Supporting Information

Simultaneous prediction of co-solvent influence on reaction equilibrium and Michaelis constants of enzyme-catalyzed ketone reductions

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Determination of calibration curves

To determine the normalized turnover frequency $v'$ the decrease of the NADH molality over time was monitored at 340 nm in an Eppendorf Biospectrometer. The calibration curves for neat conditions and under the influence of 17 wt.-% of PEG 6000 are given in Figure S1.

Figure S1. Extinction E at 340 nm plotted over the molality of NADH $m_{NADH}$ for neat (squares) and under the addition of 17 wt.-% PEG 6000 (circles). The slopes of the fit curves with an forced intercept of $[0,0]$ were used to determine the extinction coefficient of NADH in the respective system.
From the slope of the fit lines of Figure S1 the extinction coefficients of $\epsilon_{340\text{ nm}}(\text{neat}) = 5.532 \text{ kg mol}^{-1}\text{cm}$ and $\epsilon_{340\text{ nm}}(17 \text{ wt.-% PEG 6000}) = 4.570 \text{ kg mol}^{-1}\text{cm}$ were determined and used in this work.

### Predictions of the reaction equilibrium

The fundamentals of the co-solvent influence predictions were based on Equation (1).

$$K_{\text{th}}(\text{neat}) = K_{\text{th}}(\text{co-solvent}) \mid T, p, pH = \text{const.}$$

According to Equation (1), an objective functions OF1 to predict the co-solvent influence on the reaction equilibrium was formulated as shown in Equation (2):

$$\text{OF1}: \left| \frac{K_{\text{th}}(\text{neat}) - L}{K_{\text{th}}(\text{neat})} \right| \leq 10^{-5}$$

$$L = \frac{x_{\text{alcohol}} \cdot x_{\text{NAD}^+} \cdot y_{\text{alcohol}} \cdot y_{\text{NAD}^+}}{x_{\text{ketone}} \cdot x_{\text{NADH}} \cdot y_{\text{ketone}} \cdot y_{\text{NADH}}}$$

In order to allow an iteration of OF1, the initial moles $n_0$ and the reaction extent $\lambda$ are introduced into $L$ leading to Equation (4).

$$L = \frac{(n_{0,\text{alcohol}} + \lambda) \cdot (n_{0,\text{NAD}^+} + \lambda)}{(n_{0,\text{ketone}} - \lambda) \cdot (n_{0,\text{NADH}} - \lambda)} \cdot \frac{y_{\text{alcohol}} \cdot y_{\text{NAD}^+}}{y_{\text{ketone}} \cdot y_{\text{NADH}}}$$

The activity coefficients in Equation (4) are predicted for the specific composition of the reaction mixture (including the co-solvent) for each value of $\lambda$. The Iteration of OF1 was performed as illustrated in Figure S2.
**Figure S2**: Illustration of the iteration procedure used to predict co-solvent influences on $X_{\text{exp}}$ of the ADH reactions for a given $T$, $p$, and $pH$. The input parameters required are the initial moles $n_{0,i}$, the initial guess of the reaction extent $\lambda_1$ and the thermodynamic equilibrium constant determined under neat conditions $K_{th(\text{neat})}$. $X^{\text{pre}}$ is the output of the iteration procedure for the reaction under investigation. The step size $\Delta$ is chosen arbitrarily.

**Determination of kinetic constants**

In order to determine all the kinetic properties $K_{M,\text{ketone}}^{\text{obs}}$, $K_{M,NADH}^{\text{obs}}$, $k_{\text{cat}}$ and $K_{L,NADH}^{\text{obs}}$ a two-step linearization was applied in this work. Equation (5) represents the first linearization step of, in which the reciprocal normalized reaction rate represents the abscissa over the reciprocal molality of NADH:
\[
\frac{1}{n} = \left( \frac{k_{i,NADH}^{obs} \cdot K_{M,ketone}^{obs} + k_{M,NADH}^{obs}}{k_{cat} \cdot m_{ketone}^{slope}} \right) \cdot \frac{1}{m_{NADH}} + \left( \frac{K_{M,ketone}^{obs}}{k_{cat} \cdot m_{ketone}^{ordinate}} + \frac{1}{k_{cat}} \right)
\] (5)

This linear correlation is valid for different pseudo-constant molalities of the ketone, which is given in this work as the condition \( m_{ketone} \gg m_{NADH} \) is fulfilled in agreement to previous works\(^1,2\).

In order to obtain Michaelis constants, a second linearization step is required. Each fit line obtained from Equation (5) is a function of the initial pseudo-constant molality of the ketone \( m_{ketone} \). This leads to fit lines with different slopes (\( S_{\text{prim}} \)) and ordinates (\( O_{\text{prim}} \)) resulting from the linearization of \((n')^{-1}\) over \( m_{NADH}^{-1} \). These slopes (\( S_{\text{prim}} \)) and ordinates (\( O_{\text{prim}} \)) yield another linear relation that is a function of \( m_{ketone}^{-1} \) as shown in Equations (6) and (7).

\[
\frac{O_{\text{prim}}}{y} = \frac{K_{M,ketone}^{obs}}{k_{cat}^{\text{slope}}} \cdot \frac{1}{m_{ketone}^{x}} + \frac{1}{k_{cat}^{\text{ordinate}}}
\]

\[
S_{\text{prim}}^{\text{y}} = \frac{k_{i,NADH}^{obs} \cdot K_{M,ACP}^{obs}}{k_{cat}^{\text{slope}}} \cdot \frac{1}{m_{ketone}^{x}} + \frac{K_{M,NADH}^{obs}}{k_{cat}^{\text{ordinate}}}
\]

