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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.9b00666 • Publication Date (Web): 14 May 2019

Derivatize, Racemize and Analyze -

an Easy and Simple Procedure for Chiral Amino Acid Standard Preparation for Enantioselective Metabolomics

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Abstract

A simple, controllable and reproducible stereoisomerization (racemization and epimerization) protocol for the preparation of scalemic α-amino acid mixtures from stereoisomerically pure standards was developed. Simply derivatize your amino acids with a racemization-tag that incorporates a urea bond on the *N*-terminus of the target amino acid and incubate at elevated temperatures up to 95 °C for defined time periods until the targeted D-amino acid levels are obtained.

The racemization-tags investigated were 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), aminophenyl-*N*-hydroxysuccinimidyl carbamate (AC) and 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate (APC). Employing this method, it was possible to create a ready-to-use, tailor made chiral uniformly ^{13}C and ^{15}N labelled [U-$^{13}C^{15}N$]-amino acid standard with the desired D-amino acid percentage within minutes or hours without sample clean-up. A racemization time of 30 min at 95 °C will lead to a D-amino acid level of 1-5%, while 6 h at 95 °C provides 15-30% D-amino acids. Racemization occurs due to imine formation at the chiral carbon atom bound to the urea-linking group without decomposition of labile amino acids such as Asn, Gln, Trp, Cit and theanine. For amino acids possessing two chiral centers such as DL-Ile or DL-Thr, only the epimerization of isomers with different stereochemistry at the second chiral center will produce all four possible isobaric enantiomers. All measurements were performed on the zwitterionic Chiralpak ZWIX(+) column using a dual hydro-organic flow gradient combined with HPLC-ESI-QTOF-MS analysis. This new racemization method solves the problem of (enantioselective) matrix effects and inaccurate results in LC-MS based enantioselective metabolomics and warrants full MS-compatibility.

Introduction

In the continuously expanding field of omics analysis, such as proteomics, glycomics, lipidomics or metabolomics, high sample throughput and ultra-fast separation are essential, necessitating reasonably fast and simple sample preparation techniques. Ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS) combines fast analyte separation with high mass resolving power and highest possible mass-to-charge (*m/z*) accuracy. The dilute and shoot approach resembles the fastest sample preparation technique and has hence gained increasing popularity in LC-MS, ranging from the analysis of drugs [1,2] and metabolites [3] to large molecules such as insulin [4]. In this context, the use of isotopically labelled internal standards (IS) [5] to ensure highest possible accuracy in quantitative analytics is of utmost importance, since matrix effects [6] can be adequately compensated. These isotopically labelled compounds share identical chemical properties, including extraction and ionization efficiency, with their unlabelled counterparts, the target analytes. In case of the upcoming research field chiral metabolomics [7], which include chiral amino acid metabolomics [8,9], the need for chiral isotopically labelled IS to compensate for matrix effects, which effect both enantiomers differently (enantioselective matrix effects) is known. Presently, many chiral metabolomics studies still rely on external calibration [9,10] or the use of an non-proteinogenic amino acid for IS such as racemic norleucine [11]. The reasons are manifold. Isotopically labelled amino acid standards mixtures are costly and their cost increase with the number and type of labelled atoms. Nonetheless, the more expensive [^{13}C] and [^{15}N] labelled IS are preferred over [^{2}H], since the latter may behave chromatographically different compared to [^{13}C]- and [^{15}N]-labelled compounds [12,13]. Alternatively also isotopically labelled derivatization tags can be used to produce isotopically labelled IS [14]. Since in both cases the natural isotopologue pattern of organic compounds provide additional *m/z* peaks with M+1, M+2, M+3 and higher, the *m/z* difference between analyte and IS should be at least 2 and preferably higher or even uniformly [^{13}C] and [^{15}N] labelled ([U-^{13}C^{15}N]). Therefore the application of isotopically labelled IS, necessitate mass spectrometric detection to determine small *m/z* differences

between co-eluting target analyte and IS. In addition, only few isotopically labelled D-amino acid standards exist and chiral isotopically labelled DL-amino acid standard mixtures containing all proteinogenic amino acids are not at all available. Even the commercial [U-$^{13}C^{15}N$]-L-amino acid metabolomics standard does not contain all proteinogenic L-amino acids. The amino acids L-Gln, L-Asn and L-Trp are absent, since they are prone to degradationduring acid [15,16] or base [17,18] catalysed hydrolysis of peptides and proteins applied for amino acid composition determination. Also, the most common *N*-terminal amino acid racemization method involves the formation of a Schiff-base with aromatic aldehydes in the presence of concentrated acetic acids and elevated temperatures [19,20], which induces deamidation and degradation of Asn and Gln to Asp and Glu, respectively. Alternatively, Schiff base formation followed by racemization can also be induced by complex formation with an auxiliary ligand and a metal ion such as copper [21] or nickel [22,23]. In these cases the disadvantage lies in the necessity to remove the metal ions prior to mass spectrometric analysis.Besides that also C-terminal racemization of *N*-acetyl amino acids via oxazolone formation and base induced ring-opening was reported in literature [24,25]. Furthermore, several patents illustrate the racemization of *N*-acyl, *N*-phenacetyl, *N*-benzoyl [26-28] and *N*-phenacetyl-phosphino amino acid [29] derivatives as well as *N*-phenacyl-hydroxyl proline [30] in the presence of the corresponding acid and heat treatment up to 180 °C. The major disadvantage of these methods is, as previously mentioned, the decomposition of several amino acids during acid treatment.

The here presented study introduces the mild *N*-terminal heat induced stereoisomerization (racemization and epimerization [31]) of 6-aminoquinolyl-*N*-hydroxysuccinimidyl (AQC) tagged proteinogenic amino acids and some uncommon amino acids without deamidation and degradation of some metabolically interesting amino acids such as Gln, Asn Trp, Cit and theanine. Applying this racemization method on DL-Ile or DL-aIle, leads in both cases to a complete mixture of DL-Ile plus DL-aIle in one standard solution. The same accounts for DL-Thr and DL-aThr. Furthermore, this procedure allows the preparation of a scalemic DL-[U-$^{13}C^{15}N$]-isotopically labelled chiral amino acid mixture containing a defined amount of D-[U-

$^{13}C^{15}N$]- amino acids, which can be used as an internal standard for different sample types, containing varying levels of D-amino acids, to compensate for (enantioselective) matrix effects.

EXPERIMENTAL SECTION

Chemicals and standard solutions

All amino acids, L-citrulline (Cit), L-phenylglycine (Phg), ammonium formate, boric acid, hydrochloric acid, 1,3,5-tri-tert-butyl-benzene (TBB), sodium acetate, sodium hydroxide, sodium deuteroxide, diethylamine (DEA), dimethyl sulfoxide (DMSO), DMSO-d_6, acetonitrile-d_3 (ACN-d_3) and 1.5 mL Eppendorf Safe-Lock microcentrifuge tubes were from Sigma Aldrich (Schnelldorf, Germany). L-Theanine was from TCI Chemicals GmbH (Eschborn, Germany). Ultra-LC-MS grade ACN ultra-LC-MS methanol (MeOH), formic acid (FA), syringes, 0.22 µm sterile syringe filters, MµlTi®-lid locks for 1.5 mL microcentrifuge tubes and Parafilm® were from Carl ROTH (Karlsruhe, Germany). β-Phenylalanine and 7-fluoro-4-nitrobenzoxadiazole (NBD) were from TCI (Zwijndrecht, Belgium). 5-(Dimethylamino) naphthalene-1-sulfonyl-L- and DL-tryptophan (dansyl, DNS-Trp), *N*-fluorenylmethoxycarbonyl-L- and DL-phenylalanine (FMOC-Phe), *N*-carbobenzyloxy-L and DL-phenylalanine (CBZ-Phe) and *N*-3,5-dinitrobenzoyl L and DL-tryptophan (DNB-Trp) were in-house prepared analytical standards. Ultra-pure water was generated with a Water Purelab Analytics Purification System from ELGA (Celle, Germany).

