Improved phosphometabolome profiling applying isotope dilution strategy and capillary ion chromatography-tandem mass spectrometry

Marit H. Stafsnes[#], Lisa M. Røst[#], and Per Bruheim[#¤]

[#]NTNU Norwegian University of Science and Technology,

Department of Biotechnology and Food Science

Corresponding author:

[¤]Per Bruheim

Sem Sælands vei 6/8,

N-7491 Trondheim, Norway

Per.Bruheim@ntnu.no

Key words:

Phosphometabolome, capillary ion chromatography, mass spectrometry, isotope dilution

Declarations of interest: none

Abstract

The phosphometabolome is comprised of all phosphorylated metabolites including the major metabolite classes sugar phosphates and nucleoside phosphates. Phosphometabolites are invaluable in any cell as a part of primary- and energy- metabolism, and as building blocks in the biosynthesis of macromolecules. Here, we report quantitative profiling of the phosphometabolome by applying capillary ion chromatography-tandem mass spectrometry (capIC-MS/MS), ensuring improved chromatographic separation, robustness and quantitative precision. Baseline separation was achieved for six out of eight tested hexose phosphates. Quantitative precision and reproducibility was improved by introducing a fully uniformly (U) ^{13}C-labeled biological extract and applying an isotope dilution (ID) correction strategy. A ^{13}C-labeled biological extract does in principle contain internal standards (IS) for all metabolites, but low abundant metabolites pose a challenge, and solutions to this are discussed. The extreme reproducibility and reliability of this capIC-MS/MS method was demonstrated by running the instrumentation continuously for ten days.

Introduction

Metabolites comprise less than three percent of the cell dry matter, but serve critical functions in energy generation and as building blocks for macromolecules across all domains of life. Hence, the analysis of the metabolome, metabolomics, is of high interest in biological studies; especially studies of the phenotype. The metabolome is a heterogeneous collection of compounds with great variation in both abundances and physio-chemical properties, which cause significant analytical challenges. In addition, high turnover rates and chemical instability is observed for many metabolites [1, 2]. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the two most frequently used technologies in the field of metabolomics, with MS dominating due to much higher sensitivity and coverage of the metabolome.

The approaches to MS based metabolomics are many, e.g. non-target vs. target, with or without chromatographic separation prior to MS detection, fingerprinting vs. profiling, with the preferred approach depending on the biological model system and the pending research questions [3]. Target quantitative metabolite profiling aims at quantifying known metabolites from one or several metabolite groups, e.g. amino acids and other amino group containing metabolites. Usually 20-50 metabolites are included in such metabolite profiling methods [4-6], which sometimes include a stable label derivatization for increased quantitative accuracy and precision [7].

For profiling of the phosphometabolome (sugar-phosphates, nucleoside phosphates and other phosphorylated metabolites), there are mainly two methodological approaches: liquid chromatography (LC)-MS/MS using ion pair reagents [8, 9] and ion chromatography (IC)-MS/MS. The ion pair reagent improves chromatographic separation of the highly negatively charged phosphometabolites on a reverse phase (RP) LC-column, but is very sticky and hard to remove from capillaries and connections of the LC-MS/MS instrument. Thus, it is strongly recommended to dedicate a separate LC-MS/MS systems for ion pair reagent based analysis to eliminate the risk of contaminating other analyses. IC is the less frequently used alternative, but has superior separation capabilities of phosphometabolites. The instrumentation is more complex, with several units such as

membrane devises for proton-potassium exchange and a carbonate remover. Hence, the IC system requires a trained operator to ensure top separation capabilities. Yet, once running, an IC instrument is very robust with reproducible performance. IC instruments with both analytical [10] and capillary [11-13] flow modes are available, and both have been used for the analysis of the phosphometabolome. Wang and co-workers compared capIC with both HILIC and RP LC-MS/MS and concluded and superiod resolution and sensitivity of capIC for negatively charged metabolites [13].

The metabolomics workflow is elaborate and prone to variation introduced throughout the process of sampling, sample preparation and analysis, and internal standards (IS) are needed to monitor and correlate for the variation introduced [14-16]. Isotope dilution (ID) has been proposed as a strategy to collectively tackle these challenges. A fully uniformly (U) ^{13}C-labeled biological extract is introduced during sample processing stages; thereby, providing a stable isotope analogue for each metabolite. This analogue, which presumably will be degraded at the same rate and ionized at the same efficacy, can be exploited as an IS. Importantly, IS from a ^{13}C extract follow same abundance profiles as the analytes in the real extract which is also preferable compared to IS mixtures with same concentration of all analytes. Many core primary metabolites, e.g. sugar phosphates, are not commercial available in deuterated or ^{13}C labeled form either. Thus, ID enables a reliable quantification of any intracellular metabolite of interest [17].

In this short communication, we report on both increased chromatographic separation on a capillary IC (capIC) system and improved quantitative accuracy, precision and robustness combining capIC-MS/MS with ID. The ID strategy is gaining increasing popularity in the quantitative metabolomics field, yet, it poses some challenges related to low abundant metabolites, which are discussed here.

2 Materials and Methods

2.1 Standards

79 polar metabolite standards of analytical grade (Supplementary table S1) were purchased from Sigma-Aldrich and Cayman chemicals and prepared as 1 or 10 mM stock solutions in deionized water (DI-water). An external standard mixture (ES) was prepared from stock solutions by serial dilutions, aliquoted and stored at -20°C. Upon analysis, an aliquot of the standard mixture was diluted further to construct a calibration dilution series spanning the concentration range expected for most of the phosphometabolites in biological extracts at the given sampling densities: 5 to 10,000 nM.

