EBERHARD KARLS
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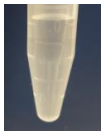
First Method for Synthesizing Chiral Isotopically labelled Internal Standards

Technology Description

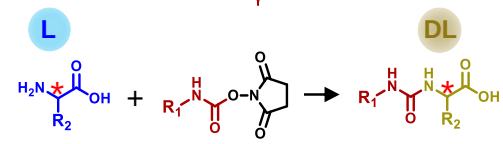


[U-^{13}C^{15}N]-AA metabolite mix

[U-^{13}C]-Algal lyophilised cells

HeLa cell pellet

applicable for standard AA-solutions, mammalian cancer cells, algal cells as well as other cell types such as yeast and bacteria cells



cell extract with amino acids and metabolites

racemization-tag

a) derivatisation at 55°C
b) racemization at 95°C

Elevated levels of D-amino acids (AA) were identified as valid biomarkers for diseases (e.g. chronic kidney disease). For this reason enantioselective (chiral) metabolomics is an emerging field in clinical diagnostic research. For accurate quantitative results and data normalization, isotopically labelled internal standards (IS) are essential, but their production remains cost-intensive and is partially even not possible so far. We have developed the first racemization procedure, which allows the production of a chiral isotopically labelled IS mixture (e.g. [U-^{13}C] or [U-^{13}C^{15}N]) of all proteinogenic AAs, their isobaric analogues and uncommon AA-type metabolites with pre-defined tailored enantiomer composition in two simple steps: Derivatize and racemize. The result is the most simple, cost-effective and labor-free racemization procedure presently available, enabling also direct MS-analysis of AA composition in any given metabolic probe.

Innovation

Up to now:

No simple racemization method available without degradation of labile AA (e.g. Asn, Gln and Trp). The classic methods involving Schiff-base formation in concentrated acid and high temperatures also required the necessity to purify the product to make it MS-compatible. No complete chiral IS for AAs in biological probes available.

Now:

Derivatize, Racemize & Analyze – simple and degradation-free racemization of 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) tagged proteinogenic and uncommon amino acids as well as peptides provides tailored R/S compositions, without purification, without degradation and offers full MS-compatibility. First-ever procedure to racemize isotopically labelled metabolites directly in biological matrices.

Advantages & Applications

- Simple & easy, fast & straight forward
- No degradation (e.g. Trp), no purification
- No degradation of labile AAs such as Asn, Gln, Trp, Cit, theanine, etc.
- Oxidation free racemization of Met
- Fully MS-compatible
- Applicable for common and uncommon AAs as well as peptides
- Applicable in buffered aqueous solution
- Applicable over a wide pH range incl. pH 7.00
- Applicable in organic solvents such as DMSO
- Preparation of scalemic DL-[U-^{13}C^{15}N]-AA metabolite IS solution enables correction of enantioselective matrix effects.
- Preparation of scalemic isotopically labelled metabolite IS solutions directly in the biological matrix (e.g. cell extract, biological fluid) allows for the first time the design of IS standard kits for accurate LC-ESI-MS/MS analysis of D-AAs.

Requested Cooperation

Industrial partners for licensing and collaboration

IP Status

Priority Application with the EPO: 18197409.8
US and JP: Application filed

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EXPERIMENTAL DATA

Preparation of an [U-$^{13}C^{15}N$]-labelled Amino Acid Metabolite Internal Standard Mixture