6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, AccQ) was purchased from Synchem (Felsberg / Altenburg, Germany). In addition, AQC, aminophenyl-*N*-hydroxysuccinimidyl carbamate (AC) and 3-aminopyridyl N-hydroxysuccinimidyl carbamate (APC) were in-house prepared as described in literature [32,33] and re-crystalized in ACN to yield yellowish off-white crystals.

Isotopically labelled L-[U-$^{13}C^{15}N$]-amino acid metabolomics standard mix (2.5 mM in 0.1 M HCl) was purchased from Euroiso-top GmbH (Saarbrücken, Germany) and does not contain Asn, Gln and Trp.

All DL- and L-amino acid stock solutions were prepared at a concentration of 50 mM in 0.1 M HCl. The single standard solutions were combined to obtain a 2.5 mM L- and DL-[U-$^{12}C^{14}N$]-amino acid standard mixtures containing all 20 proteinogenic amino acids in 0.1 M HCl, respectively. Note that the concentration of each D- and L-enantiomer in the DL-amino acid standard mixtures was 1.25 mM and 2.5 mM in the L-amino acid standard mixtures. Separate test mixtures of isobaric amino acids Leu, Ile and alle as well as Thr, aThr and Hse were prepared at concentrations of 2.5 mM per solution. If not otherwise stated, all test solutions were diluted to 0.025 mM prior to derivatization with AQC. Note that for the AQC-derivatized amino acid solutions, the stated amino acid concentrations are the concentration of the sample prior to derivatization.

Sample Preparation

Derivatization with AQC

The AQC reagent solution was prepared at a concentration of 3 mg/mL in HPLC-grade ACN (AQC: 10.5 mM) and stored at -20 °C prior to use. In general, 10 µL 0.025 mM amino acid sample solution was added to 80 µL (or 70 µL) 20 mM sodium borate buffer (pH 8.8). After addition of 10 µL AQC reagent, the reaction solution was immediately heated to 55 °C for 10 min at 800 rpm. Derivatization reactions of standard reference solutions were performed in 1.5 mL Crystal Clear microcentrifuge tubes from Starlab (Hamburg, Germany). Note that for 0.25 mM amino acid standard mixtures, 10 µL sample, 70 µL 0.2 M borate buffer (pH 8.8) and 20 µL AQC reagent solution were used.

Reagent-type dependent racemization

For comparison two additional urea-group incorporating amino acid tags, aminophenyl-*N*-hydroxysuccinimidyl carbamate (AC) [34] and 3-aminopyridyl *N*-hydroxysuccinimidyl carbamate (APC) [35] were investigated. The 0.25 mM L-[U-$^{12}C^{14}N$]- and [U-$^{13}C^{15}N$]-amino acid solution was derivatized with AC and APC as described for AQC [32,33]. The racemization experiment was performed at 95°C for 18 h, followed by chromatographic evaluation via HPLC-ESI-

QTOF-MS as described for AQC. Information about the performance of the amino acid tags FMOC, CBZ, DNB, DNS and NBD during heat treatment is provided in the ***Supporting Information***.

Racemization of compounds with two chiral centers

The test solutions 0.25 mM L-Thr, L-aThr, DL-Thr, DL-aThr, L-Ile- L-aIle, DL-Ile and DL-aIle were derivatized using 20 mM borate buffer and 10 µL AQC, and were racemized for 18 h at 95 °C. For all Ile solutions, a 1:10 dilution to 0.025mM was performed prior to chiral analysis, while the Thr solutions were injected without prior dilution.

Racemization of asparagine, glutamine, citrulline and theanine

For the racemization experiment, 20 µL of 0.025 mM L-Asn, L-Gln, L-Cit or L-theanine was added to 160 µL of 20 mM sodium borate buffer (pH 8.5) in 1.5 mL microcentrifuge tubes followed by addition of 20 µL AQC reagent (3 mg/mL in ACN) and heated to 55°C for 10 min. After ultrasonication for 1 min and purging with nitrogen for 1 min, the reaction tube was placed onto the thermoshaker for 15 h at 95°C at 800 rpm.

Note that stereoselective separation of scalemic AQC-DL-Asn and AQC-DL-Gln as well as their degradation products AQC-DL-Asp and AQC-DL-Glu was performed on a Chiralpak ZWIX(+) column (150 x 3 mm, 3 µm) using a short gradient elution method [36]. Chiral separation of scalemic AQC-DL-Cit and AQC-DL-theanine was performed on a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual-gradient elution method. Analysis was performed with HPLC-ESI-QTOF-MS.

Time and temperature dependent racemization with AQC

A standard solution, containing 0.025 mM L-[U-$^{12}C^{14}N$]- amino acids and 0.025 mM L-[U-$^{13}C^{15}N$]- amino acids was first derivatized with AQC. To ensure a tight closure during the racemization process, the lid was secured with a small strip of Parafilm® combined with a Mµlti®-lid lock **(Figure S1)**. Alternatively Eppendorf Safe-Lock microcentrifuge tubes, sealed

with Parafilm® were used. Amino acid racemization was induced by heat treatment for defined time periods. The following racemization conditions were investigated, 10 min, 30 min and 1 h at 55 °C, 75 °C and 95 °C. The shaker speed of a Grant-bio PHMT Thermo-Shaker from Grant Instruments Ltd. (Shepreth, England) was set to 600 rpm. After reaching room temperature, all samples were centrifuged and analyzed without further treatment.

In a second optimized approach, a 0.25 mM [U-$^{12}C^{14}N$]- and 0.25 mM [U-$^{13}C^{15}N$]-L-amino acid standard solution was derivatized with AQC Note that all samples were prepared in triplicates. The racemization conditions were 1 h, 6 h and 15 h at 95 °C and 600 rpm. After racemization and reaching room temperature, all samples were centrifuged and additionally derivatized with AQC (samples (A)), which resembles a 1:10 dilution. Additionally the 15 h samples were only diluted 1:10 with 0.2 M borate buffer, without additional re-derivatization with AQC (samples (B)). In a third optimization step to prevent oxidation of methionine, the amino acid test solution was ultrasonicated for 1 min and purged with nitrogen prior to stereoisomerization.

Instrumentation and Methods

HPLC-ESI-QTOF-MS

HPLC-ESI-QTOF-MS measurements were performed on an Agilent 1290 UHPLC system comprised of a binary pump and a thermostated column oven from Agilent (Waldbronn, Germany); and a CTC-PAL HTS autosampler from CTC Analytics (Zwingen, Switzerland). Furthermore, an Agilent 1100 binary pump combined with a 6-port/2-position Valco valve was used for MS-calibrant delivery. Mass calibration was performed via sodium acetate clusters (0.1 mg/mL sodium acetate in acetonitrile:water (1:1, v/v)). LC-MS analyses were performed on a TripleTOF 5600+ instrument from Sciex (Ontario, Canada) with DuoSpray Ion Source operated in positive electrospray ionization mode. MS results shown in this study were generated with TOF-MS full scans (survey scans) combined with MS/MS experiments by information dependent acquisition (IDA) in high sensitivity mode. The IDA inclusion list comprised the *m/z* of the precursor ions of all single and double AQC derivatized as well as

all un-derivatized amino acid compounds. Note that in the present sample set only Lys was double derivatized with AQC. For the QTOF measurements, the following instrument settings were used: curtain gas (CUR) 40 psi, ion source gas (nebulizing gas; GS1) 60 psi, heater gas (drying gas; GS2) 60 psi, ion spray voltage floating (ISVF) 5500 V, source temperature (TEM) 400 °C, collision energy (CE) 10 V, declustering potential 100 V. The TOF-MS full scan was set to *m/z* 30-2000 with an accumulation time of 250 ms, while for the IDA MS/MS experiments a CE of 46 V, DP of 100 V and an accumulation time of 100 ms were used in combination with an automated dynamic background subtraction.

