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Determination of pyridine and adenine nucleotide metabolites in *Bacillus subtilis* cell extract by sweeping borate complexation capillary electrophoresis

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Abstract

With a growing interest in new areas of bioanalytical research such as metabolome analysis, the development of sensitive capillary electrophoresis (CE) methods to analyze sub-μ*M* concentrations of analytes in biological samples is required. In this report, the application of CE with sweeping by borate complexation is used to analyze a group of seven pyridine and adenine nucleotide metabolites derived from bacteria *Bacillus subtilis* cell extracts. Nanomolar (n*M*) detectability of analytes by CE with UV photometric detection is achieved through effective focusing of large sample plug (~10% of capillary length) using sweeping by borate complexation method, reflected by a limit of detections ($S/N = 3$) of about 2×10^{-8} *M*. Changes in metabolites concentrations were observed in cell extracts when using either glucose or malate as the carbon source in the culture medium. Concentration of pyridine and adenine nucleotides in cell extracts varied widely from 78.6 (±7.6) μ*M* for nicotinamide–adenine dinucleotide in malate to 0.66 (±0.12) μ*M* for nicotinamide–adenine dinucleotide phosphate in glucose culture medium. Concentrations of metabolites in a single cell were also estimated at milimolar (m*M*) level. The method was validated in terms of linearity, sensitivity and reproducibility. The application of CE by sweeping borate complexation allows for sensitive and reproducible analyses of nucleotide metabolites in complex biological samples such as bacteria cell extracts.

Keywords: Bacillus subtilis; Sweeping; Borate complexation; Complexation; Dynamic pH junction; Metabolomes; Pyridine nucleotides; Adenine nucleotides; Nucleotides

1. Introduction

Upon entering a post genome sequencing era, the development of new analytical technologies for DNA, mRNA, protein, and metabolite based analyses is urgently needed [1]. As an analogy to genomics, metabolomics is a comprehensive, qualitative and quantitative analysis of metabolites present in

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living cell. The metabolome is defined as the complete set of all low molecular mass, non-peptide biomolecules synthesized by a given biological system, such as organism, organ, tissue, cell or cell compartment [2]. Since metabolites are the final products of cellular regulatory processes, their quantitative levels can be regarded as the ultimate response of biological systems to genetic and environmental changes [3]. Data obtained from metabolome analyses can be applied to various studies related to the genome area such as: simulation of the biological activity of genes; studying of functions of new genes and effects of genes mutations on metabolite levels; or application to gene technology to produce valuable metabolites [4].

The pyridine nucleotides (PNs), namely nicotinamide–adenine dinucleotide (NAD), nicotinamide–adenine dinucleotide phosphate (NADP), dihydronicotinamide–adenine dinucleotide (nicotinamide–adenine dinucleotide reduced form, NADH) and dihydronicotinamide–adenine dinucleotide phosphate (nicotinamide–adenine dinucleotide phosphate reduced form, NADPH) represent a class of coenzyme involved in a number of critical catabolic and anabolic pathways in living organisms [5]. The NADH–NAD and NADPH–NADP coenzyme pairs are synthesized from nicotinamide (niacin, vitamin B3) and function in a wide variety of redox reactions. NAD and NADP are the major electron acceptors in the oxidation of fuel molecules and in pentose phosphate pathway, respectively. Reduced forms NADH and NADPH act as major electron donors used primarily for the generation of ATP (NADH) and for reductive biosyntheses (NADPH) [5]. The adenine nucleotides (ANs) which include adenosine triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP), act as free-energy donors in most of energy-requiring processes. The free energy gain during hydrolysis of ATP or ADP is used for three major purposes: for mechanical work such as muscle contractions and other cellular movements, the active transport of molecules and ions, and the biosynthesis of molecules from simple precursors. ATP and ADP also play an important role as physiological signaling molecules which bind to membrane purine receptors [6].

