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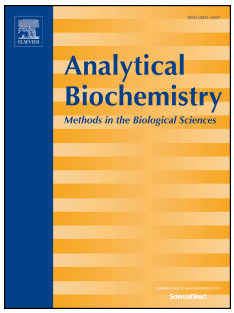

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Improved HPLC-method for estimation and correction of amino acid losses during hydrolysis of unknown samples

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Abstract

Amino acid analysis, commonly done by acid hydrolysis of proteins and HPLC analysis, faces one major problem: incomplete hydrolysis of stable amino acids and degradation of unstable amino acids are causing amino acid losses. As a result, amino acid recovery of unknown samples cannot be estimated. Some methods have been reported for correction of these factors in the past. This paper shows an improved and integrated method to overcome this problem by using stillage as an exemplary unknown sample material. Amino acid recovery from an unknown sample can be estimated by standard addition of a known protein. If the sample does not cause matrix effects during amino acid hydrolysis, recoveries of the standard protein are transferable to the sample. If the sample does cause matrix effects correction of amino acid losses can instead be done by determination of hydrolysis kinetics. Therefore, first order kinetics were used for amino acids that undergo degradation during hydrolysis. For all stable amino acids higher order kinetics were used, a novel approach to determine hydrolysis kinetics. The presented method can be a helpful tool for scientists who want to optimize amino acid analysis of a particular biomass substrate.

Keywords: amino acid analysis; HPLC; matrix effects; hydrolysis kinetics; non-linear least-squares regression

1 Introduction

The amino acid analysis procedure is divided into two parts: sample pretreatment and HPLC analysis. Sample pretreatment is applied in order to hydrolyze peptide bonds and separate amino acids into free amino acids. Amino acid hydrolysis can be achieved by acid, alkaline or enzymatic treatment. Most frequently acid hydrolysis is applied using 6 M hydrochloric acid (HCl) for 24 h at 110 °C. However, this standard acid hydrolysis leads to incomplete recoveries of certain amino acids due to varying stabilities of amino acids in acid media.

Optimization of hydrolysis parameters (i.e. temperature, hydrolysis time, composition of hydrolysis mixture) to overcome amino acid losses has been widely reported in literature (e.g. as reviewed by Fountoulakis and Lahm [1]). But so far, there is no hydrolysis method published for complete amino acid recovery; especially serine, threonine, cysteine, tryptophan and methionine can only be analyzed with losses. Additionally, hydrolysis parameters need to be adjusted for every sample type individually anyway. Thus, optimizations reported in literature are often not suitable for a wide range of sample types. For example, Simpsons et al. [2] published a method for a complete amino acid analysis from a single hydrolysate (4 M methanesulfonic acid containing 0.2 % 3-(2-aminoethyl)indole at 115 °C) by using pure lysozyme. Even though amino acid

recovery was good when using standard proteins, transferability to organic samples (e.g. blood, food, dust, biomass) is poor whenever matrix effects occur. Matrix effects are caused by a wide range of possible degradation reactions with other sample components.

Consequently, the problem of amino acid losses during hydrolysis of biomass samples cannot be solved by optimization of hydrolysis parameters. Instead, amino acid losses can be corrected subsequently, as it was shown by Darragh et al. [3]. This paper provides an improved method for estimation and correction of amino acid losses during acid hydrolysis of unknown sample material by (1) estimation of amino acid recovery by identification of matrix effects and (2) correction of amino acid losses by calculation of hydrolysis kinetics.

1.1 Amino acid losses during hydrolysis

During standard acid hydrolysis only half of the 20 proteinogenic amino acids are hydrolyzed without losses. One reason for incomplete amino acid recovery is that some amino acids are not completely hydrolyzed after 24 h. These amino acids will be called *stable amino acids* hereafter. Another source of amino acid losses during acid hydrolysis are degradation reactions. Amino acids that undergo degradation reactions will be called *unstable amino acids* hereafter.
The current state of knowledge of both groups is outlined below.

1.1.1 Stable amino acids

Hydrophobic amino acids like valine and isoleucine (in some cases also glycine) are released very slowly during hydrolysis since the peptide bonds Val-Val, Val-Gly, Val-Ile and Ile-Ile are difficult to hydrolyze due to the hydrophobicity of these amino acids [4]. A complete release depends on the protein and requires long hydrolysis times of more than 24 h.

1.1.2 Unstable amino acids

Degradation of amino acids is the main reason for incomplete recovery. Modification at its simplest is represented by racemization; in a prolonged period in hot acid even diastereomers from amino acids with two or more chiral centers can be formed [4]. Most degradations of amino acids during acid hydrolysis happen due to reactions of side chains with other components. Possible degradation reactions are outlined below.

- Cysteine and methionine, both sulfur-containing amino acids, can be degraded during acid hydrolysis due to the reaction of sulfur with residual oxygen by the formation of methionine sulfoxide / sulfone and cysteine sulfinic / sulfenic / sulfonic acid. Both sulfuric amino acids and their oxidation products can react with tyrosine and tryptophan; cystine and tryptophan for example form oxindolylalanine and tryptathionine [4].
- Glutamine and asparagine are completely converted into the corresponding carboxylic acids during acid hydrolysis. Additionally, glutamic acid can react with hydroxyl-containing amino acids like serine, threonine and tyrosine by ester formation [4].
- Tyrosine contains a phenyl ring that can easily be halogenated during hydrolysis by free chlorine or bromine to form chlorine and bromine tyrosine [4]. Furthermore, by heating tyrosine in 6 M HCl in the presence of iron ions, the reaction with methionine to bis-methylene-dityrosine was observed. High concentrations of inorganic sulfate contained in the sample material or released by the acidic hydrolysis can react with tyrosine to form sulfate esters.
- Tryptophan is the most unstable amino acid during acid hydrolysis. The pyrrole unit can easily be protonated in 6 M HCl, followed by an electrophilic addition of tryptophan molecules

[5]. The pyrrole unit can also be attacked by free radicals that are formed by residual oxygen due to high hydrolysis temperatures [6]. Transition metals have a catalytic effect on this radical formation of oxygen [7,4]. Hydroxyl radicals preferably attack the phenyl ring of the tryptophan with the formation of hydroxytryptophan and further degradation products, oxygen radicals attack the pyrrole ring leading to its opening and the formation of N-formylkynurenine and other degradation products resulting [8]. Furthermore, reactions of tryptophan with degradation products of other amino acids or with sugars contained in the sample can occur [2].

- Serine and threonine can be converted into the corresponding α-keto acids by β-elimination of water during acid hydrolysis and further react with other amino acids [9]. High concentration of sulfate can also lead to the formation of serine and threonine sulfate esters. Furthermore, during the hydrolysis of glycoproteins, where proteins are bound to oligosaccharide chains via the amino acids serine, threonine and asparagine, binding amino acids can be destroyed [4].

1.2 Method for estimation of amino acid recovery

Estimation of amino acid losses/recoveries during hydrolysis of an unknown organic sample material is not possible since there is no information about the "correct" amino acid content. Therefore, a standard protein can be used. Recoveries of amino acids from such a standard protein are transferable to an unknown sample if the latter does not cause matrix effects during amino acid hydrolysis. Investigation of matrix effects can be done by standard addition. Note that this is not valid for stable amino acids because their recovery is mainly influenced by the amount of stable peptide bonds in the sample protein fraction. Recovery of stable amino acids after 24 h can be estimated by hydrolysis kinetics.

1.3 Method for correction of amino acid losses

If the sample does cause matrix effects on the analysis of certain amino acids, recoveries for these amino acids cannot be transferred from standard protein results. In that case correction of amino acid losses can be done by determination of hydrolysis kinetics instead. Optimal hydrolysis time for each amino acid depends on the individual reaction rates of hydrolysis and degradation. During hydrolysis, protein-bound amino acids (state A) are hydrolysed (k_1) to form free amino acids (state B), while simultaneously degradation reactions (k_2) of free amino acids occur (state C).

To avoid individual sample preparation for each amino acid hydrolysis kinetics can be determined to correct amino acid concentrations after a certain hydrolysis time. Amino acid concentrations are only measurable in state B, thus, equation (4) can be formed from equations (1) – (3) by applying first order kinetics ($z = 1$). Estimates of k_1, k_2 and c_{A0} can be derived using nonlinear least-squares regression of a series of $c_B(t)$ values measured at different hydrolysis times. Darragh et al. [3] used this method based on Robel and Crane [10] to determine the hydrolysis kinetics of amino acids in egg white lysozyme.

