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The role of activity coefficients in bioreaction equilibria: Thermodynamics of methyl ferulate hydrolysis

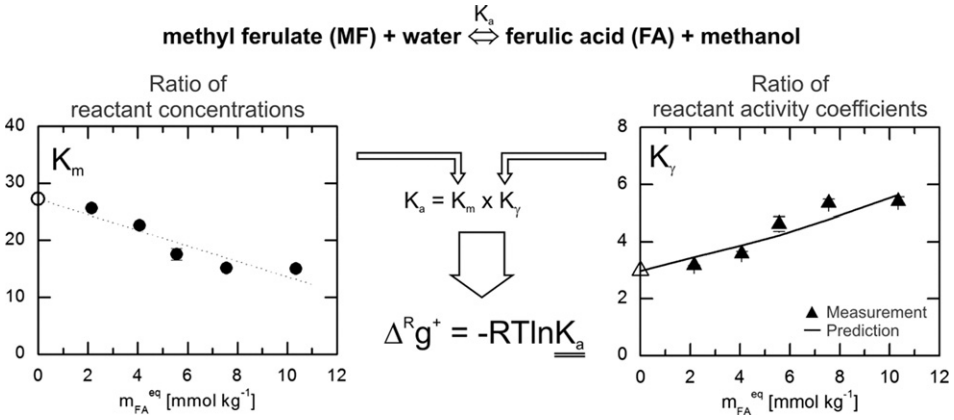
Philip Hoffmann, Matthias Voges, Christoph Held, Gabriele Sadowski *

Laboratory of Thermodynamics, Department of Biochemical and Chemical Engineering, TU Dortmund, Emil-Figge-Str. 70, 44227 Dortmund, Germany

HIGHLIGHTS

- Concentration-dependent K_m values were converted into concentration-independent K_a.
- K_γ values strongly differ from unity ($3<K_\gamma<6$), depending on the reactant concentration.
- Using K_a instead of K_m causes a deviation of 40% in $\Delta^R g^+$.
- Reactant activity coefficients (causing the deviation) cannot be assumed to be unity.
- Reactant activity coeff. can be predicted with ePC-SAFT, even in the presence of salt.

GRAPHICAL ABSTRACT



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ABSTRACT

The Gibbs energy of reaction ($\Delta^R g$) is the key quantity in the thermodynamic characterization of biological reactions. Its calculation requires precise standard Gibbs energy of reaction ($\Delta^R g^+$) values. The value of $\Delta^R g^+$ is usually determined by measuring the apparent (concentration-dependent) equilibrium constants K, e.g., the molality-based K_m. However, the thermodynamically consistent determination of $\Delta^R g^+$ requires the thermodynamic (activity-based) equilibrium constant K_a. These values (K_m and K_a) are equal only if the ratio of the activity coefficients of the reactants to the activity coefficients of the products (K_γ) is equal to unity.

In this work, the impact of K_γ on the estimation of K_a for biological reactions was investigated using methyl ferulate (MF) hydrolysis as a model reaction. The value of K_γ was experimentally determined from K_m values that were measured at different reactant concentrations. Moreover, K_γ was independently predicted using the thermodynamic model ePC-SAFT. Both the experimentally determined and the predicted K_γ values indicate that this value cannot be assumed to be unity in the considered reaction. In fact, in the reaction conditions considered in this work, K_γ was shown to be in the range of $3<K_\gamma<6$ for different reactant molalities ($2<$mmol MF $kg^{-1}<10$). The inclusion of K_γ and thus the use of the thermodynamically correct K_a value instead of K_m lead to remarkable differences (almost 40%) in the determination of $\Delta^R g^+$. Moreover, the new value for $\Delta^R g^+$ increases the concentration window at which the reaction can thermodynamically occur.

The influence of additives was also investigated both experimentally and theoretically. Both procedures consistently indicated that the addition of NaCl (0 to 1 mol kg^{-1} water) moderately decreased the value of K_γ, which means that the values of K_m increase and that a higher amount of products is obtained as a result of the addition

* Corresponding author. Tel.: +49 2317552635; fax: +49 2317552572.
E-mail address: gabriele.sadowski@bci.tu-dortmund.de (G. Sadowski).

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of salt. Additionally, K_m was found to strongly depend on pH. A ten-fold increase in the K_m values was observed in the pH range of 6 to 7; this increase corresponds to a change of more than 100% in the value of $\Delta^R g^+$.

1. Introduction

Thermodynamics has emerged as a promising tool for the refinement of metabolic network models that are developed in the scope of systems biology and for a thorough understanding of the biological reactions that comprise cellular metabolism. Although several methods exist for the thermodynamic characterization of biological reactions [1–10], these methods neglect an important thermodynamic property, namely, the activity coefficients of the reactants and the products. This quantity describes the deviation of the components from their standard state (e.g., pure component or hypothetical ideal solution) that is caused by the interactions between *all* of the present components. These interactions include molecular interactions among the reacting agents, as well as interactions of the reactants and the products with system components that do not directly participate in the reaction. Accordingly, the reactant and product activity coefficients strongly depend not only on their own concentrations but also on the nature of all of the system components. However, data on these activity coefficients are scarce, and their influence on biological and thermodynamic properties is thus largely unknown. These values are usually assumed to be unity [11], which means that these coefficients are neglected in the characterization of biological reactions.

Activity coefficients are related to the thermodynamic (activity-based) equilibrium constant K_a. K_a is the product of two quantities, both of which are concentration-dependent: the K value, which is usually calculated from the equilibrium concentrations of the reactants and the products, and a K_γ value, which accounts for the activity coefficients of these components (Eq. (1)).

$$K_a = K \cdot K_\gamma \tag{1}$$

It needs to be noted that the K values that are published in the literature, e.g., the NIST database on 'Thermodynamics of enzyme-catalyzed reactions' [12], are usually *not* the equilibrium constants K_a. Instead, these values are the concentration-dependent K values and are often even reported without declaring the units. Molality-based (K_m) or molarity-based (K_c) constants are the most commonly reported values of K. Moreover, the reported K values were usually measured at various system conditions (e.g., T, pH, ionic strength (I), and buffer) that might differ from the tabulation conditions (25 °C, pH 7, I = 0). To indicate whether the measurements were performed at non-standard system conditions, the respective K values are usually denoted by a prime and referred to as 'apparent' equilibrium constants (K′).

The complex influence of the system conditions on the apparent K′ values has been discussed and mathematically described in an extensive work by Alberty [13]. His approach allows for the comparison of K′ values from different authors (determined at different conditions).

The present work, in contrast, focuses on the thermodynamically consistent determination of the equilibrium constant K_a by measuring the molality-based K_m value and including the reactant and product activity coefficients according to Eq. (1). For this purpose, the hydrolysis of methyl ferulate (MF) to form ferulic acid (FA) and methanol (MeOH), which is catalyzed by the feruloyl esterase enzyme (Eq. (2)), was considered as a model reaction.

$$\text{methyl ferulate} + \text{water} \rightleftarrows \text{ferulic acid} + \text{methanol} \tag{2}$$

The enzyme feruloyl esterase participates in the breakdown of hemicellulose, which forms part of the plant cell wall, and catalyzes the hydrolysis of the feruloyl group from an esterified sugar in the cell wall [14]. Because MF possesses structural similarity to the esterified sugars and because efficient biomass utilization aims to efficiently break down these substrates, thermodynamics of the reactions catalyzed by feruloyl esterases is of practical importance. Moreover, a mixture of two acids (FA and MF), an organic solvent/product (MeOH), and water is expected to exhibit substantially non-ideal behavior, which makes MF hydrolysis a meaningful model reaction to demonstrate the influence of the reactant and the product activity coefficients.

