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Development and Application of a Method for Quantitative Metabolome Analysis of Various Production Strains

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I. Summary

Recent technical improvements in the area of mass spectrometry enabled quantitative measurements of small molecules. The present work used these advancements to develop a method for the quantification of metabolites of the central carbon metabolism in the nanomolar range. Sample analysis by tandem mass spectrometry hyphenated with liquid chromatography enabled measurements of 33 metabolites in 25 min. Performed measurements were validated by thermodynamic and energetic constraints, which proved that datasets were of high quality. Combination of metabolomics and fluxomics approaches enabled a holistic and systems-oriented view during analysis of the metabolic datasets. The new method was first applied to identify changes in the energy charge of *E. coli* during different cultivation modes. Analysis showed that the adenylate energy charge was actively controlled by secretion or synthesis of adenylate phosphates. Thus, a strong imbalance between energy generation and consumption was necessary to distort the energy charge permanently. The novel technique was further used to identify changes in the central carbon metabolism of microorganisms as a consequence of genetic modifications, stress inducing cultivation conditions and changes in carbon source. Surprisingly, genetic modifications and stress inducing cultivation conditions resulted only in minor changes of intracellular metabolite levels. Hence, it seems that microorganisms put great efforts into the homeostasis of metabolite ratios and fluxes.

II. Zusammenfassung

Neue Entwicklungen im Bereich der Massenspektroskopie ermöglichen quantitative Messungen von Molekülen mit sehr kleinen Massen. In der vorliegenden Arbeit wurden diese Neuentwicklungen genutzt um Metabolite des zentralen Kohlenstoffwechsels im nanomolaren Bereich nachzuweisen. Die Kopplung von Tandem-Massenspektroskopie und Flüssigchromatographie ermöglichte den Nachweis von 33 Metaboliten in 25 min. So gewonnene Datensätze wurden anschließen mittels Thermodynamik validiert, wodurch die hohe Qualität der Datensätze deutlich wurde. Bei der Analyse der Datensätze wurden dann Ergebnisse aus Metabolom- und Fluxomforschung kombiniert um einen ganzheitlichen Ansatz zu schaffen. Die neue Technik wurde zuerst angewandt um den Einfluss verschiedener Kultivierungsverfahren auf das Energielevel von *E. coli* zu untersuchen. Die Untersuchungen zeigten, dass *E. coli* seinen Energielevel aktiv durch die Sekretion und Synthese von Energiemetaboliten steuern kann. Nur extreme Ungleichgewichte bei Verbrauch und Generierung von Energie konnten den Energiehaushalt nachhaltig stören. Weitere Messungen untersuchten den Einfluss von genetischen Veränderungen, Stress und unterschiedlichen C-Quellen auf den zentralen Kohlenstoffwechsel von Mikroorganismen. Überraschenderweise führten weder genetische Veränderungen noch Stress zu starken Veränderungen der Metabolitkonzentrationen. Dies zeigt das Mikroorganismen viel Aufwand in die Homöostase von Metabolit-Verhältnissen und intrazellulären Flüsse stecken.

1 Introduction

Metabolic pathways are the very essence of life and the understanding of their functioning and their regulation has been a major goal of the natural sciences, since the discovery of metabolism itself. Interestingly, central carbon metabolism is highly conserved in most living organisms due to its efficiency (Smith & Morowitz 2004; Ebenhöh & Heinrich 2001). It channels various nutrients through pathways to provide energy and reducing power for cell maintenance and reproduction as well as precursor metabolites for biomass formation (Noor et al. 2010). As such, this core piece of metabolism is in the heart of biotechnology. Elucidation of central carbon metabolism during the past decades (Entner & Doudoroff 1952; Gunsalus et al. 1955; Krebs & Johnson 1980; Barnett 2003) laid the foundation for modern biotechnology. The meanwhile even deeper understanding of metabolism and its regulatory elements is a valuable knowledge base to enhance bio-production processes, as it enables the rational design of metabolic pathways towards high titers and yields of desired products of choice (Akinterinwa et al. 2008; Buchinger et al. 2009; Becker et al. 2011; Becker & Wittmann 2012b). Particularly, sophisticated methods, which allow the detection of imbalances in levels of energy or precursor metabolites and the identification of even unknown cellular intermediates, appear most useful to identify genetic targets for tailor-made modifications of microorganisms towards improved performance (Lee et al. 2005; Wendisch et al. 2006; Becker et al. 2007).