A number of methods have been used to analyze PNs and ANs which include enzymatic [7], fluorometric [8], bioluminescence [9], thin-layer chromatography [10] and high-performance liquid chromatography [11]. There are also several applications for determination of nucleotides with CE methods [12–17]. To the best of our knowledge, there has been no report of the analysis of all seven PNs and ANs by CE. However, analysis of submicromolar levels of nucleotide metabolites is challenging because of the poor concentration sensitivity of CE when using UV detection. Due to short detection path length and small injection volume, there is interest in the use of on-line preconcentration techniques to enhance concentration sensitivity by effective focusing of large sample injection volumes prior to detection. Previously reported on-line focusing methods in CE have included sample stacking [18,19], transient isotachophoresis [20,21] and sweeping [22,23]. Recently dynamic pH junction [24,25] and dynamic pH junction-sweeping [26] were reported as effective on-line focusing methods in CE for a variety of weakly acidic analytes enhancing sensitivity up to 1200-fold compared to conventional injections. Generally, in dynamic pH junction weakly acidic species are dissolved in an acidic or neutral sample matrix (analyte has low mobility) and separation is performed using a basic background electrolyte (BGE) where the analyte has a high negative mobility, thereby forming a discrete pH step or junction between sample and BGE zones. Since borate is used as BGE in previous dynamic pH junction reports [24–26], analyte velocity is modified by both pH and borate complexation to induce electrokinetic focusing. Quirino and Terabe [27] recently reported an on-line focusing approach for some diol compounds which was referred to as sweeping by borate complexation. Sweeping is defined as picking and accumulating of analytes by the pseudostationary phase that fills the sample zone during application of voltage [22]. In sweeping the difference in pH is not essential from the viewpoint of concentration mechanism, although in sweeping with borate ions described in Ref. [27] the sample matrix was sodium chloride solution which was required to adjust sample conductivity. The conditions given in Ref. [27] are similar to those give in Ref. [25] but the nomenclature is different and may be confusing for the audience. Since one of the authors of each relevant paper is involved in this article, we have

tried to clarify the difference as follows: the dynamic pH junction can be used if the difference in pH between the sample solution and BGE is essential for focusing analytes and the pH change will cause significant changes in migration velocities, while sweeping can be used for the system where any complexation between the analyte and the pseudo-stationary phase is the major mechanism of focusing. We will not refer to dynamic pH junction if the pH difference between the two zones is not the major focusing mechanism even if the different pH is employed between the two zones. In the current manuscript, borate complexation plays a vital role in diol focusing. Under certain conditions however, both pH differences and borate complexation may play equal roles in analyte focusing, such as catecholamines [24] or guanine and uridine nucleotides [25]. Further work is being carried out to better clarify the relationship of analyte focusing modes in CE such as dynamic pH junction, sweeping and transient isotachophoresis.

In the present study on-line analyte focusing using borate complexation is applied for analysis of seven PNs and ANs derived from *Bacillus subtilis* cell extracts. Under optimum conditions, the LOD with $S/N = 3$ is about 2×10^{-8} *M* for the PNs. Method validation of sweeping by borate complexation technique demonstrated excellent reproducibility and linearity. The concentration of PNs and ANs in cell extracts and single cell were determined. Sweeping by borate complexation CE demonstrated to be a sensitive and accurate method applicable for the analysis of submicromolar levels of metabolites in biological samples.

2. Experimental

2.1. Apparatus

All experiments were performed using an Agilent CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detection (DAD) system. Separations were carried out on fused-silica capillaries, 56 cm (48.6 cm effective length)×50 μm I.D. (Polymicro Technologies, Phoenix, AZ). New capillaries were first rinsed with 1.0 *M* NaOH (20 min), followed by 0.1 *M* NaOH (20 min), purified water (20 min), and finally with the BGE (60 min). Detection wavelengths were set on 200 or 254 nm and the capillary temperature was thermostated at 20 °C. Samples were introduced with a pressure injection of 50 mbar (5 kPa) for 2–200 s. The applied voltage was set at 20 kV during separation run. To ensure repeatability, before each injection, the capillary was preconditioned for 1 min by flushing with 0.1 *M* NaOH followed by 1 min with methanol and 1 min with water and finally by rinsing with running electrolyte for 5 min. Background electrolytes in the vials were replaced after every second run.