The same reaction was previously studied by Goldberg et al. [15], who focused on the determination of the apparent K′ values and the enthalpies of reaction at 25 °C using a citrate buffer (pH = 5). These researchers found that K′ strongly depends on the pH and that the equilibrium is strongly on the side of the products (FA and MeOH). Therefore, these researchers used an excess of MeOH to shift the reaction toward the reactant side and thus make the equilibrium concentrations analytically accessible. This excess of MeOH is expected to have a pronounced influence on the activity coefficients, particularly that of MeOH. However, Goldberg et al. did not address the dependence of the K′ values on the equilibrium concentrations nor the influence of the reactant and product activity coefficients on the K′ values. Both of these issues are considered in this study.

2. Thermodynamics of biological reactions using MF hydrolysis as an example

The following section describes the formalism for a thermodynamically consistent description of bioreactions using MF hydrolysis (Eq. (2)) as the model reaction.

The thermodynamic equilibrium constant K_a is defined as

$$K_a = \frac{a_{FA} \cdot a_{MeOH}}{a_{MF} \cdot a_w}, \tag{3}$$

where a is the activity of the reactants and the products in the MF hydrolysis reaction. The activity of a component can be written as the product of the component's concentration and its respective activity coefficient, which itself depends on the standard state as well as on the concentration units used:

$$a_i = x_i \cdot \gamma_i^x = m_i \cdot \gamma_i^m \text{ (refers to standard state "pure component")} \tag{4}$$

$$a_i = m_i \cdot \gamma_i^{*,m} \text{ (refers to standard state "hypothetical ideal solution")}. \tag{5}$$

The variables m_i and x_i represent the molality (moles of component i per kg water) and the mole fraction of component i, respectively. The γ_i value is the activity coefficient of component i. The standard state for the activity coefficients γ_i^x and γ_i^m in Eq. (4) is the pure component i. Thus, these activity coefficients become unity for the pure component i ($x_i = 1$). In contrast, the standard state for $\gamma_i^{*,m}$ in Eq. (5) is a hypothetical ideal solution of component i in a solvent (e.g., water), which is defined as a one molal solution that exhibits the same interactions as an infinite dilution of component i in the same solvent.

The activity coefficient γ_i is usually used for solvents (e.g., water and MeOH in this work), whereas the activity coefficient γ_i^* is usually used for solutes that are present at very low concentrations in the reaction mixture and thus exhibit infinite-dilution interactions (e.g., MF and FA in this work).

In analogy to the activity of a component, which can be expressed as the product of the molality of this component and the activity

coefficient of this component (Eqs. (4) and (5)), the thermodynamic equilibrium constant K_a can be written as

$$K_a = K_m \cdot K_\gamma, \tag{6}$$

where K_m (Eq. (7)) is calculated from the equilibrium molalities of the reactants and products. For the model reaction considered within this work, K_m becomes

$$K_m = \frac{m_{FA} \cdot m_{MeOH}}{m_{MF} \cdot m_w}. \tag{7}$$

The K′ values found in the literature (e.g., [15] for methyl ferulate hydrolysis) are often reported as the product of the K_m values with the molality of water:

$$K' = K_m \cdot m_w. \tag{8}$$

However, it needs to be noted that the K_m values (as well as K_γ) are required for the calculation of the value of K_a. Therefore, in this work, the K_m values were determined by explicitly accounting for the molality of water ($m_w = 55.509$ mol kg^{-1}), and the literature K′ values were converted and compared with the K_m values obtained in this work using Eq. (8). The K_γ values were calculated from the respective molality-based reactant and product activity coefficients (Eq. (9)):

$$K_\gamma = \frac{\gamma_{FA}^{*,m} \cdot \gamma_{MeOH}^{m}}{\gamma_{MF}^{*,m} \cdot \gamma_{w}^{m}}. \tag{9}$$

Because water and MeOH were present in huge excess compared with MF and FA, the "pure component" standard state was chosen for water and MeOH. Therefore, the activity coefficients γ_i^m were applied. For MF and FA, the "hypothetical ideal solution" was chosen as the standard state. Therefore, the $\gamma_i^{*,m}$ values were used for MF and FA.

At an infinite dilution of MF and FA, their activity coefficients approach unity. Therefore, under these conditions, Eq. (9) becomes

$$K_\gamma^{\infty}(m_{MF} \to 0, m_{FA} \to 0) = \frac{\gamma_{MeOH}^{m}}{\gamma_{w}^{m}}. \tag{10}$$

Because neither water nor MeOH reach their standard state (pure component) at infinitely low MF and FA molalities, their activity coefficients $\gamma^m{}_w$ and $\gamma^m{}_{MeOH}$ do not approach unity and thus need to be explicitly included in the analysis.

Using K_γ^{∞}, the thermodynamic equilibrium constant K_a can be determined from the measured K_m values, which are extrapolated to infinitely low MF and FA molalities according to Eq. (11):

$$K_a = K_m^{\infty} \cdot K_\gamma^{\infty}. \tag{11}$$

The extrapolation will be described in Section 4.2. The activity coefficients that are required in Eqs. (9) through (11) can be determined using a thermodynamic model, as described in Section 4.3.

It needs to be noted that K_a does *not* depend on the reactant concentration or on the presence of additives. This value is really an equilibrium *constant* that depends *only* on the temperature and the pH. In contrast, K_m and K_γ *do* depend on the reactant concentration and on the presence of additives (e.g., solvents, salts, and buffer) in the reaction mixture. Consequently, the reactant activity coefficients need to be considered in the analysis of additive-containing reaction mixtures. This analysis was performed in this work to study the influence of the addition of NaCl to the reaction mixture (shown in Section 4.5).

3. Experimental work

3.1. Materials

All of the relevant information on the substances used in this study is shown in Table 1. All of the substances were used as obtained without further purification. The equilibrium measurements were catalyzed enzymatically by a recombinant feruloyl esterase (EC 3.1.1.73) from a rumen microorganism (Megazyme International, Bray, Ireland). According to the supplier, the enzyme activity for methyl ferulate is ~38 U/mg.

3.2. Measurement of K_m values

The K_m values were measured in double-walled 5-ml glass reactors. The temperature was adjusted using a C12 CP Lauda thermostat (Lauda, Lauda-Königshofen, Germany) with an accuracy of ±0.1 K and controlled with temperature sensors (Pt 100) that were placed directly in the reaction mixture. Each reactor was equipped with septum-containing caps for enzyme addition. An enzyme concentration of 0.334 U/ml was used for each reaction batch. Preliminary tests (data not provided here) have shown that this amount is suitable to reach biochemical equilibrium in a reasonable time scale (between two and four hours). Each reaction mixture was prepared using a stock solution, i.e., an aqueous solution of a monosodium/disodium phosphate buffer (0.5 mol kg^{-1}) in Millipore water, the reactant MF, and a substantial excess of MeOH (5 mol kg^{-1} water). According to Le Châtelier's principle, the excess of MeOH increases the accuracy of the chromatographic analysis of MF due to the higher amounts of MF at equilibrium. This procedure has been previously applied for the analysis of the MF hydrolysis reaction [15].

The reaction mixture was continuously stirred using a magnetic stirrer at moderate speed (~300 rpm) to ensure homogeneity. An investigation of the influence of the stirrer speed on the results revealed that the K_m values were independent of the stirring speed (data not shown).

The sodium phosphate buffer used in this study was adjusted to pH 6.5 because the biological enzyme activity is very high at this pH. Because slight pH changes occurred during the reaction, the pH was recorded before and after each experiment. The maximum pH deviation was found to be 0.05 (see Table 2). To investigate the influence of pH on the reaction equilibrium, measurements at different pH were performed by varying the ratio of monosodium phosphate to disodium phosphate.

Prior to chromatographic analysis (described in the following section), the samples were centrifuged in ultrafiltration units (10 kDa) with a Hettich 'Universal 32R' centrifuge (Hettich, Tuttlingen, Germany) at 14,000 g to separate the esterase from the reactants and the products and to prevent an equilibrium shift.

The measurements with additional NaCl were performed as described above. In these experiments, NaCl, which was added in the

Table 1
Substances used in this study. The Chemical Abstracts Service (CAS) registry numbers, empirical formulae, suppliers (A = Alfa Aesar GmbH & Co. KG, M = Merck KGaA, S = Sigma-Aldrich Chemie GmbH), and approximate mass-fraction purities provided by the suppliers are included.

