

Quantitation of intracellular purine intermediates in different *Corynebacteria* using electrospray LC-MS/MS

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Abstract Intermediates of the purine biosynthesis pathway play key roles in cellular metabolism including nucleic acid synthesis and signal mediation. In addition, they are also of major interest to the biotechnological industry as several intermediates either possess flavor-enhancing characteristics or are applied in medical therapy. In this study, we have developed an analytical method for quantitation of 12 intermediates from the purine biosynthesis pathway including important nucleotides and their corresponding nucleosides and nucleobases. The approach comprised a single-step acidic extraction/quenching procedure, followed by quantitative electrospray LC-MS/MS analysis. The assay was validated in terms of accuracy, precision, reproducibility, and applicability for complex biological matrices. The method was subsequently applied for determination of free intracellular pool sizes of purine biosynthetic pathway intermediates in the two Gram-positive bacteria *Corynebacterium glutamicum* and *Corynebacterium ammoniagenes*. Importantly, no ion pair reagents were applied in this approach as usually required for liquid chromatography analysis of large classes of diverse metabolites.

Keywords Biological samples · HPLC · Mass spectrometry · Nucleic acid sampling · Quantitative extraction · Bioanalytical methods

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Introduction

Nucleotides and their corresponding nucleosides and nucleobases are essential biomolecules and fulfill various functions in the living cell. In addition to being central building blocks for DNA and RNA [1], they also act as co-substrates in enzymatic reactions, play crucial roles in activating metabolites, and serve as signal mediators [2]. Furthermore, several intermediates of the purine biosynthesis pathway exhibit additional characteristics—ranging from flavor-enhancing qualities (inosine monophosphate (IMP) and guanosine monophosphate (GMP) [3, 4]) to drug-assisted therapy (e.g., inosine and purine analogues [5, 6]). These properties make them attractive targets for the pharmaceutical and biotechnological industries. Particularly the fermentative production using *Corynebacteria* (e.g., *Corynebacterium ammoniagenes*) has been successfully performed over the past decades [7–10]. As a result, quantifying these substances from biological matrices such as bacteria is crucial to assess the potential of the underlying cellular systems.

The quantitative determination of these compounds, however, is very challenging due to their diverse chemical properties [2], the possibility for chemical and biological degradation during extraction and sample treatment, and the wide concentration range observed in biological systems [1, 11]. Over the past several decades, quantitation of nucleotide concentrations has been performed by liquid chromatography and UV detection. The poor retention of phosphorylated polar substances on conventional reversed-phase HPLC columns using mixtures of water and organic solvents as mobile phases led to the frequent application of ion-exchange chromatography (IEC). IEC using high-salt concentrations was first described in the 1950s [12]. Long run times [11, 13] and incompatibility with mass spectrometry, however, have limited its application for nucleotide quantification mostly to in vitro studies [14].

Alternatives are ion suppression HPLC [15] and ion-pairing chromatography. The latter has been used for many years with a broad application range in nucleotide analysis [2, 5, 11, 16–19]. The addition of cationic reagents such as alkylamines leads to formation of adducts between negatively charged nucleotides and positively charged ion pair reagent, increasing the retention factors for these compounds and thus improving retention. In addition to this *classical ion-pairing* model, which describes ion formation solely as a mobile phase phenomenon, an alternate *dynamic ion-exchange* model has been proposed [20] utilizing stationary phase modification processes. Here, modification of the stationary phase is attributed to active adsorption of the ion-pairing reagent to the stationary phase, in turn modifying its surface charge and providing ion-exchange sites for analyte ions [21]. The extent to which these two alternate mechanisms are contributing to analyte retention has been shown to depend on experimental conditions [22].

Although HPLC/UV systems have been successfully applied [23–25], the increasing need for higher sensitivity and improved selectivity has prompted the use of LC-MS approaches in this field.

High-salt conditions required for strong anion-exchange chromatography (SAX) and the non-volatile nature of ion-pairing reagents such as tetraalkylamines and ammonium phosphates are not readily compatible with LC-MS detection systems [26, 27]. Thus, new alternative separation methods have been developed including HPLC coupled to electrochemical detection [28] or CE-MS [29].

Nevertheless, LC-MS remains the method of choice for quantification of nucleotides in complex biological matrices. Since even low concentrations of non-volatile ion-pairing reagents such as trialkylamines and dialkylamines lead to a contamination of the ion source [30], careful choice of the ion-pairing reagent is crucial for hyphenation ion pair separations to MS [2]. Considerable efforts have been made to improve the compatibility of ion-pairing reagents for LC-MS over the recent years, usually based on volatile ion pair reagents. This led to simplified routines with significantly reduced operator intervention. Even though reduced ion pair concentrations have been reported by several groups [2, 31–33], developing a method for quantitative determination of nucleotides, nucleosides, and nucleobases from complex biological samples without any ion pairing reagent has, to our knowledge, not been reported yet.

The aim of the present study therefore was the development of a LC-MS method for targeted metabolite profiling of the purine biosynthesis pathway in bacteria, without the need for any ion-pairing reagents in the mobile phase. The assay allowed the quantitative determination of 12 intermediates of this pathway in complex biological samples in less than 15 min run time.

Materials and methods

Chemicals and reagents

Ninety-nine percent ^{13}C enriched [$\text{U-}^{13}\text{C}_6$] glucose was purchased from Cambridge Isotope Laboratories (Andover, Mass., USA). Ultrapure water was obtained from a Milli-Q purifier (Millipore, Eschborn, Germany) and HPLC-grade acetonitrile was purchased from Sigma Aldrich (Taufkirchen, Germany). All other chemicals and reagents of analytical grade were obtained from Sigma Aldrich (Taufkirchen, Germany).

