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Isotope dilution with isotopically labeled biomass: An effective alternative for quantitative metabolomics

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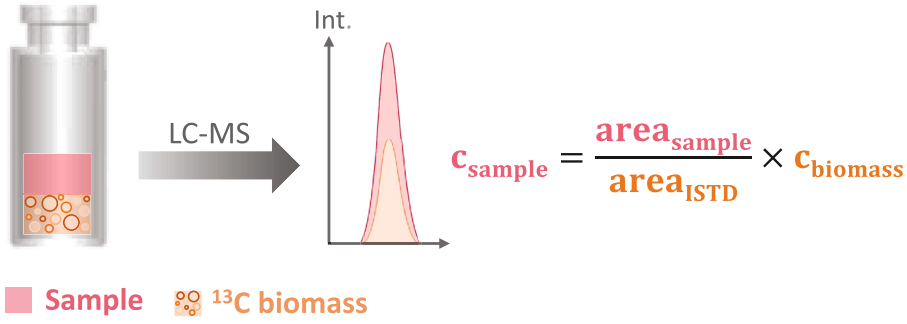
HIGHLIGHTS

- Characterized isotopically labeled biomass is used for single-spike isotope dilution.
- Exceptionally wide analyte coverage for compound-specific standardization of the hydrophilic metabolome.
- The concentrations of fully ^{13}C-labeled metabolites are measured in a commercially available ^{13}C-labeled yeast extract.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: State-of-the-art quantitative metabolomics relies on isotope dilution using internal standards (IS) derived from fully ^{13}C labeled biomass. By spiking samples and external standards with known amounts of IS, the spike characterization demands are kept to a minimum. In fact, it is sufficient to experimentally assess the isotopic enrichment of the IS. This study develops the yeast derived IS toolbox further, (1) by characterizing the concentration levels of hydrophilic metabolites in a yeast fermentation batch and (2) by exploring the analytical figures of merit of one-point IS versus multipoint external calibration using IS, the established gold-standard for quantitative metabolomics.
Results: Independent reverse isotope dilution experiments using different chromatographic methods over a period of several months, delivered a list of 83 ^{13}C-labeled metabolites with fully characterized concentration and their uncertainty, covering 5 orders of magnitude, from the nanomolar to the low millimolar range. The ^{13}C-labeled yeast-derived IS showed excellent intermediate stability with 92 % of molecules showing inter-method RSDs $\leq$30 % (75 % of molecules showed RSDs $\leq$15 %) over a timeframe of five months. One-point internal standardization with the characterized labeled biomass achieved figures of merit equivalent to multipoint calibrations for the majority of metabolites.

Abbreviations: IS, Internal standard (fully and uniformly ^{13}C-labeled biomass extract from *Komagataella phaffii*).

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Significance: The proposed calibration workflow rationalizes time and standard expenditure and is particularly beneficial for laboratories dealing with wide-target assays and small analysis batches. The present assessment serves as a seminal study for further developments of the concept towards absolute quantification from archive high-resolution MS data of U^{13}C-biomass-spiked samples and the implementation of quick biomass recalibration with each experiment, promising seamless transition between internal standards derived from different fermentation batches.

1. Introduction

Quantitative LC-IDMS-based metabolomics requires a high number of external and isotopically labeled internal standards, with ^{13}C as the preferred isotopic label due to the universal presence of carbon in metabolites and neglectable isotope effects [1–3]. While class-specific surrogate standardization and the implementation of response factors is a common approach to deal with the restricted availability of pure labeled standards in lipidomics [4,5], hydrophilic metabolites are very diverse in structure and chemical behavior and accurate LCMS-based quantification requires compound-specific standardization [6,7]. A practical solution to counter the relative unavailability of pure isotopically labeled standards (lacking commercial availability and/or high cost) is to use biomass extracts of ^{13}C-enriched microorganisms as internal standard [1]. Specifically, the yeast *Komagataella phaffii* can be grown on a single carbon source under controlled fermentation conditions and produces a whole yeast metabolome with high ^{13}C-enrichment. Fully (>98 %) and uniformly labeled metabolite extracts from *K. pfaffii* are commercially available and allow straightforward data interpretation [8]. The extensive array of structurally diverse labeled standards found in biomass extracts facilitates compound-specific standardization. This includes co-extraction, co-elution, and co-ionization with the analyte, thereby addressing variations in sample preparation procedures, matrix effects, and instrument performance across a broad

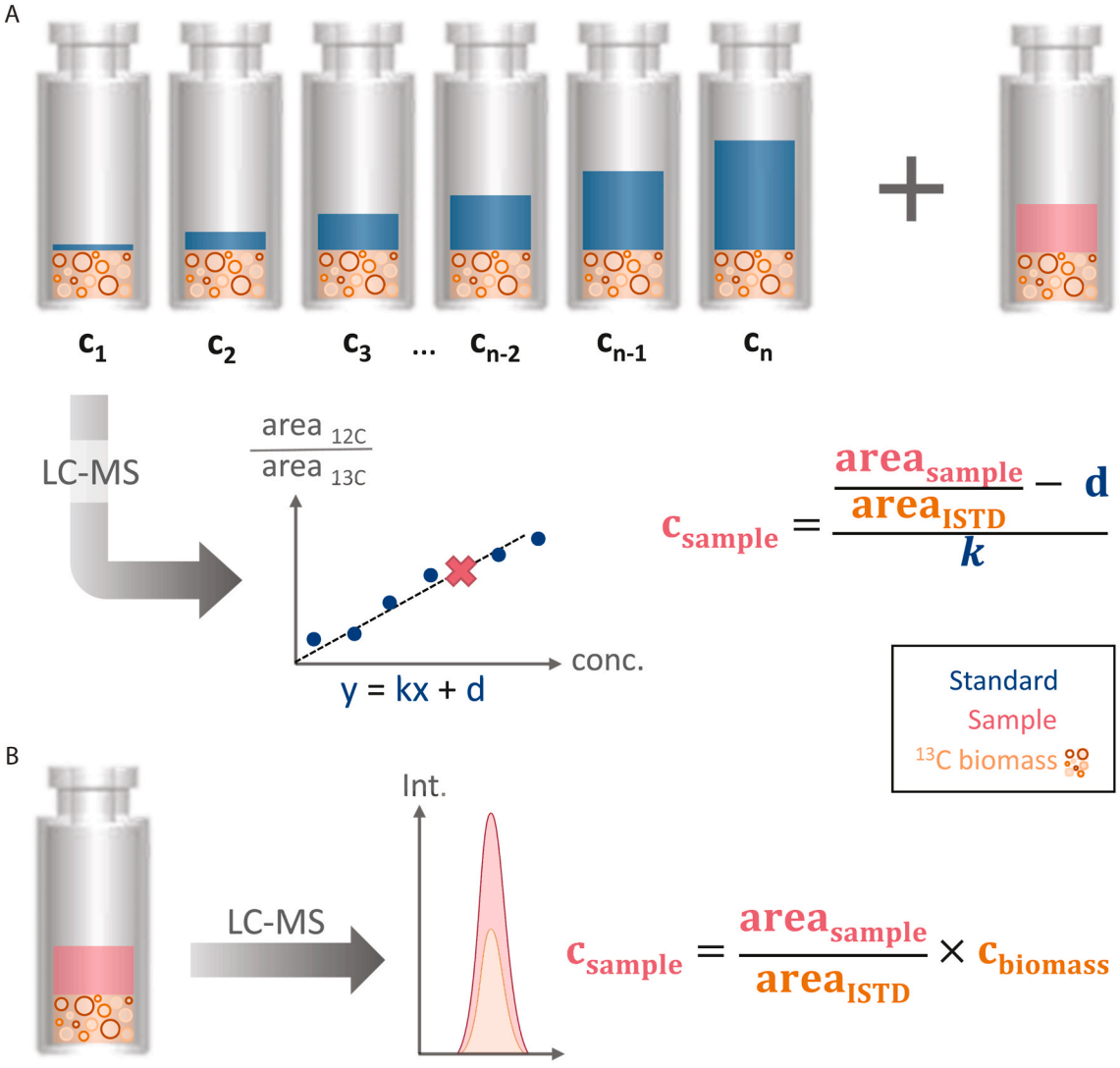


Fig. 1. Workflow of one-point calibration with characterized isotopically labeled biomass compared to the gold-standard procedure
A) IS-normalized external multi-point calibration injected with each analytical experiment (gold-standard). **B)** One-point calibration with characterized isotopically labeled biomass as internal standard. Multi-point calibration is omitted and the daily calibration workload is reduced to an IS-spiked sample injection. U^{13}C-labeled metabolites are quantified using multi-point calibration independently from sample analysis.

spectrum of analytes. This capability represents a significant advantage for the quantitative analysis of endogenous hydrophilic metabolites. Using isotopically labeled biomass extracts as an internal standard (IS), the respective concentrations of the isotopically labeled metabolites are usually not characterized. Only the fact that samples and external standards are spiked with known amounts of the ^{13}C-labeled biomass extract allows to exploit the power of isotope dilution. Each measurement sequence integrates IS-normalized multi-point calibrations and metabolite quantification is based on peak area ratios of unlabeled versus labeled metabolite assessed in each sample (Fig. 1A). This established practice has the potential of providing the highest metrological order [1], however, the required amounts of standard and measurement time still thwart the need for cost-effectiveness and scalability of quantitative metabolomics. The standards and time consumed by multi-point calibration create a cost-overhead per experiment that is particularly unfavorable for laboratories performing multi-analyte assays in small analysis batches [9], which is often seen in metabolomics: An estimated 25 % of metabolomics studies analyze 20 or less samples per study based on studies published on the MetaboLights open repository for metabolomics data (N = 1076 accessible studies) [10]. By contrast, one-point internal standardization minimizes the time and standard expenditure necessary for each experiment. The sample is spiked with a single concentration level of isotopically labeled internal standard. The concentration value of the IS is characterized via reversed isotope dilution and given with characterized uncertainty. The metabolite panel is henceforth quantified based on the respective unlabeled-to-labeled isotopologue intensity ratio and the given IS metabolite concentration (Fig. 1B). Compound-specific one-point calibration via an internal standard spike provides accurate quantification results as the analyte-to-IS area ratio is robust towards concentration and matrix-related ion suppression. It has been discussed as a means to simplify quantification procedures [11–14] and potentially improves inter-run precision compared to run-specific multi-point calibration [13, 14]. One-point calibration with isotopically labeled biomass, in principle, involves multi-point calibration because the internal standard spike is characterized via multi-point calibration in reversed isotope dilution experiments with validated analytical assays in the first place. Nevertheless, it relieves the user from the necessity of conducting external multi-point calibration with every experiment. (Fig. 1). Here, we explore if it is valid to take this shortcut and perform one-point internal standardization established through characterized isotopically labeled biomass for the quantitative assessment of hydrophilic metabolites, combining the wide selection of compound-specific labeled standards with the simplicity of single spike calibration. We (1) examine a commercially available, uniformly ^{13}C-labeled metabolite extract produced in controlled yeast fermentations, (2) retrospectively quantify uniformly ^{13}C-labeled central carbon metabolite concentrations and assemble a list of internal standards for reference, (3) investigate accuracy and precision of one-point calibration compared to the gold-standard (IS-normalized external multi-point calibration measured with each experiment) and literature, (4) discuss critical steps for successful implementation of one-point calibration using isotopically labeled biomass.

