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NOTE

# Metabolic profiling of amino acids in cellular samples via zwitterionic sub-2 μm particle size HILIC-MS/MS and a uniformly $^{13}C$ labeled internal standard

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**Abstract** A novel analytical method using hydrophilic interaction liquid chromatography combined with electrospray tandem mass spectrometry for metabolic profiling of free, underivatized amino acids is presented. The separation uses a zwitterionic modified silica-based stationary phase with 1.8-μm particle size functionalized with ammonium sulfonic acid groups. Quantification is based on external standard calibration using a *Pichia pastoris* cell extract grown on uniformly $^{13}C$ labeled glucose as an internal standard. The absolute limits of detection in the cellular matrix were in the subpicomolar range. Measurement accuracy was assessed by analyzing NIST Standard Reference Material 2389a, which provides certified values for 17 amino acids. The recovery of the amino acids ranged between 65 % (proline) and 120 % (lysine), with excellent repeatability precision below 2.5 % ($n=5$). Only, cystine showed poor recovery (29 %) and repeatability precision (13 %). Generally, the long-term precision obtained by hydrophilic interaction liquid chromatography–tandem mass spectrometry was excellent, being on average less than 9 % over 20 h of measurement time. Moreover, the novel separation method had average repeatability and reproducibility of the chromatographic peak width over time periods of 20 h and 6 months of 8 and 15 %, respectively, demonstrating its high robustness in routine analysis of cellular samples. Large concentration differences depending on the amino acid were found in the cell extracts, typically ranging from 0.002 nmol per milligram of cell dry weight (cystine) to 56 nmol per milligram of cell dry weight (arginine and glutamic acid).

**Keywords** Amino acids · Metabolic profiling · Hydrophilic interaction liquid chromatography · Tandem mass spectrometry

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## Introduction

Analytical methods targeting amino acids have a long-standing tradition, with applications in various scientific areas such as environmental, food, biomedical, and clinical sciences as well as in metabolomics and systems biology in general. Despite these facts, accurate simultaneous quantification of all 20 primary species remains a challenging task owing to the differences that exist among the amino acid subclasses with respect to their concentrations and their stability in different biological matrices.

Generally, methods addressing amino acid analysis can be divided into approaches with chemical derivatization strategies and methods targeting underivatized analysis. Traditionally, the former approach was the method of choice, since derivatization made possible fluorescence detection or separation by reversed-phase chromatography. Most importantly, derivatization procedures were elaborated for gas chromatography (GC)–mass spectrometry (MS) analysis, which was pioneered by Gehrke and Stalling with their analysis of silyl esters of amino acids with electron impact ionization. With the advent of electrospray ionization (ESI) MS, chromatographic separation methods for underivatized amino acids were developed. Ion-pairing reversed-phase chromatography, capillary

electrophoresis, and hydrophilic interaction liquid chromatography (HILIC) in combination with MS detection were successfully applied.

HILIC [1] is a highly valuable alternative to more established ion-pairing reversed-phase chromatography and ion-exchange chromatography. It is orthogonal to reversed-phase chromatography [2–4], but it is not considered to be normal-phase chromatography, because it combines the stationary phases used in normal-phase chromatography with mobile phase solvents, which are conventionally used for reversed-phase separations. Evidently, the success of HILIC in metabolomics is due to its ideal compatibility with ESI-MS detection, avoiding both ion-pairing chromatography and ion-exchange chromatography, which need high counter ion strength for elution, which is incompatible with ESI-MS. Indeed the number of publications regarding HILIC applications has been constantly increasing since 2005 [4], and nowadays these techniques are largely used [5] to separate polar compounds such as amino acids [6–10], organic acids, sugars, sugar phosphates, nucleotides, antibiotic intermediates, and coenzymes.

The aim of this work was the development of a fast, accurate, and precise method for the profiling of free, underivatized amino acids on a routine basis via HILIC–MS/MS. In this sense, the column chosen has to show high reproducibility in terms of peak shape and retention time for all analytes over a long timescale. Moreover, the matrix robustness (i.e., the property of a method to provide sensitivity independently of the cellular matrix introduced via the samples and the uniformly $^{13}C$ labeled internal standards) is a critical aspect to evaluate for the liquid chromatography (LC)–MS method tested. The method developed was validated for the analysis of amino acids in the yeast *Pichia pastoris* because of the simplicity to grow and manipulate the model organism. *P. pastoris* is an important model organism in modern systems biology with regard to heterologous human protein production [11]. To the best of our knowledge, this is the first time a zwitterionic sub-2 μm particle size HILIC column has been used for separation of all 20 primary underivatized amino acids in cellular samples from microbial organisms.