To finally get access to the Michaelis constants \( K_{M,ketone}^{obs} \) and \( K_{M,NADH}^{obs} \) two secondary plots are required, in which \( O_{\text{prim}} \) are plotted over \( m_{ketone}^{-1} \) and \( S_{\text{prim}} \) over \( m_{ketone}^{-1} \), respectively. As shown in Equations (6) and (7), both plots yield another linear relation (\( O_{\text{prim}} \) over \( m_{ketone}^{-1} \) and \( S_{\text{prim}} \) over \( m_{ketone}^{-1} \)). The linearization of Equation (6) yields an ordinate that is referred to as \( O_{\text{sec}} \)(ordinate of the linearized secondary plot of \( O_{\text{prim}} \) over \( m_{ketone}^{-1} \)) and a slope that is referred to as \( S_{\text{sec}} \)(slope of the linearized secondary plot of \( O_{\text{prim}} \) over \( m_{ketone}^{-1} \)). The linearization of Equation (7) yields an ordinate that is referred to as \( O_{\text{sec}} \)(ordinate of the
linearized secondary plot of $S_{l}^{\text{prim}}$ over $m_{\text{ketone}}^{-1}$ and a slope that is referred to as $S_{l}^{\text{sec}}$ (slope of the linearized secondary plot of $S_{l}^{\text{prim}}$ over $m_{\text{ketone}}^{-1}$). Equations (8) to (11) list the respective relations of the second linearization step to the kinetic constants obtained in this work.

$$O_{r}^{\text{sec}} = \frac{1}{k_{\text{cat}}^{r}}$$

(8)

$$S_{l}^{\text{sec}} = \frac{K_{M,\text{ketone}}^{\text{obs}}}{k_{\text{cat}}}$$

(9)

$$O_{r}^{\text{sec}} = \frac{K_{M,\text{NADH}}^{\text{obs}}}{k_{\text{cat}}}$$

(10)

$$S_{l}^{\text{sec}} = \frac{K_{L,\text{NADH}}^{\text{obs}} \cdot K_{M,\text{ketone}}^{\text{obs}}}{k_{\text{cat}}}$$

(11)

$K_{M,\text{ketone}}^{\text{obs}}, K_{M,\text{NADH}}^{\text{obs}}, k_{\text{cat}}$ and $K_{L,\text{NADH}}^{\text{obs}}$ can be directly derived from the relations in Equations (8) to (11).

**Predictions of Michaelis constants under co-solvent influence**

ePC-SAFT predictions of $K_{M,\text{ketone}}^{\text{obs}}$ and $K_{M,\text{NADH}}^{\text{obs}}$ under co-solvent influence were then based on the so-determined $K_{M,\text{ketone}}^{a}, K_{M,\text{NADH}}^{a}$ and $K_{L,\text{NADH}}^{a}$ values under neat (co-solvent free) conditions. Further, $k_{\text{cat}}$ is a factor that is solely required to predict a correct primary plot, but it was proven to cancel out for the predictions of $K_{M}^{\text{obs}}$. To create the primary plot for the predictions, two arbitrary molalities for the ketone $m_{\text{ketone}}^{\text{pre}}$ and two molalities of NADH $m_{\text{NADH}}^{\text{pre}}$ were chosen. These were used to predict the activities of the ketone and NADH by ePC-SAFT predicted activity coefficients for any given system containing the co-solvent. The activities
combined with an arbitrarily chosen value for $k_{\text{cat}}$ were used to calculate $(\nu')^{-1}$ as shown in Equation (12).

$$\frac{1}{\nu'} = \left( \frac{K_{L,NADH}^a \cdot K_{M,ketone}^a}{k_{\text{cat}} \cdot a_{\text{ketone}}} + \frac{K_{M,NADH}^a}{k_{\text{cat}}} \right) \cdot \frac{1}{a_{\text{NADH}}} + \left( \frac{K_{M,ketone}^a}{k_{\text{cat}} \cdot a_{\text{ketone}}} + \frac{1}{k_{\text{cat}}} \right)$$ (12)

The predicted values of $(\nu')^{-1}$ were plotted over the chosen reciprocal values of $(m_{NADH}^{\text{pre}})^{-1}$ for pseudo-constant molalities of the respective ketone $m_{\text{ketone}}^{\text{pre}}$. This leads to the prediction of a primary plot according to Equation (5). Note that this is possible due to the fact that $(\nu')^{-1}$ for a specific molality $(m_{NADH}^{\text{pre}})^{-1}$ is equal to $(\nu')^{-1}$ for the corresponding activity $(a_{\text{NADH}}^{\text{pre}})^{-1}$.

Equation (13) shows his relation:

$$[\nu']^{-1} \left( \frac{1}{m_{NADH}^{\text{pre}}} \right) = [\nu']^{-1} \left( \frac{1}{a_{NADH}^{\text{pre}}} \right) \text{ for constant } (m_{\text{ketone}}^{\text{pre}})^{-1} \text{ and } a_{\text{NADH}} = m_{\text{NADH}} \cdot \gamma_{\text{NADH}}^{m}$$ (13)

The linear regression of the predicted primary plot was transferred into the secondary plots as shown in Equations (6) and (7). The secondary plots finally led to the predicted Michaelis constants $K_{M,ketone}^{\text{pre}}$ and $K_{M,NADH}^{\text{pre}}$.

