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GC–QTOFMS with a low-energy electron ionization source for advancing isotopologue analysis in ^{13}C-based metabolic flux analysis

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Abstract

For the study of different levels of (intra)cellular regulation and condition-dependent insight into metabolic activities, fluxomics experiments based on stable isotope tracer experiments using ^{13}C have become a well-established approach. The experimentally obtained non-naturally distributed ^{13}C labeling patterns of metabolite pools can be measured by mass spectrometric detection with front-end separation and can be consequently incorporated into biochemical network models. Here, despite a tedious derivatization step, gas chromatographic separation of polar metabolites is favorable because of the wide coverage range and high isomer separation efficiency. However, the typically employed electron ionization energy of 70 eV leads to significant fragmentation and consequently only low-abundant ions with an intact carbon backbone. Since these ions are considered a prerequisite for the analysis of the non-naturally distributed labeling patterns and further integration into modeling strategies, a softer ionization technique is needed. In the present work, a novel low energy electron ionization source is optimized for the analysis of primary metabolites and compared with a chemical ionization approach in terms of trueness, precision, and sensitivity.

Keywords Gas chromatography–mass spectrometry · Low energy electron ionization · Metabolic flux analysis · Isotope labeling experiments · Metabolomics

Introduction

The conecpt of stable isotope labeling experiments and its development towards the estimation of in vivo intracellular metabolic rates (i.e., metabolic flux analysis (MFA)) has become a vital part for studying metabolism and its various levels of regulation within a biological entity. This approach is applicable to various research areas, such as clinical studies, metabolic engineering in biotechnological applications, and systems biology unraveling novel pathways [1]. The basic concept relies on feeding the organism of interest with a substrate, labeled at certain position(s) with heavy stable isotope(s), most commonly with [13]C. The incorporation and metabolization of the labeled substrate leads to a non-naturally distributed labeling pattern of the downstream metabolite pool [2]. To infer intracellular fluxes, these labeling patterns are measured and implemented together with data on growth, biomass composition, and uptake and secretion rates into a metabolic network. By maximizing the fit between a priori simulated and measured labeling patterns, intracellular fluxes are inferable, and after statistical analysis, this can give insight into the speed and direction of metabolic activity in a quantitative manner [3]. The analysis of the non-naturally distributed C-isotopologue pattern of *free* intracellular metabolites relies mostly on mass spectrometric (MS) detection with front-end separation via chromatography; namely, either liquid chromatography (LC) or gas chromatography (GC). To guarantee GC amenability of polar metabolites, derivatization is a required step, typically comprising a two-step reaction. In the first step, if present, keto groups are converted to alkoximes, preventing decarboxylation [4], and locking sugars and their analogues in the open-chain structure [5]. In

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the second step, volatility and thermal stability are increased by replacing active hydrogen atoms in various functional groups by trimethylsilyl (TMS) or *tert*-butyldimethylsilyl (TBDMS) groups. TBDMS derivatives are more stable and lead to characteristic fragments in electron ionization (EI) mass spectra that can be of use for identification. However, the TBDMS moiety is bulkier than the TMS group and hence vicinal active hydrogens are not fully silylated [6], leading to several derivatives, complicating the GC chromatogram. This holds especially true for sugars and sugar analogues, and hence trimethylsilylation is often the method of choice in metabolomics experiments [7].

Even though this laborious derivatization step is necessary and is accompanied by a rather extensive natural isotope interference correction [8–10] GC approaches are characterized by an excellent separation efficiency and a wide metabolite coverage within a single chromatographic run [7–13]. This leads to highly valuable information complementary to that obtained by LC-based methods. Notably, GC-based separation is still the gold standard for the analysis of certain biologically important constitutional isomers [13, 14]; for example, sugar phosphates [13]. The latter group of metabolites is particularly interesting if central carbon metabolism is the subject of the study [15, 16].

Most commonly, EI with electron energy of 70 eV is used for ionization in GC–MS based methods. Mass spectra generated with electron energy of 70 eV are characterized by extensive in-source fragmentation of the analyte. This fragmentation typically leads to selective, highly reproducible, and instrument-independent mass spectra, enabling mass spectral searches in extensive libraries, such as the NIST/Wiley library, for identification purposes. However, in MFA this harsh fragmentation complicates the ability to trace incorporation of heavy stable isotope labels of the intact molecule, since often molecular ions or fragments with an intact carbon backbone either are not present in the mass spectra or are present only in very low abundance. It is worth mentioning that in some cases information on the position of the isotope label in the carbon backbone is required [17–20]. Isotopologue-selective collision-induced-dissociation-based fragmentation is an established method to fulfill this requirement; however, this is approach is hampered when no ions with an intact carbon backbone are present.

