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Metabolomics in yeast by LC-MS

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SUMMARY

The objective of this work is the development of quantitative methods, based on LC-MS, targeting small intracellular metabolites in yeast and yeast derived products. Development covers all aspects from sample preparation to detection by complementary separation and mass spectrometric detection systems.

The first presented method is dedicated to the quantitative profiling of nucleotides, nucleosides and nucleobases by LC-MS/MS. Excellent separation of these analytes was found using a reversed phase material which is compatible with 100 % aqueous eluent and which can be applied for polar analytes. Comparison with other separations revealed superior separation efficiency than hydrophilic interaction liquid chromatography (HILIC) and lower limits of detection compared to ion pair chromatography which is the most frequently used separation of nucleotides. Moreover separation of the isomer 5', 3' and 2' ribonucleotides was achieved. Employment of ESI-triple quadrupole MS enabled selective and interference-free detection of nucleotides, nucleosides and nucleobases. The method was applied in nucleotide enriched yeast autolysates which are used as feed supplements. These products are provided in freeze dried powderous form which made sample preparation simple and straightforward. The main focus was put – due to the absence of certified reference material – on the development and validation of a complementary mass spectrometric method. The reversed phase separation was therefore combined to complementary detection via inductively coupled plasma MS and determination of nucleotides was conducted via phosphorous detection.

The second presented method deals with quantitative metabolite profiling of living yeast cells from the bioreactor. Fast sampling, the arrest of metabolic activity, efficient cell extraction and the avoidance or compensation of any losses were the main challenges here for accurate quantification. The established sample preparation, which was optimized for the yeast *Pichia pastoris*, consists of quenching with cold methanol, separation and washing of the biomass, cell extraction with boiling ethanol, evaporation of the extraction solvent, reconstitution and dilution. The multitarget analysis of a wide range of intracellular metabolites (organic acids, amino acids, nucleotides and vitamins) was carried out via LC-MS/MS employing reversed phase LC, HILIC and triple quadrupole MS. Furthermore, a fully ^{13}C labeled yeast extract was produced and its suitability as internal standard was evaluated. Within this work, this labeled cell extract was used as a tool to evaluate the recoveries and repeatability precisions of the cell extraction and the extract treatment. For this purpose the labeled cell extract was spiked to a set of cell samples at three distinct steps of sample preparation procedure and the metabolites were measured both in monoisotopic ^{12}C and fully labeled ^{13}C form. Finally the total combined uncertainties of the sample preparation procedure for yeast could be estimated using a model equation for uncertainty budgeting and error propagation for all input variables.

ZUSAMMENFASSUNG

Ziel der vorliegenden Dissertation war die Entwicklung analytischer Methoden zur Quantifizierung von Metaboliten in Hefe und Hefeprodukten. Die Methodenentwicklung umfasste hierbei sowohl die Probenvorbereitung zur Extraktion intrazellulärer Metabolite als auch die quantitative Messung dieser Metabolite mittels Flüssigkeitschromatographie (LC) in Kombination mit massenspektrometrischen Detektionsmethoden (MS).

Der erste Teil der Dissertation beschäftigt sich mit der Quantifizierung von Nukleotiden in Hefeprodukten, welche wegen ihres hohen Nukleotidgehaltes als Nahrungsergänzungsmittel in der Tierzucht zur Anwendung kommen. Verschiedenste chromatographische Trennmethoden wurden untersucht, mit dem Ziel möglichst das gesamte Spektrum von Nukleotiden, Nukleosiden und Nukleobasen mit einer Methode zu erfassen. Eine Umkehrphasen-Trennung erwies sich im Vergleich zu anderen Techniken (Hydrophile Interaktions Chromatographie (HILIC), Ionenpaarchromatographie) als überlegen, da hier hohe Robustheit, niedrigste Nachweisgrenzen und effiziente Trennung - bis hin zu Isomerentrennungen - erreicht werden konnten. Zur Validierung der quantitativen Methode, welche auf molekülmassenspektrometrischer Detektion (Triple Quadrupol Massenspektrometer) basiert, wurde eine komplementäre Methode basierend auf anorganischer Massenspektrometrie (Induktiv gekoppeltes Plasma-MS, ICP-MS) entwickelt. Die letztere Methode, eine neue Phosphor Speziationsmethode, wurde zur Vergleichsmessung herangezogen.

Der zweite Teil der Dissertation befasst sich mit der Quantifizierung von intrazellulären Metaboliten (organische Säuren, Aminosäuren, Nukleotide und Vitamine) in Hefe. Um den Stoffwechselzustand lebender Zellen beschreiben zu können, sind eine schnelle Probennahme der intakten Zellen aus dem Fermenter, sofortige und vollständige Inaktivierung aller Enzyme und effiziente Zellextraktion unumgänglich. Verluste während der Extraktion müssen gering gehalten oder berücksichtigt werden. Ein all diesen Anforderungen entsprechendes Probenvorbereitungsprotokoll wurde für die Hefe *Pichia pastoris* entwickelt. Zusätzlich schuf die Herstellung vollständig ^{13}C markierter Zellextrakte den Zugang zu ^{13}C markierten Metaboliten. Als interner Standard verwendet ermöglichen ^{13}C Extrakte die Anwendung des Prinzips der Isotopenverdünnung zur Quantifizierung. Zudem wurde der isotopenmarkierte Zellextrakt zur Bestimmung von Wiederfindungsraten sowohl einzelner Probenvorbereitungsschritte als auch der gesamten Probenvorbereitung eingesetzt. Die Messung der Proben erfolgte mittels zweier orthogonaler Trennmethoden (Umkehrphasenchromatographie und HILIC) in Kombination mit MS/MS Detektion. Die hiermit für 27 Metaboliten gewonnenen Daten zeigen Wiederfindungsraten der gesamten Probenvorbereitung von 60 % bis 100 % mit Wiederholpräzisionen von unter 10 %. Weiters wurden diese Daten zur Berechnung der Messunsicherheit herangezogen.

PREFACE

The analytical methods developed during this work imply targeted quantification of selected metabolites in yeast. The methodology can be termed quantitative metabolite profiling, an integral part of metabolomics. In the following section the relevant terms in the field of metabolomics are described including system biology. The brief glossary is followed by an introduction to metabolome analysis of microorganisms with a focus on the challenges of sampling and sample preparation. Finally recent developments in mass spectrometry and separation techniques used in metabolomic studies are explained.

1 INTRODUCTION TO METABOLOMICS

A great leap forward in biological sciences over the past few years was enabled by the fast improvement in analytical procedures and bioinformatics. Comprehensive investigation of living organisms at the molecular level was initialized by sequencing the DNA of whole genomes and was advanced by identification of all RNA molecules present in a cell (the transcriptome) and all proteins expressed by a genome (the proteome). Expansion of this hierarchic ladder proposed the analysis of all metabolites in an organism (metabolomics) because metabolite levels in living cells are regulated by the concentration and activity of enzymes that are themselves dependant on the transcription and translation of genes [1][2][3][4][5][6].

Thus, metabolomics in its original meaning is the comprehensive (qualitative and quantitative) analysis of all metabolites present in the investigated biological system. The very holistic approach demands identification and quantification of the complete set of all low molecular weight (< 1000 Da) metabolites [2][3][5][6]. This ultimate goal has not been reached yet because of the high chemical, physical and structural diversity of metabolites. The spectrum of metabolites comprises inorganic ionic species, small organic compounds like organic acids, alcohols and ketones, the monomeric units of biopolymers like monosaccharides, amino acids and nucleotides as well as hydrophobic lipids. Despite these primary metabolites, which are present in every living cell, a huge number of secundary metabolites – depending on the organism – can be added, resulting in around 1000 metabolites for *Saccharomyces cerevisiae* [7][8], or up to 200,000 metabolites in plants [2][3][9]. Furthermore, a metabolite level is a complex function of many regulatory processes inside cells e.g. regulation of transcription and translation or allosteric regulation of enzymes; it is highly dynamic and dependant to genetic and environmental changes. Summing up, the diversity of analytes makes simultaneous extraction and determination of the complete metabolome impossible and the complexity of a biological system impedes straightforward integration of metabolome data with transcriptome and proteome data.

Alternatively, various analytical strategies have been designed up to now to study the different aspects of the whole metabolism. Therefore the term metabolomics (or metabolome analysis) is considered also as the umbrella term for the different analytical approaches and data evaluation to achieve information about metabolites in a biological system [4][5]. The analytical strategies can be categorized in (1) targeted approaches; a limited number of predefined metabolites are investigated and often quantified, and (2) non targeted approaches; samples are rapidly classified by global metabolite patterns usually without quantification or even identification of individual metabolites. Further classification leads to a metabolomics related terminology which is yet not consistent in literature. Six frequently used expressions for analytical approaches in metabolomics are discussed briefly below:

(1) *Metabolite target analysis* is restricted exclusively to the substrate or the direct product of the corresponded enzyme. Extensive sample cleanup protocols can be implemented in order to improve signal to noise ratio [2][3].

(2) In *Metabolite profiling* (or sometimes *metabolic profiling*), a number of metabolites according to a class of compounds or to their association with a specific pathway are predefined. Sample preparation considers the chemical properties of the target compounds and aims mainly at reduction of matrix effects. Quantification is often included [2][3][5]. Confusingly the term metabolite profiling was also used as the synonym for non targeted approaches [4].

(3) *Metabolic fingerprinting* is a non targeted approach where a large number of intracellular metabolites are screened and metabolite patterns are compared [2][3][5].

(4) *Metabolic footprinting* is a similar approach to metabolic fingerprinting aiming at the extracellular metabolites [2][3][5]. In microbial metabolomics intra- and extracellular metabolites are also called *endometabolome* and *exometabolome* respectively [10].

(5) In *Metabolic flux analysis (MFA)*, enzymatic steady-state reaction rates are integrated in order to describe metabolic flux networks. MFA is usually accomplished by use of ^{13}C tracer experiments [11].

(6) *Metabonomics* is often used for monitoring in biofluids of non-plant systems [6] whereas it is also equivocally used as a synonym for metabolomics [12].

2 QUANTITATIVE METABOLIC PROFILING OF MICROORGANISMS

2.1. Introduction to microbial metabolomics

In industrial biotechnology, sometimes also referred to as white biotechnology, micro-organisms are used for production of a wide range of bio-products such as enzymes, antibiotics, food additives and bulk chemicals. These processes typically start with the cultivation of optimized microbial strains in bioreactors at ideal production conditions followed by the downstream processing where the desired products are separated from the cultivation broth and purified. Great potentials of optimized

biotechnological production processes are the limitation of the consumption of fossil raw materials by the replacement of established chemical production processes or the production of new and high value added products such as enzymes and pharmaceuticals. The employed microbial strains are either obtained from classical strain development based on random mutagenesis and selection procedures (e.g. citric acid from *Aspergillus niger*, penicillin from certain species of *Penicillium*) or from recombinant DNA technology (e.g. insulin from *E. coli*). The production rates and concentrations are always linked to primary metabolism independent if primary or secondary metabolic products are in demand. The primary central carbon metabolism provides precursors, cofactors and biochemical energy in the form of ATP. Hence the understanding of the primary cellular metabolism is required for optimization of biotechnological process in general and engineering of superior microbial cells (metabolic engineering) in particular [10][13].

Quantification of intracellular metabolite levels (metabolite pools), often in connection with data from metabolic flux analysis, help to indentify bottlenecks in the metabolic reaction network, and hence to predict targets for metabolic engineering. Metabolomic studies require microorganisms grown under controlled environment (temperature, pH, medium components and oxygen supply) in order to achieve reproducible results. Often continuous cultivations in a laboratory scale are used because the specific growth rate (μ) can be fixed by defining the dilution rate (D). After a sufficient cultivation time, when "steady state" is assumed, the samples are collected rapidly from the bioreactor and all enzymatic activity is arrested (quenching).

2.2. Sampling and sample preparation

The high reactivity of metabolites inside cells requires particular techniques for sampling and sample preparation, prominently rapid sampling, cell quenching and cell extraction. Rapid sampling of fermentations is achieved by devices which are predominantly in-house developments and not commercially available. The main performance parameters of these constructions are the sampling time and the reproducibility of sampling [14][15]. Rapid sampling is critical for capturing an unbiased *in vivo* snapshot of the metabolite levels of the cells, and the sampling time has to be adapted according to the analyzed metabolites and their enzymatic turnover rates. Turnover times of central carbon metabolism for example are in the range of seconds whereas for amino acids turnover is in the range of minutes. Hence quick filtration without quenching may still be suitable for amino acid analysis [16]. In pulse response experiments, where the medium composition is instantly changed and the dynamics of metabolism is recorded by subsequent sampling of the same culture, rapid sampling and instantaneous quenching is even more critical [17].

After the sampling time, the metabolism is inactivated (quenched) through rapid changes of the temperature or pH. A common technique for quenching biological samples is sampling the culture broth directly into a cold quenching solution, most popular sampling into cold methanol / water

[18][19][20][21][22]. The quenched cells and the supernatant are subsequently separated either through centrifugation or filtration whereat the cell fraction (the cell pellet) is washed, extracted and finally used for the analysis of intracellular metabolites and the supernatant fraction is used for the analysis of extracellular metabolites. This method requires that the metabolites remain in the cells during quenching whereas extracellular metabolites and interfering salts are easily removed from cell surface by washing the cells, often with similar quenching solution. Leakage into the quenching solution was checked for different microorganisms. It was found for bacteria that significant amounts of metabolites leak into cold methanol [23]. In another work leakage was entirely prevented by optimizing cold methanol quenching of *S. cerevisiae* [18].

Techniques where cells and medium are extracted together do not allow their separation, thus a sum of intracellular and extracellular metabolites are analyzed. This so called whole broth analysis has some disadvantages. The addition of extracellular medium often results in a dilution of the metabolite; hence one has to cope with either large sample volumes or low analyte concentrations. Furthermore high salt contents present in the medium, like phosphates or sulphates, may interfere with the applied analysis e.g. ion suppression in electrospray interface [10].

When intracellular metabolites are measured, those metabolites are extracted from cells usually with organic solvents. Polar solvents (water, methanol, ethanol or buffer) are used for extraction of hydrophilic metabolites and non-polar solvents (chloroform, ethylacetate or hexane) are used for extraction of lipophilic components. Extraction of acid- and alkaline- stable metabolites, require acidic or alkaline conditions respectively. Extraction solvents are often used at elevated temperatures whereas thermo labile metabolites require low temperatures. Some extraction protocols include physical principles such as mechanical disruption, freeze thaw, microwave or sonic waves. An often used extraction method that covers a wide range of polar compounds uses boiling ethanol 75 % [18][19][20]. In any extraction method, a compromise has to be made between complete extraction (high extraction efficiency) of as many metabolites as possible, and low physical and chemical modification and low biochemical degradation (high extraction recovery). The addition of proper internal standard prior to the extraction corrects for low extraction recovery. The use of fully ^{13}C labeled cell extracts was introduced in metabolic profiling because internal standardization of any desired metabolite in the proper concentration can be achieved [24][25].

After extraction, intracellular metabolites are often recovered in diluted solutions. Furthermore some chromatographic methods (reversed phase) require organic free samples (e.g. dissolved in initial mobile phase). Solvent evaporation under vacuum and subsequent reconstitution with the desired solvent is a proper solution whereat degradation of the metabolites under the temperatures required for solvent evaporation should be evaluated and – if possible – corrected for. Other options for sample concentration are lyophilization or solid-phase extraction (SPE).

3. INSTRUMENTAL PLATFORMS EMPLOYED IN METABOLOMICS

Mass spectrometry, in combination with a separation method (GC, LC, CE), is nowadays the key technology for quantification of small organic molecules. The ongoing development of efficient and novel separations on the one hand and mass spectrometers with excellent sensitivity or mass resolution on the other hand, offers new opportunities in quantitative metabolomics. The determination and identification of a large number of metabolites in a complex biological sample can be achieved in one single analysis by GC-MS or LC-MS. The frequently used enzyme-based assays for metabolite quantification avoid the use of expensive instruments [26][27], but they are limited to few metabolites per sample and they often lack reliable quantification of low intracellular concentrations; hence their application became less frequent in comprehensive metabolomic studies.

NMR is the other core technology used for metabolomic studies. It exhibits the potential of structural characterization of unknown compounds, however this can be challenging in complex biological samples. NMR is often employed for high throughput fingerprinting, and – utilized as ^{13}C NMR in ^{13}C tracer experiments – for metabolic flux analysis [28]. The benefits of NMR are minimal requirements regarding sample preparation and the character of being non-discriminating and non-destructive. MS on the other hand as key advantages features high sensitivity, selectivity and linear range – therefore it is often used for quantification – but it requires generally extensive sample clean-up. The following chapters will explain in more detail the methodologies of applied separation techniques and mass spectrometers in metabolic studies.

3.1 Separation techniques

Mass analyzers allow a selective detection of many analytes at the same time because of their ability to separate ions according their mass to charge ratio (*m/*z). In high-resolution mass spectrometry it is even possible to distinguish between compounds with the same nominal mass. Nevertheless, the use of proper separation techniques is essential in metabolomics assays, especially when metabolites are to be quantified. Chromatographic or electrophoretic separations in combination with mass spectrometers allow the detection of individual analytes without interference from other analytes or matrix components. Improved limits of detection are the result. The choice, which type/s of gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) is/are finally used in the metabolic study, depends on the initial goal of analysis (targeted / non targeted) and on the metabolite class of interest. Some metabolite classes may be discriminated by the instrumental technique or special sample preparation steps may be required. Hence the whole metabolomics experiment is planned all in one, from sample preparation to detection.

The separation technique with the longest tradition in combining with mass spectrometry based metabolomics is GC because of its high separation efficiency and the easy interfacing of GC with MS.

It is primarily used for thermal stable and volatile compounds, but if chemically derivatized, also for some non-volatile compounds whereas larger and very polar metabolites are excluded. Only a few metabolites are volatile in their native state but a wide range of the metabolome is accessible to GC after silylation or alkylation. Quantitative metabolic profiling via GC-MS was recently achieved for amino acids and organic acids based on chloroformate derivatization [29], for various intermediates of the central carbon metabolites based on trimethyl silyl derivatization [30][31] and for several sugarphosphates of the pentose phosphate pathway based on silylation [32]. Furthermore, GC-MS was used for analysis of ^{13}C labeled sugars in metabolic flux analysis [33]. A maximum of separation efficiency is achieved by combining two GC columns (GC×GC), which is applied in non targeted metabolomics [34].

The combination of HPLC and MS (LC-MS) is the most versatile technique for targeted and non-targeted metabolomics because a wide spectrum of metabolites, from neutral to polar, can be analyzed without a derivatization step. Electrospray ionization (ESI) is most commonly used in LC-MS based metabolomics, making ionic and less polar analytes accessible to MS, whereas other ionization techniques, auch as APCI are rarely used. One main limitation of ESI-MS is the need for compatible eluents. Non-volatile buffer systems and surface active additives should not be used, which impedes the straightforward combination of some separation systems with MS. Reversed phase (RP) LC is compatible with ESI-MS and it is widely used for metabolomic investigations. Because of high separation efficiency and robust retention times, RP-LC is often applied in targeted metabolite quantification [35]. However, conventional RP-LC separations lack in retention of very polar analytes. The addition of an ion-pair reagent into the mobile phase increases the selectivity for polar analytes on RP phases. Ion pair chromatography is a convenient method to analyze acidic, basic and neutral metabolites similarly and is therefore often used in metabolome analysis [36][37]. On the other hand a lower sensitivity is observed and contamination by the ion pair reagent restricts further use of MS to one polarity mode. Ion exchange chromatography is the traditional method for separation of polar and charged analytes but the combination with ESI-MS is often hindered due to non volatile mobile phase components. Nevertheless, the analysis of highly polar glycolytic intermediates was frequently achieved via anion exchange chromatography in combination with ESI-MS as published by Heijnen et al. [18][19][20][24][25][38]. Hydrophilic interaction liquid chromatography (HILIC) is the alternative LC separation method for polar and charged analytes. It is fully compatible with ESI-MS and is therefore often used in metabolic assays. Various HILIC materials exhibiting different selectivities are available today which complicates the choice of the proper separation system. Bajad et al. compared seven different RP and HILIC columns and found an aminopropyl column capable of separating 141 cellular metabolites, including amino acids, nucleotides, carboxylic acids and sugar phosphates [39]. Furthermore a comprehensive cross-platform comparison was performed where ion pair chromatography and HILIC were compared with GC and CE [40]. It was concluded that the LC methods are more suitable for a quantitative approach than GC or CE, due to their robustness and

better coverage of more metabolites. Often, orthogal separation modes are employed separately for the same sample, in order to cover as much metabolites as possible, e.g. RP-LC for less polar and zwitterionic HILIC for polar and ionic analytes [42][43] or ion pair chromatography in negative ionization mode and HILIC in positive ionization mode [41].

