver for partial differential equations (PDESol, Numerica, 1995). The injection peak was described as a block pulse. The prediction using the model is reasonable, when it is realised that acetic acid is hardly detected by the UV absorbance. The predicted peak width is wider than the experimental peak width and the component elutes later from the column. This can be explained by small differences in partition coefficients and small differences in overall volumetric mass transfer coefficients that were determined in the single pulse experiments. The

Table 7
Parameters used for the model simulations

Parameter	Fig. 8a	Fig. 8b
Flow-rate	4 ml min^{-1}	4 ml min^{-1}
Column volume	101.3 ml	101.3 ml
Hold-up	0.67 (–)	0.80 (–)
Kinetic model	Eq. (8)	Eq. (8)
E	0.20 g l^{-1}	0.20 g l^{-1}
V_{max}/K_m	0.0086 l g^{-1} s^{-1}	0.0086 l g^{-1} s^{-1}
K_{am}	4.10	4.10
K_{ac}	1.85	1.85
K_{met}	1.04	1.04
$k_o a_{am}$	0.0074 s^{-1}	0.0074 s^{-1}
$k_o a_{ac}$	0.016 s^{-1}	0.016 s^{-1}
$k_o a_m$	0.027 s^{-1}	0.027 s^{-1}
Injection volume	2 ml	2 ml
Injection time	30 s	30 s
Injection concentration		
C_{amD}	0 mol m^{-3}	35.7 mol m^{-3}
C_{amL}	35.06 mol m^{-3}	35.7 mol m^{-3}

mutual influence of the components on their partition coefficients is not examined and can be a source of errors as well.

Fig. 8b shows the results of the injection of racemic substrate. The effluent profile shows that the L-substrate is converted, because the first peak in the chromatogram corresponds to L-methionine. The unconverted D-substrate elutes from the column at an elution time that corresponds to the partition coefficient of *N*-acetyl-methionine. Small deviations are explained with the same arguments as for the previously described experiment, small errors in determination of partition coefficients, volumetric mass transfer coefficients and by bleeding of stationary phase from the column.

Fig. 8c shows the reaction chromatograms of experiments 2 and 3. Experiment 3 was performed with the same enzyme and phase system as in experiment 2, but was performed 24 h later and the column was continuously eluted with the mobile phase. Because the mobile phase was in equilibrium with both cobalt chloride and enzyme, hardly any decrease in reaction efficiency was observed.

Because the model predicts the reaction chromatograms reasonably well, it can be used to optimise the reaction–separation process. From Fig. 8b it is clear that no complete separation of the two products (acetic acid and methionine) occurs in the column. By changing the composition of the phase system, the partition coefficients are changed. Therefore it is possible to fine-tune the phase system in such a way that complete separation of methionine and acetic acid is possible. Fig. 6a and Fig. 6b show that the partition coefficient of methionine is hardly influenced by the phase composition. Acetic acid shows an increased partition coefficient at increased difference in the composition of the phases. By varying the phase composition, it appears possible to optimise the integrated reaction and separation. This item is a subject of future work.

Further purification of the chirally pure L-methionine and racemisation of unconverted *N*-acetyl-D-methionine from the PEG and salt containing mobile phase were not investigated in this study. The feasibility of a commercial process that is based on chromatographic reaction shall depend on the possibility to recycle the PEG and salt, the stability of the enzyme, and the recirculation and racemisation of the unconverted D-substrate.

5. Conclusions

This study demonstrates the possibility to perform an enzymatic reaction in the stationary phase in CPC. Aqueous two-phase systems consisting of PEG 600, potassium phosphate and water at 25°C and pH 7.0 are suitable as liquid–liquid two phase system. By independently measuring reaction kinetics, partitioning and mass transfer behaviour of the components between the two liquid phases and a method to determine the stationary phase hold-up, it is possible to adequately predict the effluent profiles with a mathematical model. With this model and a correlation between the partition coefficients and the composition of the phases, it seems possible to optimize the integrated reaction and separation process.

6. List of symbols

a	Specific area of the liquid–liquid interface (m^{-1})
C	Concentration of components in mobile phase (mol m^{-3})
E	Enzyme concentration (g/l^{-1})
k_{ic}	Inhibition constant of product (mol m^{-3})
k_{in}	Inhibition constant of substrate (mol m^{-3})
k_m	Mass transfer coefficient in mobile phase (m s^{-1})
k_o	Overall mass transfer coefficient (m s^{-1})
k_s	Mass transfer coefficient in stationary phase (m s^{-1})
K_{eq}	Equilibrium constant
K	Molar partition coefficient
K_m	Michaelis–Menten constant (mol m^{-3})
q	Concentration of components in stationary phase (mol m^{-3})
r	Specific conversion rate (mol m^{-3} s^{-1})
t	Time (s)
v	Velocity of mobile phase (m s^{-1})
V_{max}	Maximum specific conversion rate (mol g^{-1} s^{-1})
w	Weight fraction of PEG 600 (wt., %)
x	Place in the column (m)

Greek

ε_s	Stationary phase hold-up

η	Viscosity (Pa s^{-1})
μ	Stoichiometric coefficient
ρ	Density (kg m^{-3})
τ	Residence time (s)

Subscript

i	Component *i*
mob	Mobile phase
p	Product
stat	Stationary phase
0	Start

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