2.2. Reagents and samples

Pyridine and adenine nucleotides (ATP, ADP, AMP, NAD, NADP, NADH and NADPH) were all purchased from Sigma (St. Louis, MO, USA). All other reagents were purchased from Wako (Osaka, Japan). The reagents used were of analytical-reagent grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). Individual stock solutions of standards (ATP, ADP, AMP, NAD, NADP) were prepared in purified water at a concentration of 10 m*M*. NADH and NADPH are relatively unstable in acidic or neutral conditions [28]. To ensure their stability stock solutions at a concentration of 10 m*M* were prepared in 50 m*M* borate buffer pH 8.0 and stored in amber glass bottles in refrigerator at 4 °C. Prior to analysis, further dilutions to obtain appropriate concentrations of standard solutions were made using 75 m*M* phosphate buffer pH 6.0. The pH of the separation buffer was adjusted by using 1.0 *M* NaOH and boric acid (Nacalai Tesque, Kyoto, Japan) within a range of pH 9.0 to 10.2. The optimized BGE consisted of 150 m*M* borate buffer pH 9.2. A solution of acetone was used as an electroosmotic flow (EOF) marker. Buffer solutions prior to use were sonicated and filtered through 0.45 μm membrane filters. Background electrolytes in the vials were replaced after every two run. Peaks were identified by spiking the sample solution with standard solutions of each pyridine and adenine nucleotides and by comparing the UV spectra (range, 190–500 nm) of peaks obtained from DAD.

2.3. Bacillus subtilis cell culture

Bacteria *Bacillus subtilis* (strain 168) was cultured in 100 ml of S6-glucose or S6-malate medium at 37 °C by shaking. The S6 medium (100 ml) consists of 5 m*M* KH_2PO_4, 10 m*M* $(NH_4)_2SO_4$, 100 m*M* 3-(*N*-morpholino)propane sulfonate (MOPS), 0.05 mg tryptophan, 20.3 mg $MgCl_2 \cdot 6H_2O$, 10.29 mg $CaCl_2 \cdot 2H_2O$, 0.99 mg $MnCl_2 \cdot 4H_2O$, 0.014 mg $ZnCl_2$, 0.135 mg $FeCl_3 \cdot 6H_2O$, and 25 m*M* glucose or 37.5 malate as a carbon source. The number of bacteria increased with the duration of culture which was monitored by the optical density measurements. When the cell concentration reached about 4×10^8 living cells ml^{-1}, 10 ml of culture medium was withdrawn and filtered by a glass membrane filter (Whatman GF/B 1 μm, 2.4 cm). Cells on the filter were stored in −80 °C temperature until extraction.

2.4. Cell extraction

Cell extraction procedure after small modifications was similar to the method described previously [2,4]. Briefly: cells on the membrane filter were extracted two times with 1 ml of ice-cooled methanol for 20 min. Methanol extracts were combined and placed in a 15 ml plastic tube. To the tube, 1 ml of chloroform and ca. 500 μl of purified water were added and then thoroughly mixed for 1 min. After 5 min, the upper layer was withdrawn and centrifuged through a Millipore M_r 5000 cutoff filter (Ultrafree-MC) for 60 min to remove proteins and other debris. The filtrate was evaporated under a stream of nitrogen at 30 °C. Prior to CE analysis, the dried sample was dissolved in 20 μl of purified water.