Standard mixtures, quality control (QC), and biological samples

Standard mixtures and QC samples

Standards were prepared for guanosine 5'-monophosphate (GMP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), xanthosine 5'-monophosphate (XMP), adenosine, guanosine, inosine, xanthosine, adenine, guanine, hypoxanthine, and xanthine in ultrapure water (1 mM each). Subsequently all compounds were mixed and diluted with perchloric acid (PCA, final concentration 500 mM) to give a final concentration of 100 μM of each compound. A series of working standard solutions for external calibration and a set of quality control (QC) samples were prepared by adding increasing amounts of the standard mixture to the ^{13}C -labeled cell extract reaching concentration ranges from 0.001 to 5 μM .

Biological samples

Wild type strains *Corynebacterium glutamicum* (ATCC 13032) and *C. ammoniagenes* (DSM 20306) were purchased from the American Type Culture Collection (Manassas, VA, USA) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Precultures and main cultures were performed using an orbital shaker (Multitron 2, Infors AG, Bottmingen, Switzerland) at 230 rpm and 30 °C on minimal medium (pH 7.2) containing (per liter): 15 g glucose, 4 g KH_2PO_4 , 16 g Na_2HPO_4 , 500 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 300 mg 3,4-dihydroxybenzoic acid, 100 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 100 μg cyanocobalamin, 750 μg thiamine, 4 μg pyridoxal phosphate, 100 μg biotin, 400 μg calcium pantothenate, 2 μg folic acid, 400 μg nicotinic acid, 200 μg 4-aminobenzoic acid, 400 μg pyridoxine \cdot HCl, 2 mg inositol, 10 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 1 mg ZnCl_2 , 100 μg CuCl_2 , 20 μg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 20 μg $\text{Na}_2\text{MoO}_4 \cdot 2 \cdot \text{H}_2\text{O}$, and 10 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. Cultures of *C. ammoniagenes* DSM 20306 were supplied additionally with 300 mgL^{-1} methionine. *C. glutamicum* and *C. ammoniagenes* main cultures were inoculated from overnight precultures with initial optical

densities of 0.5 and 0.8, respectively (660 nm, Novospec II, Pharmacia Biotech, Uppsala, Sweden). Cultivations were carried out in 500 mL baffled shake flasks using 10 % liquid volume.

Cells were grown until an optical density of 6.5 and 11.8 for *C. glutamicum*, and 4.0 and 8.0 for *C. ammoniagenes*. During this period, cells grew exponentially under balanced growth conditions [34] and concentrations of internal metabolites were expected to be constant assuming a quasi-steady-state.

¹³C-labeled biomass extract

Wild type *C. glutamicum* ATCC 13032 precultures were performed under the same conditions as mentioned above, except using [U-¹³C₆] glucose. Cells were grown on [U-¹³C₆] glucose to an optical density of 4 and used as inoculum for the main culture. Main cultivations were carried out in a 500-mL stirred tank bioreactor (Merodos, Bovenden, Germany) containing 75 mL minimal medium and [U-¹³C₆] glucose at 30 °C and 1,000 rpm maintaining dissolved oxygen concentrations above 60 % air duration throughout the cultivation. The gas flow was controlled at 1 vvm (vessel volume per minute) (WMR Compact4, Brooks Instruments, Venedaal, Netherlands) using a nitrogen/oxygen gas mixture to avoid CO₂-derived ¹²C incorporation into biomass. Temperature, pH, and agitation were collected using the Lucillus process control system (Lucillus PIMS 2.1, Biospectra, Schlieren, Switzerland). Cells were cultivated until mid-exponential growth phase (OD_{660nm} 6.7).

Sample preparation and extraction procedure

Extraction was performed by mixing preheated PCA (55 °C, final concentration 500 mM) with the sample followed by incubation at 55 °C. Exact sample volumes were determined gravimetrically. Sampling setups and incubation times varied according to validation parameters and nature of samples.

Stability of standard compounds under extraction conditions

One milliliter standard mixture containing all 12 compounds (initial concentration 3 μM each) was mixed with 1 mL preheated extraction solution (1 M PCA). Incubation at 55 °C was carried out for 3, 5, 10, and 30 min, followed by incubation on ice (10 min). Supernatants were obtained by centrifugation (10 min, 4 °C, 6,000×g) and stored at -20 °C until further analysis.

Extraction of biological samples

Biological samples for metabolite profiling were taken at balanced growth during the mid-exponential growth phase.

To ensure instantaneous mixing, a 2-mL sample was drawn into a syringe filled with 2 mL preheated extraction solution. Generated turbulence guaranteed instantaneous mixing of sample and extraction solution as verified by colored test solutions (phenolphthalein). Incubation at 55 °C (3 min) was followed by 10 min incubation on ice. Cell debris and insoluble macromolecules were precipitated by centrifugation (10 min, 4 °C, 6,000×g). Prior to the LC-ESI-MS/MS analysis, the naturally labeled cell extracts of *C. glutamicum* and *C. ammoniagenes* were mixed with uniformly ¹³C-labeled cell extract of *C. glutamicum* treated under the same conditions (see preparation of standard solutions). As a result, the ¹³C-labeled cell extract served a dual function: firstly, by acting as internal standard (IS) providing isotope standard analytes [35] at relevant concentration levels, i.e., at levels of the analytes to be determined. The IS corrects for influences of precipitation and degradation on metabolite levels during extraction as well as for variabilities during LC-MS injection. Secondly, addition of labeled cell extract provided a suitable biological matrix with identical properties to the samples to be analyzed, while still allowing discrimination between the added analytes and those naturally present due to isotopic *m/z* differences. As a result, ¹³C-labeled cell extract was chosen as internal standard and was mixed with standard and sample solutions in all experiments, except when the biological matrix was interrogated for matrix effects (see below).

To prevent matrix effects during extraction stability studies, no cell extract was added to the samples.