More fundamentally, the exact quantification of intracellular metabolites allows describing and modeling of the complex underlying metabolic pathway networks. Such systems-oriented approaches heavily rely on suitable technologies, which provide accurate data of the studied system. Experimental techniques that provide such systems wide insights are therefore well suited to collect quantitative data to construct, adjust and validate systems biology models. These techniques are referred to as “omics” and involve genomics, transcriptomics, proteomics, fluxomics and metabolomics according to their target analytes (Romualdi & Gerolamo 2009). Hence, the field of metabolomics intends to study the profiles of metabolites, which are products of specific cellular processes (Jordan et al. 2009) and thereby provides a snapshot of cellular physiology at a certain point of time. As a subcategory, metabonomics is by definition “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modifications” (Nicholson 2006).

The recent decades have seen a tremendous progress in the field of metabolite analysis. In the pioneering days, colorimetric, manometric and enzymatic assays were employed for identification and quantification of metabolites (Entner & Doudoroff 1952; Krebs & Johnson 1980). The elucidation of the central pathways of metabolism by utilization of these methods took an enormous effort, as such techniques required large amounts of the analytes of interest and

multiple steps of purification prior to the actual measurement. The coupling of metabolite analysis to chromatographic separation processes, such as thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC), led to drastically lowered detection limits, thus displaying a milestone in the analysis of sample mixtures (Touchstone 1993). This was even improved by coupling of mass spectrometry (MS) to chromatographic separation processes. The use of highly sensitive tandem mass spectrometry for detection led to a further decrease of detection limits, while simultaneously allowing quantification of complex metabolite mixtures, due to the identification of specific metabolite fragments by their mass to charge ratio (Luo et al. 2007; Balcke et al. 2011). In addition, mass spectrometry allows distinguishing between different isotopically labeled versions of a metabolite, as a consequence of the change in mass, thereby enabling approaches like ^{13}C metabolic flux analysis (Wittmann & Heinzle 1999; Dauner & Sauer 2000). Most recently, the utilization of ion mobility mass spectrometry enables the determination the ratios of isomers, which are inseparable by chromatography (Far et al. 2014). These recent instrumental developments now provide an excellent basis to perform metabolite analysis.

Basically, it has become relatively easy to generate larger datasets of intracellular metabolite concentrations. However, remaining questions revolve around the challenge that the measured levels indeed reflect the studied *in vivo* system (Bolten et al. 2007). Particularly, the quality of metabolic datasets is strongly dependent on the preceding steps of sampling, sample treatment and processing. It is crucial to stop the metabolism instantaneously due to high turnover rates and small pool sizes of the metabolites of interest, which might otherwise be distorted (Wittmann et al. 2005). Furthermore, the avoidance of potential unwanted side effects of the sample treatment, such as metabolite leakage (Bolten et al. 2007), co-precipitation (Zakhartsev et al. 2015) and the degradation or interconversion of metabolites during the extraction, has to be considered. Combined with the largely varying physico-chemical properties of metabolites and differences in concentrations of several orders of magnitudes these problems still pose as a serious challenge when developing a reliable quantitative method for metabolite analysis.

2 Objectives

The aim of the present study was to develop a method for the quantification of intermediates from central carbon metabolism via liquid chromatography coupled with tandem mass spectrometry, suitable to be applied to a variety of different microorganisms. Among the analytes of interest were metabolites of the glycolysis, the Entner-Doudoroff (ED) pathway, the pentose phosphate (PP) pathway and the tricarboxylic acid (TCA) cycle, as well as energy metabolites and redox equivalents. Preferably, the analysis of cellular extracts should be faster as commonly reported time periods of up to 90 minutes and exhibit a high-resolution capacity for the complex cellular extracts of interest, which should be aimed for by optimization of the liquid chromatography and the coupling to mass spectrometric detection. The developed method should generate reproducible results and combine high separation efficiency with high sensitivity and short analysis times. Starting initially with standard mixtures from synthetic compounds the approach should be transferred to living cells. This should involve the estimation of the limits of detection (LOD) and quantification (LOQ) in the presence of relevant biological matrices. Additionally, different protocols for sampling and metabolite extraction should be applied and validated. Careful inspection of the obtained datasets from thermodynamic and energetic perspective should then provide a clear basis for validation.

Finally, the novel quantitative method should be applied to address relevant biological questions. Hence, the central carbon metabolome of seven biotechnological relevant Gram-negative and Gram-positive microbial strains should be studied under different growth conditions. The gained insight should then be combined with results from metabolic flux analysis to investigate the impact of genetic modifications, environmental stress or changes of substrate on selected strains.