Substance	CAS-No.	Formula	Supplier	Purity
Acetonitrile	75-05-8	C_2H_3N	A	>0.999
Ferulic acid (FA)	1135-24-6	$C_{10}H_{10}O_4$	S	>0.999
Methanol (MeOH)	67-56-1	CH_4O	M	>0.999
Methyl ferulate (MF)	2309-07-1	$C_{11}H_{12}O_4$	A	>0.99
Phosphoric acid	7664-38-2	H_3PO_4	S	>0.999
Sodium chloride	7647-14-5	NaCl	S	>0.99
Sodium dihydrogen phosphate	7558-80-7	NaH_2PO_4	S	>0.99
Sodium hydrogen phosphate	7558-79-4	Na_2HPO_4	M	>0.995
Sodium iodide	7681-82-5	NaI	M	>0.995

concentration range of 0.2 to 1.0 mol kg^{-1}, was equilibrated with the reaction mixture prior to enzyme addition. These measurements were conducted at a constant initial MF concentration of 6 mmol kg^{-1}.

All of solutions were gravimetrically prepared using a Sartorius CPA324S balance (Sartorius, Göttingen, Germany) with an accuracy of $\pm 10^{-4}$ g.

3.3. HPLC Analysis

An Agilent series 1200 HPLC (Agilent, Böblingen, Germany) equipped with a diode array detector set to a wavelength of $\lambda = 320$ nm was used to quantify the molalities of MF and FA (m_{MF} and m_{FA}) after the equilibrium measurements. MF and FA were separated using a Supelco Ascentis RP amide column (5 μm, 15 cm × 3 mm) at a flow rate of 1 ml min^{-1}. The acetonitrile and aqueous phosphoric acid mobile phases ($w_{H3PO4} = 0.00085$) were used in a linear gradient mode to reasonably separate MF and FA, as described previously [15].

4. Results and discussion

4.1. K_m as a function of pH

Methyl ferulate hydrolysis is known to strongly depend on pH. Goldberg et al. [15] observed a substantial increase in the apparent K′ value with increasing pH, particularly for the pH range that was investigated in this study (pH 6 to 7). These researchers based this finding on calculations obtained using a semi-empirical model [16]. Their pH-correlation for K′ was determined at conditions that were different from those used in this study. However, the pH correlation used by Goldberg et al. has not been validated experimentally. For these reasons and to compare the results of this work with those obtained by Goldberg et al., the value of K_m was measured at five different pH values between 6 and 7. Within this pH range, an almost 10-fold increase in the K_m value was observed (Fig. 1). The pH dependency of the K_m values could best be described by an exponential approach, as shown in Fig. 1.

Goldberg et al. measured the value of K′ (which they found to be 29.6) at 25 °C and pH 4.98 using a citrate buffer [15]. Although the qualitative pH dependence of the K_m values obtained in this work compares to that found by Goldberg et al., the quantitative values are different. This difference is likely due to various reasons, which were addressed in Section 2. One of these reasons is that Goldberg et al. used K′ instead of K_m (Eq. (8)). K′ (= 29.6) can be converted to K_m (accounting for the molality of water) using Eq. (8) to obtain $K_m = 0.533$. The application of the pH regression obtained using our experimental data (Fig. 1) yielded a K_m value of 0.425 at a pH of 4.98, which is in reasonable agreement with the K′ value obtained by Goldberg et al., particularly because the reactions were investigated at slightly different conditions (e.g., different initial MF molality and different type of buffer).

Both the pH correlation obtained by Goldberg et al. and the experimental pH dependence of the K_m values found in this work show that even small pH changes strongly influence the value of K_m. Unfortunately,

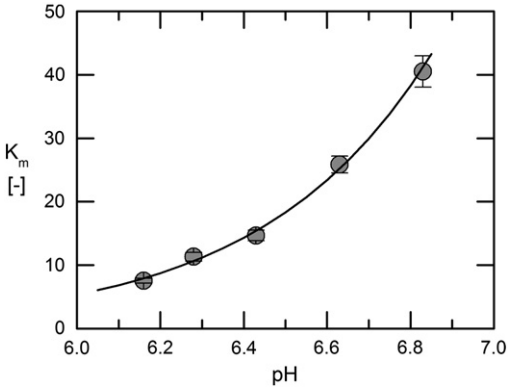

Fig. 1. K_m values of methyl ferulate hydrolysis at 25 °C in sodium phosphate buffer as a function of pH. The symbols represent the experimental data obtained in this study. The solid line represents an exponential regression of the experimental K_m values: $K_m^{reg} = 1.923 \cdot 10^{-6} \exp\{2.471 \cdot pH\}$.

during the K_m experiments (Sections 3.2 and 4.2), the pH values slightly varied. Thus, this pH change needed to be accounted for by normalizing the measured K_m value to a value at a fixed pH. As mentioned above, the pH was set to 6.5 in this work due to the high biological enzyme activity at this pH. Thus, the measured values of K_m^{exp} shown in Fig. 1 were recalculated to this pH using Eq. (12):

$$K_m(pH\ \ 6.5) = K_m{}^{exp} + \left[K_m{}^{reg}(pH\ \ 6.5) - K_m{}^{reg}(pH^{exp})\right], \quad (12)$$

where K_m^{exp} is the true measured K_m value at the true pH value (pH^{exp}, see Table 2) and K_m^{reg} is the K_m value obtained from the correlation shown in Fig. 1. Hereafter, the recalculated values of K_m (pH = 6.5) are simply referred to as K_m. This experimentally supported pH regression allows the separation of the pH influence from the net influence of the reactant concentration and the NaCl addition on the value of K_m.

4.2. Experimental determination of K_a and K_γ values

To obtain K_m values at different FA equilibrium molalities, the initial MF molality was varied between 2 mmol kg^{-1} and 10 mmol kg^{-1} using a constant MeOH molality (5 mol kg^{-1}) and a constant temperature (25 °C). The results of these equilibrium measurements are shown in Table 2.

Fig. 2 shows the experimentally obtained K_m values as a function of the FA equilibrium molality. As shown, K_m decreases to almost 50% of the initial value as the FA equilibrium molalities is increased from 2 to 10 mmol kg^{-1}. This result was observed even though the absolute molalities and the difference in the molalities (2 to 10 mmol kg^{-1}) were very small. It thus becomes clear that the corresponding molalities (although low) of the reactants and the products need to be reported when reporting K_m values.

The experimental K_m values were pH-corrected according to the information provided in Section 4.1. The extrapolation to an FA molality of zero ($m_{FA}^{eq} = 0$ mol kg^{-1}) yields a K_m^{∞} value of 27.22 (see Fig. 2). Using

Table 2
Equilibrium molalities of the reactants and products in the reaction (methyl ferulate + water ↔ ferulic acid + MeOH) at different FA equilibrium molalities (T = 25 °C, initial pH of 6.5, and sodium phosphate buffer). The experimental K_m and K_γ values are also shown.

No.	FA [mmol kg^{-1}]	MF [mmol kg^{-1}]	MeOH [mol kg^{-1}]	$H_2PO_4^-$ [b] [mol kg^{-1}]	HPO_4^{2-} [b] [mol kg^{-1}]	pH	K_m [a]	K_γ [c]
1	2.1545	0.0084	4.9169	0.4245	0.0755	6.45	25.61	3.17
2	4.0729	0.0183	4.9166	0.4260	0.0740	6.44	22.60	3.59
3	5.5633	0.0331	4.9165	0.4274	0.0726	6.43	17.57	4.62
4	7.5460	0.0546	4.9238	0.4288	0.0712	6.42	15.16	5.35
5	10.3520	0.0763	4.9391	0.4316	0.0684	6.40	14.98	5.41

[a] Calculated according to Eq. (7). The K_m values were pH-corrected using Eq. (12).
[b] The molalities of sodium phosphate were adjusted gravimetrically and assumed to be constant throughout the reaction.
[c] Calculated from the corresponding K_m and K_a using Eq. (6).