LC-MS/MS conditions

Liquid chromatography

Chromatographic separation was carried out using a Phenomenex (Torrance, CA, USA) Kinetex reversed-phase PFP core-shell column (2.6 μm pore size, 100 Å particle size, 100×2.1 mm). All separations were performed at 25 °C with a constant flow rate of 200 μLmin⁻¹ using a Shimadzu (Duisburg, Germany) UFLC Prominence system. The auto-sampler (SIL20AC) temperature was set to 4 °C. Separation of single purine intermediates was performed isocratically as proposed by Nichthäuser et al. [36] using 0.1 % formic acid in water (eluent A). Solely elution of xanthosine required an organic mobile phase (0.1 % formic acid in acetonitrile) of the following elution profile: after an initial isocratic step at 0 % B for 5.5 min, B was increased to 30 % within 10 min, followed by a flushing step at 100 % B for a further 5 min. The column was reconditioned at 0 % B (initial concentration) for 5 min. The standard injection volume was 1 μL, but was increased to 10 μL in some experiments when needed.

Mass spectrometry

LC-MS/MS analysis was carried out on an API5500 QTRAP quadrupole linear ion trap (AB Sciex, Concord, Ontario, Canada) equipped with a Turbo-V electrospray ionization source. Nitrogen was used as curtain (20 psi), nebulizer (GS1; 60 psi), and auxiliary gas (GS2; 60 psi). The ESI voltage was set to 5,500 V and a source temperature of 200 °C was used. Multiple reaction monitoring (MRM) was performed in positive mode with the following parameters: collision gas (nitrogen), medium; dwell time, 250 ms. The declustering potentials (DP) and collision energies (CE) were optimized for each MRM transition by direct injection of 5 µM standard solution in ACN/water (50/50, v/v) using a syringe pump at a constant flow rate of 10 µLmin⁻¹ for each metabolite.

Data analysis/quantitative analysis

Calibration curves were created by plotting the peak area ratios of analytes and IS, i.e., the biological ¹³C-labeled cell extract, ($\text{area}_{\text{compound}}/\text{area}_{\text{internal standard}}$) against the nominal concentration of each analyte (µM) in the cell extract and assessed by linear least square regression analysis. Analyte concentrations in unknown samples were interpolated using the calculated calibration curves.

Validation procedure

The impact of extraction was validated for metabolite stability and recovery using standard solutions. In this procedure, the values of standard concentrations were compared with experimentally determined concentrations after the extraction. Time-dependent stability of metabolites was assessed at different incubation times at 55 °C (3, 5, 10, and 30 min). In addition, the impact of varying freeze-thaw cycles on compound stability was assessed. In these experiments, up to ten freeze-thaw cycles of standard solutions mixed with ¹³C-labeled cell extract to mimic the presence of biological matrix were performed and resulting degradation determined.

The validation procedure for MS quantification included linearity, accuracy, precision, lower limit of quantification (LLOQ), and influence of matrix effects.

For all MS validation procedures, standard solutions at a concentration range between 1 nM to 5 µM were spiked with biological matrix (¹³C-labeled cell extract). Precision and lower limits of quantification were calculated by determining coefficients of variation (CV %) of standard deviation and measured concentrations of spiked QC samples. Accuracy was assessed by comparing the determined concentrations of the spiked QC samples with calculated values obtained from standard calibration curves. In this study, LLOQ was defined as the lowest concentration for which

precision values of 15 % or better and an accuracy bias of 20 % or better were observed. All calibrations were performed in the presence of a biological matrix. To investigate long-term reproducibility, analysis of precision and accuracy was performed over a period of 4 weeks.

Matrix effects were determined by a post-extraction addition approach [37]. Peak areas from extracted standards *spiked* post-extraction with an extracted biological sample (¹³C-labeled) were compared to extracted standards *spiked* with diluted extraction solution (1:1 dilution in water, v/v). Comparison was carried out for all compounds at two different concentration levels, similar to observed concentration levels in biological samples analyzed in this study.

Results and discussion

The present work describes the development of an assay aimed at the quantitation of 12 important metabolites from the purine biosynthesis pathway of bacteria, namely 5'-nucleoside monophosphates as well as their corresponding nucleosides and nucleobases.

Although several reports have investigated quantification of nucleotides and nucleosides in mammalian cells, tissues, or fluids [1, 11, 17, 23, 25, 38], this is, to the best of our knowledge, the first assay combining an extraction procedure for Gram-positive bacteria with simultaneous quantification of compounds from all three compound classes, including IMP, its nucleoside, and nucleobase. Turnover rates for intracellular metabolites are reported to be within milliseconds [39] and pool sizes are considerably small [40]. Therefore, the first essential step was the quenching of cellular metabolism maintaining actual *in vivo* concentrations and thereby yielding a representative metabolic snapshot of the current growth phase of the organism.

Validation of the extraction method

Metabolic freeze frame by acidic extraction

Since *C. glutamicum* belongs to bacteria affected by the unspecific cold shock phenomenon [40], quenching using cold methanol or other low temperature-based quenching methods should not be applied for absolute quantification of intracellular metabolite pool sizes. We have therefore developed a one-step quenching/extraction method for quantitative metabolite extraction suited to the subsequent LC-ESI-MS/MS analysis.

In this procedure, it was important to assess the acidic extraction method for intermediates with respect to their stability and recovery after incubation at 55 °C. Results clearly indicate that temperature or acid-dependent decomposition was not observed except for XMP, xanthosine, and

xanthine (Table 1). Recoveries for AMP, GMP, IMP, adenosine, guanosine, inosine, adenine, guanine, and hypoxanthine were >90 %. Only intermediates of the xanthine family (XMP, xanthosine, and xanthine) exhibited time-dependent decomposition of up to 50 % (Table 1) in 30 min. The degradation of the xanthine family intermediates was therefore studied in greater detail to assess the impact on the determination of the other compounds. Fortunately, no negative effects were observed.