**ePC-SAFT – The used version in this work**

In this work four contributions to $a^\text{res}$ were taken into account. $a^\text{hc}$ represents the reference term for the hard-chain interactions, $a^\text{disp}$ represents dispersive interactions, while $a^\text{assoc}$ describes hydrogen bonding between the chains. Finally, as an electrolyte system is present in this work the Debye-Hückel contribution $a^\text{ion}$ for interactions of charged species was accounted for. Five pure-component parameters for the description of uncharged components that form hydrogen
bonds are required. These are the segment number \( m_i^{\text{seg}} \), the segment diameter \( \sigma_i \), the dispersion-energy parameter \( u_i/k_B \), the association-energy parameter \( \varepsilon_{A_iB_i}/k_B \) and the association-volume parameter \( \kappa_{A_iB_i} \). Ions were modeled as spherical non-associating components in this work as proposed in literature\(^3\). Pure-component parameters for butanone, 1-butanol, 2-pentanone, 2-pentanol, NADH, NAD\(^+\), OH\(^-\), Na\(^+\) and water were taken from literature. The homopolymer PEG parameters were taken from Stoychev et al.\(^4\). Additionally, calculations of \( a^{\text{ion}} \) requires the medium-independent permittivity, which was set to \( \varepsilon_r = 77.56 \) (\( T = 303 \) K) in this work as proposed by Cameretti et al.\(^3\).

**Additional data for the reaction equilibrium**

To verify that reaction equilibrium was reached and no denaturation of the enzyme was present, substrate solution was added after no change in the extinction at 340 nm was observed. An exemplary plot of extinction over time with substrate addition for the reduction of butanone and 2-pentanone under neat conditions is given in Figure S2.
Figure S3. Exemplary extinction $E$ over time $t$ plot of the reduction of butanone (line) and 2-pentanone (dashed line) at 30 °C, 1bar and pH 7 catalyzed by ADH 270. The peaks at around 3600 sec for butanone and 4200 sec for 2-pentanone represent the addition of substrate solution to verify enzyme activity.

The experimental results of the equilibrium measurements for the reduction of butanone and 2-pentanone for neat conditions and under the influence of 17 wt.-% of PEG 6000 are listed in Table S1 and Table S2.
Table S1. Overview over the initial molalities of butanone $m_{\text{butanone},0}$ and NADH $m_{\text{NADH},0}$ prepared in this work to determine the equilibrium compositions $X_{\text{exp}}$ for neat conditions and under the influence of 17 wt.-% of PEG 6000 at 30 °C, 1 bar and pH of 7 catalyzed by ADH270. Additionally molalities and mole fractions at equilibrium are listed.

|                      | Neat        | 17 wt.-% PEG 6000 |
|----------------------|-------------|-------------------|
| $m_{\text{butanone},0} \left[ \frac{\text{mol}}{\text{kg}_{\text{water}}} \right]$ | 0.463 ± 0.03 | 0.492 ± 0.024     |
| $m_{\text{NADH},0} \left[ \frac{\text{mol}}{\text{kg}_{\text{water}}} \right]$    | 0.410 ± 0.001| 0.467 ± 0.06      |
| $m_{\text{butanone,eq}} \left[ \frac{\text{mol}}{\text{kg}_{\text{water}}} \right]$ | 0.081 ± 0.003| 0.056 ± 0.02      |
| $m_{\text{NADH,eq}} \left[ \frac{\text{mol}}{\text{kg}_{\text{water}}} \right]$     | 0.028 ± 0.001| 0.080 ± 0.003     |
| $m_{\text{2-butanol,eq}} \left[ \frac{\text{mol}}{\text{kg}_{\text{water}}} \right]$ | 0.382 ± 0.006| 0.412 ± 0.037     |
| $m_{\text{NAD}^+,eq} \left[ \frac{\text{mol}}{\text{kg}_{\text{water}}} \right]$      | 0.382 ± 0.006| 0.412 ± 0.037     |
| $x_{\text{butanone,eq}} \left[ \frac{\text{mol}}{\text{mol}} \right]$                | 1.355 ± 0.108 · 10^{-6} | 1.431 ± 0.339 · 10^{-6} |
| $x_{\text{NADH,eq}} \left[ \frac{\text{mol}}{\text{mol}} \right]$                    | 4.487 ± 0.497 · 10^{-7} | 1.004 ± 0.054 · 10^{-6} |
| $x_{\text{2-butanol,eq}} \left[ \frac{\text{mol}}{\text{mol}} \right]$               | 6.968 ± 0.110 · 10^{-6} | 7.427 ± 0.681 · 10^{-6} |
| $x_{\text{NAD}^+,eq} \left[ \frac{\text{mol}}{\text{mol}} \right]$                   | 6.968 ± 0.110 · 10^{-6} | 7.427 ± 0.681 · 10^{-6} |
Table S2. Overview over the initial molalities of 2-pentanone \( m_{2−\text{pentanone},0} \) and NADH \( m_{\text{NADH},0} \) prepared in this work to determine the equilibrium compositions \( X^{\text{exp}} \) for neat conditions and under the influence of 17 wt.-% of PEG 6000 at 30 °C, 1 bar and pH of 7 catalyzed by ADH270. Additionally molalities and mole fractions at equilibrium are listed.