A great potential lays in the application of new packing materials, for example sub 2 µm particles, whereupon increased chromatographic resolution provides more significant fingerprints and narrow peaks result in lower detection limits.

CE offers separation of charged metabolites with high resolution. It is fast and it requires little sample volumes. When coupled to MS, the applied electrolytes have to be compatible with CE and MS. The need of volatile buffer systems restricts the spectrum of electrophoretic separations used in combination with MS detection. CE-MS is not often used in metabolomics, however it was used for analysis of anionic and cationic metabolites as well as nucleotides and CoA compounds [44].

Direct injection of the sample into the MS while skipping the separation part is frequently used for high-throughput metabolic fingerprinting. In this case high resolution mass spectrometers (e.g. FT-ICR-MS) are the instruments of choice [45][46].

3.2. Mass spectrometry

Various types of mass spectrometers are currently used in metabolomics. Simply put, they differ on the one hand in the sample introduction system and the ion source, and on the other hand in the implemented mass analyzer. For metabolomics, the most relevant ion sources are ESI and electron impact ionization (EI). ESI, denoted as soft ionization source, is used in connection with LC or CE. Here the liquid sample is nebulised, evaporated and ionized under atmospheric pressure in a strong electric field. Small metabolites are usually ionized to single charged molecule ions, either positively charged or negatively charged depending on the applied polarity. A well known problem in ESI is ion suppression which is ideally alleviated by the use of isotope dilution mass spectrometry (IDMS), e.g. by use of fully ^{13}C labeled metabolites as internal standard [24][25]. EI is used in connection with GC, where the gaseous eluent and analytes are introduced into the ion source. Positive ionization of molecules is achieved under vacuum pressures by use of an electron beam at specific and reproducible energy. A typical fragmentation of the analytes occurs which is reproducible between instruments.

A mass analyzer, as an integral part of a mass spectrometer, provides the separation in time or space, of ions according their mass-to-charge ratio (*m/z*). Criteria for mass analyzers are sensitivity, mass resolution, mass accuracy, scan speed or acquisition rate and MS/MS capability.

A robust, simple to use and relative cheap mass analyzer is the single quadrupole. It can act as mass filter allowing only ions of a certain *m/z* to reach the detector (single ion monitoring, SIM), or as scanning instrument where ions of different *m/z* are detected consecutively and the mass spectrum is obtained (full scan mode). The instrument sensitivity is lower in full scan mode compared to SIM

mode. Generally single quandrupole instruments offer only nominal mass resolution, low mass accuracy and limited sensitivity. They are frequently coupled to GC because the obtained EI spectra can easily be compared with well established databases [29][30].

As comparably reviewed elsewhere [47], the state of the art techniques in metabolomic studies, utilize either selected reaction monitoring MS (SRM-MS/MS) or high resolution MS (HRMS). The former technique employs triple quadrupoles or triple quadrupoles utilizing linear ion trap functionality and is generally used for quantitative approaches whereas the latter tool set addresses fingerprinting by e.g. time of flight or Orbitrap instruments.

In principle triple quadrupole mass spectrometers (QQQ) consist of two quadrupole mass analyzers and one collision cell in between. The collision cell is a quadrupole or multipole filled with low pressure collision gas such as nitrogen, helium or argon. The molecular ions of the analytes (precursors) undergo gas phase fragmentation in the collision cell and the fragment ions are subsequently analyzed. This type of analysis is termed tandem MS or MS/MS or MS^2. A similar measurement strategy is achieved by ion trap instruments where even multiple tandem MS (MS^n) experiments by multiple gas phase fragmentation are possible. The linear quadrupole ion trap (Qtrap) is a hybrid instrument that combines a quadrupole mass analyzer, a collision cell and a linear ion trap (LIT). Qtrap and QQQ instruments allow comparable analysis strategies. They can be run in selected reaction monitoring or multiple reaction monitoring (SRM, MRM) respectively. Here, the third mass filter is set on distinct fragments of certain precursors and not scanned through the fragment pattern. SRM methods provide low limits of detection, high dynamic ranges and increased selectivity in quantification studies. Accordingly, QQQ and Qtrap instruments are ideal for targeted and quantitative metabolomics. E.g. a QQQ instrument was used for quantification of glycolytic intermediates in yeasts [19][25][38], and a Qtrap instrument was used for quantification of intracellular metabolites in fermentation broths from antibiotics production [42][43]. Modern QQQ instruments with faster scan rates can achieve quantification of up to hundreds of metabolites in one chromatographic run. Because triple quadrupole and quadrupole ion trap mass spectrometers offer only nominal mass resolution they are generally not employed for untargeted global metabolomics. However, beside accurate and sensitive quantification, these instruments offer additional tools for metabolic studies. Product ion scanning reveals the specific fragment spectrum of a precursor ion and MS^n provides detailed fragmentation information which is used for metabolite identity confirmation. Tandem MS methods are also applied to screen for metabolites exhibiting a common structural motif. For example, a Qtrap instrument in this mode was used to screen for glucuronide metabolites as potential biomarkers [48].

Time of flight (TOF) mass analyzers determine *m/z* by measurement of the time the ions need to pass the distance to the detector. TOF instruments provide high mass resolution and mass accuracy that allows an estimation of molecular formula by accurate mass determination but they provide comparably low dynamic range for quantification. In contrast to quadrupole mass analyzers all ions are detected simultaneously and are not scanned. Because TOF instruments exhibit high acquisition

rates they are ideal detectors for high resolution chromatography, thus GC-TOF-MS systems for example are used for fast metabolite detection [34][49]. A hybrid instrument which employs quadrupole and TOF mass analyzers as well as a collision cell is the quadrupole-time-of-flight MS (Q-TOF). It can be operated only as TOF instrument or as tandem mass spectrometer where a precursor ion is isolated by a quadrupole mass analyzer, fragmented in the collision cell and the fragment spectrum is acquired in the TOF mass analyzer. A Q-TOF instrument combines the high mass resolution of a TOF instrument with the MS/MS capability and low chemical noise of triple quadrupole instument.

A major technological breakthrough was the introduction of Orbitrap hybrid instruments [50][51]. Orbitraps are very versatile instruments providing a high level of mass accuracy and mass resolution as well as MS^n capability. The highly detailed fragmentation information obtained is used for metabolite identity confirmation. TOF, Q-TOF an Orbitrap instruments are high resolution mass spectrometers and are therefore dedicated to untargeted analysis and unknown screening [52][53][54]. However their application in quantitative metabolomics was also recently reported [47].

In the course of this Ph.D work novel methods for quantitative metabolite profiling of yeast and yeast products by LC-MS have been developed, which resulted in two scientific papers presented in the following. Method development included comprehensive sample preparation procedures for yeast products as well as for living yeast cells from the bioreactor. Moreover, LC separations (reversed phase LC, HILIC) were developed to cover a wide range of small polar metabolites (nucleotides, nucleosides, nucleobases, amino acids, organic acids and vitamins). The quantification strategies included SRM-MS/MS methods employing a triple quadrupole mass spectrometer and isotope dilution mass spectrometry employing isotopically labeled metabolites as internal standards. In publication II an in-house produced fully labeled ^{13}C yeast cell extract was characterized and proved to serve as ideal internal standard. It was furthermore implemented as a tool to evaluate the recoveries and repeatability precisions of the cell extraction and the extract treatment. Calculation of measurement uncertainty allowed establishing the main components of uncertainty contributors in this complex quantitative task.

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PUBLICATIONS

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ORIGINAL PAPER

Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases—application to feed supplements

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Abstract In this work, accurate MS-based methods for quantitative profiling of nucleotides, nucleosides, and nucleobases in yeast extracts used as additives in animal feedstuff are presented. Reversed-phase chromatography utilizing a stationary phase compatible with 100 % aqueous mobile phases resulted in superior analytical figures of merit than HILIC or ion-pair reversed-phase separation. The novel separation method was combined with both molecular and elemental mass spectrometry. By use of RP–LC–MS–MS, excellent limits of detection <1 μmol L^{-1} could be obtained for all the compounds investigated. The elemental speciation analysis approach enabled determination of nucleotides by phosphorus detection. Sensitivity of LC–ICP–MS was 1–2 orders of magnitude lower than that of LC–MS–MS. Quantitative analysis of yeast products using complementary MS detection furnished values in good agreement.

Keywords Nucleotide · Nucleoside · Nucleobase · LC–MS–MS · LC–ICP–MS · Feed supplement

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Abbreviations

ACN	Acetonitrile
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
As	Adenosine
ATP	Adenosine triphosphate
Cd	Cytidine
CE	Capillary electrophoresis
CMP	Cytidine monophosphate
DNA	Deoxyribonucleic acid
DRC-QMS	Dynamic reaction cell–quadrupole mass spectrometry
ESI	Electrospray ionization
FV	Fragmentor voltage
GMP	Guanosine monophosphate
Gs	Guanosine
HILIC	Hydrophilic-interaction liquid chromatography
HPLC	High-performance liquid chromatography
IC	Ion chromatography
ICP	Inductively coupled plasma
IMP	Inosine monophosphate
IP	Ion pair

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IS	Internal standard
Is	Inosine
k′	Capacity factor
LC	Liquid chromatography
LOD	Limit of detection
MeOH	Methanol
MRM	Multiple reaction monitoring
MS-MS	Tandem mass spectrometry
N	Number of theoretical plates
NDP	Nucleoside diphosphate
NMP	Nucleoside monophosphate
NTP	Nucleoside triphosphate
RP	Reversed phase
tR	Retention time
Ud	Uridine
UMP	Uridine monophosphate

Introduction

Nucleotide-enriched yeast products, used as flavour enhancers in foods and beverages (high concentrations of 5′GMP, and 5′ IMP) [1–3] are currently used for their beneficial effects as feed supplements. Feed supplementation was mainly proposed because of reported evidence of the effect of nucleotides on the metabolism of fatty acids and the contribution of nucleotides to iron absorption in the gut [1, 4].

The production of nucleotide-enriched yeast is based either on chemical treatment or, more commonly, on autolysis. Yeast cells contain a range of different nucleases, and autolytic degradation of polynucleotides—basically RNA—yields different isomers of nucleotides, and nucleosides and nucleobases. As a consequence, optimization of nucleotide enrichment and characterization of the yeast products requires quantitative methods including not only nucleotides, but also nucleosides and nucleobases. More specifically, the metabolites of interest are the ribonucleotides CMP, UMP, AMP and GMP—the breakdown products of RNA—and IMP, and the corresponding nucleobases and nucleosides. In the past, different nucleotide patterns have been found in yeast autolysates depending on yeast strain or autolysis conditions [1, 5]. Moreover, yeast autolysis resulted not only in the well-known 5′ NMPs but also in isomers phosphorylated at other positions (3′ NMPs and 2′ NMPs) [1, 5].

Table S1 (Electronic Supplementary Material) gives an overview of the state of the art of chromatography-based bioanalytical methods and applications for nucleotide, nucleobase, and nucleoside quantification [1–32]. Alternative techniques include enzymatic assay [6] or separation by capillary electrophoresis and capillary electrochromatography [7]. A comprehensive overview of recent liquid chromatographic methods for determination of endogenous nucleotides in cancer therapy was recently published [8]. Since the advent of mass spectrometry, its combination with chromatography has developed into an important technique for quantitative profiling of metabolites. Accordingly, the chromatographic separations initially developed for UV detection [1, 4, 9–11], for example ion chromatography or reversed-phase chromatography, utilizing non-volatile, saline buffers, have been replaced by separation methods compatible with electrospray ionization. As can be readily observed, the predominant separation principle used for nucleotide analysis in combination with MS detection was, and still is, ion-pair chromatography. Nucleotides—comprising phosphate esters—are negatively charged in a wide pH range. Accordingly, tetraalkylammonium or trialkylammonium salts could be used as cationic ion-pair reagents, especially in combination with UV detection. Different volatile ion-pair reagents, for example dibutylammonium formate [12], dihexylammonium acetate [2], triethylamine [3], *N*,*N*-dimethylhexylamine [13], and dibutylamine [14], have been used for MS detection. As a general drawback, the combination of IP-RP-LC with ESI–MS, resulted in adverse effects, for example limited robustness regarding the stability of retention times. Moreover, compromised sensitivity by ion suppression and contamination is often regarded as a limitation of this technique. The parallel use of negative and positive ionization for optimum detection of nucleotides, nucleosides, and nucleobases is not possible, because of suppression/interference by the introduced counter ion. However (also reflected in Table S1), few studies have focused on alternative modes of chromatographic separation. As a matter of fact, ion-pair chromatography is unrivalled in terms of separation efficiency, and reversed-phase chromatography, the most common separation method combined with ESI–MS was limited to the analysis of nucleobases and nucleosides [15]. Alternatively, HILIC [16] or adsorption chromatography utilizing porous graphitic carbon as stationary phase [17, 18] could be applied to nucleotides, nucleosides, and nucleobases. Preinerstorfer et al. showed the potential of HILIC in a comprehensive metabolomic study using HILIC at two different pHs [16]. In adsorption chromatography high buffer concentrations up to 100 mmol L^{-1} ammonium acetate were needed for elution of nucleotides. This, again, limited the compatibility of the separation method with electrospray ionization techniques.

Table S1 also lists recent elemental speciation methods based on ICP–MS. Evidently, this type of analysis was limited to phosphorus-containing nucleotides and was only rarely applied. The studies concerned extracted DNA samples, with the objective of quantification of deoxy nucleotides dCMP, dTMP, dAMP, and dGMP. ICP–MS was combined with μHPLC and capillary electrophoresis [19–21]. Previous LC–ICP–MS studies had focused on

analysis of DNA adducts [22, 23] or use of the octapole collision cell for interference-free measurement of phosphorus by CE–ICP–MS and LC–ICP–MS applied to calf thymus DNA [24]. Pioneering work in 1999 utilized complementary LC–ICP–MS and LC–ESI–MS for quantification of DNA adducts [25].

In this work, quantitative profiling of nucleotides, nucleobases and nucleosides in nucleotide-enriched yeast extracts used as additives in animal feedstuffs was addressed by use of LC–MS–MS. Recent developments in RP phases, for example materials with lower ligand density tolerating highly aqueous conditions, offered unique selectivity for analysis of these polar compounds compared with conventional C_{18} phases. Hence, as a novelty, reversed-phase separation of nucleotides, nucleosides, and nucleobases could be obtained. Analytical figures of merit of RP–LC–MS–MS are discussed and compared with those of ion-pair chromatography–MS–MS and HILIC–MS–MS. Moreover, because of the absolute lack of reference materials, a reference method for nucleotide quantification was implemented. The reversed-phase separation was combined with complementary ICP–MS detection. For the first time, LC–ICP–MS has been used for quantification of free nucleotides in yeast products.

Experimental

Chemicals and standards

Acetonitrile and methanol of LC–MS-grade were purchased from Fisher Scientific (Loughborough, UK). Water was filtered and deionized in two steps. After reverse osmosis, filtration, and UV treatment as the first step, the electrical conductivity of the water was <0.2 μS cm. The second step was use of a high-purification system from Millipore (Billerica, USA), which furnished water with electrical resistance >18 MΩ. Suprapur formic acid (98–100 %, *v*/*v*) and glacial acetic acid (100 %) from Merck (Darmstadt, Germany) and tributylamine (p.a. ≥99.5 %) from Fluka (Vienna, Austria) were used for chromatography. Sodium chloride, tris (hydroxymethyl)aminomethane (Tris), and hydrochloric acid (30 % Ultrapur), used for sample preparation, were purchased from Merck (Vienna, Austria).

Cytosine, uracil, adenine, guanine, cytidine, uridine, adenosine, guanosine, inosine, 5′CMP (cytidine 5′-monophosphate disodium salt), 5′UMP (uridine 5′-monophosphate disodium salt), 5′AMP (adenosine 5′-monophosphate sodium salt), 3′AMP (adenosine 3′-monophosphoric acid), 2′AMP (adenosine 2′-monophosphate), 5′GMP (guanosine 5′-monophosphate disodium salt hydrate), 2′GMP and 3′ GMP (guanosine 2′ and 3′-monophosphate, mixed isomers 63:36, supplied as the sodium salts), and 5′IMP (inosine 5′-monophosphate disodium salt) were purchased from Sigma or Fluka (Vienna, Austria). Adenosine $^{15}N_5$5′AMP ($^{15}N_5$ 5′-monophosphate, supplied as the sodium salt) and $^{15}N^{13}C_2$ uracil from Euriso-top (Saint-Aubin Cedex, France) were used as internal standards. Each substance (0.5 to 2.0 mg) was weighed and 1 mL HPLC-grade water was added (for guanine, guanosine, 3′AMP and 2′AMP 1 mL 0.1 mol L^{-1} NaOH was added instead of water) resulting in 5 mmol L^{-1} stock solutions. Further dilution was performed with pipettes with disposable tips from Eppendorf (Vienna, Austria). The final dilution step was conducted by diluting with water. Stock solutions were kept at −20 °C.

Sample preparation

The samples investigated were provided by the Biomin research center (Tulln, Austria). These preparations were from a variety of companies; most are used as additives in animal feedstuff. Dry powdered feed supplement (50 mg) was dissolved in 50 mL saline buffer (150 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris adjusted to pH 7.4 with hydrochloric acid) and treated ultrasonically at room temperature for 15 min. Centrifugal filtration was conducted using Amicon Ultra filter units (0.5 mL, 10 kDa cut off, cat no: UFC501096) from Millipore and the MiniSpin plus micro centrifuge from Eppendorf (Hamburg, Germany). Filter units were loaded with 0.5 mL sample solution and centrifuged for 5 min at 14,100 rcf. For LC–ICP–MS analysis, the filtrate was diluted by a factor of 10, and five standard additions were prepared, resulting in additional concentrations of 1, 2, 5, 10, and 20 μmol L^{-1} of 5′NMPs. For LC–MS–MS analysis $^{15}N_5$ 5′ AMP and $^{15}N^{13}C_2$ uracil were added to each sample as internal standards before filtration, resulting in final concentrations of 2 μmol L^{-1}, and samples were diluted by a factor of 100 after filtration. For standard addition experiments with LC–MS–MS four spikes were prepared resulting in additional concentrations of 0.5, 1, 2, and 5 μmol L^{-1} NMPs.

Chromatography

HILIC–MS–MS was performed with a ZicHILIC separation column (150 mm×4.6 mm, 3.5 μm particle size, 100 Å pore size) from SeQuant (Marl, Germany) and a ZicHILIC guard column (20 mm×2.1 mm, 5 μm particle size). The mobile phase was a gradient prepared from water–ACN–formic acid 98:1:1 (%*v*/v) (eluent A) and ACN–water–formic acid 98:1:1 (%*v*/v) (eluent B). Mobile phase composition was held constant at 90 % B for 2 min, then B was reduced to 10 % within 7 min and was held for 1 min. Subsequent reconstitution of the starting conditions within 0.1 min and re-equilibration with 90 % B for 9.9 min resulted in a total analysis time of 20 min. The flow rate was 0.6 mL min^{-1}, the injection volume 3 μL, and the column temperature 40 °C.

IP-RP-LC–MS–MS was performed as published elsewhere [26]. In brief a Synergi Hydro-RP column (150 mm × 2.0 mm, 3 μm particle size, 100 Å pore size) from Phenomenex (Aschaffenburg, Germany) was used. The mobile phase was a gradient prepared from an aqueous 10 mmol L^{-1} solution of tributylamine, pH adjusted to 4.95 with acetic acid (eluent A) and methanol (eluent B). Mobile phase composition was held constant at 0 % B for 5 min, then B was increased to 10 % within 15 min and was held for 2 min. Subsequent reconstitution of the starting conditions within 0.1 min and re-equilibration with 0 % B for 12.9 min resulted in a total analysis time of 35 min. The flow rate was 0.2 mL min^{-1}, the injection volume 3 μL, and the column temperature 40 °C.

RP-LC–MS–MS and RP-LC–ICP–MS were performed with an Atlantis T3 C_{18} reversed-phase column (150 mm × 4.6 mm, 3 μm particle size) from Waters (Milford, USA) with an Atlantis T3 guard column (20 mm × 4.6 mm, 3 μm particle size). The mobile phase was a gradient prepared from water containing 0.1 % (*v*/*v*) formic acid (eluent A) and methanol (eluent B). The gradient used for LC–MS–MS was: 0 % B held constant for 2 min, then increased to 40 % within 8 min and held for 2 min. Subsequent increase to 100 % within 0.1 min and held for 1.9 min to flush the column, subsequent reconstitution of the starting conditions within 0.1 min, and re-equilibration with 0 % B for 5.9 min resulted in a total analysis time of 20 min. The gradient used for LC–ICP–MS was: 0 % B held constant for 2 min, then increased to 40 % within 8 min and held for 2 min. Subsequent reconstitution of the starting conditions within 0.1 min and re-equilibration with 0 % B for 7.9 min resulted in a total analysis time of 20 min. For both detection methods the flow rate was 1.0 mL min^{-1}, the injection volume 12.5 μL, and the column temperature 40 °C.