Unspecific metabolite leakage and secretion during the cultivation were also investigated for the purine intermediates studied in this work. Sole intracellular localization of the quantified metabolites is a prerequisite for application of a whole culture quenching procedure as performed in this work and neglecting this fact may otherwise result in significant errors. Therefore, medium supernatants were analyzed at different time points throughout the entire cultivation process. However, no detectable amounts were observed for the minimal medium used for cultivation. Thus, the described extraction procedure was well suited for the extraction and quantitation of *in vivo* free intracellular concentrations of the metabolites analyzed in this work. Importantly, unspecific leakages or secretions have to be considered when medium compositions are changed or different organisms are analyzed.

Application of PCA as quenching/extraction solution resulted in precipitation of cell debris and insoluble macromolecules. The obtained sample solution was of sufficient purity for direct LC-ESI-MS/MS analysis. However, extraction with PCA has to be validated for each substance due to varying chemical stabilities. Thus, PCA extraction is only suitable for stable analytes during acidic extraction.

To assess the extent of degradation of the individual purine intermediates caused by multiple freeze–thaw cycles, up to ten freeze–thaw cycles using a standard solution were

consistently performed on ice (concentrations, 1 μ M each in PCA as described in the “Materials and methods” section). The recovery rate (as percentage normalized to the relative initial concentrations) was then determined. As matrix effects from the complex biological samples might interfere with stability properties, the standard solution was mixed with IS (i.e., the ^{13}C -labeled cell extract, 1/1; *v/v*). As is clearly seen in Table 2, the freeze–thaw procedure was found to trigger a significant reduction in the concentration levels, particularly for AMP, GMP, IMP, and XMP. The recovery rates for AMP were found to exhibit the greatest loss at 12 % after 10 cycles, while even for 2 cycles showed a significant reduction. The susceptibility of the individual analytes to degradation was determined to be highly variable, as neither nucleosides nor nucleobases showed any freeze–thaw-related degradation (Table 2). An extended extraction procedure at 55 °C (Table 1) for 30 min, however, yielded increased degradation mainly for xanthine, xanthosine, and XMP. Therefore, to obtain reproducible and quantitative results, unnecessary freeze–thaw cycles should be avoided and extreme care should be taken in terms of unnecessary extraction cycles.

Validation of the LC-ESI-MS/MS method

Separation and MS-MS optimization

Several studies have attempted the simultaneous quantification of nucleosides and nucleotides [2, 23, 31, 41, 42]. No method for all purine nucleoside monophosphates, nucleosides, and nucleobases without the necessity for ion-pairing reagents, however, has yet been reported in the literature.

The MRM chromatograms for the 12 purine intermediates are shown in Fig. 1, which demonstrates the simultaneous analysis by LC-ESI-MS/MS.

Table 1 Extraction recoveries and stabilities of standard solutions

| Compound | Extraction times | | | |
|--------------|------------------|-----------------|-----------------|-----------------|
| | 3 min | 5 min | 10 min | 30 min |
| AMP | 99.5 \pm 1.7 | 100.3 \pm 2.3 | 98.3 \pm 0.9 | 99.0 \pm 1.6 |
| Adenosine | 100.5 \pm 2.6 | 93.2 \pm 2.1 | 93.3 \pm 3.2 | 94.1 \pm 1.2 |
| Adenine | 101.4 \pm 4.6 | 101.6 \pm 2.2 | 101.3 \pm 3.1 | 102.1 \pm 0.9 |
| GMP | 115.5 \pm 6.3 | 110.0 \pm 3.2 | 112.3 \pm 1.2 | 120.3 \pm 4.3 |
| Guanosine | 102.1 \pm 2.7 | 105.4 \pm 2.7 | 104.9 \pm 0.4 | 101.8 \pm 1.1 |
| Guanine | 95.0 \pm 2.2 | 94.7 \pm 2.1 | 93.4 \pm 3.8 | 95.5 \pm 4.8 |
| IMP | 103.2 \pm 2.3 | 100.8 \pm 1.1 | 103.4 \pm 0.7 | 103.3 \pm 2.3 |
| Inosine | 108.3 \pm 0.9 | 102.1 \pm 3.1 | 104.1 \pm 2.1 | 111.3 \pm 1.8 |
| Hypoxanthine | 105.9 \pm 3.9 | 98.1 \pm 8.6 | 96.6 \pm 1.2 | 90.3 \pm 2.3 |
| XMP | 93.8 \pm 2.1 | 90.2 \pm 2.5 | 82.8 \pm 1.5 | 60.8 \pm 1.0 |
| Xanthosine | 89.7 \pm 1.1 | 82.8 \pm 2.1 | 69.5 \pm 0.8 | 46.3 \pm 2.3 |
| Xanthine | 92.6 \pm 1.6 | 94.9 \pm 2.9 | 92.2 \pm 5.3 | 81.1 \pm 2.8 |