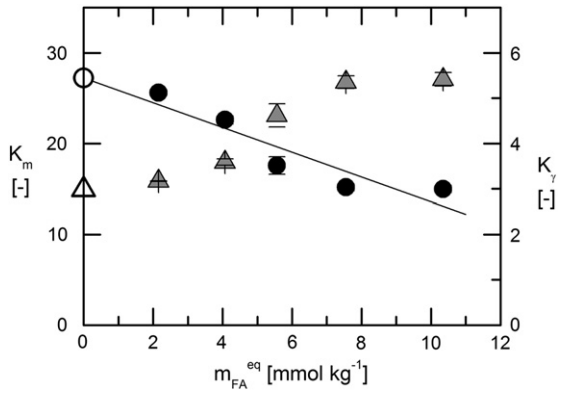

Fig. 2. Experimental K_m values (full circles, left ordinate) and K_γ values (triangles, right ordinate) for the methyl ferulate hydrolysis reaction at 25 °C as a function of the ferulic acid equilibrium molality. These values were calculated according to Table 2 and Eqs. (6) and (9). The line is shown to guide the eye. The open circle represents K_m^∞ (by extrapolating K_m to $m_{FA}^{eq}=0$ mmol kg^{-1}), and the open triangle represents K_γ^∞.

this value, as well as the activity coefficients of water and MeOH (i.e., $\gamma^x{}_w$ and $\gamma^x{}_{MeOH}$ in the water/MeOH system), allows the calculation of K_a (see Eq. (11)). The values of $\gamma^x{}_w$ and $\gamma^x{}_{MeOH}$ are available in the literature [17] and can be converted into $\gamma^m{}_w$ and $\gamma^m{}_{MeOH}$ using Eq. (4). For the binary water/MeOH mixture with $m_{MeOH}=5$ mol kg^{-1} and $m_w=55.509$ mol kg^{-1} ($m_{MF}^{eq}=0$ and $m_{FA}^{eq}=0$) at 25 °C, $\gamma^x{}_w$ ($\gamma^m{}_w$) and $\gamma^x{}_{MeOH}$ ($\gamma^m{}_{MeOH}$) are 1.02 (0.0166 kg mol^{-1}) and 2.99 (0.0496 kg mol^{-1}), respectively. These data yield a K_γ^∞ of 2.98 according to Eq. (10). Using Eq. (11), the thermodynamic equilibrium constant K_a (the product of K_m^∞ and K_γ^∞) is thus calculated to be 81.1.

It should be noted that 81.1 is the value for K_a at pH 6.5. Due to a change in the amount of species existing at pH values other than 6.5, K_a is expected to be different at other pH values. Using the pH regression obtained in this work (Section 4.1) to recalculate the value of K_m^∞ at pH 7 (which was found to be 71.6) in addition to $K_\gamma^\infty=2.98$ gives K_a (pH 7) = 213.3, which is the K_a at biological standard conditions. However, because the main intention of this work was to investigate the general influence of the reactant and the product activity coefficients on the calculation of $\Delta^R g^+$ (at any pH), all of following K values refer to a pH of 6.5.

Accordingly, $K_a=81.1$ was then used to calculate the K_γ values at various equilibrium molalities through the rearrangement of Eq. (6). The results are also shown in Fig. 2. The value of K_γ increases from the value of K_γ^∞ (lowest value) with the addition of MF/FA. This increase strongly depends on the FA equilibrium molality. In the range of FA equilibrium molalities considered in this study (between 2 and 10 mmol kg^{-1}), an increase of more than 80% was observed in the K_γ values. These experimentally determined K_γ values are also given in Table 2.

4.3. Estimation of K_γ from a thermodynamic model

The K_γ values can be not only determined through reaction experiments, as described in the previous section but also estimated using thermodynamic models, e.g., g^E models, such as UNIFAC [18,19] and eNRTL [20], or equations of state.

However, the models that are suitable for the estimation of activity coefficients in biosystems should explicitly account for specific interactions due to hydrogen bonding or charges and for pH effects. Therefore, in this work, the reactant and the product activity coefficients were predicted using the electrolyte Perturbed-Chain Statistical Associating Fluid Theory (ePC-SAFT) [21], which has been proven to be able to precisely calculate the activity coefficients of the components of aqueous solutions that contain electrolytes and biomolecules [21–28]. In some of these studies, the model was shown to be predictive, i.e., the activity coefficients of the components of multi-solute solutions could be calculated based on the experimental data of single-solute solutions only.

The ePC-SAFT model calculates the residual Helmholtz energy of a system (a^{res}) as the sum of different contributions (Eq. (13)):

$$a^{res} = a^{hc} + a^{disp} + a^{assoc} + a^{ion}. \quad (13)$$

These contributions are assumed to be additive, i.e., independent. A molecule is assumed to consist of m_i^{seg} spherical segments of diameter σ_i. The hard-chain contribution (a^{hc}) accounts for the repulsion of the molecules. The term a^{disp} represents the attractive van der Waals forces among the molecules, which are characterized by the dispersion-energy parameter u_i/k_B. The association term a^{assoc} accounts for the formation of hydrogen bonds between associating molecules. Here, a molecule is considered to have a certain number of association sites that can form these hydrogen bonds. The interaction between two association sites is characterized by two additional parameters: the association-energy parameter ε^{AiBi}/k_B and the association-volume parameter κ^{AiBi}.

In this work, the expressions for a^{hc}, a^{disp}, and a^{assoc} were used as in the original PC-SAFT model [24]. The energy contribution for charged species (a^{ion}) was calculated according to a Debye-Hückel term (ePC-SAFT). Once the expression for a^{res} is known, all of the other thermodynamic properties, e.g., enthalpies, densities, osmotic coefficients and activity coefficients, can be derived by applying textbook thermodynamic relationships (see, e.g., [25]).

The ePC-SAFT model requires the availability of the abovementioned model parameters for each of the system components. These parameters are not usually available for biomolecules (e.g., MF and FA in this work). However, osmotic coefficients have been proven to be a reliable database for the estimation of these parameters for biological components (e.g., [26]). Therefore, this type of data for MF and FA was measured in this study (see Appendix A for details). The resulting pure-component parameters, as well as the model parameters for water and MeOH [27,28], are shown in Table 5 of the Appendix A.

Based on these parameters, ePC-SAFT was used to predict the activity coefficients of the four reactants and products ($\gamma^m{}_{MeOH}$, $\gamma^m{}_w$, $\gamma^{*,m}{}_{MF}$, and $\gamma^{*,m}{}_{FA}$) of the reaction mixtures considered in this work. These coefficients are listed in Table 3. It is obvious that most of the activity coefficients strongly deviate from unity, which emphasizes the need to explicitly account for them in the calculation of K_γ. Moreover, it can be observed that the activity coefficient of FA ($\gamma^{*,m}{}_{FA}$) strongly depends on the equilibrium composition, whereas the changes in the activity coefficients of the other components are rather small.

According to Eq. (9), the predicted activity coefficients were used to calculate K_γ at the various concentrations. The predicted K_γ values, as well as the deviation from the experimental values reported in Fig. 2, are also given in Table 3. The comparison of the predicted K_γ values to the experimental data (Fig. 3) yields very good agreement between the two methods, with a maximum relative deviation of only 10%. This is an excellent result keeping in mind the scatter in the experimental data and the fact that these data were predicted without any knowledge of the reaction data.

Table 3
ePC-SAFT-predicted activity coefficients of FA and MF ($\gamma_i^{*,m}$) and of MeOH and water (γ_i^m) as a function of the equilibrium molalities in the reaction mixtures (Table 2). The values of the resulting water activities (a_w), K_γ (calculated using Eq. (9)), and the deviations AAD and ARD [in %] between the predicted and the experimental K_γ values are also shown.