3 Theoretical Background

3.1 Quantitative Measurements in Microbial Metabolomics

Generally, metabolomics investigates the endo- and exo-metabolome by qualitative, semi-quantitative and quantitative approaches respectively. Hereby, quantitative analysis gives the most accurate picture. In contrast to semi-quantitative approaches (Zamboni et al. 2008), quantitative datasets can be validated by thermodynamic inspection and used for kinetic and metabolic modeling (de Jonge et al. 2014; Wiechert & Noack 2011). Metabolomics itself is a relatively young component in the field of systems biology. Since the year 2000 more and more studies involve metabolomic measurements, indicating a fast-growing interest in this field (Figure 3.1). Obviously, metabolomics is becoming a valuable tool of research in the fields of metabolic engineering (Trethewey 2004; Toya & Shimizu 2013) and bioprocess optimization (Sonntag et al. 2011) over the last decade. However, compared to e.g. transcriptomics, genomics and proteomics, the number of metabolomic studies seems still rather low (Kohlstedt et al. 2010). Particularly, open questions remain and involve suitable protocols for sampling, quenching and extraction procedures as well as analytical methods to guarantee validity and comparability of metabolic datasets. Without doubt, metabolome analysis remains an analytical challenge (van der Werf et al. 2007).

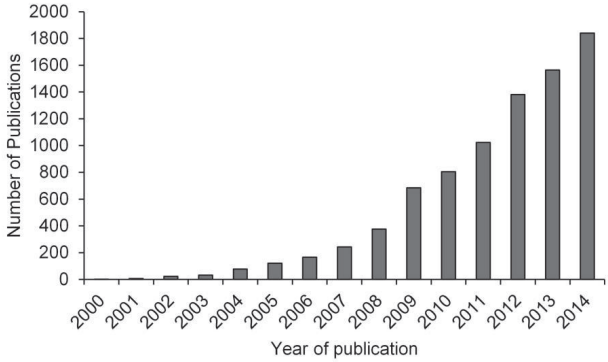

Figure 3.1: Increase in the number of publications in the field of metabolomics over the past 14 years according to the PubMed database (2015).

A powerful and robust quantitative method for the measurement of intracellular metabolites has to meet several key criteria. The method should provide high resolution, short measurement time and high reproducibility. The first challenge, which has to be overcome to achieve this goal, is the complexity of biological samples, which comprise multiple compounds with great variance in concentration and chemical properties. As example, the metabolome of

E. coli consists out of about 1000 different metabolites, whereby intracellular concentrations range from picomolar to millimolar (Dunn & Ellis 2005; Feist et al. 2007). Thus, the applied analytical instruments have to enable high separation efficiency, while at the same time providing a high dynamic detection range.

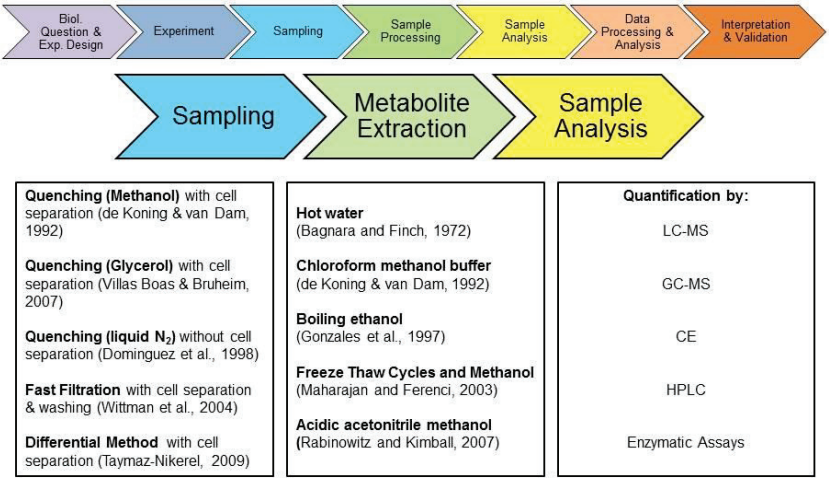

Figure 3.2: Standard workflow of a metabolomics experiment with emphasis on critical choices during method development.

Immediately emerging from the general metabolomics workflow (Figure 3.2) the establishment of reproducible sampling and extraction methods becomes mandatory. Turnover rates of metabolites of the central carbon metabolism are in the range of seconds to sub-seconds, making it crucial to stop all enzyme activities immediately during sampling, while at the same time avoiding metabolite leakage (Wittmann et al. 2004; van Gulik 2010). Subsequently, an extraction method has to be chosen, which combines reproducibility and extraction efficacy, while simultaneously avoiding degradation of metabolites as a consequence of harsh extraction conditions (Villas-Bôas et al. 2005; Canelas et al. 2009). Furthermore, the application of a uniformly ^{13}C labeled internal standard, prior to the extraction process, has been proven a powerful tool to compensate for effects of biological matrices in the sample, as well as effects of degradation during the extraction process (Mashego et al. 2007).

