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Simplified absolute metabolite quantification by gas chromatography–isotope dilution mass spectrometry on the basis of commercially available source material

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ABSTRACT

In the field of metabolomics, GC–MS has rather established itself as a tool for semi-quantitative strategies like metabolic fingerprinting or metabolic profiling. Absolute quantification of intra- or extracellular metabolites is nowadays mostly accomplished by application of diverse LC–MS techniques. Only few groups have so far adopted GC–MS technology for this exceptionally challenging task. Besides numerous and deeply investigated problems related to sample generation, the pronounced matrix effects in biological samples have led to the almost mandatory application of isotope dilution mass spectrometry (IDMS) for the accurate determination of absolute metabolite concentrations. Nevertheless, access to stable isotope labeled internal standards (ILIS), which are in many cases commercially unavailable, is quite laborious and very expensive. Here we present an improved and simplified gas chromatography–isotope dilution mass spectrometry (GC–IDMS) protocol for the *absolute* determination of intra- and extracellular metabolite levels. Commercially available ^{13}C-labeled algal cells were used as a convenient source for the preparation of internal standards. Advantages as well as limitations of the described method are discussed.

1. Introduction

1.1. Absolute quantification of metabolites

Quantitative measurements of intra- and extracellular metabolites rose to prominence in the early nineties as a highly directed and targeted analysis of selected compounds during so-called "stimulus–response" experiments designed for the identification of enzyme kinetics under *in vivo* conditions [1–7]. The analytical tools utilized in these earlier targeted, model driven metabolite measurements were either enzymatic assays or HPLC. The application of more powerful instrumental separation technology such as LC–MS [8–20], GC–MS [21–32] as well as capillary electrophoresis [33–37] and NMR [38–42] for analysis of the metabolites was an important extension of the approach.

With the power of today's technologies in instrumental analytics the focus has shifted from targeted analysis of selected compounds towards rich and detailed determination of a tremendous number of compounds comprising thousands of peaks, which enable realization of the conceptual strategy of 'metabolome', a term originally proposed by Oliver et al. [43]. In contrast to the strong precision requirements of targeted metabolite analysis, global strategies like metabolite fingerprinting, footprinting, profiling, etc. are characterized by qualitative or at least semi-quantitative measurements [44]. The broad application of this strategy in diverse fields, such as plants, blood plasma and microbial systems has been extensively reviewed [14,26,27,45–53].

As long as these approaches are driven by different purposes and applications, the differences in the precision of the results and quantification are acceptable. But as far as the aforementioned application of metabolite measurement for dynamic modeling of metabolism is concerned, reliable quantification of absolute concentrations is of outermost importance. Liquid chromatography coupled to mass spectrometry (LC–MS) became accepted as the most widespread analytical technology in absolute metabolite quantification today [10,18,23,54–59].

1.2. GC–MS in metabolome analysis

In contrast to LC–MS, gas chromatography–mass spectrometry (GC–MS) has rather established itself as a tool for qualitative and semi-quantitative strategies in metabolomics (e.g. metabolic

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fingerprinting, GC-TOF) [21,24,29–31,60–72]. The first report on application of this technology for metabolic profiling in plants was presented by Sauter et al. [73] and later extended to the simultaneous determination of large number of metabolites in a range of plant species [25,48,74,75]. Further application of this analysis in microbial systems, mammalian cells and clinical chemistry is discussed by [22,30,47,56,76–78]. The first suggestion to do global metabolomics of yeast by GC–MS was made by Villas-Boas et al. [21]. A first detailed discussion of the derivatization method in the application of GC–MS analysis as a tool for the determination of highly polar metabolites in non-clinical biological samples has been provided by Roessner et al. [25]. Their approach rests upon derivatization procedures involving sequential methoximation and trimethylsilylation [79] to simultaneously determine a large number (150) of polar metabolites in potato tuber.

The need to derivatize polar metabolites in the presence of varying biological matrices prior to analysis is probably one of the many reasons for observed uncertainties in the reliability of quantification of metabolites utilizing GC–MS analysis. GC–MS analysis is, on the other side, attractive because of the superior separation power after proper derivatization of the metabolites that often co-elute in LC–MS [17,57,80]. Higher sensitivity with simple quadrupole technology, excellent ionization efficiency for a wide spectrum of derivatized metabolites, the information rich EI-mass spectra as well as comparatively easy operation and robust technology pertain to the further benefits.

1.3. Isotope dilution mass spectrometry (IDMS)

Uniformly (U) ^{13}C-labeled metabolites used as internal standards (isotope labeled internal standards, ILIS) are able to compensate for losses during sample generation, varying derivatization yields or ion suppression in MS ion sources. Therefore, ILIS allow for accurate absolute quantification of metabolites in the presence of complex biological matrices. But such compounds are immensely expensive or unavailable at all. One feasible approach uses the metabolic productivity of microorganisms to gain access to mixtures of uniformly ^{13}C-labeled metabolites [10,81]. For the first time this was demonstrated in the yeast *Saccharomyces cerevisiae* using LC–MS/MS [9]. The authors cultivated a yeast strain on stable isotope-labeled C-sources and the resulting cell extracts were directly used as an IS-mix allowing the quantification of any intracellular metabolite of interest in *S. cerevisiae* (isotope dilution MS, "IDMS"). This methodology was recently applied to intracellular metabolite measurements in aerobic yeast culture using GC–IDMS by Cipollina et al. [81]. But still this strategy causes considerable costs and is rather laborious.

Here, we present the results of systematic investigations regarding absolute quantitative metabolite measurements by GC–IDMS using commercially available U–^{13}C-labeled algal extracts as internal standards. The yeast *S. cerevisiae* cultivated at physiologically defined conditions served as an example. An improved protocol is suggested which permits reliable and accurate measurement of intra- and extracellular metabolites.

2. Materials and methods

2.1. Solvents and chemicals

HPLC-grade water and methanol were supplied by VWR (Darmstadt, Germany). Reagents, metabolite standards and lyophilized algal cells were supplied by Sigma–Aldrich (Taufkirchen, Germany).

2.2. Strain, medium and pre-culture conditions

The yeast *S. cerevisiae* haploid strain CEN.PK 113-7D (MATa, Ura3, His3, Leu2, Trp1, Mal2, Suc2)[4] was used in the research. The yeast from glycerol stock was cultivated on agar in sealed plates at 30 °C. Some colonies were picked up and inoculated into 5 mL liquid anaerobic CEN.PK medium prepared according to Verduyn [82] with 15 g/L glucose and incubated overnight. It results in the pre-culture with $OD_{660} \sim 0.1$ O.U.

The final composition of the minimal growth medium for anaerobic cultivation of yeast *S. cerevisiae* CEN.PK 113-7D according to [82] was: $(NH)_2SO_4$ 15 g/L, KH_2PO_4 9 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, EDTA-Na_2 45 mg/L, $ZnSO_4{\cdot}7H_2O$ 13.5 g/L, $MnCl_2{\cdot}4H_2O$ 3.0 mg/L, $CoCl_2{\cdot}6H_2O$ 0.9 mg/L, $CuSO_4{\cdot}5H_2O$ 0.9 g/L, $Na_2MoO_4{\cdot}2H_2O$ 1.2 mg/L, $CaCl_2{\cdot}2H_2O$ 13.5 mg/L, $FeSO_4{\cdot}7H_2O$ 9.0 mg/L, H_3BO_3 3.0 mg/L, KI 0.3 mg/L, D-biotin 0.15 mg/L, Ca-D(+) panthotenate 3.0 mg/L, nicotinic acid 3.0 mg/L, myo-inositol 75.0 mg/L, thiamine hydrochloride 3.0 mg/L, pyridoxol hydrochloride 3.0 mg/L, p-aminobenzoic acid 0.6 mg/L. Additionally, ergosterol (10 mg/L) and Tween 80 (420 mg/L) were dissolved in ethanol (2.84 g/L) and added to CEN.PK medium as anaerobic supplement.