3. Results and discussion

3.1. Separation optimization of pyridine and adenine nucleotides

On-line focusing of large injection volumes by borate complexation was performed by using 75 m*M* phosphate pH 6.0 in the sample matrix based on a previous report [26]. Because of the lack of weakly acidic functional groups on PNs and ANs, analyte focusing is primarily mediated by borate complexation. In sweeping by borate complexation method, a long sample injection plug is introduced directly into the capillary. During all preliminary experiments with standard samples, a 50-s injection was used. Initially, borate buffer in concentration range from 50 to 150 m*M* (pH 9.2) was used to select the most suitable concentration for separation optimization. Beside its role as an alkaline buffer, borate was chosen also for its ability to act as selective complexation agent for vicinal diol groups. Borate ions can interact with the vicinal diol moiety of analytes to form anionic complexes with higher electrophoretic mobility [29]. Optimal borate concentration for resolution of analytes was determined to be 150 m*M*. A lower concentration of borate using a long injection plug (~10% of capillary length) resulted in co-migration of the most peaks of interest. The aim of this study was to determine an optimum separation conditions for sensitive analysis of pyridine and adenine nucleotides using borate complexation effect. In order to investigate the influence of borate pH on separation performance, different borate buffers in the pH range from 9 to 10.2 were used. Electrophoretic mobilities of PNs and ANs changed significantly reflected by longer migration times when pH of borate buffer increased from 9.0 to 10.2 (Fig. 1A). Fig. 1B shows the measured electrophoretic mobilities of nucleotides and adenine phosphates as a function of borate pH. The mobility of analytes slightly increases as the pH of borate was increased from 9.0 to 10.2 due to enhanced borate complexation with vicinal hydroxyl groups [29]. However, at the same time with an increase of borate pH, the EOF of BGE decreases significantly from 5.49×10^{-4} cm^2 at pH 9.0 to 4.94×10^{-4} cm^2 at pH 10.2. A weaker EOF with an increase of pH is caused by higher ionic strength of BGS due to sodium hydroxide base used for adjustment of BGE pH. As a result migration times of analytes increase despite small changes in their electrophoretic mobilities. Also note in Fig. 1 the influence of borate pH on peak resolution. In pH 9.0, analytes migrate the fastest however three peaks are unresolved (ATP, NADPH, ADP). In pH 9.2 all seven analytes are separated in less than 15 min. At pH values above 9.5 resolutions among most of the analytes increased with an exception of NADH and NADP as the peaks co-migrated. Observed migration order for adenine

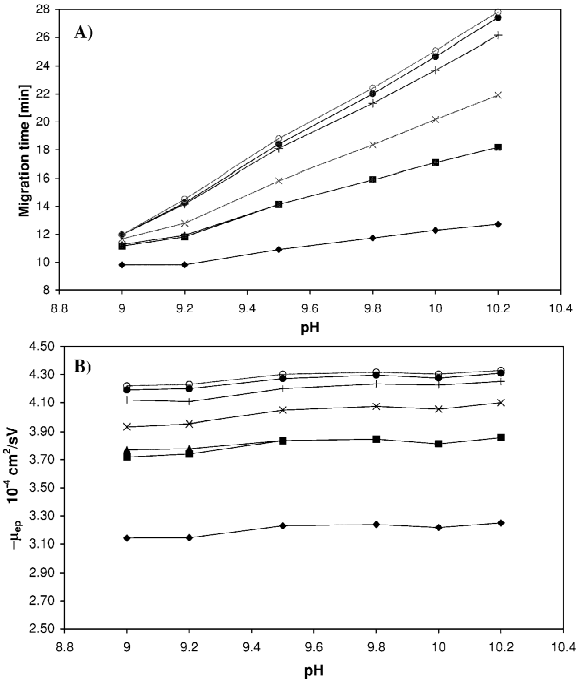

Fig. 1. Effect of borate pH on migration time (A) and electrophoretic mobility (B) of pyridine and adenine nucleotides. Experimental conditions: 150 m*M* borate, applied potential, 20 kV; injection pressure, 50 mbar, 50 s; capillary temperature 20 °C; detection, 200 nm; fused-silica capillary 56.0 cm (48.6 cm effective length)×50 μm I.D. Sample identification: ♦, NAD; ■, NADP; ▲, NADH; ×, AMP; +, ATP; ●, NADPH; and ○, ADP. All samples at concentration 1×10^{-5} *M* were dissolved in 75 m*M* phosphate buffer pH 6.0.

nucleotides from mono-, tri- and diphosphate is similar to that reported by Uhrova et al. [30]. A possible explanation of this migration behavior is due to incomplete dissociation (higher acidity) of adenosine triphosphate resulting in lower mobility in comparison with adenosine diphosphate. Incomplete dissociation could come from the terminal –OH group of ATP which has a weaker acidic properties than other –OH moieties of nucleoside.