Standard concentrations were 3 μ M each. Results from $n=3$ for each compound

Table 2 Recovery for standard solutions (1 μM each) after freeze–thaw cycles

| | 0 | 1 | 2 | 3 | 5 | 10 |
|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| AMP | 102.1 \pm 2.3 | 105.0 \pm 3.0 | 94.0 \pm 1.8 | 90.6 \pm 1.0 | 89.2 \pm 0.8 | 87.7 \pm 0.5 |
| Adenosine | 100.2 \pm 0.6 | 100.6 \pm 0.1 | 101.6 \pm 0.1 | 102.1 \pm 0.1 | 101.6 \pm 0.2 | 103.0 \pm 0.4 |
| Adenine | 98.9 \pm 1.3 | 100.0 \pm 0.7 | 99.2 \pm 0.8 | 99.5 \pm 0.2 | 99.9 \pm 0.2 | 98.5 \pm 0.3 |
| GMP | 99.8 \pm 0.7 | 98.2 \pm 0.3 | 92.0 \pm 0.3 | 92.0 \pm 0.2 | 92.2 \pm 0.5 | 93.8 \pm 0.5 |
| Guanosine | 98.6 \pm 2.0 | 98.1 \pm 3.6 | 104.7 \pm 2.1 | 104.1 \pm 1.1 | 99.6 \pm 0.1 | 101.7 \pm 0.6 |
| Guanine | 103.1 \pm 2.3 | 99.7 \pm 1.5 | 101.4 \pm 0.7 | 100.7 \pm 0.1 | 101.0 \pm 0.3 | 101.2 \pm 0.2 |
| IMP | 99.7 \pm 0.6 | 98.9 \pm 0.1 | 96.7 \pm 0.2 | 96.4 \pm 0.7 | 95.2 \pm 0.2 | 96.5 \pm 0.2 |
| Inosine | 99.6 \pm 3.8 | 103.0 \pm 2.8 | 101.9 \pm 0.9 | 105.1 \pm 2.5 | 94.2 \pm 2.8 | 99.0 \pm 0.3 |
| Hypoxanthine | 98.9 \pm 1.9 | 101.2 \pm 2.0 | 97.8 \pm 0.5 | 98.3 \pm 0.1 | 98.7 \pm 0.2 | 98.4 \pm 0.9 |
| XMP | 100.3 \pm 0.1 | 98.0 \pm 0.6 | 98.6 \pm 0.7 | 96.9 \pm 1.3 | 95.8 \pm 3.9 | 96.1 \pm 0.6 |
| Xanthosine | 101.9 \pm 1.8 | 100.4 \pm 1.5 | 101.3 \pm 0.3 | 100.6 \pm 0.2 | 98.3 \pm 0.9 | 99.8 \pm 1.9 |
| Xanthine | 98.8 \pm 2.2 | 101.7 \pm 2.5 | 101.5 \pm 0.1 | 100.5 \pm 0.8 | 100.0 \pm 0.4 | 99.5 \pm 0.4 |

Results derived from $n=3$ and determined in the presence of a biological matrix

To describe active adsorption processes and retention effects for the different analytes, retention factors (k) were calculated ($k=(t_a-t_0)/t_0$). The net analyte retentions (t_a) were corrected using the void volume ($t_0=0.95$) of the column and k values determined as shown in Table 3. These values were found to be the lowest for guanine (1.3), adenine (1.4), and AMP (1.6), whereas all other analytes exhibited retention factors $k>2.0$, indicating active adsorption by the stationary phase.

Fragmentation of protonated molecules of the investigated nucleotides and nucleosides by collision-induced dissociation (CID) resulted in the highly specific protonated nucleobase as major product ions, allowing selection of specific product ions for quantitation by MRM (Table 4). The CID spectra, fragmentation patterns, as well as specific product ions for quantification of IMP, inosine, and hypoxanthine are shown in Fig. 2 as illustrative examples.

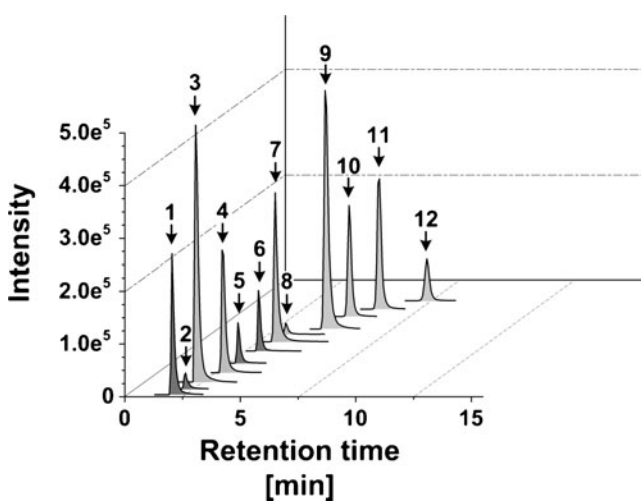


Fig. 1 MRM chromatograms for the 12 investigated purine intermediates. 1 guanine, 2 adenine, 3 AMP, 4 GMP, 5 hypoxanthine, 6 xanthine, 7 IMP, 8 XMP, 9 adenosine, 10 inosine, 11 guanosine, 12 xanthosine

Linearity, sensitivity, and long-term reproducibility

Calibration curves were obtained from a series of working standard solutions over a wide concentration range, using ^{13}C -labeled cell extract as internal standards (IS). To ensure thorough incorporation of ^{13}C -labeled carbon (derived from the sole carbon source [$^{13}\text{C}_6$] glucose) into cellular components and intermediates, the mass isotopomer distributions of the nucleotide IMP were determined (Fig. 3). The ^{13}C -labeled cell extract was shown to exhibit the uniformly labeled isotopomer at 90 %, whereas the remaining 10 % comprised incorporation of 1 molecule of ^{12}C carbon (m_{+9}) due to the 99 % enrichment of [$^{13}\text{C}_6$] glucose. The non-labeled cell extract was found to exhibit the natural distribution of the corresponding isotopes from the natural presence of ^{13}C (1.1 % [43]; Fig. 3). In addition to utilizing glucose as carbon source, the purine biosynthesis comprises incorporation of 1 molecule of CO_2 from hydrogencarbonate. Thus, to preclude any ^{12}C assimilation, carbon dioxide-

Table 3 Retention factors (k) of the purine analytes

| Compound | k |
|--------------|-----|
| AMP | 1.6 |
| Adenosine | 4.0 |
| Adenine | 1.4 |
| GMP | 2.0 |
| Guanosine | 5.1 |
| Guanine | 1.3 |
| IMP | 2.6 |
| Inosine | 4.2 |
| Hypoxanthine | 2.3 |
| XMP | 2.7 |
| Xanthosine | 6.6 |
| Xanthine | 2.4 |

