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Dispersive solid phase extraction combined with ion-pair ultra high-performance liquid chromatography tandem mass spectrometry for quantification of nucleotides in *Lactococcus lactis*

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ABSTRACT

Analysis of intracellular metabolites in bacteria is of utmost importance for systems biology and at the same time analytically challenging due to the large difference in concentrations, multiple negative charges, and high polarity of these compounds. To challenge this, a method based on dispersive solid phase extraction with charcoal and subsequent analysis with ion-pair liquid chromatography coupled with electrospray ionization tandem mass spectrometry was established for quantification of intracellular pools of the 28 most important nucleotides. The method can handle extracts where cells leak during the quenching. Using a Phenyl-Hexyl column and tributylamine as volatile ion-pair reagent, sufficient retention and separation was achieved for mono-, di-, and triphosphorylated nucleotides. Stable isotope labeled nucleotides were used as internal standards for some analytes. The method was validated by determination of the recovery, matrix effects, accuracy, linearity, and limit of detection based on spiking of medium blank as well as standard addition to quenched *Lactococcus lactis* samples. For standard addition experiments, the isotope-labeled standards needed to be added in similar or higher concentrations as the analytes. *L. lactis* samples had an energy charge of 0.97 ± 0.001 which was consistent with literature, whereas some differences were observed compared with legacy data based on ^{33}P labeling.

The nucleotides are particularly important parts of the intracellular metabolome not only because of their role as substrates in the synthesis of RNA and DNA but also because they are involved in virtually every metabolic pathway either directly as providers of energy or as allosteric effectors. Cyclic nucleotides have been shown to be a part of many signaling pathways, guanine nucleotides are involved in protein synthesis, and adenosine 5′-triphosphate serves as a primary energy supply for transport, cell motion, and many biosynthetic processes [1–4]. Thus, knowing the intracellular concentration of nucleotides is important for understanding many biological processes within the cell.

For quantitative measurement of nucleotide pools, the sample preparation is of utmost importance because instant quenching of the cell metabolism is required due to the very fast turnover rates of the nucleotide pools within the cell [5,6]. This step can easily be a source of errors due to the possible enzymatic change of nucleotide pools as well as cell leakage in cases where the cells are separated from the growth medium [7]. Thus, merging the quenching and extraction steps is a solution for leaky cells, as shown by Martinussen and coworkers [8], where formic acid was added to *Lactococcus lactis* cells and subsequently three freeze–thaw cycles were used to extract the intracellular metabolites [9].

Purification of the extracted intracellular metabolites is a difficult task, considering both the wide range of concentrations and the diverse physiochemical properties of the intracellular metabolites. Solid phase extraction (SPE)[1] is a widely used method for either sample cleanup or trace enrichment in a variety of matrices, from environmental to biological samples [10–12]. Anastassiades and coworkers [13] proposed an SPE method for cleanup of food and environmental samples called dispersive solid phase extraction (DSPE). In DSPE, the sorbent material is added to the extract to separate the compounds of interest from the matrix components and then is removed from the extract by centrifugation. One of the main advantages of using DSPE is the small amounts of sorbent and solvent that are required, reducing handling and costs [11]. In this regard, charcoal has been proven to be a suitable sorbent that is able

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[1] *Abbreviations used:* SPE, solid phase extraction; DSPE, dispersive solid phase extraction; TLC, thin-layer chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TBA, tributylamine; HCl, hydrochloric acid; DBAA, dibutylamine acetate; ESI, electrospray ionization; IS, internal standard; SA, synthetic amino acid; UHPLC, ultra high-performance liquid chromatography; QTOF, quadrupole time-of-flight; LOD, limit of detection; RSD, relative standard deviation.

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to retain nucleotides from biological extracts [9,14]. Nucleotide adsorption on charcoal is based on interaction of the aromatic ring electrons from the purine or pyrimidine moiety with the π electrons from the charcoal.

Quantification methods for nucleotides, given in the literature, involve incorporation of radioactive phosphate followed by one- or two-dimensional thin-layer chromatography (TLC) [9,15] as well as less sensitive and selective ion-pair, ion-exchange, and ion chromatography with fluorescence, ultraviolet, and conductivity detection [16–19]. In addition, the combination of liquid chromatography (LC) and mass spectrometry (MS) has been shown to be a powerful analytical technique for quantitative analysis of nucleotides in *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Escherichia coli*, human plasma, and animal tissue [20–23]. In addition, LC–MS/MS (tandem mass spectrometry) with porous graphitic carbon as a stationary phase has been used for analysis of araCTP, CTP, and dCTP [24]. LC–MS/MS fulfills three important requirements when dealing with analysis of intracellular metabolites: (i) sensitivity, (ii) wide linear range, and (iii) no need to form thermally stable derivatives of the tri-, di-, or monophosphorylated nucleotides as required for gas chromatography mass spectrometry [6,25]. Due to the high polarity and multiple charges, nucleotides are not well retained under reversed phase conditions, and therefore methods such as hydrophilic interaction chromatography (HILIC) and ion-exchange chromatography are needed for their separation [26,27]. A subtype of the latter is ion-pair chromatography that is made by dynamic modification of a reversed phase separation by coating the surface with charged but still hydrophobic ion-pair reagent added to the mobile phase [20,21,28,29]. Ion-pair chromatography is a promising alternative especially when dealing with isomeric compounds that any other chromatography technique fails to separate. Furthermore, the hydrophobic parts of the nucleotides can also interact with the hydrophobic parts of the reversed phase column, thereby giving mixed-mode conditions. For compatibility with atmospheric ionization techniques used for LC–MS, volatile ion-pairing reagents such as tributyl-, dibutyl-, and triethylamine are required because the ion source will clog within minutes of operation if a nonvolatile reagent such as tetrabutylamine is used [30].