The separation of PNs and ANs in the presence of the anionic surfactant sodium dodecyl sulfate (SDS), was next investigated. It was observed that addition of SDS to the background electrolyte did not change overall selectivity of separation but affected migration time of nucleotides. Analysis times using micellar electrokinetic chromatography (MEKC) were longer than those in capillary zone electrophoresis, and the resolution was not significantly improved since analyte–SDS partitioning is relatively weak.

To enhance selectivity, host–guest complexation with β-cyclodextrin (β-CD) in borate buffer, with or without presence of SDS, was also investigated. Theoretically, the addition of β-CD to BGE enhances mobility differences for analytes with similar charge to mass ratios, resulting in better resolution. According to Kawamura [14] the complexation of nucleotides with β-CD is stronger than that with γ-CD or α-CD. In our studies, 5 and 10 m*M* β-cyclodextrin in BGE at different pH was used to verify the influence of complexation on selectivity. The addition of β-CD to borate buffer increased migration time of all analytes. The increase in apparent migration time is due to relatively small decrease in analyte mobility from specific CD binding superimposed on increase in buffer viscosity with CD addition. Stronger increase of migration could be observed when a concentration of 10 m*M* β-CD instead of 5 m*M* β-CD in BGE was used. After thorough optimization of separation conditions, 150 m*M* borate buffer pH 9.2 without micelles or CDs was selected as the optimum.

Since solutions of NADH and NADPH are unstable in phosphate buffer at acidic or neutral conditions [28], it was necessary to investigate the influence of analysis time on NADH and NADPH peak area. During CE analysis, after 5 h NADH decreased to about 60% of its initial peak area (after 12 h to 25%) and NADPH, which is quantitatively less stable than NADH, after 5 h had decrease to less than 20% of initial peak area. For comparison, sample solutions of NAD and NADP changed by less than 1% after 5 h under the same conditions. During further studies only freshly prepared, ex tempore, sample solutions of NADH and NADPH were used.

3.2. Optimization of sample matrix composition

The composition of the sample matrix relative to the BGE plays a vital role in on-line preconcentration techniques in CE. Optimization of sample matrix composition consisted of selection of optimum ionic strength, pH and injection length to achieve the highest preconcentration effect. Ionic strength of sample matrix was verified at the con-

centration of 0 m*M* phosphate (pure water), 25, 50, 75, 100 and 150 m*M*. Using large injection plug (~5 cm or 10% of capillary length), when analytes are dissolved in pure water sample zones migrate as broad and unresolved peaks (Fig. 2A). With an increase of phosphate ions in sample matrix, peak resolution was greatly improved because of extremely narrow peak widths (Fig. 2B). However when the ionic strength of phosphate was greater than 100 m*M*, NADPH and ADP peaks overlapped due to increase effect of preconcentration and extended sample zone width. Using continuous buffer (which has the same composition as the BGE) as a sample matrix no preconcentration effect was observed. This could be explained since there is no electrokinetic mechanism counteracting diffusion, so that analytes migrate as broad, overlapping peaks due to sample overloading [26]. No further significant improvement in preconcentration or peak separation could be noted when the pH of sample matrix solutions was varied from 6.0 to 8.0. One could expect that with changes of sample matrix pH the changes in ionization states of analytes would occur. However, PNs and ANs are mostly ionized in pH above 6, thus there are no significant changes in their charges. Optimum sample matrix conditions consisted of 75 m*M* phosphate pH 6.0 in sample matrix (Fig. 2B). Next, the dependence of injection plug length on nucleotide focusing was studied when using an injection time of 2 to 200 s, which represents 0.4 to 40% of capillary length. When injections plug length exceeded 10% of capillary length (50 s), resolution among NADPH and ADP peaks decreased (Fig. 2B) and with further increase of injection time peaks overlapped. Therefore, in subsequent studies of metabolites in *B. subtilis* cell extracts, a 40 s injection time was applied. Detector bandwidths (w_{det}) of analytes were calculated from nucleotide migration time, peak baseline width and length of capillary to detector in order to normalize measured bandwidths for differential analyte migration velocities [26]. Using sweeping by borate complexation and large sample injection (injection bandwidth, $w_{inj}=5$ cm), PN and AN peaks are extremely sharp and w_{det} range from 0.57 to 0.69 cm for AMP and NADH, respectively. This represents about a 4-fold enhancement in narrowing of nucleotides peak width in comparison to stacking with a large injection plug and water as a sample matrix. In previous reports [24–26] the detector to injection bandwidth ratio (DIBR) was used as a quantitative measure for assessing analyte focusing that corrects for different migration velocities. Analyte focusing is defined when DIBR values are less than 1. NAD peak on Fig. 2B has a w_{det} of only 0.63 cm, which results in DIBR value less than 0.13, meaning that the original sample plug for NAD is narrowed almost 8-fold during focusing when using a borate complexation.