## Experimental

### Chemicals

LC–MS-grade acetonitrile, LC–MS-grade water, and ammonium acetate were purchased from Sigma-Aldrich (Vienna, Austria).

### Standards

Amino acid standards for L-aspartic acid, L-histidine, L-cystine, L-alanine, L-serine, L-threonine, L-lysine, L-proline, L-asparagine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tyrosine, L-tryptophan, L-valine, L-citrulline, and L-ornithine were purchased from Sigma-Aldrich (Vienna, Austria). Glycine, L-glutamine, and L-glutamic acid were obtained from Merck (Vienna, Austria), and L-arginine was purchased from SAFC (Vienna, Austria).

Amino acid Standard Reference Material 2389a was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The amino acid solution was diluted by a factor of 100 in LC–MS-grade water and afterwards was spiked with the same amount of uniformly $^{13}C$ labeled *P. pastoris* extracts as for the calibration levels and quality control samples.

### *P. pastoris* growth, quenching, and extraction of target compounds and the uniformly $^{13}C$ labeled internal standard

The methylotrophic yeast *P. pastoris* was used as a model organism to produce both the unknown samples for amino acid profiling and the uniformly $^{13}C$ labeled internal standard. The protocols used for yeast fermentation, the consecutive quenching, and extraction of both target metabolites and $^{13}C$ internal standards are reported in detail in Neubauer et al. [8]; however, a short summary is given in the following sections.

### Chemostat cultivation on naturally labeled glucose

Cryostock (750 μL) of *P. pastoris* CBS7435 from the working cell bank was added to 100 mL preculture medium and grown at 28 °C and 150 rpm overnight. This culture was used for inoculation of the bioreactor at an optical density at 600 nm of 1.0. Subsequently, the yeast was grown in a working volume of 400 mL in a 1.4-L benchtop bioreactor. After a batch phase of approximately 24 h, the feed for the continuous chemostat cultivation was started. The cells were grown under glucose-limited conditions with a dilution rate of 0.1 $h^{-1}$ for at least seven residence times before sampling.

### Fed-batch cultivation for production of uniformly $^{13}C$ labeled internal standards

The fed-batch cultivation was performed in a benchtop reactor at 28 °C and 150 rpm on [U-$^{13}C$]glucose as the only carbon source. The preculture used for inoculation of the bioreactor had an optical density at 600 nm of 1.0 and was grown on [U-$^{13}C$]glucose. After a batch phase of approximately 35 h, the exponential feed (0.1 $h^{-1}$) was started. Before the first sampling round, the cells were grown for at least 2 h to ensure exponential growth.

### Sampling, quenching, and extraction of intracellular metabolites

The cells of both the samples and the internal standards grown on $^{nat}$C-glucose and [U-$^{13}$C]glucose, respectively, were harvested by using a peristaltic pump and directly quenched in 60 % (v/v) methanol at −27 °C [100 mL fermentation broth in 400 mL 60 % (v/v) methanol]. The quenched cells were aliquoted to 10-mL portions and washed via centrifugation, and the metabolites were extracted with 75 % (v/v) ethanol at 85 °C. The ethanolic extract was divided into 1-mL portions and evaporated to complete dryness and resuspended in 250 μL of LC–MS-grade water.

### Amino acid profiling via HILIC–ESI-MS/MS

Standard mixtures and levels for external calibration as well as quality control standards were independently prepared by mixing single standard solutions prepared from solid amino acids. All calibration levels and quality control standards were spiked with the same uniformly $^{13}$C labeled *P. pastoris* extract as the internal standard, which was added to the sample prior to extraction. The former samples were constantly analyzed throughout the 20 h measurement time to monitor the performance of the analytical platforms. After ethanolic extraction, drying, and reconstitution in water, cell extracts, quality control samples, and calibration standards were diluted by a factor of 10 and analyzed. It is noteworthy that the internal standard (i.e., $^{13}$C cell extract) was added to the real samples before ethanolic extraction of the cells, whereas it was added to the calibration standards and quality control samples prior to the LC–ESI-MS/MS measurement.