MS Software Information and Data Analysis

The Analyst®TF Software was utilized for data acquisition, while for qualitative HPLC-ESI-QTOF-MS data analysis the PeakView® and MasterView™ software and for quantitative analysis the MultiQuant™ from SCIEX (Ontario, Canada) was used. MultiQuant™ was only used for peak integration, for which individual pre-defined integration time sections for each amino acid enantiomer were employed. Note that the same time section width of baseline noise (closely neighboring the enantiomer peak) was integrated for each amino acid enantiomer and subtracted from the corresponding enantiomer peak. An overall smoothing factor of 2 was used prior to peak integration. Due to the use of a dual gradient elution, changing the mobile phase composition as well as the flow rate, the peak areas of the D- and L-enantiomers of a racemic mixture are not identical. For the calculation of the percent D-amino acid value, the D/L ratios of the commercial [U-$^{12}C^{14}N$]-amino acids was used for normalization. Since these normalization factors are highly dependent on chromatographic separation conditions, the analysis of a racemic DL amino acid standard mixture must accompany each sample measurement sequence. Since a racemic mixture of isotopically labelled amino acid was not available, the D/L ratios of the [U-$^{13}C^{15}N$]-amino acids were assumed to be identical to that of their unlabeled counterparts.

Chiral Separation of AQC, APC and AC

Chiral Separation was performed on a Chiralpak ZWIX(+) (150 mm x 4 mm i.d., 3 µm) column and Chiralpak ZWIX(+) (150 mm x 3 mm i.d., 3 µm) from Chiral Technologies Europe (Illkirch, France) using the following chromatographic dual gradient elution conditions. Mobile phase A (MP-A) was 9.4 mM NH_4FA and 9.4 mM FA in ACN:MeOH:H_2O (75:25:2; (v/v)), while mobile phase B (MP-B) contained MP-A:9.4 mM NH_4FA and 9.4 mM FA in water (1:1, (v/v)). The gradient consists of the following steps: i) isocratic run: MP-A 0-35 min at 0.2 mL/min; ii) dual gradient mode: gradient 1 (flow rate): 0-45 min 0.2 mL/min, 45-50 min 0.2-0.5 mL/min and 50-65 min 0.5 mL/min; iii) gradient 2 (hydro-organic): 35-45 min 0% MP-B to 40% MP-B, 45-50 min to 60% B, 50-60 min to 100% B and 60-65 min 100% B, followed by iv) column re-equilibration to 100% MP A at 0.2 mL/min. Column temperature was kept at 30 °C and sample injection volume were uniformly 2.5 µL. Note that for the NBD-derivatized sample the same chromatographic condition was used **(Figure S3)**.

For the short gradient elution method [36] an oven temperature of 30 °C was employed. The gradient setting was 0-9 min with MP-A containing 2% water in MeOH with 9.4 mM NH_4FA and 9.4 mM FA; and 9-20 min to 100% MP-B containing MP-A:water with 9.4 mM NH_4FA and 9.4 mM FA (1:1, (v/v)), followed by 20-25 min at 100% MP-B and re-equilibration to 100% MP-A. For the Chiralpak ZWIX(+) column (150 x 4 mm, 3µm) a flow rate of 0.7 mL/min and an injection volume of 2.5 µL was used, while for the ZWIX(+) column (150 x 3 mm, 3µm) a flow rate of 0.45 mL/min and an injection volume of 1 µL was used.

RESULTS AND DISCUSSION

The reaction of an amino functional compound with *N,N'*-disuccinimidyl carbonate (DSC) leads to an *N*-hydroxysuccinimidyl carbamate activated reagent [33], such as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, AccQ), aminophenyl-*N*-hydroxysuccinimidyl carbamate (AC) [34] and 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate (APC) [35]. These reagents are known to easily react with the N-terminus of amino acids or any amine containing substances, incorporating thereby an aromatic side chain via a urea linking group. The addition of an aromatic ring system enhances not only the detectability of amino acids, but provides also an additional hydrophobic and shape selective interaction site for improved reversed phase and mixed mode liquid chromatographic separation. Since the chiral stationary phase with zwitterionic (*S,S*)-trans-2-aminocyclohexanesulfonic acid (*S,S*-ACHSA) modified quinine type chiral selector (Chiralpak ZWIX(+)) [37] is actually a mixed mode phase, the attachment of AQC to amino acids leads also to improved chiral separation of enantiomers [36,38-40]. The formation of the urea linking group between the amino group of the aromatic tag and the N-terminus of the amino acid provides a close proximity between the urea group and the chiral center of the amino acid. Upon heating to 95 °C for elevated time periods racemization (for amino acids with one stereogenic center) and epimerization (for amino acids with more than one center of chirality) occurs. It can be expected that a heat-induced movement of delocalized electrons within the electron clouds of the urea link leads to a reversible and short term transient imine formation at the carbon atom of the chiral center (see TOC Graphic) facilitated by keto-enol tautomerism [41,42] and charge-transfer effects [43]. The planar imine structure induces the loss of chirality, hence providing an *N*-terminal stereointerconversion of the attached amino acid.

The occurrence of a stereoisomerization event of the model compound AQC-phenylglycine (AQC-Phg) in a variety of different solutions including aqueous buffer and DMSO as well as in the presence of acid and base catalysis could be verified by a hydrogen-deuterium exchange at the chiral center using HPLC-ESI-QTOF-MS combined with chiral separation on

the Chiralpak ZWIX(+) column (**Figure S2**). Information about the investigated solvent systems and racemization conditions is provided in the **Supporting Information**.

< add Figure 1 >

A comparison of different N-terminal amino acid tags such as AQC, APC, AC and NBD as well as protective groups such as DNS, DNB and CBZ in **Figure 1** and **Figure S3** show that only those derivatives that carry a urea linking group can induce racemization of the attached α-amino acid. **Figure 1** shows the racemization results for AQC-L-[U-$^{13}C^{15}N$]- Phe, APC-L-Phe and AC-L-[U-$^{13}C^{15}N$]-Phe after heat treatment at 95 °C for 18 h. Note that the slight retention time shift of scalemic AQC-DL-[U-$^{13}C^{15}N$]-Phe compared to AQC-L-[U-$^{13}C^{15}N$]-Phe was due to a too short column equilibration time. The generated D-enantiomers were all verified via their MS-spectrum. In comparison, **Figure S3** summarizes the failed racemization for DNS-Trp, DNB-Trp, CBZ-Phe and NBD-Arg. In case of FMOC-Phe in **Figure S4**, the heat treatment has led to a complete loss of the protective group under the investigated condition. Note that β-amino acids cannot be stereoisomerized by this method, since the keto-enol tautomerism between urea group and the attached amino acid is disrupted by the additional methylene group in the β-amino acid **(Figure S5)**..

In case of amino acids possessing two or more chiral centers, only the chiral center in position 2 will be epimerized. In order to substantiate this hypothesis, it was necessary to chromatographically separate the isobaric isomers of Leu and Thr. During method optimization the L-[U-$^{13}C^{15}N$]-amino acid standard was used as a chiral peak assignment standard to determine the L-enantiomers within a single run **(Figure S6)** [36]. Additionally, single DL-standard solutions of Leu and Thr isomers were analyzed to determine the correct elution order of all D- and L-isomers. Hereby, the best result was obtained using a dual gradient elution method, which included a low flow rate of 0.2 mL/min with 2% water in the mobile phase A at the beginning to separate the DL-Ile and DL-Leu isomers followed by a gradient elution to a flow rate of 0.5 mL/min with a water content of 52% in the mobile phase B, which elute all other amino acids. The latter allows the baseline separation of all six Thr

isomers, namely DL-Thr, DL-aThr and DL-Hse **(Figure S7)**. Note that D-Ile and D-aIle as well as L-Leu and L-aIle coelute on the ZWIX(+) column.