|                               | Neat               | 17 wt.-% PEG 6000 |
|-------------------------------|--------------------|-------------------|
| \( m_{2−\text{pentanone},0} \) \[ mol \text{ kg}_{\text{water}} \] | 0.478 ± 0.06       | 0.424 ± 0.011     |
| \( m_{\text{NADH},0} \) \[ mol \text{ kg}_{\text{water}} \]       | 0.423 ± 0.013       | 0.415 ± 0.005     |
| \( m_{2−\text{pentanoleq},0} \) \[ mol \text{ kg}_{\text{water}} \] | 0.076 ± 0.006       | 0.095 ± 0.024     |
| \( m_{\text{NADHeq}} \) \[ mol \text{ kg}_{\text{water}} \]     | 0.021 ± 0.001       | 0.057 ± 0.01      |
| \( m_{2−\text{pentanoleq}} \) \[ mol \text{ kg}_{\text{water}} \] | 0.401 ± 0.013       | 0.350 ± 0.005     |
| \( m_{\text{NADHeq}} \) \[ mol \text{ kg}_{\text{water}} \]     | 0.401 ± 0.013       | 0.350 ± 0.005     |
| \( x_{2−\text{pentanoleq}} \) \[ mol \text{ mol}^{-1} \]         | 1.374 ± 0.118 \cdot 10^{-6} | 1.715 ± 0.429 \cdot 10^{-6} |
| \( x_{\text{NADHeq}} \) \[ mol \text{ mol}^{-1} \]              | 3.826 ± 0.124 \cdot 10^{-7} | 1.033 ± 0.187 \cdot 10^{-6} |
| \( x_{2−\text{pentanoleq}} \) \[ mol \text{ mol}^{-1} \]         | 7.235 ± 0.228 \cdot 10^{-6} | 6.312 ± 0.091 \cdot 10^{-6} |
| \( x_{\text{NADHeq}} \) \[ mol \text{ mol}^{-1} \]              | 7.235 ± 0.228 \cdot 10^{-6} | 6.312 ± 0.091 \cdot 10^{-6} |
Table S3 lists the mole fractions, activity coefficients predicted with ePC-SAFT based on the parameters from Table 1 and Table 2 in the main text and the resulting activities for the reduction of butanone and 2-pentanone at neat conditions used to determine $K_{th} \cdot a_{H^+}$.

### Table S3. Overview over the mean mole fractions $x_i$ used for activity coefficient predictions with the ePC-SAFT parameters from Table 1 and Table 2 of the main text, the predicted activity coefficients $\gamma_i$ and the resulting activities $a_i$ for the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by AFH 270

| Component      | $x_i$ [mol/mol] | $\gamma_i$ [mol/mol] | $a_i$ [-]     |
|----------------|-----------------|----------------------|---------------|
| butanone       | $1.355 \cdot 10^{-6}$ | 1540.987             | 0.00209       |
| NADH           | $4.487 \cdot 10^{-7}$ | 0.00369              | $1.656 \cdot 10^{-9}$ |
| 2-butanol      | $6.968 \cdot 10^{-6}$ | 179.466              | 0.00125       |
| NAD$^+$        | $6.968 \cdot 10^{-6}$ | 0.00129              | $8.989 \cdot 10^{-9}$ |
| 2-pentanone    | $1.374 \cdot 10^{-6}$ | 6826.968             | 0.00938       |
| NADH           | $3.826 \cdot 10^{-7}$ | 0.00369              | $1.412 \cdot 10^{-9}$ |
| 2-pentanol     | $7.235 \cdot 10^{-6}$ | 675.310              | 0.00489       |
| NAD$^+$        | $7.235 \cdot 10^{-6}$ | 0.00129              | $9.333 \cdot 10^{-9}$ |

**Neat data for reaction kinetics**

The experimental data for the normalized reciprocal reaction rate $(\nu')^{-1}$ including the respective molalities of ketone and NADH are listed in Table S4 for the reduction of butanone and in Table S5 for the reduction of 2-pentanone.
Table S4. Experimentally determined values for the normalized reciprocal reaction rate \((v')^{-1}\) for pseudo-constant molalities of butanone and different initial molalities of NADH at 30 °C, 1 bar and pH 7 catalyzed by ADH 270.

| \(m_{\text{butanone}} = 30 \frac{\text{mmol}}{kg_{\text{water}}}\) | \(m_{\text{butanone}} = 50 \frac{\text{mmol}}{kg_{\text{water}}}\) | \(m_{\text{butanone}} = 250 \frac{\text{mmol}}{kg_{\text{water}}}\) |
|---|---|---|
| \(m_{\text{NADH}}^{-1} \quad \left[ \frac{kg_{\text{water}}}{mmol} \right]\) | \(m_{\text{NADH}}^{-1} \quad \left[ \frac{kg_{\text{water}}}{mmol} \right]\) | \(m_{\text{NADH}}^{-1} \quad \left[ \frac{kg_{\text{water}}}{mmol} \right]\) |
| \((v')^{-1} \quad [10^{-3}s]\) | \((v')^{-1} \quad [10^{-3}s]\) | \((v')^{-1} \quad [10^{-3}s]\) |
| 8.35 | 2.73 ± 0.03 | 8.35 | 2.50 ± 0.06 | 8.33 | 1.59 ± 0.01 |
| 6.66 | 2.12 ± 0.09 | 6.56 | 1.93 ± 0.09 | 6.67 | 1.33 ± 0.04 |
| 4.00 | 1.52 ± 0.03 | 5.00 | 1.46 ± 0.02 | 5.00 | 0.98 ± 0.06 |
| 3.33 | 1.17 ± 0.01 | 3.99 | 1.32 ± 0.05 | 3.33 | 0.72 ± 0.01 |
| 3.10 | 0.97 ± 0.03 | 3.33 | 1.11 ± 0.01 | 2.86 | 0.64 ± 0.01 |
| - | - | 2.85 | 0.95 ± 0.03 | - | - |