No.	FA	MF	MeOH	Water[a]	a_w	K_γ	AAD[b]	ARD[b]
1	1.1227	0.9813	0.0497	0.0165	0.9159	3.46	0.2927	9.24
2	1.2650	0.9813	0.0498	0.0166	0.9215	3.87	0.2808	7.82
3	1.3789	0.9813	0.0498	0.0166	0.9215	4.22	0.3967	8.59
4	1.5682	0.9813	0.0499	0.0167	0.9270	4.78	0.5706	10.66
5	1.8631	0.9812	0.0499	0.0168	0.9326	5.64	0.2251	4.16

[a] The apparently strong deviation of the γ_w^m values from unity is caused by the use of the molality scale. The water activity (a_w) is close to unity.
[b] Calculated according to Eq. (15) (Appendix A).

Moreover, due to the validity of Eq. (6), it can be stated that the dramatic change in the measured K_m values with increasing FA equilibrium molalities (Table 2) can be ascribed to the non-constant activity-coefficient ratio K_γ.

4.4. Calculation of the standard Gibbs energy of reaction $\Delta^R g^+$

The key quantity in the thermodynamic description of reaction equilibria is the Gibbs energy of reaction ($\Delta^R g$). The value of $\Delta^R g$ is equal to zero for a reaction at equilibrium. Accordingly, at equilibrium, $\Delta^R g^+$ is obtained from the thermodynamic (activity-based) equilibrium constant:

$$\Delta^R g = \Delta^R g^+ + RT \ln K_a = 0. \quad (14)$$

Using the K_a value of 81.1 which was obtained by extrapolating the measured K_m values to $m_{Fa}^{eq} = 0$, and the value of K_γ^∞, the standard Gibbs energy of reaction was calculated to be $\Delta^R g^+ = -10.90$ kJ mol^{-1}. This result indicates that the reaction equilibrium of the MF hydrolysis reaction strongly lies on the side of the products, as previously described [15]. However, the exact quantity of $\Delta^R g^+$ has not been previously determined and strongly depends on the type of equilibrium constant K used in Eq. (14).

In the literature, Eq. (14) is usually applied through the use of different K values that are either measured or found in databases (e.g., [12]). To illustrate the differences in the values of $\Delta^R g^+$ obtained when either the thermodynamic equilibrium constant K_a or the apparent K is used, two cases are discussed:

1. State of the art. K_m is usually measured at an arbitrarily chosen reactant concentration. This K_m is applied in Eq. (14), and K_γ is assumed to be unity. This procedure would yield a $\Delta^R g^+ = -6.71$ kJ mol^{-1} for the MF hydrolysis reaction (e.g., reaction batch No. 5 in Table 2 ($K_m = 14.98$)), which corresponds to a relative deviation of almost 40% from the correct value of -10.90 kJ mol^{-1}. Based on our results, this difference becomes more pronounced if K_m values at even higher FA molalities are used (Fig. 2). Accordingly, the potential error in the determination of $\Delta^R g^+$ as a result of the use of the K_m value instead of K_a is very significant. Moreover, this difference factually adds up to the general uncertainty in standard Gibbs energies.
2. Accounting for the concentration dependence of K_m. In this case, the K_m values are measured at certain reactant concentrations; K_γ is assumed to be unity. Extrapolating these K_m values to zero reactant concentrations yields K_m^∞ (note that this is *not* K_a for MF hydrolysis due to the excess of MeOH). For MF hydrolysis, K_m^∞ becomes 27.22 (according to Fig. 2), which would yield $\Delta^R g^+ = -8.19$ kJ mol^{-1}. Accordingly, the potential error in the determination of $\Delta^R g^+$ based on the use of the K_m^∞ value is lower than that obtained in case 1 but still significant because it corresponds to a relative difference of more than 20% of the correct value of -10.90 kJ mol^{-1}.

To obtain the thermodynamically correct $\Delta^R g^+$ value, the activity coefficients of the reactants and the products, particularly those of water and MeOH, must be included in the analysis (as described in Section 4.2).

Next to the 'naked values' of $\Delta^R g^+$, these values impact feasibility studies on biological reactions. Therefore, $\Delta^R g^+$ was used to calculate the possible operation window for MF hydrolysis (Fig. 4). This operation window defines the molality ratio of MF to FA that ensures that $\Delta^R g$ is negative (which makes the reaction feasible with respect to the forward direction). As shown, the use of the value of $\Delta^R g^+$ obtained using the K_a value determined in this work (81.1 kJ mol^{-1}) strongly increased the possible operation window of MF hydrolysis compared with the use of the value of $\Delta^R g^+$ obtained using the value of K_m^∞ (27.22 kJ mol^{-1}, as described in case 2).

The approach described within this work is thus not only of particular interest in the study of MF hydrolysis. The general methodology shown here becomes even more relevant for reactions for which, following the state-of-the-art procedure, the obtained $\Delta^R g^+$ values are positive. An example of such a reaction, which is heavily discussed in the context of reaction feasibility, is the 6th step of the well-investigated glycolysis pathway: the conversion of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-BPG). The state-of-the-art value of $\Delta^R g^+$ for this reaction is positive (6.28 kJ mol^{-1}), and thermodynamic calculations of the feasibility of this reaction appear to be inconsistent with experimental observations. Thus, the use of the activity coefficients in the analysis is expected to help overcome these inconsistencies [29,30].

4.5. Influence of additives

Although it is known that salts influence the K_m values, their influence is often neglected (e.g., [31]). In this work, NaCl was used to investigate the influence of an additive on the K_m and K_γ values of MF hydrolysis. For the experimental investigation, the methods that were used to determine K_a in the additive-free reaction system (Section 4.2) were also applied in this analysis. In these experiments, the initial MF molality was maintained constant (6 mmol kg^{-1}), whereas the NaCl concentration was varied between 0.1 and 1.0 mol kg^{-1}. The results of the equilibrium measurements in the presence of NaCl are summarized in Table 4. It was found that the reaction equilibrium is shifted to the product side upon the addition of NaCl. For an initial MF molality of 6 mmol kg^{-1} and in the absence of salt, K_m had a value of 17.57 (see reaction batch No. 3 in Table 2). The successive addition of NaCl causes this value to increase to

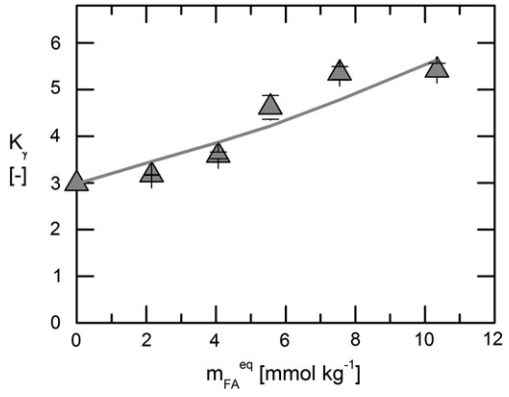

Fig. 3. Comparison of the experimentally determined K_γ values from the equilibrium measurements (symbols; shown in Fig. 2) and the ePC-SAFT-predicted K_γ values (line) as a function of the FA equilibrium molality.

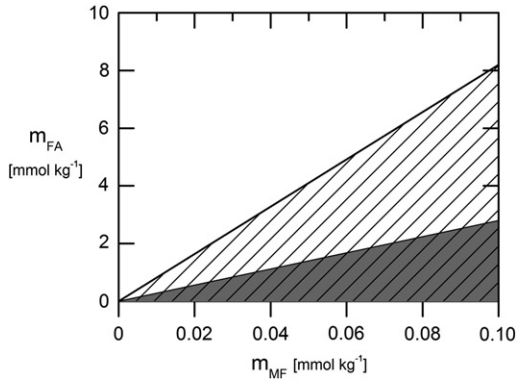

Fig. 4. Possible operation windows for methyl ferulate hydrolysis calculated using K_m^∞ (dark gray area) or K_a, i.e., accounting for K_γ^∞ (striped area).

20.66 with an NaCl concentration of 1.0 mol kg^{-1} in the reaction mixture (Fig. 5), which corresponds to a relative deviation of more than 15%.