< add Figure 2 >

As shown in **Figure 2** and **Figure S8**, the epimerization of L-Thr, L-aThr, DL-Thr and DL-aThr as well as L-Ile, L-aIle, DL-Ile and DL-aIle, has led to the generation of the following new epimers, namely D-aThr, D-Thr, DL-aThr, DL-Thr as well as D-aIle, D-Ile, DL-aIle and DL-Ile, respectively. Hence, only the epimerization of DL-Thr or DL-aThr as well as DL-Ile or DL-aIle will ultimately provide the complete set of all 4 possible epimers of Thr and Ile. Note that the apparent difference in epimerization ratio for isobaric amino acids shown in **Figure 2** is due to differences in analyte ionization caused by an enantioselective matrix effect.

< add Figure 3 >

Another point that needed to be addressed was whether this stereoisomerization method was mild enough to racemize deamidation prone amino acids such as Asn [44] and Gln [45], which are also of metabolic interest. **Figure 3a** shows the XICs of AQC derivatized DL-Asn and DL-Gln as well as their deamidation products DL-Asp and DL-Glu. **Figure 3b** shows the XICs of L-Asn and L-Gln and their potential degradation products prior to racemization and **Figure 3c** shows their XICs after racemization for 15 h at 95 °C. While the XICs of AQC-Asn and AQC-Gln show additional peaks for their respective D-enantiomers, the corresponding XICs of their degradation products were absent of AQC-Asp and AQC-Glu.

< add Figure 4 >

To show the applicability of this method for uncommon amino acids, this experiment was repeated for theanine [46,47] and citrulline (Cit) [48]. **Figure 4a** shows the XICs of AQC derivatized L-theanine and L-Cit as well as their respective degradation products AQC-Glu and AQC-ornithine (Orn). **Figure 4b** shows clearly that theanine and Cit can be racemized without being decomposed. Note that after the stereoisomerization event all reaction solutions were additionally derivatized with AQC to counterbalance the possible loss of AQC during heat treatment and to show that really no Asp, Glu or Orn was produced.

The following experiment show the racemization result for the other proteinogenic amino acids as well as their [U-$^{13}C^{15}N$]-labelled analogs.

< add Figure 5 >

Figure 5 shows the XICs of AQC derivatized [U-$^{13}C^{15}N$]-labelled amino acids as well as the XICs of Asn, Gln and Trp after stereoisomerization of a 0.025 mM L-[U-$^{12}C^{14}N$]- amino acid (bold red) and 0.025 mM L-[U-$^{13}C^{15}N$]- amino acid mixture (without Asn, Gln and Trp) at 95 °C for 6 h. An overlay of XICs of all labelled and unlabeled amino acids is provided in **Figure S9** and illustrates that the [U-$^{13}C^{15}N$]-labelled amino acids are racemized to the same extent as their unlabeled counterparts. For the calculation of retention factors and separation factors 1,3,5-tri-tert-butyl-benzene (TBB) was used as a void volume marker. A comparison with 4-(dimethylamino)azobenzene showed that TBB was the better choice (**Figure S10)** [36]. Note that the separation factors are displayed beside the corresponding XICs in **Figure 5**. In case of [U-$^{13}C^{15}N$]-Met ([AQC-[U-$^{13}C^{15}N$]-Met + H]$^+$: *m/z* 326.1202) the displayed peaks at 52.66 min and 59.08 min are not corresponding to AQC-[U-$^{13}C^{15}N$]-Met, but to isobaric AQC-His ([AQC-His + H]$^+$: *m/z* 326.1248). Both, AQC-[U-$^{13}C^{15}N$]-Met and AQC-[U-$^{12}C^{14}N$]-Met were oxidized in the presence of oxygen in the reaction tube. Even after re-derivatization no AQC-[U-$^{13}C^{15}N$]-Met or AQC-[U-$^{12}C^{14}N$]-Met was found. Met was oxidized to methionine sulfoxide ([AQC-[U-$^{13}C^{15}N$]-Met-O + H]$^+$: *m/z* 342.1151) and methionine sulfone ([AQC-[U-$^{13}C^{15}N$]-Met-O_2 + H]$^+$: *m/z* 358.1100). To overcome this problem, the experiment was repeated after ultasonication and purging of the microcentrifuge tube with nitrogen. The result was a racemized and intact solution containing AQC-[U-$^{13}C^{15}N$]-Met and AQC-[U-$^{12}C^{14}N$]-Met (**Figure S11**).

A potential application product of this simple stereoisomerization process is the generation of an isotopically labelled chiral metabolite standard mixture for internal standardization, which includes the chiral peak assignment of the L- as well as the D-enantiomers. The application of AQC-L-[U-$^{13}C^{15}N$]-amino acids for chiral elution order assignment [36] was shown for the isobaric amino acids DL-Ile, DL-Leu and DL-Thr in **Figure S6**. In addition a chiral DL-[U-$^{13}C^{15}N$]-amino acid standard mixture containing all amino acids would also allow a most

accurate quantification of both enantiomers of every amino acid by counterbalancing an enantioselective matrix effect induced by sample background matrix or the chromatographic gradient elution conditions used. Due to the fact that an isotopically labeled internal standard is subjected to the same matrix effects and ionization effects such as ion suppression or ion enhancement as the target analyte, it corrects and normalizes quantitative results. A comparison between an isocratic separation of racemic DL-Phe and AQC-DL-Phe [36] with the separation of AQC-DL-Phe using a dual elution gradient, shows that under gradient elution condition the first eluting AQC-D-Phe peak exhibits a much higher and broader peak compared to its counterpart AQC-L-Phe **(Figure S12)**.

Considering that different types of real samples contain different percentages of D-amino acids, the D-amino acid content within an isotopically labelled internal standard should meet these requirements. Non-ribosomal lipopeptides, for instance contain not only an unexpectedly large number of proteinogenic D-amino acids [49], which may even exceed the racemic 50%-level [50], but also contain many uncommon amino acids [51], such as allo-threonine (aThr) [52], homoserine (Hse) [53], allo-isoleucine (alle) [54] and hydroxylproline (Hyp) [55], just to name a few. On the other hand, physiological fluids may contain trace amounts of D-amino acids and require isotopically labelled IS with below 1% to 10% D-amino acids [56,57].

< add Figure 6 >

Therefore, the preparation of scalemic DL-amino acid standards with defined D-amino acid content was investigated. For this purpose two separate temperature and stereoisomerization time studies were performed. **Figure 6** shows the result for the racemization of an amino acid standard test solution containing L-[U-$^{13}C^{15}N$] and L-[U-$^{12}C^{14}N$]-amino acids at 55 °C, 75 °C and 95 °C for 10 min, 30 min and 60 min, while **Figure 7** illustrates the results for the racemization of the same test solution at 95 °C for 1 h, 6 h and 15 h. In both cases the final concentration was 0.025 mM of scalemic mixtures containing DL-[U-$^{12}C^{14}N$]- amino acid and DL-[U-$^{13}C^{15}N$]-amino acids.

In general, only a slight stereoisomerization (below 1%) occurred for Val, Thr, Tyr, His, Lys and Arg after incubation at 55 °C for 30 min. Surprisingly high values were found for [U-

$^{13}C^{15}N$]-Ser, reaching above the 1% level. Therefore a derivatization time of 10 min at 55°C should not be exceeded. Note that under this condition no racemization was observed. However, significant racemization was only observed for the incubation experiment at 95 °C for 30 min and 60 min, providing the highest values for Asn, Tyr, Glu, Gln, His and Val, which went beyond the 5% D-amino acid level with highest values obtained for [U-$^{12}C^{14}N$]-Asn with 8% and [U-$^{13}C^{15}N$]-Tyr with 7%. Lowest values were obtained for Pro, which racemized rather poorly.