The free amino acids were separated via zwitterionic HILIC on a Nucleodur® silica-based column (100 mm× 2.0 mm, 1.8-μm particle size) equipped with a guard column (20 mm×2.0 mm, 1.8-μm particle size), both purchased from Macherey-Nagel (Düren, Germany). The aqueous and organic mobile phases were LC–MS-grade water with 10 mM ammonium formate, pH 3.25 (eluent A) and 100 % LC–MS-grade acetonitrile (eluent B), respectively. The separation was performed in a total run time of 15 min, including a 5-min reequilibration step. The chromatographic gradient was set as follows: 10 % eluent A and a flow rate of 300 μL min$^{-1}$, hold for 0.1 min, increase to 40 % eluent A in 7.9 min, increase to 90 % eluent A in 0.1 min, hold for 1.9 min, return to the initial conditions (10 % eluent A) in 0.1 min and increase the flow rate to 450 μL min$^{-1}$, hold for 4.8 min, and then decrease the flow rate to the original 300 μL min$^{-1}$ in 0.1 min. At this flow rate, the backpressure of the system was in the range of 120 bar.

Reconstituted cell extract (5 μL) was injected with a CTC PAL autosampler from Thermo Fisher Scientific (Dreieich, Germany), and the chromatographic gradient was operated by an Accela 1259 pump from Thermo Fisher Scientific, (Dreieich, Germany). For MS detection, a TSQ Vantage tandem mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany) featuring a heated electrospray interface was used in single reaction monitoring (SRM) mode. The ion source parameters for positive ESI were set as follows: vaporizer temperature 400 °C, ion transfer tube temperature 380 °C, auxiliary gas pressure 25 arbitrary units, sheath gas pressure 60 arbitrary units, ion sweep gas pressure 0 arbitrary units, declustering voltage 10 V, spray voltage 3,000 V, collision gas argon with a relative pressure of 1.5 mTorr. The dwell time was 50 ms per SRM transition. The selected precursor ions, product ions, and the corresponding collision energies and ionization polarity are listed in Table 1.

The uniformly $^{13}$C labeled internal standards of glycine and cystine showed low sensitivity; therefore, [U-$^{13}$C]alanine and [U-$^{13}$C]methionine, respectively, were used as substitutes. Glycine did not show any satisfactory SRM transitions in HILIC–MS/MS; therefore, a pseudo-SRM was performed without fragmentation by setting $m/z$ of the selected precursor ion as the product ion at the second quadrupole [6,7]. This explains the poor results which were obtained for glycine in this study.

## Results and discussion

### HILIC separation of amino acids

The aim of this work was to develop a derivatization-free, rapid, and robust HILIC–MS/MS method for analysis of cytosolic amino acids from cellular samples. As already discussed, HILIC provides retention for amino acids, but drawbacks such as the matrix affecting the retention time shift, the short column lifetime, and poor stability have been reported. We have reduced such drawbacks and further shortened the total method cycle time to 15 min by the implementation of a HILIC stationary phase with sub-2-μm particle diameter and an ideal, fully $^{13}$C labeled internal standard produced by *P. pastoris* using [U-$^{13}$C]glucose as a single carbon source. In our experience, 1.8 μM HILIC Nucleodur® resulted in high stability of chromatographic performances even after several hundred injections over 6 months (see later), so overcoming one of the main drawbacks of HILIC separation. Moreover, the power of uniformly $^{13}$C labeled internal standards compensates for the variability introduced by sample preparation, the integration process, and LC–MS/MS performances (Fig. 1).

### Analytical figures of merit

Table 2 lists the analytical figures of merit obtained for the novel HILIC–MS/MS method; the results are discussed as follows.

**Table 1** Single reaction monitoring transitions of amino acids and corresponding uniformly $^{13}C$ labeled internal standards of the hydrophobic interaction liquid chromatography (HILIC)–tandem mass spectrometry (MS/MS) method developed. The electrospray ionization (ESI) polarity and the precursor and fragment ions ($m/z$) are listed together with the corresponding collision energy