2.2 Preparation of IS; ^{13}C-labeled biomass

S. cerevisiae CEN PK was cultivated in a shake flask (0.1 l) at 30°C, 200 rpm, in a mineral medium [18] containing U-^{13}C-labeled glucose (> 99%, Cambrigde isotope laboratories) as the sole carbon source. At OD_{660} of 1, 10 ml aliquots were withdrawn from the flask and processed as described in 2.4.

2.3 Cultivation of microorganisms and human cells for metabolite profiling

The cultivation conditions were optimized to obtain good quality inoculum and for completely usage of the ^{13}Clabeled glucose since it is an expensive substrate. *E. coli* was grown in M9 media both in inoculum (shake flasks) and experiment (1L fermenters). *S. cerevisiae* was grown in YNB without amino acids in inoculum (shake flasks) and Verduyn media [18] during experiment (1 L fermentors), both supplemented with 10 g/L glucose. Each experiment started with inoculating shake flasks from cryo vials and grown overnight. 1L media in fermentors were thereafter inoculated to a starting OD_{660} of 0.1 – 0.15 and cultured under controlled conditions until sampling between OD 1 and 2.

The human monocytic leukemia cell line THP-1 (ATCC) was cultured in in RPMI-1640 medium (ATCC) supplemented with 50 mL fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2.

2.4 Sampling and sample preparation

A fast filtration method was applied to separate microbial cells from the media. 10 ml samples were withdrawn from a port in the fermenter, harvested onto 47 mm low protein binding filters compatible with ACN, and fast filtered with controlled vacuum pressure. The vacuum pressure was controlled by CVC 3000 and VSK 3000 units (Vacuubrand Gmbh) and optimized for rapid filtration without drying the filters. *S. cerevisiae* was quickly filtered with a pore size of 0.65 µm (Durapore Membrane Filter, PVDF), whereas a two stack filter (Whatman glass fiber filter type GF/C; 1 µm pore size; 47 mm diameter on top of a Pall Supor membrane filter 800; 0.8 µm pore size) was used for *E. coli*. The filters were quickly rinsed with 10 ml of cold (0 °C) mineral media followed by a rapid 10-ml cold (0 °C) DI-water rinse lasting less than two seconds, and transferred to a 50 ml centrifuge tube with a 13 ml cold (0°C) solution of 50% Acetonitrile (ACN) in DI-water, which again was transferred to liquid N_2. The adherently growing human cell line was sampled at a cell density of $6*10^6$ cells/dish with quickly removal of the growth medium, washing with cold PBS and DI water, before quenched using a cold ACN-DI water solution. The cells were scraped off and transferred to 50 ML centrifuge tubes and frozen. Metabolite extraction from both human and microbial cells was performed by three freeze-thaw cycles with liquid N_2 and cooling bath kept at 0 °C. The supernatant was aliquoted into four tubes, frozen at -80 °C, further cooled in liquid N_2, lyophilized and stored at -80°C awaiting analysis.

Upon analysis, metabolite extracts were dissolved in 500 µL of cold (0°C) DI-water and centrifuged at 14,000rpm, 4 °C for 10 min. The supernatants were carefully transferred onto 3-kD-molecular-weight spin cut-off filters (#516-0228, VWR) and centrifuged at 14,000 rpm, 4 °C for 10 minutes.

2.5 CapIC separation

A Thermo Scientific Dionex ICS-4000 capillary IC was operated in external mode with DI-water delivered by an external AXP pump at a flow rate of 30 µl/min. To assist desolvation for better electrospray, a makeup solvent of 90 % ACN in DI-water containing 0.01% ammonium hydroxide (NH_4OH) was delivered by an external AXP-MS pump at 30 µl/min, combined with the eluent via a low dead volume mixing tee and passed through a grounding union before entering the MS. A minimized length of peak tubing (0.08 mm/0.003 in. ID) (#P/N 049715) was used for the capIC-MS interphase. The capIC analysis was performed with a IonPac AS11HC-4 µm, 0.4 × 250 mm column (2000 Å) and a IonPac AG11-HC 4um 0.4x50mm guard column. IC flow rate was 16 µl/min at 40 °C. The gradient conditions were as follows: an initial 4 mM KOH was held for 1 min, increased to 12 mM at 5 min, to 20 mM at 13 min, and 70 mM at 22 min, held at 70 mM for 7.5 min, followed by a rapid increase to 100 mM at 31 min, held 100 mM for 5 min and decreased to 4 mM in 4 min, and finally held for 10 min to re-equilibrate the column. The total run time was 50 min.

2.7 MS analysis

A Waters Xevo TQ-S triple-quadrupole MS was operated in negative electrospray ionization (ESI) mode with a capillary voltage of 2.5 kV and ion-source temperature of 150 °C. The desolvation gas was nitrogen, and the flow was set to 800 l/h at a temperature of 300 °C. The collision energy for each MRM transition was optimized for each compound both manually and using the “Intellistart”-function in MassLynx 4.1. The MS was run in dynamic MRM mode, and the retention time (RT) window for each compound was set to ±2 min of the expected RT. Downstream data processing was performed in MassLynx V4.1.