In the present proof of concept, a novel low-energy EI (LEI) source is tested for its ability to preserve an intact carbon backbone of the native molecule during ionization and thereby facilitate stable isotope tracing. The concept of applying electron energies lower than 70 eV is not novel and was investigated in the early days of GC–EI-MS approaches [21, 22]. However, at that time, because of drawbacks in sensitivity and robustness (low ion source temperatures were applied, causing adsorption to the ion source walls), chemical ionization (CI) approaches prevailed. Here, we used an EI source that was modified in terms of the axial magnet and the lens geometry to render the application of lower electron energies possible without facing the aforementioned issues to the same extent. We optimized this commercially available state-of-the-art LEI source to meet the requirements of isotopologue distribution (ID) analysis of amino acids, organic acids, sugars, and sugar phosphates. The data obtained with this GC–LEI time-of-flight MS (TOFMS) approach were then compared with the data obtained with a CI-based approach in terms of sensitivity, trueness, and precision under repeatability conditions of measurement.

Materials and methods

Chemicals and reagents

2-Phosphoglyceric acid disodium salt hydrate, 3-phosphoglyceric acid disodium salt, *cis*-aconitic acid, α-ketoglutaric acid sodium salt, L-alanine, dihydroxyacetone phosphate lithium salt, fructose 6-phosphate disodium salt hydrate, D-(+)-fructose, fumaric acid, D-(+)-glucose, glucose 6-phosphate dilithium salt, glyceraldehyde 3-phosphate solution, DL-isocitric acid trisodium salt hydrate, L-isoleucine, L-lysine, L-methionine, D-(+)-mannose, mannose 6-phosphate disodium salt hydrate, mannitol 1-phosphate lithium salt, L-proline, ribulose 5-phosphate sodium salt, ribose 5-phosphate disodium salt hydrate, DL-serine, succinic acid, and L-threonine were purchased from Sigma-Aldrich (St Louis, MO, USA). L-Aspartic acid, citric acid monohydrate, L-glutamic acid, glycine, L-leucine, DL-malic acid, and L-valine were purchased from Merck Millipore (Darmstadt, Germany). For calculation of retention indices, fatty acid methyl esters from C_8 to C_{30} were purchased as part of the Fiehn GC/MS metabolomics standards kit (Agilent Technologies, Santa Clara, CA, USA).

Stock standard solutions of all analytes were prepared by dissolving an appropriate amount of solid standard in LC–MS-grade water or 0.1 M HCl. An analyte mixture solution of all single standards with a concentration of 25 μM was prepared in LC–MS-grade water by appropriate dilution. Methoxyamine hydrochloride and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich (St Louis, MO, USA). The methoxyamine solution was prepared daily by dissolving an appropriate amount of solid substance in water-free pyridine (Sigma-Aldrich, St Louis, MO, USA). After addition of an appropriate amount of methoxyamine hydrochloride, the working standards were evaporated to complete dryness in a vacuum centrifuge operating at low pressure, crimped, and stored at -80 °C. Dried working standards were stable for a minimum of 8 weeks. Before analysis, the prepared analyte mixtures were dried again for 0.5 h.

Pichia pastoris cell extracts

To test the robustness and selectivity of the optimized method, apart from solvent standards, two different cell extracts of *P. pastoris* were measured. In both yeast cell extracts, the intracellular metabolites exhibit a defined isotopologue pattern and hence were also suitable for the assessment of trueness: either the yeast was fed with natural glucose, and therefore also the metabolites exhibit a naturally distributed isotope pattern, or the cells were cultivated on 50:50 ^{12}C-labeled and ^{13}C-labeled methanol as the sole carbon source. In the latter case the defined ^{13}C distribution follows the binomial coefficients found in Pascal's triangle, a concept that was introduced by Millard et al. [23]. Both cell extracts were obtained from ISOtopic Solutions (Vienna, Austria). Depending on the injection (10:1 split or splitless), the final sample intake (i.e., the amount of initially extracted cell dry weight injected on-column) was approximately 1.5 μg and 15 μg cell dry weight, respectively.