LC–MS–MS system

Agilent Technologies G1312A series 1200 binary pump, G1367B high-performance autosampler, and G1316A column compartment were used for HPLC. MS detection was performed with an Agilent Technologies 6410 LC–MS–MS featuring an ESI interface. Source conditions in negative and positive-ionization modes for IP-RP-LC and HILIC were: drying gas temperature 300 °C, drying gas flow 10 L min^{-1}, nebulizer pressure 25 psi, and capillary potential 4000 V. Source conditions in negative and positive-ionization modes for RP-LC using the Atlantis T3 were: drying gas temperature 350 °C, drying gas flow 10 L min^{-1}, nebulizer pressure 50 psi, and capillary potential 4000 V. MRM transitions of four nucleobases, five ribonucleosides and nine ribonucleoside monophosphates were determined by flow injection of 20 μmol L^{-1} single standards (isocratic conditions: 0.05 % formic acid in MeOH–H_2O 50:50 *v*/*v*). For optimization, Mass Hunter Optimizer Software from Agilent was used in four steps, in the order:

1. optimization of isolation of the selected precursor ion by varying the fragmentor voltage;
2. determination of the four most abundant product ions;
3. optimization of the collision energies for each of these product ions; and
4. determination of the exact *m*/*z* value of the product ions.

The transition with the highest signal-to-noise ratio was chosen for quantification. For some cases the transition with the second highest signal-to-noise ratio was chosen for identification (qualifier). Precursor and product ions, and the specific values of the fragmentor voltage and collision energy are listed in Table S2 in the Electronic Supplementary Material.

Phosphorus speciation via LC–ICP–DRC-QMS

For speciation analysis a metal-free chromatographic system consisting of an AS 50 autosampler (including a custom-made temperature-control device) and an ICS-3000DP dual pump system, all from Dionex (Sunnyvale, CA, USA), was used. Chromatographic separation was performed on an Atlantis T3 C_{18} reversed-phase column by using the water–methanol gradient described above. HPLC was connected to the ICP-QMS (Elan 6100 DRC II; Perkin Elmer Sciex, Ontario, Canada) The Meinhard nebulizer (Elemental Scientific, Omaha, NE, USA) with the cyclonic spray chamber (PE-SCIEX) were used as the sample-introduction system. Phosphorus was measured as PO^+ at *m*/*z*=47 by use of the dynamic reaction cell (DRC) technique with oxygen at a flow rate of 0.8 mL min^{-1}. Finally, a downscaling experiment with 2.1 and 1 mm Atlantis T3 columns and the PFA nebulizer (Elemental Scientific) was used for accommodating HPLC flow rates of 50 μL min^{-1} and 200 μL min^{-1}.

Data evaluation

Generation and export of HPLC–ICP–QMS chromatograms was performed by use of Chromlink (Version 2.1; PE Sciex) in combination with Totalchrom (Version 6.2, PE-Sciex). Chromeleon software (Version 6.7; Dionex, Sunnyvale, CA, USA) was used for integration of all chromatographic data from ICP–MS detection. Agilent LC–ESI–MS–MS data were evaluated by use of Agilent Mass Hunter Qualitative and Quantitative Analysis software modules.

Results and discussion

Chromatographic separation

Chromatographic separation of nucleotides, nucleobases, and nucleosides was studied for analysis of yeast-derived samples which have been processed to enrich nucleotides. The major

objective was the development of a novel separation method suitable for complementary combination with molecular and elemental mass spectrometry, avoiding use of ion-pair reagents. In the past, ion-pair reversed-phase chromatography was the method of choice for analysis of polar (and charged) nucleotides by LC–MS. However, a severe drawback of the use of ion-pair reagents is the impaired sensitivity of LC–MS instrumentation for other applications, because of contamination. In a first step, two orthogonal chromatographic separation methods were compared with ion-pair chromatography using tributylammonium acetate as ion-pair reagent: HILIC using a zwitterionic phase and a novel reversed-phase separation using a 100 % wettable C_{18} phase were tested in combination with MS–MS-based detection. Retention times and capacity factors of the three implemented separations principles are listed in Table 1. As can be readily observed, the ion-pair RP-LC separation resulted in the expected good separation efficiency and capacity factors ranging from approximately 1 to 10. However, for some analytes (adenine, NMPs) peak tailing was observed. By use of the Atlantis T3 column for reversed-phase separation, not only could ion-pair reagents be avoided, but also separation time could be significantly reduced. By adopting a linear water–methanol gradient with initial conditions of 100 % aqueous mobile phase, an excellent separation could be achieved, increasing the number of compounds studied from 14 to 18 (compare Table 2 with Table 3, and Figs. 1 and 2). As an outstanding feature, excellent separation of the 5′, 3′, and 2′ isomers of nucleotides could be achieved. The isomers of AMP eluted after 6.2, 7.3, and 8.2 min and the isomers of GMP eluted after 6.5, 8.1, and 9.2 min. The isomers of CMP and UMP were not included in the analysis because standards were not available. Nevertheless, in analysis of samples of nucleotide-enriched yeast we found signals corresponding to isomers of CMP (t_R=3.7, 6.0, and 6.2 min) and isomers of UMP (t_R=5.9, 7.3, and 7.6 min). The separation efficiency of HILIC was, however, relatively poor, because all the compounds investigated eluted in the time window from 7.3 to 9.1 min.

In this specific application, separation of nucleosides from corresponding nucleobases and nucleotides is a crucial criterion. Because of in-source fragmentation, a phenomenon which often occurs in electrospray ionization, ESI–MS based unambiguous detection and, consequently, accurate quantification could be hampered. More specifically, in-source fragments of a nucleoside could simulate the respective nucleobase and in-source fragments of nucleotides could simulate the corresponding nucleosides, if not chromatographically separated. This criterion is met by ion-pair chromatography and reversed-phase chromatography. However, using HILIC, adenine and adenosine were not separated to baseline and the two compounds uracil and cytosine could not be detected.

As a matter of fact, compared with the other separations studied, RP chromatography was also superior in respect of the sensitivity obtained, which ultimately resulted in superior signal-to-noise ratios. Indeed, significantly lower LODs by 1–2 orders of magnitude were obtained compared with the other two methods investigated after MS optimization. Finally, the water–methanol gradient used in RP-LC enables straightforward combination with both ESI–MS and ICP–MS detection.

Optimization of ESI–MS-MS detection in combination with reversed-phase chromatography

In principle both, negative and positive ESI ionization modes with corresponding MS-MS transitions could be used for nucleobases, nucleosides, and nucleotides (cytosine and

Table 1 Comparison of three separation methods for nucleobases, nucleosides, and nucleotides

Compound	t_R (min)			k'		
	ZicHILIC	Ion pair	RP (Atlantis)	ZicHILIC	Ion pair	RP (Atlantis)
Cytosine	–	1.5	2.1	–	0.07	0.21
Uracil	–	2.5	5.0	–	0,79	1.83
Adenine	8.3	5.5	4.1	1.86	2.93	1.33
Guanine	8.6	3.1	4.5	1.97	1.21	1.56
Cytidine	8.9	2.6	4.3	2.07	0.86	1.47
Uridine	7.0	3.6	7.2	1.41	1.57	3.09
Adenosine	8.2	12.4	7.6	1.83	7.86	3.36
Inosine	7.3	6.7	8.2	1.52	3.79	3.66
Guanosine	8.0	8.5	8.2	1.76	5.07	3.66
5′CMP	9.1	14.2	3.7	2.14	9.14	1.10
5′UMP	8.2	15.2	5.9	1.83	9.86	2.40
5′AMP	8.7	16.1	6.2	2.00	10.50	2.57
5′IMP	8.3	15.5	7.0	1.86	10.07	3.01
5′GMP	8.8	15.5	6.8	2.03	10.07	2.87

Table 2 Analytical figures of merit for RPLC–MS–MS and RPLC–ICP–MS method development for separation and detection of nucleobases, nucleosides, and nucleotides in aqueous solution

Compound	t_R (min)		k'		N		S/N[a]		LOD (nmol L^{-1})		LOQ (nmol L^{-1})		Precision of LC–MS–MS, RSD (%; n=6)	
	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS
Cytosine	2.1	–	0.21	–	2260	–	11900	–	0.30	–	1.0	–	13	–
Uracil	5.0	–	1.83	–	8750	–	133	–	38	–	130	–	15	–
Adenine	4.1	–	1.33	–	4970	–	3030	–	1.4	–	4.7	–	15	–
Guanine	4.5	–	1.56	–	6240	–	4990	–	0.87	–	2.9	–	8	–
Cytidine	4.3	–	1.47	–	5650	–	48690	–	0.082	–	0.27	–	5	–
Uridine	7.2	–	3.09	–	44180	–	7180	–	0.67	–	2.2	–	3	–
Adenosine	7.6	–	3.36	–	45230	–	19060	–	0.17	–	0.56	–	8	–
Inosine	8.2	–	3.66	–	49670	–	9360	–	0.33	–	1.1	–	6	–
Guanosine	8.2	–	3.66	–	52680	–	20020	–	0.20	–	0.67	–	7	–
5′CMP	3.7	3.2	1.10	0.92	4790	2890	1800	30	2.6	170	8.7	550	3	4
5′UMP	5.9	5.1	2.40	2.08	18110	5030	5750	21	0.94	240	3.1	790	3	5
5′AMP	6.2	6.0	2.57	2.57	31680	15760	5070	81	0.93	61	3.1	200	2	6
3′AMP	7.3	7.1	3.18	3.26	42840	43400	12450	73	0.39	68	1.3	230	2	5
2′AMP	8.2	8.0	3.69	3.78	54330	57480	16910	101	0.28	49	0.93	160	3	5
5′IMP	7.0	6.7	3.01	3.02	33610	24070	2880	44	1.8	110	5.9	370	2	5
5′GMP	6.8	6.5	2.87	2.89	37370	25490	3980	52	1.3	96	4.3	320	3	6
3′GMP	8.1	7.7	3.60	3.61	43400	41840	3410	25	0.98	130	3.3	430	3	6
2′GMP	9.2	8.7	4.24	4.22	53330	41100	2400	8	0.85	220	2.8	730	4	5

[a] Signal-to-noise ratio from 10 μmol L^{-1} standards except 3′GMP (6.3 μmol L^{-1}) and 2′GMP (3.6 μmol L^{-1})

uracil transitions were found in positive mode only and 5′ IMP transitions in negative mode only). However, optimum sensitivity was obtained by use of the positive-ionization mode for nucleobases and nucleosides, and the negative mode for nucleotides, resulting two methods. In both methods, the optimum transitions of quantifier and qualifier ions of analytes and internal standards were segmented into three time intervals, as illustrated in Figs. 1 and 2. Table S2 in the Electronic Supplementary Material summarizes the optimized measurement routine used in this study. With regard to MRM settings, for all nine nucleobases and nucleosides the selected precursor ions corresponded to $[M+H]^+$ ions. Product ions for nucleoside measurement corresponded to the entire or fragmented nucleobase. For the nucleotides, all selected precursor ions corresponded to $[M-H]^-$ ions. Selected product ions were derived from the nucleobase, from the phosphate (m/z=79 or 97), or from the ribose phosphate (m/z=211). Quantifier and qualifier fragments could be implemented for most of the compounds investigated. Moreover, for internal standardization, isotopically labelled $^{15}N^{13}C_2$ uracil and $^{15}N_5$ 5′AMP were included in the MS method as given in Table S2. Dwell times were optimized, depending on the number of concurrent transitions per time segment required to achieve 15 to 30 time points per peak. Figs. 1 and 2 show chromatograms resulting from optimized RP–LC–MS–MS determination of a standard mixture.

Table 3 Downscaling of normal bore RP–LC–ICP–DRC–MS to microbore RP–LC–ICP–DRC–MS

Compound	Column diameter	Flow (μL min^{-1})	S/N	LOD (nmol L^{-1})	LOQ (nmol L^{-1})
AMP	1	50	6	1683	5612
	2.1	200	35	310	1032
	4.6	1000	81	61	203
CMP	1	50	2	4832	16106
	2.1	200	8	1240	4134
	4.6	1000	30	170	567

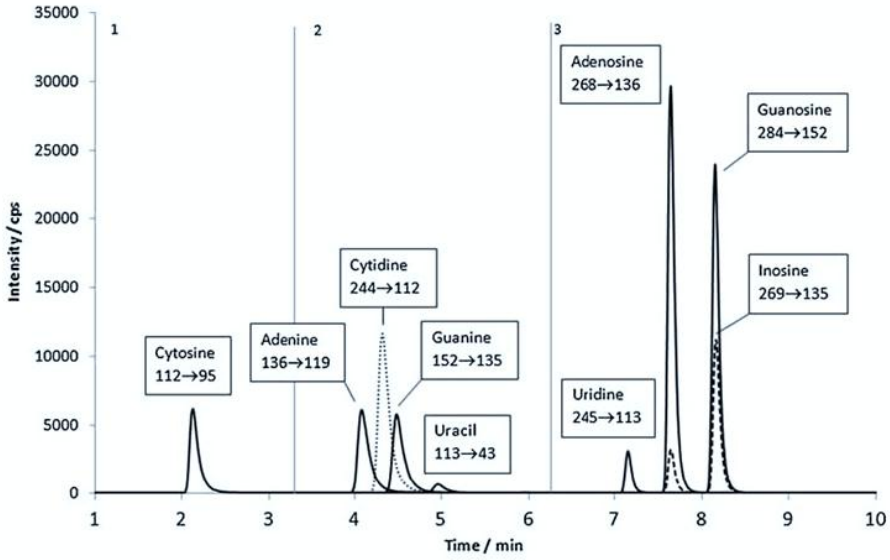

Fig. 1 Extracted chromatograms of nine MRM transitions obtained by RPLC–MS–MS in positive-ionization mode from aqueous standard mixtures of four nucleobases and five nucleosides. Concentrations are 10 μmol L^{-1} for uracil, 1 μmol L^{-1} for adenosine, and 2 μmol L^{-1} for the other substances

As already mentioned above, interferences arising from in-source fragments were avoided by chromatographic separation. In nearly all cases, interferences from compounds differing in one mass unit and with analogous quantifier ions could also be avoided by reversed-phase chromatographic separation. In these cases, the isotopologue M+1 of the lighter analyte could simulate the heavier analyte. A number of pairs of analytes differ by one mass unit cytosine and uracil, cytidine and uridine, adenosine and inosine, CMP and UMP, and AMP and IMP. After reversed-phase separation, only one problematic case was left. At high concentrations of 3′AMP, the separation between the 3′AMP and 5′ IMP was not sufficient, as can be observed in Fig. 2. As a consequence, the isotopologue M+1 of 3′AMP (m/z=347, t_R=7.3) simulated 5′IMP (m/z=347, t_R=7.0) having the corresponding transition 347→79. Moreover, two nucleosides, i.e. inosine and guanosine, were not separated by the developed reversed-phase chromatographic method (Fig. 1). However, in this case, specific fragments enabled interference-free detection and accurate quantification.

Excellent signal-to-noise ratios could be obtained, resulting in LODs in the sub-nmol L^{-1} range for all the investigated nucleosides, nucleobases, and nucleotides summarized in Table 2. More specifically, for nucleobases (measured in positive-ionization mode) LODs were between 0.3 and 1.4 nmol L^{-1} with the exception of uracil (LOD 40 nmol L^{-1}). LODs between 0.08 and 0.7 nmol L^{-1} and between 0.3 and 2.6 nmol L^{-1} were obtained for nucleosides (measured in positive-ionization mode) and nucleotides (in negative-ionization mode), respectively.

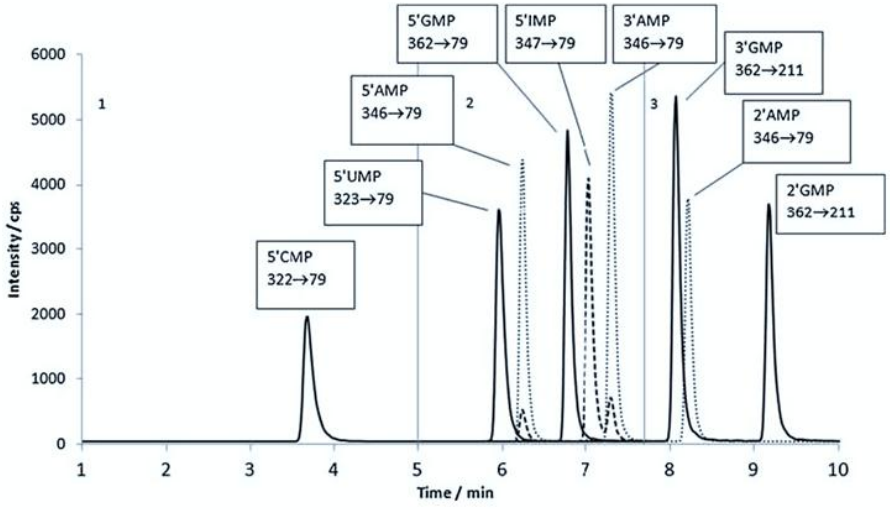

Fig. 2 Extracted chromatograms of six MRM transitions obtained by RPLC–MS–MS in negative-ionization mode from nine nucleotides in a 2 μmol L^{-1} aqueous standard mixture (except 3′GMP 1.26 μmol L^{-1} and 2′GMP 0.72 μmol L^{-1})

Reversed-phase chromatography and detection by ICP MS

No reference materials are available for this type of bioanalytical application. Therefore, for a subset of target compounds a complementary measurement method was implemented. The method entailed quantification of nucleotides containing the hetero-element phosphorus by LC ICP MS. Figure 3 presents the LC ICP MS chromatogram of nine nucleotides in a 10 µmol L^{-1} aqueous standard mixture. The retention times, corresponding capacity factors, and theoretical number of plates (*N*) of the investigated nine nucleotide standards are in agreement with those obtained by LC MS MS (Table 2), taking into consideration the extra-column conditions of the different HPLC systems used (i.e. dead volume of HPLC, capillary length, and interface dead volume). For all peaks, resolution, calculated in accordance with the European Pharmacopeia, was >1.60.

Phosphorus detection was achieved by applying ICP MS with the dynamic reaction cell technique. Thus, phosphorus was measured via the $^{31}P^{16}O^{+}$ molecular ion. As shown elsewhere, the sensitivity achieved in dynamic reaction cell mode is comparable with that of ^{31}P measurement in the standard mode of the quadrupole ICP MS [27]. Detection by ICP DRC MS was preferred in this study to tackle spectral interferences inherent to N-containing analytes, for example $(^{15}N^{16}O)^{+}$ or $(^{14}N^{16}O^{1}H)^{+}$. The application of sector-field mass spectrometry would be beneficial in terms of sensitivity. Becker et al. reported off line LOD of 0.02 µg P L^{-1} when applying mass resolution R=4,000 [28]; this exceed the LODs routinely obtained by ICP DRC MS by a factor of 10 20. One disadvantage of combining sector-field MS with reversed-phase chromatography was lower robustness and more severe matrix suppression effects than for the combination of quadrupole ICP MS and reversed-phase LC. In this work, as is apparent from Table 2, comparable signal-to-noise ratios were obtained for all the nucleotides investigated, despite the fact that gradient elution was used, because signal suppression was compensated by superior peak shape of the late eluting compounds. The limits of detection were approximately 100 nmol L^{-1}, 2 3 orders of magnitude higher than in LC MS MS. As a consequence, fundamental experiments were performed to address the question of whether sensitivity could be improved by down-scaling narrow-bore LC ICP DRC MS to capillary LC ICP DRC MS. A previous study showed that sensitivity loss because of matrix suppression effects exerted by organic solvents could be compensated to some extent by down-scaling LC, i.e. reducing the amount of HPLC eluent introduced to the plasma [29]. Hence, reversed-phase separation of two selected lead compounds was combined to ICP DRC MS using different column diameters, i.e. 4.6 mm, 2.1 mm, and 1 mm, with the respectively down-scaled LC flow rates and injection volumes. Table 3 summarizes the *S/N* ratios obtained and resulting limits of detection for 5′AMP and 5′CMP. No sensitivity improvement could be achieved for either substance eluting under different conditions.