Table S5. Experimentally determined values for the normalized reciprocal reaction rate \((v')^{-1}\) for pseudo-constant molalities of 2-pentanone and different initial molalities of NADH at 30 °C, 1 bar and pH 7 catalyzed by ADH 270.

| \(m_{\text{2-pentanone}} = 30 \frac{\text{mmol}}{kg_{\text{water}}}\) | \(m_{\text{2-pentanone}} = 50 \frac{\text{mmol}}{kg_{\text{water}}}\) | \(m_{\text{2-pentanone}} = 100 \frac{\text{mmol}}{kg_{\text{water}}}\) |
|---|---|---|
| \(m_{\text{NADH}}^{-1} \quad \left[ \frac{kg_{\text{water}}}{mmol} \right]\) | \(m_{\text{NADH}}^{-1} \quad \left[ \frac{kg_{\text{water}}}{mmol} \right]\) | \(m_{\text{NADH}}^{-1} \quad \left[ \frac{kg_{\text{water}}}{mmol} \right]\) |
| \((v')^{-1} \quad [10^{-3}s]\) | \((v')^{-1} \quad [10^{-3}s]\) | \((v')^{-1} \quad [10^{-3}s]\) |
| 9.01 | 2.23 ± 0.07 | 8.33 | 1.78 ± 0.08 | 8.45 | 1.47 ± 0.04 |
| 7.24 | 1.84 ± 0.02 | 6.69 | 1.46 ± 0.06 | 6.75 | 1.21 ± 0.01 |
| 5.42 | 1.37 ± 0.06 | 5.00 | 1.14 ± 0.05 | 5.06 | 0.94 ± 0.02 |
| 4.33 | 1.11 ± 0.03 | 4.00 | 0.97 ± 0.05 | 4.05 | 0.77 ± 0.04 |
| 3.61 | 0.98 ± 0.05 | 3.34 | 0.80 ± 0.04 | 3.38 | 0.68 ± 0.01 |
| 3.10 | 0.97 ± 0.03 | - | - | - | - |
The resulting secondary plots for the reduction of butanone and 2-pentanone are given in Figure S3 and Figure S4.

**Figure S4.** Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure 4 in the main text plotted over the reciprocal pseudo-constant molalities of butanone $m_{\text{butanone}}^{-1}$ for the reduction of butanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270.

**Figure S5.** Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure 4 in the main text plotted over the reciprocal pseudo-constant molalities of 2-pentanone $m_{2\text{-pentanone}}^{-1}$ for the reduction of butanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270.

The raw data for the secondary plots is given in Table S6.
Table S6. Raw data for the Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in the main manuscript for the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

| Butanone | 2-Pentanone |
|----------|-------------|
| $m_{\text{butanone}}^{-1}$ | $m_{\text{2-pentanone}}^{-1}$ |
| [kg$_{\text{water}}$/mol] | [kg$_{\text{water}}$/mol] | [kg$_{\text{water}}$/mol] | [kg$_{\text{water}}$/mol] |
| Or [10$^{-4}$s] | SL [10$^{-4}$s] | Or [10$^{-4}$s] | SL [10$^{-4}$s] |
| 32.83 | 1.85±0.72 | 3.03±0.12 | 33.05 | 1.98±0.45 | 2.24±0.08 |
| 19.40 | 1.74±0.53 | 2.74±0.09 | 19.99 | 1.74±0.49 | 1.93±0.08 |
| 4.00 | 1.33±0.24 | 1.76±0.04 | 10.13 | 1.50±0.23 | 1.56±0.04 |

Table S7 lists the determined kinetic parameters.

Table S7. Overview over the determined kinetic parameters of the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

| Butanone | 2-Pentanone |
|----------|-------------|
| $k_{\text{cat}}$ [1/s] | 7708.93 |
| $K_{M,\text{butanone}}^{\text{obs}}$ [mmol/kg$_{\text{water}}$] | 14.06 |
| $K_{M,NADH}^{\text{obs}}$ [mmol/kg$_{\text{water}}$] | 1.29 |
| $K_{I,NADH}^{\text{obs}}$ [mmol/kg$_{\text{water}}$] | 2.44 |
| $k_{\text{cat}}$ [1/s] | 7666.25 |
| $K_{M,2-\text{pentanone}}^{\text{obs}}$ [mmol/kg$_{\text{water}}$] | 15.82 |
| $K_{M,NADH}^{\text{obs}}$ [mmol/kg$_{\text{water}}$] | 0.99 |
| $K_{I,NADH}^{\text{obs}}$ [mmol/kg$_{\text{water}}$] | 0.70 |
Activity-based data for reaction kinetics

The data for the activity-based primary plots is given in Table S8 for the reduction of butanone and in Table S9 for the reduction of 2-pentanone.