Derivatization

To guarantee GC amenability of polar metabolites, a two-step derivatization procedure consisting of methoxymation in the first step and trimethylsilylation using MSTFA in the second step was used. The derivatization procedure followed the parameters stated in the Fiehn GC/MS metabolomics retention time locked library [7]: In the first step, 10 μL of methoxyamine hydrochloride (40 mg/mL in water-free pyridine) was added to the sample, and the mixture was vortexed and incubated at 40 °C for 90 min. In the second derivatization step, 40 μL of MSTFA (with 1% TMCS) was added, and the mixture was vortexed and incubated at 40 °C for 30 min.

GC–QTOFMS analysis and data evaluation

For the analysis of the isotopologue distribution of selected metabolites, including amino acids, organic acids, sugars, and sugar phosphates, a 7250 GC/Q-TOF MS system (Agilent Technologies, Santa Clara, CA, USA) was used. The system consisted of an Agilent 7890B network gas chromatograph equipped with a split/splitless injector and an Agilent 7250 accurate-mass quadrupole QTOFMS instrument with an LEI source. Before analysis the mass axis of the TOFMS instrument was calibrated automatically with an internal calibrant solution containing perfluorotributylamine. A sample volume of 1 μL was injected in split mode, ratio 10:1, using a deactivated straight split liner, without glass wool (Agilent Technologies, USA). The inlet was kept at 250 °C. A 10-μL syringe was used for injection and was washed with pyridine before and after injection, each time twice. The system was conditioned by injecting MSTFA and derivatized samples. Depending on the matrix load of injected samples, the liner

was exchanged after approximately every 100 injections. The guard column and gold seal were exchanged in the event of peak shape distortion.

The derivatized metabolites were separated on a 30-m Agilent DB-5ms analytical column (30 m × 250-μm inner diameter, 0.25-μm film thickness), with an additional 10-m guard column employing the GC settings recommended in the Fiehn GC/MS metabolomics retention time locked library [7].

To obtain a selective fragment (i.e., most commonly [M − CH₃]•⁺ for TMS- derivatives) with relative as well as absolute high intensity, different ion source temperatures (140, 150, 180, and 200 °C) and electron energies (10, 12, and 15 eV) were tested. The optimized LEI parameters were as follows: the ion source temperature was 140 °C and the electron energy was 15 eV. After "*auto tuning*" the ion source, the voltages of the repeller, source body, and extractor lens were optimized manually to maximize abundance and softness. By doing so, fragments of perfluorotributylamine, namely, with m/z 218.9851. 263.9866, and 501.9706, showed approximately similar mass spectral peak height intensity. The quadrupole temperature was set at 150 °C. An acquisition rate of 5 Hz was used.

To compare the LEI approach with another soft ionization strategy, CI was used. CI experiments were conducted with a 7200B GC/Q-TOF MS system (Agilent Technologies, Santa Clara, CA, USA). A dual-rail multipurpose sampler (MPSII, GERSTEL, Mülheim an der Ruhr, Germany) was used for injection. For chromatographic separation, the same conditions were used [7], with the exception of using a splitless injection of 1 μL at 250 °C, with use of a deactivated single-taper splitless liner, without glass wool. To maintain the system, the same conditioning actions were taken as mentioned before. CI parameters can be found in [20].

For confirmation of the identity of derivatized metabolites, retention indices were determined with use of fatty acid methyl esters and used for library search in the Fiehn library in MassHunter Unknowns Analysis (B.09.00, Agilent Technologies, Santa Clara, CA, USA). For evaluation of the high-resolution MS data set, MassHunter Quantitative Analysis (TOF) (B08, Agilent Technologies, Santa Clara, CA, USA) was used. Mass spectral data were acquired in profile mode and were converted after acquisition to centroid data with use of MassHunter software. Extracted m/z values and the mass extraction windows used for the LEI approach can be found in Table S1, whereas for the CI approach, information can be found in [20].

After manual inspection and reintegration where needed, the ID of the derivatives were calculated for quality control. Data obtained from cell extracts of *P. pastoris* that was fed with labeled methanol were subsequently corrected for interferences stemming from the contributions of naturally distributed heavy stable isotopes (^{29}Si, ^{30}Si, ^{13}C, ^{2}H, ^{15}N, ^{18}O, ^{34}S) with ICT (isotope correction toolbox) [10]. After heavy stable

isotope interference correction, the labeling pattern of the carbon backbone was calculated.