$k=(t_a-t_0)/t_0$, t_a —net analyte retention time, t_0 —void volume retention ($t_0=0.95$) of the column

Table 4 MRM transitions and settings for LC-ESI-MS/MS

| ID | Monoisotopic mass (amu) | Q1 (amu) | Q3 (amu) | DP | CE |
|--------------|-------------------------|----------|----------|-----|----|
| Adenine | 135.0545 | 136 | 119 | 234 | 42 |
| Adenosine | 267.0968 | 268 | 136 | 16 | 25 |
| AMP | 347.0631 | 348 | 136 | 72 | 25 |
| GMP | 363.0580 | 364 | 152 | 26 | 19 |
| Guanine | 151.0494 | 152 | 135 | 18 | 27 |
| Guanosine | 283.0917 | 284 | 152 | 42 | 17 |
| Hypoxanthine | 136.0385 | 137 | 110 | 147 | 29 |
| IMP | 348.0471 | 349 | 137 | 64 | 19 |
| Inosine | 268.0808 | 269 | 137 | 42 | 32 |
| Xanthine | 152.0334 | 153 | 136 | 50 | 21 |
| Xanthosine | 284.0757 | 285 | 153 | 32 | 19 |
| XMP | 364.0420 | 365 | 153 | 21 | 23 |

For each compound, the following parameters are indicated: compound name (ID), monoisotopic mass, selected ions on quadrupoles Q1 and Q3 (Q1 mass and Q3 mass, respectively), declustering potential [V] (DP) and collision energy [V] (CE)

free synthetic air was used. This was validated by mass spectrometric analysis of the CO₂ released during the whole fermentation process. This released CO₂ was found to consist exclusively of ¹³CO₂. Therefore, the mass isotopomer distribution, as exemplarily determined for IMP, did not possess any detectable amounts of naturally occurring mass isotopomers (i.e., *m*₀, *m*₊₁, and *m*₊₂). This allowed non-interfered analyte quantitation using ¹³C-labeled cell extract as internal standard. Peak area ratios of each analyte relative to the IS, i.e., the biological matrix, were plotted against the standard concentrations and linear regression was applied. The correlation coefficients (*r*²) were consistently >0.99 for concentration levels >0.001 μM for GMP and IMP and 0.0075 μM for AMP, adenosine, inosine, and guanosine. The highest LLOQ of 0.015 μM were observed for XMP, xanthosine, adenine, hypoxanthine, guanine, and xanthine. Applying QC samples at the lower limit of quantification (LLOQ), the precision was always better than 15 % and accuracy deviated less than 20 % (Table 5) for all analytes. This was also true for all concentrations above LLOQ. To accurately quantify in vivo concentrations in biological samples, all validation steps, including linearity, precision, and accuracy, were performed in the presence of a biological matrix. Standard working solutions containing ¹³C-labeled cell extract were used for the calibration, which allowed for compensation of matrix effects. Matrix effects, which are specified as any interference of ionization efficiency caused by co-eluting substances [37], usually results in a signal attenuation. To analyze the influence of possible matrix effects on the 12 analytes, a post-extraction–addition experiment was performed and the recovery for all analytes was

determined. The resulting recoveries are summarized in Table 4 and did not show any significant matrix effects for all analytes. This recovery study not only evaluated possible matrix effects, it also illustrated quantitative determination of purine intermediate pool concentrations in *Corynebacteria*, since the concentration range (Table 6) covered the observed biological concentration levels (Table 7) except for guanosine. To ensure accurate and precise quantitation of guanosine and other low level analytes or of those exhibiting lower detection sensitivities, the injection volume was adapted accordingly (see the “Materials and methods” section).

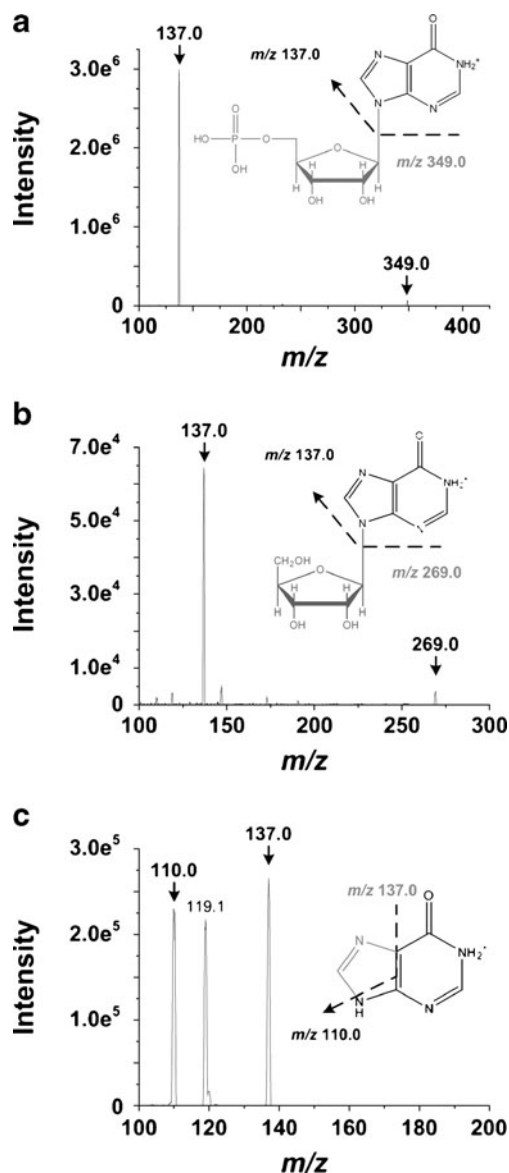


Fig. 2 CID spectra, fragmentation patterns, and product ions of the [M + H]⁺ ions of IMP (a), inosine (b), and hypoxanthine (c). Chemical structures shown in gray represent the parent ions, and chemical structures shown in black represent the corresponding product ions