< add Figure 6 >

The follow-up study, employing 95 °C for racemization combined with increased incubation times up to 15 h was performed in triplicates (n=3). Since a cleavage of the AQC-tag was observed, all samples were re-derivatized with AQC prior to HPLC-ESI-QTOF-MS analysis (sample (A)). To evaluate, if there was a general trend, whether the original L-amino acid or the generated D-amino acid lost their AQC-tag predominantly, the 15 h incubated samples were only diluted 1:10 with 0.2 M borate buffer without re-derivatization (sample (B)). A comparison of D-amino acid levels of re-derivatized (A) and diluted (B) samples in **Figure 7** showed that an overall larger percentage of L-amino acids had lost their AQC-tags, since the diluted samples provided higher AQC-D-amino acid levels.

Hence it can be assumed that with increasing incubation time and temperature, more and more AQC-tags will be cleaved leading to a natural recession in stereoisomerization, since only AQC-tagged amino acids are isomerized. Furthermore note that the stereoisomerization results for Met could only be achieved due to ultasonication of the samples after derivatization with AQC and performing the stereoisomerization reaction in the absence of air in inert nitrogen atmosphere **(Figure S11)**. The same experiment as shown in **Figure 7** performed in the presence of oxygen had led to the absence of AQC-Met as earlier shown in **Figure 5**.

In general, an increase in incubation time from 1 h to 6 h and 15 h, increased the racemization rate of all amino acids by 10% to 15%, with exception of Met, for which 20% D-Met was found after 6 h of heating. Increasing the treatment time further had only led to the

complete oxidation of Met. In actual fact, if a chiral [U-$^{13}C^{15}N$]-amino acid standard solution with D-amino acid levels below 5% would be favored, 30 min incubation at 95 °C was sufficient. For 1 h heat treatment at 95 °C, overall racemization rates from 2-10% were observed, while for D-amino acid levels around 15-25%, which included Met, 6 h incubation at 95 °C was adequate. An incubation time of 15 h, however, led to D-amino acid values between 20% and 45%, depending on the amino acid, but this increase went hand in hand with total loss of Met. Pro however showed maximum D-amino acid values around 3.5% with little improvement with time. An additional study concerning the influence of sample solute type, buffer type, as well as molarity and pH-value of the borate buffer on stereoisomerization rate and amino acid composition will be discussed separately in a follow-up article [58].

CONCLUSIONS

The here described stereoisomerization method is simple, derivatize your sample with AQC, which takes 10 min, then ultrasonicate for 1 min, purge with inert gas for 1 min and heat the sample for 30 min or up to 15 h at 95 °C on a thermoshaker. Stereoisomerization takes place without loss of Gln, Asn or Trp. The generated D-amino acid levels depend on the racemization temperature and the incubation time. If a D-amino acid content between 1-5% is required, a stereoisomerization time of 30 min at 95 °C is sufficient. Increasing the incubation time to 60 min provides [U-$^{13}C^{15}N$]-labeled amino acid standard mixtures containing 2-10% D-amino acids, 6 h and 15 h incubation time, increases the D-amino acid levels to 15-30% and 20-45%, respectively. Note that a slight racemization of <0.5% was observed for 30 min to 60 min racemization time at 55 °C. Hence for chiral separation of AQC-derivatized amino acids, the derivatization condition of 55 °C for 10 min should not be exceeded in temperature nor derivatization time.

Chiral separation of all proteinogenic amino acids and some uncommon amino acids such as aIle, aThr, Hse, Cit and theanine has been performed on a chiral zwitterionic Chiralpak ZWIX(+) column using a dual elution gradient, which included a hydro-organic gradient from 2% to 52% water content and a flow gradient from 0.2 mL/min to 0.5 mL/min. This use of a

dual gradient for enantiomer separation and fast elution leads unfortunately to enantioselective matrix effects providing enantiomer peaks with different peak height and peak areas, which may cause inaccurate results in LC-MS based enantioselective metabolomics if only an L-enantiomer containing isotopically labelled metabolite solution is used as an internal standard for the quantification of both L- and D-enantiomers. The use of a chiral uniformly isotopically labelled internal standard would overcome such enantioselective matrix effect issues in quantitative chiral analysis. The here presented package of an easy and simple procedure to generate tailor made internal standard with desired D-amino acids composition of natural and uncommon amino acids with desired D-amino acid levels, combined with an universal chiral separation protocol for the zwitterionic Chiralpak ZWIX(+) column, increases flexibility and will in the near future facilitate highly accurate quantitative chiral amino acid metabolomics studies performed in a simple derivatize, racemize & analyze approach.

ASSOCIATED CONTENT

Supporting Information

Reaction tubes sealed with Mμlti®-lid locks and Parafilm, stereoisomerization of AQC-L-Phg in various solvent systems, stereoisomerization conditions tested on DNS, DNB, CBZ, FMOC and NBD amino acids as well as AQC-β-D-Phe, chiral separation of isobaric AQC-Thr and AQC-Ile isomers, XICs of scalemic mixtures of AQC-[U-$^{12}C^{14}N$]- and AQC-[U-$^{13}C^{15}N$]-amino acids, void volume markers for the Chiralpak ZWIX(+) column, and stereoisomerization of AQC-Met under oxygen free condition and enantioselective matrix effect under different elution conditions (PDF).

Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes: The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1. Stereoselective separation of scalemic AQC-DL-[U-$^{13}C^{15}N$]-Phe, APC-DL-Phe and AC-DL-[U-$^{13}C^{15}N$]-Phe on a Chiralpak ZWIX(+) column (150 x 4 mm; 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS, after racemization at 95 °C for 18 h. Note that only representative chromatograms are shown, since racemization was performed with sample mixtures containing L-[U-$^{12}C^{14}N$]- and L-[U-$^{13}C^{15}N$]- amino acids.

Figure 2. Stereoselective separation of scalemic mixtrures of isobaric isomers of **a)** AQC-Thr ([AQC-Thr + H]$^+$: *m/z* 290.1135) and **b)** AQC-Ile ([AQC-Ile + H]$^+$: *m/z* 302.1499) on a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS, after epimerization of L-Thr, L-aThr, DL-Thr and DL-aThr as well as L-Ile, L-aIle, DL-Ile and DL-aIle for 18 h at 95 °C. Note that newly generated isomers are highlighted in bold red.

Figure 3. XICs of AQC derivatized **a)** DL-Asn, DL-Asp, DL-Gln and DL-Glu; **b)** XIC of AQC derivatized L-Asn and L-Gln before racemization and **c)** after racemization for 15 hours at 95 °C under nitrogen gas. The XIC traces for Asp and Glu in **b)** and **c)** show that no Asp and no Glu were present before nor after the racemization process. Stereoselective separation was performed with a Chiralpak ZWIX(+) column (150 x 3 mm, 3 µm) using a short gradient elution method in combination with HPLC-ESI-QTOF-MS analysis.

Figure 4. XICs of AQC derivatized **a)** L-theanine and L-Cit as well as their deamidation products AQC-Glu and AQC-Orn before racemization and **b)** after racemization for 15 hours at 95 °C under nitrogen gas. Note that no deamidation products were observed before nor after the racemization process. Stereoselective separation was performed with a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method in combination with HPLC-ESI-QTOF-MS.

Figure.5. XICs of scalemic AQC-derivatized [U-$^{13}C^{15}N$]-amino acids (black; without Asn, Gln and Trp) together with three [U-$^{12}C^{14}N$]-amino acids, namely the missing amino acids Asn, Gln and Trp (red; the remaining amino acids are shown in the **Supporting Information**) after racemization for 6 h at 95 °C. Separation factors α(L-enantiomer/D-enantiomer) are provided using a void time of 7.041 min. Note that for L-Ile and L-Thr, racemization will provide L-aIle and L-aThr, hence α(L/D) are shown for α(L-Ile/D-Ile), α(L-Ile/D-aIle), α(L-Thr/D-Thr) and α(L-Thr/D-aThr). Reference chromatograms for the separation of DL-aIle and DL-aThr are provided in the **Supporting Information**. Stereoselective separation was performed on Chiralpak ZWIX(+) (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS in positive ionization mode. Gaussian Smoothing Factor 2.5.