Results and discussion

Optimization of LEI source conditions

Obtaining information on ^{13}C labeling patterns of the metabolites' intact carbon backbone is central in ^{13}C-based metabolic flux analysis experiments. To meet this requirement, the optimization, which aimed at low fragmentation at reasonable intensity, was performed with a set of model metabolites, representing different compound classes of the central carbon metabolism. The selection of model compounds for amino acids was based on their polarity (aspartic acid and lysine as positively charged and negatively charged amino acids, respectively, and valine as a nonpolar and branched-chain amino acid) and length of the carbon backbone (C_4–C_6). In the case of organic acids and sugar phosphates, the carbon backbone length and the presence of keto groups were a determining factor. For hexoses, one ketose, namely fructose, and one aldose, namely glucose, was selected.

This necessary derivatization introduces an additional source of variance and generally increases the complexity of analysis regarding the number of derivatives generated as well as the matrix-dependent derivatization efficiency [24]. As stated before, two different instruments were used for reasons of comparison. In the CI approach, a dual-rail multipurpose sampler was used for just-in-time online two-step derivatization. After incubation with the silylation agent, the sample was stored until subsequent injection (within a maximum of 10 min) in a cool tray at 6 °C. The derivatization for the LEI approach was performed off-line and samples were stored at room temperature until injection (within 24 h). It is noteworthy that the reaction was not stopped. Hence, differences in the relative ratio of two (or more) derivatives of a single metabolite depended not only on the (clean) status of the GC–MS instrument but also on the actual incubation time.

Aiming at an ion with an intact carbon backbone with appropriate signal intensity, we optimized the source conditions on the basis of the relative and absolute signal intensity of the selective fragment (i.e., $[M - CH_3]^{\bullet+}$). Since there is a clear correlation between temperature and the extent of fragmentation, different ion source temperatures were tested. The lowest temperature that could be kept constant during the chromatographic run was empirically found to be 140 °C. Besides, measurement at lower temperatures might lead to adsorption of substances on the ion source wall and thereby potentially decrease robustness as well as sensitivity over time. The highest temperature tested in the optimization process was a standard temperature for this EI source (i.e., 200 °C). For

electron energies, three different voltages were tested: 10, 12, and 15 eV. Higher electron energies were not comprehensively tested, since preliminary data (not shown) indicated no substantial gain either in softness or in absolute intensity for the selected set of compounds. The results of testing the different parameters for the selected organic acids, amino acids, sugar phosphates, and sugars are summarized in Fig. 1. The relative intensity of the $[M - CH_3]^{\bullet+}$ ion to the base peak (i.e., the most intense mass spectral peak) is depicted on the primary y-axis (bars shown in blue, green, yellow, and orange). The absolute intensity is shown as the sum of the mass spectral peak intensities of the two ions on the secondary y-axis (depicted as overlapping white/transparent bars). If the selective fragment (i.e., $[M - CH_3]^{\bullet+}$) was the base peak, then the second most intense peak was chosen to calculate the ratio and absolute signal intensities. For each model compound, the same two ions were always compared for the conditions tested.

To account for variability of the ionization process itself, a rather overestimated standard deviation of 15% was applied to the relative intensities. The absolute signal intensities depicted on the secondary y-axis need to be considered as rough estimates because no just-in-time derivatization was used and hence neither the derivatization reactions nor compound-dependent decomposition was standardized [24].

Although the extent of fragmentation is highly compound dependent, there is evidently an opposing trend in terms of softness (corresponding to the colored bars) and absolute signal intensity (corresponding to the overlapping white/transparent bars). As shown in Fig. 1, this general trend is observable for all selected model compounds, except for valine, where no clear conclusion can be drawn because of the low relative intensity of the selective fragment. As optimum source conditions, 15 eV and a source temperature of 140 °C were chosen since here both softness (corresponding to the dark-blue bar and the primary y-axis) and absolute signal intensity (corresponding to the white bar overlapping the dark-blue bar and the secondary y-axis) show good results.