2. Experimental

We analyze SRM 1950 plasma metabolite reference material spiked with a fully (>98 %) and uniformly ^{13}C-labeled hydrophilic yeast metabolite extract on two pH-complementary LC platforms (alkaline and acidic separation conditions) coupled to high-resolution orbitrap MS, focusing a panel of 149 hydrophilic central carbon metabolites.

2.1. Solvents, standards, and internal standard

Chemicals. Acetonitrile (ACN), methanol (MeOH), and water were of LC-MS grade and ordered at Fisher Scientific (Vienna, Austria) or Sigma Aldrich (Vienna, Austria). Ammonium acetate and ammonium hydroxide were ordered as the eluent additive for LC-MS at Sigma Aldrich. Formic acid was also of LC-MS grade and ordered at VWR International (Vienna, Austria). ISO1, a uniformly ^{13}C-labeled metabolite dry extract obtained from the yeast *Komagataella phaffii* with a metabolite labeling degree >98 %, was obtained from ISOtopic solutions e.U. (Vienna, Austria) and stored at −80 °C. One vial of the labeled dry extract was reconstituted and used as an internal standard spike. Reconstitution volumes and solvent differed in the data sets: For the alkaline LC data sets, one tube of ISO1 was reconstituted in 3 mL methanol/water 1:1 (v/v) and aliquots were stored at −80 °C until further use. An aliquot was thawed at room temperature and used as an internal standard for each experiment. For the runs using acidic LC conditions, one tube of ISO1 was reconstituted in 2 mL water and used as recommended by the vendor. All experiments used internal standard from the same biomass fermentation (lot number 20211007). Non-labened metabolite standards were purchased from Sigma Aldrich (Vienna, Austria) or Carbosynth (Berkshire, UK). All standards were weighed and dissolved in an appropriate solvent and volumetrically joined to obtain a mix of 149 metabolites. The mix was aliquoted in HPLC vials, evaporated to dryness, and stored at −80 °C. For each experiment, one aliquot was reconstituted in 200 µL water or ACN/water 1:1 (v/v), giving a multi-metabolite stock solution with a concentration of 50 µmol L^{-1} for each metabolite. The stock solution was further diluted with LC-appropriate solvent to prepare a calibration curve. Molecules and sum formulas of the assessed metabolites are listed in Table A1 of Appendix A. The number and concentrations of the calibrators depend on the data set. For the experiments using alkaline LC, seven calibration levels (0.001 µmol L^{-1}, 0.01 µmol L^{-1}, 0.1 µmol L^{-1}, 0.5 µmol L^{-1}, 1 µmol L^{-1}, 5 µmol L^{-1}, 10 µmol L^{-1}) plus a zero calibrator (0 µmol L^{-1}) were prepared, each containing IS stock solution in a ratio of 1:8 (*v/v*) compared to the final calibrator volume and a final solvent composition of 70 % (*v/v*) isopropanol in water. For the experiments using acidic LC, 13 calibration levels (0.001 µmol L^{-1}, 0.005 µmol L^{-1}, 0.01 µmol L^{-1}, 0.025 µmol L^{-1}, 0.05 µmol L^{-1}, 0.075 µmol L^{-1}, 0.1 µmol L^{-1}, 0.25 µmol L^{-1}, 0.5 µmol L^{-1}, 0.75 µmol L^{-1}, 1 µmol L^{-1}, 5 µmol L^{-1}, 10 µmol L^{-1}) plus a zero calibrator (0 µmol L^{-1}) were prepared, each containing IS stock solution in a ratio of 1:10 (*v/v*) compared to the final calibrator volume and a final solvent composition of 80 % (*v/v*) acetonitrile in water.

2.2. Plasma samples and extraction

For the two experiments using alkaline LC separation, SRM 1950 human plasma reference material was allowed to thaw at room temperature for 5 min. Three 50 µL aliquots were placed in Eppendorf vessels and mixed 1:1 (v/v) with 50 µl yeast IS. 29 µL 8.4 mmol L^{-1} N-Ethylmaleimide in 10 mmol L^{-1} ammonium formate pH 7.0 were added to protect thiol groups prone to oxidation, followed by 371 µL cooled methanol for protein precipitation and metabolite extraction. The samples were vortexed and placed on wet ice for 30 min. After vortexing again, samples were centrifuged (14 000×*g*, 15 min, 4 °C) and 125 µl supernatant was transferred to an HPLC vial for each of the three aliquots. The solvent was completely evaporated in a vacuum centrifuge and samples were stored at −80 °C until they were reconstituted in 200 µL isopropanol/water 7:3 (*v/v*) for LC-MS analysis. For the two experiments using acidic LC separation, SRM 1950 was sampled with a 20 µl Mitra volumetric sampling device (Neoteryx, Trajan Scientific Australia Pty Ltd.). The dried Mitra tip was removed from the plastic holder and placed into a 2 ml Eppendorf vessel. 40 µL water and 20 µL IS stock solution were added, vortexed, and followed by the addition of 140 µL acetonitrile with a subsequent vortex step. The sample was vortexed at 1200 rounds per minute for 15 min at room temperature. The supernatant was filtered directly into an HPLC vial and injected without further processes.

2.3. Liquid chromatography

For the two experiments using alkaline LC conditions, separation was carried out on an iHILIC-(P) Classic HPLC Column (2.1 × 100 mm, 5 μm, Hilicon) equipped with a Viper inline filter and an iHILIC-(P) Classic pre-column (2.1 × 20 mm, 5 μm, Hilicon). Eluent A was a 9:1 mixture of water with 15 mM ammonium acetate adjusted to pH 9.4 with ammonium hydroxide and acetonitrile and eluent B was a 1:9 mixture of water with 15 mM ammonium acetate adjusted to pH 9.4 with ammonium hydroxide and acetonitrile. The injection volume was set to 5 μL and the flow rate to 200 μL min^{-1}. The column compartment temperature was 40 °C and sample and standards were kept in the autosampler at 10 °C. The injection needle was washed for 10 s with a mixture of acetonitrile, methanol, and water 1:1:1 (v/v/v) between injections. The following gradient was applied: 0–12 min linear gradient from 100 % to 20 % B, 12–14 min hold at 20 % B, 14–17 min linear decrease to 0 % B, 17–19 min hold at 0 % B, 19–20 min switch to 100 % B, 20–34 min re-equilibration at 100 % B. For the two experiments using acidic LC conditions, separation was carried out on a HILIC-RP dual-LC configuration. The same extract was first injected and eluted from the HILIC column, followed by injection and elution from the RP column while the HILIC effluent was directed to waste using a switching valve during re-equilibration. HILIC separation was performed with an Acquity UPLC BEH Amide (2.1 × 150 mm, 1.7 μm, Waters) equipped with an Acquity UPLC BEH Amide VanGuard precolumn (5 × 2.1 mm, 1.7 μm, Waters) using water with 10 mM ammonium formate and 0.125 % formic acid as eluent A and acetonitrile/water 95:5 (*v/v*) with 10 mM ammonium formate and 0.125 % formic acid as eluent B. Injection volume was set to 5 μL and the flow rate to 400 μL min^{-1} for separation and re-equilibration. The following gradient was applied: 0–7.7 min linear decrease from 100 % to 70 % B and 7.7–10.25 min linear decrease to 30 % B, 10.25–12.75 min increase to 100 % B, 12.75–14 min flush at 100 % B. The flow rate was reduced to 50 μL min^{-1} during RP separation (13–26 min) to save on eluents. RP separation was performed with an Acquity UPLC HSS T3 (2.1 mm × 150 mm, 1.8 μm, Waters) equipped with an Acquity UPLC HSS T3 VanGuard Pre-column (2.1 mm × 5 mm, 1.8 μm, Waters) using water with 0.1 % formic acid as eluent A and 95 % acetonitrile mixed with 5 % eluent A as eluent B. The injection volume was set to 5 μL and the flow rate to 250 μL min^{-1} for separation and re-equilibration. The flow rate was reduced to 50 μL min^{-1} during HILIC separation to save on eluents. The column compartment temperature was 45 °C and sample and standards were kept in the autosampler at 4 °C. The injection needle was washed for 5 s with a mixture of acetonitrile, methanol, and water 1:1:1 (v/v/v) between the injections. The following gradient was applied: 13–24 min linear increase from 0 % to 100 % B, 24–26 min hold at 100 % B, 26 min switch to 0 % B, followed by 5 min re-equilibration to starting conditions. The flow rate was reduced to 50 μL min^{-1} during HILIC separation to save on eluents. The gradients applied to each separation are tabulated in Table A2 of Appendix A.