Figure6. D-Amino acid content in percent after stereoisomerization of a solution containing 0.025 mM AQC-derivatized **a)** L-[U-$^{12}C^{14}N$]-amino acids (20 amino acids) and **b)** L-[U-$^{13}C^{15}N$]-amino acids (17 amino acids without Asn, Gln and Trp) for 10 min, 30 min and 1 h at 55 °C, 75 °C and 95 °C. D-Amino acid results were normalized using the peak areas of the corresponding enantiomers from a racemic AQC-[U-$^{12}C^{14}N$]-DL-amino acid mixture. Stereoselective separation was performed on the Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS.

Figure 7. D-Amino acid content in percent after stereoisomerization of an AQC-derivatized amino acid solution containing 0.025 mM of **a)** L-[U-$^{12}C^{14}N$]-amino acids (20 amino acids) and **b)** L-[U-$^{13}C^{15}N$]-amino acids (without Asn, Gln and Trp) for 1 h, 6 h and 15 h at 95 °C. Experiments were performed in triplicates (n=3). All samples were centrifuged after reaching room temperature, their volumes were adjusted to 100 µL and they were additionally derivatized with AQC (samples (A)), which resembles a 1:10 dilution. Additionally, the 15 h samples were only diluted 1:10 with 0.2 M borate buffer, without additional re-derivatization (samples (B)). Sample (B) showed higher D-amino acid levels compared to sample A, which illustrates the cleavage of the AQC tag from the L-enantiomer and not from the

stereoisomerized D-enantiomers. Stereoselective separation was performed on a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS.

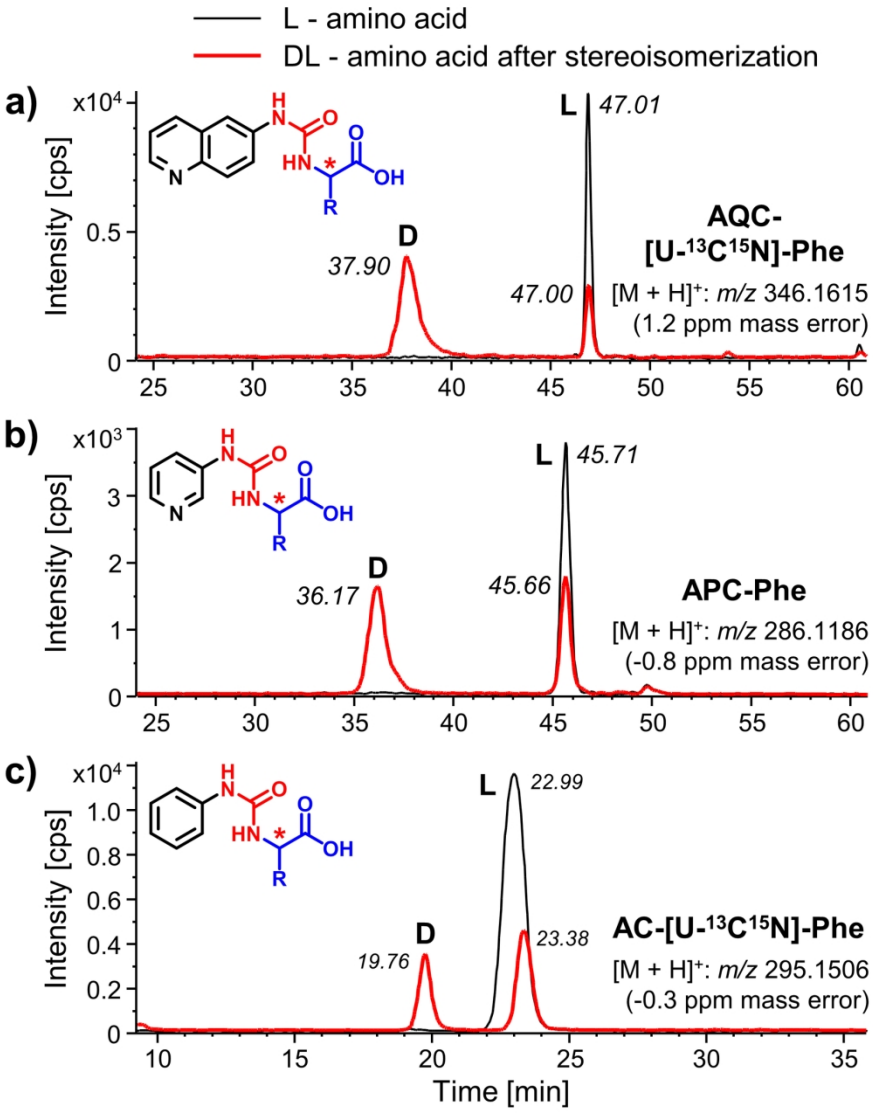

Figure 1. Stereoselective separation of scalemic AQC-DL-[U-13C^{15}N]-Phe, APC-DL-Phe and AC-DL-[U-^{13}C^{15}N]-Phe on a Chiralpak ZWIX(+) column (150 x 4 mm; 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS, after racemization at 95 °C for 18 h. Note that only representative chromatograms are shown, since racemization was performed with sample mixtures containing L-[U-^{12}C^{14}N]- and L-[U-^{13}C^{15}N]- amino acids.

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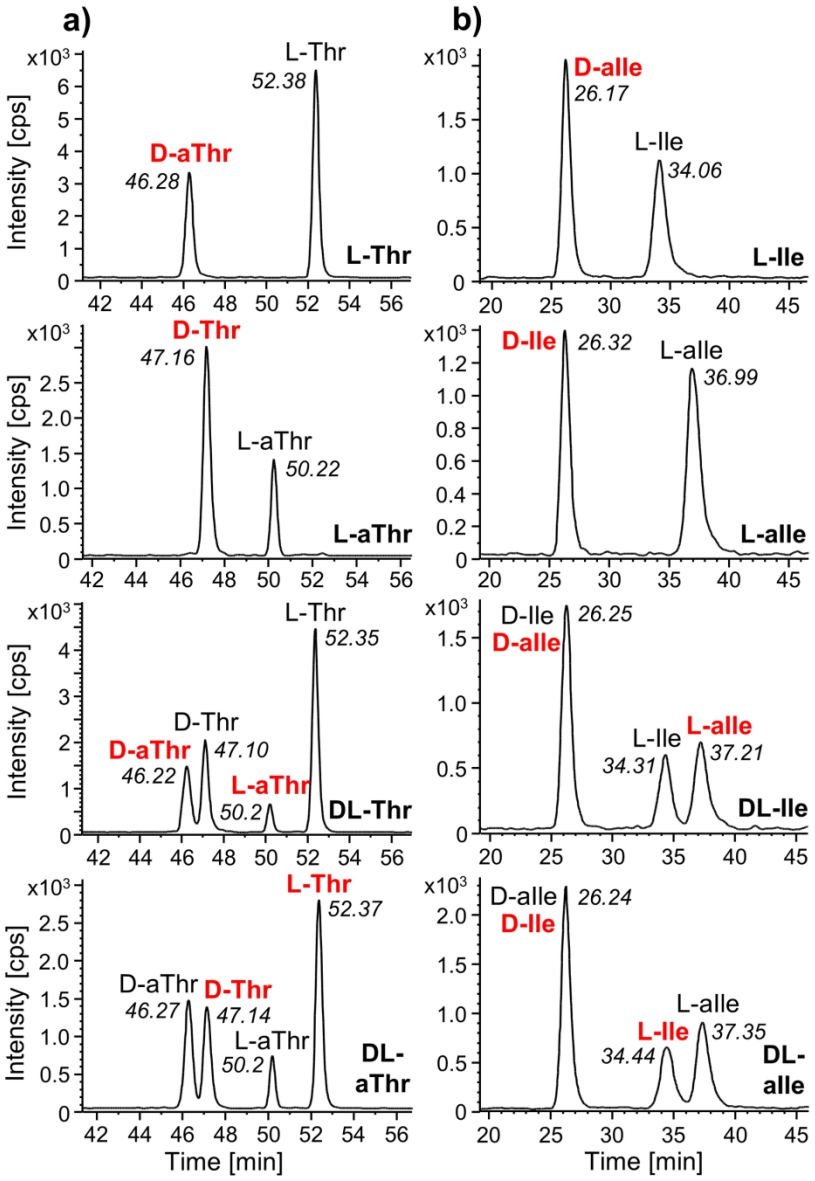