Analogous to Eq. (6), the value of K_γ at each salt concentration can be obtained by dividing the universally valid equilibrium constant K_a for MF hydrolysis (81.1, see Fig. 2) by the value of K_m at the respective salt molality (between 0.1 and 1.0 mol kg^{-1}). At a NaCl molality of zero, K_γ is identical to the value at $m_{FA}^{eq}=6$ mmol kg^{-1} shown in Table 2 ($K_\gamma=4.62$). As illustrated in Fig. 5, the experimental K_γ values start at 4.62 (zero NaCl molality) and decrease to 3.93 at 1 mol kg^{-1} of NaCl, which corresponds to a relative change of 15%. Accordingly, due to the constant K_a, the shift in K_m is caused by the change in K_γ. Moreover, this change in K_γ indicates that NaCl influences the activity coefficients of all of the reactants and products. Thus, in cases in which salts are unavoidably present in enzymatic reactions (e.g., as co-factors), the salt influence on the reaction equilibria should be studied to distinguish between the influence of the reactants and the products and the influence of the salts (or other additives). Moreover, it needs to be noted that NaCl was considered as a 'model additive' in this work and that the method described here for NaCl could also be applied for the investigation of other salts.

To validate the experimental results obtained with NaCl, the salt influence on the value of K_γ at $m_{FA}^{eq}=6$ mmol kg^{-1} was modeled with ePC-SAFT. Because the parameters for Na^+ and Cl^- are available (see Table 5 of the Appendix A), the modeling results are pure predictions. In accordance with the experimentally observed decrease in the K_γ value as a result of NaCl addition, ePC-SAFT also predicts a decrease in K_γ (Fig. 5). The predicted and experimentally determined K_γ data are in almost-quantitative agreement. Due to this agreement, the salt influence on the K_m value could also have been purely predicted using Eq. (6) from the K_a value and the predicted K_γ values.

Finally, other biomolecules (not only salts) influence the K_γ values. Cells, which are the place where biological reactions take place, are not empty containers but rather fully packed with proteins, nucleic acids, and metabolites. This phenomenon is usually called 'macromolecular crowding', which could be investigated by the addition of macromolecules, e.g., the addition of model proteins to in vitro equilibrium experiments. Although macromolecular crowding was not investigated in this study, it is strongly suggested to have an influence on the reactant and product activity coefficients and thus on $\Delta^R g^+$ and $\Delta^R g$ [32–34].

5. Conclusion

The thermodynamics of biological reactions can be analyzed in terms of the Gibbs energies of reaction or the equilibrium constants. These key quantities are affected by many parameters, including the activity coefficient of the reactants and the products, which are often discussed and certainly of relevance. However, the influence of these activity coefficients is usually neglected due to (1) the low concentration of the reactants and the products in biological reactions and (2) the lack of data for these activity coefficients in biological reactions.

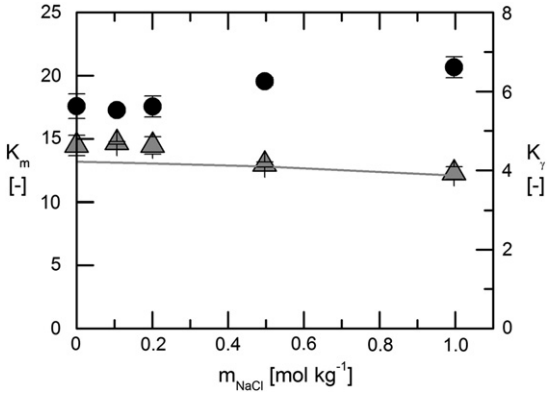

Fig. 5. Experimental K_m (circles, left ordinate) and K_γ (triangles, right ordinate) of MF hydrolysis at 25 °C and $m_{MF,init}=6$ mmol kg^{-1} as a function of the sodium chloride molality. The predicted K_γ values using ePC-SAFT (solid line) are also shown.

In this work, the influence of the activity coefficients (accounted for by K_γ) on a biological model reaction (the hydrolysis of methyl ferulate) was investigated. Thus, equilibrium measurements at an excess of MeOH and at different initial methyl ferulate molalities were performed. The resulting K_m values were found to strongly depend on the concentration, i.e., the MF and the FA equilibrium molalities.

An extrapolation of these K_m values to an infinite dilution of MF and FA allowed the determination of K_m^∞. Accounting for the ratio of the MeOH activity coefficient to the activity coefficient of water (K_γ^∞, MF→0 and FA→0) allowed the calculation of the value of the concentration-*independent* K_a. This value can subsequently be used to experimentally determine the K_γ values as a function of the reactant and the product concentrations. In addition, the ePC-SAFT thermodynamic model was used to estimate these activity coefficients and therefore K_γ. This calculation was performed completely independently from the reaction-equilibrium measurements, and no parameters were fitted to the reaction data. Thus, the application of ePC-SAFT provides a truly predictive procedure for the calculation of reactant and product activity coefficients. It was found that the K_γ values predicted by ePC-SAFT also depend on the MF and the FA equilibrium molalities. Furthermore, the absolute relative deviation between the experimental and the predicted K_γ values was found to be very small (<6%).

Thus, the experimental data, as well as the ePC-SAFT model, consistently indicate that the activity coefficients cannot be neglected in the determination of K_a (which is required to calculate $\Delta^R g^+$). In the considered reactant concentration range, K_γ varied more than 80%. Accordingly, K_γ needs to be included in the thermodynamic calculations to prevent unintended errors in the determination of consistent $\Delta^R g^+$ values or even in the evaluation whether a biological reaction is 'feasible' or 'not feasible'.

If the activity coefficients and the absolute equilibrium concentrations of all of the reactants and products from equilibrium measurements are known (e.g., available in the literature), it is possible to

Table 4
Equilibrium molalities of all of the components present in the reaction (methyl ferulate + water ↔ ferulic acid + MeOH) at different NaCl molalities (T = 25 °C, initial pH of 6.5, and sodium phosphate buffer). The experimental K_m and K_γ values and the deviations AAD and ARD [in %] between the predicted and experimental K_γ values are also shown.

NaCl [mol kg^{-1}]	Ferulic acid [mmol kg^{-1}]	Methyl ferulate [mmol kg^{-1}]	MeOH [mol kg^{-1}]	$H_2PO_4^-$ [mol kg^{-1}]	HPO_4^{2-} [mol kg^{-1}]	pH	K_m[a]	K_γ	AAD[b] for K_γ	ARD[b] for K_γ
0.106	5.4559	0.0414	4.9027	0.4380	0.0619	6.35	17.27	4.70	0.4999	10.64
0.201	5.3494	0.0465	4.8993	0.4452	0.0548	6.29	17.55	4.63	0.4421	9.58
0.497	5.5206	0.0519	4.9080	0.4573	0.0427	6.17	19.55	4.15	0.0457	1.10
0.997	5.4356	0.0648	4.9263	0.4715	0.0284	5.98	20.66	3.93	0.0444	1.10

[a] Calculated according to Eq. (7). The K_m values were pH-corrected using Eq. (12).
[b] Calculated according to Eq. (15) (Appendix A).

calculate the thermodynamically correct values of K_a and thus $\Delta^R g^+$. The activity coefficients required for the determination of the K_γ values might be calculated with ePC-SAFT for known reaction conditions (temperature, buffer, and initial reactant concentration) if the pure-component ePC-SAFT parameters are available. This finding particularly emphasizes the importance of a detailed and accurate documentation of equilibrium data.

In addition to the influence of the reactant concentrations, the effect of sodium chloride as a model additive was also investigated at a constant FA equilibrium molality (6 mmol kg^{-1}) through both experimentation and modeling. Compared with the influence of the MF and FA equilibrium molalities on K_γ, sodium chloride was found to have only a moderate influence. An increase in the NaCl molality from 0 to 1.0 mol kg^{-1} results in a decrease of 15% in the measured K_γ values. This change in the K_γ value resulted in an increase in K_m and thus a shift of the reaction equilibrium toward the product side. The prediction of the K_γ values by ePC-SAFT revealed an almost-quantitative agreement between the experimentation and modeling results.