Quantitative study in nucleotide enriched yeast

Finally, both methods LC MS MS and LC ICP MS were applied to a quantitative study of nucleotide-enriched yeast products. Because the aqueous solubility of nucleotides and related substances is high, sample treatment was straightforward and included dilution in saline buffer followed by

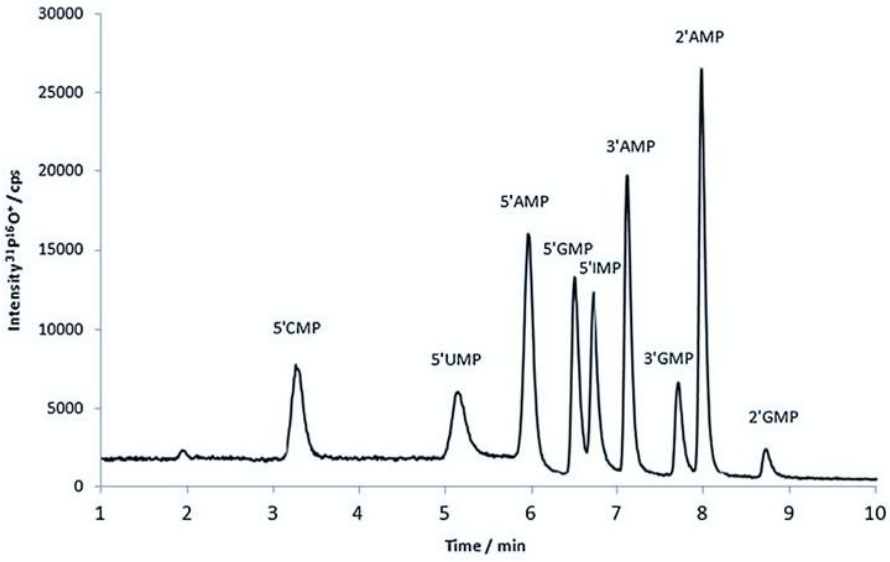

Fig. 3 Extracted chromatogram of PO^{+} at *m/z*=47 obtained by RPLC–ICP–DRC–MS of nine nucleotides in a 10 µmol L^{-1} aqueous standard mixture (except 3′GMP 6.3 µmol L^{-1} and 2′GMP 3.6 µmol L^{-1})

Table 4 Concentrations (μmol g_{DW}^{-1}) of nucleotides in one sample of feed supplement measured by LC–MS–MS and LC–ICP–MS via external calibration and standard addition, (standard uncertainty of external calibration calculated from n=3 determinations; standard uncertainty of standard addition experiments calculated according to DIN 32645)

Compound	External calibration LC–MS–MS	Standard addition LC–MS–MS	Standard addition LC–ICP–MS
5′CMP	132±4	131±1	131±3
5′UMP	162±5	161±2	144±7
5′AMP	2.44±0.04	3.76±0.54	4.50±2
3′AMP	2.78±0.04	3.43±0.43	n.a.[a]
2′AMP	0.77±0.02	0.83±0.45	n.a.[a]
5′IMP	249±5	232±3	240±12
5′GMP	198±6	180±2	185±4
3′GMP	4.41±0.15	4.88±0.32	n.a.[a]
2′GMP	1.62±0.07	1.67±0.11	n.a.[a]

[a]Not analysed

centrifugal filtration (10 kDa cut-off filtration) for removing high-molar-mass biomolecules (proteins, RNA) and particles. In the past, centrifugal ultrafiltration without further treatment steps was applied to infant formulas [11] and filtration through a 0.45 μm membrane was used for sample preparation of cell-free yeast autolysates [1].

For almost all the nucleoside and nucleobase compounds the approach implemented in this work resulted in satisfactory recovery (>90 %; determined by studying, by LC–MS–MS, the recovery of a sample spiked with 5 μmol L^{-1} standard). The exceptions were adenine and adenosine for which recovery was approximately 75 %. Corresponding experiments with nucleotide standards revealed average recovery was 95 %. Hence, internal standardization for accurate quantification by LC–MS–MS was implemented as given in Table S2 in the Electronic Supplementary Material. Using this approach, losses during the sample preparation could be corrected for and the standard uncertainty obtained for n=6 individually prepared standards by LC–MS–MS was approximately 4 %. Analysis of n=4 individually prepared samples revealed a repeatability was 10–15 %. Table 4 summarizes the comparative quantification of nucleotides by the two complementary MS-based methods using a nucleotide enriched yeast sample provided by our industry cooperation partner. The sample was prepared as described above, and the filtered aliquots were measured by use of different detection methods and quantification strategies. Because isotope dilution analysis could not be used for the monoisotopic phosphorus, standard addition by LC–ICP–MS was compared with external calibration and standard addition by LC–MS–MS, (both quantitative strategies using internal standardization as given in Table S2 in the Electronic Supplementary Material). As can be readily observed, the quantitative values obtained were in good agreement. Moreover, LC–MS–MS determinations revealed concentrations of nucleosides and nucleobases of approximately <15 μmol g_{DW}^{-1} for all the samples investigated (for methodological details see Electronic Supplementary Material Table S2).

Conclusion

Quantitative profiling of nucleotides, nucleobases, and nucleosides by different methods was investigated. RP–LC–MS–MS was the method of choice for accurate and sensitive quantification. The separation method was rapid and versatile, overcoming the technical restriction associated with ion-pair chromatography. Moreover, combination of the implemented reversed-phase separation with complementary ICP–MS detection was straightforward. In this way, a reference method for nucleotide quantification could be established and proved the validity of LC–MS–MS based quantification.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases—application to feed supplements

Stefan Neubauer, Ariana Rugova, Dinh Binh Chu, Hedda Drexler, Anja Ganner, Michael Sauer, Diethard Mattanovich, Stephan Hann and Gunda Koellensperger

Table S1. Selected publications on nucleotide analysis

Analytes	Detection Limits	Application, Samples	Chromatography	Analysis Time /min	Detection	Ref.	Year publ.
AMP, ADP, GDP, UTP, ATP		acid soluble extracts of human cell extracts	IC, solution of KH_2PO_4 and KCl	70	UV detection, full scale absorption	[4]	1973
20 isomers (5', 3' and 2' isomers) of NMPs and dNMPs		yeast autolysates	IP-RP-LC, 0.05 M tetrabutylammmonium-phosphate, phosphate buffer, pH 5.45	40	UV detection at 245 and 280 nm	[1]	1996
18 nucleobases, nucleosides and nucleotides	3 – 29 pmol on column	pharmacodynamic monitoring, human blood cells	isocratic IP-RP-LC, H_3PO_4/triethylamine/$MgSO_4$	55	UV detection at 254 nm	[9]	2003
Cd, Ud, As, Is, Gs, CMP, UMP, AMP, IMP, GMP	0.05 – 0.68 $mg*g^{-1}$	bovine and human milk, infant formulas	RP-LC, KH_2PO_4 buffer,pH 5.6	130	UV detection at 250, 260 and 270 nm	[10]	2007
CMP, UMP, AMP, IMP, GMP	0.01– 0.05 $\mu g*mL^{-1}$	infant formulas	IC, 50 mM $(NH_4)_2HPO_3$ buffer, pH 4.0	30	UV detection at 254 nm	[11]	2008
16 nucleosides, NMPs, NDPs and NTPs		method development, standard solutions	adsorption chromatography porous graphitic carbon, H_2O/ACN, NH_4AC	18	ESI(+,-) LC-MS	[17]	2004
nucleotides, sugar nucleotides, sugar bisphosphates and coencyme A esters	0.01 – 0.06 $\mu mol*mL^{-1}$	microbial metabolomics	IP-RP-LC, H_2O/MeOH, hexylamine, pH 6.3	51	ESI(-) LC-MS, linear ion trap	[30]	2006
AMP, CDP, UDP, ADP, GDP, CTP, UTP, ATP, GTP, NAD, FAD	0.25 – 4 pmol on column	cells and tissues of rats	IP-RP-LC, H_2O/MeOH, dibutylammonium formate	20	ESI(-) LC-MS	[12]	2007
23 nucleosides and nucleotides	LLOQ < 2.1 µM	dietary foods and beverages	IP-RP-LC, H_2O/ACN, dihexylammonium acetate, pH 5.0	50	ESI(+,-) LC-MS	[2]	2010
21 isomers (5',3', and 2' isomeres) of NMPs and dNMPs		Champagne wine	IP-RP-LC, H_2O/MeOH, triethylamine, phosphate buffer, pH 4.45	90	ESI(-) LC-MS-MS, triple quadrupole MS	[3]	2001
mono-, di and triphosphorylated nucleotides of Cd, Ud, As, Gs	ca. 1 pmol on column	method development, standard solutions	capillary IP-RP-LC, H_2O/MeOH, N,N-dimethylhexylamine	60	ESI(+,-) LC-MS, MS-MS, triple quadrupole MS	[31]	2002
more than 30 nucleobases and nucleosides	0.1 – 10 µM	purine and pyrimidine metabolic profile, urine	RP-LC, H_2O/MeOH, ammonium acetate	17	ESI(+) LC-MS-MS, triple quadrupole MS	[15]	2006
29 metabolites including AMP, cyclic GMP, ADP, ATP, NAD, NADH, NADP, NADPH	2.6 – 60 nM	cell extracts of *Escherichia coli*	IP-RP-LC, H_2O/MeOH, tributylammonium acetate	90	ESI(-) LC-MS-MS, quadrupole ion trap MS	[26]	2007
Cd, Ud, As, Is, Gs and a number of modified nucleosides		urine (cancer markers)	capillary HILIC, ACN/H_2O, $HCOONH_4$, HCOOH	60	ESI (+) LC-MS-MS, quadrupole ion trap MS	[32]	2008
24 nucleotides and related compounds	100 – 250 nM	cultured Chinese hamster ovary cells	IP-RP-LC, H_2O/MeOH, N,N-dimethylhexylamine	35	ESI(-) LC-MS-MS, tripple quadrupole MS	[13]	2008
16 nucleotides	0.03 – 0.62 pmol on column	intracellular nucleotides in microorganisms	IP-RP-LC, H_2O/ACN, dibutylamine, pH6.7	25	ESI(-) LC-MS-MS, triple quadrupole MS	[14]	2009
200 intracellular metabolites including		fermentation broths of	HILIC pH 3.5, HILIC pH 7.5	45	ESI(+/-) LC-MS-MS,	[16]	2010

nucleobases, nucleosides and nucleotides		antibiotics production			quadrupole ion trap MS		
dAMP, dTMP, dGMP and dCMP	8 – 73 ng*mL^{-1}	standard solutions	capillary electrophoresis, isocratic micro-HPLC, 20 mM CH_3COONH_4 and MeOH, pH 8.6	7	ICP-MS, mass of $^{31}P^+$	[19]	2009
dCMP, dTMP, dAMP, dGMP	3.1 – 26 ng*mL^{-1}	enzymatically digested DNA	capillary electrophoresis	23	ICP-MS, mass of $^{31}P^+$	[20]	2010
dCMP, 5mdCMP, dTMP, dAMP, dGMP	0.05 – 0.15 µM	salmon testes DNA, oligonucleotides	isocratic RP-LC, ammonium acetate buffer pH3/MeOH	15	ICP-MS, mass of $^{31}P^+$	[21]	2010

Table S2. MRM settings of the LC-MS-MS instrument

Compound	Polarity / time segment	Dwell time / ms	Precursor ion *m/z*	Quantifier *m/z*	Qualifier *m/z*	FV / V	$CE_{Quantifier}$ / V	$CE_{Qualifier}$ / V	IS for quantification
Cytosine	+ / 1	500	112.1	94.9	52.0	104	17	33	$^{15}N^{13}C_2$ Uracil
Uracil	+ / 2	100	113.0	43.0	-	90	21	-	$^{15}N^{13}C_2$ Uracil
$^{15}N^{13}C_2$ Uracil	+ / 2	100	116.0	70.9	97.9	100	13	13	-
Adenine	+ / 2	100	136.1	118.9	91.9	124	25	33	$^{15}N^{13}C_2$ Uracil
Guanine	+ / 2	100	152.1	134.9	109.9	104	17	21	$^{15}N^{13}C_2$ Uracil
Cytidine	+ / 2	100	244.1	111.9	94.9	70	9	40	$^{15}N^{13}C_2$ Uracil
Uridine	+ / 3	100	245.1	112.9	-	70	9	-	$^{15}N^{13}C_2$ Uracil
Adenosine	+ / 3	100	268.1	135.9	118.9	90	17	40	$^{15}N^{13}C_2$ Uracil
Inosine	+ / 3	100	269.1	136.9	-	80	9	-	$^{15}N^{13}C_2$ Uracil
Guanosine	+ / 3	100	284.1	151.9	134.9	80	13	40	$^{15}N^{13}C_2$ Uracil
5'CMP	- / 1	500	322.0	79.0	97.0	-124	-40	-21	$^{15}N_5$ 5'AMP
5'UMP	- / 2	100	323.0	78.9	96.9	-114	-40	-21	$^{15}N_5$ 5'AMP
5'AMP	- / 2	100	346.0	78.9	134.0	-114	-40	-40	$^{15}N_5$ 5'AMP
3'AMP	- / 2	100	346.0	211.0	79.1	-114	-13	-40	$^{15}N_5$ 5'AMP
2'AMP	- / 3	200	346.0	211.0	134.0	-114	-13	-40	$^{15}N_5$ 5'AMP
5'IMP	- / 2	100	347.0	79.1	-	-114	-40	-	$^{15}N_5$ 5'AMP
$^{15}N_5$ 5'AMP	- / 2	100	351.0	79.0	139.0	-104	-33	-37	-
5'GMP	- / 2	100	362.0	79.0	211.0	-124	-21	-13	$^{15}N_5$ 5'AMP
3'GMP	- / 3	200	362.0	211.0	79.0	-124	-13	-21	$^{15}N_5$ 5'AMP
2'GMP	- / 3	200	362.0	211.0	79.0	-124	-13	-21	$^{15}N_5$ 5'AMP

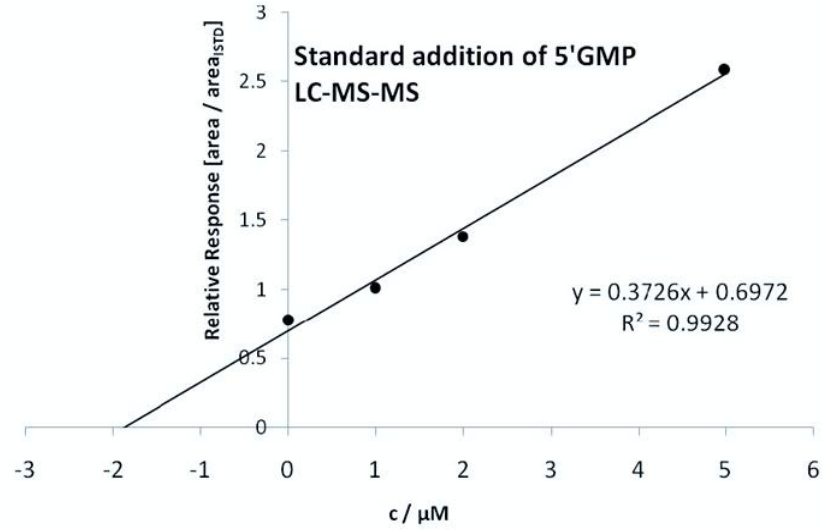

Figure S1. Calibration curve of standard addition experiment. The relative standard deviation for the procedure calculated according to DIN 32645 is 1.2 %.

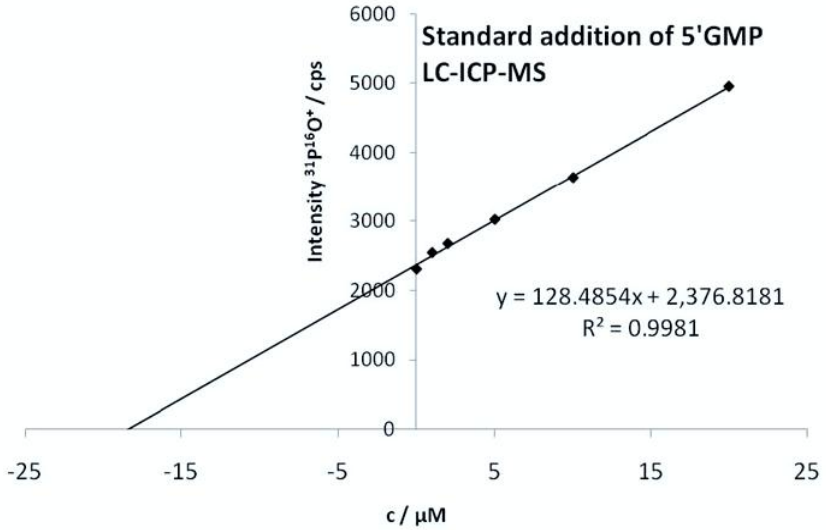

Figure S2. Calibration curve of standard addition experiment. The relative standard deviation for the procedure calculated according to DIN 32645 is 2.0 %.

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Research Article

$U^{13}C$ cell extract of *Pichia pastoris*—A powerful tool for evaluation of sample preparation in metabolomics

Quantitative metabolic profiling is preceded by dedicated sample preparation protocols. These multistep procedures require detailed optimization and thorough validation. In this work, a uniformly ^{13}C-labeled ($U^{13}C$) cell extract was used as a tool to evaluate the recoveries and repeatability precisions of the cell extraction and the extract treatment. A homogenous set of biological replicates ($n = 15$ samples of *Pichia pastoris*) was prepared for these fundamental experiments. A range of less than 30 intracellular metabolites, comprising amino acids, nucleotides, and organic acids were measured both in monoisotopic ^{12}C and $U^{13}C$ form by LC-MS/MS employing triple quadrupole MS, reversed phase chromatography, and HILIC. Recoveries of the sample preparation procedure ranging from 60 to 100% and repeatability precisions below 10% were obtained for most of the investigated metabolites using internal standardization approaches. Uncertainty budget calculations revealed that for this complex quantification task, in the optimum case, total combined uncertainty of 12% could be achieved. The optimum case would be represented by metabolites, easy to extract from yeast with high and precise recovery. In other cases the total combined uncertainty was significantly higher.

Keywords: Extraction / Metabolic profiling / *Pichia pastoris* / Recovery / Sample preparation
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1 Introduction

The knowledge of the microbial metabolome and its regulations facilitates advanced metabolic engineering and cell factory design [1]. Metabolomics is per definition a global analysis—including both identification and quantification—of the complete metabolome of a given biological system [1–6]. The analytical strategies can be categorized in (i) nontargeted approaches (metabolic fingerprinting, metabolic footprinting) and (ii) targeted approaches. The quantification of metabolite levels, often referred to as quantitative metabolic profiling is based on targeted approaches, e.g. the quantitative determination of selected metabolites sharing the same metabolic pathway or chemical properties. Typical metabolites are intermediates from central carbon metabolism, purine and pyrimidine nucleotides, and amino acids. The aim of quantitative metabolite profiling is to obtain an unbiased in vivo snapshot of the metabolic state of the investigated biological system.

The key points in any analytical quantitative process regard representative sampling, efficient extraction, and subsequent analysis. When quantifying intracellular metabolites in a cell culture, the main focus is on three steps: (i) rapid sampling, (ii) cell quenching, and (iii) cell extraction. The sampling time is defined as the time needed to take cells from their environment (e.g. bioreactor) into quenching solution. Quenching aims at instantly stopping the enzymatic activity of the sample by an abrupt change of sample temperature either to low temperatures (e.g. $< -20°C$) or, like e.g. in the case of whole broth analysis, when quenching and extraction are combined in a single step, at elevated temperatures (e.g. $> 80°C$) [7]. And finally, during extraction the cell walls are chemically and/or thermally permeabilized or mechanically disrupted to exhaustively extract the relevant metabolites into the liquid phase.