Table 3.1: Strengths and weaknesses of the hyphenation of gas and liquid chromatography to mass spectrometry for metabolomics

	GC	LC
Advantages	- electron impact (EI) ionization highly reproducible and robust	- high variety of separation mechanisms enable detection of a wide range of analytes
	- high separation efficiency	- applicable to non-volatile, thermally labile and polar compounds
	- spectral libraries available	- electrospray-ionization (ESI) is a soft ionization type (no fragmentation)
Disadvantages	- limited applicability to non-volatile, thermally labile and polar compounds	- sensible to matrix dependent suppression effects
	- derivatization necessary	- limited flexibility in eluent composition due to ESI
	- fragmentation during the ionization step	

As stated above, microbial extracts contain up to 1000 different low molecular weight compounds, thereby requesting for efficient separation mechanisms prior to quantification of selected molecules of interest. Different separation techniques, hyphenated with mass spectrometry (MS), have emerged in quantitative metabolomics. Gas chromatography (GC) and liquid chromatography (LC) are the most prominent separation mechanism, which offer complementary advantages (Table 3.1). The GC unites the benefits of a wide compound range and high separation efficiency with the high reproducibility of electron impact (EI) ionization. As a consequence, a variety of spectral libraries can be found, which enable the easy identification of a huge variety of metabolites. Due to its high sensitivity, mass accuracy and reproducibility GC-MS has become a standard tool, which is widely applied to study fluxes in prokaryotic (Park et al. 1997) and eukaryotic cells (Christensen et al. 2000) or to elucidate biosynthetic pathways (Hellerstein et al. 1991; Kelleher et al. 1994). GC of nonvolatile or polar metabolites, however, requires an additional derivatization step to render these compounds amendable to gas phase separation, thus adding a potential bias due to incomplete derivatization (Büscher et al. 2009). Moreover, the subsequent vaporization hinders the successful measurement of thermally labile metabolites and the hard ionization techniques limit the gain of additional structural information.

However, these drawbacks can be overcome by coupling liquid chromatography to mass spectrometry. In contrast to GC-MS, LC coupled MS uses soft ionization techniques, such as electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI). Thus, it is especially useful for the analysis of thermally labile, polar and nonvolatile compounds, of which many are found in the central carbon metabolism. Consequently, recent technical improvements in the coupling of liquid chromatography and mass spectrometry led to an increasing

interest in this technology, due to its broad application potential in dynamic flux analysis (Oldiges et al. 2004; Kiefer et al. 2007) and high throughput screenings (Sauer 2004). A major drawback of LC methods is the susceptibility of separation and ionization quality to matrix effects. Fortunately, matrix effects can be lowered by addition of ion-pairing reagents, which prolong the retention times of metabolites on the column, thereby enhancing the separation process. As a consequence, the analytical method should be chosen carefully. The quantitative approach of targeted metabolomics focuses on the measurement of defined sets of intermediates or end products of metabolic pathways as in studies of drug induced metabolism (Li et al. 2012; Spaggiari et al. 2014). Further application fields are the identification of biomarkers in cells, tissues or body fluids (Alberice et al. 2013; Quinones & Kaddurah-Daouk 2009) and the utilization in forensic sciences. Here, the LC-MS is used to identify synthetic drugs in blood, urine or hair samples which cannot be identified by GC-MS (Thevis et al. 2008; Maurer 2005; Broecker et al. 2012) or to detect trace levels of chemical warfare agents (Hayes et al. 2004) and explosives (DeTata et al. 2013) due to its high sensitivity.

3.2 Tandem Mass Spectrometry hyphenated with Liquid Chromatography

3.2.1 Liquid Chromatography for Metabolite Separation

As stated above, the coupling of liquid chromatography to mass spectrometers allows the analysis of nonvolatile, polar, charged and thermally labile metabolites. Meanwhile, the development of ultra-high performance liquid chromatography (UHPLC) enables shorter analysis times (Plumb et al. 2005), while also improving chromatographic resolution (Guillarme et al. 2010) and peak capacity (Wilson et al. 2005). Liquid chromatography offers different kinds of stationary phases providing a highly versatile platform for the separation of chemicals with different properties. To date, reversed phase (RP) columns, combined with a gradient elution, represent the most favored separation mode (Theodoridis et al. 2012). However, when applied to polar or ionic metabolites, such as organic acids and amino acids, it is important to use additional separation mechanisms. One possibility is the use of specific stationary phases like hydrophilic interaction liquid chromatography (HILIC) (Spagou et al. 2011), aqueous normal phase chromatography (ANP) (Pesek & Matyska 2005; Callahan et al. 2009) or reversed phase pentafluorophenylpropyl (PFPP) columns (Yang et al. 2010), respectively. HILIC columns combine the separation mechanisms of ion chromatography, normal phase and reversed phase and perform well in the analysis of uncharged highly hydrophilic and amphiphilic metabolites. The disadvantages of this technique are time consuming re-equilibration, column bleeding, decreasing resolution and instable retention times of metabolites (Walker et al. 2012; Snyder et al. 2010).