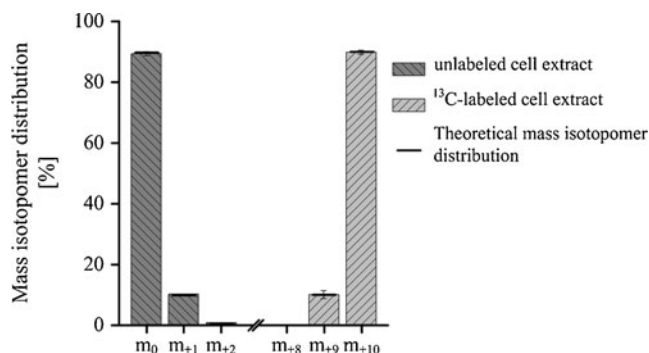


Fig. 3 Mass isotopomer distributions of the intracellular purine metabolite IMP, unlabeled (extracted from unlabeled cell extract) and uniformly labeled (extracted from ^{13}C -labeled cell extract). The theoretical distributions were corrected for the natural abundance of ^{13}C with 1.1 % (unlabeled cell extract) and for the 99 % enrichment of $[\text{U-}^{13}\text{C}_6]$ glucose (labeled cell extract)

All compounds exhibited a linear dynamic range of at least two orders of magnitude, which was considered sufficient for quantitation in this study.

Application to pathway analysis

Samples from two Gram-positive *Corynebacterium* species, *C. glutamicum* and *C. ammoniagenes*, were extracted and quantitation of intracellular purine pools was performed. Multiple reaction monitoring (MRM) chromatograms of purine biosynthetic pathway intermediates in biological samples are shown in Fig. 4. The corresponding concentra-

Table 5 Lower limits of quantification (LLOQ), accuracy and precision of QC samples

| Compound | LLOQ mean [nM] | Accuracy [%] | Precision [CV %] |
|--------------|----------------|--------------|------------------|
| AMP | 9.4 | 106.0 | 13.7 |
| Adenosine | 8.2 | 108.5 | 13.6 |
| Adenine | 16.1 | 97.2 | 10.0 |
| GMP | 0.8 | 103.8 | 13.2 |
| Guanosine | 7.2 | 109.3 | 12.4 |
| Guanine | 17.5 | 101.8 | 7.9 |
| IMP | 0.8 | 107.4 | 7.6 |
| Inosine | 7.0 | 99.4 | 6.2 |
| Hypoxanthine | 15.2 | 90.6 | 7.3 |
| XMP | 18.8 | 94.8 | 5.9 |
| Xanthosine | 16.1 | 93.0 | 5.1 |
| Xanthine | 19.0 | 98.3 | 10.9 |

LLOQ corresponds to lower limits of quantification; acceptance criteria for LLOQs were 15 CV % or less for precision and a bias of 20 % or less for accuracy. Results derived from $n=4$ and determined in the presence of a biological matrix

Table 6 Recovery for standard solutions

| Compound | Conc [μM] | Recovery [%] |
|--------------|------------------------|-----------------|
| AMP | 3.1 | 102.0 \pm 3.5 |
| | 0.2 | 102.1 \pm 5.1 |
| Adenosine | 2.7 | 102.8 \pm 4.7 |
| | 0.1 | 106.7 \pm 3.9 |
| Adenine | 2.2 | 105.0 \pm 1.1 |
| | 0.1 | 101.7 \pm 6.6 |
| GMP | 2.7 | 98.1 \pm 6.9 |
| | 0.1 | 95.1 \pm 2.5 |
| Guanosine | 2.4 | 104.0 \pm 1.1 |
| | 0.1 | 105.7 \pm 1.8 |
| Guanine | 2.4 | 100.1 \pm 4.1 |
| | 0.1 | 103.6 \pm 3.3 |
| IMP | 2.9 | 99.8 \pm 1.7 |
| | 0.1 | 97.9 \pm 0.4 |
| Inosine | 2.3 | 101.8 \pm 1.7 |
| | 0.1 | 104.4 \pm 0.2 |
| Hypoxanthine | 2.1 | 99.0 \pm 3.7 |
| | 0.1 | 92.3 \pm 4.6 |
| XMP | 2.6 | 100.2 \pm 1.5 |
| | 0.1 | 98.8 \pm 0.1 |
| Xanthosine | 2.2 | 96.7 \pm 0.5 |
| | 0.1 | 99.6 \pm 1.3 |
| Xanthine | 2.6 | 101.0 \pm 2.0 |
| | 0.1 | 98.8 \pm 2.4 |

Determined recoveries for standard solutions in two different concentrations in order to estimate matrix effects

tion levels for detected nucleotides, nucleosides, and nucleobases normalized to the cell volume [44] are summarized in Table 7. Concentrations in μM , obtained from LC-MS quantitations, are additionally provided to allow evaluation of an adequate and suitable concentration range. For *C. glutamicum*, all compounds except for XMP and xanthosine (which were below the detection limit) were quantified with precision values of less than 12 %. AMP and GMP, central building blocks for DNA and RNA and co-substrates for activation of metabolites, exhibited the highest intracellular nucleotide concentrations of 4.1 and 1.3 $\mu\text{mol mL}^{-1}$, respectively. Other purine pathway intermediates showed a broad range of concentration levels varying between 0.02 and 1.7 $\mu\text{mol mL}^{-1}$. However, nucleosides exhibited the lowest levels of all analyzed metabolites at 0.02 $\mu\text{mol mL}^{-1}$. The observed concentrations for all quantified analytes were 1 to 3 orders of magnitude above the determined quantification limits, indicating that the investigated sample volumes could be further reduced if required.