This study can be regarded as a contribution to the substantial and diligent work performed by Alberty, Goldberg et al., and others in the field of biological thermodynamics. We are aware that this study focused on a model reaction that is a rather less-critical candidate in terms of reaction feasibility because the equilibrium is far on the side of the products. Therefore, the general conclusions of our findings need to be validated by transferring the methodology to other reactions. Nevertheless, this study emphasizes the risk of the use of molalities (or concentrations) instead of activities in the determination of K values and the calculation of $\Delta^R g^+$. The potential influence of the reactant and of the product activity coefficients was found to be of crucial importance in the thermodynamic analysis of biological reactions.

List of symbols

Roman symbols

a_i	[–] activity of component i
a	[J mol^{-1}] Helmholtz energy
c_i	[mol l^{-1}] concentration of component i
$\Delta^R g$	[J mol^{-1}] Gibbs energy of reaction
$\Delta^R g^+$	[J mol^{-1}] standard Gibbs energy of reaction
k_B	[J K^{-1}] Boltzmann constant, $1.38065 \cdot 10^{-23}$ J K^{-1}
k_{ij}	[–] binary interaction parameter
K_a	[–] activity-based (thermodynamic) equilibrium constant
K_c	[–] concentration-based equilibrium constant
K_γ	[–] ratio of activity coefficients
K_m	[–] molality-based equilibrium constant
m_i	[mol kg^{-1}] molality (moles of solute i per kg of water, except in the appendix and in Fig. 6 where it is moles of solute per kg of MeOH)
m_i^{seg}	[–] number of segments
N	[–] total number of molecules
NP	[–] number of data points
R	[J mol^{-1} K^{-1}] ideal gas constant, 8.31446 J mol^{-1} K^{-1}
R	[–] measurement signal
T	[K] temperature
u_i/k_B	[K] dispersion-energy parameter
U	[mol s^{-1}] enzyme units
x	[–] mole fraction
y	[–] measured value

Greek symbols

ε^{AiBi}/k_B	[K] association-energy parameter
γ_i	[–] generic activity coefficient of component i (related to pure component)
γ_i^*	[–] rational activity coefficient of component i (related to hypothetical ideal solution)
κ^{AiBi}	[–] association-volume parameter
λ	[nm] wave length
ν_i	[–] stoichiometric factor of component i
ϕ	[–] osmotic coefficient
σ_i	[Å] temperature-independent segment diameter of molecule i

Subscripts

a	based on activity
c	based on concentration
cal	calibration component
eq	equilibrium
init	initial
i, j	component indices
m	based on molality
reg	regression
seg	segment
solv	solvent
w	water
x	based on mole fraction

Superscripts

assoc	association
calc	calculated
disp	dispersion
exp	experimental
hc	hard chain
ion	ionic interaction
m	based on molality
res	residual
x	based on mole fraction
∞	infinitely diluted
′	apparent quantity (at defined system conditions)
*	related to hypothetical ideal solution

Abbreviations

1,3-BPG	1,3-bisphosphoglycerate
AAD	absolute average deviation
ARD	absolute relative deviation
CAS	chemical abstracts service
EC	enzyme commission
ePC-SAFT	electrolyte Perturbed-Chain Statistical Associating Fluid Theory
FA	ferulic acid
G3P	glyceraldehyde-3-phosphate
HPLC	high performance liquid chromatography
MeOH	methanol
MF	methyl ferulate
NaCl	sodium chloride
NaI	sodium iodide
NIST	National Institute of Standards and Technology
RP	reversed phase
TFA	thermodynamic feasibility analysis

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Appendix A. Estimation of model parameters for ePC-SAFT

To model water, MeOH, MF, and FA (all of which were treated as associating molecules), ePC-SAFT requires five parameters for each component (see Section 4.3 and Table 5). Only two parameters are required for the modeling of inorganic ions: the hydrated ion diameter σ_{ion} and the dispersion-energy parameter u_{ion}/k_B.

Whereas the pure-component parameters for water, MeOH, and the buffer species are available [28], the ePC-SAFT parameters for MF and FA were not available. Therefore, the MF and FA pure-component parameters were fitted to the osmotic-coefficient data of binary MeOH/MF and MeOH/FA solutions obtained in this work (see Fig. 6). The measurements were performed using an Osmomat O70 vapor pressure osmometer (Gonotec, Berlin, Germany), as described previously [28]. Prior to each measurement, the Osmomat was calibrated with solutions of sodium iodide in MeOH [36].

The modeling results are shown in Fig. 6. As shown, ePC-SAFT is capable of representing the experimental data reasonably well using the fitted parameters for MF and FA (see Table 5).

The absolute average deviations (AAD) and the absolute relative deviations (ARD) between the experimental (exp) and the modeled (calc) osmotic coefficients are 0.002 and 1.4% for MF and 0.064 and 5.6% for FA, respectively. These were calculated using the following equations:

$$\mathrm{AAD} = \frac{1}{\mathrm{NP}}\sum_{k=1}^{\mathrm{NP}}\left|\left(y_k^{\mathrm{calc}} - y_k^{\mathrm{exp}}\right)\right| \quad \text{and} \quad \mathrm{ARD} = 100\cdot\frac{1}{\mathrm{NP}}\sum_{k=1}^{\mathrm{NP}}\left(1-\frac{y_k^{\mathrm{calc}}}{y_k^{\mathrm{exp}}}\right) \tag{15}$$

where y represents the considered quantity (i.e., the osmotic coefficient).

To describe the mixture properties, the conventional mixing rules described by Lorentz and Berthelot were applied for each set of two components (i and j):

$$\sigma_{ij} = 0.5\left(\sigma_i + \sigma_j\right) \tag{16}$$

and

$$u_{ij} = \left(u_i u_j\right)^{0.5}\left(1-k_{ij}\right) \tag{17}$$

Table 5
ePC-SAFT pure-component parameters for all components present in the experiments. Their binary interaction parameters with water and MeOH are also shown.

Component	m_i^{seg}	σ_i	u_i/k_B	ε^{AiBi}/k_B	κ^{AiBi}	k_{ij} (H_2O)	k_{ij} (MeOH)
MF[a]	10.2982	3.5124	414.71	1936.6	0.000100	–	−0.080
FA[a]	15.9236	3.9985	360.15	617.2	0.022700	−0.180[e]	−0.298
water[b]	1.2047	2.7927	353.94	2425.7	0.045099		–
MeOH[c]	1.5255	3.2300	188.90	2899.5	0.035176	−0.085	
Na^{+}[b]	1.0000	2.4122	646.05	–	–	–	–
Cl^{-}[b]	1.0000	3.0575	472.88	–	–	–	–
$H_2PO_4^{-}$[d]	1.0000	3.7026	–	–	–	–	–
HPO_4^{2-}[d]	1.0000	4.4608	–	–	–	–	–

[a] This work.
[b] [27].
[c] [28].
[d] [22].
[e] Fitted to the solubility of FA in water [35].

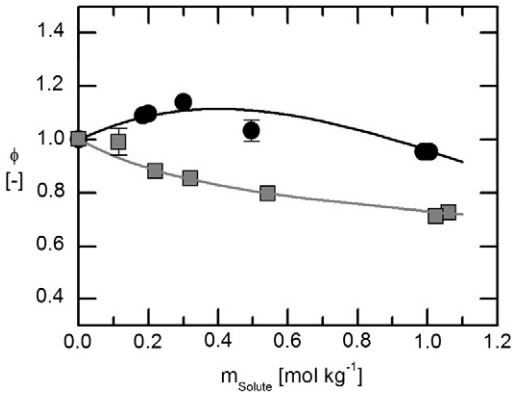

Fig. 6. Osmotic coefficients of MeOH/MF (squares) and MeOH/FA (circles) solutions at 25 °C as a function of the reactant molality. The experimental data are represented by symbols, and the osmotic coefficients modeled with ePC-SAFT are represented by lines.