2.3. Anaerobic chemostat cultivation

A steel KLF bioreactor (Bioengineering, Switzerland) with 2.5 L working volume (V_r) was used in the research (Fig. 1). The growth conditions were: anaerobic CEN.PK medium, 30 °C, pH 5.0 ± 0.1 (adjusted by 3 M KOH), stirring at 400 rpm, antifoam (1 mL per 3 h) and constant nitrogen sparging through the culture with 1.8 L/min and 0.1 bar headspace overpressure during cultivation and 0.7 bar during sampling.

2.0 L of anaerobic CEN.PK medium containing 15 g/L of glucose were inoculated with pre-culture and the bioreactor was maintained in batch mode for ~24 h whilst the biomass reaches ca. ~3 gDW/L. Then the bioreactor is switched into chemostat mode being fed by anaerobic CEN.PK medium containing 50 g/L glucose with a dilution rate (D) of 0.1 h^{-1}. Fermentation parameters (weight of the bioreactor, temperature, stirring, pH, pressure, etc.) were controlled by a control block (Hardi Instruments, San Diego, USA, Type 0582-5420-11) supplied by Bioengineering. CO_2 in the off-gas was measured on-line by an Innova 1313 fermentation monitor (LumaSense Technologies, Denmark). The fermentation parameters (time, pressure, pH, %CO_2) were computer-controlled via Interface 1 (Fig. 1) using the interface-program Biolog 1.3 which was developed by A. Freund (IBVT, University of Stuttgart, Germany) in Labview 8.6 (National Instruments, USA). After ~72 h the biomass reaches steady state at 3.5 gDW/L. The culture was maintained in the chemostat mode no longer than one week, otherwise adaptation processes take place [83].

2.4. Measurement of concentration of dry biomass (Cx)

Prior to metabolite sampling, 10 mL of broth from the bioreactor were sampled by over-pressure through sampling port 1 (Fig. 1) for gravimetric dry weight biomass [gDW/L] determination. The sample was immediately filtered out on pre-weighed membrane filter (0.45 μm, 47 mm, Supor®-450, Pall Gelman Laboratory) under vacuum. The filter was twice washed with 10 mL of ice-cold 0.9% NaCl and dried out at 115 °C overnight in a drying oven. Prior to weighing the filters were placed in a desiccator until they reached room temperature.

[4] The <fn0005>strain was kindly provided by Dr. Peter Kötter, Institute for Molecular Biosciences, Goethe University of Frankfurt, Germany.

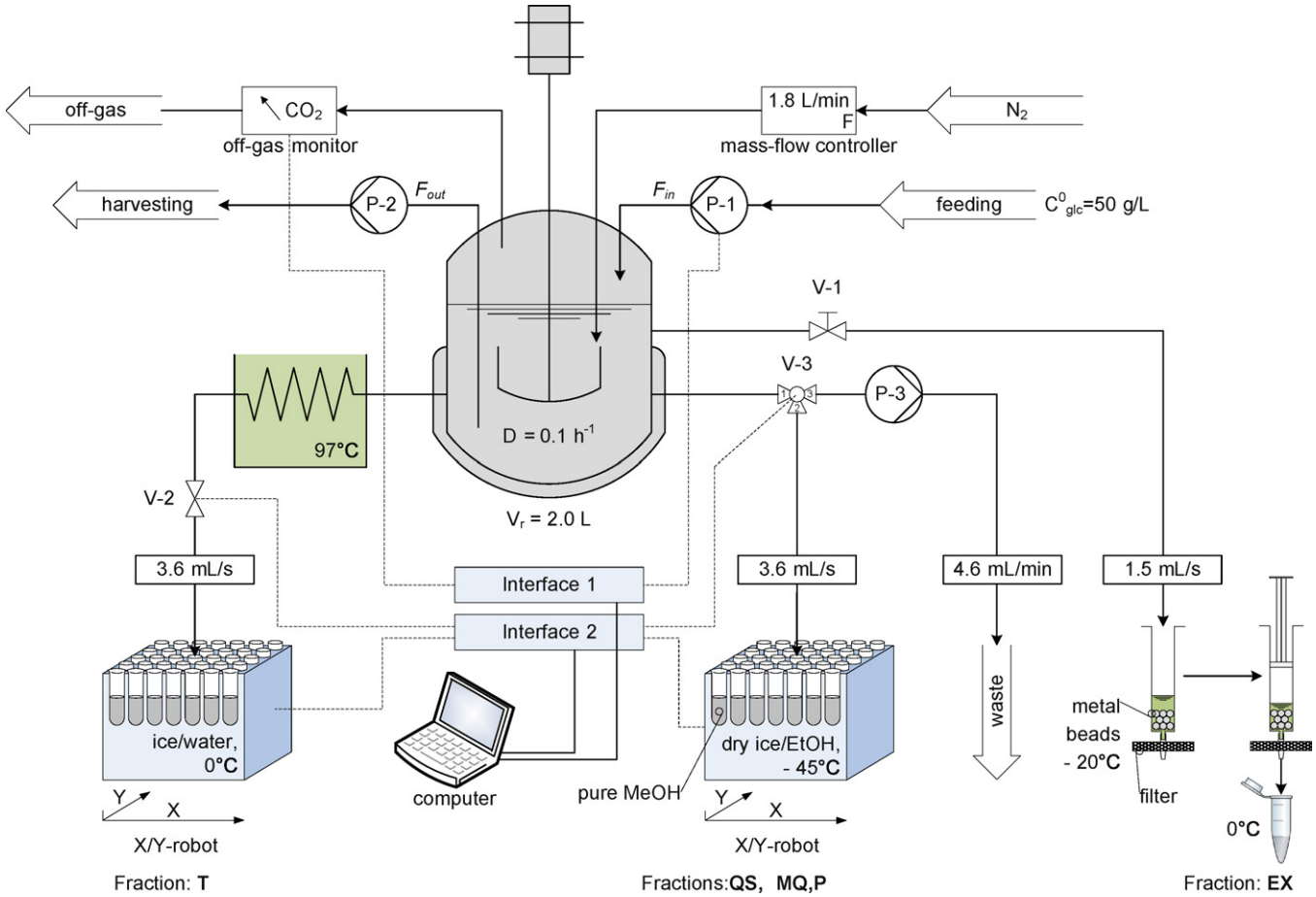

Fig. 1. Scheme of the experimental setup.

2.5. Sampling techniques

Steady state samples were collected using a setup which was designed for forthcoming stimulus–response experiments. The bioreactor was equipped with three ports for rapid sampling (Fig. 1): (V-1) manual sampling for extracellular metabolites by fast filtering, (V-2) robotic sampling for total metabolites (from both biomass and medium) by a fast heat exchanger at 97 °C and (V-3) robotic sampling for intracellular metabolites by classical 'cold methanol quenching' at −45 °C. V-3 was not in use in the present context.