The sampling time is critical and has to be adapted according to the analyzed metabolites and their enzymatic turnover

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Abbreviations: AMP, adenosine monophosphate; **CDW**, cell dry weight; **CE**, collision energy; **CMP**, cytidine monophosphate; **FV**, fragmentor voltage; **GMP**, guanosine monophosphate; **IS**, internal standard; **k**, coverage factor; **k'**, capacity factor; **N**, number of theoretical plates; **NAD**, nicotinamide adenosin dinucleotide; **NADP**, nicotinamide adenosine dinucleotide phosphate; **PEP**, phosphoenol pyruvate; **RSD**, relative standard deviation; **S/N**, signal to noise ratio; **SRM**, selected reaction monitoring; **SU**, standard uncertainty; t_R, retention time; $U^{12}C$, monoisotopic signal from cell sample or standard solution; $U^{13}C$, uniformly carbon thirteen labeled; **UMP**, uridine monophosphate

times [8]. Recently, a comprehensive overview on sampling strategies was published [9]. The development of quenching and extraction methods, which can also be combined in a single step, depends on the investigated metabolites as well as on the investigated sample (organism). Once the cells are quenched, quick separation of the cells from the quenching solution is necessary to avoid leakage of intracellular metabolites, which occurs when metabolites diffuse out of the cell into the quenching solution. Moreover, metabolites present in the extracellular medium need to be efficiently removed by rapid washing. Hence quenching and washing conditions have to be thoroughly optimized for the investigated organism with respect to leakage [8]. After separation of the quenched (and washed) cells from the supernatant, either by centrifugation or filtration, the cells are subjected to extraction in order to release the relevant metabolites, which is besides rapid sampling and quenching one of the most critical steps. Generally, the implemented protocols depend on the investigated microorganism, which show highly variable properties of cell wall or no cell wall at all. In fact, fragile mammalian cells require specialized techniques, which consider the lack of cell walls and the fact, that these cells are often grown adherently [10, 11]. Recently, detailed summaries on developed sample preparation protocols were published [7, 12]. Moreover, quenching and extraction of microbial samples and reported uncertainties were comprehensively summarized [13]. For yeasts, e.g. most commonly, sampling into cold methanol quenching solution was applied [8, 14–17]. Extraction implied both, cold (subambient temperatures) as well as hot methods (e.g. boiling ethanol) [8, 14, 15].

For each investigated metabolite, extraction efficiency, and recovery has to be considered. The extraction efficiency was defined as the ability of a certain extraction method to release metabolites from the cells and could/still can—due to the lack of reference materials—only be obtained by direct comparison of several extraction methods based on different physical and chemical principles [15, 18, 19]. In contrast to this, extraction recovery considers metabolite losses during the application of the extraction method (losses due to adsorption, losses due to degradation, or interconversion of metabolites due to the presence of not fully inactivated enzymes), but does not consider incomplete extraction yields [15].

In this work, the multitarget analysis of a wide range of intracellular metabolites (organic acids, amino acids, nucleotides, and vitamins) was carried out via LC-MS/MS employing triple quadrupole MS. The high number of small metabolites ($m/z < 500$) and the nominal mass resolution of the employed triple quadrupole MS, demanded for the development of powerful chromatographic separations in order to avoid isobaric interferences. Reversed phase liquid chromatography (RP-LC) is a good choice for multitarget quantitative metabolic profiling due to high separation efficiency, high robustness of chromatography and unconfined compatibility with ESI-MS. However, the separation of polar metabolites e.g. polar amino acids is hampered. One way to circumvent this problem is to increase retention of polar analytes on RP-LC by the use of ion-pair reagents like alkyl-ammonium salts or perfluorinated carboxylic acids. Although often applied in metabolic assays [20, 21], the use of an ion pair reagent leads to several severe drawbacks, such as compromised sensitivity by ion suppression, enduring contamination of the HPLC and of the interface may restricting the use of the MS instrument to one polarity and poor robustness regarding retention times. Alternatively, additional derivatization steps were introduced making polar compounds amenable to RP-LC-MS analysis [22]. In other approaches, the metabolite set was divided according to polarity, and the samples were analyzed by complementary separation methods (e.g. RP-LC, adsorption chromatography using porous graphitized carbon, hydrophilic interaction chromatography (HILIC), or weak ion exchange) in separated analytical runs. In this work, a novel C_{18} bonded silica phase having lower ligand density and tolerating highly aqueous conditions and a HILIC column, employing a zwitterionic-bonded silica phase were used for evaluation of cell extraction and sample preparation.

A well-founded evaluation of all critical aspects (e.g. leakage, extraction recovery, storage stability) is mandatory for quantitative analysis of microbial metabolome due to its outstanding challenges. The analyst is confronted with highly reactive target analytes in a complex cellular matrix. Furthermore, the expected intracellular concentrations, in other words the pool sizes of the different metabolites in the observed cells, may range over several orders of magnitude. Hence, the reliability of the analytical data in terms of accuracy and precision has to be verified carefully before making any biological interpretation. Parameters like recoveries and storage stabilities need to be assessed in an early state of method development and determine further steps of optimization. The calculation of total combined uncertainties is, although not yet established in quantitative metabolomics, the most powerful tool to evaluate the introduced analytical process (from sampling to analysis). Comprehensive uncertainty budgeting again pinpoints the major uncertainty contributions.

In this work, we present an approach for evaluation of the sample preparation in microbial metabolomics using the example of the procedure, we optimized for the yeast *Pichia pastoris*. In particular, we assessed metabolite recoveries and repeatability precisions of the cell extraction, of the extract treatment, and of the overall sample preparation using uniformly ^{13}C-labeled ($U^{13}C$) metabolites as tracer. For the present work, a $U^{13}C$ cell extract was produced in our laboratory, it was characterized regarding purity of the metabolite labeling and regarding metabolite pattern and it was spiked to cell samples at three distinct steps of sample preparation procedure.

2 Materials and methods

2.1 Chemicals

For cultivation media, following substances were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany):

$MgSO_4.7H_2O$ 99%, KCl 99.5%, K_2HPO_4 99%, KH_2PO_4 98%, glycerol 99%, glucose monohydrate for microbiology, and citric acid 99.5%. $(NH_4)_2HPO_4$ p.a. from AppliChem (Gatersleben, Germany), $CaCl_2.2H_2O$ p.a, ammonia (25% v/v), KOH pellets pure and hydrochloric acid (30% ultrapure) from Merck KGaA (Darmstadt, Germany) and H_3PO_4 85% (v/v) and biotin 99% from Sigma-Aldrich (Vienna, Austria) were also used for cultivation media. Trace salt stock solution contained per liter: 6.0 g $CuSO_4.5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4.H_2O$, 0.2 g $Na_2MoO_4.2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2.6H_2O$, 20.0 g $ZnCl_2$, and 5.0 mL H_2SO_4 (95–97% w/w), all from Merck as well as 5.0 g $FeSO_4.7H_2O$ 99.5% from Carl Roth. $U^{13}C_6$ D-glucose 98% was used as substrate for the fed batch cultivation medium for production of the $U^{13}C$ cell extract and was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

For rapid sampling and quenching dry ice pellets from Linde Gas GmbH (Vienna, Austria), ethanol 96% from Merck, ethylene glycol, and methanol (> 99%) from Carl Roth were used. For cell extraction were used: ethanol 99.8% and LC-MS grade water from Sigma-Aldrich. Hydrochloric acid (30% ultrapure) and sodium hydroxide monohydrate Suprapure® from Merck and LC-MS grade water from Sigma-Aldrich were used for dissolution of standard substances. For chromatography LC-MS grade water and LC-MS grade acetonitrile from Sigma-Aldrich, LC-MS grade methanol from Fisher Scientific (Loughborough, UK) and formic acid 98–100% Suprapur® from Merck were used.

2.2 Standards

The following standard substances were purchased from Sigma-Aldrich or Fluka (Vienna, Austria): cis aconitic acid 98%, L-alanine 99.5%, adenosine 3′monophosphoric acid 97%, adenosine 5′ monophosphate sodium salt 99%, L-arginine 98.5%, L-asparagine 98%, biotin 99%, cytidine 5′-monophosphate sodium salt 99%, fumaric acid 99.5%, guanosine 3′-monophosphate sodium salt 99%, guanosine 5′-monophosphate disodium salt 99%, DL-histidine 99%, DL-isocitric acid sodium salt 98%, L-isoleucine 98%, L-lysine 98%, β-nicotinamide adenine dinucleotide sodium salt 95%, β-nicotinamide adenine dinucleotide phosphate disodium salt 97%, phospho(enol)pyruvate 99%, L-proline 99.5%, (-) riboflavin 98%, DL-serine 99%, succinic acid 99.5%, L-threonine 98%, L-tyrosine 99%, and uridine 5′-monophosphate disodium salt 98%. The following substances were purchased from Merck KGaA (Darmstadt, Germany): L-aspartic acid 99%, citric acid 99.5–100.5%, L-glutamic acid 99%, L-glutamine 99%, L-leucine 99%, DL-malic acid 99.5%, L-methionine 99%, L-phenylalanine 99%, and L-valine 99%.

A range of 0.5–2.0 mg of each substance were weighed and 1 mL of LC-MS grade water was added—in the case of amino acids 1 mL of 0.1 mol L^{-1} HCl was added instead of water—and in case of 3′AMP 1 mL of 0.1 mol L^{-1} NaOH was added instead of water—resulting in 5 mmol L^{-1} stock solutions. Further dilution steps were achieved with pipettes with disposable tips from Eppendorf (Vienna, Austria). The final dilution step was conducted by diluting in LC-MS grade water. Stock solutions were kept at −20°C.

2.3 Fully labeled ^{13}C cell extract

The wild-type strain of *P. pastoris* was cultivated in fed batch using a 1.4 L benchtop bioreactor (DASGIP Parallel Bioreactor System, Germany) with a working volume of 400 mL using a minimal medium with $U^{13}C_6$ glucose as sole carbon source. The preculture medium (per liter: 0.5 g $MgSO_4.7H_2O$, 0.9 g KCl, 0.022 g $CaCl_2.2H_2O$, 10.98 mL 85% (v/v) H_3PO_4, 4.6 mL trace salt stock solution, 2 mL biotin solution (c = 0.2 g L^{-1}), 10 g $U^{13}C_6$ D-glucose, and 100 mL K_2HPO_4/KH_2PO_4 buffer (1 mol L^{-1}, pH 6), pH set to 5.0 with 25% (v/v) ammonia) was inoculated with 200 μL cryostock of *P. pastoris* CBS 7435. The preculture was grown at 28°C and 150 rpm overnight and was used for inoculation of the bioreactor with an optical density (OD600) of 0.2.

After a batch phase of approximately 35 h the feed was started. The fermentation strategy was a fed batch under carbon-limited conditions with a growth rate of 0.1 h^{-1}. The pH was controlled at 5.0 with 25% (v/v) ammonia, the temperature was controlled at 25°C and the dissolved oxygen was kept constant at 20% by controlling the stirrer speed and inlet gas flow. To ensure complete ^{13}C labeling the CO_2 from the inlet air using an in-house made CO_2 remover with aerosol scrubber were used (two bottles with 4 mol L^{-1} KOH and one bottle with water connected with silicon tubes and frits in series; liquid level is approximately 20 cm and corresponds to the distance the air bubbles through the solution).

Batch and fed batch medium contained per liter: 0.5 g $MgSO_4.7H_2O$, 0.9 g KCl, 0.022 g $CaCl_2.2H_2O$, 10.98 mL 85% (v/v) H_3PO_4, 4.6 mL trace salt stock solution, 2 mL biotin solution (c = 0.2 g L^{-1}), and 10 g $U^{13}C_6$ glucose. The pH was adjusted to 5.0 by using 25% (v/v) ammonia.

The cells were grown until a final biomass concentration of approximately 4.5 g_{CDW} L^{-1}. Before the first sampling round the cells were grown at least 2 h to ensure exponential growth. The cultivation broth was then sampled immediately into the four-fold volume of cold quenching and washing solvent (QS = 60% v/v methanol) using a peristaltic pump and silicone tubes (diameter 5 mm, length 81 cm) at a pumping speed of 5 mL s^{-1}. QS and quenched cell suspension were tempered to −30°C ± 3°C by a cooling mixture (= ethylene glycol:ethanol = 70 : 30 + dry ice pellets). Biomass was determined by drying triplicates of 2 mL chemostat culture to constant weight at 105°C in preweight glass tubes and was 4.03 g_{CDW} L^{-1} (cell dry weight) with a standard deviation of 0.08 g_{CDW} L^{-1} ($n = 3$).

The quenched cell suspension was aliquoted in 10 mL portions into 15 mL sample tubes from Greiner (Frickenhausen, Germany) and pelleted via centrifugation at 4000 g and −20°C for 10 min using a Sorvall RC 6+ centrifuge from Thermo Scientific (Waltham, MA, USA). A second aliquot of

10 mL was added on top of each cell pellet and centrifuged again. The cell pellets were washed twice by resuspension in 5 mL of tempered QS and centrifugation at 4000 *g* and −20°C. Four milliliters of extraction solvent (= 75% v/v ethanol, tempered to 85°C) was added to each pellet, the cells were suspended by vortexing and the cells were extracted for 3 min at 85°C whereat vortexed a second time after 1.5 min of extraction and vortexed a third time after 3 min of extraction. The extracted pellets were rapidly cooled down but not frozen. The ethanolic extracts were separated from the cell debris by centrifugation at 4000 *g* and −20°C and decanting; they were pooled and stored at −80°C. The pooled ethanolic extract was evaporated to complete dryness in a vacuum centrifuge (Savant RVT400 from Thermo Scientific) operating at pressures below 1 mbar and resuspended with LC-MS grade water to a final volume that is one-fourth of the primary ethanolic extract. Unsoluble particles were removed via centrifugation at 4000 *g* at 5°C for 10 min using a table centrifuge from Hettich (Tuttlingen, Germany) and the aqueous extract was pooled. The aqueous $U^{13}C$ cell extract was kept at −80°C.

2.4 Chemostat cultivation for cell samples

The chemostat cultivation was performed in a 1.4 L bentchtop bioreactor (DASGIP Parallel Bioreactor System, Germany) with a working volume of 400 mL. Hundred milliliters preculture medium (per liter: 10 g yeast extract, 20 g peptone, 10 g glycerol) was inoculated from the working cell bank (750 μL cryostock of *P. pastoris* CBS7435). The preculture was grown at 28°C and 150 rpm overnight and was used for inoculation of the bioreactor at an optical density (OD_{600}) of 1.0.

After a batch phase of approximately 24 h the feed and harvest for the continuous chemostat cultivation was started. The cells were grown under glucose limited conditions with a dilution rate of 0.1 h^{-1} for at least seven residence times before taking the sample. Temperature, pH and dissolved oxygen were maintained at 25°C, 5.0 (with 8 mol L^{-1} KOH) and 20% (by controlling the stirred speed and inlet gas flow), respectively.

Batch medium contained per liter: 0.5 g $MgSO_4.7H_2O$, 0.9 g KCl, 0.022 g $CaCl_2.2H_2O$, 4.6 mL trace salt stock solution, 2 mL biotin solution (c = 0.2 g L^{-1}), 1.8 g citric acid, 12.6 g $(NH_4)_2$ HPO_4, and 39.9 g glycerol. The pH was set to 5.0 with 32% (w/w) HCl.

Chemostat medium contained per liter: 1.0 g $MgSO_4.7H_2O$, 2.5 g KCl, 0.04 g $CaCl_2.2H_2O$, 2.43 g trace salt stock solution, 2 g biotin solution (c = 0.2 g L^{-1}), 2.3 g citric acid, 21.75 g $(NH_4)_2$ HPO_4, and 55 g glucose monohydrate. The pH was set to 5.0 with 32% (w/w) HCl.

2.5 Rapid sampling and quenching

Samples for analysis of intracellular metabolites were taken immediately using a peristaltic pump and silicone tubes (diameter 5 mm, length 81 cm) at a pumping speed of 5 mL s^{-1}. Approximately 50 mL (47.0 g ± 0.1 g) fermentation broth was quenched in 200 mL of cold quenching and washing solvent (QS = 60% v/v methanol). QS and quenched cell suspension were tempered to −30°C ± 3°C by a cooling mixture (= ethylene glycol:ethanol = 70 : 30 + dry ice pellets). After quenching, 15 portions of 5 mL quenched cell suspension (approximately 4.50 g ± 0.01 g, corresponding to approximately 5 mg of CDW) were pelleted in 15 mL sample tubes (Greiner, Frickenhausen, Germany) by centrifugation at 4000 *g* at −20°C for 10 min using a Sorvall RC 6+ centrifuge from Thermo Scientific (Waltham, MA, USA). All tubes were weighed before and after the sampling procedure (± 0.01 g) in order to determine the exact amount of sample taken. The cells were washed two times by adding 5 mL of QS to the cell pellet, resuspended and centrifuged at −20°C and were kept on dry ice until extraction.

Biomass was determined by drying five replicates of 2 mL chemostat culture to constant weight at 105°C in preweight glass tubes and was 5.12 g_{CDW} L^{-1} (cell dry weight) with a standard deviation of 0.15 g_{CDW} L^{-1} ($n = 5$).

2.6 Boiling ethanol extraction and extract treatment

Prior to extraction, five samples were spiked with 200 μL of aqueous fully ^{13}C-labeled ($U^{13}C$) cell extract and ten samples were spiked with 200 μL of LC-MS grade water. A 3.8 mL of extraction solvent (= 79% v/v ethanol, tempered to 85°C,) was added to each pellet, the cells were suspended by vortexing and the cells were extracted for 3 min at 85°C, vortexed a second time after 1.5 min of extraction and vortexed a third time after 3 min of extraction. The extracted pellets were rapidly cooled down but not frozen. The ethanolic extracts were separated from the cell debris by centrifugation at 4000 *g* at −20°C for 10 min using a Sorvall RC 6+ centrifuge from Thermo Scientific (Waltham, MA, USA) and decanting. Five unspiked samples were spiked with 200 μL of aqueous $U^{13}C$ cell extract and to ten samples 200 μL of LC-MS grade water was added. The ethanolic extracts were stored on dry ice until they were evaporated to complete dryness in a vacuum centrifuge (Savant RVT400 from Thermo Scientific) operating at pressures below 1 mbar. Five unspiked samples were spiked with 200 μL of aqueous $U^{13}C$ cell extract. All 15 samples were filled up to a final volume to 1000 μL using LC-MS grade water and resuspended via vortexing. Unsoluble particles were removed via centrifugation at 4000 *g* at 5°C using for 10 min a table centrifuge from Hettich (Tuttlingen, Germany). For HILIC measurement, one additional dilution step of 1:10 was carried out using LC-MS grade water.

2.7 Chromatography

RP-LC was performed using an Atlantis T3® C_{18} reversed phase column (150 × 4.6 mm, 3-μm particle size) from Waters® (Milford, USA), an Atlantis T3® guard column (20 × 4.6 mm, 3-μm particle size), eluent A (water, 0.1% v/v

formic acid), and eluent B (methanol). Following gradient was applied: 0% B was constant for 2 min, then B was increased to 40% within 8 min and was held for 2 min. Subsequent increase to 100%, within 0.1 min and holding for 1.9 min to flush the column, subsequent reconstitution of the starting conditions within 0.1 min and reequilibration with 0% B for 5.9 min resulted in a total analysis time of 20 min. A flow rate of 1.0 mL min^{-1}, an injection volume of 12.5 μL and a column temperature of 40°C were applied.

HILIC was performed using a ZicHILIC® separation column (150 × 4.6 mm, 3.5-μm particle size, 100 Å pore size) from SeQuant (Marl, Germany) a ZicHILIC® guard column (20 × 2.1 mm, 5-μm particle size), eluent A (98% v/v water, 1% v/v acetonitrile, 1% v/v formic acid), and eluent B (98% v/v acetonitrile, 1% v/v water, 1% v/v formic acid) applying the following gradient: 90% B was constant for 2 min, then B was reduced to 10% within 7 min and was held for 1 min. Subsequent reconstitution of the starting conditions within 0.1 min and reequilibration with 90% B for 9.9 min resulted in a total analysis time of 20 min. A flow rate of 0.5 mL min^{-1}, an injection volume of 12.5 μL and a column temperature of 40°C were applied.