2.4. Mass spectrometry

For the two experimental runs using alkaline LC conditions, high-resolution mass spectrometry was performed on a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific™) equipped with a heated electrospray ion source (HESI). The HESI source parameters were the following: sheath gas 40, auxiliary gas 3, spray voltage 2.8 kV in negative and 3.5 kV in the positive mode, capillary temperature 280 °C, S-Lens RF level 50, and probe heater temperature 320 °C. Full mass scan data were acquired in profile mode in a scan range of 65–900 *m/z*. Positive and negative mode switching was applied with a resolution of 120 000 at m/z 200. The automatic gain control target was set to 10^6 and the maximum injection time is 200 ms. For the two experimental runs using acidic LC conditions, high-resolution mass spectrometry was performed on the same Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific™) equipped with a heated electrospray ion source (HESI). The HESI source parameters were the following: sheath gas 60, auxiliary gas 25, spray voltage 3.1 kV in the positive and negative mode, capillary temperature 300 °C, S-Lens RF level 30, and probe heater temperature 370 °C. Full mass scan data were acquired in profile mode in a scan range of 60–900 *m/z*. Positive and negative mode switching was applied with a resolution of 60 000 at m/z 200. The automatic gain control target was set to 10^6 and the maximum injection time was 100 ms. Acidic and alkaline LC data sets were acquired by different experimenters.

2.5. Data timeline, centroiding, and ion chromatogram extraction

Two data sets, one using alkaline and one using acidic LC conditions, were acquired with five months in between and comprised a multipoint calibration curve, repeated SRM 1950 injections, and system blank injections. A second set of multipoint calibration data was acquired four (alkaline) or six days later (acidic) following the same experimental procedure as the corresponding previous data set. Raw data were centroided and converted to mzML format with msConvert GUI (version 3.0.19014-f9d5b8a3b) [15]. Then, extracted ion chromatograms of 149 target metabolites (Table A1) were generated in Skyline [16] with a mass extraction window of 5 ppm. The evaluation focused on $[M+H]^+$ and $[M-H]^-$ adducts of the monoisotopic and fully ^{13}C-labeled masses. The molecules, including isomers, were identified by retention time comparison with authentic standards. A. *csv* file stating area values, raw intensities, and mass error was further processed with R [17].

2.6. Assessing concentrations of IS compounds – biomass characterization

The dried biomass extract used as IS is commercially available as "ISO1" from ISOtopic Solutions EU and Cambridge Isotope Laboratories Inc. Each vial, containing hydrophilic metabolite extract from ~20 billion *K. phaffii* cells, was reconstituted in different volumes in the four data sets. For the acidic dual-LC data sets, the experimenter followed the vendor-recommended reconstitution procedure using 2 ml LC-MS grade water per falcon tube. For the iHILIC data sets, 3 ml MeOH/water were used as reconstitution solvent. For comparing IS concentrations across the data sets, the volumetric reconstitution factor was considered mathematically and reported IS concentrations to refer to a reconstitution volume of 2 ml per falcon tube. The concentrations of the different fully ^{13}C-labeled internal standard metabolites were assessed from the IS-spiked calibrators, i.e. by standard addition multi-point calibration. Calibrator concentration was limited to max. 5 μmol L^{-1} for most of the molecules. For a low-concentrated subset of IS molecules (alpha-ketoisovaleric acid, mevalonic acid, uridine, xanthine), the calibrator concentration was limited to < 1 μmol L^{-1}. An in-house R-script was used for estimating linear range, LLOQ, and ULOQ via linear regression based on the peak areas of unlabeled standards, and calibrator concentrations falling outside of the linear range were detected and rejected by applying a slope filter according to Schoeny et al. (2021) [18]. Calibration curves were accepted if they had a minimum of three calibration points. All calibrators of a calibration curve contained the same amount of biomass spike. Chromatographic peaks of $U^{13}C$-biomass metabolites were filtered by stringent quality criteria designed to reject the majority of sample-unrelated noise signals while allowing a variety of peak shapes and possible $U^{13}C$-signal suppression at higher standard concentrations. The peak filter criteria are listed in Appendix A, Table A3. Automatic peak filtering was followed by manual curation. Molecules were accepted only if they complied with the mentioned criteria in both corresponding data sets (i.e., they were sufficiently detected in both acidic or both alkaline LC data sets, or all four). Equations for IS-metabolite quantification are given in Table A5 of Appendix A. In the first step, IS metabolite concentration was calculated per data as the median concentration over all calibrator injections in the linear range

(Equations A1 and A2). Then, concentration mean and RSD were calculated over all data sets (N = 4 for IS metabolites that were quantified in all data sets and N = 2 for IS metabolites that could only be quantified with one of the analytical platforms) using the best MS polarity for each LC method (usually the one with the highest signal intensity) (Equation A3). The results of IS quantification are listed in Table B1 of Appendix B.

2.7. Determination of sample metabolite concentrations

Metabolite concentrations in the spiked SRM 1950 samples were calculated by one-point calibration via the quantified isotopically labeled biomass spike and results were compared to the gold-standard calibration procedure (IS-normalized external multi-point calibration injected with each experiment). Briefly, for the gold-standard calibration procedure, samples and standards were spiked with a defined volume of isotopically labeled biomass. The fully ^{13}C-labeled biomass metabolites were used as an internal standard for compound-specific normalization but concentrations of the IS metabolites remained unknown. Contrary, the one-point calibration approach was performed as a standard-addition experiment relying on internal standard concentrations of labeled biomass that had been characterized beforehand. Measuring a full calibration curve in each experiment was omitted. Fig. 1 gives an overview of the two calibration procedures. Formulas for calculating SRM 1950 concentrations via the two different calibration procedures (i.e., standard-addition one-point calibration and IS-normalized external multi-point calibration) are given in Appendix A, Equations A4 and A5. Before metabolite quantification, the chromatographic signals of sample and U^{13}C-labeled IS metabolites (intensity of extracted ion chromatograms of monoisotopic masses) in IS-spiked SRM 1950 were filtered by stringent peak quality criteria listed in Table A3 of Appendix A, followed by manual curation.

2.8. Assessing the four requirements for internal one-point calibration [14]

The isotopically labeled biomaterial was assessed with respect to the four requirements for internal one-point calibration stipulated by Nilsson and Eklund [14]. **Requirement 1:** The relative response should not be concentration-dependent. SRM 1950 and IS were mixed 1:1 (v/v), diluted to 5 %, 10 %, and 20 % in the analytical sample, and analyzed using the acidic HILIC-RP-LC platform. RSD of the relative response (i.e., analyte-to-IS area ratio) was calculated per metabolite for the dilution series (5 %, 10 %, 20 %, each N = 1) and compared to the technical system variability (RSD over N = 3 injections of the 10 % sample). **Requirement 2:** The relative response should be constant between experiments/days. We compared sample data from the alkaline iHILIC data set acquired in May and the acidic dual-LC data set acquired in October. The sample was SRM 1950 spiked 1:1 with IS and both operators used IS from the same production lot. Analyte-to-IS area ratios were mathematically corrected for differing IS re-constitution volumes (i.e., IS peak area was multiplied by 1.5 for the iHILIC data) and RSDs of the isotopologue ratios (i.e., analyte-to-IS peak area ratio) was calculated for SRM 1950 metabolites that were quantified with both methods: RSDs were calculated within each LC-MS method (N = 4 analytical replicates for alkaline iHILIC and N = 7 for acidic dual LC) and compared to RSDs calculated for all sample injections regardless of the LC-MS method (N = 11 replicates). Agreement between the two data sets was calculated as % bias of the mean isotopologue ratio obtained under alkaline LC conditions compared to the mean isotopologue ratio obtained under acidic LC conditions. **Requirement 3**: The level of analyte in the internal standard should not be detectable. Smaller molecules in the target list could have a relevant overlap of natural isotopologue pattern and fully labeled IS analogon, leading to quantitative interferences between analyte and IS. A) Theoretical assessment: Isotopologue patterns were calculated for all 149 target molecules using the enviPat R-package [19] assuming a uniform ^{13}C-labeling degree of 98 % as specified by the vendor (^{13}C abundance was set to 0.98) to flag suspect molecules where the theoretical isotopologue distribution of the fully labeled IS molecule would interfere relevantly with the monoisotopic mass trace of the natural analyte. B) Experimental verification: Analytical replicate injections of IS stock solution (re-constitution volume 3 mL) diluted 1:2 (N = 3) and pure solvent injections (N = 6) were analyzed using the alkaline LC platform. Signal intensities in the monoisotopic mass trace were extracted with Skyline [16] for each IS molecule that was quantified with the alkaline LC-MS platform. Suspect molecules with significantly altered monoisotopic mass trace compared to the system background were narrowed down by a non-paired, one-tailed T-test followed by manual inspection of the suspects. Null hypothesis: The difference between the mean signal intensity of the IS (m_{IS}) and the mean signal intensity of pure solvent injections (i.e., system background, $m_{solvent}$) is lower than or equal to zero (H_0: $m_{IS} \leq m_{solvent}$, H_a: $m_{IS} > m_{solvent}$). To evaluate if the signal contribution of the IS to the monoisotopic mass trace of the analyte, if any, was relevant for quantification, the detected signal intensity was mathematically corrected to match the IS concentration in the real sample (i.e., divided by 8 as IS concentration in the real sample was 1:16 vs. 1:2 for the IS cross-contribution challenge) and compared to the signal intensity of the U^{12}C-sample analyte. A signal contribution greater than five percent was rated problematic for quantification. Correcting experimental U^{13}C-metabolite peak areas with a correction factor to mimic lower IS concentration in the sample neglects possible signal suppression caused by the higher IS concentration and is thus an approximation. **Requirement 4**: There should be no influence from naturally occurring isotopes of the analyte on the internal standard peak area. A) Theoretical assessment: We again calculated isotopologue patterns for all molecules in our target list using the enviPat R-package [19], this time assuming natural isotope abundances, and checked the mass traces of the corresponding fully labeled IS molecules. B) Experimental verification: Authentic pure standards (c = 10 μM) were injected under acidic elution conditions (N = 1) and the mass trace of the fully labeled analogon was checked for signals for each of the 130 standard metabolites detected with the respective LC-MS platform.