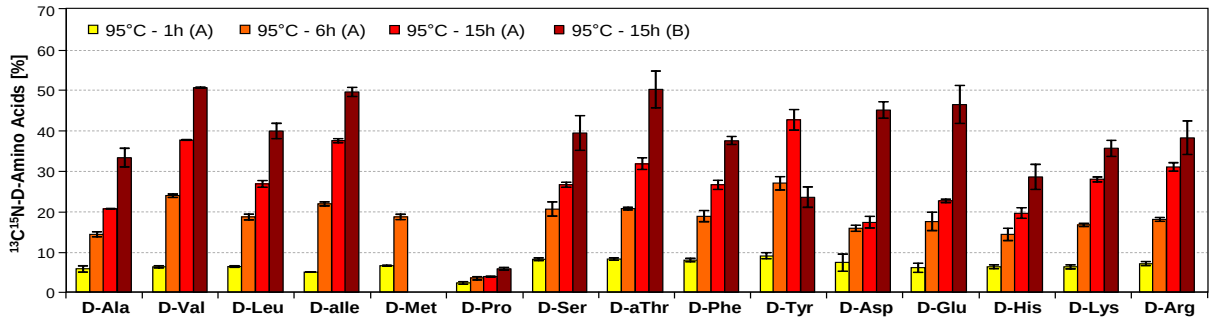

Figure 1: *D-amino acid content [%] of a scalemic AQC-[U-$^{13}C^{15}N$]-AA metabolite mixture after stereoisomerization at 95°C for 1h, 6h and 15h. Note that prior to racemization, the samples were derivatized with AQC at 55 °C for 10 min. Sample (A) shows the generated D-AA percentage after 1:10 dilution with buffer solution, while sample (B) illustrates the result after re-derivatization with AQC, which resembles a 1:10 dilution.*

Figure 1 shows that the amount of generated D-AAs was directly correlated with the racemization time and the racemization temperature, which was employed. If a chiral [U-$^{13}C^{15}N$]-AA standard solution with D-AA content ≤ 5% was desired, 30 min racemization at 95 °C would be sufficient. In case of 1h racemization at 95 °C, an overall D-AA content of 2-10% was obtained, while for D-amino acid levels around 15-25%, 6h heating at 95 °C was enough. For D-AA values between 20% and 45%, a racemization time of 15h was needed. However, an increase in racemization time had led to the complete loss of Met due to oxidation. This problem was overcome by performing the racemization reaction under oxygen free condition, after ultrasonication and purging with nitrogen. Furthermore, only low racemization rates of max. 3.5 % were found for Pro, which did not improve with an increase in racemization time [1, 2].

N-Terminal Epimerization of Tri-Peptide Arg-Gly-Asp

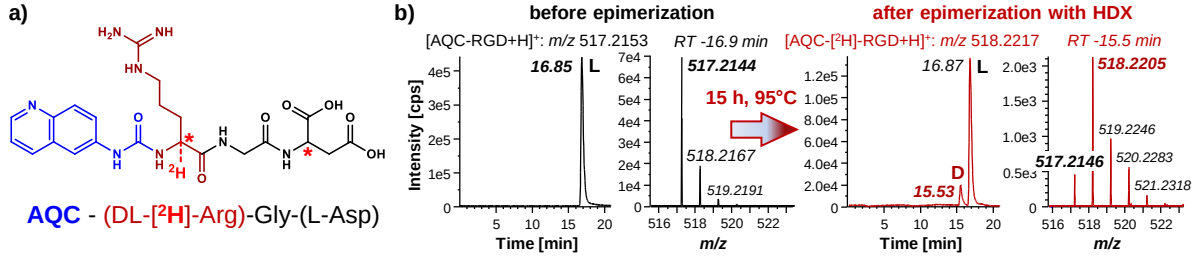

Figure 2: *Epimerization of the tripeptide AQC-Arg-Gly-Asp in deuterated borate buffer for 15h at 95 °C. Chiral separation was performed on a Chiralpak ZWIX(+) column (150 x 4 mm; 3µm).*

Figure 2a shows chemical structure of the AQC-tagged tripeptide Arg-Gly-Asp with a hydrogen-deuterium exchange (HDX) at the chiral center of Arg. Figure 2b shows the extracted ion chromatograms (XICs) and the mass spectra of AQC-Arg-Gly-Asp before and after racemization. The comparison of the before and after XICs shows clearly the generation of an additional peak at 15.53 min, which was proven to be the D-enantiomer peak via comparison of the mass spectra of L- (16.9 min) and D-AQC-Arg-Gly-Asp (15.5 min). Note that the HDX exchange, which takes place during the racemization event (and hence is a mass spectrometric proof that racemization took place) leads to the addition of 1 Da (*m/z* 517 → *m/z* 518) for the mono-isotopic peak.

REFERENCES

1 Horak J. and M. Lämmerhofer M.; Derivatize, Racemize and Analyze - an Easy and Simple Procedure for Chiral Amino Acid Standard Preparation for Enantioselective Metabolomics; *Anal. Chem.* (2019), 91, 7679 DOI: 10.1021/acs.analchem.9b00666

2 Horak J. and Lämmerhofer M.; Racemization without deamidation: Effect of racemization conditions on 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate tagged amino acids; J. Chromatog. A Volume 1604, 25 October 2019, 460492, doi.org/10.1016/j.chroma.2019.460492