This model approach has been further developed here because for stable peptide bonds requiring longer hydrolysis times first order kinetics are not applicable any more. Thus equation (7) was formed from equations (5) and (6) by using higher order kinetics ($z \neq 1$) and simultaneously neglecting degradation reactions ($k_2 = 0$). Estimates of k_1, z and c_{A0} were again derived using nonlinear least-squares regression of the collected data.

first order kinetics ($z = 1$):

$$\frac{dc_A}{dt} = -c_A \cdot k_1 \quad (1)$$

$$\frac{dc_B}{dt} = c_A \cdot k_1 - c_B \cdot k_2 \quad (2)$$

$$\frac{dc_C}{dt} = c_B \cdot k_2 \quad (3)$$

$$c_B(t) = \frac{c_{A0} \cdot k_1}{k_1 - k_2} \cdot (e^{-k_2 t} - e^{-k_1 t}) \quad (4)$$

higher order kinetics ($k_2 = 0$)

$$\frac{dc_A}{dt} = -c_A^z \cdot k_1 \quad (5)$$

$$\frac{dc_B}{dt} = c_A^z \cdot k_1 \quad (6)$$

$$c_B(t) = c_{A0} - ({c_{A0}}^{1-z} - k_1 t(1-z))^{\frac{z}{1-z}} \quad (7)$$

where

$_A(t)$ = amino acid concentration in state A at time *t* (in %-wt)
$_B(t)$ = amino acid concentration in state B at time *t* (in %-wt)
c_{A0} = amino acid concentration in state A at *t* = 0 (in %-wt)
k_1 = reaction rate of hydrolysis (in s^{-z})
k_2 = reaction rate of degradation (in s^{-z})
z = reaction order (-)

2 Experimental

2.1 Materials

Stillage from an alcohol fermentation plant was used as an exemplary unknown sample material. Stillage in this case resulted from a single decantation of whole stillage coming from the distillation column. The stillage sample was freeze dried (12.5 % DM), ground in a mortar and sieved to particle sizes below 0.5 mm. Amino acid standard solution (AAS18) as well as internal standards L-norvaline (N7627) and sarcosine (S7672) were purchased from Sigma Aldrich. Standard protein egg white lysozyme (8259.2) was obtained from Carl Roth.

2.2 Hydrolysis

For standard hydrolysis procedure, 25 mL hydrolysis mixture (6 M HCl) were added to the sample in a 100 mL glass bottle with screw cap (protein to 6 M HCl ratio approx. 1:250, w:v). The bottle was incubated at 110 °C in a ventilated oven for 24 h, while the cap was loose for the first hour to avoid bottle damage. Right after incubation the bottle was cooled in an ice-water bath. Neutralization was performed by adding ultrapure water and adjusting the solution pH with 10 M NaOH to pH 1 at 20 °C. The hydrolysate was transferred to a 200 mL round-bottomed flask, 2 mL of 20 mM internal standard were added, the flask was filled up with 0.1 M HCl and thor-

oughly mixed. Then 2 mL hydrolysate were filtered (PVDF, 0.45 µm) and stored for HPLC analysis.

For determination of cysteine, oxidation to cysteic acid was performed prior to acid hydrolysis by incubating the sample for 26 h at 0 °C in performic acid (0.5 mL 30 % hydrogen peroxide, 3.88 mL formic acid and 0.6 mL water containing 0.02 g phenol). Right afterwards 0.84 g sodium metabisulfite were added to stop oxidation reactions and the sample was hydrolyzed for 16 h as mentioned above. Variation of hydrolysis mixture was performed by changing the hydrolysis agent (4.2 M NaOH, 4 M MSA) or by adding different additives (phenol, 2-mercaptoethanol, thiodiglycol) to the hydrolysis mixture. For determination of hydrolysis kinetic 22 hydrolysis times (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 40, 50, 60, 70, 95, 120 and 140 h) were studied.

2.3 HPLC-Analysis

Amino acid analysis was carried out on Agilent 1260 HPLC Series. Pre-column derivatization of primary amino acids was done with o-phthaldialdehyde and 3-mercaptopropionic acid (OPA/MPA), while secondary amino acids were derivatized with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl). Amino acid separation was achieved using a Poroshell LC HPH-18 column (4.6 x 100 mm, 2.7 µm) and a binary gradient system. Solvent A was an aqueous buffer at pH 8.4 and Solvent B contained methanol, acetonitrile and water (45/45/10, v/v/v). Detection was done by a variable-wavelength FDL-detector. Extinction wavelengths for OPA/MPA and FMOC-Cl derivates were set to 338 and 266 nm, respectively. Emission wavelengths were adjusted to the particular detected intensity of each sample.

2.4 Modelling

Calculation of hydrolysis kinetics was done by Matlab using nonlinear least-squares regression of amino acid analysis results measured at different hydrolysis times. Modelling was done by applying Matlab function *gradient(f)* on equation (4) or (7) to calculate partial derivatives to c_{A0}, k_1 and k_2 or c_{A0}, k_1 and z, respectively. Equation (4) was applied to all amino acids that undergo degradation reactions ($k_2 \neq 0$) during hydrolysis by assuming first order kinetics ($z = 1$). For stable peptide bonds like Val-Val, Val-Ile or Ile-Ile that require longer hydrolysis times degradation reactions can be neglected ($k_2 = 0$). For these amino acids equation (7) was used as first order kinetics are not applicable any more ($z > 1$).

3 Results

With the applied method all 20 amino acids could be quantified (asparagine and glutamine were detected together with aspartic acid and glutamic acid). Table 1 shows the results of amino acid concentrations in lysozyme compared with the theoretical amino acid concentrations (note that for lysozyme 100 %-wt protein equals 116.2 %-wt amino acids due to water addition). The average amino acid recovery in lysozyme was 87 %-wt with standard hydrolysis procedure. The average relative statistical error was 1.0 % (1 x sigma). Cysteine and tryptophan recovery in lysozyme with acid hydrolysis was poor (60 and 40 %, respectively) due to degradation reactions.

In stillage, an exemplary unknown sample material, total amount of amino acids was 311 mg/g (DM) by applying standard hydrolysis procedure (table 1). In contrast to lysozyme, tryptophan is totally destroyed during standard acid hydrolysis of stillage. Neither the addition of

additives (phenol, 2-mercaptoethanol) to the hydrolysis mixture nor the variation of acid hydrolysis agents (HCl, MSA) did show any stabilization of tryptophan. The only effective method to detect tryptophan in stillage is by alkaline hydrolysis. Tryptophan concentration in stillage was 3.3 mg/g (DM) by hydrolysis with 4.2 M NaOH containing 3.3 % thiodiglycol. Tryptophan recovery in lysozyme at these conditions was 69 %.

At this stage, estimation of amino acid recovery in stillage is not possible because stillage is an unknown mixture of fermented cereals; i.e. there are no literature values that define the "correct" amino acid content. Therefore, lysozyme recoveries will be used in section 3.1 for amino acids that are not affected by stillage matrix effects during amino acid hydrolysis. For all other amino acids correction of amino acid losses will be done by determination of hydrolysis kinetics in section 3.2.

3.1 Identification of matrix effects

For amino acids that are totally hydrolyzed after 24 h recoveries from a standard protein are transferable to an unknown biomass sample if the latter does not cause matrix effects during amino acid hydrolysis. Thus, matrix effects of stillage on the recovery of amino acids in lysozyme were investigated. To a constant amount of lysozyme (0.1 g) different amounts of stillage (0, 0.1, 0.2 and 0.4 g) were added. Standard acid hydrolysis was applied to both pure samples and mixtures to measure amino acid contents. In figure 1 bars indicate the deviation of mixture results from the sum of pure sample results and error bars indicate the 99 % confidence interval. If matrix effects do not affect hydrolysis or degradation reactions of an amino acid, data point of the mixture equals the sum of pure lysozyme and pure stillage. The more the mixture deviates from the sum of pure samples, the higher the matrix effect of stillage is. Matrix effects are significant if they exceed the 99 % confidence interval (2.5 x sigma).

The results in figure 1 show that the addition of stillage to lysozyme during acid hydrolysis increases degradation of arginine, methionine, and especially tryptophan and tyrosine. For these amino acids it is not possible to transfer the recovery reached with lysozyme due to matrix effects of the stillage. Possible reasons for these matrix effects are the following:

- In case of tyrosine it can be assumed that high concentrations of glutamic acid (28 %-wt of total amino acids) and/or inorganic sulfate (5 %-wt (DM) SO_4^{2-}) in stillage result in ester formation with lysozyme's tyrosine.
- Methionine can react with tyrosine by heating tyrosine in 6 M HCl in the presence of iron ions.
- Arginine is probably degraded to ornithine, which is usually known to occur at higher temperatures [11], but the stillage matrix seems to favor the reaction to occur already at 110 °C.
- Tryptophan recovery in pure lysozyme is 36 % for acid hydrolysis, but when stillage is added, tryptophan is no longer detectable. Sulfur-containing components and residual sugars in stillage are probably the main reason for total tryptophan degradation. But for alkaline hydrolysis the opposite effect is noticeable: tryptophan is stabilized by addition of stillage, thus tryptophan recovery in pure stillage is probably higher that in pure lysozyme.