Figure 2. Stereoselective separation of scalemic mixtrures of isobaric isomers of a) AQC-Thr ([AQC-Thr + H]$^+$: m/z 290.1135) and b) AQC-Ile ([AQC-Ile + H]+: m/z 302.1499) on a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS, after epimerization of L-Thr, L-aThr, DL-Thr and DL-aThr as well as L-Ile, L-aIle, DL-Ile and DL-aIle for 18 h at 95 °C. Note that newly generated isomers are highlighted in bold red.

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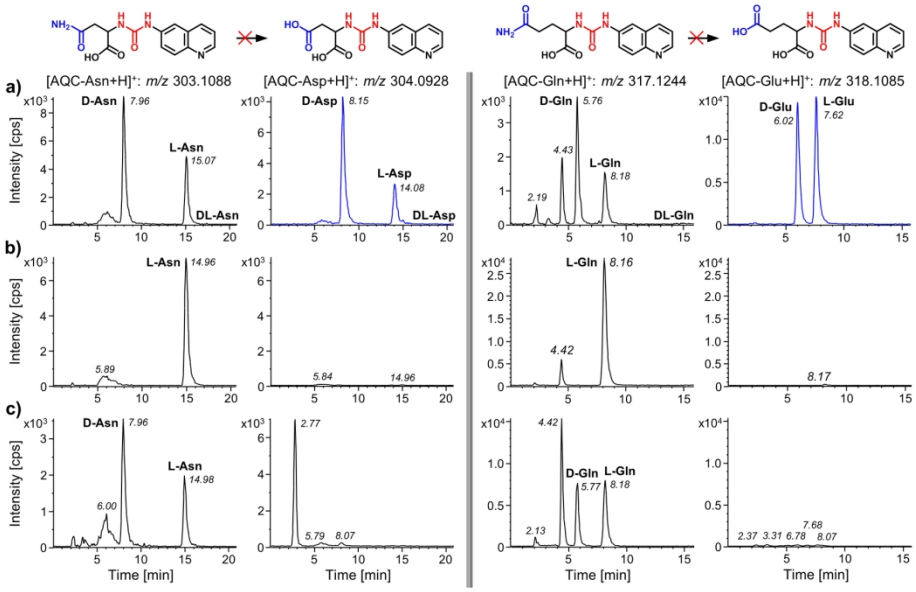

Figure 3. XICs of AQC derivatized a) DL-Asn, DL-Asp, DL-Gln and DL-Glu; b) XIC of AQC derivatized L-Asn and L-Gln before racemization and c) after racemization for 15 hours at 95 °C under nitrogen gas. The XIC traces for Asp and Glu in b) and c) show that no Asp and no Glu were present before nor after the racemization process. Stereoselective separation was performed with a Chiralpak ZWIX(+) column (150 x 3 mm, 3 µm) using a short gradient elution method in combination with HPLC-ESI-QTOF-MS analysis.

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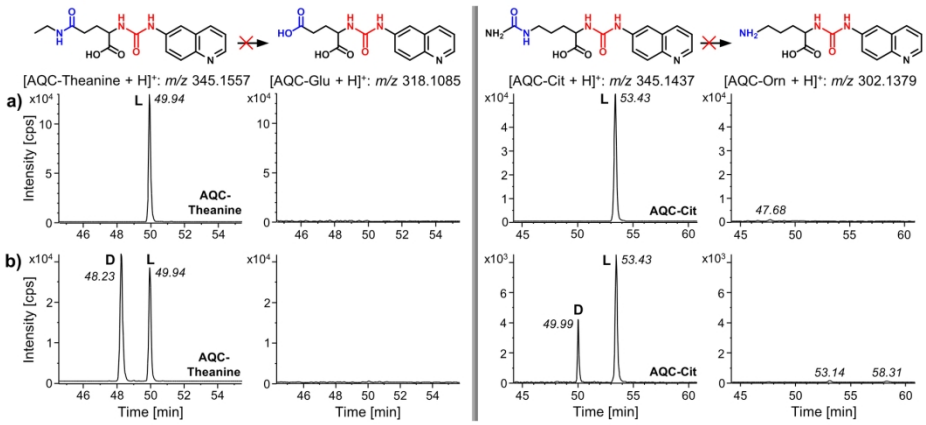

Figure 4. XICs of AQC derivatized a) L-theanine and L-Cit as well as their deamidation products AQC-Glu and AQC-Orn before racemization and b) after racemization for 15 hours at 95 °C under nitrogen gas. Note that no deamidation products were observed before nor after the racemization process. Stereoselective separation was performed with a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method in combination with HPLC-ESI-QTOF-MS.

174x74mm (300 x 300 DPI)