2.5.1. Dilution rate control

During collection of the samples, the harvesting peristaltic pump (P-2; Fig. 1) was turned off (i.e. $F_{out} = 0$) and consequently the weight control of the bioreactor was turned off as well, since total sampling flow rate was more than the harvesting itself. Therefore, the feeding rate (F_{in}) must be dynamically corrected via a computer-aided peristaltic pump 1 (P-1; Fig. 1) to compensate a loss of the V_r and to keep the dilution rate $D = const. = 0.1\ h^{-1}$ throughout the entire sampling period.

2.5.2. Robotic sampler

The computer-aided robotic sampler [6,57] consists of: (i) a gate-valve and (ii) a Cartesian position system (X/Y-robot) with a mounted thermo-insulated tray accommodating a rack for 60 tubes of 15 mL each (Fig. 1). The Cartesian position system (Bahr Modultechnik, Luhden, Germany) and solenoid gate-valves (Fluid Automation Systems, Versoix, Switzerland) are coupled with a computer via interface control unit SM-300 (Interface 2 in Fig. 1; SM Elektronik, Schwenningen, Germany). The X/Y-robot moves the rack with sample tubes in a step-wise manner relative to a valve fixed in position. The interface program was developed by A. Hauck (IBVT, University of Stuttgart, Germany) in Labview 8.6 (National Instruments, USA). The interface program operates with two time-variables: t_1 – opening time of the gate-valve; t_2 – time difference between two successive samples. The combination of the headspace pressure (P) and t_1 define the sample volume, whereas t_2 defines sampling frequency. The dead volume of the sampling port was more than 5% of the sample volume; therefore the sampling port had to be flushed before the actual sampling to avoid broth stagnation in the dead volume.

2.5.3. Bioreactor pressure control

As headspace of the bioreactor increases during the sampling, the gas pressure and mass-flow had to be stabilized ($P = 0.7$ bar and 1.8 L/min by a mass flow controller, type GFC-171S, Analyt Mtc. GmbH, Germany).

2.5.4. Differential sampling approach

All operations concerning sampling and sample processing were monitored gravimetrically in order to accurately match measured metabolite concentrations in different fractions to the respective biomass dry weights.

Sample fractions were notated according to the assumption that the total mass (T) of any metabolite is distributed both extra- (EX) and intracellularly (IN). The system (cells + medium) can then be described by:

$$T = EX + IN \quad \text{or} \quad IN = T - EX$$

Table 1
Quantification of intra- and extracellular metabolites by GC–IDMS.

Analyte	Calibration range (pmol)	SIM mass analyte (m/z)	Internal standard (IS)	SIM mass IS (m/z)
Pyr	10–1000	174	U-^{13}C–Pyr	177
Lac	10–1000	219	U–^{13}C–Lac	222
Suc	25–2500	247	U–^{13}C–Suc	251
Fum	10–1000	245	U–^{13}C–Fum	249
Mal	25–2500	245	U–^{13}C–Mal	249
aKG	10–1000	304	U–^{13}C–aKG	309
G3P	10–1000	445	U–^{13}C–G3P	448
2PG	10–1000	369	U–^{13}C–2PG	372
3PG	25–2500	459	U–^{13}C–3PG	462
Pep	10–1000	369	U–^{13}C–Pep	372
Cit	100–10,000	273	U–^{13}C–Cit	278
Glc	100–10,000	319	U–^{13}C–Glc	323
R5P	10–1000	217	U–^{13}C–R5P	220
F6P	10–1000	459	U–^{13}C–F6P	462
G6P	25–2500	471	U–^{13}C–G6P	475
6PG	10–1000	333	U–^{13}C–6PG	337

2.5.5. EX: extracellular mass of metabolites

The samples for measurements of extracellular metabolite concentrations (EX) were collected manually through V-1 sampling port. 3 mL of the broth were quickly sampled by over-pressure through sampling port 1 (manual valve V-1; Fig. 1) into a syringe (5 mL Braun® with Luer lock) containing ~3.85 g of pre-cooled (−20 °C) steel beads (4 mm, 15 beads per syringe). This method allows decreasing sample temperature down to ~0 °C in less than a second [1,84]. The broth was immediately filtered through an attached syringe filter (Ratilabo®, CME, 0.45 μm, outer diameter 33 mm, with Luer lock, Carl Roche GmbH) directly into a pre-cooled (0 °C) tube and stored at −70 °C. For a skilled team the sampling procedure takes about 2 s per sample which corresponds to a sampling rate of ~1.5 mL/s.

2.5.6. T: total mass of metabolites

The samples for measurements of total mass of metabolites (T) were collected using a rapid heat exchanger with a robotic sampler [57] through V-2 sampling port (Fig. 1). The sampling system was designed as a single coiled steel tube (with 0.5 mm wall thickness and 2 mm inner diameter) which is submerged into a 97 °C thermostat (Fig. 1). The overall internal volume of the sampling system was 12.7 mL and the sampling flow rate was 3.6 mL/s under P = 0.7 bar. The heating rate of the sample during sampling is estimated to be ~135 °C/s (from 30 up to 97 °C in ~0.5 s) [57] and the residence time of the sample in the heat exchanger is 3.5 s. To avoid excessive loss of broth due to the high sampling flow rate, the V-2 was opened only during sampling. Consequently, the samples were always taken in a pair: (1) 11.15 ± 0.27 mL (with t_1 = 3.1 s) to flush the dead volume of the heat exchanger and then immediately (2) 2.89 ± 0.08 mL of sample (with t_1 = 0.8 s). The exact amount of each obtained sample was determined gravimetrically. The collecting tray with sample tubes in the robotic sampler was filled with ice/water at 0 °C and it takes ~1 min to cool the sample down to 0 °C after the heat exchanger. The withdrawn samples were additionally boiled at 95 °C for 5 min for complete extraction, then immediately cooled to 0 °C and centrifuged at 4 °C and 20 × 10^3 g for 5 min. Then the supernatant was collected, aliquoted and stored at −70 °C until further analysis.

2.6. Preparation of U–^{13}C-labeled internal standard

U–^{13}C-labeled lyophilized algal cells (>99 atom% ^{13}C, lot no. 06741EB, Isotec) were weighed into polypropylene tubes (screw cap, 12 mL; Greiner, Frickenhausen, Germany) in portions of 60–75 mg. The scale of the extractions was intentionally kept small in order to enable quick heating and cooling. About 5 mL of boiling water was added to each tube and the algal cells were extracted for 3 min at 95 °C whilst shaking. The tubes were chilled in ice-water, the resulting suspensions were pooled and centrifuged (0 °C, 4000 × g, 15 min). The clear supernatant was divided into 500-μl-aliquots and stored at −70 °C. Extracts from non-labeled algal cells (lot no. TV1530, Isotec) were prepared in the exact same manner.

2.7. Sample processing for GC–IDMS analysis

To 40 μl of sample (contains approx. 3 μmol of phosphate, see Section 3.1.1.4) were added 50 μl of internal standard (see above) and 50 μl of O-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine). The samples were evaporated to dryness in 1.5-mL Eppendorf tubes using a rotary vacuum concentrator (SpeedDry 2-33IR, Christ, Osterode, Germany). Sample temperature was kept below 10 °C during evaporation by means of sample-specific drying programs (controlled drying-pressure and -temperature). Furthermore, residence time of dried samples in the concentrator was kept as short as possible (≤15 min). 60 μl of O-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine) was added to the evaporated samples and the properly sealed tubes were incubated at 30 °C for 90 min during vigorous shaking. Insoluble matter was separated by centrifugation (r.t., 14,000 × g, 5 min) and 40 μl of clear supernatant were transferred to a brown glass V-shaped micro-vial (0.8 mL, CS Chromatographie Service, Langerwehe, Germany) with screw cap and silicone/PTFE septa. After addition of 64 μl of N,O-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS 99:1, Supelco), the vials were placed in a heating shaker (Eppendorf, Hamburg, Germany) equipped with an appropriate heater block suitable for standard autosampler vials (11.5 mm × 32.0 mm). Each well in the heater block was equipped with a dedicated PTFE support sleeve (Supelco) which ensured a proper position of the V-shaped vial. The silylation reaction was conducted at 50 °C with shaking for 60 min. The vials were cooled to r.t., vortexed and analyzed by GC–IDMS within 10 h.