2.8 LC-triple quad system

An Agilent G1312A Binary Pump 1200 series from Agilent Technologies (Santa Clara, CA, USA) together with an Agilent G1367B high performance autosampler and an Agilent G1316A column compartment was employed for HPLC. For MS detection an Agilent 6410 Triple Quad LC-MS from Agilent Technologies was used featuring an ESI interface. Source parameters in negative and positive ionization mode for RP-LC using Atlantis T3® were set as follows: drying gas temperature 350°C, drying gas flow 10 L min^{-1}, nebulizer pressure 50 psi and capillary voltage 4000 V. Source parameters in positive ionization mode for HILIC were set as follows: drying gas temperature 300°C, drying gas flow 10 L min^{-1}, nebulizer pressure 25 psi and capillary voltage 4000 V. Selected reaction monitoring (SRM) transitions of the metabolites using the monoisotopic ^{12}C isotopologue ($U^{12}C$) have been determined via flow injection of 5 μmol L^{-1} single standard (isocratic conditions: 0.05% formic acid in methanol : H_2O = 50 : 50 v/v). For optimization Mass Hunter Optimizer Software from Agilent was applied processing the following four steps in order: (i) optimization of the isolation of the selected precursor ion by varying the fragmentor voltage, (ii) determination of the four most abundant product ions, (iii) optimization of the collision energies for each of these product ions, and (iv) determination of the exact m/z value of the product ions. The transition featuring the highest signal to noise ratio was chosen for recovery evaluation (quantifier) and if available, the transition featuring the second highest transition was chosen for identification (qualifier). The optimized SRM transitions of the fully labeled ^{13}C ($U^{13}C$) metabolites were acquired by calculating the number of carbon atoms in the fragments and adopting the fragmentation parameters. Precursor and product ions as well as specific values for fragmentor voltage and collision energy are listed in Table 1. The instrument was set to one polarity mode per run resulting in three methods. For RPLC (+), for RPLC (−) and for HILIC (+), SRM methods were performed setting the retention times of the analytes, a time window of 2 min and a cycle time of 1000 ms. These settings resulted for RPLC and positive ionization in a total of 49 transitions with a minimum dwell time of 39.98 ms (23 concurrent transitions) and a maximum dwell time of 329.83 ms. For RPLC and negative ionization they resulted in a total of 69 transitions with a minimum dwell time of 22.14 ms (39 concurrent transitions) and a maximum dwell time of 996.15 ms and for HILIC and positive ionization they resulted in a total of 43 transitions with a minimum dwell time of 27.75 ms (32 concurrent transitions) and a maximum dwell time of 996.50 ms. LC-ESI-MS/MS data was evaluated employing Agilent Mass Hunter Qualitative and Quantitative Analysis software modules. Peak integration was based on ± 0.5 m/z peak with for precursor and product ion.

2.9 LC-MS/MS analysis and evaluation of recoveries

From our initial set of 34 metabolites, 19 were measured only on RP-LC-MS/MS (Table 2), ten polar amino acids were measured only on HILIC-MS/MS (Table 3) and five amino acids comprising apolar side chains (met, phe, pro, tyr, val) were measured on both platforms. Low abundant metabolites (S/N $U^{13}C < 10$) were excluded from recovery evaluation (Table 4) and five highly abundant amino acids (arg, gln, glu, his, lys) where measured on HILIC-MS/MS in an additional 1:10 dilution. Metabolite recoveries were evaluated by comparing peak areas of $U^{13}C$ metabolites deriving from three different spikes and the $U^{13}C$ cell extract was characterized from a control sample:

① = addition of 200 μL aqueous $U^{13}C$ cell extract onto cell pellet prior to extraction ($n = 5$)
② = addition of 200 μL aqueous $U^{13}C$ cell extract to cooled (−20°C) ethanolic cell extract ($n = 5$)
③ = addition of 200 μL aqueous $U^{13}C$ cell extract to dried extract after evaporation ($n = 5$)

ctrl = 200 μL aqueous $U^{13}C$ cell extract, 800 μL LC-MS grade water ($n = 4$)

The recoveries of the extraction, of the sample treatment (evaporation) and of the overall sample preparation is then calculated according to Eqs 1, 2, and 3 (see also Fig. 1).

$$R_{ex} = area\ U^{13}C①/area\ U^{13}C② \quad (1)$$

$$R_{ev} = area\ U^{13}C②/area\ U^{13}C③ \quad (2)$$

$$R_{sp} = area\ U^{13}C①/area\ U^{13}C③ \quad (3)$$

Furthermore, the monoisotopic signal ($U^{12}C$) deriving from the cell sample was used as internal standard and $U^{12}C$ corrected recoveries were determined correcting by

Table 1. Measurement conditions and SRM settings of of the LC-MS/MS instrument are shown for all the measured metabolites. Precursor ions ($[M+H]^+$,$[M-H]^-$) and fragment ions (quantifier, qualifier) are shown for the monoisotopic molecule deriving from standard or cell sample ($U^{12}C$) and for the one deriving from fully labeled cell extract ($U^{13}C$)

Compound	Polarity	Chromatography	Dilution	Precursor	Quantifier	Qualifier	FV / V	$CE_{Quantifier}$ / V	$CE_{Qualifier}$ / V	Comp. for corr.
$U^{12}C_6$ cis-Aconitate	–	RP-LC	1:1	173	85	129	−80	−5	−5	–
$U^{13}C_3$ Alanine	+	HILIC	1:1	93	46	–	40	9	–	$U^{12}C_3$ Alanine
$U^{12}C_3$ Alanine	+	HILIC	1:1	90	44	–	40	9	–	–
$U^{13}C_{10}$ 3′AMP	–	RP-LC	1:1	356	216	79	−114	−13	−40	–
$U^{12}C_{10}$ 3′AMP	–	RP-LC	1:1	346	211	79	−114	−13	−40	–
$U^{13}C_{10}$ 5′AMP	–	RP-LC	1:1	356	79	139	−118	−40	−40	$U^{12}C_{10}$ 5′AMP
$U^{12}C_{10}$ 5′AMP	–	RP-LC	1:1	346	79	134	−118	−40	−40	–
$U^{13}C_6$ Arginine	+	HILIC	1:1, 1:10	181	74	–	80	25	–	$U^{12}C_6$ Arginine
$U^{12}C_6$ Arginine	+	HILIC	1:1, 1:10	175	70	60	80	25	13	–
$U^{13}C_4$ Asparagine	+	HILIC	1:1	137	76	90	60	13	5	$U^{12}C_4$ Asparagine
$U^{12}C_4$ Asparagine	+	HILIC	1:1	133	74	87	60	13	5	–
$U^{13}C_4$ Aspartate	+	HILIC	1:1	138	76	73	60	9	13	$U^{12}C_4$ Aspartate
$U^{12}C_4$ Aspartate	+	HILIC	1:1	134	74	70	60	9	13	–
$U^{12}C_{10}$ Biotin	+	RP-LC	1:1	245	227	97	90	9	33	–
$U^{13}C_6$ Citrate	–	RP-LC	1:1	197	116	–	−90	−5	–	$U^{12}C_6$ Citrate
$U^{12}C_6$ Citrate	–	RP-LC	1:1	191	111	–	−90	−5	–	–
$U^{13}C_9$ 5′CMP	–	RP-LC	1:1	331	79	97	−124	−40	−21	–
$U^{12}C_9$ 5′CMP	–	RP-LC	1:1	322	79	97	−124	−40	−21	–
$U^{13}C_4$ Fumarate	–	RP-LC	1:1	119	74	–	−80	−9	–	–
$U^{12}C_4$ Fumarate	–	RP-LC	1:1	115	71	–	−80	−9	–	–
$U^{13}C_5$ Glutamate	+	HILIC	1:1, 1:10	153	88	59	70	13	25	$U^{12}C_5$ Glutamate
$U^{12}C_5$ Glutamate	+	HILIC	1:1, 1:10	148	84	56	70	13	25	–
$U^{13}C_5$ Glutamine	+	HILIC	1:1, 1:10	152	88	135	80	17	9	$U^{12}C_5$ Glutamine
$U^{12}C_5$ Glutamine	+	HILIC	1:1, 1:10	147	84	130	80	17	9	–
$U^{13}C_{10}$ 3′GMP	–	RP-LC	1:1	372	216	79	−118	−17	−21	–
$U^{12}C_{10}$ 3′GMP	–	RP-LC	1:1	362	211	79	−118	−17	−21	–
$U^{13}C_{10}$ 5′GMP	–	RP-LC	1:1	372	79	216	−118	−21	−17	–
$U^{12}C_{10}$ 5′GMP	–	RP-LC	1:1	362	79	211	−118	−21	−17	–
$U^{13}C_6$ Histidine	+	HILIC	1:1, 1:10	162	115	87	80	13	25	$U^{12}C_6$ Histidine
$U^{12}C_6$ Histidine	+	HILIC	1:1, 1:10	156	110	83	80	13	25	–
$U^{13}C_6$ Isocitrate	–	RP-LC	1:1	197	116	179	−90	−5	−5	$U^{12}C_6$ Isocitrate
$U^{12}C_6$ Isocitrate	–	RP-LC	1:1	191	111	173	−90	−5	−5	–
$U^{13}C_6$ Isoleucine	+	RP-LC	1:1	138	91	–	70	9	–	$U^{12}C_6$ Isoleucine
				91	–	61	130	–	20	
$U^{12}C_6$ Isoleucine	+	RP-LC	1:1	132	86	–	70	9	–	–
				86	–	57	130	–	20	
$U^{13}C_6$ Leucine	+	RP-LC	1:1	138	91	–	70	9	–	$U^{12}C_6$ Leucine
				91	–	46	130	–	20-	
$U^{12}C_6$ Leucine	+	RP-LC	1:1	132	86	–	70	9	–	–
				86	–	43	130	–	20	
$U^{13}C_6$ Lysine	+	HILIC	1:1, 1:10	153	89	136	60	13	9	$U^{12}C_6$ Lysine
$U^{12}C_6$ Lysine	+	HILIC	1:1, 1:10	147	84	130	60	13	9	–
$U^{13}C_4$ Malate	–	RP-LC	1:1	137	119	–	−80	−9	–	$U^{12}C_4$ Malate
$U^{12}C_4$ Malate	–	RP-LC	1:1	133	115	–	−80	−9	–	–
$U^{13}C_5$ Methionine	+	RP-LC, HILIC	1:1	155	59	–	70	13	–	–
$U^{12}C_5$ Methionine	+	RP-LC	1:1	150	56	61	70	13	25	–
$U^{13}C_{21}$ NAD^+	–	RP-LC	1:1	683	555	–	−94	−13	–	$U^{12}C_{21}$ NAD^+
$U^{12}C_{21}$ NAD^+	–	RP-LC	1:1	662	540	273	−94	−13	−37	–
$U^{13}C_{21}$ $NADP^+$	–	RP-LC	1:1	763	635	418	−104	−13	−33	$U^{12}C_{21}$ $NADP^+$
$U^{12}C_{21}$ $NADP^+$	–	RP-LC	1:1	742	620	408	−104	−13	−33	–
$U^{13}C_3$ PEP	–	RP-LC	1:1	170	79	–	−70	−9	–	$U^{12}C_3$ PEP
$U^{12}C_3$ PEP	–	RP-LC	1:1	167	79	–	−70	−9	–	–
$U^{13}C_9$ Phenylalanine	+	RP-LC, HILIC	1:1	175	128	111	80	9	29	$U^{12}C_9$ Phenylalanine
$U^{12}C_9$ Phenylalanine	+	RP-LC, HILIC	1:1	166	120	103	80	9	29	–

Table 1. Continued.

Compound	Polarity	Chromatography	Dilution	Precursor	Quantifier	Qualifier	FV / V	$CE_{Quantifier}$ / V	$CE_{Qualifier}$ / V	Comp. for corr.
$U^{13}C_5$ Proline	+	RP-LC, HILIC	1:1	121	74	46	80	13	33	$U^{12}C_5$ Proline
$U^{12}C_5$ Proline	+	RP-LC, HILIC	1:1	116	70	43	80	13	33	–
$U^{12}C_{17}$ Riboflavin	–	RP-LC	1:1	375	255	–	−124	−13	–	–
$U^{13}C_3$ Serine	+	HILIC	1:1	109	62	44	60	9	21	$U^{12}C_3$ Serine
$U^{12}C_3$ Serine	+	HILIC	1:1	106	60	42	60	9	21	–
$U^{13}C_4$ Succinate	–	RP-LC	1:1	121	76	–	−80	−9	–	–
$U^{12}C_4$ Succinate	–	RP-LC	1:1	117	73	–	−80	−9	–	–
$U^{13}C_4$ Threonine	+	HILIC	1:1	124	77	69	60	9	13	$U^{12}C_4$ Threonine
$U^{12}C_4$ Threonine	+	HILIC	1:1	120	74	56	60	9	13	–
$U^{13}C_9$ Tyrosine	+	RP-LC, HILIC	1:1	191	144	98	80	9	29	$U^{12}C_9$ Tyrosine
$U^{12}C_9$ Tyrosine	+	RP-LC, HILIC	1:1	182	135	91	80	9	29	–
$U^{13}C_5$ Valine	+	RP-LC, HILIC	1:1	123	76	59	70	9	21	$U^{12}C_5$ Valine
$U^{12}C_5$ Valine	+	RP-LC, HILIC	1:1	118	72	55	70	9	21	–
$U^{13}C_9$ 5′UMP	–	RP-LC	1:1	332	79	97	−114	−40	−21	–
$U^{12}C_9$ 5′UMP	–	RP-LC	1:1	323	79	97	−114	−40	−21	–

Table 2. Chromatographic separation of 24 metabolites using Atlantis® reversed phase column

Compound	Polarity	t_R/min	k′	N
cis-Aconitate	–	7.4	3.23	38,400
3′AMP	–	7.2	3.09	48,000
5′AMP	–	6.2	2.54	36,000
Biotin	+	13.2	6.54	92,900
Citrate	–	6.3	2.60	17,900
5′CMP	–	3.6	1.06	13,500
Fumarate	–	6.7	2.83	24,400
3′GMP	–	7.8	3.46	44,600
5′GMP	–	6.6	2.77	46,600
Isocitrate	–	3.6	1.04	4700
Isoleucine	+	6.6	2.75	23,800
Leucine	+	6.8	2.90	24,300
Malate	–	3.4	0.95	5700
Methionine	+	4.4	1.52	6300
NAD^+	–	6.6	2.80	50,100
$NADP^+$	–	6.5	2.72	41,800
PEP	–	2.8	0.64	3300
Phenylalanine	+	9.0	7.28	55,800
Proline	+	2.5	0.43	2700
Riboflavin	–	13.9	6.94	12,6700
Succinate	–	6.9	2,94	36,600
Tyrosine	+	6.9	2.91	37,800
5′UMP	–	5.8	2.31	10,900
Valine	+	3.3	0.90	3900

Table 3. Chromatographic separation of 15 metabolites using ZicHILIC© separation column

Compound	Polarits	t_R / min	k′	N
Alanine	+	9.4	2.24	46,100
Arginine	+	11.8	3.06	52,600
Asparagine	+	9.8	2.38	68,900
Aspartate	+	9.7	2.33	55,000
Glutamate	+	9.4	2.26	47,600
Glutamine	+	9.6	2.31	54,400
Histidine	+	11.3	2.89	80,100
Lysine	+	11.6	3.01	76,600
Methionine	+	8.4	1.90	21,200
Phenylalanine	+	7.9	1.71	27,400
Proline	+	8.9	2.07	40,600
Serine	+	9.9	2.40	63,700
Threonine	+	9.5	2.28	52,400
Tyrosine	+	8.9	2.05	36,000
Valine	+	8.5	1.95	34,100

the corresponding monoisotopic metabolite response (area $U^{12}C/m_{sample}$) (Eq 4–7).

$$f = area\ U^{13}C * m_{sample}/area\ U^{12}C \quad (4)$$

$$R_{ex\,corr} = f①/f② \quad (5)$$

$$R_{ev\,corr} = f②/f③ \quad (6)$$

$$R_{ep\,corr} = f①/f③ \quad (7)$$

The sequence was set up by measuring the first replicate of the spikes and the control sample as block and measuring five blocks consecutively. Individual recoveries were calculated from each block and afterwards averaged.

2.10 Evaluation of total combined uncertainties

The terms measurand, standard uncertainty, and coverage factor are used according to the Guide to the Expression of Uncertainty in Measurement [23]. All combined uncertainties were calculated according to the ISO/GUM guide [24] using the uncertainty propagation procedure. Dedicated software (GUMworkbench software, Metrodata GmbH,

Table 4. Characterization of $U^{13}C$ cell extract by signal to noise ratio and peak area (dilution 1:5 in order to obtain same concentration as in the spike)

Compound	Polarity	Chromatography	S/N ($U^{12}C$, $n = 4$)	S/N ($U^{13}C$, $n = 4$)	RSD of area /% ($U^{13}C$, $n = 4$)	Used for recovery
cis-Aconitate	–	RP-LC	<2	n.a.	n.a.	x
Alanine	+	HILIC	<2	43	26	√
3′AMP	–	RP-LC	<2	8.2	14	x
5′AMP	–	RP-LC	<2	56	9	√
Arginine	+	HILIC	2.2	1400	20	√
Asparagine	+	HILIC	<2	57	17	√
Aspartate	+	HILIC	<2	370	23	√
Biotin	+	RP-LC	6.9	n.a.	n.a.	x
Citrate	–	RP-LC	4.3	67	10	√
5′CMP	–	RP-LC	<2	2.1	20	x
Fumarate	–	RP-LC	<2	2.7	23	x
Glutamate	+	HILIC	3.3	4800	18	√
Glutamine	+	HILIC	<2	2200	17	√
3′GMP	–	RP-LC	<2	5.1	16	x
5′GMP	–	RP-LC	<2	2.4	19	x
Histidine	+	HILIC	4.4	1000	24	√
Isocitrate	–	RP-LC	<2	10	10	√
Isoleucine	+	RP-LC	<2	190	12	√
Leucine	+	RP-LC	3.0	220	7	√
Lysine	+	HILIC	5.6	1800	25	√
Malate	–	RP-LC	<2	110	3	√
Methionine	+	RP-LC	3.8	6.5	18	x
Methionine	+	HILIC	<2	4.2	21	x
NAD^+	–	RP-LC	<2	9400	5	√
$NADP^+$	–	RP-LC	<2	410	3	√
PEP	–	RP-LC	<2	140	21	√
Phenylalanine	+	RP-LC	<2	180	9	√
Phenylalanine	+	HILIC	<2	400	20	√
Proline	+	RP-LC	<2	100	10	√
Proline	+	HILIC	<2	50	8	√
Riboflavin	–	RP-LC	<2	n.a.	n.a.	x
Serine	+	HILIC	5.3	77	21	√
Succinate	–	RP-LC	<2	2.5	23	x
Threonine	+	HILIC	2.1	200	22	√
Tyrosine	+	RP-LC	5.3	960	10	√
Tyrosine	+	HILIC	7.6	120	13	√
5′UMP	–	RP-LC	<2	5.2	7	x
Valine	+	RP-LC	2.2	44	11	√
Valine	+	HILIC	<2	280	21	√

Grenzach-Wyhlen, Germany) was employed for the calculations based on the numerical method of differentiation [25]. Accordingly, the uncertainty of the measurement result was determined from quantities through a functional relation called the measurement equation. The final uncertainty arises from the input uncertainties that enter the equation that were categorized in (i) Type A evaluation, i.e. method of evaluation of uncertainty by the statistical analysis of series of observations and (ii) Type B evaluation, i.e. method of evaluation of uncertainty by means other than the statistical analysis of series of observations. Hence, a Type B evaluation of standard uncertainty is usually based on scientific judgment using all of the relevant information available, which may include: previous measurement data, experience with, or general knowledge of, the behavior and property of relevant materials and instruments, manufacturer's specifications, data provided in calibration and other reports, and uncertainties assigned to reference data taken from handbooks.

3 Results and discussion

3.1 Optimization of LC-MS/MS

The assembled set of target analytes comprised a broad range of primary metabolites such as amino acids, organic acids,

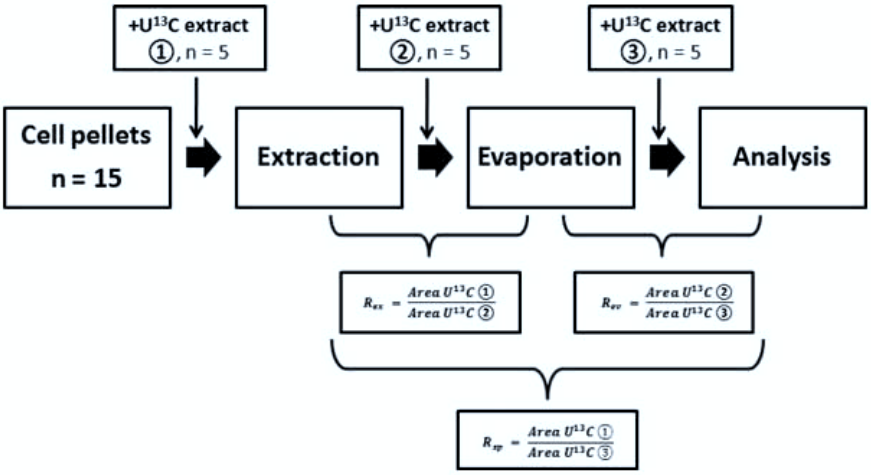

Figure 1. Experimental setup for determination of recoveries of cell extraction, evaporation of the extraction solvent, and overall sample preparation.

nucleotides, cofactors, and vitamins. The complete list of target metabolites is given in Table 4. One objective of the present work was the development of two orthogonal chromatographic separation methods, covering as much of these analytes as possible while avoiding the use of ion pair reagents.