Table S8. ePC-SAFT predicted reciprocal activity coefficients of NADH ($\gamma_{NADH}^m$)$^{-1}$ and resulting reciprocal activities of NADH $a_{NADH}^{-1}$ for the pseudo constant molalities of butanone of the butanone reduction at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

| $m_{\text{butanone}}$ | $a_{NADH}^{-1}$ | ($\gamma_{NADH}^m$)$^{-1}$ | $(v')^{-1}$ | $m_{\text{butanone}}$ | $a_{NADH}^{-1}$ | ($\gamma_{NADH}^m$)$^{-1}$ | $(v')^{-1}$ | $m_{\text{butanone}}$ | $a_{NADH}^{-1}$ | ($\gamma_{NADH}^m$)$^{-1}$ | $(v')^{-1}$ |
|-----------------------|-----------------|-----------------------------|-------------|-----------------------|-----------------|-----------------------------|-------------|-----------------------|-----------------|-----------------------------|-------------|
| 30 mmol/kg water      | 12.07           | 10.08                       | 2.73        | 12.02                 | 10.03           | 2.50                        | 11.46       | 9.55                  | 1.59            | 9.18                        | 6.13        |
|                       | 9.66            | 6.43                        | 2.12        | 9.46                  | 6.21            | 1.93                        | 9.18        | 6.13                  | 1.33            | 6.87                        | 3.43        |
|                       | 5.80            | 2.32                        | 1.52        | 7.21                  | 3.61            | 1.46                        | 6.87        | 3.43                  | 0.98            | 4.59                        | 1.53        |
|                       | 4.84            | 1.61                        | 1.17        | 5.77                  | 2.31            | 1.32                        | 4.59        | 1.53                  | 0.72            | 3.94                        | 1.13        |
|                       | -               | -                           | -           | -                     | 4.81            | 1.60                        | 3.94        | 1.13                  | 0.64            | -                           | -           |
|                       | -               | -                           | -           | -                     | 4.12            | 1.18                        | -           | -                     | -               | -                           | -           |
Table S9. ePC-SAIT predicted reciprocal activity coefficients of NADH \((\gamma_{NADH}^m)^{-1}\) and resulting reciprocal activities of NADH \(a_{NADH}^{-1}\) for the pseudo constant molalities of 2-pentanone of the 2-pentanone reduction at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

| \(m_{2\text{-pentanone}} = 30\) mmol kg\(_{\text{H2O}}\) | \(m_{2\text{-pentanone}} = 50\) mmol kg\(_{\text{H2O}}\) | \(m_{2\text{-pentanone}} = 100\) mmol kg\(_{\text{H2O}}\) |
|---|---|---|
| \(a_{NADH}^{-1}\) [10\(^7\)] | \((\gamma_{NADH}^m)^{-1}\) [10\(^8\)mol/kg] | \((\gamma_{NADH}^m)^{-1}\) [10\(^8\)mol/kg] | \((\gamma_{NADH}^m)^{-1}\) [10\(^8\)mol/kg] | \((\gamma_{NADH}^m)^{-1}\) [10\(^8\)mol/kg] | \((\gamma_{NADH}^m)^{-1}\) [10\(^8\)mol/kg] |
| 13.00 | 11.71 | 2.23 | 11.97 | 9.98 | 1.78 | 11.96 | 10.10 | 1.47 |
| | | ± 0.07 | | | ± 0.08 | | ± 0.04 | |
| 10.44 | 7.56 | 1.84 | 9.59 | 6.41 | 1.46 | 9.55 | 6.45 | 1.21 |
| | | ± 0.02 | | | ± 0.06 | | ± 0.01 | |
| 7.84 | 4.25 | 1.37 | 7.17 | 3.59 | 1.14 | 7.15 | 3.62 | 0.94 |
| | | ± 0.06 | | | ± 0.05 | | ± 0.02 | |
| 6.27 | 2.71 | 1.11 | 5.74 | 2.30 | 0.97 | 5.73 | 2.32 | 0.77 |
| | | ± 0.03 | | | ± 0.05 | | ± 0.04 | |
| 5.23 | 1.89 | 0.98 | 4.81 | 1.61 | 0.80 | 4.78 | 1.61 | 0.68 |
| | | ± 0.05 | | | ± 0.04 | | ± 0.01 | |
| 4.50 | 1.39 | 0.97 | - | - | - | - | - | - |
| | | ± 0.03 | | | | | | |

The resulting activity-based secondary plots for the reduction of butanone and 2-pentanone are given in Figure S5 and Figure S6.
Figure S6. Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure 5 in the main text plotted over the reciprocal ePC-SAFT predicted activities of butanone $a^{-1}_{\text{butanone}}$ for the reduction of butanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

Figure S7. Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure 5 in the main text plotted over the reciprocal ePC-SAFT predicted activities of 2-pentanone $a^{-1}_{\text{2-pentanone}}$ for the reduction of 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

The ePC-SAFT predicted activity coefficients and the resulting activities of butanone and 2-pentanone including the values for the slopes and ordinates of the activity-based primary plots are given in Table S10
Table S10. Overview over the ePC-SAFT predicted reciprocal activity coefficients of butanone \( (\gamma_{\text{butanone}}^m)^{-1} \) and 2-pentanone \( (\gamma_{\text{2-pentanone}}^m)^{-1} \) and the resulting reciprocal activities \( a_{\text{butanone}}^{-1} \) and \( a_{\text{2-pentanone}}^{-1} \). Additionally the ordinates (Or) and slopes (Sl) of the activity-based primary plot are listed.

| butanone       | 2-pentanone       |
|----------------|-------------------|
| \( a_{\text{butanone}}^{-1} \)  | \( a_{\text{2-pentanone}}^{-1} \) |
| \( (\gamma_{\text{butanone}}^m)^{-1} \) | \( (\gamma_{2-\text{pentanone}}^m)^{-1} \) |
| \( \text{Or} \ [10^4 \text{s}] \) | \( \text{Or} \ [10^4 \text{s}] \) |
| \( \text{Sl} \ [10^{-11} \text{s}] \) | \( \text{Sl} \ [10^{-11} \text{s}] \) |
| 3.43           | 0.56              |
| 112.64         | 18.43             |
| 1.81±0.73      | 1.83±0.50         |
| 2.10±0.09      | 1.57±0.06         |
| 2.06           | 0.37              |
| 39.99          | 7.34              |
| 1.70±0.53      | 1.73±0.50         |
| 1.91±0.07      | 1.35±0.06         |
| 0.50           | 0.19              |
| 1.99           | 1.89              |
| 1.46±0.18      | 1.66±0.20         |
| 1.27±0.02      | 1.08±0.02         |

The activity-based kinetic constants determined in this work are listed in Table S11.