Assessment of trueness and precision under repeatability conditions of measurement of LEI and CI for the analysis of isotopologue distributions

To assess the fitness for purpose of the optimized LEI method, the trueness and precision of isotopologue distributions were assessed with three sample types, as described in "Materials and methods." Additionally, this approach was compared with a previously published CI-based approach [13, 20]. In addition to trueness and repeatability, limits of detection (LODs) were determined for the set of model compounds in Fig. 1. For reasons of comparability, earlier published data on an EI approach for isotopologue analysis [20] were included as well (data were acquired with a 7200 GC–QTOFMS instrument, although with
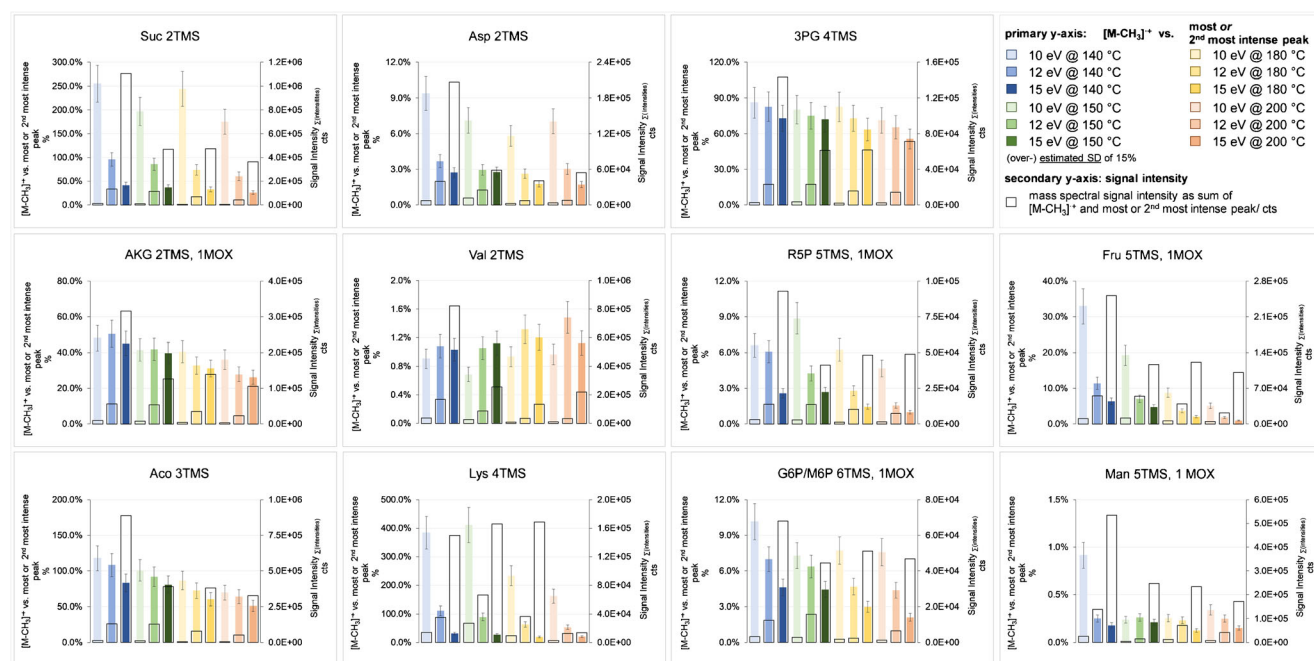
Fig. 1 Optimization of ion source parameters on the basis of selected metabolites. Metabolites are arranged according to their compound classes and within the columns with increasing number of carbons in the backbone. The first column shows organic acids; namely, derivatives of succinic acid (Suc), α-ketoglutaric acid (AKG), and cis-aconitic acid (Aco). The second column shows derivatized amino acids: L-aspartic acid (Asp) and L-lysine (Lys) as representatives of amino acids with polar side chains and L-valine (Val) as an example of an amino acid with a hydrophobic side chain. The third column shows derivatives of sugar phosphates: namely, 3-phosphoglyceric acid (3PG), ribose 5-phosphate (R5P), and two aldohexose 6-phosphates, glucose 6-phosphate (G6P) and mannose 6-phosphate (M6P) (combined data), since these two isomers are not chromatographically separated. In the fourth column results for the ketose -fructose (Fru) and the aldose - mannose (Man) are shown. On the primary y-axis, the ratio between the selective fragment (i.e., $[M - CH_3]^{\bullet+}$) and the base peak (or if the selective fragment represents the base peak, the second most intense peak) is depicted as a percentage, whereas on the secondary y-axis the signal intensity as the sum of the two ions is shown in counts. Absolute intensities are indicated in black framed transparent columns for each data point. For the ratios on the primary y-axis, an estimated standard deviation (SD) of 15% was applied. For the ion source temperatures tested, 140 °C is shown in different shades of blue, 150 °C is shown in different shades of green, 180 °C is shown in different shades of yellow, and 200 °C is shown in different shades of orange. Electron energies of 10, 12, and 15 eV are shown in decreasing transparency depending on the ion source temperature settings applied. MOX, methoxyamine TMS trimethylsilyl