2.8 ID correction

The ^{13}C-labelled *S. cerevisiae* extract was added to the ES calibration mixture and the naturally labeled extracts from *S. cerevisiae, E.coli* and the human cell line THP-1 at a ratio of 1:5. The ratio of the response area of unlabeled to $U^{13}C$-labeled metabolites was used for correction.

3 Results and Discussion

3.1 Optimization of capIC elution conditions

In principle, optimization of the capIC separation is straightforward as there is only one variable to change; the KOH gradient. Yet, to improve separation of hexose phosphates we also found it necessary to change the column from the recommended Ion Swift MAX 100 column [11, 13] to a AS11-HC column. The latter has higher loading capacity and tolerates higher flow rates. When changing the column and flow rate it was also necessary to optimize the make-up flow, composition of make-up mobile phase, capillary dimensions and back-pressures for stable performance throughout the sequence run [13]. Figure 1A shows the chromatographic separation of an eight hexose phosphate standard mixture on the capIC-MS/MS system with the optimized configuration and elution conditions. Baseline separation was obtained for six out of eight analytes over a seven-minute period, only Mannose 1-phosphate (M1P) and Glucose-1-phosphate (G1P) co-eluted regardless of settings. Importantly, all hexose 6-phosphates were baseline separated, also in biological extracts from three different species; *S. cerevisae* (Figure 1B), *E. coli* and the human cell line THP-1 (data not shown).

3.2 Improved quantitation by ID strategy

IC presents quite stable ionization conditions throughout the gradient run compared to standard LC in both RP and normal phase (NP) where changes in mobile phase composition has a significant effect on ionization. Nevertheless, use of ISs are highly recommended regardless of chromatographic separation technique, as an IS added in the first step of sampling allows for correction of variation introduced throughout the elaborate sample preparation workflow.

To evaluate the performance of the ID strategy, an initial test with four injections from parallel samples (individual vials, *S. cerevisiae* extract spiked with ^{13}C *S. cerevisiae* extract) was performed. The relative standard deviation (RSD) decreased from 5 to 1 % when introducing correcting for AMP, and from 4 to 1 % for ATP. As this correction strategy significantly increased quantitative precision for the two tested metabolites, ^{13}C MS/MS transitions were established for all 79 metabolites included in the method (Supplementary Table S1: RT, limit of quantitation (LOQ), ^{12}C and ^{13}C MS/MS transitions). By exploiting the U^{13}C-isotope analogue of each metabolite for correction, linearity of most calibration curves was improved (Supplementary Table S2), and the linear range was extended to span all concentrations relevant for the biological extracts.

3.3 Challenges related to ID correction

Low abundant metabolites. The LOQ, ranging from 1-50 nM for most phosphorylated metabolites and 200-500 nM for most organic acids on the capIC-MS/MS system (Supplementary table S1), is an important parameter to consider for ^{13}C ID correction. For quantitation from biological extracts, the ^{13}C isotope analogue of each metabolite was used for correction when it's concentration was above LOQ. However, when the concentration of a U^{13}C-labeled metabolite is below LOQ in the ^{13}C extract, using this metabolite for correction of the corresponding ^{12}C analogue could lead to erroneous adjustments, as it's concentration is uncertain and varies much more than the concentration of high abundant metabolites. For choosing an alternative ^{13}C IS when the isotope analogue was below LOQ, two criteria was introduced: 1) Similar physico-chemical properties, and 2) similar RT. A metabolite with similar physicochemical properties and RT to the preferred IS metabolite will be degraded at a comparable rate throughout sample preparation, and enter the concentration dependent ion source at approximately the same time, making it the best candidate without severely compromising the properties of the ID correction strategy.

The analysis should preferably be run with a complete ES mix, including all metabolites balanced according to their abundance in biological extracts. If the analysis is performed with a

209 reduced ES mix, a third aspect must be considered for selecting candidate IS. The IS mix is a
210 biological extract, with metabolite concentrations varying over several orders of magnitude. Hence,
211 the magnitude of the correected responses, and the slope of the calibration curves will vary
212 accordingly. Thus, the selected IS and ES must be matched for each metabolite. Ignoring this can
213 cause inaccurate results, deviating in orders of magnitude from the true concentration.

214 **Effect of ID on chromatographic separation.** RT and chromatographic separation was
215 maintained in both ES mixtures and natural labeled biological extracts from *S. cerevisiae*, *E. coli* and
216 human THP-1 cells when added ^{13}C *S. cerevisiae* extract for close to all metabolites, the exception
217 being hexose phosphates. Surprisingly, several of the hexose phosphates that were baseline
218 separated in natural labeled ES mixtures and biological extracts (lower panel Figure 2A and B,
219 respectively) co-eluted when spiked with ^{13}C *S. cervisiae* extract (Figure 2, upper panel). Figure 2A
220 depicts how Fructose 6-phosphate (F6P) (RT 11.5) has merged with the G6P (RT 11.1), with a slight
221 shift in RT. The hexose 1-phosphates are seemingly unaffected. The effect was consistent across
222 pure ES and biological extracts from the three tested species, and could not be counteracted by
223 altering chromatographic conditions nor sample preparation. Spiking with a ^{13}C *S. cerevisiae* extract
224 does not exceed the column loading capacity as the natural labeled extract is reduced
225 proportionately. Hence, if individual quantitation of hexose-6-phosphates is required, samples must
226 be re-run without ^{13}C *S. cerevisiae* extract. ID has recently been introduced in non-target
227 Metabolomics [10], and these results demonstrates that great care must be taken with this strategy,
228 as mixing extracts can introduce bias and artefacts which is harder to identify in a non-target
229 approach.