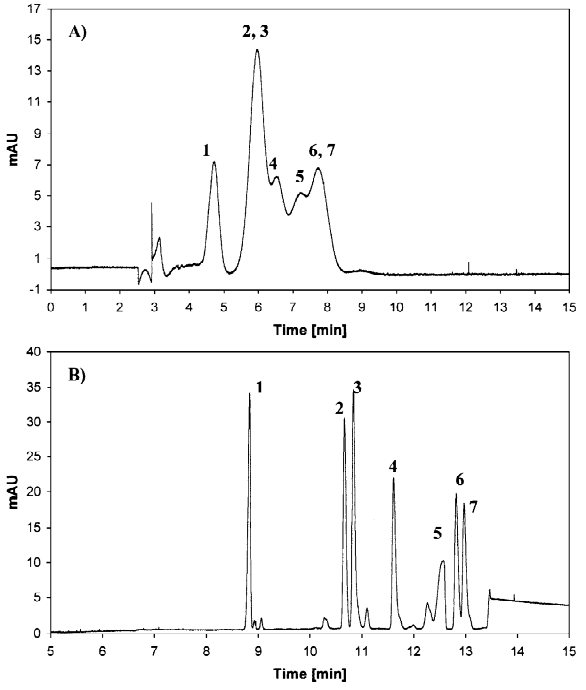

Fig. 2. Electropherograms comparing nucleotides and adenosine phosphates focusing using large injection plugs (5 cm) with: (A) pure water as sample matrix, (B) sweeping borate complexation in CE. The BGE used was 150 m*M* borate at pH 9.2. Sample solutions at concentration 1×10^{-5} *M* dissolved in either: (A) purified water, (B) 75 m*M* phosphate, pH 6.0. Identification: 1, NAD; 2, NADP; 3, NADH; 4, AMP; 5, ATP; 6, NADPH; and 7, ADP. Other conditions as in Fig. 1.

3.3. Repeatability, linearity and LODs

The reproducibility, linearity as well as limits of detection (LODs) and limits of quantitation (LOQs) of method for the analysis of PNs and ANs were evaluated. Table 1 presents the reproducibility data obtained for migration time, peak area and peak

Table 1
Repeatability, linearity and sensitivity for pyridine and adenine nucleotides with sweeping borate complexation

Name	RSD ($n=6$), (%)			Calibration line		LOD (mol/l) ($S/N=3$)
	Migration time	Peak height	Peak area	$y=ax+b$	R	
NAD	0.33	1.2	1.1	$y=1.066x+0.01$	0.9999	2.45×10^{-8}
NADH	0.33	–[a]	–	$y=1.071x-2\times10^{-5}$	0.9999	2.44×10^{-8}
NADP	0.32	1.1	2.4	$y=1.1x+0.009$	0.9999	2.37×10^{-8}
NADPH	0.28	–	–	$y=1.161x-0.004$	0.9999	2.25×10^{-8}
AMP	0.31	1.6	1.0	$y=0.796x+0.004$	1.0	3.28×10^{-8}
ADP	0.27	1.8	1.2	$y=0.996x+0.0003$	0.9999	2.62×10^{-8}
ATP	0.27	2.4	1.2	$y=0.729x+0.003$	0.9999	3.58×10^{-8}