Calibration standards were prepared from 50 μl of properly adjusted metabolite composition (solution in water, see Table 1), 50 μl of internal standard and 5 μl of O-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine). The calibration standards were processed in the exact same manner as described above for samples.

2.8. Metabolite analysis by GC–IDMS

GC–IDMS analysis was performed on a TurboMass GC–MS system (PerkinElmer LAS, Rodgau, Germany) consisting of a TurboMass quadrupole mass selective detector coupled to an AutoSystem

Table 2
Failure[a,b,c] of GC–IDMS quantification of important metabolites (measurement as methoxime-tms-derivatives).

Metabolite	Observed reason for failure			
	Stability[d]	Derivatization[e]	MS fragmentation[f]	Internal standard[g]
Oxaloacetate[a]	×			×
Glyceraldehyde-3-phosphate[a]	×			×
Dihydroxyacetonephosphate[b,c]	×	×		
Fructose-1,6-bisphosphate[b,c]		×	×	
Sugar-1-phosphates[h]		×		

[a] In biological samples: measured amounts near detection limit, sometimes not detected.
[b] Poor calibration curve (R^2 < 0.98).
[c] Poor analytical recovery (<80%).
[d] Decomposition during sample evaporation.
[e] Poor derivatization yield and/or unstable derivatives [24,79].
[f] No suitable *m/z*-values for IDMS quantification are observed in the EI-mass spectrum.
[g] Not detectable in U–^{13}C-labeled algal cell extracts.
[h] No detectable derivatization.

XL gas chromatograph. System control, acquisition and analysis of data were performed using TurboMass 5.4 GC-MS software. The spectrometer was operated in the EI mode (70 eV); the ion source was heated to 180 °C and the transfer line to 280 °C. Measurements for quantification purposes were conducted using single ion monitoring. GC was performed on a Zebron ZB-5ms column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Aschaffenburg, Germany) with helium as carrier gas at 1.0 mL/min (constant flow mode). Injections were carried out with an integrated AutoSystem XL autosampler in the "fast" mode, standard injection volume was 1 μl. The injector temperature was set to 230 °C (isothermal, PerkinElmer PSS injector); sample transfer to the column was performed in the splitless mode with application of a pressure pulse (20 psi, 1.3 min). The inlet liner (FocusLiner, 2.0 mm i.d. × 86.2 mm; Supelco) was factory-packed with deactivated glass wool and was disposed after replacement. The oven temperature was initially held at 70 °C for 5 min. Thereafter, the temperature was raised (6 °C/min) to 320 °C and held there for 10 min.

2.9. Validation of GC–IDMS analysis

Calibration standards were prepared from 50 μl of properly adjusted metabolite composition (solution of non-labeled pure metabolites in water, see Table 1), 50 μl of U–^{13}C-labeled internal standard solution (extract from U–^{13}C-labeled lyophilized algal cells, see above) and 5 μl of O-methylhydroxylamine-hydrochloride (20 mg/mL in pyridine, see Section 3). The calibration standards were processed in the exact same manner as described above for samples. The amount of internal standard was strictly kept constant in all samples and calibration standards. Calibration curves based on internal standard calibration were obtained by linear regression for the peak–area ratio of the analyte to the respective internal standard against the amount of analyte. The concentration of analytes in unknown samples was obtained from the regression functions. Assay accuracy and precision were determined by analyzing spiked samples and quality controls. The latter were selected standard mixes prepared in the same way as calibration standards and which were interspersed into the sample sequence in order to check the validity of the actual calibration curve. The spiking experiments were performed to determine analytical recoveries connected with the GC–IDMS procedure. Spiked samples ("SpS") were produced by adding 50 μl of properly adjusted metabolite composition (solution of non-labeled pure metabolites in water, see Table 1) to cell extracts collected by the integrated sampling device (fraction "T") directly after withdrawal from the heat exchanger. Comparison specimen consisted of analogous samples containing an equal volume of water instead of metabolite composition (non-spiked reference, "nSpR") or instead of cell extract (control reference, "CR"). Recovery regarding a certain metabolite "M" was calculated from the determined metabolite levels c_M (μmol/g_{DW}) of the involved samples "SpS", "nSpR" and "CR" according to the following equation:

$$(\text{Analytical Recovery})_M = \frac{100 \cdot [C_M(SpS) - C_M(nSpR)]}{C_M(CR)}$$

In addition, selected samples were subjected to parallel determinations in order to identify the reproducibility of different steps of the measurement workflow.

3. Results and discussion

3.1. GC–IDMS analysis

3.1.1. Derivatization

3.1.1.1. Stability of metabolites. The applied "all-purpose" derivatization procedure [25] involves a trimethylsilylation step which exclusively proceeds under anhydrous conditions – both the silylating reagents and the formed trimethylsilyl-derivatives are water-labile. It is therefore imperative to remove water from bioreactor samples, one of the major difficulties connected to this procedure. As a matter of fact, we observed distinct differences between evaporated standard mixtures and biological samples. This is not a surprise, since many metabolites are potentially reactive compounds, and reactivity as well as the rate of degradation increases during the concentration/evaporation process. One possible degradation reaction occurring in biological material is the well-known Maillard reaction, which initially involves the condensation of metabolites possessing a carbonyl-moiety and amino-compounds. An obvious implication is that protection of carbonyl-groups prior to evaporation should result in less degradation of metabolites. According to the original method, the aqueous sample is first evaporated and afterwards reacted with methoxyamine hydrochloride in pyridine [60]. Yang et al. proposed the addition of methoxyamine hydrochloride directly to the aqueous sample [85] in order to protect keto- and aldehyde-groups via methoximation. We also found, that considerably larger peaks of derivatized metabolites were detected, when about 1 mg of methoxyamine hydrochloride was added to cell extracts before evaporation (Fig. 2). Certain metabolites (OAc,[5] GAP) could not be

[5] *Abbreviations used*: 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; aKG, 2-oxo-glutarate; Cit, citrate; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; EtOH, ethanol; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; Fum, fumarate; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; IDMS, isotope dilution mass spectrometry; IS, internal standard; ILIS, isotope labeled internal standard;

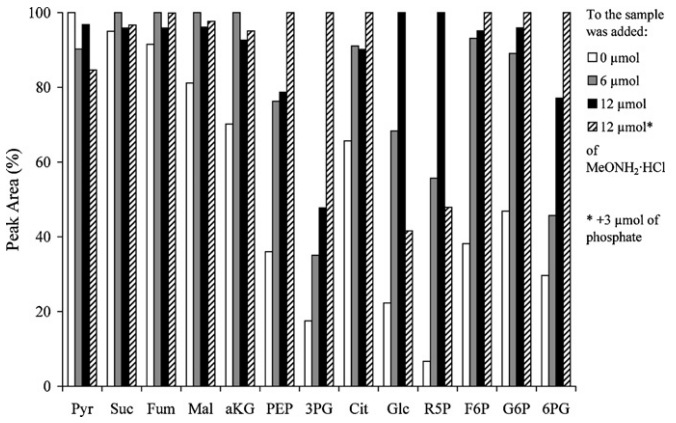

Fig. 2. Influence of O-methylhydroxylamine-hydrochloride ($MeONH_2 \cdot HCl$) on the derivatization yield of different metabolites in cell extracts ("real samples"). Varying amounts of O-methylhydroxylamine-hydrochloride solution (20 mg/mL pyridine) were added to a constant amount of algal cell extracts (boiling water extraction non labeled lyophilized algal cells). The total sample volume was kept constant by addition of water. Samples were evaporated to dryness, derivatized and analyzed by GC–IDMS as described in the Section 2. Peak area percentages refer to the largest peak of each metabolite.