Table S11. Overview over the determined activity-based kinetic parameters of the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270

| butanone       | 2-pentanone       |
|----------------|-------------------|
| \( k_{\text{cat}} \ [\frac{1}{L} \text{s}] \) | \( k_{\text{cat}} \ [\frac{1}{L} \text{s}] \) |
| \( K_{M,\text{butanone}}^a [-] \) | \( K_{M,\text{2-pentanone}}^a [-] \) |
| \( K_{M,NADH}^a [-] \) | \( K_{M,NADH}^a [-] \) |
| \( K_{I,NADH}^a [-] \) | \( K_{I,NADH}^a [-] \) |
| 7039.10        | 6392.55           |
| 0.084          | 0.301             |
| 8.36 \cdot 10^{-8} | 5.40 \cdot 10^{-8} |
| 2.40 \cdot 10^{-7} | 2.78 \cdot 10^{-7} |
17 wt.-% PEG 6000 data for reaction kinetics

The experimental data for the normalized reciprocal reaction rate \((\nu')^{-1}\) including the respective molalities of ketone and NADH under the influence of 17 wt.-% of PEG 6000 are listed in Table S12 for the reduction of butanone and in Table S13 for the reduction of 2-pentanone.

**Table S12.** Experimentally determined values for the normalized reciprocal reaction rate \((\nu')^{-1}\) for pseudo-constant molalities of butanone and different initial molalities of NADH at 30 °C, 1 bar and pH 7 catalyzed by ADH 270 under the influence of 17 wt.-% of PEG 6000

| \(m_{\text{butanone}}\) = \(30 \frac{\text{mmol}}{\text{kg}_{\text{water}}}\) | \(m_{\text{butanone}}\) = \(50 \frac{\text{mmol}}{\text{kg}_{\text{water}}}\) | \(m_{\text{butanone}}\) = \(100 \frac{\text{mmol}}{\text{kg}_{\text{water}}}\) |
|---------------------------|---------------------------|---------------------------|
| \(m_{\text{NADH}}^{-1}\) | \((\nu')^{-1}\) [10^{-3}s] | \(m_{\text{NADH}}^{-1}\) | \((\nu')^{-1}\) [10^{-3}s] | \(m_{\text{NADH}}^{-1}\) | \((\nu')^{-1}\) [10^{-3}s] |
| \(\frac{\text{kg}_{\text{water}}}{\text{mmol}}\) | \(\frac{\text{kg}_{\text{water}}}{\text{mmol}}\) | \(\frac{\text{kg}_{\text{water}}}{\text{mmol}}\) | \(\frac{\text{kg}_{\text{water}}}{\text{mmol}}\) | \(\frac{\text{kg}_{\text{water}}}{\text{mmol}}\) | \(\frac{\text{kg}_{\text{water}}}{\text{mmol}}\) |
| 6.65 | 2.30 ± 0.03 | 8.94 | 2.68 ± 0.10 | 8.37 | 2.05 ± 0.09 |
| 4.97 | 1.87 ± 0.05 | 7.07 | 2.07 ± 0.09 | 6.77 | 1.76 ± 0.06 |
| 3.95 | 1.45 ± 0.08 | 5.21 | 1.75 ± 0.02 | 5.08 | 1.38 ± 0.07 |
| 3.27 | 1.32 ± 0.03 | 4.16 | 1.49 ± 0.10 | 3.98 | 1.17 ± 0.06 |
| 2.78 | 1.21 ± 0.07 | 3.43 | 1.28 ± 0.04 | 3.29 | 1.00 ± 0.01 |
| - | - | 2.91 | 1.01 ± 0.02 | - | - |
Table S13. Experimentally determined values for the normalized reciprocal reaction rate \((v')^{-1}\) for pseudo-constant molalities of 2-pentanone and different initial molalities of NADH at 30 °C, 1 bar and pH 7 catalyzed by ADH 270 under the influence of 17 wt.-% of PEG 6000

| \(m_{\text{2-pentanone}} = 30 \frac{\text{mmol}}{\text{kg water}}\) | \(m_{\text{2-pentanone}} = 50 \frac{\text{mmol}}{\text{kg water}}\) | \(m_{\text{2-pentanone}} = 100 \frac{\text{mmol}}{\text{kg water}}\) |
|-------------------|------------------|------------------|
| \(m_{\text{NADH}}^{-1}\) \text{[kg water mmol]} | \((v')^{-1}[10^{-3} \text{s}]\) | \(m_{\text{NADH}}^{-1}\) \text{[kg water mmol]} | \((v')^{-1}[10^{-3} \text{s}]\) | \(m_{\text{NADH}}^{-1}\) \text{[kg water mmol]} | \((v')^{-1}[10^{-3} \text{s}]\) |
| 6.71 | 1.85 ± 0.07 | 8.41 | 1.97 ± 0.13 | 8.69 | 1.72 ± 0.05 |
| 5.02 | 1.47 ± 0.02 | 6.71 | 1.55 ± 0.16 | 6.88 | 1.43 ± 0.01 |
| 4.03 | 1.23 ± 0.02 | 5.02 | 1.43 ± 0.06 | 5.20 | 1.19 ± 0.04 |
| 3.31 | 1.08 ± 0.02 | 4.13 | 1.12 ± 0.05 | 4.14 | 0.97 ± 0.05 |
| 2.84 | 0.98 ± 0.04 | 3.32 | 0.90 ± 0.07 | 3.44 | 0.84 ± 0.02 |

The primary plots for the reduction of butanone and 2-pentanone under the influence of 17 wt.-% of PEG 6000 are given Figure S7.