2.9. Statistical analysis

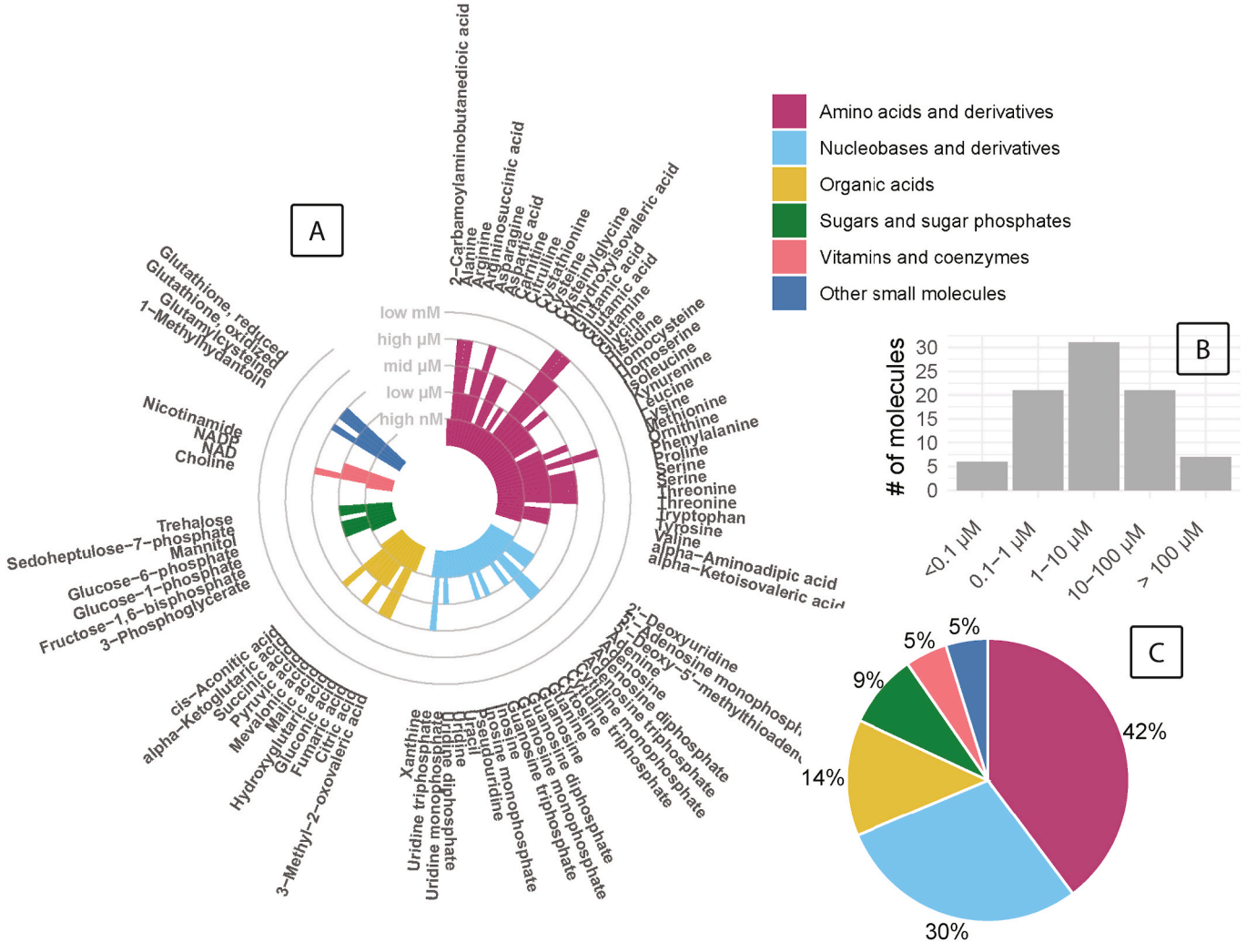
The performance of IS metabolite quantification was characterized by the precision of concentration values across four experimental runs, including pH-complementary LC methods (method cross-validation). Accuracy of standard-addition one-point calibration via quantified isotopically labeled biomass was assessed by comparing SRM 1950 metabolite concentrations to the gold-standard calibration procedure and to reference values provided by NIST or literature, if available [20,21]. Formulas for statistical analysis are stated throughout the text and in figure captions, where appropriate.

3. Results

3.1. Characterization of U^{13}C-labeled biomass

First, we characterized a batch of uniformly and fully ^{13}C-labeled biomass extract by applying a reverse isotope dilution strategy in four independent experiments (independently prepared and measured by two different operators, different methods and time points). Multi-point calibrations of unlabeled standards spiked with ^{13}C-labeled biomass were evaluated as standard addition. An in-house R-script was used for linear regression estimating LLOQ and ULOQ. 83 fully ^{13}C-labeled central carbon metabolites were quantified via two independent standard addition experiments using either alkaline or acidic LC conditions and both MS polarities. A subset of 51 metabolites was amenable to quantitative analysis under both pH regimes and was thus quantified in four independent experiments including different LC-high-resolution MS

methods, spanning a time frame of five months. The reverse isotope dilution exercise is summarized in Appendix B. Table B1 reports the average concentrations and their uncertainty, Table B2 contains the concentrations obtained for the individual data sets respectively, Table B3 states within-method repeatability. Intermediate stability (inter-method variability over five months) for the subset of 51 IS metabolites is given in Table B4. The panel of quantified ^{13}C metabolites comprised amino acids and derivatives (42 % of the detected molecules), nucleobases and derivatives (30 %), organic acids (14 %), sugars and sugar phosphates (9 %), vitamins and co-enzymes (5 %) and other small organic molecules (5 %) (Fig. 2A). The obtained concentrations spanned over 5 orders of magnitude from nanomolar to low millimolar with most molecules (66 %) in the micromolar range (Fig. 2B and C and Table B1 of Appendix B). Several hexose-isomers were detected but excluded from quantification due to co-elution. Intermediate stability was assessed based on a subset of molecules that was quantified with both pH-complementary LC-MS methods over a period of five months (51 molecules): The major fraction of metabolites (47 molecules – 92 %) showed inter-method variation (expressed as concentration RSD) below 30 %. Three-quarters of the assessed metabolites (38 molecules – 75 %) showed excellent reproducibility across methods with RSD <15 % as recommended by the US FDA guideline for bioanalytical method validation [22]. The molecules showing an inter-method variation >30 % were the amino acid alanine, the pyrimidines cytosine, uracil, and uridine, as well as the purine-derivative xanthine. The multi-point calibration for alanine was hampered in one of the analytical methods and concentration data from this method were discarded. Uracil and xanthine were prone to progressive signal suppression of standard and IS signals with increasing concentration. Despite the high uncertainty, these metabolites were included in the wide targeted panel in the following. NEM-derivatization was employed to protect primary thiols (cysteine, cysteinylglycine, glutamylcysteine, homocysteine, and reduced glutathione) in the experiments using alkaline LC, but not in the experiments using acidic LC. These metabolites were excluded from the inter-method comparison, while we included cystathionine and oxidized glutathione, dimerization products of the primary thiols cysteine and reduced glutathione, with inter-method RSDs of 5.3 % and 29 %, respectively. To achieve highest accuracy by isotope dilution using a single spike approach, the internal standard requires to fulfill the following criteria (see Fig. 3)[14]: (1) The isotopologue ratio should be independent of the sample concentration and (2) constant over independent experiments, (3) there should be no signal contribution from the labeled IS to the un-labeled analyte (4) and no signal contribution from the un-labeled analyte to the IS. The here studied yeast-derived IS fulfilled criterion 1, as analyte-to-IS area ratios showed high agreement across different dilutions using one method. (Fig. 4). The isotopologue ratio was independent of analyte, IS, and matrix concentration in the investigated subset of 42 metabolites measured in the SRM 1950 sample.



Figs. 2. 83 $U^{13}C$-labeled metabolites quantified in isotopically labeled yeast extract
A) $U^{13}C$-metabolite concentrations. **B)** Concentration distribution of $U^{13}C$-metabolites. **C)** $U^{13}C$-metabolite classes. Concentrations refer to a vial of IS dry extract (2 billion cells) reconstituted in 2 mL hydrophilic solvent. High nM: <1 µM, low µM: 1–10 µM, mid µM: 10–100 µM, high µM: 100–1000 µM, low mM: >1000 µM.