different GC separation conditions). In the case of sugar phosphates, only results for 3-phosphoglyceric acid and ribose 5-phosphate are given, since glucose 6-phosphate and mannose 6-phosphate are not chromatographically separated by the Fiehn method. Since data are missing for D-(+)-mannose in the case of EI, D-(+)-glucose was added as an additional representative for an aldohexose. The LODs are given in femtomole on column, accounting also for different injection modes, including the respective split ratio, and are depicted in Fig. S1. LODs were determined following the EURACHEM validation guidelines [25], using the integrated peak areas of the isotopologue with the highest measurable mass increment. The sensitivities of the different approaches are compound dependent but are in general approximately of the same order of magnitude. Absolute LODs are in the low femtomole range and meet the requirements for the analysis of labeling patterns from labeling experiments with heavy stable isotopes [20].

The amount of data acquired with the different sample types as well as the two ionization approaches varies (Fig. 2), which can be explained by the differences in metabolite concentration present in the cell extracts and by the different injection types used. Split injection in the case of CI would not have been suitable in terms of sensitivity, whereas for practical reasons of metabolite identification by means of a retention index–EI library [7], splitless injection for the LEI approach was not considered as reasonable.

Solvent standards and a yeast cell extract with natural glucose as the substrate were measured to assess the trueness and precision for naturally distributed metabolite derivatives. To assess trueness for isotopologue fractions (IFs) of heavier isotopologues that naturally have low abundance, P. pastoris was fed with labeled methanol and was measured, natural isotope interference corrected [10], and evaluated. Yeast cell extracts were derivatized once and injected in total six times. The sample will change over the elapsed time; however, if the signal intensity is sufficient (visually estimated signal-to-noise ratio of 10), it will not influence the isotopologue ratios. Metabolites that did not meet the signal-to-noise ratio requirement were omitted. Solvent standards were derivatized in triplicate, and one sample was injected three times.