| | Ionization polarity | $^{nat}C$ amino acids | Uniformly $^{13}C$ labeled internal standards | Collision energy (eV) |
|---|---|---|---|---|
| Glycine | Positive | 76→76 | 78→78 | 2 |
| Leucine | Positive | 86→43 | 91→46 | 20 |
| Isoleucine | Positive | 86→57 | 91→60 | 20 |
| Alanine | Positive | 90→44 | 93→46 | 10 |
| Serine | Positive | 106→60 | 109→62 | 10 |
| Proline | Positive | 116→70 | 121→74 | 15 |
| Valine | Positive | 120→74 | 124→77 | 21 |
| Threonine | Positive | 132→41 | 137→44 | 15 |
| Ornithine | Positive | 133→116 | 138→121 | 5 |
| Asparagine | Positive | 133→74 | 137→76 | 14 |
| Aspartic acid | Positive | 134→74 | 138→76 | 13 |
| Lysine | Positive | 147→84 | 153→89 | 15 |
| Glutamine | Positive | 147→84 | 152→88 | 15 |
| Glutamic acid | Positive | 148→84 | 153→88 | 5 |
| Methionine | Positive | 150→56.1 | 155→59 | 13 |
| Histidine | Positive | 156→10 | 162→115 | 12 |
| Phenylalanine | Positive | 166.1→120 | 175→128 | 9 |
| Arginine | Positive | 175→70 | 181→74 | 24 |
| Citrulline | Positive | 176→70 | 181→74 | 22 |
| Tyrosine | Positive | 182→135.9 | 191→144 | 9 |
| Tryptophan | Positive | 205→188 | 216→199 | 7 |
| Cystine | Positive | 241→74 | 247→76 | 27 |

## Retention time stability

The retention times of the analytes, calculated over five injections, were stable from a minimum relative standard deviation (RSD) of 0.17 % (±0.02 s) for lysine to a maximum RSD of 1.2 % (±0.11 s) for alanine. With $t_0$ equal to approximately 1 min, all analyzed metabolites exhibited a retention time greater than 4.4 min (i.e., phenylalanine) as expected because hydrophobic amino acids (e.g., leucine, isoleucine, phenylalanine) are the early eluted species—between 4.4 min for phenylalanine and 6.0 min for alanine—whereas the basic polar amino acids (e.g., lysine, arginine, histidine) are the late eluted species, with retention times of approximately 9 min. On the other hand, acidic polar compounds such as aspartic acid and glutamic acid and moderately charged species (e.g., serine, threonine) showed intermediate elution times between 6.1 and 7 min.

## Repeatability and reproducibility of the chromatographic peak width

The average width of the chromatographic peaks obtained at 50 % of the peak height was approximately 15 s. However, the repeatability and reproducibility of the chromatographic peak widths were evaluated at peak base widths, as these values provided a more reliable criterion for the assessment of the method robustness. The measurements revealed an excellent repeatability of less than 10 % ($n$ = 5). The only exception was aspartic acid (peak width of 50 s), which probably required a higher ionic strength of the mobile phase. The peak widths of leucine and isoleucine revealed RSDs of 17 and 21 %, respectively. This could be explained by the comparably lower sensitivity of the SRM leading to a higher uncertainty of measurement [12]. The reproducibility of the peak widths calculated in an investigational time range of 6 months was greater by a factor of only approximately 2, highlighting the robustness of the separation column used.

## Limits of detection

The limits of detection (nmol $L^{-1}$) were comparable with the values reported by Schiesel et al. [7] in 2009, where amino acids and other metabolites extracted from fermentation broths of penicillin synthesis were separated on a commercially available zwitterionic HILIC column. The on-column limits of detection of our working range were from 1.3 fmol for histidine to 470 fmol for serine. The only amino acid with a limit of detection beyond the femtomolar range was glycine, with an absolute value of 1.6 pmol owing to the moderate sensitivity of the pseudo-SRM applied.