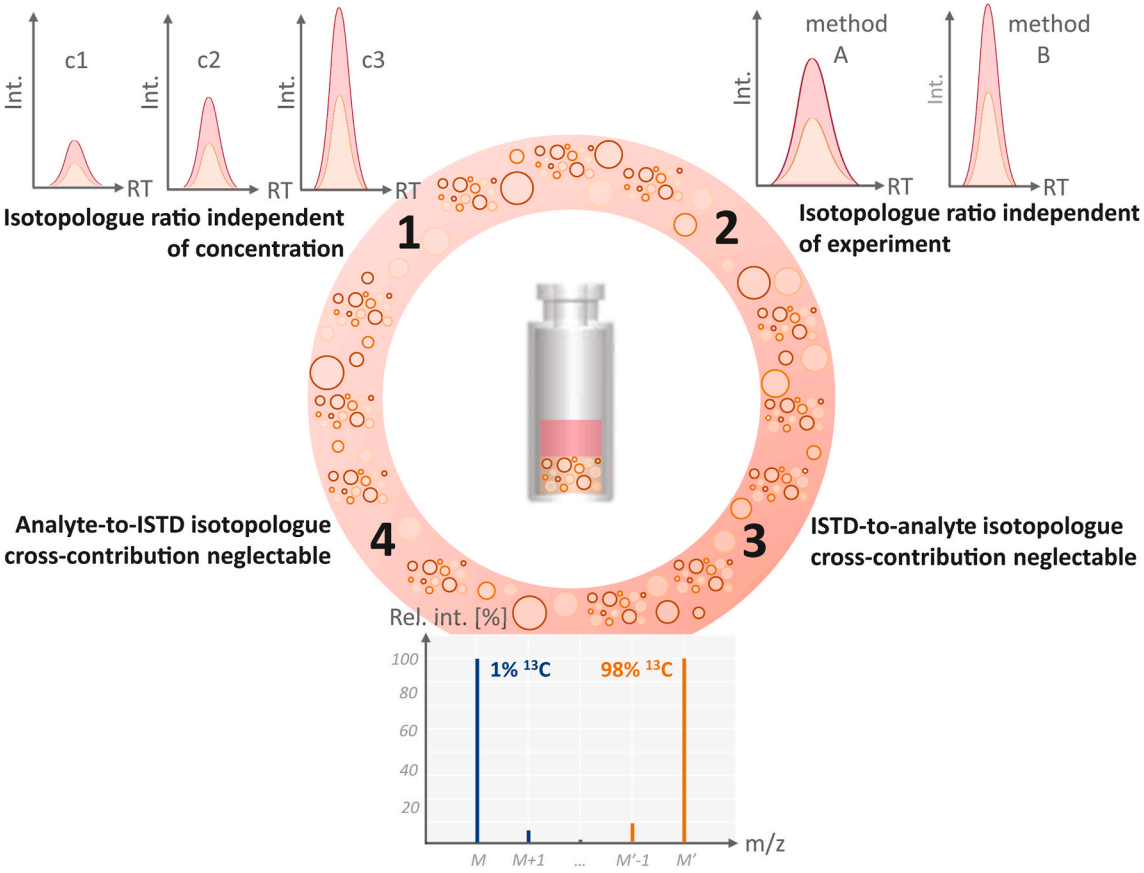


Fig. 3. The four requirements for sample-addition one-point calibration (Nilsson and Eklund, 2007)[14]

To test whether requirement 2 was fulfilled, the isotopologue ratios in the reference material SRM1950 were scrutinized in a "worst-case scenario". Two independent data sets were compared, stemming from different operators acquired on two different days with five months in between, using different sample extraction protocols, sample concentration, and different LC methods (see Experimental section). In both cases, the reference material was SRM 1950 spiked 1:1 with IS, and both operators used IS from the same fermentation lot. Signal intensity of the fully labeled isotopologue was mathematically corrected for the differing IS re-constitution volumes used in the two analytical methods and bias [%] of the mean isotopologue ratio obtained in the alkaline LC setup (N = 4) was calculated with respect to the mean isotopologue ratio obtained using the acidic LC setup (N = 7) for a panel of 36 SRM 1950 metabolites that was quantified with both LC methods. 24 metabolites (67 %) showed an inter-method bias below ±20 %, while the remaining metabolites exceeded 20 % bias. This is reflected by elevated median analyte-to-IS area ratio RSD including analytical replicates from both analytical methods (7.9 %, N = 11) as compared to within-method RSDs (median 3 % (N = 4) and median 4.4 % (N = 7)) (Fig. 5). For the twelve metabolites with inter-method bias >20 %, we uncovered discrepancies between the analytical platforms regarding the chromatographic resolution of isomers in sample and IS and chromatographic resolution of parent and daughter ions for metabolites prone to in-source fragmentation, as well as sensitivity and background noise. A detailed description is given in Table A4 of Appendix A. Summarizing, the isotopologue ratio was constant between analytical runs and overall robust towards deliberate modifications of the analytical method for two-thirds of investigated molecules and affected by chromatographic challenges for one-third, underlining the necessity for thorough method characterization. Finally, the isotopically labeled biomass was fit for purpose regarding requirements 3 and 4. The high ^{13}C-labeling degree of 98 % (specified by the vendor) ensured that interference of the IS molecule with the monoisotopic (natural analyte) mass trace was minimal. Considering the concentration ratio of sample and IS and the overall low concentration of IS in the measurement solution (6.25 %), none of the investigated labeled IS molecules showed a significant interference experimentally, as only a signal contribution >5 % is rated as problematic for quantification. However, calculations of isotopologue patterns revealed a theoretical abundance of the fully un-labeled ($U^{12}C$) isotopologue of around 2 % compared to the fully labeled ($U^{13}C$) metabolite species for urea (one carbon atom). Vice versa, the influence of naturally occurring heavy isotopologues of the analyte on the internal standard (requirement 4) was again checked theoretically by generating isotopologue patterns with natural isotope abundances, followed by experimental verification. From our target list of 149 metabolites, again only the natural isotopologue pattern of urea with one carbon atom would interfere with its labeled analog (natural abundance of ^{13}C-urea ~1 %). Authentic pure standards (c = 10 μM) were injected under acidic elution conditions (N = 1) and the mass trace of the fully labeled analogon was checked for signals for each metabolite. For all of the standards assessed experimentally (130 target metabolites detected with acidic LC conditions), there was no contribution to the fully labeled isotopologue mass trace from the standard. While a concentration of 10 μM (in the measurement solution) used for the isotopologue cross-contribution challenge can be rated as the safety margin for many sample metabolites, the abundance of some metabolites largely exceeds this concentration depending on the sample. Thus, the plasma sample SRM 1950, where the concentration of urea was roughly estimated at around 200 μM in the measurement solution, was also checked for isotopologue cross-contribution: A measurable contribution from the natural sample analyte to the fully labeled ($U^{13}C$) IS mass trace was detected, amounting to 1 % compared to the intensity of the fully un-labeled ($U^{12}C$) isotopologue.

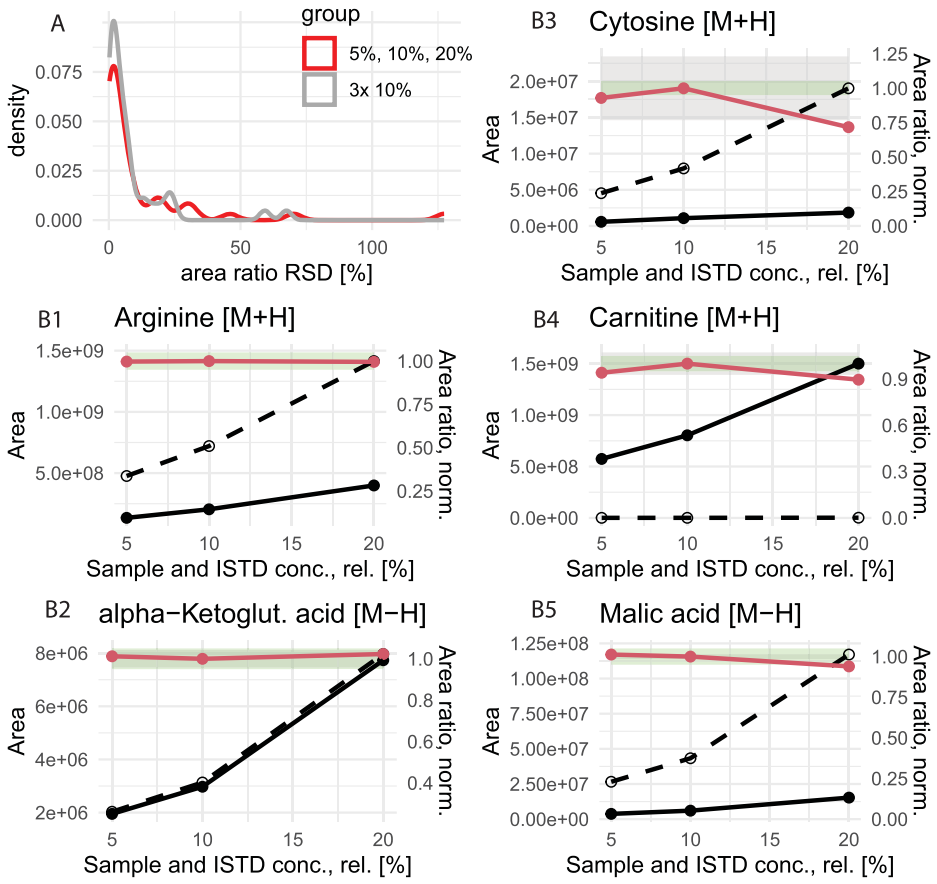


Fig. 4. Relative response variability depending on the sample concentration
SRM 1950 and ISTD were mixed 1:1 (v/v), diluted 1:5, 1:10 and 1:20 and analyzed using the acidic HILIC-RP-LC platform (each N = 1 injection). **A)** Analyte-to-ISTD area ratio RSD was calculated per metabolite (42 molecules) for the dilution series (each N = 1) and compared to the RSD obtained from analytical replicates (N = 3 injections) of the 1:10 dilution. The median analyte-to-ISTD area ratio RSDs were 3.3 % for the dilution series and 3.5 % for replicate injections of the same concentration, respectively. **B1–B5)** Analyte area (black solid line), ISTD area (black dashed line) and analyte-to-ISTD area ratio (red line) of different sample dilutions for selected metabolites. Analyte-to-ISTD area ratios were normalized to the 1:10 dilution and bias is given in %. The acceptable variability margin for analytical replicates (±5 %) is indicated in green. The technical base variability (analyte-to-ISTD area ratio RSD of the 1:10 dilution, N = 3 injections) is indicated in grey.