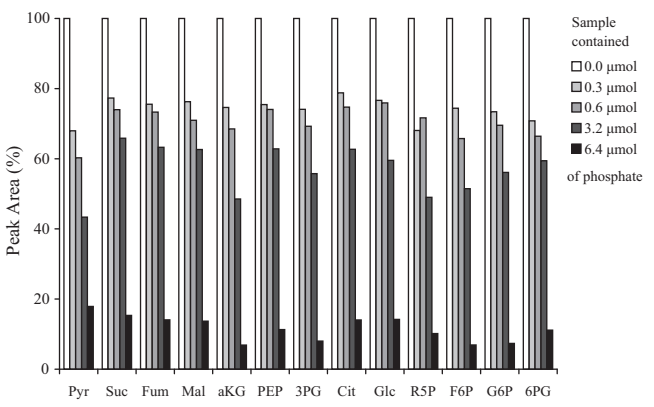

Fig. 3. Influence of phosphate on the derivatization yield of different metabolites. Varying amounts of aqueous potassium phosphate solution (pH 7.0) were added to a non-varying amount of aqueous metabolite mix. The total sample volume was kept constant by addition of water. Samples were evaporated to dryness, derivatized and analyzed by GC–IDMS as described in Section 2. Peak area percentages refer to the largest peak of each metabolite.

detected at all in cell extracts, if the protection step was omitted. Nonetheless, a few metabolites from central carbon metabolism (OAc, GAP, DHAP) seem to be too unstable to be precisely quantified by the GC–IDMS method discussed here (Table 2). Considerable loss during the evaporation step could not be prevented.

3.1.1.2. Stability of methoxime-trimethylsilyl-derivatives. The stability of metabolite methoxime-tms-derivatives was already tested in a previous work in our group [86]. Derivatized samples are sufficiently stable if they are analyzed within 10 h after derivatization. However, peak sizes of the derivatives of GAP, DHAP and FBP are diminishing in significantly shorter periods of time (Table 2).

3.1.1.3. Derivatization efficiency. It is generally very difficult to accurately measure the derivatization efficiency of a certain compound, because the respective derivatives are hardly obtainable in pure form. Moreover, in the case of labile – non-derivatized – metabolites (e.g. OAc or GAP), it is virtually impossible to distinguish between metabolite loss during sample workup and low derivatization yield. A rough estimation for stable metabolites can be deduced from GC–MS total ion chromatograms as has been done by Koek et al. [24]. In accordance with their results, the authors found higher derivatization efficiencies for organic acids (>70%) than for phosphorylated compounds (<60%). A few metabolites display fairly poor derivatization yields (<10% for FBP or sugar 1-phosphates, Table 2). This results in a lack of sensitivity and large rSDs.

3.1.1.4. Sample matrix effects on derivatization. Of particular concern for the analysis is the impact of sample matrix (biotic and abiotic) which in turn depends on the particular sequence of sampling, quenching and extraction methods.

In the case of the integrated sampling procedure applied here, the extracellular fluid could not be removed from the biomass and therefore the analytical samples contained the entire amount of extracellular components. We observed, on the other hand, that the applied derivatization procedure fails, if the ratio between sample matrix and derivatization reagents is not properly adjusted, e.g. evaporated samples of >100 µl of fraction *T* resulted in small to disappearing metabolite peaks, mainly for phosphorylated compounds. We assumed, that primarily the high salt contents of the fermentation media led to extensive residues (salt precipitates) after evaporation and consequently to failure of the derivatization reaction. We investigated the effect of potassium phosphate as a "representative salt precipitate" since phosphate is a main component in the used fermentation media. Aqueous standard metabolite mixtures (50 µM each) were treated with different amounts of potassium phosphate, pH 7.0, evaporated and derivatized. The results of the GC–IDMS measurements are shown in Fig. 3. The detrimental effect of phosphate on derivatization yield is obvious. It has to be noted, that in the case of GC–MS no inorganic phosphate will attain the ion source in the mass spectrometer. Therefore, the effect of "ion suppression", which is well known from LC–MS, cannot explain the influence of phosphate observed here. On the other hand, large amounts of silylated orthophosphate [m/z 299;$OP(Otms)_3$], were observed in the chromatograms. According to the data presented in Fig. 3 we have established as a rule of thumb, that no more than 3 µmol of phosphate should be present in any sample to assure sufficient derivatization efficiency. This will restrict the useable volume of a given sample depending on its phosphate content and consequently will directly determine the lowest detectable metabolite concentration in this sample. Incidentally, we found that the addition of O-methylhydroxylamine-hydrochloride to the aqueous sample will not only protect certain metabolites during evaporation (see above) but also can effectively reduce the detrimental effect of phosphate on derivatization (Fig. 3). Consequently, limited amounts (<3 µmol) of phosphate can be tolerated in the samples without considerably losing sensitivity.

In summary, the sample matrix effects on derivatization yield lead to small useable sample volumes (generally <100 µl) and make great demands on the sensitivity of the analytical system.

3.1.2. Quantification with GC–IDMS

As already mentioned, derivatization of metabolites as described here does generally not proceed quantitatively, and the derivatization yield is clearly matrix-dependent. Additionally, the stability of the formed derivatives varies significantly between different metabolites. In light of these difficulties, the use of internal standards is mandatory. Stable isotope labeled derivatives of the analytes to be measured are almost ideal internal standards

Lac, lactate; Mal, malate; OAc, oxaloacetate; Pep, phosphoenolpyruvate; PPP, pentose phosphate pathway; Pyr, pyruvate; r.t., room temperature; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; Suc, succinate; TCA, tricarboxylic acid; tms, trimethylsilyl; X5P, xylulose-5-phosphate.

Table 3
Failure of conventional internal standards in absolute metabolite determination with GC–MS.

Metabolite	Pyr	Suc	Fum	aKG	PEP	DHAP	3PG	Cit	Glc	R5P	F6P	G6P	6PG
Recovery[a] (%)	71.5	100.3	249.8	144.6	355.6	3.0	218.5	427.7	121.8	15.8	191.3	180.3	151.3
Internal standard[b]	A	B	B	B	C	C	D	C	E	D	D	D	D

Recovery experiments were performed using spiked microbial cell extracts.
[a] Recovery was calculated as described in Section 2.9.
[b] A, 2-oxobutyric acid; B, glutaric acid; C, L-(+)-tartaric acid; D, 2-deoxy-D-glucose 6-phosphate; E, 2-deoxy-D-glucose. No natural contents of the selected IS compounds could be detected in non-spiked cell extracts via the described GC–MS method.