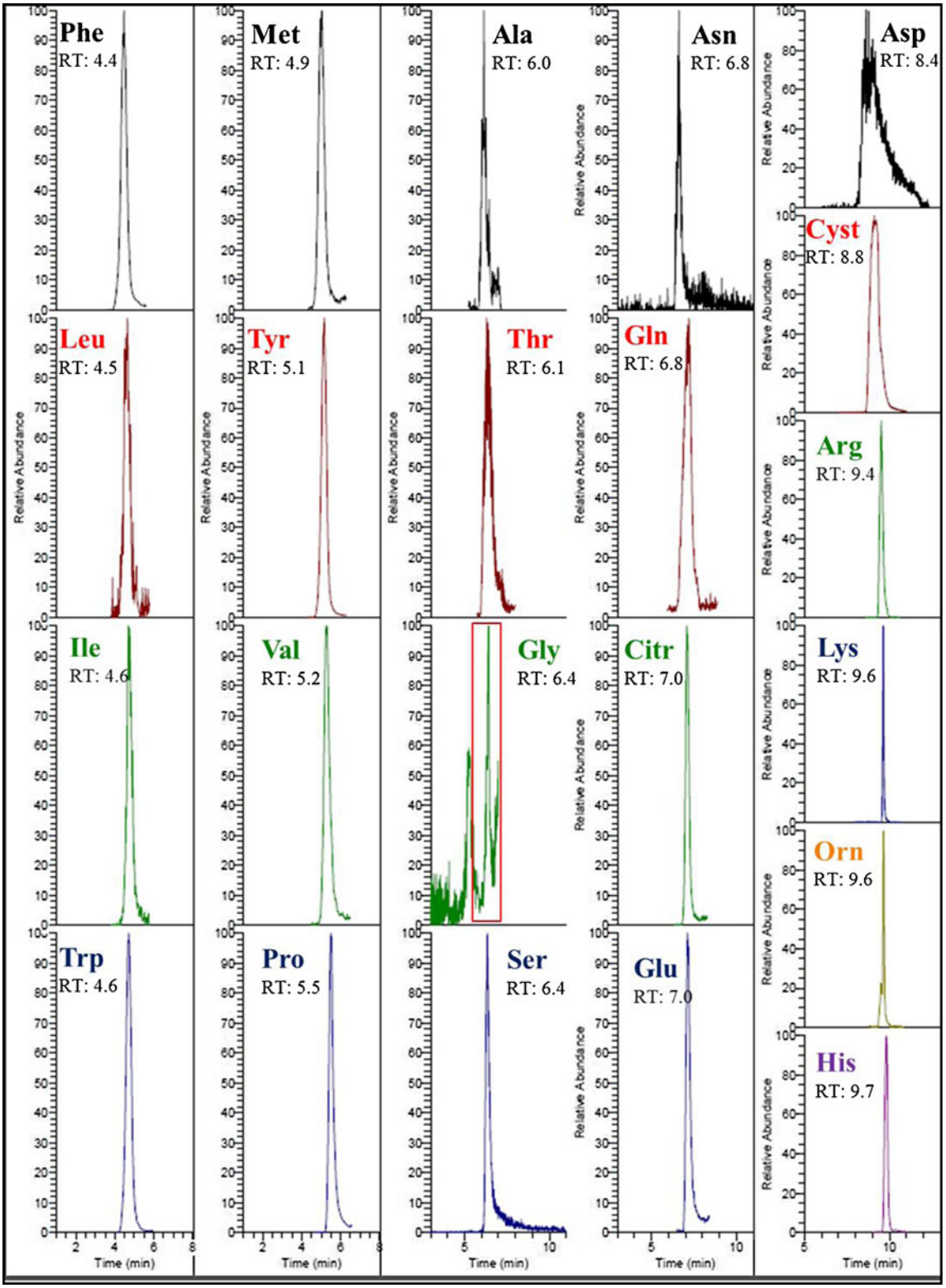


**Fig. 1** Extracted ion chromatograms of amino acid standard solution (concentration range from 1.25 to 25 μmol $L^{-1}$) separated on a zwitterionic sub-2 μm particle size hydrophobic interaction liquid chromatography column. *Ala* L-alanine, *Arg* L-arginine, *Asn* L-asparagine, *Asp* L-aspartic acid, *Citr* L-citrulline, *Cyst* L-cystine, *Gln* L-glutamine, *Glu* L-glutamic acid, *Gly* glycine, *His* L-histidine, *Ile* L-isoleucine *Leu* L-leucine *Lys* L-lysine, *Met* L-methionine, *Orn* L-ornithine, *Phe* L-phenylalanine, *Pro* L-proline, *RT* retention time, *Ser* L-Serine, *Thr* L-threonine, *Trp* L-tryptophan, *Tyr* L-tyrosine, *Val* L-valine