For all other amino acids (except for the stable amino acids Val, Ile and Gly) recoveries reached with lysozyme are transferable to recoveries of the corresponding amino acids in stillage. For example, alanine recovery in lysozyme reached 94 % (table 1), and since stillage does not cause matrix effects on alanine it can be assumed that alanine recovery in stillage is 94 % as well. Thus, the measured alanine concentration in stillage can be corrected by dividing by 0.94.

For the stable amino acids glycine, isoleucine and valine recoveries are mainly influenced by the amount of stable peptide bonds, thus recoveries of these amino acids in stillage need to be estimated by hydrolysis kinetics (section 3.2).

Table 1: Results of amino acid analysis by standard hydrolysis for lysozyme and stillage.

		Lysozyme (mol amino acids/mol protein)				**Stillage** (mg/g (DM))		
AA	**Amino Acid**	**Actual**	**Results**[b]	**1 x sigma**[c]	**Recovery**	**Results**[b]	**1 x sigma**[c]	**Estimated Recovery**
Ala	Alanine	12	11.7	0.5 %	94%	15.47	0.6 %	94%
Arg	Arginine	11	10.6	0.4 %	93%	(15.97)	0.9 %	?
Asp	Aspartic Acid	21	21.3	0.5 %	98%	23.38	0.9 %	98%
Cys	Cysteine	8	4.8	4.9 %	58%	4.39	2.5 %	58%
Glu	Glutamic Acid	5	4.9	0.4 %	96%	77.56	0.5 %	96%
Gly	Glycine	12	11.7	0.5 %	94%	(14.83)	0.5 %	?
His	Histidine	1	1.0	0.7 %	97%	7.47	1.7 %	97%
Ile	Isoleucine	6	(5.5)	0.4 %	89%	(13.09)	0.6 %	?
Leu	Leucine	8	7.7	0.5 %	94%	21.00	0.5 %	94%
Lys	Lysine	6	6.2	0.4 %	99%	14.38	0.4 %	99%
Met	Methionine	2	1.8	0.3 %	87%	(4.88)	3.1 %	?
Phe	Phenylalanine	3	2.9	0.6 %	94%	15.63	0.6 %	94%
Pro	Proline	2	1.8	2.3 %	88%	28.46	1.5 %	88%
Ser	Serine	10	(8.7)	0.5 %	85%	(15.65)	0.8 %	85%
Thr	Threonine	7	(6.4)	0.5 %	89%	(13.12)	0.9 %	89%
Trp	Tryptophan	6	(2.4) / 4.1[a]	3.2 %/ 4.0 %	39% / 69%[a]	0 / 3.33[a]	3.0 %	?
Tyr	Tyrosine	3	2.9	0.5 %	93%	(9.89)	1.2 %	?
Val	Valine	6	(5.2)	0.3 %	85%	(16.24)	0.4 %	?
Σ		**116.2**	**101.3**		**87%**	**311.4**		

All data points are means of triplicates.
[a] Alkaline hydrolysis.
[b] Results in brackets will later be corrected with hydrolysis kinetics.
[c] Relative standard deviation (n = 4)
[d] Recoveries of amino acids that are not effected by matrix effects are transferable from lysozyme recoveries (section 3.1).

3.2 Correction of amino acid losses during hydrolysis

Instead of using matrix effects, correction of amino acid losses can also be done by determination of hydrolysis kinetics. Therefore, sample preparation was done with varying hydrolysis times and amino acid analysis results were modeled in order to calculate kinetic parameters of hydrolysis and degradation for each amino acid. Results of modelled and measured data for lysozyme and stillage are shown in table 2 and table 3.

For unstable amino acids ($k_2 \neq 0$) first order kinetics according to equation (4) were used to correct amino acid concentrations $c_B(24)$ after 24 h hydrolysis by extrapolation to zero time (c_{A0}). In stillage this applies to arginine, methionine, serine, threonine, tryptophan[1] and tyrosine, where the concentrations of free amino acids $c_B(t)$ continuously decreased after a certain time (figure 3). Concentrations of these amino acids could be increased by correction with first order kinetics by 3, 3, 9, 4 and 8 %, respectively. In lysozyme, methionine, serine, threonine and tryptophan were affected by degradation during hydrolysis; extrapolation to zero time could increase concentra-

[1] Correction of tryptophan concentration in stillage was done by determination of kinetic parameters of alkaline hydrolysis, data not shown.

tions by 1, 9, 5 and 76 %, respectively. When comparing the degradation rates of unstable amino acids in lysozyme (figure 2) and stillage (figure 3) it becomes clear that stability of amino acids towards degradation strongly depends on the sample matrix. For arginine, methionine, tryptophan and tyrosine degradation in stillage happens significantly faster than in lysozyme. This corresponds to the matrix effects that were detected in section 3.1 and verifies the presented method. By determination of hydrolysis kinetics, concentrations of arginine, methionine, tryptophan and tyrosine in stillage can at least be corrected (not knowing if it covers 100 %).

For all amino acids that are not affected by degradation during hydrolysis ($k_2 = 0$), higher order kinetics according to equation (7) were used to correct measured amino acid concentrations after 24 h hydrolysis. Peptide bonds between hydrophobic amino acids are difficult to hydrolyze. Thus, for stable amino acids corrections of $c_B(24)$ by extrapolation to $t \rightarrow \infty$ resulted in significant increases of glycine, isoleucine and valine concentrations in stillage by 3 %, 11 % and 6 %, respectively. In lysozyme, recovery of isoleucine and valine increased about 5 % each to 93 % and 89 %, respectively. It can be assumed that for stable amino acids recovery in standard protein at $t \rightarrow \infty$ equals recovery in sample material. Consequently, recoveries for glycine, isoleucine and valine in stillage can be estimated by 94 %, 93 % and 89 %, respectively, and the measured concentrations can be corrected accordingly.

When comparing the effects of first and higher order kinetics for stable amino acids in table 2 and table 3 it is obvious that first order kinetics are not applicable any more for stable bound amino acids. Thus, the modification of the model described by Robel and Crane [10] that was done in this study significantly improves calculation of kinetic parameters for amino acids that require longer hydrolysis times for total hydrolysis.

Table 2: Kinetic parameters for hydrolysis and degradation during acid hydrolysis of lysozyme.

		First order kinetics (z = 1)					Higher order kinetics ($k_2 = 0$)					Corrected
AA	$c_B(24h)$[a]	c_{A0}[b]	k_1	k_2	R^2	rel. Δ[c]	c_{A0}[b]	k_1	z	R^2	rel. Δ[c]	Recovery[d]
Ala	7.05	7.05	1.0292	0	0.72	0%	7.06	0.6898	1.5604	0.80	<1%	94%
Arg	12.50	12.50	0.5411	0	0.96	0%	12.51	0.3908	1.1929	0.96	<1%	93%
Asp	19.12	19.12	0.6297	0	0.87	0%	19.12	0.5223	1.0905	0.87	0%	98%
Glu	4.92	4.92	0.4572	0	0.94	0%	4.94	0.3542	1.3074	0.95	<1%	96%
Gly	5.94	5.94	1.2043	0	0.67	0%	5.94	1.2030	1.0106	0.67	0%	94%
His	1.05	1.05	0.6114	0	0.26	(0%)[e]	1.20	2.3534	3.6888	0.52	(15%)[e]	97%
Ile	4.88	4.92	0.1994	0	0.89	<1%	5.13	0.1293	1.4223	0.98	**5%**	93%
Leu	6.87	6.87	0.5337	0	0.92	0%	6.92	0.3127	1.4790	0.97	<1%	94%
Lys	6.10	6.10	0.4612	0	0.94	0%	6.15	0.3118	1.4019	0.97	<1%	99%
Met	1.83	1.84	1.0681	0.0003	0.40	<1%					0%	87%
Phe	3.25	3.25	0.3960	0	0.73	0%	3.31	0.3061	1.5529	0.96	2%	94%
Ser	6.23	6.79	0.9594	0.0037	0.97	**9%**					0%	92%
Thr	5.19	5.34	0.4991	0.0013	0.93	**3%**					0%	92%
Trp	3.33	5.87	0.5188	0.0257	0.98	**76%**					0%	69%
Tyr	3.55	3.55	0.6627	0	0.86	0%	3.55	0.6320	1.1714	0.87	0%	93%
Val	4.16	4.22	0.1781	0	0.97	1%	4.39	0.1300	1.3421	0.98	**5%**	89%

[a] Measured amino acid concentration after 24 h hydrolysis time (in mg/100 mg lysozyme)

[b] Modelled amino acid concentration (in mg/100 mg lysozyme). Calculation of c_{A0} according to equation 4 (first order kinetics) and equation 7 (higher order kinetics).

[c] rel. Δ indicates the relative increase of c_{A0} in comparison with $c_B(24)$

[d] Corrected recovery = recovery from table 1 increased by rel. Δ

[e] Very low His content in lysozyme caused high statistical error, thus model R^2 is insufficient

Table 3: Kinetic parameters for hydrolysis and degradation during acid hydrolysis of stillage.