Fig. 2 Assessment of trueness of the results obtained for isotopologue fractions (IFs) obtained by different ionization approaches: distribution of IFs versus bias obtained by gas chromatography (GC)–low-energy electron ionization (EI) time-of-flight mass spectrometry (TOFMS) (a) and GC–chemical ionization (CI) TOFMS (b). Blue squares represent IFs measured in a solvent standard (n = 5) and green diamonds show the IFs measured in a *Pichia pastoris* cell extract, with natural glucose as the substrate (n = 6). The red circles represent results obtained with a yeast cell extract that was fed with 50:50 $^{13}CH_3OH$ and $^{12}CH_3OH$, leading to a labeling pattern according to the binomial coefficients of Pascal's triangle (n = 6)
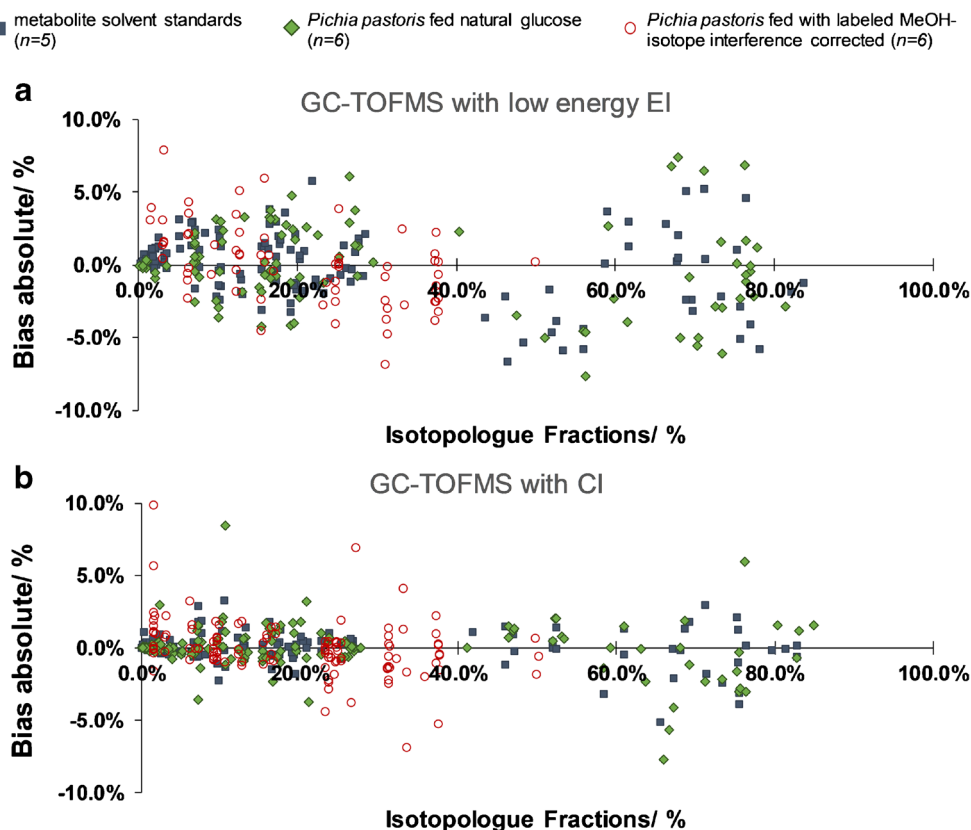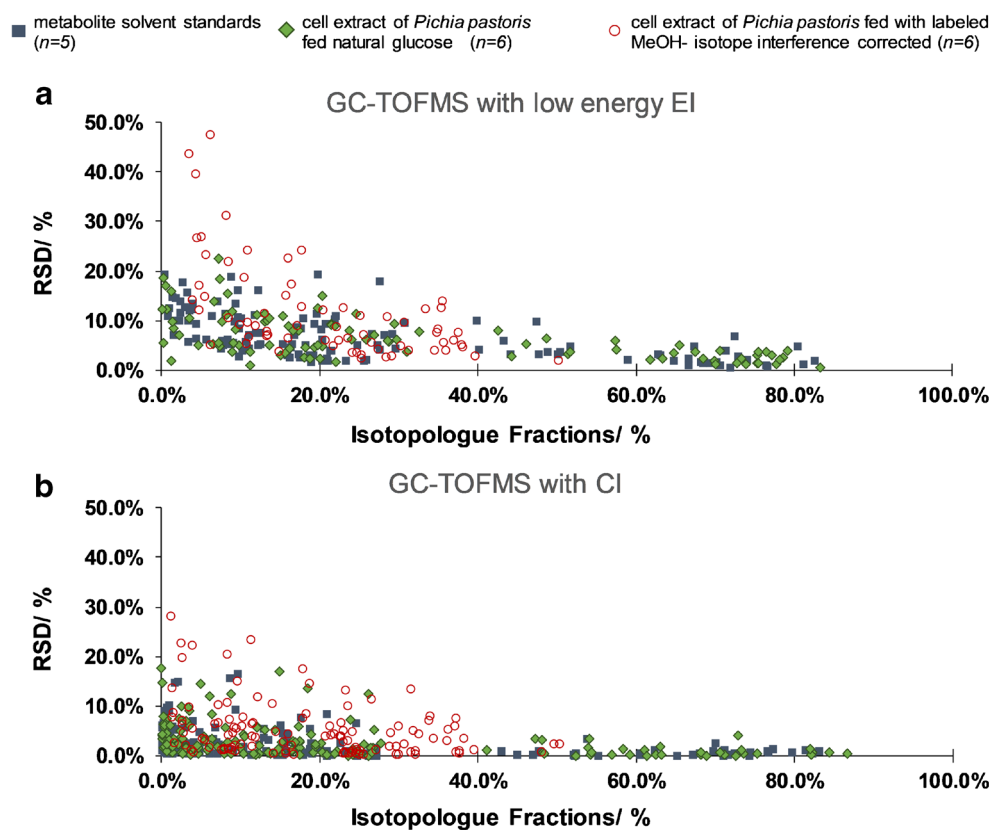

Trueness was determined by assessing the difference between the measured IF and the theoretical IF and is depicted as percent bias in Fig. 2 plot a for GC–TOFMS with LEI and in Fig. 2 plot b for GC–TOFMS with CI. Importantly, assessment of trueness of isotopologue patterns by using only naturally distributed compounds will not fully resolve possible interferences, especially on naturally small fractions. An elegant way of avoiding this was demonstrated by the Portais group in a very comprehensive manner [23, 26]. Hence also a cell extract of *P. pastoris* fed with 50:50 $^{13}CH_3OH$ and $^{12}CH_3OH$, was used, since here the isotopologue distribution follows the binomial coefficients of Pascal's triangle [23]. Use of this approach also proved to be highly beneficial for interplatform comparison [27].