**Table 2** Analytical figures of merit of the novel HILIC–ESI-MS/MS method for amino acid profiling in cellular extracts. The average retention time and the relative standard deviation (*RSD*) of the retention time obtained within a 20-h measurement sequence, the limit of detection (*LOD*) and the limit of quantification (*LOQ*), and the absolute LOD on the column ($LOD_{col}$) and LOQ on the column ($LOQ_{col}$) are reported. The LODs and LOQs were determined by calculating the concentration (μmol $L^{-1}$) corresponding to three and ten standard deviations of the baseline signal, respectively. The absolute $LOD_{col}$ and $LOQ_{col}$ were calculated for an injection volume of 5 μL. The average chromatographic peak width calculated at 50 % of the height is reported for each analyte together with the repeatability and reproducibility calculated over a time period of 20 h and 6 months, respectively. The calibration ranges and determination coefficients calculated over three repetitive injections of six calibration levels over 20 h are shown as well. The repeatability and reproducibility precision of the HILIC–MS/MS method expressed as the percentage RSD were calculated from five injections of the matrix-matched quality control (*QC*) standards analyzed over a time period of 20 h and 6 months, respectively. In the *last column*, concentration ranges measured in cellular extracts of *Pichia pastoris* are reported

| | Retention time (min) | Retention time RSD (%) | Peak width at 50 % peak height (min) | Repeatability of peak base width over 20 h ($n$=5) (%) | Reproducibility of peak base width over 6 months ($n$=9) (%) | LOD (nmol $L^{-1}$) | LOQ (nmol $L^{-1}$) | $LOD_{column}$ (fmol) | $LOQ_{column}$ (fmol) | Calibration range (μmol $L^{-1}$) | Determination coefficient ($r^2$) | Repeatability of QC standards over 20 h ($n$=5) (%) | Reproducibility of QC standards over 6 months ($n$=9) (%) | Concentration range measured in *P. pastoris* extracts (μmol $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alanine | 6.0 | 1.2 | 0.18 | 7.4 | 18 | 25 | 83 | 120 | 410 | 4-400 | 0.9980 | 6.7 | 7.8 | 56-78 |
| Arginine | 9.4 | 0.25 | 0.16 | 4.6 | 18 | 3.5 | 12 | 17 | 58 | 10-1,000 | 0.9996 | 3.9 | 5.6 | 550-580 |
| Asparagine | 6.8 | 1.1 | 0.35 | 9.6 | 10 | 74 | 250 | 370 | 1200 | 2-200 | 0.9938 | 9.6 | 8.9 | 21-24 |
| Aspartic acid | 8.4 | 0.29 | 0.42 | 7.4 | 15 | 58 | 190 | 290 | 960 | 8-800 | 0.9990 | 3.8 | 13 | 28-30 |
| Citrulline | 7.0 | 0.30 | 0.17 | 4.5 | 10 | 4.3 | 14 | 22 | 72 | 0.5-50 | 0.9989 | 5.0 | 11 | 31-36 |
| Cystine | 8.8 | 0.17 | 0.12 | 7.7 | 15 | 1.7 | 5.6 | 8.4 | 28 | 0.5-50 | 0.9946 | 7.6 | 15 | $7.6\times10^{-3}$-$34\times10^{-3}$ |
| Glutamine | 6.8 | 0.12 | 0.26 | 4.1 | 16 | 83 | 280 | 420 | 1400 | 8-800 | 0.9992 | 2.5 | 5.0 | 270-280 |
| Glutamic acid | 7.0 | 0.44 | 0.17 | 5.0 | 17 | 50 | 170 | 250 | 830 | 8-800 | 0.9968 | 2.2 | 5.6 | 530-590 |
| Glycine | 6.4 | 0.29 | 0.25 | 8.3 | 16 | 320 | 1,000 | 1600 | 5200 | 4-400 | 0.8929 | 20 | 30 | <LOD |
| Histidine | 9.7 | 0.84 | 0.23 | 7.0 | 14 | 0.25 | 0.84 | 1.3 | 4.2 | 8-800 | 0.9986 | 4.6 | 6.7 | 36-38 |
| Isoleucine | 4.7 | 0.73 | 0.32 | 21 | 34 | 16 | 52 | 78 | 260 | 1-100 | 0.9954 | 6.9 | 6.5 | 1.3-1.4 |
| Leucine | 4.5 | 0.49 | 0.33 | 17 | 28 | 52 | 174 | 260 | 870 | 1-100 | 0.9905 | 4.3 | 7.5 | 2.0-2.5 |
| Lysine | 9.6 | 0.17 | 0.14 | 4.9 | 5.8 | 4.2 | 14 | 21 | 70 | 8-800 | 0.9970 | 3.7 | 5.7 | 33-36 |
| Methionine | 4.9 | 0.61 | 0.25 | 8.4 | 15 | 3.2 | 11 | 16 | 54 | 1-100 | 0.9882 | 4.7 | 8.2 | 3.3-3.6 |
| Ornithine | 9.6 | 0.26 | 0.12 | 5.1 | 5.9 | 3.4 | 11 | 17 | 57 | 0.5-50 | 0.9903 | 1.8 | 3.0 | 200-230 |
| Phenylalanine | 4.4 | 0.66 | 0.29 | 5.2 | 6.1 | 1.6 | 5.3 | 8.0 | 27 | 1-100 | 0.9996 | 2.9 | 7.4 | 1.5-1.7 |
| Proline | 5.5 | 0.18 | 0.31 | 4.2 | 10 | 3.5 | 12 | 18 | 59 | 4-400 | 0.9993 | 1.1 | 5.1 | 95-110 |
| Serine | 6.4 | 0.37 | 0.21 | 7.5 | 13 | 95 | 316 | 470 | 1600 | 4-400 | 0.9939 | 7.9 | 7.2 | 28-31 |
| Threonine | 6.1 | 0.45 | 0.21 | 9.9 | 10 | 21 | 69 | 100 | 346 | 2-200 | 0.9946 | 2.0 | 8.3 | 7.9-9.5 |
| Tryptophan | 4.6 | 0.65 | 0.27 | 8.7 | 11 | 3.9 | 13 | 20 | 66 | 1-100 | 0.9978 | 9.0 | 11.4 | 0.40-0.44 |
| Tyrosine | 5.1 | 0.59 | 0.24 | 8.0 | 18 | 10 | 32 | 48 | 160 | 2-200 | 0.9963 | 4.2 | 9.0 | 1.7-1.9 |
| Valine | 5.2 | 0.33 | 0.23 | 6.9 | 13 | 19 | 62 | 93 | 310 | 1-100 | 0.9921 | 7.1 | 7.2 | 7.2-8.3 |