		First order kinetics (z = 1)					Higher order kinetics (k_2 = 0)					Estimated Recovery
AA	$c_B(24h)$[a]	c_{A0}[b]	k_1	k_2	R^2	rel. Δ[c]	c_{A0}[b]	k_1	z	R^2	rel. Δ[c]	
Ala	15.47	15.47	0.8394	0	0.81	0%	15.48	1.0269	1.3428	0.86	<1%	94%
Arg	15.97	16.44	0.5505	0.0013	0.92	**3%**						?
Asp	23.38	23.43	0.7369	0.0001	0.89	0%	23.38	0.7618	1.1771	0.90	0%	98%
Glu	77.56	77.73	0.6847	0.0001	0.94	<1%	77.56	0.6999	10000	0.94	0%	96%
Gly	14.83	14.83	0.9199	0	0.60	<1%	15.24	2.2204	2.3426	0.89	**3%**	94%[e]
His	7.47	7.47	0.4536	0	0.92	0%	7.54	0.6868	1.4149	0.96	1%	
Ile	13.09	13.47	0.1483	0	0.97	3%	14.52	0.1493	1.4857	0.99	**11%**	93%[e]
Leu	21.00	21.00	0.4451	0	0.96	0%	21.07	0.4424	1.2624	0.97	<1%	94%
Lys	14.38	14.38	0.4609	0	0.94	0%	14.46	0.5195	1.3280	0.96	<1%	99%
Met	4.88	5.02	0.5842	0.0012	0.69	**3%**[d]						?
Phe	15.63	15.63	0.4409	0	0.96	0%	15.67	0.4664	1.2027	0.96	<1%	94%
Ser	15.65	17.09	0.7207	0.0039	0.97	**9%**						85%
Thr	13.12	13.61	0.4001	0.0017	0.96	**4%**						89%
Tyr	9.89	10.72	0.4346	0.0037	0.95	**8%**						?
Val	16.24	16.38	0.1975	0	0.97	<1%	17.18	0.1901	1.4279	0.99	**6%**	89%[e]

[a] Measured amino acid concentration after 24 h hydrolysis time (in mg/g stillage)

[b] Modelled amino acid concentration (in mg/g stillage). Calculation of c_{A0} according to equation 4 (first order kinetics) and equation 7 (higher order kinetics) (note that $c_B(24)$ needs to be inserted in mg/100 mg stillage (DM))

[c] rel. Δ indicates the relative increase of c_{A0} in comparison with $c_B(24)$

[d] rel. Δ is within the statistical error 1 x sigma

[e] Recovery of stable amino acids in stillage = recovery in lysozyme at $t \rightarrow \infty$ (table 2)

4 Conclusion

With help of the presented method the problem of incomplete amino acid analysis in unknown samples can be overcome and amino acid losses can be quantified, as verified on the example of stillage. Instead of optimizing hydrolysis parameters, amino acid losses are corrected subsequently. The results of matrix effect investigation show that stillage matrix favors degradation reactions of methionine, arginine, threonine and tyrosine during acid hydrolysis. All other amino acids are not affected by matrix effects, thus estimation of amino acid losses could be done by analyzing the recoveries of the corresponding amino acids from a standard protein. For all amino acids that are affected by matrix effects, incomplete results could be successfully corrected by determination of hydrolysis kinetics. It could be shown that for amino acids that undergo degradation during hydrolysis first order kinetics should be used. For all amino acids not affected by degradation higher order kinetics are necessary. Especially for the highly instable amino acid tryptophan the recovery rate could be increased by 76 % due to correction with first order kinetics, which is a novel method to correct tryptophan losses during hydrolysis. The results have shown that the presented method can be a helpful tool for scientists who want to optimize amino acid analysis of a particular biomass substrate.

Acknowledgement

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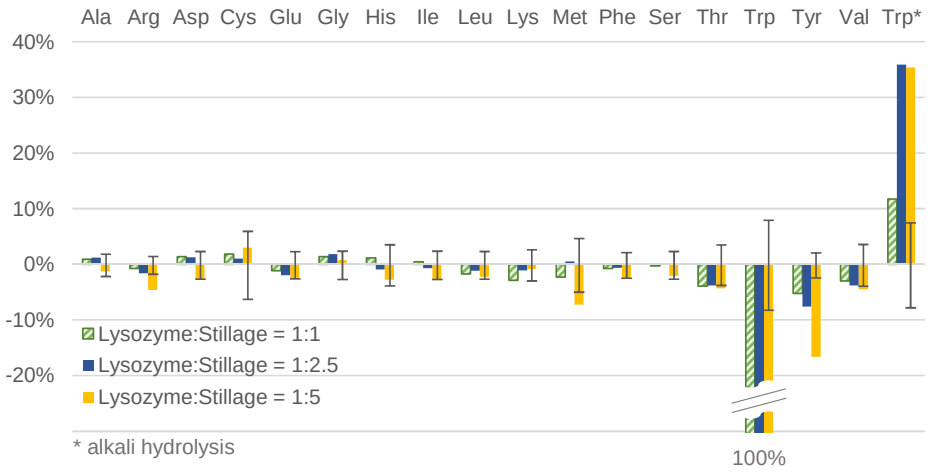
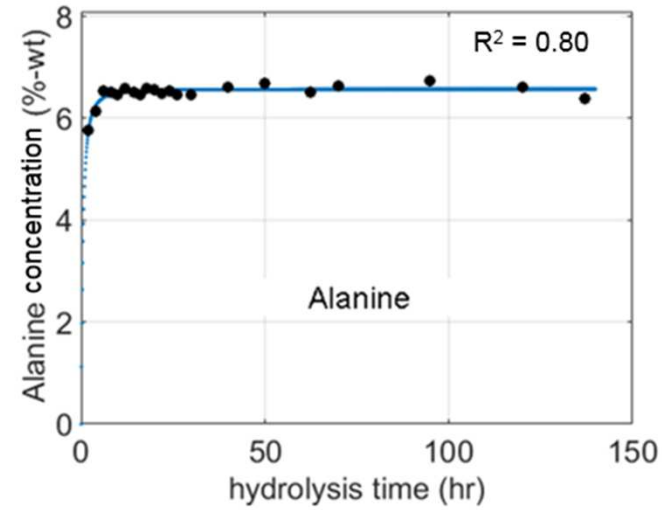
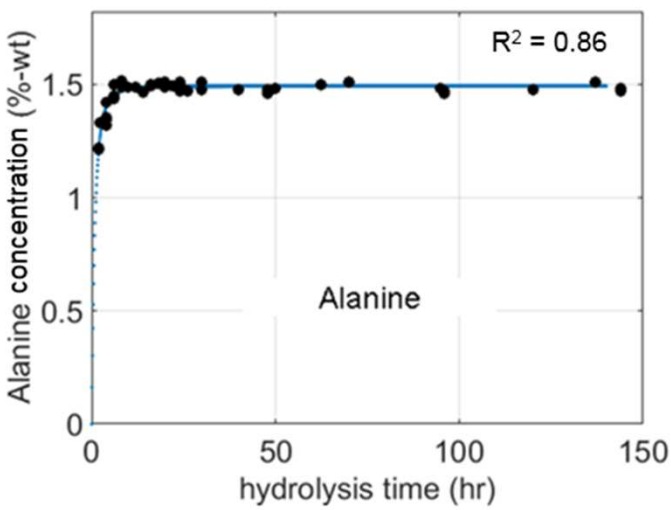


Figure 1: Matrix effects of stillage on amino acid recovery in lysozyme. To a constant amount of lysozyme (0.1 g) different amounts of stillage were added (0, 0.1, 0.2 and 0.4 g) resulting in lysozyme:stillage ratios (w:w) of 1:1, 1:2.5 and 1:5. Standard acid hydrolysis was applied to pure samples and mixtures. Bars = deviation of mixture results from the sum of pure sample results. Error bars = 2.5 x standard deviation (99 % confidence interval). A matrix effect is significant if the bar exceeds the error bar.