230

231 **3.4 Assessment of long term stability**

232 Metabolite profiling with capIC-MS/MS is not high throughput; a sample run is 30-60 minutes long
233 depending on the metabolites included in the method and the required level of separation.
234 Comprehensive studies can result in sample lists with over 100 injections including biological and

technical replicates from each sampled condition, blanks, ES calibration series and quality controls (QCs), implying that the analysis will last for days/week(s). Thus, maintained precision of the system is critical, and the final step in this method upgrade project was to monitor and evaluate the system performance over a 10-day period with >200 injections. Individual samples are not presented, as they cannot be used to evaluate technical precision solely, but an average RSD of 23% between biological replicas was measured for the human cell line THP-1, this including biological variation between separate culture dishes and variation introduced during sample preparation and capIC-ID-MS/MS analysis. Table 1 lists the RSD between 10 injections, each separated in time by one day, for a 5 µM ATP standard sample and ATP, ADP and AMP in five THP-1 QC cell extracts. The RSD between samples was drastically reduced, especially for ATP and AMP. Clearly, ^{13}C ID correction improves the quantitative precision.

In conclusion, the presented capIC-MS/MS method including ID correction and covering almost 80 metabolites provides high chromatographic separation capabilities and a reliable quantitative profiling of the phosphometabolome. Figure 3 visualizes the comprehensive coverage of primary metabolite pools by the capIC-MS/MS and the heat mapping indicates the large variation in concentration among the primary metabolites.

Acknowledgements

This project was supported by the Norwegian Research Council (grant number 237165) as part of the Era-IB project Terpenosome (MHS) and by NTNU Enabling Biotechnology PhD grant (LMR).

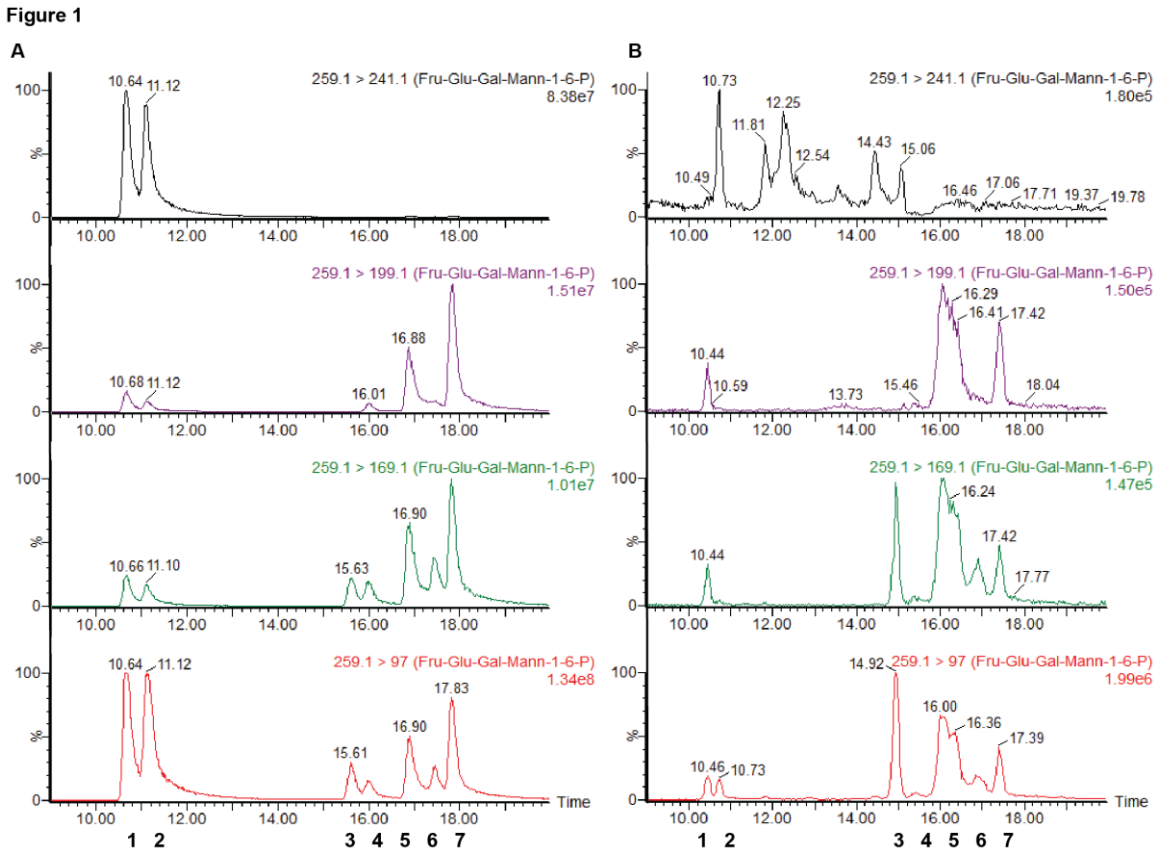
References

[1] A. Gil, D. Siegel, H. Permentier, D.J. Reijngoud, F. Dekker, R. Bischoff, Stability of energy metabolitesAn often overlooked issue in metabolomics studies: A review, Electrophoresis, 36 (2015) 2156-2169.

[2] D. Siegel, H. Permentier, D.J. Reijngoud, R. Bischoff, Chemical and technical challenges in the analysis of central carbon metabolites by liquid-chromatography mass spectrometry, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 966 (2014) 21-33.