### Repeatability and reproducibility of quality control standards

The repeatability of quality control samples did not exceed 10 %. Only, glycine showed a variability of 20 %, again owing to the low sensitivity of the pseudo-SRM transition. The reproducibility of the HILIC–MS/MS method was calculated for matrix-matched quality control standards analyzed on the same analytical column within 6 months. It is noteworthy that the reproducibility was of the same order of magnitude as the repeatability precision, Except for glycine, which exhibited a reproducibility precision of 30 %, all other species exhibited reproducibilities below 15 %. These results are further confirmation of the outstanding robustness over a long time.

### Linearity of external calibration

A matrix-matched calibration containing six concentrations each spiked with the same amount of uniformly $^{13}C$ labeled internal standard was performed. The determination coefficient ($r^2$) of the curves represents the linear regression calculated for three injections of each concentration performed within the 20-h time range. Except for glycine, which had $r^2$ of only 0.8929, and methionine, which was contradistinguished by $r^2$ of 0.9882 because of low stability, all amino acids had $r^2$ above 0.9900.

Measurement accuracy: analysis of matrix-spiked amino acid Standard Reference Material (Standard Reference Material 2389a)

To assess the purity and stability of the standard substances used and the trueness of the quantitative results obtained by the calibration performed, the amino acid Standard Reference Material (Standard Reference Material 2389a) [13] was diluted by a factor of 100 and subjected to the same preparation procedure as the samples and was analyzed five times within one measurement sequence (duration 20 h). The results are listed in Table 3.

The average recovery of the HILIC–MS/MS method was 90 %, with an average precision ($n$ = 5, 20 h) of 8 %. Cystine showed a low recovery of 29 %, which can be explained by uncontrolled oxidation the reference material prior to spiking with the internal standard. Instability and oxidation may have also caused the low recovery of methionine (85 %). Also, proline and threonine showed moderate recovery of only 65 % and 74 %, respectively. Consequently, the low recoveries could be explained only by losses during storage or preparation of the Standard Reference Material and were not related to the measurement technique.

