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Alkaline conditions in hydrophilic interaction liquid chromatography for intracellular metabolite quantification using tandem mass spectrometry

Attila Teleki [1], Andrés Sánchez-Kopper [1], Ralf Takors *

Institute of Biochemical Engineering, University of Stuttgart, 70569 Stuttgart, Germany

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ABSTRACT

Modeling of metabolic networks as part of systems metabolic engineering requires reliable quantitative experimental data of intracellular concentrations. The hydrophilic interaction liquid chromatography–electrospray ionization–tandem mass spectrometry (HILIC–ESI–MS/MS) method was used for quantitative profiling of more than 50 hydrophilic key metabolites of cellular metabolism. Without prior derivatization, sugar phosphates, organic acids, nucleotides, and amino acids were measured under alkaline and acidic mobile phase conditions with pre-optimized multiple reaction monitoring (MRM) transitions. Irrespective of the polarity mode of the acquisition method used, alkaline conditions achieved the best quantification limits and linear dynamic ranges. Fully 90% of the analyzed metabolites presented detection limits better than 0.5 pmol (on column), and 70% presented 1.5-fold higher signal intensities under alkaline mobile phase conditions. The quality of the method was further demonstrated by absolute quantification of selected metabolites in intracellular extracts of *Escherichia coli*. In addition, quantification bias caused by matrix effects was investigated by comparison of calibration strategies: standard-based external calibration, isotope dilution, and standard addition with internal standards. Here, we recommend the use of alkaline mobile phase with polymer-based zwitterionic hydrophilic interaction chromatography (ZIC–pHILIC) as the most sensitive scenario for absolute quantification for a broad range of metabolites.

Metabolomics addresses the identification and quantification of small molecule metabolites, in essence the reactants of biological systems, and enables the comprehensive analysis of complex biochemical networks systemic studies. Whereas the genome and proteome represent upstream biochemical events, the metabolome reveals underlying regulatory mechanisms and therefore reflects, most closely, the actual cellular physiological state [1–3]. As such, metabolome analysis can be subdivided into targeted and nontargeted approaches. Nontargeted high-throughput applications such as *metabolic fingerprinting* aim at the rapid and qualitative or semiquantitative analysis of whole-cell metabolic patterns, reducing the analytical effort considerably [4–6]. On the contrary, targeted applications such as *quantitative metabolic profiling* are focused on the nonbiased absolute quantification of a subset of metabolites of predefined metabolic pathways or classes of compounds [2,4,6]. In this context, monitoring of intracellular metabolism dynamics as a result of rapid changes in the extracellular environment within stimulus–response experiments can be used to investigate in vivo enzyme kinetics offering insights into underlying regulatory mechanisms [7–10]. Considering that the enormous number of intracellular metabolites within various biological matrices and concentrations range over several orders of magnitude (pM–mM), a variety of different analytical methods have been investigated [2,6]. Used analytical methods vary widely, from enzymatic assays [11] for targeted measurements to modern and more powerful soft ionization mass spectrometry (SIMS)[2] coupled to liquid chromatography (LC) [12–19], gas chromatography

* Corresponding author. Fax: +49 0711 685 65164.
E-mail address: ralf.takors@ibvt.uni-stuttgart.de (R. Takors).
[1] Contributed equally to this manuscript.

[2] *Abbreviations used:* SIMS, soft ionization mass spectrometry; LC, liquid chromatography; GC, gas chromatography; CE, capillary electrophoresis; NMR, nuclear magnetic resonance; LC–MS, liquid chromatography combined with mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; LC–ESI–MS/MS, liquid chromatography–electrospray ionization–tandem mass spectrometry; MRM, multiple reaction monitoring; ZIC–pHILIC, polymer-based zwitterionic hydrophilic interaction chromatography; LB, Luria–Bertani; MDL, method detection limit; ANOVA, analysis of variance; RPLC, reversed phase liquid chromatography; WWR, wrong-way-round.

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(GC) [20–25], or capillary electrophoresis (CE) [26–28] as well as nuclear magnetic resonance (NMR) [29,30]. For comprehensive metabolite quantification studies, a combination of multiple analytical methods is usually indispensable, resulting in data sets with varying precision and accuracy [1,2,6,31]. However, for dynamic modeling of cellular metabolism, in connection with stimulus–response studies, reliable quantifications of absolute intracellular concentrations in defined cellular states and time points are of crucial importance [7,10,25]. Compared with GC, CE, and NMR, liquid chromatography combined with mass spectrometry (LC–MS) offers inherent advantages; LC–MS does not require further derivatization like GC, is able to separate polar and nonpolar compounds unlike CE with selectivity depending on column material, and offers a higher sensibility compared with NMR. Consequently, LC–MS approaches are becoming highly accepted as the most universal analytical platform for reliable quantification of intracellular metabolites [2,3,25]. Recently, hydrophilic interaction liquid chromatography (HILIC) has become increasingly popular because it enables the separation of charged and polar analytes and exhibits excellent compatibility with MS detection [32–34]. Related studies focused mainly on silica-based stationary HILIC phases, modified by various functional groups, enabling comprehensive quantitative analysis of metabolites under acidic and neutral mobile phase conditions [12,13,15,16]. Motivated by these encouraging studies, we developed the approach further for exploiting its potential, concentrating on so far nonaddressed modes of operation.