R² = 0.80
Alanine
Alanine concentration (%-wt)
hydrolysis time (hr)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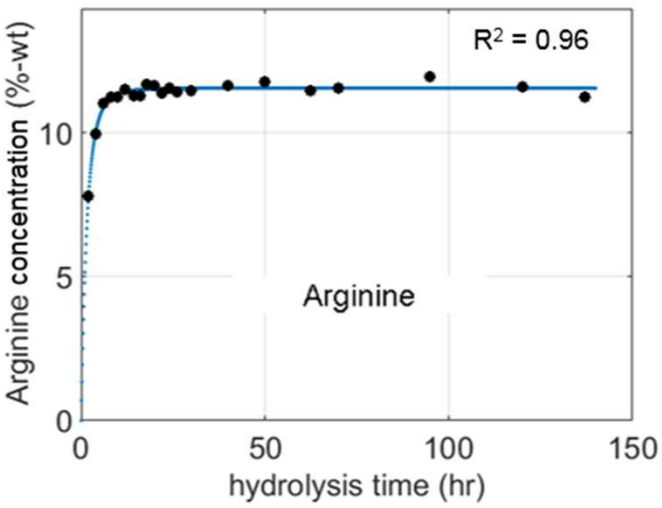
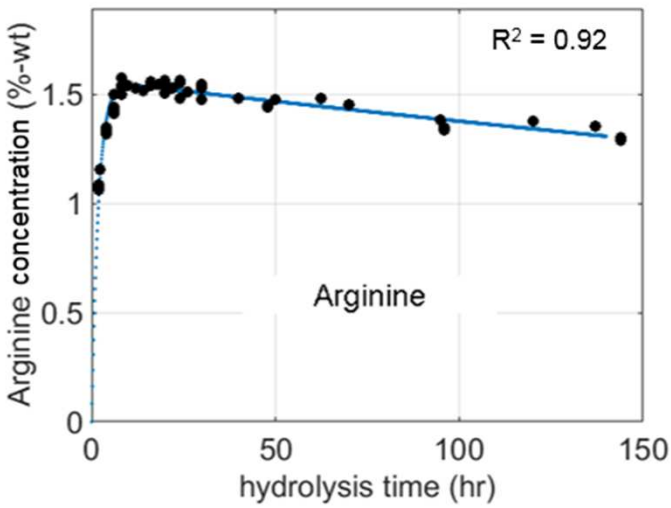
Alanine
Alanine concentration (%-wt)
hydrolysis time (hr)


R² = 0.96
Arginine
Arginine concentration (%-wt)
hydrolysis time (hr)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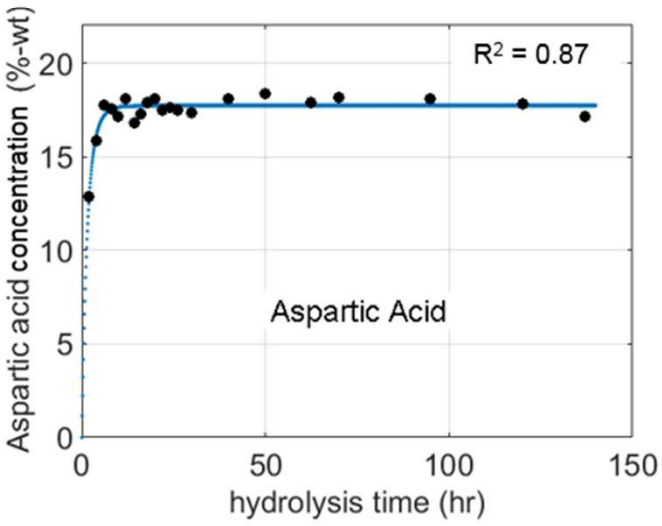
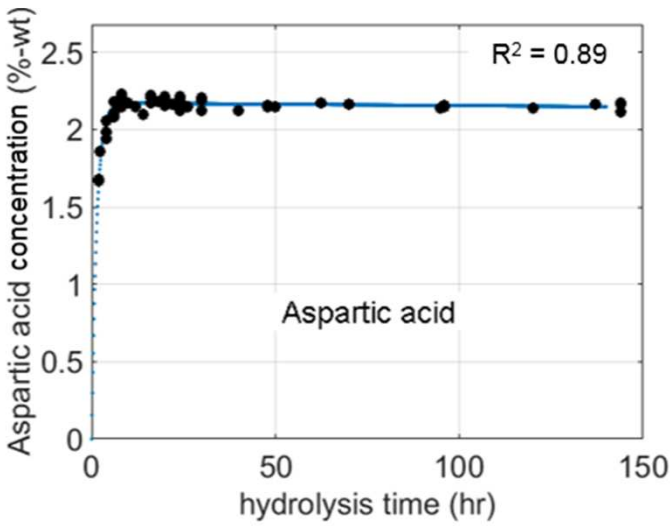
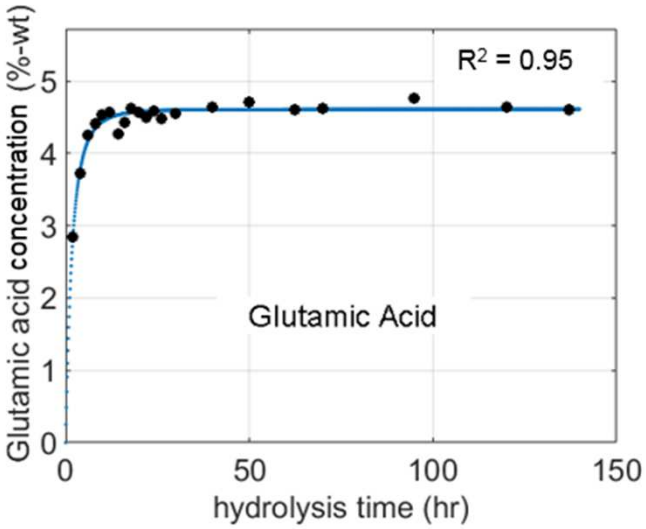
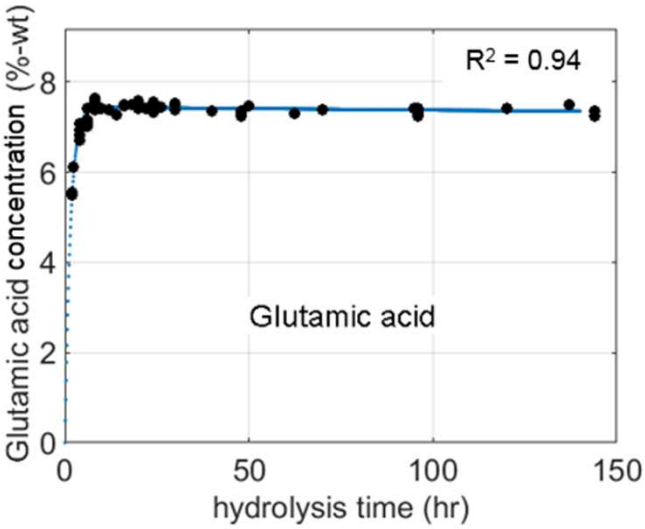
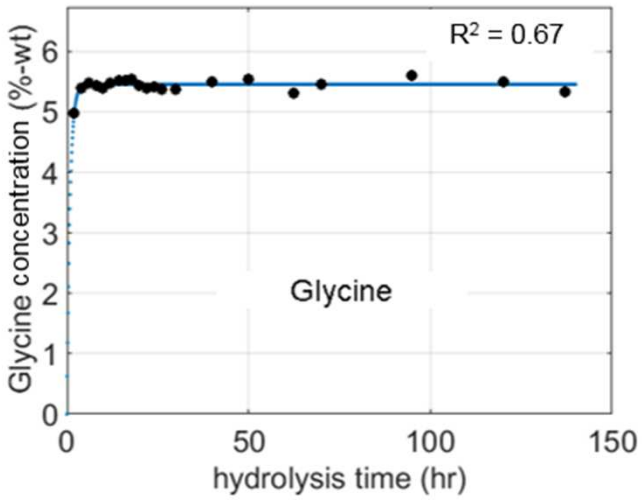
Arginine
Arginine concentration (%-wt)
hydrolysis time (hr)


R² = 0.87
Aspartic Acid
Aspartic acid concentration (%-wt)
hydrolysis time (hr)


R² = 0.89
Aspartic acid
Aspartic acid concentration (%-wt)
hydrolysis time (hr)


R² = 0.95
Glutamic Acid
Glutamic acid concentration (%-wt)
hydrolysis time (hr)