3.2. $U^{13}C$-labeled biomass as IS for SRM 1950

A panel of 49 metabolites commonly present in the yeast derived IS and in the standard reference material for human plasma metabolomics the SRM1950, devised by the US National Institute of Standards and Technology [21], was quantified by "single spike" isotope dilution, using the now characterized internal standard as one-point calibration. The scrutinized metabolite panel covered amino acids, small organic acids, and nucleobase derivatives. Several hexose-isomers were detected in both materials but excluded from the quantification exercise due to overly poor chromatographic separation. The resulting SRM1950/IS blends featured $U^{12}C$-to-$U^{13}C$-isotopologue ratios over a broad range (Fig. 6). To evaluate whether this concentration mismatch between sample and standard affected the repeatability of the isotopologue ratio assessment, a (rank-based) Spearman correlation test was performed. Concentration similarity was defined as the unlabeled-to-labeled isotopologue ratio or labeled-to-unlabeled isotopologue ratio, whichever was greater. The correlation was weak and non-significant (Spearman's rho 0.116 ($p = 0.4564$) and 0.221 ($p = 0.1583$) for alkaline and acidic LC, respectively). However, repeatability of the isotopoplogue ratio was significantly correlated with analyte signal intensity (Spearman's rho −0.605 ($p = 3x10^{-5}$) and −0.705 ($p = 6x10^{-7}$) and IS signal intensity (Spearman's rho −0.585 ($p = 5x10^{-5}$) and −0.600 ($p = 4x10^{-5}$) for alkaline and acidic LC, respectively). A tailored $U^{12}C/U^{13}C$ area ratio and, thus, the similarity of concentration profiles between sample and IS, was of minor importance for quantification repeatability as long as analyte and internal standard signal were well above LOD (Fig. 7A–C). Finally, the accuracy of the one-point internal standardization was scrutinized by comparing SRM 1950 quantification results to reference values available from the certificate of analysis and literature (22 metabolites) [20,21]. The two data sets using different instrumental methods were analyzed separately. For both, quantification results obtained via single-spike isotope dilution agreed with the reference values within 20 % for 17 out of 22 metabolites (Fig. 8). Using the gold-standard procedure (IS-normalized external multipoint calibration injected in the same run as the sample), 18 (acidic LC) and 14 (alkaline LC) molecules were within 20 % agreement with the reference values (Fig. 9). For the remaining share of quantified metabolites, no certified values were available. In these cases, comparison with the gold-standard, i.e. multipoint calibration with internal standardization served for evaluation of the accuracy. For 32 (74 %) and 33 (79 %) molecules, the quantification results from one-point calibration agreed within ±20 % with the quantification results obtained by multi-point calibration for the alkaline and acidic LC-MS condition, respectively (Fig. 10). Using a (rank-based) Spearman correlation test, the correlation between the measured $^{12}C/^{13}C$ isotopologue ratio (expressed as concentration similarity between SRM 1950 and IS) and the observed agreement of the two standardization approaches was scrutinized. The calculated correlation was weak and non-significant (Spearman's rho −0.042 ($p = 0.784$) and 0.307 ($p = 0.0516$) for alkaline and acidic LC,

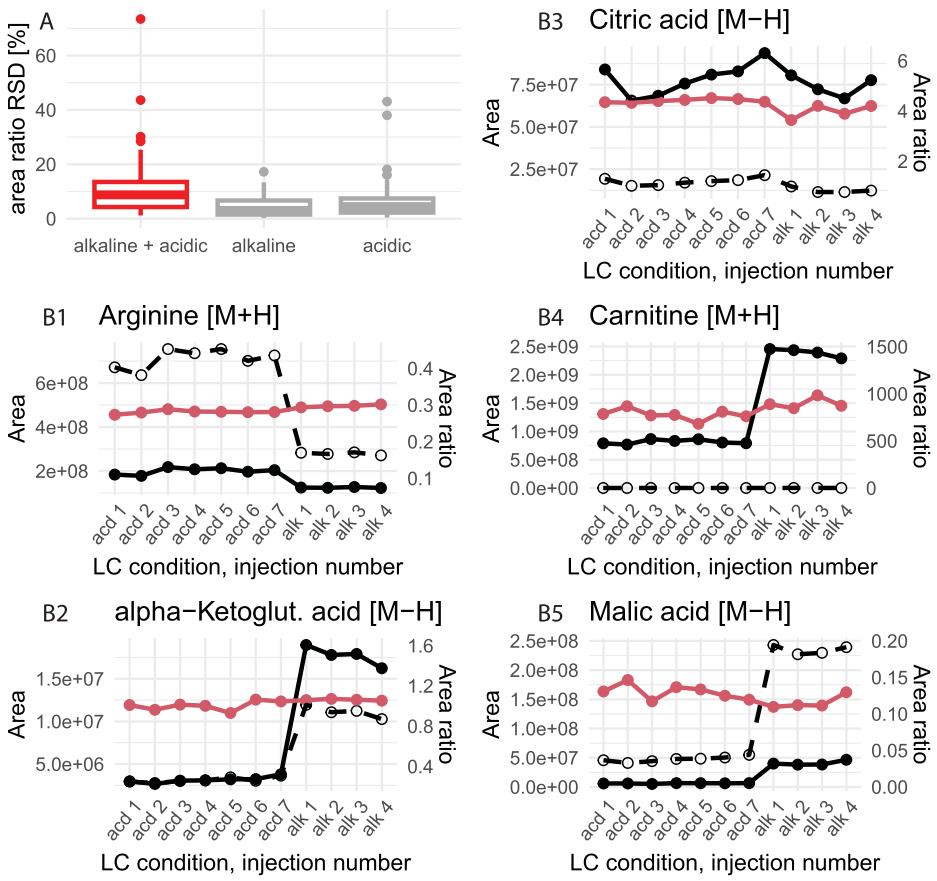
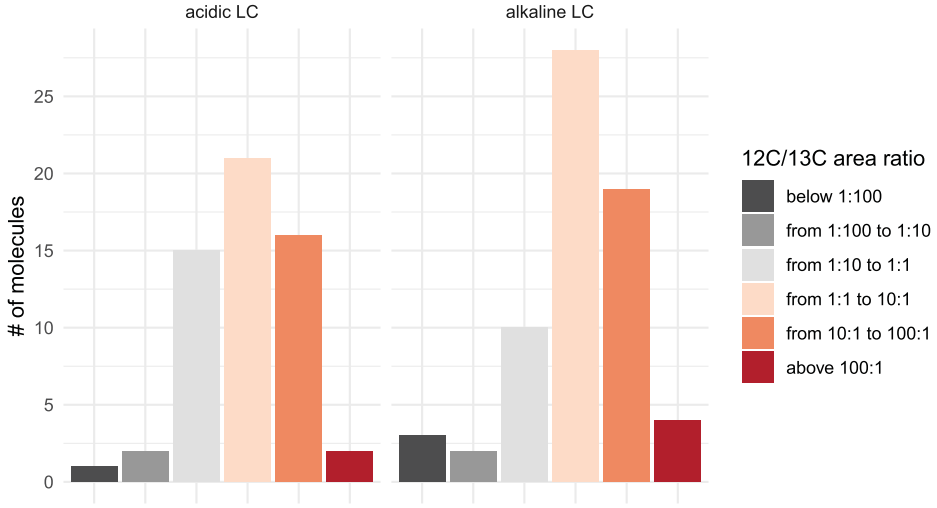


Fig. 5. Variability of the isotopologue ratio within and between independent analytical methods
Two separate aliquots of SRM 1950 were spiked with IS 1:1 (v/v) followed by protein precipitation, dilution (1:16 or 1:10) and LC-HRMS analysis on two different days using alkaline or acidic elution conditions (see details in Experimental section). The sample was repeatedly injected to obtain N = 4 (alkaline conditions) and N = 7 (acidic conditions) analytical replicates. **A)** Inter-method variability (N = 11) of the relative response was assessed for 36 SRM 1950 metabolites quantified with both analytical methods and compared to intra-method variability of each analytical method. Before calculating inter-batch RSD, ISTD peak area was mathematically corrected for the differing IS reconstitution volumes used in the compared data sets. **B1–B5)** Analyte area (black solid line), IS area (black dashed line) and relative response (analyte-to-ISTD area ratio, red line) per injection for selected metabolites. acd 1–7 and alk 1–4: Replicate injections of SRM 1950 using the acidic and alkaline LC platform, respectively.



Figs. 6. $^{12}C/^{13}C$ peak area ratios in quantified SRM 1950 metabolites
Average relative response is given for 44 molecules that were quantified using alkaline LC (N = 4 analytical replicates) and for 42 molecules that were quantified using acidic LC (N = 7 analytical replicates), including metabolites that were quantified in only one of the methods.