[3] I. Aretz, D. Meierhofer, Advantages and Pitfalls of Mass Spectrometry Based Metabolome Profiling in Systems Biology, International journal of molecular sciences, 17 (2016).
[4] H.F.N. Kvitvang, T. Andreassen, T. Adam, S.G. Villas-Boas, P. Bruheim, Highly Sensitive GC/MS/MS Method for Quantitation of Amino and Nonamino Organic Acids, Analytical Chemistry, 83 (2011) 2705-2711.
[5] O.I. Savolainen, A.-S. Sandberg, A.B. Ross, A Simultaneous Metabolic Profiling and Quantitative Multimetabolite Metabolomic Method for Human Plasma Using Gas-Chromatography Tandem Mass Spectrometry, Journal of Proteome Research, 15 (2016) 259-265.
[6] Y. Shen, T. Fatemeh, L. Tang, Z. Cai, Quantitative metabolic network profiling of Escherichia coli: An overview of analytical methods for measurement of intracellular metabolites, TrAC Trends in Analytical Chemistry, 75 (2016) 141-150.
[7] P. Bruheim, H.F.N. Kvitvang, S.G. Villas-Boas, Stable isotope coded derivatizing reagents as internal standards in metabolite profiling, Journal of Chromatography A, 1296 (2013) 196-203.
[8] B. Luo, K. Groenke, R. Takors, C. Wandrey, M. Oldiges, Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography-mass spectrometry, Journal of Chromatography A, 1147 (2007) 153-164.
[9] R.M. Seifar, C. Ras, J.C. van Dam, W.M. van Gulik, J.J. Heijnen, W.A. van Winden, Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry, Analytical biochemistry, 388 (2009) 213-219.
[10] M. Schwaiger, E. Rampler, G. Hermann, W. Miklos, W. Berger, G. Koellensperger, Anion-Exchange Chromatography Coupled to High-Resolution Mass Spectrometry: A Powerful Tool for Merging Targeted and Non targeted Metabolomics, Analytical Chemistry, 89 (2017) 7667-7674.
[11] H.F.N. Kvitvang, K.A. Kristiansen, P. Bruheim, Assessment of capillary anion exchange ion chromatography tandem mass spectrometry for the quantitative profiling of the phosphometabolome and organic acids in biological extracts, Journal of Chromatography A, 1370 (2014) 70-79.
[12] K. Burgess, D. Creek, P. Dewsbury, K. Cook, M.P. Barrett, Semi-targeted analysis of metabolites using capillary-flow ion chromatography coupled to high-resolution mass spectrometry, Rapid Commun. Mass Spectrom., 25 (2011) 3447-3452.
[13] J. Wang, T.T. Christison, K. Misuno, L. Lopez, A.F. Huhmer, Y. Huang, S. Hu, Metabolomic Profiling of Anionic Metabolites in Head and Neck Cancer Cells by Capillary Ion Chromatography with Orbitrap Mass Spectrometry, Analytical Chemistry, 86 (2014) 5116-5124.
[14] H.F.N. Kvitvang, P. Bruheim, Fast filtration sampling protocol for mammalian suspension cells tailored for phosphometabolome profiling by capillary ion chromatography - tandem mass spectrometry, Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 998 (2015) 45-49.
[15] S.G. Villas-Boas, P. Bruheim, Cold glycerol-saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells, Analytical biochemistry, 370 (2007) 87-97.
[16] T.J. Causon, S. Hann, Review of sample preparation strategies for MS-based metabolomic studies in industrial biotechnology, Analytica Chimica Acta, 938 (2016) 18-32.
[17] L. Wu, M.R. Mashego, J.C. van Dam, A.M. Proell, J.L. Vinke, C. Ras, W.A. van Winden, W.M. van Gulik, J.J. Heijnen, Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly 13C-labeled cell extracts as internal standards, Analytical biochemistry, 336 (2005) 164-171.
[18] C. Verduyn, A.H. Stouthamer, W.A. Scheffers, J.P. Vandijken, A Theoretical evaluation of growth yields of yeasts, Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 59 (1991) 49-63.

Figure legends



313

314 Figure 1. Extracted ion chromatogram for hexose phosphate MS/MS transitions for an ES mixture (A)

315 and a *S.cerevisiae* extract (B). The hexose phosphates included are 1: GAL1P; 2: M1P+G1P; 3: F1P; 4:

316 GAL6P; 5: G6P; 6:F6P; 7: M6P (Abbreviations listed in Supplementary Table S3).

317

318

319

320

321

Figure 2

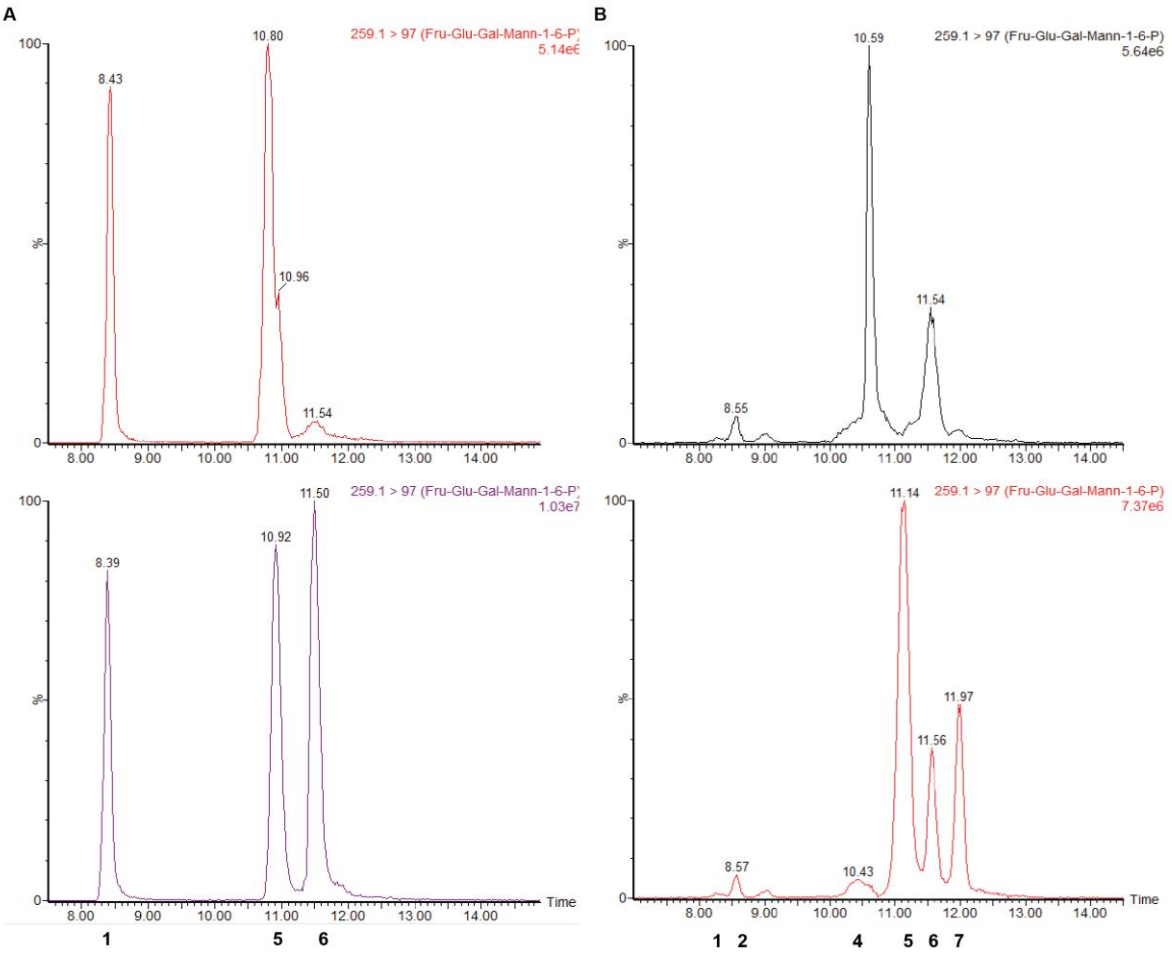
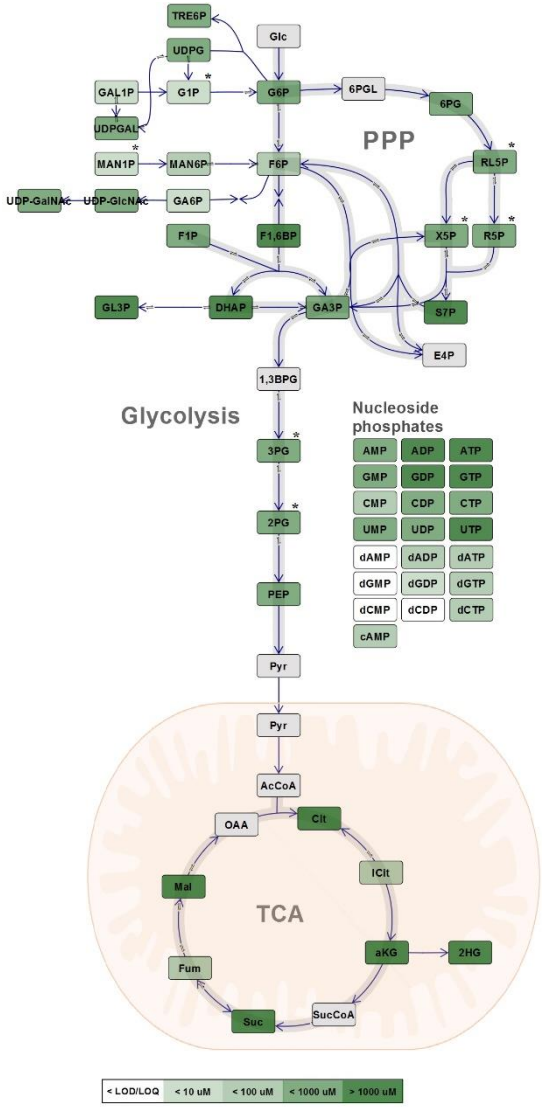


Figure 2. Extracted ion chromatogram for hexose phosphates (m/z 259.1 → 97 transition) of a three-hexose phosphate mixture (A) and an *E. coli* DH5α extract (B) with (upper panel) and without (lower panel) ^{13}C *S.cerevisiae* extract added. The hexose phosphates included are 1: GAL1P; 2: M1P+G1P; 3: F1P; 4: GAL6P; 5: G6P; 6:F6P; 7: M6P (Abbreviations listed in Supplementary Table 3).