[a] Not determined due to poor stability, see Section 3.1.

height as reflected by relative standard deviation (RSD). Excellent reproducibility of analyte migration time indicates proper choice of BGE and analytical conditions as well as effectiveness of preconditioning steps. The RSDs for the migration times with six consecutive injections ($n=6$) are in the range from 0.27 to 0.33%. Similarly, good reproducibility was obtained for both peak height and peak area with the RSD in % in the range of 1.1 to 2.4 and 1.0 to 2.4, respectively. Concentration of sample during reproducibility studies was 1×10^{-5} M and CE analyses were performed with pressurized injection (50 mbar in 40 s). The reproducibility of peak parameters was not measured for NADH and NADPH due to their low stability in sample solution and graduate decomposition with time. However, during linearity studies both analytes were included to the test because before every injection new sample solution had been prepared.

To determine the linearity of method eight standard samples at different concentrations ranging from 1×10^{-5} to 5×10^{-8} mol/l were measured. The correlation coefficients of calibration line equations for all analytes were at the level $R=0.9999$. The concentration detection limits for analyzed nucleotides and adenine phosphates were between 2.2×10^{-8} and 3.6×10^{-8} M with pressure injection of 50 mbar for 40 s at a signal-to-noise (S/N) ratio of 3. LODs obtained by sweeping by borate complexation are about two orders of magnitude better than when the pure water is used as a sample matrix using conventional injection 2 s. Note, that with water as a sample matrix, long injection plug cannot be applied due to band broadening and resulting overlapped peaks. The limit of quantitation (LOQ) was determined based on two criteria: (i) the S/N ratio of analyzed peak was greater than 10; and (ii) the RSD of the area for replicate analysis of samples at the LOQ was less than 10%. The LOQ for analyzed peaks were found to be 5.3×10^{-8} and $6.5–7.8\times10^{-8}$ M for PNs and ANs, respectively.

3.4. Analysis of nucleotide metabolites in cell extracts from B. subtillis

Table 2 presents the data of measured concen-

Table 2
Estimated concentrations of pyridine and adenine nucleotides in *Bacillus subtilis* cell extracts and single cell from different culture media (CM)

Carboxylic acid	Concentration in cell extract from malate CM (μM)	Concentration in cell from malate CM (mM)	Concentration in cell extract from glucose CM (μM)	Concentration in cell from glucose CM (mM)
NAD	78.6 (±7.6)	94.7	17.3 (±7.25)	20.8
NADP	3.46 (±0.46)	4.17	0.66 (±0.12)	0.8
AMP	35.0 (±1.21)	42.2	7.71 (±0.71)	9.29
ADP	13.9 (±0.99)	16.7	2.61 (±1.06)	3.15
ATP	5.4 (±0.78)	6.51	2.33 (±0.42)	2.81