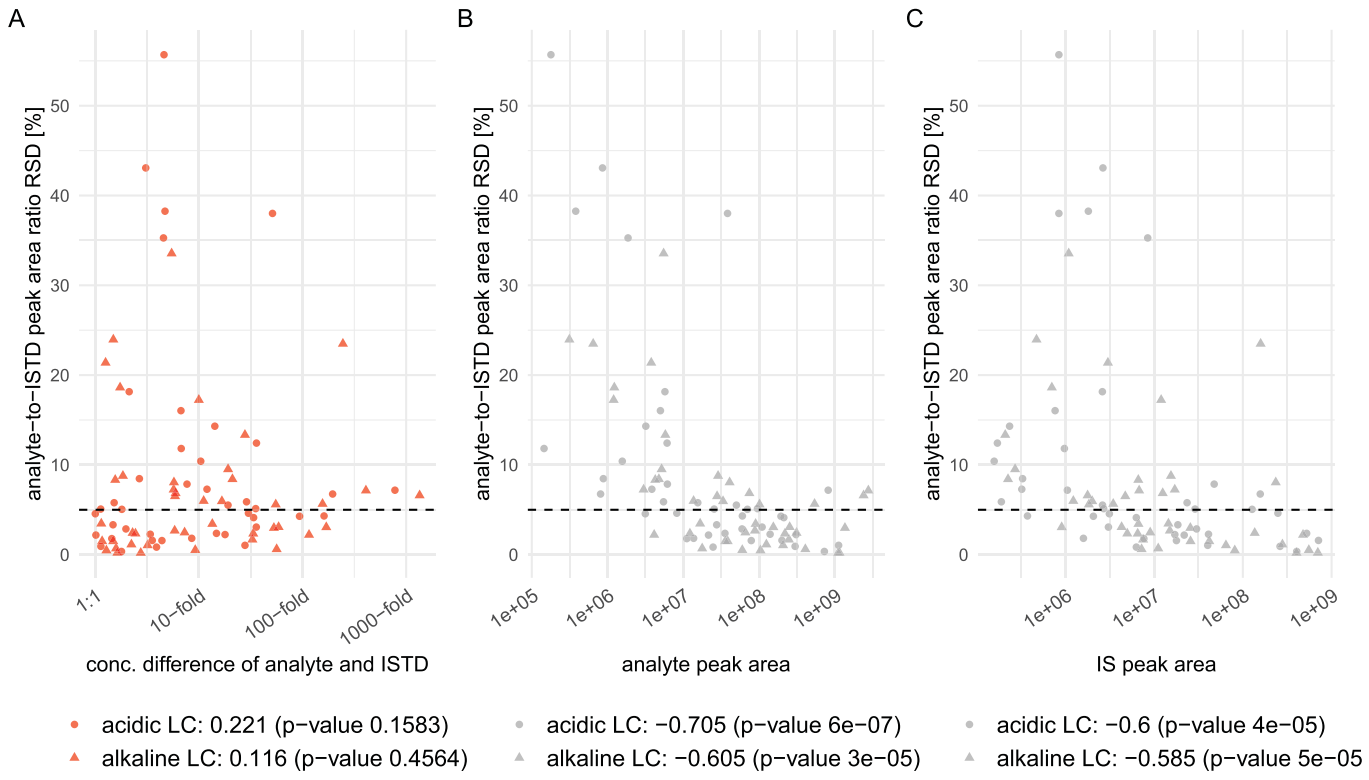


Fig. 7. The repeatability of isotopologue ratios does not correlate with concentration similarity but with signal intensity
Analytical replicates of SMR 1950 were analyzed with two different analytical methods (acidic and alkaline LC conditions). Repeatability of analyte-to–IS–area ratios (N = 7 and N = 4 repeated injections, respectively) was calculated per analytical method for all quantified SRM 1950 metabolites. **A)** Correlation of isotopologue ratio repeatability and analyte–IS–concentration similarity. **B)** Correlation of isotopologue ratio repeatability and analyte signal intensity. **C)** Correlation of isotopologue ratio repeatability and IS signal intensity. Spearman correlation coefficients and p-value are given below each panel. The dashed line indicates 5 % RSD (i.e., acceptance limit for the repeatability of technical replicates).

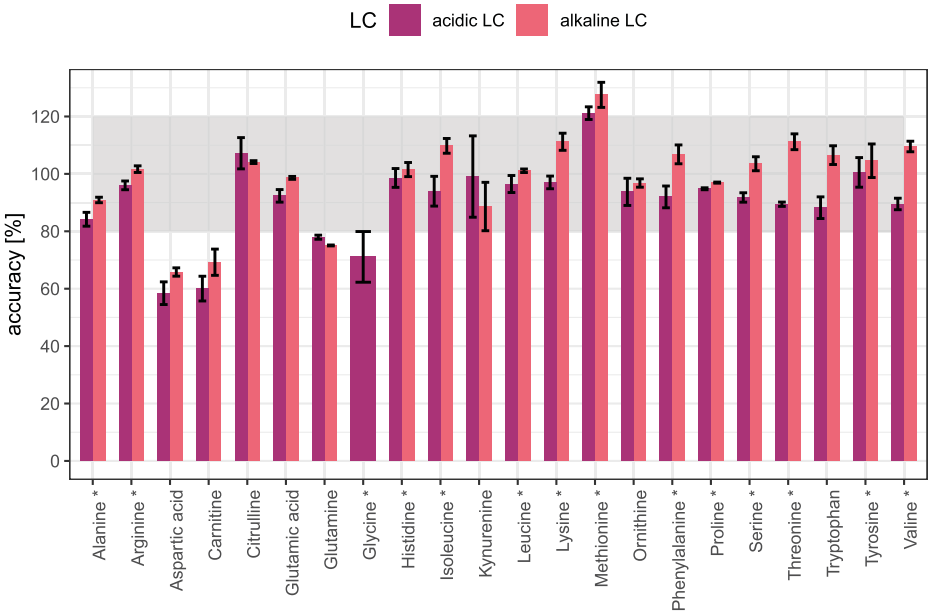


Fig. 8. Accuracy of one-point calibration compared to literature
SRM 1950 metabolites were quantified using in-sample one-point calibration with characterized isotopically labeled biomass in two analytical methods (acidic and alkaline LC, see Experimental section). Isotopically labeled metabolites had been quantified beforehand using both analytical methods (four analytical runs in total) and concentrations were averaged for one-point calibration. Results were compared to literature and accuracy was calculated as % of the reference value. The acceptable accuracy margin is shaded in grey (±20 %). Error bars indicate standard deviation of analytical replicates (N = 7 and N = 4, respectively). The asterisk indicates that quantification results were compared to the official certificate of analysis issued by NIST. All other molecules were compared to Thompson et al., 2019.

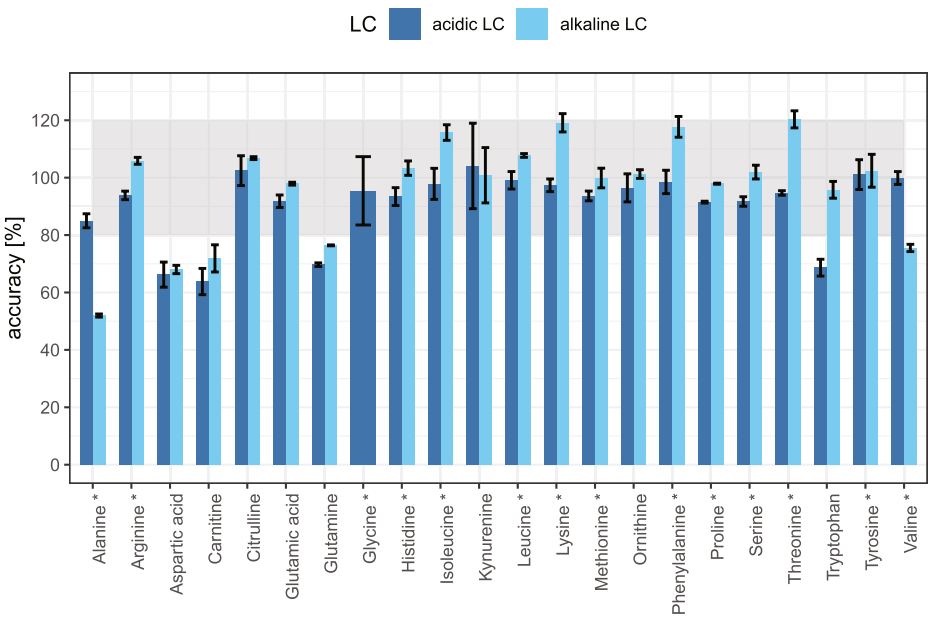


Fig. 9. Accuracy of ISTD-normalized external multipoint calibration (gold-standard) compared to literature
SRM 1950 metabolites were quantified using IS-normalized external multipoint calibration with isotopically labeled biomass as IS in two analytical methods (acidic and alkaline LC, see Experimental section). A dilution series of IS-spiked external standards was injected in the same run as the sample to obtain contemporaneous multi-point calibration. Results were compared to literature and accuracy was calculated as % of the reference value. The acceptable accuracy margin is shaded in grey (±20 %). Error bars indicate standard deviation of analytical replicates (N = 7 and N = 4, respectively). The asterisk indicates that quantification results were compared to the official certificate of analysis issued by NIST. All other molecules were compared to Thompson et al., 2019. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

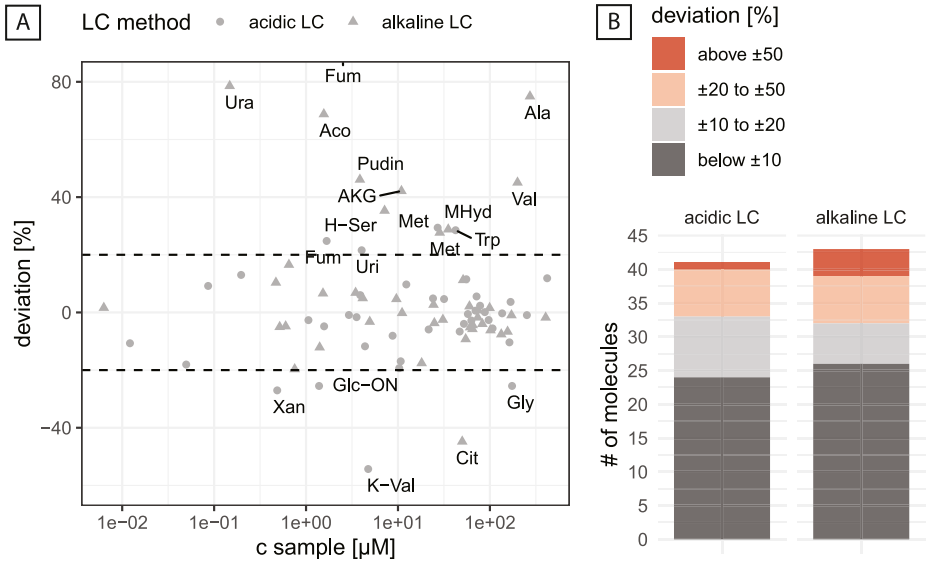


Fig. 10. Agreement of SRM 1950 quantification results obtained by one-point calibration vs. IS-normalized external multi-point calibration
A) Correlation with analyte concentration. The y-axis displays the relative deviation of the quantification result obtained using one-point calibration compared to multi-point calibration. Deviation [%] is calculated as stated below. The names of molecules exceeding 20 % deviation are displayed: MHyd: 1-methylhydantoin, Ala: alanine, Cit: citric acid, Fum: fumaric acid, Glc-ON: gluconic acid, Gly: glycine, H-Ser: homoserine, Met: methionine, Pudin: pseudouridine, Trp: tryptophan, Ura: uracil, Uri: uridine, Val: valine, Xan: xanthine, AKG: alpha-ketoglutaric acid, K-Val: alpha-ketoisovaleric acid, Aco: cis-Aconitic acid. Fumaric acid measured with alkaline LC was removed from the plot (deviation of one-point results compared to multi-point >150 %). **B)** Deviation of quantification results including number of molecules.

$$\textit{deviation}\ [\%] = \left(\frac{c\ \textit{one point}}{c\ \textit{gold std.}} - 1\right) \times 100.$$

respectively). Again, the agreement was lower for low concentrations, except for the high abundant amino acids glycine, alanine and valine (Fig. 10). The two analytical methods yielded markedly different concentrations for these amino acids using the gold-standard calibration procedure. A root cause analysis is given in Table A4 of Appendix A.