The k_{ij} term in Eq. (17) is a binary interaction parameter that is introduced to correct for the deviations in the dispersion-energy parameter between the two components from the mean.

The binary interaction parameters k_{ij} between MeOH/MF and MeOH/FA were also fitted to the osmotic coefficient data (Fig. 6), and the k_{ij} between MeOH and water has been previously published [28]. However, no binary interaction parameters have been fitted to any experimental data of the reaction system.

Table 5 summarizes the pure-component parameters for MF and FA and the parameters for water, MeOH, the buffer components $H_2PO_4^{-}$ and HPO_4^{2-}, and the Na^{+} and Cl^{-} ions. In addition, the k_{ij} values between FA and water, between FA and MeOH, and between MF and MeOH are also shown.

References

[1] R. Schuster, S. Schuster, Refined algorithm and computer program for calculating all non-negative fluxes admissible in steady states of biochemical reaction systems with or without some flux rates fixed, Bioinformatics 9 (1) (1993) 79–85.
[2] H.P. Bonarius, G. Schmid, J. Tramper, Flux analysis of underdetermined metabolic networks: the quest for the missing constraints, Trends in Biotechnology 15 (8) (1997) 308–314.
[3] K.C. Soh, V. Hatzimanikatis, Network thermodynamics in the post-genomic era, Current Opinion in Microbiology 13 (3) (2010) 350–357.
[4] D.A. Beard, S.-d. Liang, H. Qian, Energy balance for analysis of complex metabolic networks, Biophysical Journal 83 (1) (2002) 79–86.
[5] H. Qian, D.A. Beard, S.-d. Liang, Stoichiometric network theory for nonequilibrium biochemical systems, European Journal of Biochemistry 270 (3) (2003) 415–421.
[6] A. Kümmel, S. Panke, M. Heinemann, Putative regulatory sites unraveled by network-embedded thermodynamic analysis of metabolome data, Molecular Systems Biology 2 (2006).
[7] C.S. Henry, M.D. Jankowski, L.J. Broadbelt, V. Hatzimanikatis, Genome-scale thermodynamic analysis of *Escherichia coli* metabolism, Biophysical Journal 90 (4) (2006) 1453–1461.
[8] C.S. Henry, L.J. Broadbelt, V. Hatzimanikatis, Thermodynamics-based metabolic flux analysis, Biophysical Journal 92 (5) (2007) 1792–1805.
[9] M.L. Mavrovouniotis, Identification of qualitatively feasible metabolic pathways, Artificial Intelligence and Molecular Biology, AAAI Press, 1993, pp. 325–364.
[10] M.L. Mavrovouniotis, Identification of localized and distributed bottlenecks in metabolic pathways, Proc. Int. Conf. Intell. Syst. Mol. Biol, Proceedings, 1, 1993, pp. 275–283.
[11] J. Villadsen, J. Nielsen, G. Lidén, Thermodynamics of Bioreactions, Springer US, Boston, MA, 2011.
[12] R.N. Goldberg, Y.B. Tewari, T.N. Bhat, Thermodynamics of enzyme-catalyzed reactions — a database for quantitative biochemistry, Bioinformatics 20 (16) (2004) 2874–2877.
[13] R.A. Alberty, Thermodynamics of Biochemical Reactions, Wiley-Interscience, Hoboken N.J., 2003
[14] E.C. Webb, Enzyme nomenclature. Published for the International Union of Biochemistry and Molecular Biology by Academic Press, San Diego, 1992.
[15] R.N. Goldberg, B.E. Lang, M.J. Selig, S.R. Decker, A calorimetric and equilibrium investigation of the reaction {methyl ferulate(aq) + H_2O(1) = methanol(aq) + ferulic acid(aq)}, Journal of Chemical Thermodynamics 43 (3) (2011) 235–239.
[16] D.L. Akers, BioEqCalc: a package for performing equilibrium calculations on biochemical reactions, The Mathematica Journal 8 (2001) 86–113.

[17] D.J. Hall, Vapour–liquid Equilibrium for the Systems Water + Methanol, Water + Ethanol, Methanol + Ethanol and Water + Methanol + Ethanol at 298.15 K, National Physical Laboratory, Teddington Middlesex, 1979.
[18] A. Fredenslund, R.L. Jones, J.M. Prausnitz, Group-contribution estimation of activity coefficients in nonideal liquid mixtures, AICHE Journal 21 (6) (1975) 1086–1099.
[19] A. Fredenslund, J. Gmehling, P. Rasmussen, Vapor-liquid Equilibria using UNIFAC, Elsevier Scientific, Amsterdam, 1977. (etc.).
[20] H. Renon, J.M. Prausnitz, Local compositions in thermodynamic excess functions for liquid mixtures, AICHE Journal 14 (1) (1968) 135–144.
[21] L.F. Cameretti, G. Sadowski, J.M. Mollerup, Modeling of aqueous electrolyte solutions with perturbed-chain statistical associated fluid theory, Industrial and Engineering Chemistry Research 44 (9) (2005) 3355–3362.
[22] C. Held, L.F. Cameretti, G. Sadowski, Modeling aqueous electrolyte solutions — part 1. Fully dissociated electrolytes, Fluid Phase Equilibria 270 (1-2) (2008) 87–96.
[23] C. Held, G. Sadowski, Modeling aqueous electrolyte solutions. Part 2. Weak electrolytes, Fluid Phase Equilibria 279 (2) (2009) 141–148.
[24] J. Gross, G. Sadowski, Perturbed-chain SAFT: an equation of state based on a perturbation theory for chain molecules, Industrial and Engineering Chemistry Research 40 (4) (2001) 1244–1260.
[25] C. Held, T. Neuhaus, G. Sadowski, Compatible solutes: thermodynamic properties and biological impact of ectoines and prolines, Biophysical Chemistry 152 (1–3) (2010) 28–39.
[26] C. Held, L.F. Cameretti, G. Sadowski, Measuring and modeling activity coefficients in aqueous amino-acid solutions, Industrial and Engineering Chemistry Research 50 (1) (2011) 131–141.
[27] D. Fuchs, J. Fischer, F. Tumakaka, G. Sadowski, Solubility of amino acids: influence of the pH value and the addition of alcoholic cosolvents on aqueous solubility, Industrial and Engineering Chemistry Research 45 (19) (2006) 6578–6584.
[28] C. Held, A. Prinz, V. Wallmeyer, G. Sadowski, Measuring and modeling alcohol/salt systems, Chemical Engineering Science 68 (1) (2012) 328–339.
[29] T. Maskow, U. von Stockar, How reliable are thermodynamic feasibility statements of biochemical pathways? Biotechnology and Bioengineering 92 (2) (2005) 223–230.
[30] V. Vojinović, U. von Stockar, Influence of uncertainties in pH, pMg, activity coefficients, metabolite concentrations, and other factors on the analysis of the thermodynamic feasibility of metabolic pathways, Biotechnology and Bioengineering 103 (4) (2009) 780–795.
[31] N.W. Cornell, M. Leadbetter, R.L. Veech, Effects of free magnesium concentration and ionic strength on equilibrium constants for the glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase reactions, Journal of Biological Chemistry 254 (14) (1979) 6522–6527.
[32] R. Ellis, Macromolecular crowding: obvious but underappreciated, Trends in Biochemical Sciences 26 (10) (2001) 597–604.
[33] A.P. Minton, The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media, Journal of Biological Chemistry 276 (14) (2001) 10577–10580.
[34] A.P. Minton, How can biochemical reactions within cells differ from those in test tubes? Journal of Cell Science 119 (14) (2006) 2863–2869.
[35] F.L. Mota, A.J. Queimada, S.P. Pinho, E.A. Macedo, Aqueous solubility of some natural phenolic compounds, Industrial and Engineering Chemistry Research 47 (15) (2008) 5182–5189.
[36] P. Tomasula, G.J. Czerwienski, D. Tassios, Vapor pressures and osmotic coefficients: electrolyte solutions of methanol, Fluid Phase Equilibria 38 (1–2) (1987) 129–153.