### HILCI–MS/MS analysis of cellular extracts of *P. pastoris*

Quantification of intracellular concentrations of free amino acids extracted from a *P. pastoris* culture was performed by HILIC–MS/MS. Ten samples with approximately 10 mg of cell dry weight per fraction were rapidly sampled, filtered, quenched in cold methanol, and extracted with boiling ethanol

**Table 3** Measurement accuracy obtained for the certified amino acid Standard Reference Material 2389a ($n$ = 5, time range of 20 h)

| | Certified concentration (Standard Reference Material 2389a) | | | Measured concentration (HILIC–MS/MS) | | | | |
|---|---|---|---|---|---|---|---|---|
| | Certified concentration (mmol $L^{-1}$) | Relative expanded uncertainty (%) | Certified range (dilution 1:100) (μmol $L^{-1}$) | Average ($n$ = 5) (μmol $L^{-1}$) | RSD ($n$ = 5) (%) | Low limit (μmol $L^{-1}$) | High limit (μmol $L^{-1}$) | Recovery (%) |
| Alanine | 2.501 | 2.900 | 24.28–25.74 | 25.05 | 6.7 | 23.37 | 26.73 | 100 |
| Arginine | 2.507 | 2.800 | 24.37–25.77 | 25.54 | 4.0 | 24.53 | 26.55 | 100 |
| Aspartic acid | 2.502 | 2.900 | 24.29–25.75 | 23.63 | 11 | 21.04 | 26.22 | 94 |
| Cystine | 1.231 | 4.500 | 11.76–12.86 | 3.57 | 13 | 3.10 | 4.04 | 29 |
| Glutamic acid | 2.504 | 2.900 | 24.31–25.77 | 27.44 | 4.2 | 26.28 | 28.59 | 110 |
| Glycine | 2.520 | 2.900 | 24.47–25.93 | 19.13 | 47 | 10.12 | 28.14 | 76 |
| Histidine | 2.516 | 2.800 | 24.46–25.86 | 23.17 | 3.4 | 22.37 | 23.97 | 92 |
| Isoleucine | 2.440 | 4.500 | 23.30–25.50 | 23.85 | 3.2 | 23.08 | 24.63 | 98 |
| Leucine | 2.436 | 4.500 | 23.26–25.46 | 22.73 | 7.0 | 21.13 | 24.33 | 93 |
| Lysine | 2.414 | 6.500 | 22.57–25.71 | 29.83 | 1.2 | 29.47 | 30.19 | 120 |
| Methionine | 2.505 | 2.900 | 24.32–25.78 | 21.19 | 6.5 | 19.81 | 22.58 | 85 |
| Phenylalanine | 2.549 | 3.000 | 24.73–26.25 | 22.20 | 3.4 | 21.45 | 22.94 | 87 |
| Proline | 2.456 | 4.500 | 23.45–25.67 | 15.89 | 2.5 | 15.49 | 16.29 | 65 |
| Serine | 2.441 | 3.500 | 23.56–25.26 | 25.69 | 11 | 22.87 | 28.51 | 110 |
| Threonine | 2.490 | 2.900 | 24.18–25.62 | 18.47 | 11 | 16.36 | 20.57 | 74 |
| Tyrosine | 2.539 | 2.900 | 24.65–26.13 | 26.16 | 4.4 | 25.00 | 27.32 | 100 |
| Valine | 2.506 | 3.300 | 24.23–25.89 | 23.77 | 4.6 | 22.66 | 24.87 | 95 |

as described elsewhere in detail [8]. The cellular extracts were diluted 1:10 and analyzed by HILIC–MS/MS ($n=3$). The results are given in Table 2. The intracellular levels obtained in our work were compared with those reported by Klavins et al. [9]. In the latter study, different metabolites of the primary carbon metabolism, including amino acids, were profiled in the same strain of *P. pastoris* as used in this work. Good agreement of the intracellular levels was found for all species except for aspartic acid, glutamic acid, and glutamine, which exhibited concentrations higher by a factor of 2 and 3. This is most probably because fed-batch cultivation was used in the previous study [9], whereas in this work batch-mode fermentation was implemented.

## Conclusions

A novel method for the analysis of 22 underivatized amino acids in cell extracts via a sub-2 μm particle size zwitterionic HILIC stationary phase combined with ESI-MS/MS has been developed and evaluated regarding accuracy, precision, and long-term robustness. The figures of merit obtained for the analysis of cell extracts from a *P. pastoris* cultivation were found to be highly promising for the currently ongoing application of the novel method in the context of optimization of fermentation performance and yield of product, as small variations of the amino acid profile can be determined in a comparative experimental setup. The excellent short-term and long-term precision is due to both the comprehensive implementation of the concept of isotope dilution analysis via the $^{13}C$-labeled extract and the high robustness of the HILIC stationary phase used.

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