4. Discussion

Isotope dilution utilizing isotopically labeled biomass featuring characterized metabolite concentrations and known isotopic enrichment holds great potential for quantitative metabolomics. The single spike strategy reduces the daily calibration workload combined with a wide physicochemical coverage. While gold-standard calibration, i.e., a full calibration curve measured with each experiment, necessitates batch-mode operation and hampers spontaneous, random access to the LC-MS platform [9], the proposed one-point calibration workflow rationalizes time and standard expenditure and is particularly profitable for laboratories dealing with wide-target assays and small analysis batches. In this study, we assessed the suitability of U^{13}C-labeled biomass for internal one-point calibration. We quantified 83 fully ^{13}C-labeled metabolites in a commercially available biomass extract from U^{13}C-labeled *K. pfaffii*, including mostly amino acids, nucleobases, organic acids, and sugars, and their respective derivatives, suitable for compound-specific standardization. The panel of detected metabolites is closely connected to the LC-MS methods chosen (we combined both LC-MS polarities and pH-complementary elution conditions) and can be further modified by a different combination of experimental procedures.

4.1. Suitability of U^{13}C-labeled biomass for standard-addition one-point calibration

High and uniform isotopic enrichment is necessary to obtain suitable internal standards for straightforward data interpretation [8]. As overlooked interference from insufficient labeling would be detrimental for quantification [1,8], we checked the reproducibility of the isotopologue ratio and isotopologue cross-contribution using fully U^{13}C-labeled yeast extract [14]. We found that (1) the magnitude of the relative response was not related to the analytical sample concentration and thus independent of sample, IS, and matrix concentration for the investigated subset of metabolites, (2) relative response was constant between independent experiments and robust towards deliberate modifications of the analytical method for most metabolites (compound-by-compound assessment and method cross-validation is advisable to uncover analyte- and method-specific interferences affecting the isotopologue ratio), (3) none of the IS molecules showed relevant isotopologue interference with natural sample (SRM 1950) metabolites and (4) for most of the molecules, there was no contribution to the IS mass trace from the natural analyte. By contrast, we found signal contribution from the natural analyte to the IS mass trace for urea in SRM 1950 samples due to its high concentration in blood plasma (3.9 mmol L-1 according to the official certificate of analysis [21]). While we did not detect urea in the internal standard and consequently omitted this metabolite from quantification, this example underlines that the tolerable concentration ratio between the sample and IS metabolite critically depends on the molecular composition and ^{13}C-enrichment degree, and resulting isotopologue patterns. Thus, analyte–IS–pairs should be experimentally checked for interference in the sample of interest at least for suspect metabolites (which are identified based on their theoretical isotopologue distribution) and for metabolites with expectably high concentration in the analytical sample. For example, assuming a natural ^{13}C-abundance of 1 %, a biomass labeling degree of 98 %, and an acceptable isotopologue cross-contribution of max. 5 % for quantification, the concentration ratio of urea analyte and IS should not exceed min. 2:5 and max. 5:1. For comparison, a C_2-molecule would tolerate analyte-to–IS–area ratios of approximately 1:125 to 500:1. Summarizing, the employed labeled biomass extract in the used concentrations conforms with the four requirements for internal one-point calibration, which builds the rationale for pursuing the simplified calibration procedure.

4.2. Accuracy and precision

Whether one-point calibration gives more, less, or equally accurate results as multi-point calibration has been discussed in the literature with varying outcomes [11–13,23]. One-point calibration assumes a zero y-intercept while multi-point calibration and linear regression produces calibration with slope and y-intercept. It is thus conceivable that quantification results obtained with one-point calibration and multi-point calibration differ, especially for concentrations at the low end of the calibration line. Analyzing a well-studied sample (the official NIST reference material SRM 1950) allowed us to verify the accuracy of the different calibration procedures based on reference values from literature. We found that the accuracy of the proposed one-point calibration procedure was equivalent to IS-normalized external calibration (the gold-standard procedure). However, reference values for hydrophilic metabolites are limited and mostly available for higher abundant compounds. Therefore, we compared results from one-point calibration to our results from IS-normalized external calibration (the gold-standard procedure) and found good agreement between one-point and gold-standard calibration results for the higher abundant compounds (micromolar range), while lower analyte concentrations were associated lower agreement. Multi-level calibration lines were extended over several orders of magnitude in an attempt to reconcile a wide concentration coverage and acceptable calibration workload. This balancing act is inherent to quantitative metabolomics experiments since analyte concentrations are not known beforehand but are expected to cover a wide dynamic range. Calibration over several orders of concentration magnitude leads to increased residual error at the extreme ends of the calibrated range, adding to lower agreement between one-point to gold-standard calibration. Moreover, in the present study, IS metabolite concentrations used for one-point calibration were averaged over different analytical methods, yielding the averaged accuracy of both analytical methods. For a handful of metabolites where the two analysis platforms produced discrepant results, averaging across different experiments and analytical methods increased the uncertainty of IS quantification but attenuated the systematic bias of each assay. Cross-validation between the two different LC-MS methods and comparison with reference values allowed us to expose systematic bias to some degree, underlining that method validation is of utmost importance for accurate quantification results irrespective of the calibration mode. In the long run, focusing analytical efforts on IS quantification including method cross-validation has the potential to improve the trueness of IS quantification and the one-point calibration procedure compared to relying on a single IS-normalized calibration curve measured with a single LC method in each experiment (i.e., the gold-standard procedure). In line with the findings of Khamis et al. (2018) [12], we conclude that IS characterization is the most critical step to achieve accurate quantification using one-point calibration with characterized isotopically labeled biomass. Wasito et al. assessed the reproducibility of the fully controlled in vivo standard synthesis by scrutinizing metabolite concentration levels obtained from different fermentation in an interlaboratory comparison [24]. Intra-batch variation was found to be acceptable for a large panel of metabolites. Thus, accurate isotope dilution relies on the thorough characterization of the individual fermentation batches, however, the highly reproducible biomass derived standard production allows for the application of streamlined recalibration strategies. Implementing one-point calibration frees resources (external and internal standards and time) that can be focused on the critical step of IS quantification.

4.3. Similarity of sample and IS concentration profiles is not a critical factor

Labeled biomass extract is a readily available source for a customizable panel of chemically diverse internal standards [1]. Different concentration profiles of sample and IS metabolites, within physiological constraints, are to be expected due to different sample origins (human vs. yeast, body fluid vs. whole cells, etc.) and different extraction procedures. While Bennett et al. (2008) recommended adjusting the

IS concentration for analyte-IS peak pairs differing in size more than 10-fold [8], we obtained good quantification results independent of the specific concentration similarity. This finding is in line with Khamis et al. (2018), who compared the accuracy and precision of one-point calibration to multi-point calibration for targeted metabolomic analysis [12]. Our data suggest that a rough approximation of sample and IS metabolite concentrations (within 100-fold as a rule of thumb) is adequate, assuming near-complete (98 %) isotopic enrichment in the IS and provided signal intensities of monoisotopic and $U^{13}C$-labeled isotopologue are both well above LOD.

4.4. Chemical stability of IS metabolites

Evaluating measurement series separated by five months demonstrated intermediate stability for a subset of IS metabolites. Interestingly, we obtained inter-method concentration RSD over 30 % for $U^{13}C$-alanine, which was related to compromised multi-point calibration in the alkaline iHILIC setup. The pyrimidines cytosine, uracil, and uridine belong to compound classes known for their chemical instability and in-source fragmentation under typical (pre-)analytical conditions [25–27]. Sulfhydryl containing metabolites (cysteine, homocysteine, cysteinyl glycine, γ-glutamyl cysteine, and reduced glutathione), which are prone to oxidation [28], were stabilized using NEM-derivatization (N-ethylmaleimide), and isomeric and interconverting [25–27] metabolites were chromatographically separated as far as possible. While the stability of all labeled IS metabolites cannot be definitively judged from our data, we did not detect time-related intensity drift that would hint at degradation. Generally, the stability of labeled metabolites in *K. phaffii* biomass extract has been demonstrated for >70 metabolites over a period of six months [29], and metabolomics samples are mostly stable facing storage at −80 °C and a low number of freeze-thaw-cycles [30]. We report a reference library estimating the concentrations of 83 $U^{13}C$-metabolites in commercially available yeast extract which is subject to continuous updating.

4.5. Labeled biomass enables retrospective quantification on full-scan high-resolution MS data

The scope of internal standardization with pure labeled compounds is usually strictly limited and needs to be planned in advance. By contrast, labeled biomass in conjunction with full-scan high-resolution MS, allows one to select (the best) internal standards from a broad spectrum of $U^{13}C$-labeled metabolites in a data mining approach, thus offering fit-for-purpose post-acquisition normalization without sample re-measurement.

5. Conclusion

Our results suggest suitable concentration, stability, and isotopic purity of the implemented labeled biomass extract for absolute quantification of a wide metabolite panel. The performance of one-point calibration was comparable to the gold standard (IS-normalized external multi-point calibration), with accurate biomass characterization and analytical method validation as neuralgic points. The present assessment serves as a seminal study for further developments of the concept towards a) absolute quantification from archive high-resolution MS data of $U^{13}C$-biomass-spiked samples and b) combination of one-point calibration with “rolling” biomass quantification [13], in which the IS is re-calibrated with each experiment while at the same time being used for sample quantification.

Notes

The authors declare no competing financial interest.

CRediT authorship contribution statement

Veronika Fitz: Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lisa Panzenboeck:** Writing – review & editing, Methodology, Investigation. **Harald Schoeny:** Software, Methodology, Investigation. **Elisabeth Foels:** Investigation. **Gunda Koellensperger:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.342909.

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