trations of PNs and ANs in *B. subtilis* cell extracts. NAD and NADP were quantitated in cell extracts from both malate and glucose culture (Fig. 3). Measured concentrations of NAD and NADP in glucose and malate culture were 17.3, 0.66 and 78.6 and 3.46 μ*M*, respectively. NADH and NADPH were not measured in cell extracts with optimized method. It is not clear if this is due to low concentrations of analytes in cell extracts or due to the their poor stability. Among nucleotide metabolites, AMP was determined to be at the highest concentration, 7.71 and 35.0 μ*M* in glucose and malate culture, respectively. ADP and ATP were measured at significantly lower concentrations in both types of cell extracts. The trend in metabolite concentrations from the highest in case of AMP to the lowest for ATP is in agreement with those recently reported by Soga et al. [31] using CE–electrospray ionization MS. These preliminary experiments demonstrated that nucleotide metabolite concentration levels are sensitive to environmental conditions, such as type of carbon source or other nutrients as reported for flavin coenzymes [32]. With malate as a carbon source, concentrations of PNs and ANs are approximately five times greater than in glucose culture. This fact could be related to the role of malate in cell specific metabolic pathways. Malic acid is an intermediate in the tricarboxylic acid (TCA) cycle which can be directly incorporated to the biochemical pathway, whereas glucose (during glycolysis) is converted to pyruvate and later to acetyl-CoA upon entering the citric acid cycle. The influence of carbon source in culture medium on number and amounts of metabolites was also reported during previously studied metabolites from the TCA cycle [2]. Based on the number of bacteria cells per ml of culture medium (4×10^8 cell/ml) and considering the total volume of the cells in 1 ml medium as 0.83 μl, the concentrations of PNs and ANs per single bacteria cell was estimated (Table 2). Concentrations of metabolites in single cell were at m*M* level. The highest concentrations in the cell were found for NAD and AMP 94.7 and 42.2 m*M* from malate and 20.8 and 9.3 m*M* from glucose culture medium, respectively.

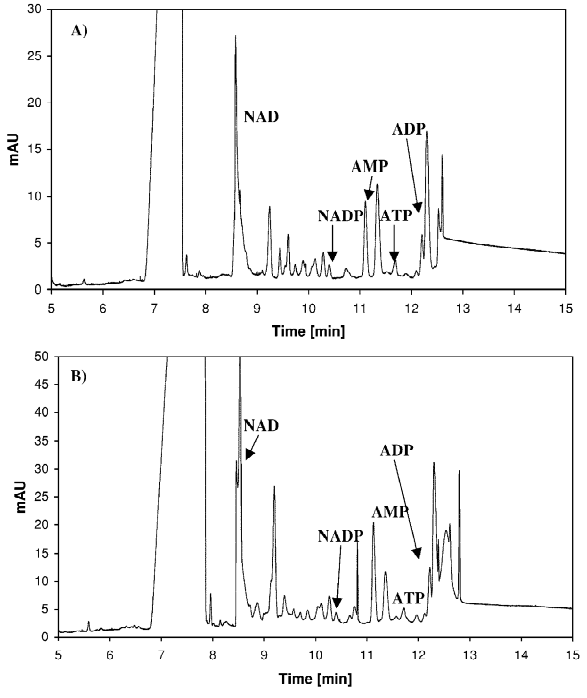

Fig. 3. Electropherograms presenting sweeping borate complexation in analysis of *B. subtilis* cell extracts from: (A) glucose and (B) malate, as culture mediums. Experimental conditions as in Fig. 1 except pressure injection: 50 mbar, 40 s.

4. Concluding remarks

Sweeping by borate complexation represents a useful on-line focusing method in CE to preconcentrate diol analytes, reflected by over 4-fold enhancement in band narrowing of nucleotides zones in comparison to other preconcentration methods. Using sweeping by borate complexation CE, sensitive analysis of PNs and ANs in *B. subtilis* cell extracts was achieved. LODs of nucleotide metabolites were less then 20 n*M* and LOQs were between 50 and 80 n*M* using UV detection. From the total cell volume at ml of cell extract, concentrations of PNs and ANs per single bacterium cell were estimated at m*M* level. The optimized method demonstrated excellent linearity and reproducibility. NADH and NADPH were found to be unstable in alkaline conditions thus requiring preparation of fresh sample solutions ex tempore. Concentrations of intracellular metabolites were determined to be different in cell extracts derived from glucose or malate culture media. This observation highlights the role of en-

vironmental conditions (like, e.g. culture medium or carbon source) on intracellular metabolite levels and metabolite profiling. *B. subtilis* which is a bacteria commercially used for the production of several important industrial enzymes, is still rarely studied in the area of metabolome and its relationships to the genome and the environment. Further studies of CE using sweeping by borate complexation on other classes of metabolites present in *B. subtilis* are needed for better understanding of complex cellular processes.

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