(isotope dilution MS, IDMS). But such compounds are immensely expensive or unavailable at all. One feasible approach uses the metabolic productivity of microorganisms to gain access to mixtures of uniformly ^{13}C-labeled metabolites [10,81]. The authors cultivated a yeast strain on stable isotope-labeled C-sources and the resulting cell extracts were directly used as an IS-mix. But still this strategy causes considerable costs and is rather laborious. Therefore, we tried to reduce the effort by using conventional ISs and tested carefully selected compounds for their suitability [86]. The correlation coefficients of calibration curves prepared from standard mixes were good ($R^2 > 0.99$), but recovery experiments using spiked microbial cell extracts resulted in recoveries ranging from 3% to 400%, depending on the respective metabolite (see Table 3). This demonstrates that yield after sample processing and derivatization is a distinctive property of each individual metabolite in each individual sample (matrix). As LC–MS methods do not need a derivatization step, they are – without regard to sample generation concerns – generally operational with conventional ISs [56,57] and the application of IDMS merely increases the accuracy of quantification. In contrast, absolute quantification by GC–MS as described here strictly relies on the employment of stable isotope labeled ISs. In other words, GC–IDMS seems to be the only possibility to achieve accurate absolute metabolite quantification in the present context. Cipollina et al. [81] argue that the usage of an IDMS approach would eliminate the need to perform recovery tests. In contrast, we observed incorrect recoveries for a few metabolites even when using ^{13}C-labeled internal standards (see Tables 2 and 4). The recovery of DHAP was particularly low (<10%). Since our attempts to quantify DHAP resulted in similar concentrations (~0.3 μmol/g_{DW}) compared to [81], the accuracy of the reported DHAP level may have to be verified by complementary methods.

3.1.2.1. Application of U–^{13}C-labeled lyophilized algal cells. We tested the applicability of commercially available U–^{13}C-labeled lyophilized algal cells as a convenient source for the production of stable isotope labeled IS-mixes suitable for IDMS. Several extraction procedures were evaluated: water, aqueous methanol, pure methanol and pure ethanol in conjunction with boiling or ultrasonication. Boiling water extraction turned out to be the easiest as well as the most efficient method. Especially phosphorylated metabolites were most efficiently extracted. The resulting extracts were characterized by GC–IDMS concerning their metabolite content. Table 5 lists metabolites which could be identified in amounts that are sufficient for the usage as ISs (>0.1 μmol/g_{DW}). Observed mass fragments which were used (Table 4) or which are potentially useful (Table 5) as quantification masses for IDMS are additionally included. Note that, compared to a recently published GC–IDMS method, we use different SIM masses for the quantification of F6P (*m*/*z* 459 in place of *m*/*z* 217) and G6P (*m*/*z* 471 in place of *m*/*z* 387) [81]. Although the abundance of the fragments we used is rather low compared to the intensity of the previously employed masses (43% in case of F6P; 28% in case of G6P), they provide sufficient sensitivity for accurate measurements of F6P and G6P in biological samples. And as they are comparatively large and more specific for these metabolites, one encounters fewer problems with signal interferences between analytes and internal standards or matrix compounds. The exact contents of metabolites from glycolysis, PPP and TCA cycle, which can favourably be quantified by the GC–IDMS method presented here, are given in Fig. 4. They could be deduced from suitable calibration curves prepared using non-labeled metabolite standards and the algal extract as IS. As expected, the metabolite levels are quite unbalanced; actually they differ by two orders of magnitude. The minimum amount of IS-mix which has to be added to each individual sample (calibration standard, quality control, etc.) is predetermined by the metabolites with the lowest contents, i.e. PEP, 2PG and 6PG. This means that even if the most sensitive GC–MS mode is used (splitless injection/SIR), minimum 50 μl of IS-mix have to be added to each sample. Apart from these minor drawbacks, extracts from commercially available U–^{13}C-labeled lyophilized algal cells proved to

Table 4
Averaged intra- and extracellular steady state metabolite concentrations (±SD) measured by GC–IDMS. Sampling was performed using an integrated sampling procedure (*T*) in conjunction with quick filtration (*EX*).

Metabolite	Total *T* (μmol/g_{DW})[a]	Extracellular *EX* (μmol/g_{DW})[a]	Intracellular *T* − *EX* (μmol/g_{DW})[a]	Recovery[b] (%)
Glc	410 ± 5.74	395 ± 6.29	14.7 ± 8.51[c]	101.2 ± 1.5
Cit	40.3 ± 1.85	13.6 ± 0.79	26.7 ± 2.02	99.0 ± 1.3
Suc	328 ± 6.22	320 ± 4.12	7.53 ± 7.46	109.3 ± 6.7
Fum	2.13 ± 0.04	1.33 ± 0.02	0.80 ± 0.05	103.0 ± 4.6
Mal	26.2 ± 0.50	23.8 ± 0.24	2.41 ± 0.55	97.3 ± 4.6
aKG	10.9 ± 0.21	10.2 ± 0.08	0.69 ± 0.22	102.3 ± 1.8
PEP	0.43 ± 0.04	0.05 ± 0.01	0.38 ± 0.04	85.8 ± 22.0
G3P	4.83 ± 0.18	1.80 ± 0.03	3.03 ± 0.19	89.4 ± 14.9
3PG	1.14 ± 0.06	0.61 ± 0.02	0.53 ± 0.06	113.9 ± 7.9
R5P	1.29 ± 0.03	0.08 ± 0.01	1.21 ± 0.03	98.5 ± 10.5
F6P	2.00 ± 0.12	0.19 ± 0.01	1.81 ± 0.12	114.4 ± 8.7
G6P	4.42 ± 0.31	0.40 ± 0.01	4.02 ± 0.31	101.6 ± 13.0
6PG	0.88 ± 0.05	0.00	0.88 ± 0.05	176.7 ± 25.5

Samples were from a culture at $D = 0.1\ h^{-1}$ and 3.4 g_{DW}/L.
[a] Values are averages ± standard error of two replicate samples each analyzed at least in duplicate.
[b] Determined by comparison of spiked and non-spiked total broth samples, each analyzed at least in triplicate.
[c] The ±SD for $T - EX$ was calculated according to $SD_{(T-EX)} = [(SD_T)^2 + (SD_{EX})^2]^{1/2}$.

Table 5
Metabolites identified in extracts of uniformly ^{13}C-labeled lyophilized algal cells.