For the LEI approach, the average bias measured in solvent standards was 1.9%, with a maximum bias of 6.6%, whereas in the naturally distributed yeast cell extract an average bias of 2.3%, with a maximum bias of 7.6%, was obtained. Similar results were obtained for the yeast cell extract, where labeled methanol was used as the tracer: an average value of 2.1%, with a maximum of 7.9%. For the soft ionization technique using CI, slightly lower biases were obtained: an average of 0.6% and a maximum bias of 5.1% in solvent standards and an average of 0.9% and a maximum bias of 8.4% for the naturally distributed derivatives in yeast cell extract. For the extract with the labeling pattern according to Pascal's triangle, an average bias of 1.3% and a maximum of 9.8% were obtained.

For the two ionization techniques used, results for repeatability of measuring IFs in different sample matrices are depicted in Fig. 3. Figure 3 plot a shows the results obtained with the LEI source, whereas Fig. 3 plot b shows CI data. As recently demonstrated, because of error propagation, especially low-abundant fractions are characterized by a significant increase of measurement uncertainty after isotope interference correction [28]. This can also be observed in Fig. 3 plot a for the smaller IFs of the isotope-interference-corrected yeast cell extract, depicted as red circles. The average relative standard deviation obtained for the LEI approach is 6.9% for solvent standards and is 6.5% for the naturally distributed yeast cell extract. Lower precision was achieved for the cell extract with methanol as the substrate; namely, 11.0%. This can be explained, on the one hand, by the different distribution, since a normal distributed pattern most often shows the highest precision on the monoisotopic mass, and, on the other hand, by isotope interference correction performed on this data set [28]. The average relative standard deviation for the CI approach was 2.6%, 3.0%, and 5.2% respectively, as can be seen in Fig. 3 plot b. Both approaches result in values in a good range and depending on the type of analytes (e.g., branched-chained amino acids or aldohexoses) are fit for purpose for ^{13}C-based metabolic flux analysis experiments since they are comparable with values obtained with a previously published method [15, 16, 20, 28]. Differences for the two ionization

Fig. 3 Assessment of precision under repeatability conditions of measurement: distribution of isotopologue fractions (IFs) versus relative standard deviation (RSD) obtained by gas chromatography (GC)–low-energy electron ionization (EI) time-of-flight mass spectrometry (TOFMS) (a) and GC–chemical ionization (CI) TOFMS (b). Blue squares represent IFs measured in a solvent standard ($n = 5$) and green diamonds show the IFs measured in a *Pichia pastoris* cell extract with natural glucose as the substrate ($n = 6$). The red circles represent results obtained with a yeast cell extract that was fed with 50:50 $^{13}CH_3OH$ and $^{12}CH_3OH$

Conclusion

A novel LEI source was successfully optimized in terms of softer fragmentation and thereby for most metabolites delivered sufficient relative and absolute signal intensity of the selective fragment $[M - CH_3]^{•+}$. Because of the compound-specific behavior it is obvious that for certain analytes (e.g., derivatives of branched-chain amino acids such as valine or aldohexoses such as mannose), information on labeling patterns of the intact carbon backbone will be difficult to obtain. However, there might be other possibilities (e.g., changing the derivatization from TMS to TBDMS derivatives for amino acids or increasing the amount of sample on column) to circumvent these issues. Clearly, CI, as a soft ionization approach, is still favorable if it is of utmost importance that the protonated molecule represents the base peak. However, the assessment of trueness and precision under repeatability conditions demonstrated the suitability of the LEI approach in ^{13}C-based metabolic flux analysis experiments. In terms of sensitivity, both approaches, LEI and CI, result in values within

techniques can also be explained by the general lower absolute signal intensity due to the different amount of sample on column.

approximately the same range. Besides, the advantage of performing harsh and semisoft ionization in one ion source is striking.

Notably, the optimized settings are not limited to ^{13}C-based metabolic flux analysis, but can be directly extended to identification workflows, since molecular ions or fragments with an intact native molecule, such as $[M - CH_3]^{•+}$, are produced and primary fragmentation is favored. Both lead to more easily interpretable mass spectra. Together with the availability of retention indices in libraries, we consider the LEI approach as highly promising, especially for future GC–MS-based nontargeted work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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