Analysis of *C. ammoniagenes* revealed the same distribution pattern of intracellular concentrations of purine pathway

Table 7 Concentrations of purine intermediates ($\mu\text{mol mL}_{\text{cell volume}}^{-1}$) in *Corynebacterium glutamicum* and *Corynebacterium ammoniagenes*

| Compound | <i>C. glutamicum</i> | | <i>C. ammoniagenes</i> | |
|--------------|----------------------|---------------------|------------------------|---------------------|
| AMP | 4.09±0.20 | (4.33) ^a | 1.19±0.12 | (1.41) ^a |
| GMP | 1.35±0.08 | (1.40) ^a | 0.15±0.01 | (0.20) ^a |
| IMP | 0.23±0.02 | (0.26) ^a | 0.06±0.00 | (0.08) ^a |
| XMP | – ^b | | – ^b | |
| Adenosine | 0.05±0.00 | (0.06) ^a | 0.17±0.01 | (0.20) ^a |
| Guanosine | 0.02±0.00 | (0.02) ^a | – ^b | |
| Inosine | 0.05±0.00 | (0.07) ^a | – ^b | |
| Xanthosine | – ^b | | – ^b | |
| Adenine | 1.47±0.08 | (1.40) ^a | 0.78±0.05 | (1.05) ^a |
| Guanine | 1.67±0.11 | (1.74) ^a | 1.16±0.05 | (1.50) ^a |
| Hypoxanthine | 0.13±0.01 | (0.15) ^a | 0.07±0.01 | (0.09) ^a |
| Xanthine | 0.06±0.00 | (0.06) ^a | 0.05±0.00 | (0.06) ^a |

Concentrations and corresponding standard deviations were determined using four biological and two technical replicates for each compound and standardized to the cell volume [44]

^a Concentrations in micromolar from LC-MS quantitation, not related to biomass

^b Below detection limit

intermediates; however, all quantified compounds (except for adenosine) exhibited significantly reduced intracellular concentration levels as compared to *C. glutamicum*. Furthermore, no free intracellular nucleosides were detected, except for adenosine. The relatively decreased intracellular purine concentration levels detected in *C. ammoniagenes* could indicate differing regulatory mechanism of these species, eventually favoring reduced intracellular concentrations

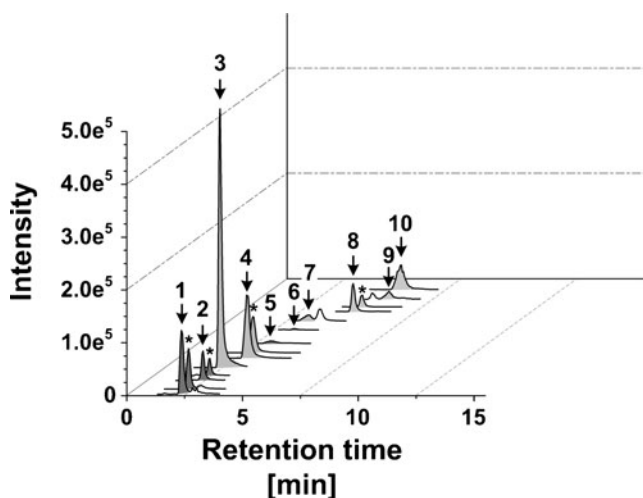


Fig. 4 MRM chromatogram of a *C. glutamicum* extract. 1 guanine, 2 adenine, 3 AMP, 4 GMP, 5 hypoxanthine, 6 xanthine, 7 IMP, 8 adenosine, 9 inosine, 10 guanosine. Isotope peaks (exemplary for 1, 2, 4, and 8) are marked with an asterisk

in *C. ammoniagenes*. This might be linked to the increased capability of *C. ammoniagenes* for nucleotide and nucleoside secretion [9], resulting in decreased intracellular concentrations. This organism, which is characterized by its potential for extracellular purine accumulation, has been exploited for large-scale production of diverse compounds, i.e., IMP, GMP, XMP, and inosine [29], whereas *C. glutamicum*, an outstanding model organism in the field of systems biotechnology and producer of various substances such as amino acids, polymers, or biofuels [45] only possesses poor secretion capabilities [46, 47]. A further deduction of the information based on the inter-species variations, however, has to be performed carefully, as the resulting pool sizes do not allow any interpretation of the participating enzymes; the alteration of intracellular metabolite pool sizes cannot directly be linked to varying enzyme activities and can only provide indications of differing underlying mechanisms.

Additional investigation of extended extraction times up to 15 min did not result in increased metabolite concentrations (data not shown), indicating that a temperature of 55 °C in combination with an incubation time of 3 min was sufficient for complete metabolite extraction. Incubating for more than 3 min caused degradation of analytes belonging to the xanthine family and should therefore be avoided.

Conclusions

In this study, a LC-ESI-MS/MS approach in conjunction with a single-step extraction procedure was developed and validated for quantitation of 12 major purine biosynthetic pathway intermediates from biological samples. Chromatographic separation of polar nucleotides and non-polar nucleosides and nucleobases was achieved in less than 15 min using reversed-phase HPLC-MS without using any ion-pairing reagents. The method exhibited excellent selectivity, precision and accuracy in the presence of a biological matrix. As no ion-pairing reagents were used, the method was much more rugged than existing methodologies and required significantly less instrument maintenance and operator intervention. Other advantages over previous methods include the simplified extraction procedure, the straightforward quantitation routine, and the exclusive use of inexpensive reagents, making this approach particular suitable for high throughput, multi-user laboratories. Even though addition of ion-pairing reagents might sometimes have a positive effect on retention performance, in particular for phosphorylated compounds, the separations achieved in this work were found to fully meet the requirements in this study, without the need for troublesome ion-pairing reagents.

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