Metabolite	Derivative contains		Retention index[a]	SIM mass analyte (m/z)[b]	SIM mass IS (m/z)[b]
	tms-Groups	N-OMe-groups			
Pyr	1	1	1045.5	174	177
Lac	2		1054.9	219	222
Hydroxyacetate	2		1071.8	205	207
Ala	2		1099.6	218	221
Gly	2		1119.0	102	103
Oxalate	2		1137.2	219	221
β-Hydroxybutyrate	2		1157.5	233	237
Val	2		1211.9	144	148
Phosphate	3		1268.1	299	299
Leu	2		1269.0	158	163
Glycerol	3		1272.1	218	221
Ile	2		1289.4	158	163
Pro	2		1292.2	142	146
Gly	3		1300.9	174	175
Suc	2		1313.2	247	251
Glycerate	3		1327.9	292	295
Fum	2		1350.7	245	249
Ser	3		1357.5	306	309
Thr	3		1382.6	218	221
Homoserine	3		1447.3	218	221
Mal	3		1487.4	245	249
Met	2		1513.5	176	180
Asp	3		1517.4	232	235
aKG	2	1	1575.8	304	309
Pep	3		1596.0	369	372
Glu	3		1617.7	246	250
Phe	2		1619.2	192	200
Ribose	4	1	1674.1	217	220
Isocitrate-lactone	2		1703.2	157	161
Lys	3		1704.2	156	161
Aconitate	3		1748.0	229	235
DHAP	3	1	1752.0	400	403
G3P	4		1758.7	445	448
2PG	4		1775.4	369	372
3PG	4		1803.4	459	462
Cit	4		1815.1	273	278
Isocitrate	4		1817.9	273	278
Fructose	5	1[c]	1866.1	307	310
Fructose	5	1[c]	1876.0	217	220
Glc	5	1[c]	1890.1	319	323
Glc	5	1[c]	1908.2	319	323
E4P	4	1	1908.2	357	359
Lys	4		1917.1	317	322
Tyr	3		1932.9	280	288
X5P	5	1[c]	2095.5	357	359
Ru5P	5	1[c]	2096.2	357	359
R5P	5	1[c]	2101.9	217	220
R5P	5	1[c]	2104.5	217	220
Ru5P	5	1[c]	2112.7	357	359
X5P	5	1[c]	2112.7	357	359
Trp	3		2202.0	202	211
F6P	6	1	2297.9	459	462
G6P	6	1[c]	2309.4	471	475
G6P	6	1[c]	2326.8	471	475
6PG	7		2409.0	333	337
S7P	7	1	2537.5	471	475
Sucrose	8		2627.4	361	367
FBP	7	1[c]	2698.3	217	220
FBP	7	1[c]	2705.8	217	220
Maltose	8	1[c]	2724.2	361	367
Trehalose	8		2730.0	361	367
Maltose	8	1[c]	2750.8	361	367

[a] Modified Kovats index according to Van den Dool and Kratz [90].
[b] Potentially useful as quantification mass for IDMS.
[c] Two peaks deriving from corresponding *syn*- and *anti*-methoximes were observed.

be very useful IS-mixes for IDMS-based GC–MS quantification of central carbon metabolism intermediates. They did neither interfere with the employed derivatization procedure nor with GC–MS analysis. A large number of metabolites can be calibrated at relatively low operating and financial expense. The contribution of labeled algal cells to the total expense for analysis is comparable to that resulting from IS preparation according to an established method published by Wu et al. [10] which uses cell extracts from home-made U–^{13}C-labeled *S. cerevisiae* (pricing at Sigma–Aldrich, 2009). But (financial) burden is only one aspect. The presented "algae approach" will also enable more labs to accomplish accurate absolute metabolite quantification by IDMS. There might be

Table 6
Precision data for the analysis of different sample fractions.[a] Values are relative standard deviations rSD (%) of measured metabolite concentrations.

Metabolite	*T*, *n* = 4	*EX*, *n* = 4	*T* − *EX*, *n* = 4[b]	Extracellular fraction *EX*/*T* (%)
Glc	1.4	1.6	57.9	96.4
Cit	4.6	5.8	7.5	33.7
Suc	1.9	1.3	99.1	97.2
Fum	2.0	1.5	5.9	62.5
Mal	1.9	1.0	22.9	90.8
aKG	1.9	0.8	32.2	93.7
PEP	8.4	27.5	9.9	11.0
G3P	3.8	1.9	6.1	36.8
3PG	5.3	2.7	12.0	53.5
R5P	2.5	8.5	2.8	6.4
F6P	5.8	5.4	6.4	9.5
G6P	7.0	3.3	7.7	9.0
6PG	6.0	–	6.0	0.0

[a] *T*: levels measured in total broth after integrated sampling procedure; *EX*: extracellular levels measured after quick filtration; *T* − *EX*: intracellular level calculated from corresponding mean values of *T* and *EX*.
[b] The relative rSD (%) of *T* − *EX* were calculated from the absolute ± SD for *T* − *EX* and the respective averaged concentrations (see Table 4).

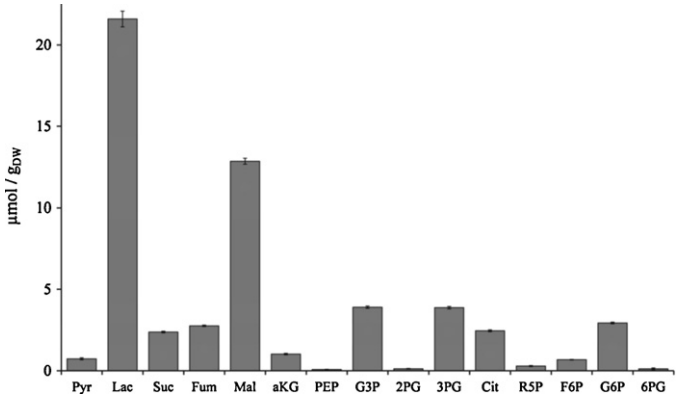

Fig. 4. Measured averaged metabolite levels in U–^{13}C-labeled lyophilized algal cells after boiling water extraction. Error indicators display standard deviations ($n = 7$).

scientists who are interested in using a GC– or LC–IDMS approach, but who do not have access to fermentation equipment, i.e. who will not be able to prepare their own ^{13}C-labeled internal standards. It is self-evident that he need for metabolite determination is not restricted to microbial Systems Biology.

3.1.2.2. Quality of quantification. The sensitivity of the present method strongly depends on the properties of the sample matrix (see above); therefore specification of values for limits of detection (LOD) would be questionable. Anyhow, LOD was invariably below 10 pmol per sample (corresponding to <0.1 pmol on column) for any quantified metabolite. Calibration curves which are generated using the mentioned algal extracts display good ($R^2 \geq 0.99$) to excellent ($R^2 \geq 0.999$) coefficients of determination. Quality control data from repeatability and recovery experiments using microbial cell extracts are listed in Tables 4, 6 and 7. Based on the given data, it can be stated that the GC–IDMS approach presented in this paper provides similar performance in absolute quantification compared to previously published LC-MS methods [10,55–57,59]. The quantification of phosphorylated sugar compounds by GC–IDMS poses a particular challenge. Derivatization yields of suchlike compounds tend to be low compared to non-phosphorylated species (see above). Furthermore, EI-spectra of the respective derivatives are dominated by fragments resulting from the trimethylsilylated phosphate group and which do not contain any information about the C-skeleton [79]. $[M]^+$or $[M-15]^+$ ions are generally very weak or absent at all. And due to the introduction of Si-atoms by derivatization, the complexity of isotopic patterns increases drastically, which in turn leads to interferences between signals of the quantification masses of analyte and U–^{13}C-labeleled IS. Thus, it can be difficult to find suitable fragments whose mass difference between analyte and ILIS is large enough. If this difference is too small $[\Delta(m/z) < 3]$, the signal interferences provoke strongly nonlinear regression curves which in turn will decrease sensitivity of the measuring signal at higher analyte levels.

Table 7
Comparison of analytical precision data resulting from independent series of measurement[a] (long term performance). Values are relative standard deviations rSD (%) of measured metabolite concentrations.