In this study, we present an analytical platform based on two liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) methods for quantitative profiling of more than 50 key metabolites of the cellular metabolism, comprising amino acids, sugar phosphates, organic acids, nucleotides, and coenzymes. Metabolites were detected by MS/MS on a triple quadrupole instrument in the multiple reaction monitoring (MRM) mode with high selectivity based on pre-optimized MRM transitions. Chromatographic separation was performed by polymer-based zwitterionic hydrophilic interaction chromatography (ZIC–pHILIC), and the mobile phase pH effect with respect to analyte sensibility was evaluated. Special focus placed on method quantification limits, linear dynamic range, and repeatability for a representative cross section of 56 common intracellular metabolites in acidic and alkaline mobile phase conditions. Finally, the applicability of the method was demonstrated by absolute quantification of selected metabolites in intracellular extracts of *Escherichia coli* showing consistency with previously published endogenous steady-state concentrations. In addition, occurrence of quantification bias caused by matrix effects is presented, comparing quantitative results obtained by standards-based external calibration, isotope dilution, and standard addition as calibration strategies.

Materials and methods

Chemicals

Metabolite standards, reagents, and U-^{13}C-labeled lyophilized algal cells were supplied by Sigma–Aldrich (Taufkirchen, Germany). MS-grade water was purchased from VWR (Darmstadt, Germany). MS-grade acetonitrile was purchased from Carl Roth (Essen, Germany). Standard stock solutions were prepared in LC–MS water and stored at −70 °C.

Strains and growth condition

E. coli K-12 MG1655 (purchased from DSMZ, Leipzig, Germany) was grown in minimal medium with 0.75% (w/v) D-glucose as sole carbon source. The minimal medium contained the following components: 63.1 mM NaH_2PO_4, 26.9 mM K_2HPO_4, 10.0 mM $(NH_4)_2HPO_4$, 20.3 mM $(NH_4)_2SO_4$, 6.2 mM Na_2SO_4, 1.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01 mM thiamine hydrochloride. Overnight precultures (12 h, 37 °C, 120 rpm) were inoculated from a cryoculture (50% glycerol and Luria–Bertani [LB] medium) and were grown in 100-ml baffled shake flasks with 20 ml of minimal medium (12 h, 37 °C, 120 rpm). Main cultures (37 °C, 150 rpm) were inoculated in 1:100 dilution and grown in triplicates in 500-ml baffled shake flasks with 60 ml of minimal medium (10 h, 37 °C, 150 rpm).

Sampling, quenching, and metabolite extraction

Cells were sampled during the exponential growth phase at a biomass concentration of approximately 2 g L^{-1}. Amounts of approximately 4 mg biomass were sampled by fast centrifugation (20,000*g*, 20 s) and washed with 2 ml of isotonic 0.9% (w/v) sodium chloride solution (20,000*g*, 20 s). Biomasses were quenched by liquid nitrogen (−196 °C) and temporarily stored at −70 °C. Subsequently, a defined amount of water (MS grade) was added to obtain an extraction concentration of 15 g L^{-1}. Resulting suspensions were immediately pre-incubated for 1 min at 100 °C in a water bath for enzymatic inactivation and resuspended by short-time vortexing. Subsequently, samples were incubated for 5 min at 100 °C in a water bath and afterward chilled on ice water. Metabolite extracts were separated from cell debris by centrifugation (20,000*g*, 10 min) and stored at −70 °C [35].

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

Commercially available U-^{13}C-labeled lyophilized algal cells (>99 atom% ^{13}C, lot no. 487945, Sigma–Aldrich) were weighed into 2-ml reaction vessels in small amounts. Preheated water (100 °C) was added aiming for an extraction concentration of 90 g L^{-1}. The resulting suspensions were incubated at 100 °C for 2 min in a water bath and resuspended by short-time vortexing. The procedure was repeated two more times, and the resulting samples were chilled on ice water. ^{13}C-labeled metabolite extracts were separated from algal cells by centrifugation (20,000*g*, 10 min) and stored at −70 °C [25].

Optimization of chromatographic and source conditions

Development of chromatography was performed on a Sequant ZIC–pHILIC column (150 × 2.1 mm, 5 µm) with guard column (Sequant ZIC–pHILIC, 20 × 2.1 mm, 5 µm). The optimization of chromatographic conditions was based on a selection of highly polar intracellular metabolites within the central metabolism (6-phophogluconate, fructose 1,6-bisphosphate, glucose 6-phosphate, phosphoenolpyruvate, malate, succinate, and citrate). Bicratic chromatographic runs were evaluated in regard to selectivity and sensitivity of applied standard mixtures. Optimization was focused on pH of mobile phases (5.0, 7.0, and 9.0), buffer concentration (2–20 mM ammonium acetate), flow rate (0.05–0.20 ml/min), column temperature (20–60 °C), and gradient slope of polar eluent B (2,5–5% B/min) within a bicratic elution mode. In addition, source parameters (ESI) were optimized with respect to maximal signal intensities, focusing on the nitrogen gas flow (7–13 L/min), nebulizer pressure (15–60 psi), and capillary voltage (±3000–4000 V). An overview of optimized chromatographic and source parameters is shown further below.

Sample processing and optimized chromatographic conditions

Standards and endogenous cellular extracts were analyzed on an Agilent 1200 high-performance liquid chromatography (HPLC) system (Agilent, Waldbronn, Germany) consisting of a degasser, a