R² = 0.94
Glutamic acid
Glutamic acid concentration (%-wt)
hydrolysis time (hr)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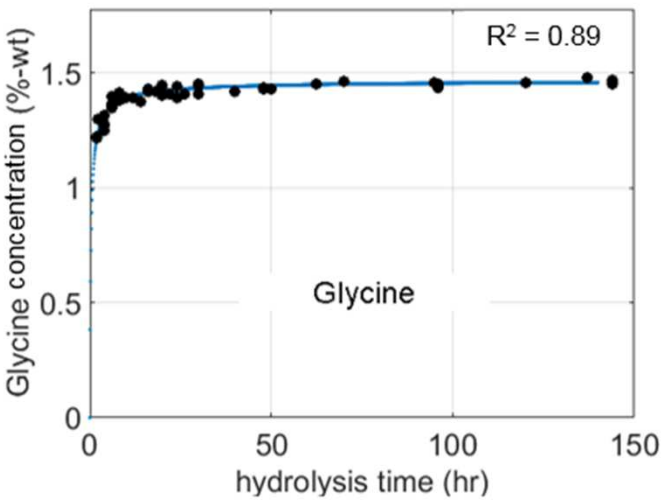
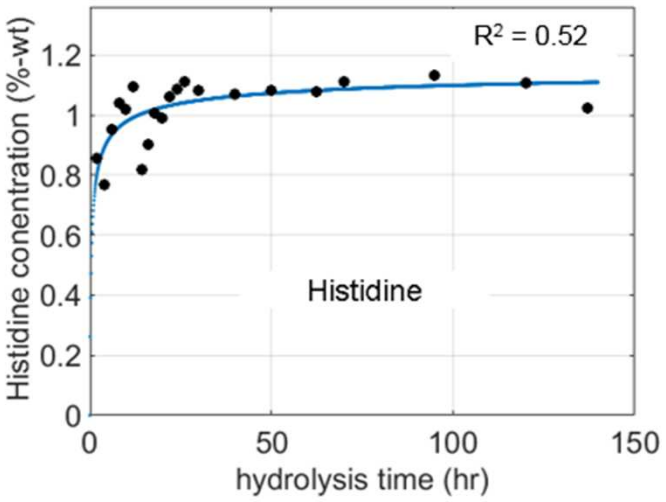
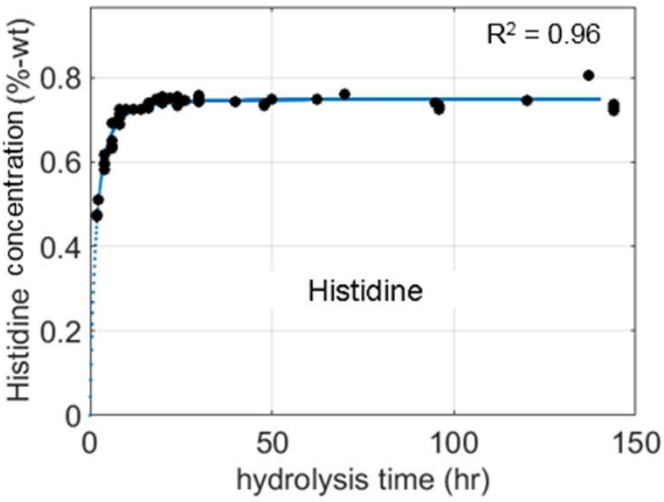
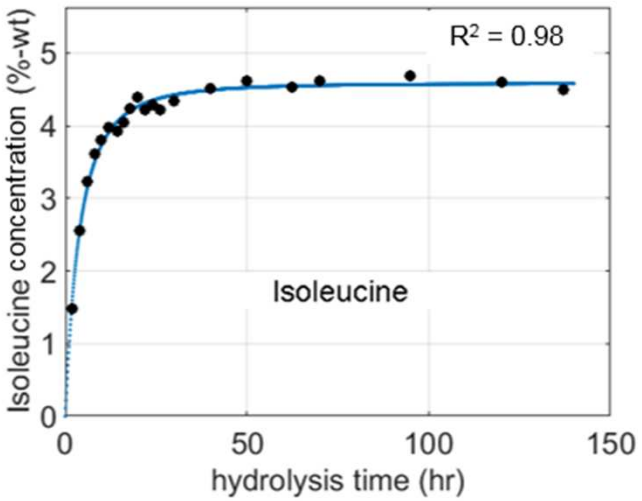
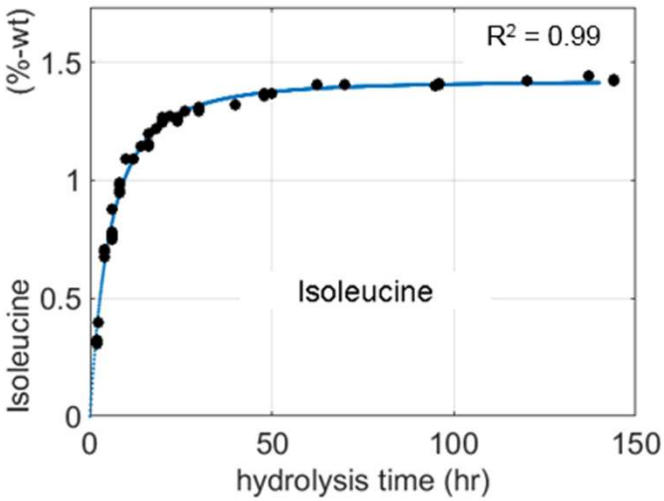
Glycine
Glycine concentration (%-wt)
hydrolysis time (hr)


R² = 0.89
Glycine
Glycine concentration (%-wt)
hydrolysis time (hr)


R² = 0.52
Histidine
Histidine conentration (%-wt)
hydrolysis time (hr)


R² = 0.96
Histidine
Histidine concentration (%-wt)
hydrolysis time (hr)


R² = 0.98
Isoleucine
Isoleucine concentration (%-wt)
hydrolysis time (hr)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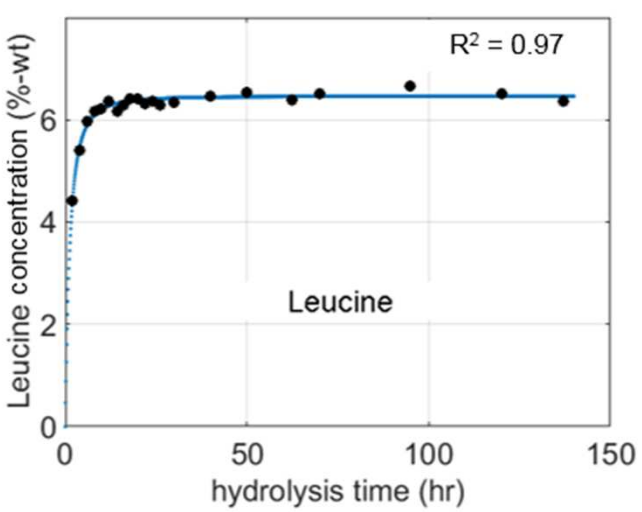
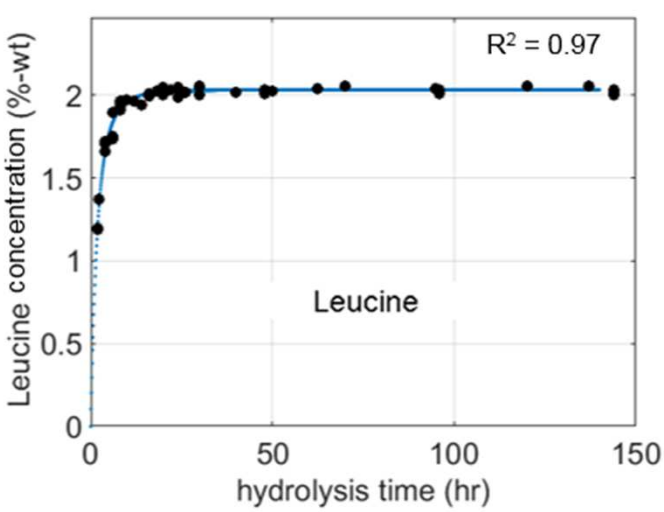
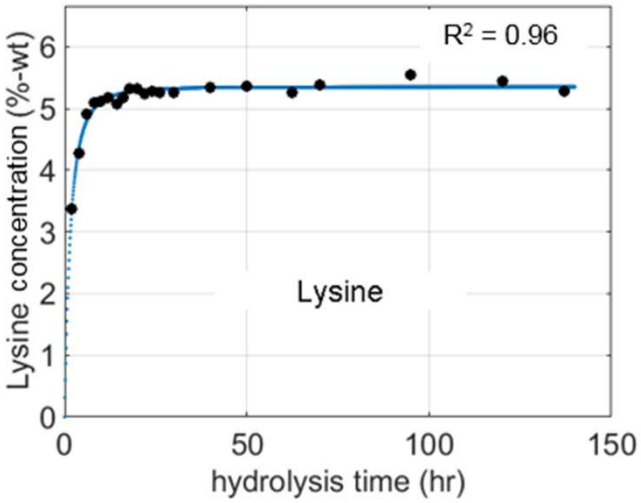
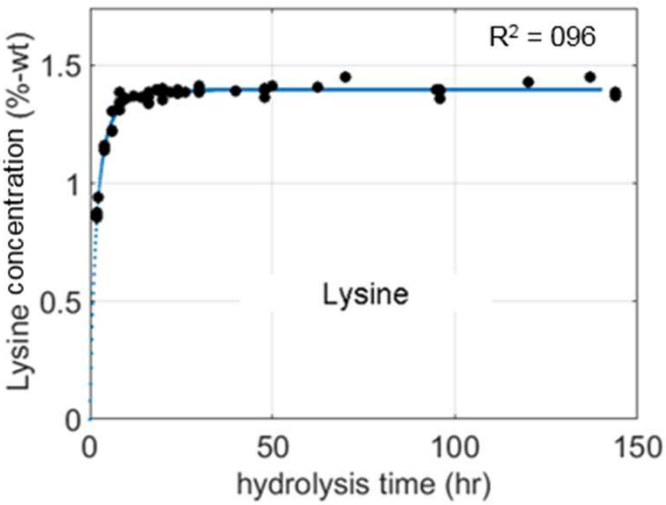
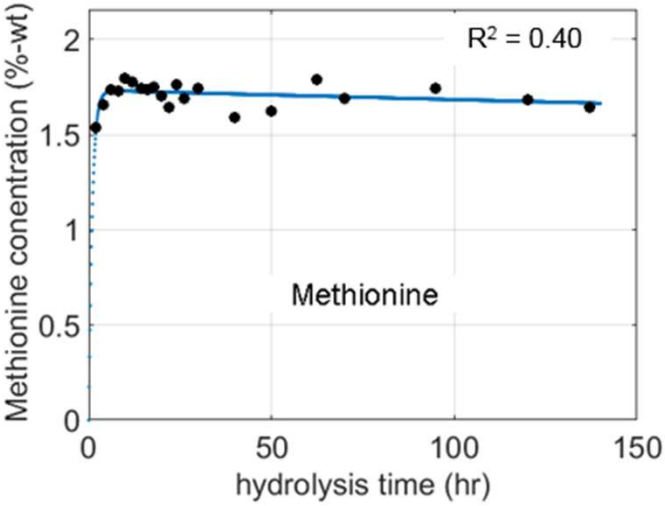
Isoleucine
(%-wt)
Isoleucine
hydrolysis time (hr)