Metabolite	This study		Independent pulse experiment[a]		
	Steady state $n = 4$	Steady state $n = 7$	Pulse, t_1 $n = 3$	Pulse, t_2 $n = 3$	Pulse, t_3 $n = 3$
Cit	4.6	1.2	1.3	2.1	1.5
Suc	1.9	1.1	2.5	1.5	2.4
Fum	2.0	1.9	0.0	2.8	3.1
Mal	1.9	1.4	1.3	2.0	2.4
aKG	1.9	1.8	5.2	1.4	2.9
PEP	8.4	7.2	7.1	4.7	0.0
G3P	3.8	1.8	1.5	4.6	3.5
3PG	5.3	4.9	7.1	3.8	4.2
R5P	2.5	5.0	6.3	0.9	5.4
F6P	5.8	2.1	2.6	1.1	4.2
G6P	7.0	1.5	2.0	1.9	1.5
6PG	6.0	n.d.[b]	n.d.[b]	3.6	1.4

[a] Collection of samples was performed at different stages before (steady state) and during ($t_1 \cdot t_2 \cdot t_3$) a long term pulse experiment with the yeast *Saccharomyces cerevisiae* cultivated at physiologically defined anaerobic conditions. All metabolite levels (absolute values not shown) were measured in total broth after integrated sampling procedure.
[b] Not determined.

Table 8
Advantages of the presented GC–IDMS approach.

Advantage	Practical observations
Easy operation and robust technology	MS maintenance was only necessary after 6 months of continuous operation. GC column stayed operable for more than two years and several thousand injections.
Superior chromatographic separation power after derivatization of small and similar metabolites	Very similar metabolites often co-elute with LC [17,57,80]. Respective derivatives could be separated by GC without difficulty: e.g. 2PG/3PG, F6P/G6P, R5P/Ru5P/X5P, DHAP/GAP, Cit/iso-Cit, Suc/Fum/maleate, all proteinogenic amino acids.
High sensitivity with simple single quadrupole technology	Most compounds could be detected in the fmole-range (on-column).
Good ionization efficiency for a wide spectrum of derivatized metabolites	In contrast to LC–MS/MS methods, no optimization of ionization conditions was needed. Standard EI-parameters were applicable for all measured metabolite derivatives.
Information-rich mass spectra	Most metabolites could be identified by comparison with database EI-spectra (NIST library).
Acceptable sensitivity, accuracy and precision	Determined performance parameters are comparable to those of published LC–MS/MS methods [10,55–57,59] (see Tables 4, 6 and 7).

Regardless of these GC–IDMS specific difficulties, several phosphorylated metabolites could quantitatively be measured in biological samples by means of carefully chosen suitable quantification masses (Tables 1 and 4). A few exceptions (rSD > 30% and/or recovery <80%) are listed in Table 2. Even though failing for individual phosphorylated compounds, the presented GC–IDMS method offers some attractive advantages compared to LC–MS methodology (Table 8).

3.2. Application of GC–IDMS for analysis of anaerobic yeast chemostat cultures

The presented GC–IDMS approach allowed the absolute metabolite quantification in extracellular samples (fraction "EX") as well as in particularly challenging total broth samples (fraction "T"). A carefully balanced sample preparation and derivatization protocol as well as the application of U–^{13}C-labeleled IS are indispensable in order to overcome detrimental sample matrix effects. Our experiments were performed with anaerobic chemostat cultures of *S. cerevisiae*, which differ from aerobic conditions used by [8]. In particular, some extracellular metabolites are present in huge amounts. Results from measurements of total and extracellular metabolites are summarized in Table 4. Almost all intracellular metabolites, including phosphorylated ones from central carbon metabolism are present extracellularly in detectable amounts. Some analytes like Pyr or Lac were present at extracellular concentrations that were too high to allow accurate intracellular determinations by GC–IDMS via a differential approach. On the one hand, the concentrations were far beyond a useful calibration range of the GC–IDMS method. On the other hand, the differential method possesses the intrinsic drawback of being unable to predict accurate intracellular data if the extracellular fraction is too high (Table 6) [58]. If the mass fraction of an extracellular pool of metabolite ($100 \times EX/T$) is more than 90%, the rSD of the calculated intracellular metabolite content ($IN = T - EX$) may exceed 20% (Fig. 5).

U–^{13}C-labeleled IS were added to the sample after the applied integrated sampling procedure and there might be heat induced loss of metabolites in the heat exchanger. But it is justifiable with good reason to add ISs directly *after* sample treatment in the heat exchanger and yet to expect accurate results. Heat induced loss of metabolites was carefully tested by Schaub et al. [57] and was found to be negligible during the very short time of exposure to heat. Several publications support this standpoint [56,87–89]. Analogous tests performed by the authors invariably confirmed the previously published results.

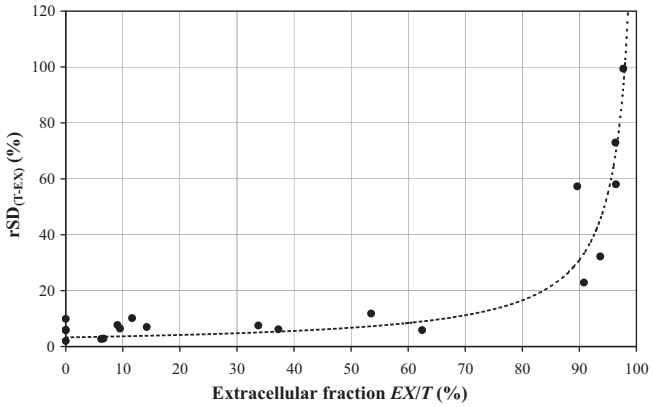

Fig. 5. Observed rSD deriving from 4 independent determinations of $(T - EX)$ against extracellular metabolite fraction $100 \cdot (EX/T)$ (see Table 6). Each dot (•) represents an individual metabolite. The dashed line (- - -) displays non-linear regression $[y = a(b + x)/(c - x);\ R^2 = 0.91]$ of the data points.

4. Conclusions

In this work, we present the application of gas chromatography–mass spectrometry for the measurement of absolute intracellular metabolite levels in *S. cerevisiae* growing in chemostat culture under anaerobic conditions. The isotope dilution mass spectrometry (IDMS) approach, which makes use of stable isotope labeled metabolites as internal standards, was to date predominantly used in connection with LC–MS measurements in the field of metabolome analysis (LC–ID-MS/MS) and was only recently adopted for GC–MS measurements (GC–IDMS) [81]. We modified this methodology and extended it to a set of additional metabolites belonging to compound classes other than phosphorylated sugars (e.g. sugars and organic acids). Sample workup and derivatization procedures have been optimized with regard to the applicability for "real" fermentation samples. Furthermore, we demonstrated the applicability of commercially available U–^{13}C-labeled lyophilized algal cells as a convenient source for the production of stable isotope labeled internal standard (ILIS) mixes suitable for IDMS. Consequently, costs and labour input could be significantly reduced compared to the established strategy of cultivating microorganisms on uniformly ^{13}C-labeled C-sources. A differential approach for the estimation of intracellular metabolite levels ("IN") was applied. This method calculates the difference between total ("T", acquired from a fast heat exchanger-based integrated sampling device) and extracellular ("EX", acquired from fast filtration) metabolite contents ($IN = T - EX$). Precision and

accuracy of the presented GC–IDMS approach were assessed and turned out to be comparable to currently published LC-ID–MS/MS methods. Among the most important advantages compared to LC–MS methodology, superior chromatographic separation capacity towards small and similar metabolites, excellent ionization efficiency for a wide spectrum of analytes, and minimum maintenance of robust GC–MS technology are to be mentioned. On the other hand, a few important metabolites refuse accurate determination (rSD > 10%) using the presented GC–IDMS approach.

We are currently applying this strategy to high resolution time series analysis of extra- and intracellular metabolites during anaerobic pulse experiments with chemostat culture of *S. cerevisiae*.

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