330

331 Figure 3. Visaluzation of the comprehensive coverage of primary metabolite pools by the present

332 capIC method. Data is taken from a *Saccharomyces cerevisiae* batch cultivation in mineral medium

333 and the heat mapping shows how the metabolite pools are varying over 4 orders of magitude.

Table 1. Instrument performance over a 10-day period for three selected metabolites (ATP, ADP and AMP). Each sample (a 5 µM ES mixture and 5 identical biologal samples; (QCs) was injected once per day over a 10-day period. The table lists RSDbetween the ten injections for non-correlated results (Area) and results correlated with ^{13}C IS (Response factor).

	ATP		ADP		AMP	
	Area	Response factor	Area	Response factor	Area	Response factor
ES mix (5µM)	39	13	22	24	14	15
QC1	23	14	19	23	41	11
QC2	25	6	48	18	15	5
QC3	47	4	23	11	41	3
QC4	26	3	35	25	26	3
QC5	30	3	17	14	49	11

Supplementary Table S1

Complete MS/MS settings for metabolites included in the method. LOQ is determined according to Kvitvang et al [11].. Abbreviationsare listed in Supplementary Table S3.

			^{12}C		U ^{13}C-IS		
Metabolite	RT (min)	Collision energy (V)	[M-H] (Q1)	Q3	[M-H] (Q1)	Q3	LOQ (nM)
PYR	5.0		87.0		90.0		
LAC	3.4		89.0		90.0		
FUM	15.4	8	115.1	71.1	119.0	75.0	200
ACTP	20.8	15	139.0	79.0	141.0	79.0	50
SUC	11.9	10	117.1	73.1	121.0	76.0	500
IA	13.5	10	129.1	85.0	131.0	85.0	25
MAL	11.9	14	133.1	71.1	137.0	74.0	300
aKG*	11.7	8	145.0	101.0	150.0	106.0	300
2-HG	11.6	16	147.1	85.1	152.0	89.0	300
PEP	22.1	12	167.0	79.0	170.0	79.0	5
DHAP	24.1	8	169.0	97.0	172.0	79.0	1
G3P	22.9	14	169.0	79.0	172.0	79.0	1
GL3P	10.6	14	171.0	79.0	174.0	79.0	50
2-IPPMAL	14,1	14	175.1	85.0	182.1	120.0	500
2PG+3PG	21.4	12	185.0	97.0	188.0	97.0	400
ICIT	22.4	18	191.0	73.1	197.0	116.0	25
CIT	21.2	16	191.1	87.1	197.0	116.0	75
DOXP	11.2	23	213.0	139.0	218.0	79.0	1
MEP	10.6	13	215.0	79.0	220.0	79.0	1
RL5P+R5P+X5P	16.2	10	229.1	96.8	234.0	97.0	5
DMAPP	23.2	14	245.0	79.0	250.0	79.0	1
IPP	23.4	14	245.0	79.0	250.0	79.0	1
GA1P	9.3	16	258.0	97.0	264.0	97.0	0,5
GA6P	15.3	16	258.0	97.0	264.0	97.0	2
F1P	14.8	12	259.1	97.0	265.0	97.0	1
F6P	16.7	14	259.1	97.0	265.0	97.0	20
G1P	10.6	20	259.1	241.0	265.0	247.0	10
M1P	10.6	20	259.1	241.0	265.0	247.0	1
G6P	16.3	14	259.1	97.0	265.0	97.0	20
GAL1P	10.0	14	259.1	97.0	265.0	97.0	20
GAL6P	15.5	14	259.1	97.0	265.0	97.0	1
M6P	17.4	16	259.1	97.0	265.0	97.0	5
1-IP1	10.0	14	259.1	97.0			1
3-IP1	10.0	14	259,1	97.0			1
4-IP1	10.6	14	259,1	97.0			1
6PG	20.5	16	275.1	97.0	281.1	97.0	20
S7P	17.8	20	289.0	97.0	296.0	97.0	10
dUMP	21.3	16	307.1	195.0	316.0	200.0	1

GPP	36.0	18	313.1	79.0	318.1	79.0	0,5
dTMP	21.9	16	321.1	195.0	331.1	200.0	25
CMP	12.7	20	322.0	79.0	331.0	79.0	10
UMP	23.0	20	323.2	97.0	332.1	97.0	25
cAMP	13.8	26	328.1	134.2	338.1	139.1	10
dAMP	14.7	14	330.1	195.0	340.0	200.0	25
F1,6BP	25.0	20	339.1	97.0	345.1	97.0	50
4,5-IP2	24.5	15	339.1	241.0	345.1	97.0	1
1,4,5-IP3	30.8	15	339.1	241.0	419.1	339.1	0,5
cGMP	24.5	22	344.1	150.0	354.0	152.0	10
AMP	17.8	30	346.1	79.0	356.1	79.0	5
IMP	25.2	22	347.2	79.0	357.0	79.0	50
GMP	25.4	24	362.1	79.0	372.0	79.0	1
FPP	36.6	16	381.1	79.0			1
PRPP	27.4	14	388.9	177.1	394.0	177.1	1
dTDP	26.0	22	401.1	159.0	411.1	158.9	100
CDP	21.7	26	402.1	159.0	411.1	158.9	5
UDP	27.0	20	403.1	110.9	412.1	115.1	10
dADP	22.7	24	410.1	159.0	420.1	158.9	25
T6P	10.4	23	422.0	241.0	434.0	242.0	1
ADP	23.9	26	426.1	159.0	436.1	139.1	25
dGDP	27.5	38	426.1	275.1	436.1	280.1	10
GDP	32.0	18	442.1	344.2	452.1	155.1	100
GGPP	24.5	25	449.0	79.0			5
dCTP	23.9	34	466.1	159.0	175.1	158.9	1
dUTP	30.4	24	467.1	159.0	476.1	158.9	10
dTTP	31.6	34	481.1	159.0	491.1	159.0	25
CTP	25.1	34	482.1	159.0	491.1	158.9	25
UTP	33.7	34	483.1	159.0	492.1	158.9	25
dATP	26.1	30	490.1	159.0	500.1	158.9	2
ATP	27.6	32	506.1	159.0	516.1	158.9	50
dGTP	34.1	32	506.1	159.0	516.1	158.9	1
ITP	34.6	36	507.1	159.0	517.1	158.9	10
GTP	35.9	30	522.1	159.0	532.1	158.9	10
UDPG+UDPGAL	(22+)22,4	22	565.0	323.0	580.0	323.0	1
UDP-GlcNAc+UDP-GalNAc	21.4	26	606.2	385.1	623.6	394.1	10