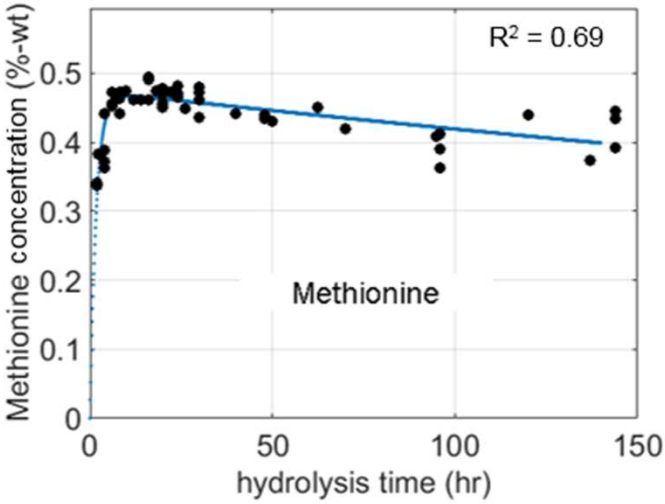

R² = 0.97
Leucine
Leucine concentration (%-wt)
hydrolysis time (hr)


R² = 0.97
Leucine
Leucine concentration (%-wt)
hydrolysis time (hr)


R² = 0.96
Lysine
Lysine concentration (%-wt)
hydrolysis time (hr)
0
1
2
3
4
5
6
0
50
100
150


R² = 096
Lysine
Lysine concentration (%-wt)
hydrolysis time (hr)
0
0.5
1
1.5
0
50
100
150


R² = 0.40
Methionine
Methionine conentration (%-wt)
hydrolysis time (hr)
0
0.5
1
1.5
2
0
50
100
150


R² = 0.69
Methionine
Methionine concentration (%-wt)
hydrolysis time (hr)
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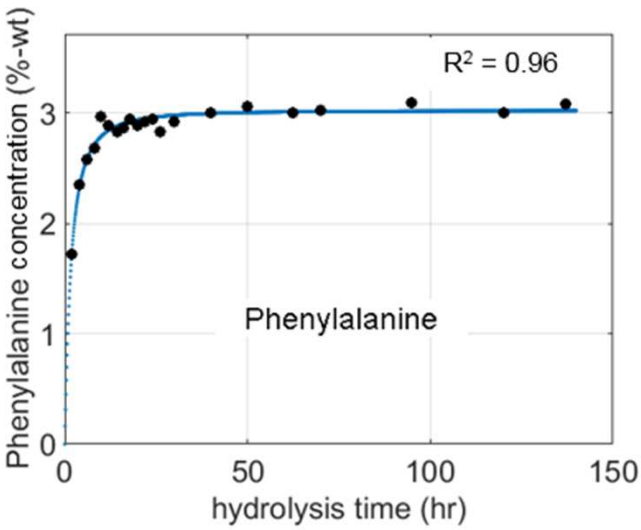
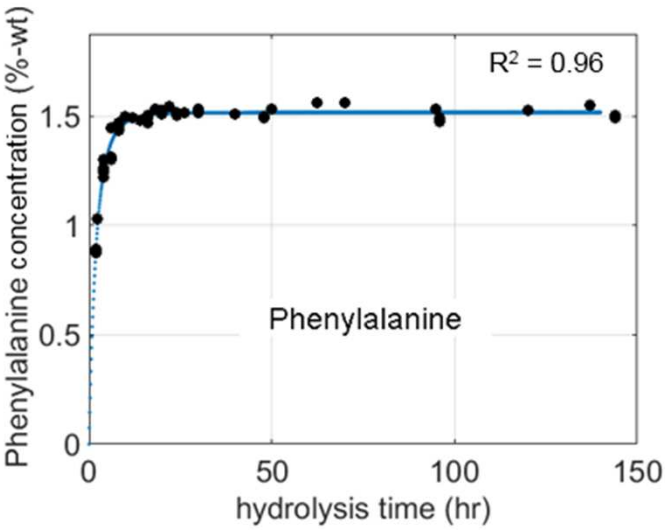
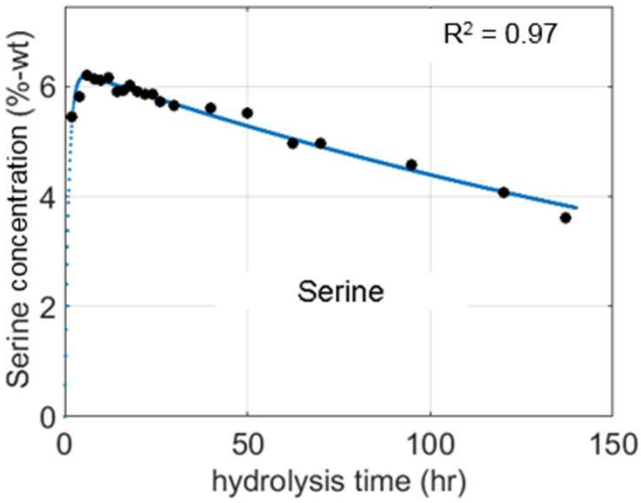
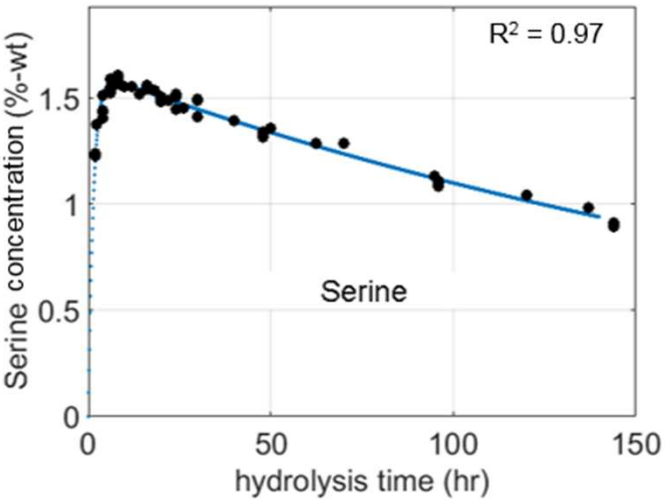
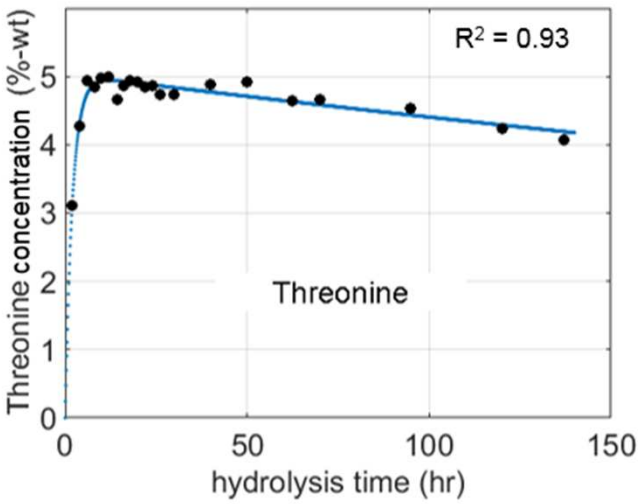
0.1
0.2
0.3
0.4
0.5
0
50
100
150


R² = 0.96
Phenylalanine
Phenylalanine concentration (%-wt)
hydrolysis time (hr)
0
1
2
3
0
50
100
150