First, the specific SRM transitions for recovery evaluation (quantifier) and for identification (qualifier) were optimized on the LC Triple Quad using single standards and monoisotopic ^{12}C ($U^{12}C$) mass isotopolgues. The transitions for the corresponding uniformly ^{13}C-labeled ($U^{13}C$) metabolites were not optimized individually but calculated from the $U^{12}C$ transitions. For few compounds the $U^{13}C$ transitions ($U^{13}C_6$ cis-aconitate, $U^{13}C_{10}$ biotin, $U^{13}C_{17}$ riboflavin) could not be confirmed because of very low concentrations in the cell extract. All SRM settings and the chosen polarity for the measured metabolites are listed in Table 1 and *m/z* values of $U^{12}C$ and $U^{13}C$ precursor ions and fragment ions are shown.

A screening procedure of various chromatographic phases was performed. Among the tested stationary phases were silica based HILIC phases and silica-based phases tested under RP gradient conditions as well as phases based on graphitized carbon (Luna NH_2® from Phenomenex®, Mediterranea™ Sea_{18} from Teknokroma, Atlantis T3® from Waters®, ZicHILIC© from SeQuant, and Hypercarb™ from Thermo Scientific). A reversed phase separation using a 100% wettable C_{18} phase (Atlantis T3®) showed promising peak capacity for acidic compounds (organic acids, nucleotides) and for less hydrophilic metabolites (amino acids with apolar side chains, vitamins). A comparable method based on the same chromatographic system was developed in our group for analysis of nucleotides, nucleosides, and nucleobases in feed supplements [26]. Applying the optimized chromatographic condition, and using the gradient program described before, resulted an excellent chromatography for a wide range of small analytes having apolar or acidic nature within a total runtime of 20 min. The retention times, capacity factors, and theoretical plate numbers are listed in Table 2. As an outstanding feature, this method is able to separate many isobaric compounds. Ribonucleotides that differ in their phosphorylation site but lack-specific transitions are baseline separated. This is shown for 5′AMP and 3′AMP—retention times are 6.2 min and 7.2 min, and for 5′GMP and 3′GMP—retention times are 6.6 min and 7.8 min, respectively. One pair of isomer metabolites prominent in the central carbon metabolism comprises citrate and isocitrate. These organic acids are well separated (t_R = 6.3 and 3.6) avoiding isobaric interferences. The isomer amino acids leucine and isoleucine can almost be baseline separated (t_R = 6.8 and 6.6) that allows accurate peak integration of both amino acids from the same transition. The distinction of the two isomers via monitoring of selective transitions (*m/z* 86.0 → 43 and *m/z* 86 → 57 for leucine and isoleucine, respectively) was not utilized since 86.0 is no $[M+H]^+$ -ion but an in-source fragment and loss in sensitivity was observed when compared with transition from the respective $[M+H]^+$ -ion. The optimized RP-LC method was finally applied to the aqueous $U^{13}C$-labeled cell extract. The resulting chromatograms are presented in Fig. 1 in the Supporting Material.

Despite of the outstanding performance of the described RP chromatography for metabolic profiling, it cannot be used as a single analysis method due to weak retention of highly polar analytes. The narrow elution window close to the void volume renders analysis susceptible to matrix effects due to ion suppression. Additionally poor selectivity has to be considered due to isobaric interferences in the void volume. This is even more relevant when analyzing in parallel the isotopically labeled metabolome and the metabolome in its natural monoisotopic form (uniformly ^{12}C, ^{1}H, ^{14}N, ^{16}O, ^{32}S, ^{31}P). The screening of the initial set of target analytes on the Atlantis T3® column revealed poor retention (with $k' < 1$) of mainly two substance groups: (i) phosphorylated sugars and phosphorylated hydroxy acids and (ii) polar amino acids. A comprehensive separation of phosphorylated sugars and hydroxy acids—which is required for their investigation—could not be achieved and thus only phosphoenolpyruvate (PEP) was

further surveyed. For amino acid analysis, a HILIC column was used, employing a zwitterionic-bonded silica phase. A comparable method based on the same chromatographic system was developed in our group for analysis for soil solutions [27]. Applying the optimized chromatographic condition, and using the gradient program described above, resulted in superior retention of amino acids, whereupon 15, including the positive charged arginine, leucine, and histidine could be separated within a total runtime of 20 min. The retention times, capacity factors, and theoretical plate numbers are listed in Table 3. HILIC separation of isobaric leucine and isoleucine was not in the focus of our work because of the satisfactory reversed phase separation. The optimized HILIC method was finally applied to the aqueous $U^{13}C$-labeled cell extract. The resulting chromatograms of $U^{13}C$ transitions are presented in Fig. 2 in the Supporting Material.

3.2 Characterization of the $U^{13}C$ cell extract

The in vivo synthesis of $U^{13}C$ metabolites using proper microorganisms cultivated on $U^{13}C$ substrates was introduced in metabolomics within the last decade for use as multifunctional internal standard [28] with the advantageous possibility of internal standardization of any desired metabolite in the proper concentration. In our lab a fully labeled ^{13}C cell extract was produced providing labeled metabolites used as tracer for evaluation of recoveries. Therefore the wild-type strain of the yeast *P. pastoris* was grown in aerobic, glucose limited fed batch culture. The fermentation strategy was an exponential fed batch under carbon-limited conditions with a growth rate of 0.1 h^{-1}. An isotopically labeled glucose ($U^{13}C_6$, purity = 98%) was used as sole carbon source for batch and fed batch medium as well as for the preculture medium to minimize ^{12}C impurities. Furthermore, other sources of ^{12}C contamination were excluded: the citrate used to buffer media was replaced by phosphate buffer and carbon dioxide was removed from inlet air to prevent the carbon dioxide fixation. Cell quenching was carried out by rapid sampling into cold methanol at conditions optimized for *P. pastoris* by Carnicer et al. [14] and cell extraction was carried out by boiling ethanol extraction comparable to the method proposed for *Saccharomyces cerevisiae* by Canelas et al. [15]. The cell extraction via boiling ethanol has become a standard method for investigation of yeasts and was recently applied to *P.pastoris* [14].

The direct addition of ethanolic cell extract to the measurement solution turned out to impede the reversed phase separation starting with 100% aqueous mobile phase. More precisely, the peaks in the retention time window from 5 to 7 min were shifted unpredictably. Therefore the ethanolic extract was evaporated under vacuum to dryness and reconstituted with water. The reconstitution step was also used to concentrate the extract ($f_{conc} = 4$). This aqueous $U^{13}C$ cell extract was used as spike in the recovery evaluation experiment. For quality assessment it was diluted as described above and measured as control sample in replicates. The extract was characterized according to three parameters—the signal to noise ratio (S/N) of the $U^{13}C$ metabolite, the relative standard deviation (RSD) of the $U^{13}C$ area, and the signal to noise ratio of the $U^{12}C$ impurity—which are summarized for all measured metabolites in Table 4. Looking at the $U^{13}C$ S/N values, the high dynamic range of intracellular concentrations can be readily observed (S/N range from < 2 to 10^4). In fact the $U^{13}C$ S/N value is a significant criterion to assess the applicability as metabolite standard. For the recovery study, compounds with ratios < 10 were excluded (Table 4). A number of ribonucleotides and organic acids did not fulfill the requirement whereas cis-aconitate, biotin, and riboflavin were < LOD. Minor $U^{12}C$ impurities (S/N < 10) are found for citrate, serine, and tyrosine while biotin is found only as unlabeled vitamin (essential for *P. pastoris*, component of the media) (Table 4).

3.3 Evaluation of recoveries of single sample preparation steps and overall sample preparation

A general sample preparation workflow for intracellular metabolic profiling in *P. pastoris* was established in our laboratories, considering the experience of recently published studies. It briefly consisted of rapid sampling from the bioreactor and quenching into cold methanolic solution, separation of quenched cells from supernatant by centrifugation, cell extraction using boiling ethanol, evaporation of the ethanolic extract, and reconstitution in water. Optimized quenching conditions (methanol content and temperature) were adopted from Carnicer et al. [14]. The contact time of cells with the quenching solution was not controlled and was up to 30 min with the applied cold centrifugation method. The boiling ethanol extraction was adapted from Mashego et al. [28] with addition of 4 mL of 75% ethanol instead of 5 mL. Cell extraction via boiling ethanol was already evaluated according to extraction efficiency and extraction recovery for *S. cerevisiae* and rated as one of two best extraction methods (beside cold chloroform–methanol) out of five methods for *S. cerevisiae* [15].

In this work, a fundamental study on extraction recoveries was carried out for *P. pastoris*. The addition of isotopically labeled metabolites at different stages of sample preparation and the comparison of the corresponding signals is a straightforward approach to evaluate metabolite specific recoveries of the sample preparation steps. As mentioned before the fully ^{13}C-labeled cell extract served as an ideal standard in the spiking experiments. This valuable tool provided a full list of labeled metabolites that matched the concentration pattern in the samples. Moreover, a homogeneous set of 15 cell pellets allowed investigating the 3-step procedure (extraction, extract evaporation, and LC-MS/MS analysis) in five replicates. Figure 1 shows the experimental setup used to address the recovery of the cell extraction (R_{ex}), of the extract treatment (evaporation of ethanolic solvent, R_{ev}) and of the overall sample preparation (R_{sp}). The $U^{13}C$ cell extract was spiked to $n = 5$ cell samples at three different steps at sample preparation and the obtained $U^{13}C$ peak areas were

Table 5. Observed recoveries and repeatability precisions of cell extraction (R_{ex}), evaporation of the extraction solvent (R_{ev}) and overall sample preparation (R_{sp}) obtained from ratios of $U^{13}C$ responses (no correction) and by correction with the corresponding $U^{12}C$ response and CDW of extracted cell pellet ($U^{12}C$ correction)

Compound	Chromatography	No correction			$U^{12}C$ correction		
		R_{ex} /%	R_{ev} /%	R_{sp} /%	$R_{ex\,corr}$ /%	$R_{ev\,corr}$ /%	$R_{sp\,corr}$ /%
Alanine	HILIC	105 ± 32	123 ± 60	115 ± 24	100 ± 6	115 ± 15	115 ± 18
5′AMP	RP-LC	94 ± 4	111 ± 5	105 ± 6	110 ± 13	123 ± 17	135 ± 19
Arginine	HILIC	116 ± 61	123 ± 71	115 ± 41	76 ± 1	105 ± 3	79 ± 2
Arginine	HILIC, dilution	85 ± 13	99 ± 15	83 ± 6	75 ± 2	105 ± 4	78 ± 2
Asparagine	HILIC	122 ± 56	123 ± 75	119 ± 23	92 ± 3	102 ± 5	94 ± 4
Aspartate	HILIC	99 ± 23	107 ± 37	100 ± 13	94 ± 3	102 ± 3	96 ± 5
Citrate	RP-LC	74 ± 5	100 ± 3	74 ± 5	74 ± 3	102 ± 5	75 ± 1
Glutamate	HILIC	101 ± 23	109 ± 40	103 ± 14	92 ± 2	102 ± 4	94 ± 4
Glutamate	HILIC, dilution	89 ± 16	100 ± 10	88 ± 13	92 ± 2	102 ± 6	94 ± 5
Glutamine	HILIC	107 ± 35	110 ± 54	104 ± 19	88 ± 2	96 ± 9	84 ± 7
Glutamine	HILIC, dilution	85 ± 21	96 ± 14	80 ± 17	87 ± 3	96 ± 10	83 ± 6
Histidine	HILIC	130 ± 76	132 ± 91	125 ± 41	85 ± 3	101 ± 4	85 ± 3
Histidine	HILIC, dilution	90 ± 19	95 ± 20	83 ± 12	87 ± 3	98 ± 8	85 ± 8
Isocitrate	RP-LC	83 ± 10	103 ± 14	86 ± 15	88 ± 11	113 ± 8	98 ± 7
Isoleucine	RP-LC	93 ± 4	99 ± 3	91 ± 4	98 ± 4	100 ± 6	98 ± 8
Leucine	RP-LC	90 ± 5	99 ± 2	89 ± 4	94 ± 3	100 ± 5	94 ± 7
Lysine	HILIC	95 ± 44	117 ± 62	94 ± 27	62 ± 3	107 ± 6	66 ± 5
Lysine	HILIC, dilution	73 ± 12	101 ± 15	72 ± 6	61 ± 3	109 ± 3	67 ± 2
Malate	RP-LC	85 ± 8	108 ± 4	92 ± 9	90 ± 10	109 ± 3	98 ± 13
NAD^+	RP-LC	79 ± 4	100 ± 4	78 ± 3	84 ± 12	92 ± 20	76 ± 10
$NADP^+$	RP-LC	75 ± 4	101 ± 5	76 ± 5	66 ± 5	92 ± 16	61 ± 10
PEP	RP-LC	81 ± 7	96 ± 7	77 ± 4	105 ± 8	90 ± 15	95 ± 22
Phenylalanine	RP-LC	86 ± 7	100 ± 2	86 ± 8	92 ± 1	104 ± 5	95 ± 4
Phenylalanine	HILIC	146 ± 86	133 ± 102	133 ± 34	93 ± 4	101 ± 7	94 ± 5
Proline	RP-LC	81 ± 14	103 ± 5	83 ± 16	94 ± 6	106 ± 7	99 ± 12
Proline	HILIC	112 ± 38	118 ± 66	115 ± 29	91 ± 4	109 ± 13	99 ± 13
Serine	HILIC	122 ± 58	122 ± 70	118 ± 22	90 ± 6	105 ± 8	95 ± 10
Threonine	HILIC	103 ± 40	112 ± 53	99 ± 15	91 ± 2	101 ± 4	92 ± 4
Tyrosine	RP-LC	87 ± 7	99 ± 2	86 ± 8	92 ± 3	103 ± 3	94 ± 3
Tyrosine	HILIC	109 ± 39	120 ± 71	111 ± 26	90 ± 3	106 ± 3	95 ± 2
Valine	RP-LC	83 ± 11	101 ± 2	83 ± 10	91 ± 5	102 ± 8	93 ± 8
Valine	HILIC	114 ± 57	125 ± 82	110 ± 22	94 ± 4	103 ± 8	96 ± 8

compared (Eqs 1–3). Furthermore, internal standardization with the monoisotopic signal ($U^{12}C$) deriving from the cell sample was applied (Eqs 4–7) and recoveries without correction and $U^{12}C$ corrected recoveries were compared. A prerequisite for $U^{12}C$ correction was the availability of homogenous samples and knowledge of the amount of sampled biomass; both requirements were fulfilled in this study. In this way, assessed recoveries could be corrected for instrumental drifts and sample volume losses during sample preparation.

The resulting recoveries—of cell extraction, of evaporation, and of overall sample preparation—are summarized for all the investigated metabolites, without and with $U^{12}C$ correction in Table 5. Several amino acids are listed twice: amino acids comprising apolar side chains (phe, pro, tyr, val) were measured on RP-LC-MS/MS as well as on HILIC-MS/MS; and high abundant amino acids (arg, glu, gln, his, lys) showing signal to noise ratios above 10^3 (Table 4) were measured in an additional 1:10 dilution prior to analysis. As can be readily observed, the investigated metabolites showed excellent recoveries and repeatability precision over the sample preparation procedure. Even the extract treatment step, necessary for subsequent RP-LC-MS/MS measurement showed no significant influence. The values obtained by complementary HILIC and RP-LC-MS/MS analysis were in excellent agreement after internal standardization. Moreover, the relatively poor standard uncertainty obtained by HILIC-MS/MS confirmed the necessity of internal standardization. This regarded especially results obtained from not corrected peak areas measured via HILIC without a further dilution step (see also Table 4, poor precision of $U^{13}C$ areas obtained by HILIC). Higher matrix effects in HILIC compared to RP-LC were the cause. Only five metabolites showed overall recoveries ranging from 60 to 80%: arginine, citrate, lysine, NAD^+, and $NADP^+$. Nevertheless they may be extracted via boiling ethanol and quantified

Table 6. Characterization and quantification of uncertainty sources for sample preparation and quantification of intracellular 5′AMP

Input quantities	Values	RSD	Uncertainty	Unit	Type, distribution	SU	Rel. contr. to comb. unc.
$c_{released}$	0.16	15%[a]	0.024	μmol g_{CDW}^{-1}	A, normal	0.024	35.4%
CDW	4.5	2.2%[b]	0.099	g_{CDW} L^{-1}	A, normal	0.099	0.8%
V_{Sample}	0.002		$2\,10^{-5}$ [c]	L	B, triangular	$8.16\,10^{-6}$	0.0%
n_{IS}	0.00156		$3.12\,10^{-5}$ [c]	μmol	B, triangular	$1.56\,10^{-5}$	0.1%
L	1		0.02[d]		B, rectangular	0.0115	0.2%
R	1.35		0.19		A, normal	0.19	31.1%
R_{IS}	1.35		0.19		A, normal	0.19	32.4%

a) Estimated from published data.
b) Based on the repeatability of the CDW determination, $n = 5$.
c) Uncertainties derived from the manufacturers specification of the pipettes.
d) Experiments in our lab addressing leakage showed that its contribution is negligible (≤ 2%) if quenching is optimized and cells are separated instantaneously.

correctly when adding the specific internal standard prior to extraction. It has to be underlined here that the sample preparation was not optimized for accurate and simultaneous measurement of redox pairs e.g. NAD/NAD^+ or NADP/$NADP^+$.

3.4 Calculation of sample preparation uncertainties for intracellular metabolite quantification in yeast

The accuracy of LC-MS/MS-based quantitative analysis in metabolomics, though rarely discussed, is a major issue. There is a complete lack of reference materials. Only recently, an overview of uncertainty of measurement in quantitative metabolomics including practical examples was discussed by Guerrasio et al. [13]. Uncertainty calculations are valuable tools for systematically addressing the total combined uncertainty of a quantification task, assessing the uncertainty of each step in the analytical process and its contribution to the total combined uncertainty. Different approaches can be used to calculate the uncertainty of a system: the GUM [24] provides a very detailed bottom-up approach to estimate the combined standard uncertainty of the method. The stepwise procedure starts with the definitions of the measurand (i.e. the particular quantity subject to measurement), of the input quantities, and of the model equation. In this way, all possible sources of uncertainty are identified and the standard uncertainties of each input quantity are evaluated. The value of the measurand and the combined standard uncertainty of the result are calculated, the expanded uncertainty is calculated using a selected coverage factor (k) and finally the result is reported with the expanded uncertainty. Furthermore the uncertainty contributions are analyzed.

In this work, the total combined uncertainty of sample preparation was estimated based on the experimental data obtained in the extraction recovery studies. Accordingly, the compiled model equation took into account the sources of uncertainty of the sample preparation but not of LC-MS/MS measurement. In Eq 8, the measurand was defined as the ratio of the metabolite concentration versus concentration of the corresponding labeled metabolite in the obtained measurement solution ($c_{final}/c_{final\,IS}$). This ratio is given by the concentration released from the cell upon extraction ($c_{released}$), hence by the extraction efficiency, by the cell dry weight of the quenched cell suspension (CDW), by the volume of sampled quenched cell suspension (V_{Sample}), by the amount of added internal standard (n_{IS}), by leakage (L), and by the recoveries of the sample preparation procedure of the metabolite and the labeled metabolite (R/R_{IS}).

$$\frac{c_{final}}{c_{final\,IS}} = c_{released} \times CDW \times \frac{V_{Sample}}{n_{IS}} \times L \times \frac{R}{R_{IS}} \qquad (8)$$

c_{final}	μmol L^{-1}	Final concentration of the metabolite in the measurement solution
$c_{final\,IS}$	μmol L^{-1}	Final concentration of the internal standard in the measurement solution
$c_{released}$	μmol g_{CDW}^{-1}	Intracellular concentration of the metabolite released by extraction
CDW	g_{CDW} L^{-1}	Cell dry weight of quenched cell suspension
V_{Sample}	L	Volume of quenched cell suspension, which is sampled
n_{IS}	μmol	Amount of internal standard spiked before extraction
L		Leakage factor
R		Recovery of sample preparation of the metabolite
R_{IS}		Recovery of sample preparation of the internal standard

Uncertainty budgeting was carried out using the example of three representative metabolites showing different extraction efficiencies and extraction recoveries, namely 5′AMP, citrate, and phenylalanine. The assumed values for $c_{released}$ are the expected intracellular concentrations of the metabolite released by the applied boiling ethanol extraction and the corresponding standard uncertainties (SU) are estimations of the extraction efficiencies of published data [15].