When using LC–MS as a detection technique for analysis of complex biological extracts, enhancement or suppression of the ionization is often observed, resulting in different responses for the compounds of interest than are seen from pure standard solutions [31,32]. This occurs when target compounds coelute with matrix interferences and, therefore, the modulation of the LC–MS signal (i) affects reproducibility, (ii) leads to systematic errors, and (iii) can obscure the detection of the target compound in extreme cases.

To prevent the signal modulation especially observed in electrospray ionization (ESI), four alternatives exist: (i) development of more selective chromatographic separation, (ii) better sample purification (e.g., by SPE), (iii) use of more sensitive MS instruments that allow sample dilution, and (iv) use of stable isotope-labeled internal standards (ISs) that can compensate for matrix effects [33]. Due to the similar physicochemical properties, the isotope-labeled ISs also correct for losses and decomposition during sample preparation.

In this study, we present an ion-pair LC isotope dilution MS/MS method combined with charcoal sample purifications for analysis of nucleotide pools. Tributylamine (TBA) was used as ion-pair reagent to modify the mobile phase and facilitate the retention of the nucleotides. Subsequently the applicability of the method was demonstrated by analysis of nucleotide pools in *L. lactis*. To the best of the authors' knowledge, there is no previous report regarding the combination of DSPE purification using charcoal and ion-pair LC–MS/MS analysis for determination of nucleotide pool sizes.

Materials and methods

Materials

Nucleotide standards were purchased from Sigma–Aldrich (Steinheim, Germany). Uniformly isotope-labeled (^{13}C and/or ^{15}N) nucleotides were used as ISs and were purchased from Silantes (Munich, Germany) and Sigma–Aldrich. The chemical purity and isotope enrichment of the isotope-labeled nucleotides were more than 90 and 98%, respectively, except for [U-^{13}C]ATP, [U-^{13}C]GTP, and [U-$^{13}C^{15}N$]CTP, which had a chemical purity of 95%. Amino acids, vitamins, glucose, inorganic salts, active charcoal (C3345), hydrochloric acid (HCl), TBA (puriss. plus grade), 0.5 M dibutylamine acetate (DBAA) concentrate (LC–MS grade), ethanol, methanol, acetonitrile, and acetic acid (LC–MS grade) were obtained from Sigma–Aldrich. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Stock solutions

Stock solutions of the nucleotides with concentration of 1 mg/ml were prepared in water and stored under –20 °C until use. Aliquots of the stock solutions were used to prepare the daily working solutions by further dilution in 10 mM TBA and 10 mM acetic acid solution. Stock solutions (1 and/or 0.2 mg/ml) of labeled ISs were prepared in water and kept under –20 °C until use. The concentration of the labeled nucleotides used to prepare the IS mixture for spiking the calibration standards and the extracts is given in Table 1.

Cell growth and sampling

L. lactis wild-type strain was grown overnight at 30 °C on agar plates containing M17 broth medium obtained from Oxoid supplied with 1% glucose (GM17) [34]. Ten single colonies were used to inoculate synthetic amino acid (SA) medium [35] containing 0.5% glucose. The culture was subjected to 10-fold dilution series (up to 10^{-7}) and grown overnight at 30 °C. Then 50 ml of culture from the dilution where the cells were still in exponential growth was transferred into 500 ml of preheated SA medium at 30 °C. The OD_{450} of the cultures was measured continuously, starting from 0.03 until 0.5, when 5 ml of culture was quenched with 1 ml of 10 M cold formic acid, followed by the addition of 65 μl of IS mixture. After vigorous mixing, the cultures were stored at –80 °C. For extraction of the metabolites, the cultures were subjected to three freeze–thaw cycles by placing the samples from a –80 °C freezer to an icewater bath, followed by mixing. Then the samples were centrifuged at 4248*g* and the supernatant (6.0 ml) was transferred to a chilled Falcon tube.

Charcoal sample cleanup

Activated charcoal (0.75 g) was suspended in 5 ml of 96% ethanol and 45 ml of water and was vortexed. The suspension was centrifuged for 20 min at 4248*g* and 4 °C, and the supernatant was discarded. The pellet was then mixed with 1.5 ml of 1 M HCl and centrifuged. After discarding the supernatant, the charcoal pellet was washed with 10 ml of water three times and finally resuspended in 1 ml of water.

The quenched bacterial sample was transferred to a chilled tube containing 0.6 ml of the activated charcoal suspension and was vigorously mixed and kept on ice. The suspension was centrifuged for 20 min at 4248*g* and 4 °C. The supernatant was transferred to a chilled Falcon tube and stored at –80 °C. The charcoal pellet was washed two times with ice-cold water and centrifuged for