Figure S8. Primary plots for the determination of \(K_{M,ketone}^{\text{obs}}\) and \(K_{M,NADH}^{\text{obs}}\) under the influence of 17 wt.-% of PEG 6000 for the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270. Reciprocal normalized reaction rate \((v')^{-1}\) plotted over the reciprocal initial molality of NADH \(m_{\text{NADH}}^{-1}\) for the pseudo-constant molalities of butanone (triangles: 30 mmol(kg\text{water})\(^{-1}\); squares: 50 mmol(kg\text{water})\(^{-1}\); circles: 100 mmol(kg\text{water})\(^{-1}\)) in the left and pseudo-constant molalities of 2-pentanone (triangles: 30 mmol(kg\text{water})\(^{-1}\); squares: 50 mmol(kg\text{water})\(^{-1}\); circles: 100 mmol(kg\text{water})\(^{-1}\)) in the right diagram.
The resulting secondary plots for the reduction of butanone and 2-pentanone under the influence of 17 wt.-% of PEG 6000 are given in Figure S8 and Figure S9.

**Figure S9.** Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure S7 over the reciprocal pseudo-constant molalities of butanone $m_{\text{butanone}}^{-1}$ for the reduction of butanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270 under the influence of 17 wt.-% of PEG 6000

**Figure S10.** Secondary plots of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure S7 plotted over the reciprocal pseudo-constant molalities of 2-pentanone $m_{2-\text{pentanone}}^{-1}$ for the reduction of butanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270 under the influence of 17 wt.-% of PEG 6000

The raw data for the secondary plots is given in Table S14. Table S15 lists the determined kinetic parameters.
**Table S14.** Raw data for the *Secondary plots* of the slope (SL) and ordinates (Or) of the respective fit lines in the primary plot given in Figure S7 for the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270 under the influence of 17 wt.-% PEG 6000

|          | butanone                  | 2-pentanone                |
|----------|---------------------------|----------------------------|
|          | $m_{\text{butanone}}^1$  | $m_{2\text{-pentanone}}^1$|
| $[\text{kg}_{\text{water}}/\text{mol}]$ | Or [10$^4$s] | SL [10$^4$s] | Or [10$^4$s] | SL [10$^4$s] |
| 34.68    | 3.69±0.65                | 2.92±0.14                 | 34.11        | 3.27±0.30    | 2.27±0.07    |
| 19.13    | 3.52±0.74                | 2.57±0.12                 | 20.37        | 3.04±1.05   | 1.97±0.18   |
| 10.07    | 3.37±0.53                | 2.06±0.09                 | 10.21        | 2.83±0.34   | 1.67±0.06   |

**Table S15.** Overview over the determined kinetic parameters of the reduction of butanone and 2-pentanone at 30 °C, 1 bar and pH 7 catalyzed by ADH 270 under the influence of 17 wt.-% of PEG 6000

|          | butanone                  | 2-pentanone                |
|----------|---------------------------|----------------------------|
|          | $k_{\text{cat}} [1/s]$   |                           | 3080.25                  |
|          | $K_{M,\text{butanone}}^{\text{obs}} [\text{mmol}/\text{kg}_{\text{water}}]$ |   | 4.03                |
|          | $K_{M,NADH}^{\text{obs}} [\text{mmol}/\text{kg}_{\text{water}}]$ |   | 0.55                |
|          | $K_{I,NADH}^{\text{obs}} [\text{mmol}/\text{kg}_{\text{water}}]$ |   | 2.57                |
|          |                           |                           | 3774.49                  |
|          | $k_{\text{cat}} [1/s]$   |                           |                           |
|          | $K_{M,2\text{-pentanone}}^{\text{obs}} [\text{mmol}/\text{kg}_{\text{water}}]$ |   | 6.93                |
|          | $K_{M,NADH}^{\text{obs}} [\text{mmol}/\text{kg}_{\text{water}}]$ |   | 0.54                |
|          | $K_{I,NADH}^{\text{obs}} [\text{mmol}/\text{kg}_{\text{water}}]$ |   | 0.74                |
**Chemical Provenance table**

**Table S16.** Sample provenance table. S = Sigma Aldrich Chemie GmbH, E = evoxx technologies GmbH, VWR = VWR International, A = Alpha Aesar.

| Compound | Purification method | CAS-number | Purity, mass% based | Supplier |
|----------|---------------------|------------|---------------------|----------|
| 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) | none | 7365-459 | > 99 | VWR |
| 2-pentanone | none | 107-87-9 | > 99 | S |
| alcohol dehydrogenase (ADH 270) | none | 9028-12-0 | 31 | E |
| butanone | none | 78-93-3 | > 99 | A |
| sodium hydroxide (NaOH) | none | 1310-73-2 | > 97 | A |
| β-nicotinamide adenine dinucleotide reduced disodium salt dihydrate (NADHNa₂·2H₂O)* | none | 606-68-8 | > 97 | S |
| polyethylene glycol 6000 (PEG 6000) | none | 25322-68-3 | - | VWR |

* was purchased as dihydrate

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