R² = 0.96
Phenylalanine
Phenylalanine concentration (%-wt)
hydrolysis time (hr)
0
0.5
1
1.5
0
50
100
150


R² = 0.97
Serine
Serine concentration (%-wt)
hydrolysis time (hr)
0
2
4
6
0
50
100
150


R² = 0.97
Serine
Serine concentration (%-wt)
hydrolysis time (hr)
0
0.5
1
1.5
0
50
100
150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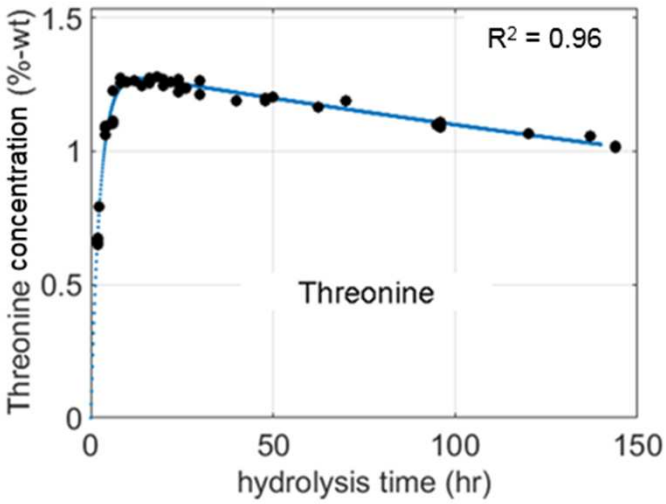
Threonine concentration (%-wt)
hydrolysis time (hr)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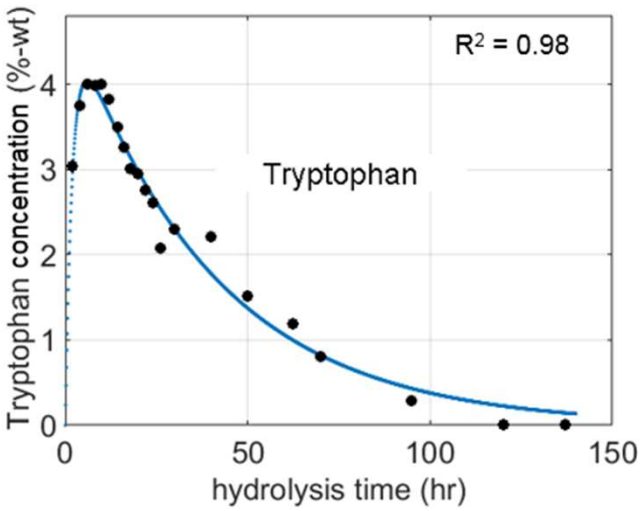
Threonine
Threonine concentration (%-wt)
hydrolysis time (hr)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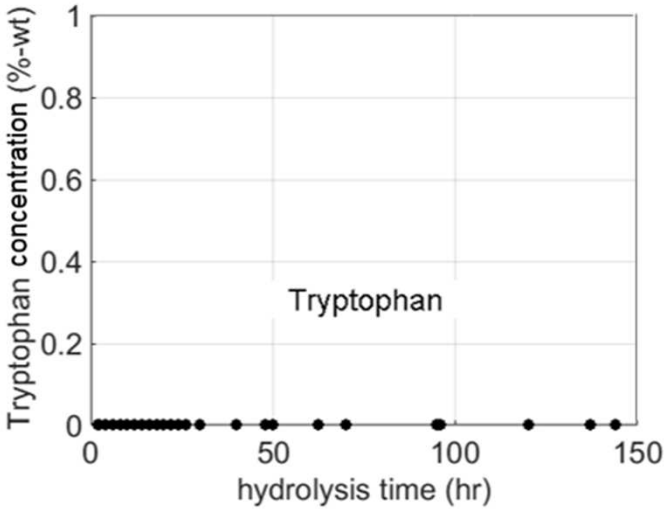
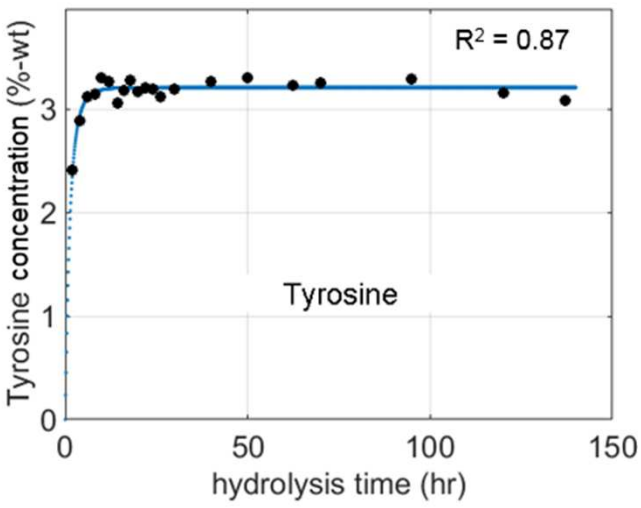
Tryptophan
Tryptophan concentration (%-wt)
hydrolysis time (hr)


Tryptophan
Tryptophan concentration (%-wt)
hydrolysis time (hr)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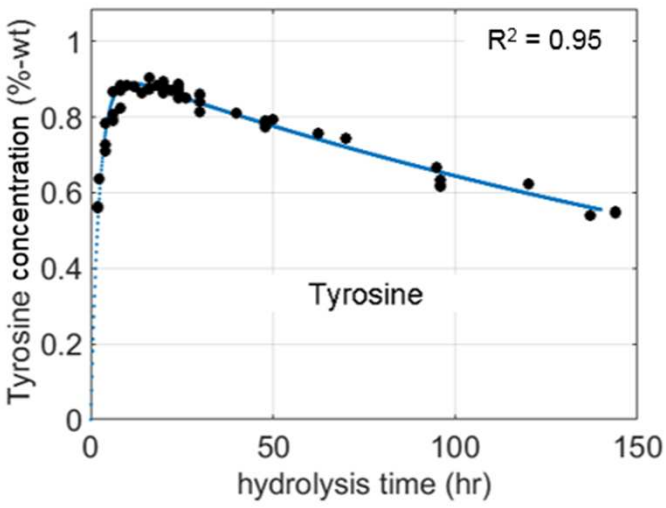
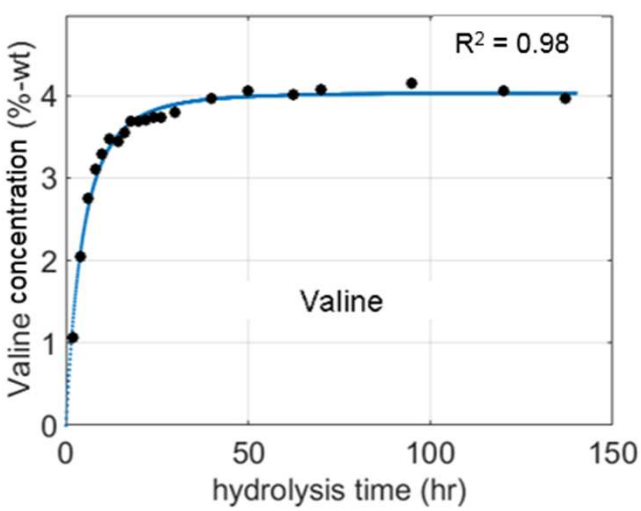
Tyrosine
Tyrosine concentration (%-wt)
hydrolysis time (hr)


R² = 0.95
Tyrosine
Tyrosine concentration (%-wt)
hydrolysis time (hr)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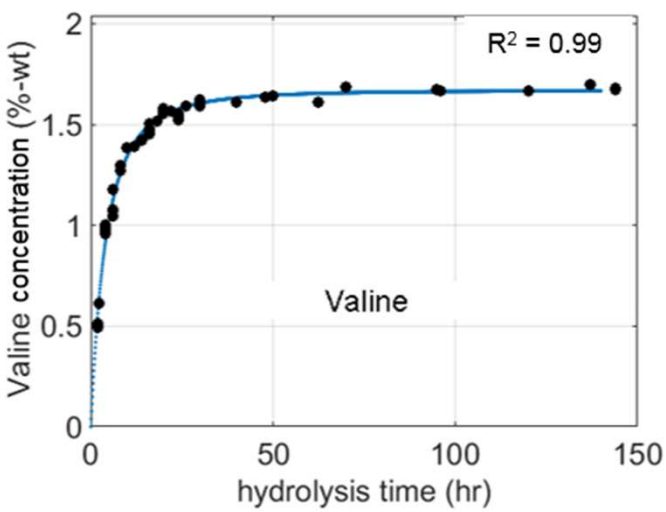
Valine
Valine concentration (%-wt)
hydrolysis time (hr)


R² = 0.99
Valine
Valine concentration (%-wt)
hydrolysis time (hr)