344

345

Supplementary Table S2

Coefficients of determination (R^2) obtained from non-correlated and correlated ^{13}C IS calibration lines.

	R^2 calibration curve	
Metabolite	Without ^{13}C IS	With ^{13}C IS
ADP	0.988	0.996
AMP	0.997	0.998
ATP	0.998	0.998
CIT	0.992	0.912
CMP	0.996	0.999
dADP	0.968	0.999
dAMP	0.999	0.989
dATP	0.973	0.999
DOXP	0.999	0.999
F1P	0.988	0.999
FUM	0.996	0.998
GAL1P	0.993	0.999
GTP	0.997	0.999
IA	0.973	0.997
ICIT	0.972	0.854
M6P	0.998	0.999
RL5P+R5P+X5P	0.999	0.998
S7P	0.996	0.999
TTP	0.997	0.997
UDP-Glc/Gal-Nac	0.988	0.999

Supplementary Table 3. List of abbreviations

1,4,5-IP_3	Inositol-1,4,5-triphosphate
1-IP_1	Inositol 1-phosphate
2-HG	2-Hydroxyglutarate
2-IPPMAL	2-Isopropylmalate
2PG+3PG	2-Phosphoglycerate, 3-Phosphoglycerate
3-IP_1	Inositol 3-phosphate
4,5-IP_2	Inositol 4,5-diphosphate
4-IP_1	Inositol 4-phosphate
6PG	6-phosphogluconate
ACTP	Acetyl-phosphate
ADP	Adenosine diphosphate
aKG	α-Ketoglutarate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CIT	Citrate
CDP	Cytidine diphosphate
CMP	Cytidine monophosphate
CTP	Cytidine triphosphate
DHAP	Dihydroxyacetone phosphate
DMAPP	Dimethylallyl pyrophosphate
DOXP	1-deoxy-D-xylulose 5-phosphate
dADP	Deoxy-adenosine diphosphate
dAMP	Deoxy-adenosine monophosphate
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytidine triphosphate
dGDP	Deoxy-guanosine diphosphate
dGTP	Deoxy-guanosine triphosphate
dTDP	Deoxy-thymidine diphosphate

383	dTMP	Deoxy-thymidine monophosphate
384	dTTP	Deoxy-thymidine triphosphate
385	dUMP	Deoxy-uridine monophosphate
386	dUTP	Deoxy-uridine diphosphate
387	F1,6BP	Fructose 1,6-bisphosphate
388	F1P	Fructose 1-phosphate
389	F6P	Fructose 6-phosphate
390	FPP	Farnesyl pyrophosphate
391	FUM	Fumarate
392	G1P	Glucose 1-phosphate
393	G6P	Glucose 6-phosphate
394	GA1P	Glucoseamine 1-phosphate
395	G3P	Glyceraldehyde 3-phosphate
396	GA6P	Glucoseamine 6-phosphate
397	GAL1P	Galactose 1-phosphate
398	GAL6P	Galactose 6-phosphate
399	GDP	Guanosine diphosphate
400	GGPP	Geranylgeranyl pyrophosphate
401	GL3P	Glycerol 3-phoshate
402	GMP	Guanosine monophosphate
403	GPP	Geranyl pyrophosphate
404	GTP	Guanosine triphosphate
405	IA	Itaconic acid
406	ICIT	Isocitrate
407	IMP	Inosine monophosphate
408	IPP	Isopentenyl pyrophosphate
409	ITP	Inosine triphosphate
410	LAC	Lactate
411	M1P	Mannose 1-phosphate
412	M6P	Mannose 6-phosphate
413	MAL	Malate
414	MEP	2-C-methyl-D-erythritol 4-phosphate

415	PEP	Phosphoenolpyruvate
416	PRPP	Phosphoribosyl pyrophosphate
417	PYR	Pyruvate
418	RL5P+R5P+X5P	Ribulose 5-phosphate, Ribose 5-phosphate, Xylulose 5-phosphate
419	S7P	Sedoheptulose 7-phosphate
420	SUC	Succinate
421	T6P	Trehalose-6-phosphate
422	UDP	Uridine diphosphate
423	UDPG+UDPGAL	UDP Glucose, UDP Galactose
424	UDP-GlcNAc+UDP-GalNAc	UDP N-acetylglucoseamine, UDP N-acetylgalactoseamine
425	UMP	Uridine monophosphate
426	UTP	Uridine triphosphate