Table 7. Characterization and quantification of uncertainty sources for sample preparation and quantification of intracellular citrate

Input quantities	Values	RSD	Uncertainty	Unit	Type, distribution	SU	Rel. contr. to comb. unc.
$c_{released}$	4.35	5%[a]	0.218	$\mu mol\ g_{CDW}^{-1}$	A, normal	0.218	70.3%
CDW	4.5	2.2%[b]	0.099	$g_{CDW}\ L^{-1}$	A, normal	0.099	13.6%
V_{Sample}	0.002		$2\ 10^{-5}$[c]	L	B, triangular	$8.16\ 10^{-6}$	0.5%
n_{IS}	0.0235		$4.70\ 10^{-4}$ [c]	µmol	B, triangular	$1.92\ 10^{-4}$	1.9%
L	1		0.02[d]		B, rectangular	0.0115	3.7%
R	0.75		0.01		A, normal	0.01	5.0%
R_{IS}	0.75		0.01		A, normal	0.01	5.0%

a) Estimated from published data.
b) Based on the repeatability of the CDW determination, $n = 5$.
c) Uncertainties derived from the manufacturers specification of the pipettes.
d) Experiments in our lab addressing leakage showed that its contribution is negligible (≤ 2%) if quenching is optimized and cells are separated instantaneously.

The assumed values of n_{IS} are the expected concentrations of labeled metabolite in the extract and the SU, as well as the SU of the quenched sample volume, derive from the manufacturers specification of the pipettes. The SU of cell dry weight determination is based on the repeatability of the CDW determination ($n = 5$). Values and SU of recoveries are taken from present experiment and leakage was assumed to be minimal when quenching conditions and contact time of cells and quenching solution is optimized. The identified sources of uncertainty which are present in the model equation are characterized and quantified for the three exemplary metabolites (Tables 6–8) and the resulting absolute and relative standard uncertainties for sample preparation for the three representative intracellular metabolites are summarized in Table 9. In order to illustrate the contribution of extraction efficiency and recovery, we selected exemplary metabolites with reportedly high uncertainty in extraction efficiency and assessed extraction recovery (5′AMP), low uncertainty in both efficiency and recovery (citrate) and moderate uncertainty in extraction efficiency and low uncertainty in recovery (phenylalanine). For 5′AMP a relative high standard uncertainty for extraction efficiency of approximately 15% was reported. Recovery SU was found to range at 20%. The obtained expanded total combined uncertainty of 50% ($k = 2$) deriving from sample preparation

Table 8. Characterization and quantification of uncertainty sources for sample preparation and quantification of intracellular phenylalanine

Input quantities	Values	RSD	Uncertainty	Unit	Type, distribution	SU	Rel. contr. to comb. unc.
$c_{released}$	0.33	10%[a]	0.033	$\mu mol\ g_{CDW}^{-1}$	A, normal	0.033	70.2%
CDW	4.5	2.2%[b]	0.099	$g_{CDW}\ L^{-1}$	A, normal	0.099	3.4%
V_{Sample}	0.002		$2\ 10^{-5}$ [c]	L	B, triangular	$8.16\ 10^{-6}$	0.1%
n_{IS}	0.00226		$4.52\ 10^{-5}$ [c]	µmol	B, triangular	$1.85\ 10^{-5}$	0.5%
L	1		0.02[d]		B, rectangular	0.0115	0.9%
R_{U12C}	0.95		0.04		A, normal	0.04	12.4%
R_{U13C}	0.95		0.04		A, normal	0.04	12.5%

a) Estimated from published data.
b) Based on the repeatability of the CDW determination, $n = 5$.
c) Uncertainties derived from the manufacturers specification of the pipettes.
d) Experiments in our lab addressing leakage showed that its contribution is negligible (≤ 2%) if quenching is optimized and cells are separated instantaneously.

Table 9. Uncertainties for sample preparation for three representative intracellular metabolites

Compound	C_{final} / $C_{final\ IS}$	Unit	Expanded uncertainty	Expand. unc. (relative)	Coverage factor (k)
5′AMP	0.92	$\mu mol\ L^{-1}/\mu mol\ L^{-1}$	0.47	50%	2.0
Citrate	1.67	$\mu mol\ L^{-1}/\mu mol\ L^{-1}$	0.20	12%	2.0
Phenylalanine	1.31	$\mu mol\ L^{-1}/\mu mol\ L^{-1}$	0.31	24%	2.0

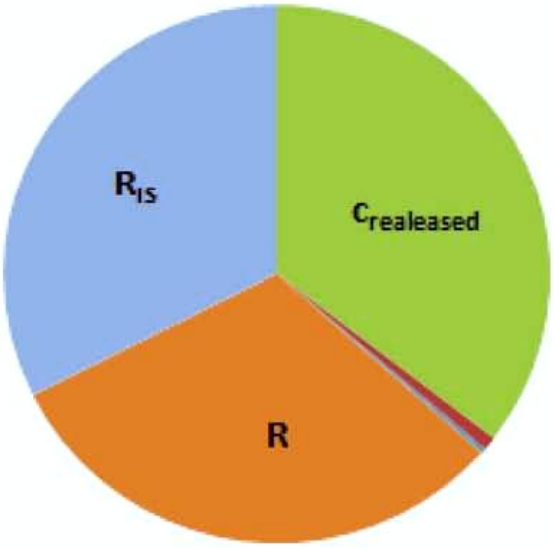

Figure 2. Relative contributions to combined standard uncertainty for 5′AMP.

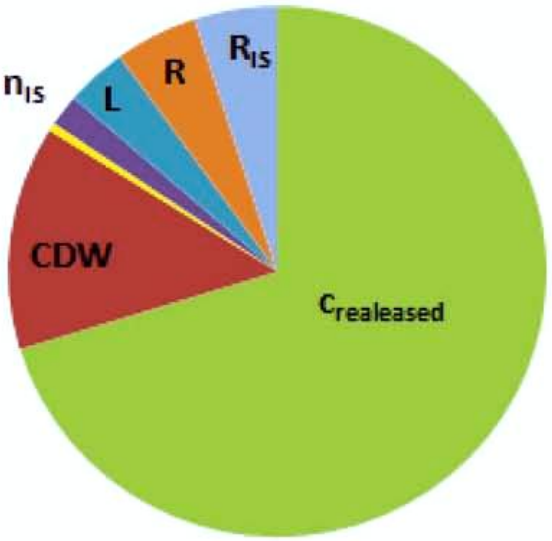

Figure 3. Relative contributions to combined standard uncertainty for citrate.

procedure characterized AMP as a very critical compound in sample preparation. For citrate a SU of approximately 5% was reported for extraction efficiency and the recovery of sample preparation was found to be 75% but with high precision. Accordingly, the obtained expanded total combined uncertainty of 12% corresponded to the minimum contribution to be expected from sample preparation in quantitative metabolic profiling. Finally, approximately 10% SU in extraction efficiency was reported for phenylalanine. The experimentally assessed recovery of sample preparation was found to be 95% ± 4%. Uncertainty calculation revealed a total combined uncertainty of 24% ($k = 2$). This was considered as an average uncertainty stemming from sample in quantitative metabolic profiling. The relative contributions to total combined standard uncertainties are illustrated in Figs. 2–4. In all three cases, the major contribution derives from extraction efficiency ($c_{released}$) whereas recovery is critical for 5′AMP and phenylalanine.

Result (5′AMP): $$c_{final}/c_{final\ IS} = 0.92\,\frac{\mu mol_{metabolite} \times L^{-1}}{\mu mol_{IS} \times L^{-1}}$$

Expanded uncertainty (5′ AMP, $k = 2$):

$$U = 0.47\,\frac{\mu mol_{metabolite} \times L^{-1}}{\mu mol_{IS} \times L^{-1}}$$

Result (citrate): $$c_{final}/c_{final\ IS} = 1.67\,\frac{\mu mol_{metabolite} \times L^{-1}}{\mu mol_{IS} \times L^{-1}}$$

Expanded uncertainty (citrate, $k = 2$):

$$U = 0.20\,\frac{\mu mol_{metabolite} \times L^{-1}}{\mu mol_{IS} \times L^{-1}}$$

Result (phenylalanine):

$$c_{final}/c_{final\ IS} = 1.31\,\frac{\mu mol_{metabolite} \times L^{-1}}{\mu mol_{IS} \times L^{-1}}$$

Expanded uncertainty (phenylalanine, $k = 2$):

$$U = 0.31\,\frac{\mu mol_{metabolite} \times L^{-1}}{\mu mol_{IS} \times L^{-1}}$$

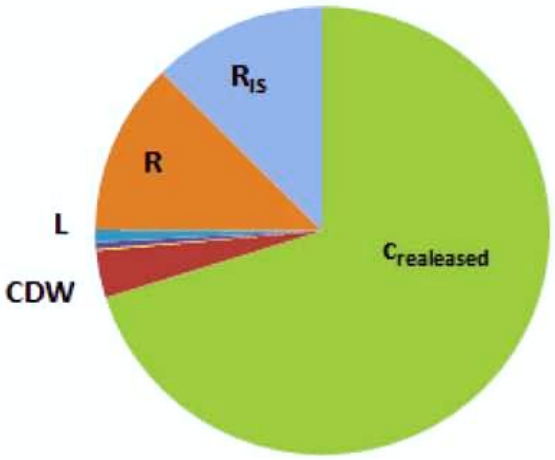

Figure 4. Relative contributions to combined standard uncertainty for phenylalanine.

4 Concluding remarks

The use of two orthogonal chromatographic modes (RP-LC, HILIC) and highly selective triple quadrupole MS enabled the measurement of a wide range of intracellular metabolites (amino acids, organic acids, nucleotides, and cofactors). At the stage of method implementation, $U^{13}C$-labeled cell extract was used as a tool for studying the recovery and standard uncertainty of each step in the sample preparation process. We obtained overall repeatability precisions less than 10% for most of the investigated metabolites using internal standardization approaches. Setting up model equations for uncertainty budgeting, error propagation for all input variables for sample preparation procedure, pinpointed the extraction efficiency, and the extraction recovery as the main sources contributing to total combined uncertainty.

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Supporting Material

$U^{13}C$ cell extract of *Pichia pastoris* – a powerful tool for evaluation of sample preparation in metabolomics

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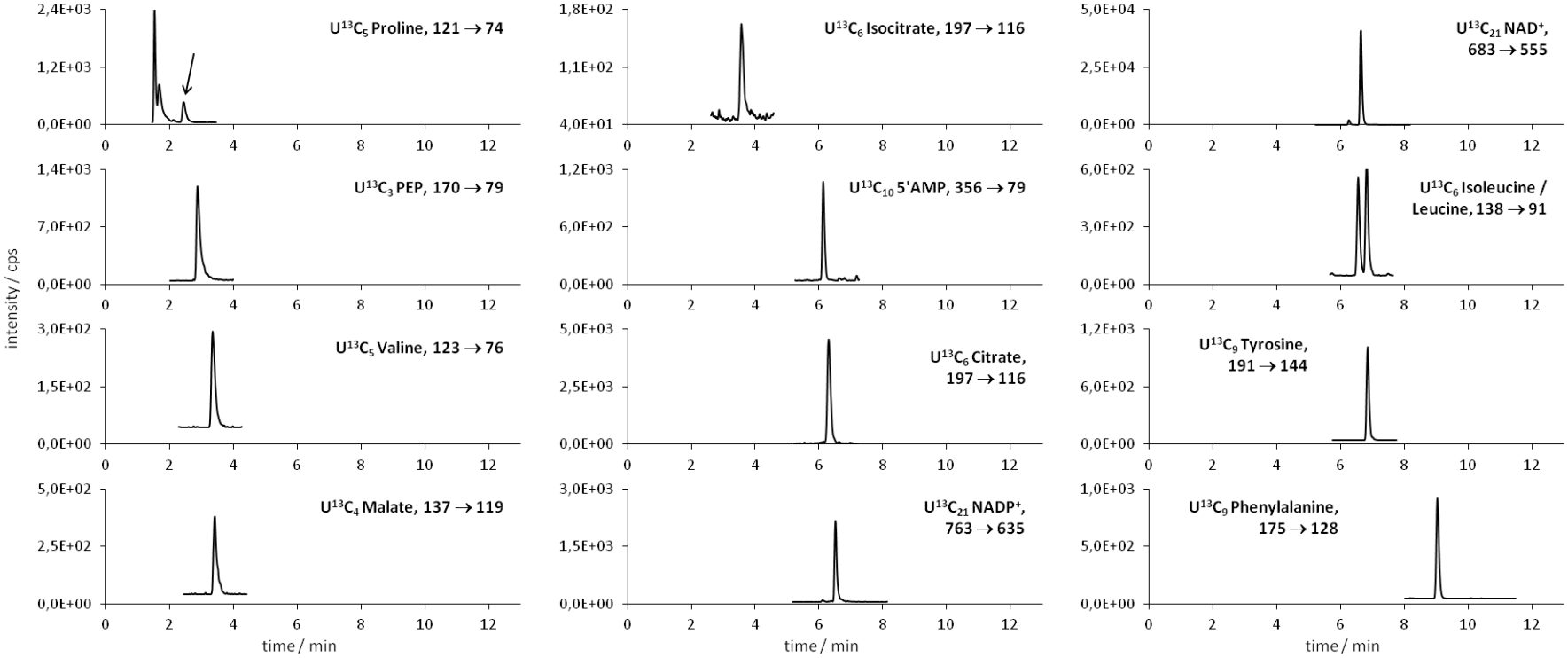

Fig.1. Extracted chromatograms of $U^{13}C$ transitions from a fully labeled cell extract applying the proposed RP-LC-MS/MS method.

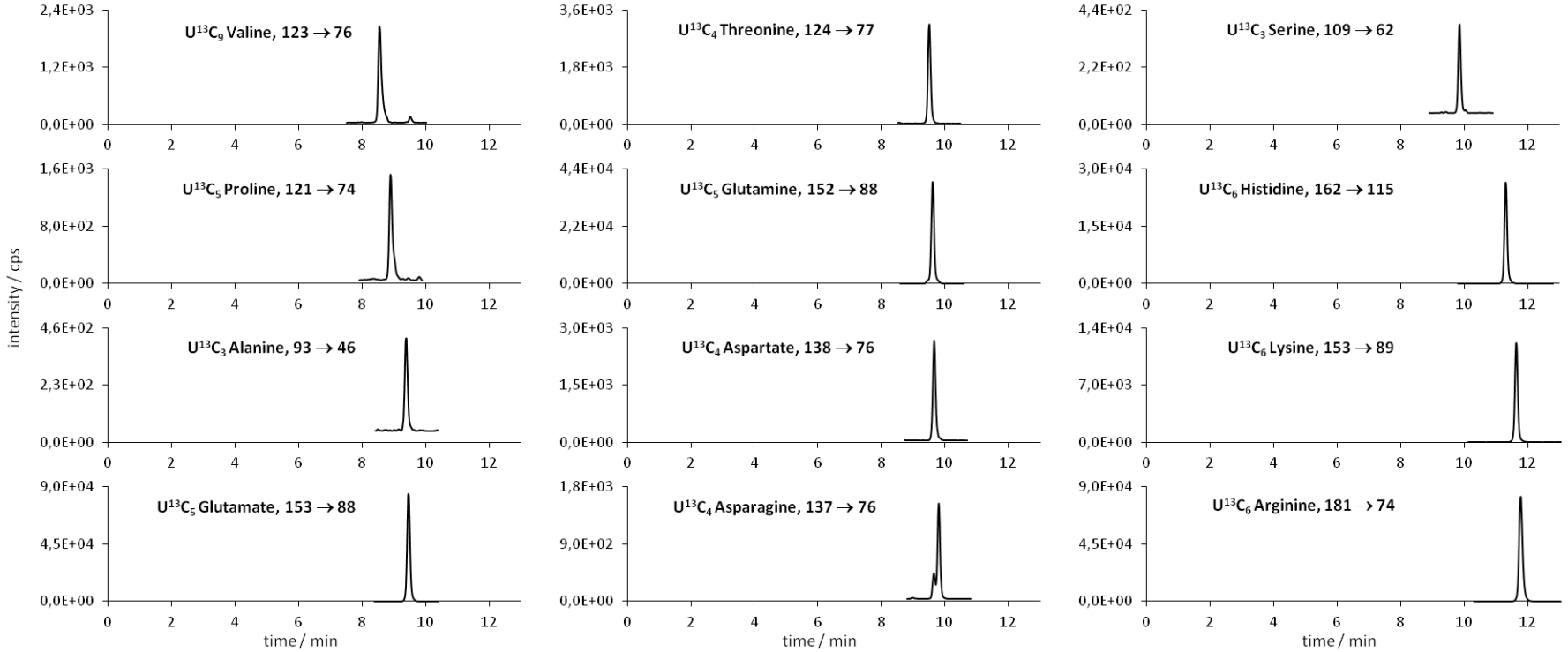

Fig.1. Extracted chromatograms of $U^{13}C$ transitions from a fully labeled cell extract and applying the proposed HILIC-MS/MS method.

CONCLUSION

The two presented methods deal with quantification of microbial metabolites in different sample matrices. The first presented method is applied in nucleotide enriched yeast autolysates provided in freeze dried powderous form. Sample preparation is simple and straightforward, and the main focus is put, due to the absence of certified reference material, on the development and validation of two complementary mass spectrometric methods for nucleotide determination. A rapid and efficient RP-LC separation was found and coupled to ESI-MS/MS and phosphorus detection via ICP-DRC-MS. Although the obtained detection limits were 1 to 2 orders of magnitude higher for elemental MS compared to molecular MS, quantitative results were in good agreement. Furthermore, the LC-MS/MS method is able to determine nucleosides and nucleobases, the breakdown products of nucleotides.

The second method deals with quantification of various intracellular metabolites (amino acids, organic acids, nucleotides, vitamins). In this case, the reliable and accurate quantification requires sophisticated protocols for sampling, sample preparation and analysis. A workflow procedure is presented for the yeast *Pichia pastoris* comprising fast sampling from the bioreactor, cell quenching, sample preparation including cell extraction as well as analysis using RP-LC, HILIC and triple quadrupole MS. Furthermore a $U^{13}C$ labeled cell extract is produced and used as a tool for studying the recovery and standard uncertainty of cell extraction and extract treatment. The obtained recoveries of the sample preparation procedure (extraction and extract treatment) are between 60 % and 100 % with repeatability precisions below 10 % for most of the investigated metabolites. Finally, the sample preparation process was evaluated by assessment of measurement uncertainty. For the optimum case (metabolite easy to extract) a total combined uncertainty of 12 % could be achieved. It could be shown that the extraction efficiency and the extraction recovery are the main uncertainty sources contributing to the total combined uncertainty.

Curriculum Vitae

Personal Data

Name	Stefan Neubauer
Date of Birth	May 18th 1981
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Father	Franz Neubauer, commercial executive
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Tertiary Education

Sep. 2009 – present	PhD studies at the University of Natural Resources and Life Sciences – BOKU Vienna, Department of Chemistry, Division of Analytical Chemistry under supervision by Ao. Prof. Dr. Gunda Koellensperger. Work title: "Metabolomics in yeast by LC-MS"
Sep. 2001 – Sep. 2008	Diploma studies in Food Technology and Biotechnology at the University of Natural Resources and Life Sciences – BOKU Vienna.
Sep. 2008 – Sep. 2009	Diploma thesis under supervision by Ao. Prof. Dr. Gunda Koellensperger at the University of Natural Resources and Life Sciences – BOKU Vienna, Department of Chemistry, Division of Analytical Chemistry, topic: LC-MS analysis of nucleobases, nucleosides and nucleotides in yeast extracts.

Language Skills

German	Native speaker
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Work experience

July 2005 – Aug. 2008	Austrian Research Centers Seibersdorf. Assistance during study (ca. 2 months per year). Chemical laboratory, stability tests of pharmaceutical products, working under good manufacturing practice (GMP) system
Sep. 2003	Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau Klosterneuburg. Holiday work in chemikal laboratory, testing wine and fruit juices.

Conference Contributions and Publications

Oral Presentations

Neubauer S., Guerrasio R., Klavins K., Haberhauer-Troyer C., Koellensperger G., Hann S., *Metabolomics in Pichia pastoris*. Pumpaya Meeting 2011, May 6 – 7 2011, Köszeg, Hungary.

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Poster Presentations

Neubauer S., Klavins K., Haberhauer-Troyer C., Steiger M.G., Gasser B., Sauer M., Mattanovich D., Hann S., Koellensperger G., *Evaluation of recoveries of cell extraction and overall sample preparation using a $U^{13}C$ cell extract.* 60th ASMS Conference on Mass Spectrometry and Allied Topics, May 20 – 24 2012, Vancouver, Canada.

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Guerrasio, R., Haberhauer-Troyer, C., Neubauer, S., Klavins, K., Werneth, M., Koellensperger, G., Hann, S., *Uncertainty of Measurement in Quantitative Metabolomics*. In: M. Lämmerhofer, W. Weckwerth /eds.), Metabolomics in Practice, Wiley, Weinheim, 2012.