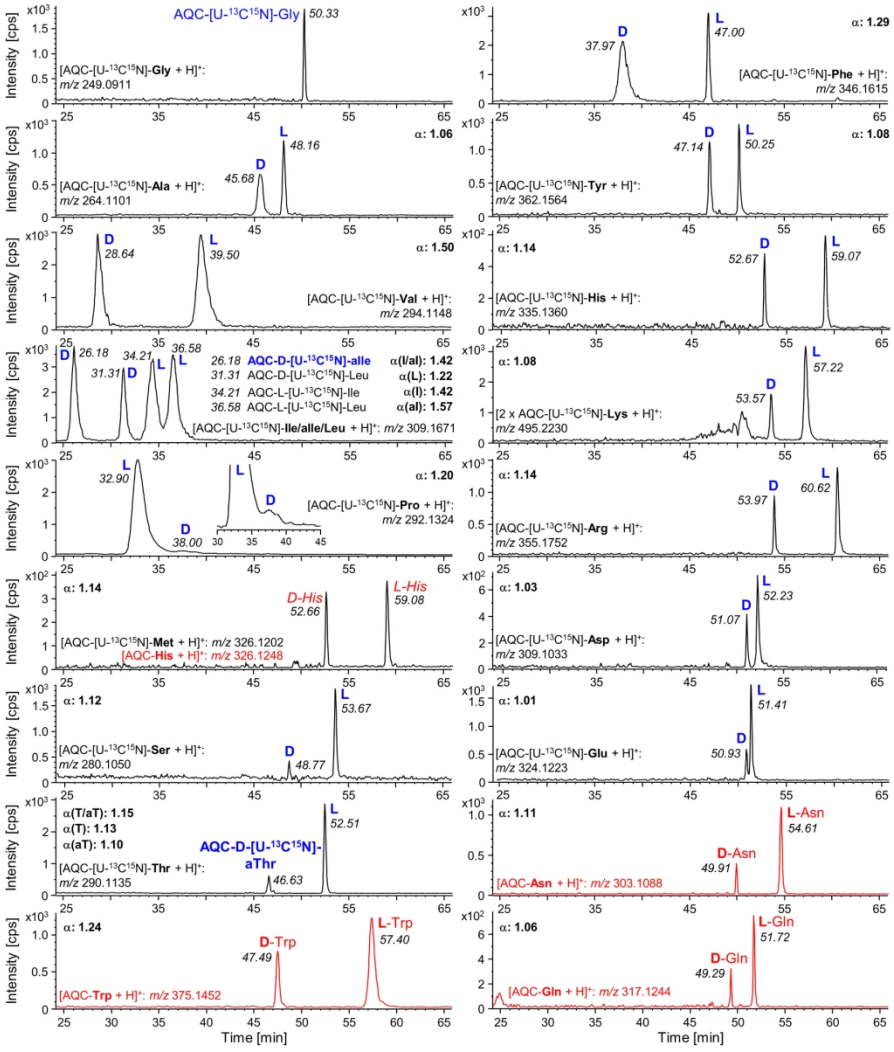

Figure.5. XICs of scalemic AQC-derivatized [U-13C15N]-amino acids (black; without Asn, Gln and Trp) together with three [U-12C14N]-amino acids, namely the missing amino acids Asn, Gln and Trp (red; the remaining amino acids are shown in the Supporting Information) after racemization for 6 h at 95 °C. . Separation factors α(L-enantiomer/D-enantiomer) are provided using a void time of 7.041 min. Note that for L-Ile and L-Thr, racemization will provide L-aIle and L-aThr, hence α(L/D) are shown for α(L-Ile/D-Ile), α(L-Ile/D-aIle), α(L-Thr/D-Thr) and α(L-Thr/D-aThr). Reference chromatograms for the separation of DL-aIle and DL-aThr are provided in the Supporting Information. Stereoselective separation was performed on Chiralpak ZWIX(+) (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS in positive ionization mode. Gaussian Smoothing Factor 2.5.

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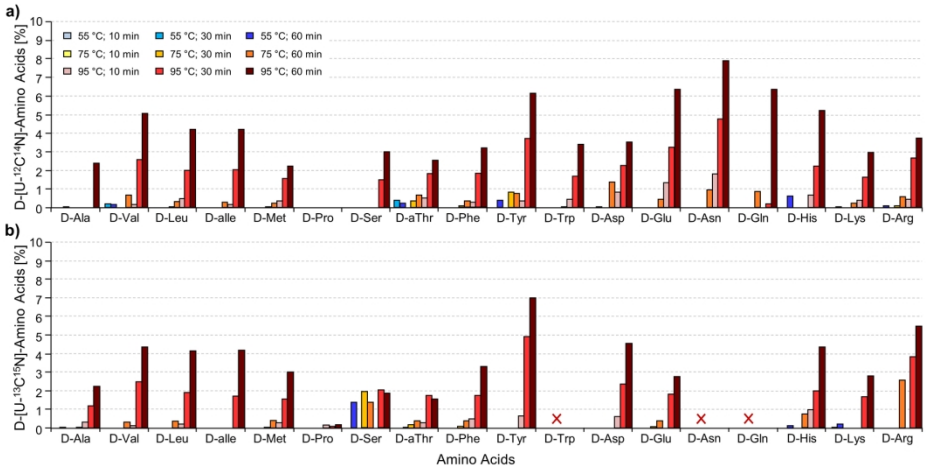

Figure6. D-Amino acid content in percent after stereoisomerization of a solution containing 0.025 mM AQC-derivatized a) L-[U-^{12}C14N]-amino acids (20 amino acids) and b) L-[U-^{13}C15N]-amino acids (17 amino acids without Asn, Gln and Trp) for 10 min, 30 min and 1 h at 55 °C, 75 °C and 95 °C. D-Amino acid results were normalized using the peak areas of the corresponding enantiomers from a racemic AQC-[U-^{12}C^{14}N]-DL-amino acid mixture. Stereoselective separation was performed on the Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS.

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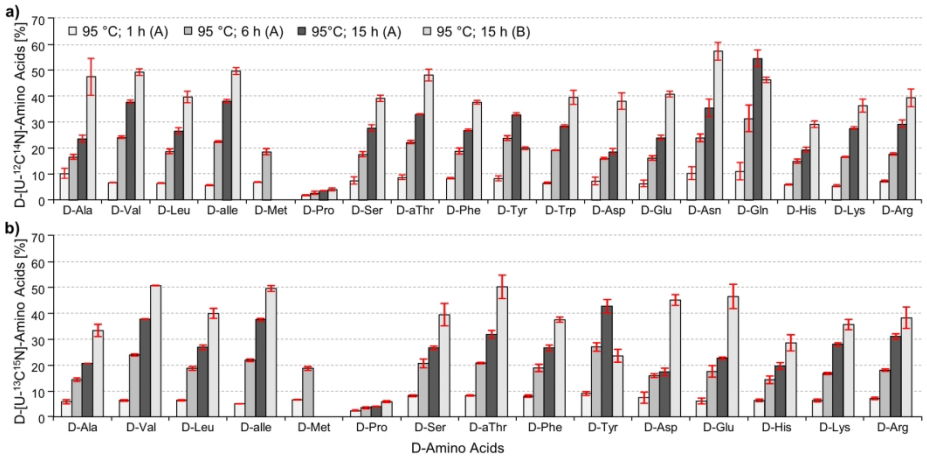
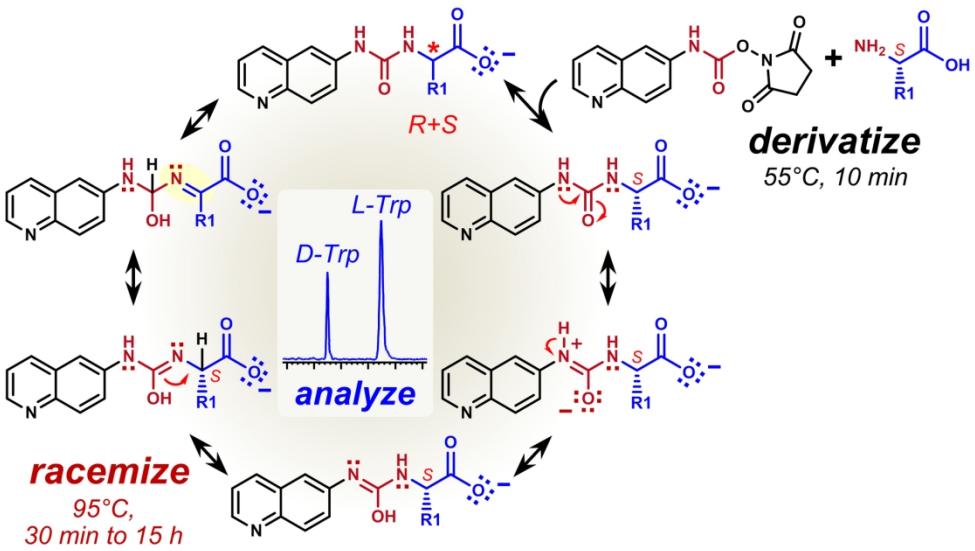


Figure 7. D-Amino acid content in percent after stereoisomerization of an AQC-derivatized amino acid solution containing 0.025 mM of a) L-[U-^{12}C14N]-amino acids (20 amino acids) and b) L-[U-^{13}C^{15}N]-amino acids (without Asn, Gln and Trp) for 1 h, 6 h and 15 h at 95 °C. Experiments were performed in triplicates (n=3). All samples were centrifuged after reaching room temperature, their volumes were adjusted to 100 µL and they were additionally derivatized with AQC (samples (A)), which resembles a 1:10 dilution. Additionally, the 15 h samples were only diluted 1:10 with 0.2 M borate buffer, without additional re-derivatization (samples (B)). Sample (B) showed higher D-amino acid levels compared to sample A, which illustrates the cleavage of the AQC tag from the L-enantiomer and not from the stereoisomerized D-enantiomers. Stereoselective separation was performed on a Chiralpak ZWIX(+) column (150 x 4 mm, 3 µm) using the dual gradient elution method with HPLC-ESI-QTOF-MS.

174x84mm (300 x 300 DPI)



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