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Analysis of carboxylic acid metabolites from the tricarboxylic acid cycle in *Bacillus subtilis* cell extract by capillary electrophoresis using an indirect photometric detection method

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Abstract

With a growing interest in metabolome analysis, there is a need for developing robust methods for analysis of intracellular metabolites profiles in real samples like e.g., bacteria cell. Due to their weak absorbance properties, tri- and dicarboxylic acids from TCA cycle (citric, isocitric, 2-oxoglutaric, succinic, fumaric, malic) as well as carboxylic acid metabolites from glycolysis pathway, urea cycle and metabolism of amino compounds (formic, pyruvic, lactic, acetic, glutamic) were analyzed by capillary electrophoresis (CE) with indirect UV detection. Using 4 m*M* 2,6-pyridinedicarboxylic acid as a highly UV absorbing carrier electrolyte, 0.2 m*M* cetyltrimethylammonium bromide, 10% ethylene glycol and 10% acetonitrile, pH 3.5, carboxylic acids metabolites were analyzed in *Bacillus subtilis* cell extract from two different cultures: glucose and malate. CE with an electrokinetic injection mode achieved limits of detection in the range of 13–54 ppb ($1.12\cdot10^{-7}$–$5.96\cdot10^{-7}$ *M*). The reproducibility and linearity of method was investigated with RSD for migration time less than 1.3% and acceptable correlation coefficients. The optimized CE method was used to compare metabolome content of cell extract derived from two different culture media containing either glucose or malate as a carbon source. The changes in carboxylic acid metabolites profile were observed depending from used culture medium. Carboxylic acid concentrations ranged: in cell extract from malate culture from 59 to 0.5 μ*M* for lactate and citrate, respectively, and in cell extract from glucose culture from 133 to 0.5 μ*M* for glutamate and citrate, respectively. Appropriate concentrations of carboxylic acid in the single bacterium cell were estimated at m*M* and sub-m*M* levels.
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Keywords: *Bacillus subtilis*; Indirect detection; Detection, electrophoresis; Bacteria; Tricarboxylic acid cycle; Carboxylic acids

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1. Introduction

Studying metabolites from the tricarboxylic acid (TCA) cycle in the real sample (like e.g., bacteria cell extract) can be considered as a part of metabolome analysis [1]. The metabolome is defined

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as the complete set of metabolites synthesized by a given biological system, such as organism, organ, tissue, cell or cell compartment. After major advances in projects involving the genome and proteome, metabolome analysis seems to be the next emerging field in bioscience. Metabolome analysis can be described as a comprehensive, qualitative and quantitative analysis of all low molecular mass compounds present in living cells. Metabolites are the final products of cellular regulatory processes, and their quantitative levels can be regarded as the ultimate response of biological systems to genetic and environmental changes [2]. Data obtained from metabolome analysis can be used in different purposes like: simulation of the biological activity with genes coded in genome; studying of functions of new genes and effects of gene mutation on metabolite levels; or applied to gene technology to produce valuable metabolites [1].

Recently, two reports were published on analysis of metabolome in bacteria cell extracts [3,4]. Dynamic pH junction-sweeping CE with laser-induced fluorescence (LIF) detection was applied to analyze trace amounts of flavin derivatives in *Bacillus subtilis* at picomolar concentrations [3]. Significant differences in flavin concentrations were measured in cell extracts derived from culture media of different carbon sources. CE with UV photometric detection with sweeping by borate concentration was used for analysis of pyridine and adenine nucleotides at nanomolar level [4]. 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 biological samples such as *B. subtilis* cell extracts.

The bacteria, *Bacillus subtilis*, due to its culture properties has been commercially applied for the production of several industrial enzymes (e.g., α-amylase, alkaline protease, β-glucanase). In 1997, the genome of *B. subtilis* was completely sequenced [5] however; there is still relatively little development in the area of bacteria metabolome and its relations to the genome and the environment.

The main metabolites (intermediates) in the TCA cycle are di- and tricarboxylic acids (Fig. 1). TCA cycle also known as a citrate cycle or Krebs cycle is the final common catabolic pathway for the oxidation of fuel molecules like e.g., amino acids, fatty acids or carbohydrates. During one course of citric acid cycle for each molecule of acetyl-coenzyme A (CoA) which enter the cycle, a total of 12 high-energy phosphate bonds of adenosine triphosphates (ATP) are generated [6]. Comparative qualitative and quantitative analysis of carboxylic acids from the TCA cycle in different cell types and under different

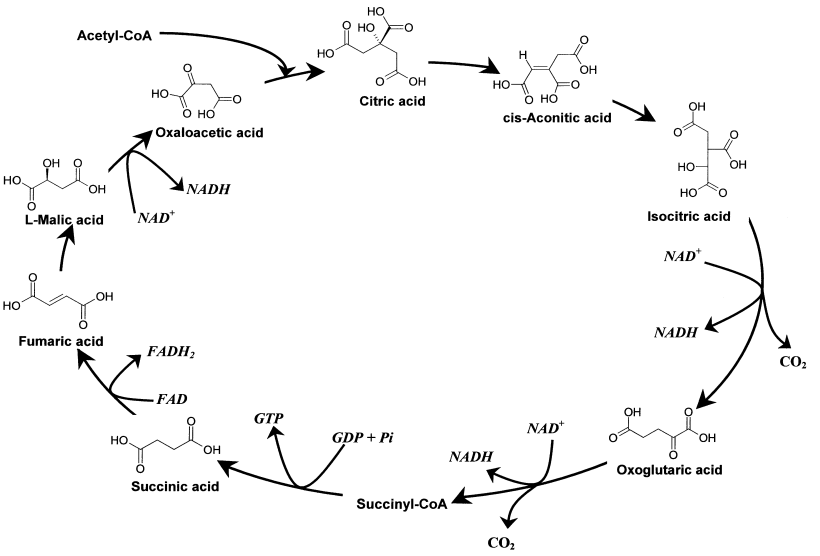

Fig. 1. Schematic representations of TCA cycle metabolic pathway with chemical structures of analyzed carboxylic acids.

growth conditions could be of utmost importance for understanding of external environment influences on metabolic output.

Most of the aliphatic carboxylic acids, which have a low molar absorptivity, are UV transparent and thus are poorly detectable compounds by spectrophotometry. This is due to a lack of strong chromophores in their chemical structure. To overcome this difficulty the UV indirect photometric detection method has been often applied. Principles of indirect UV detection method has been described elsewhere [7–11].

Although many reports exist on analysis of carboxylic acids [12–17], to the best of our knowledge, there is no report in the literature which deals with the analysis of the set of mono-, di- and tricarboxylic acids forming intercellular metabolites by CE.

In our study, tri- and dicarboxylic acids from the TCA cycle as well as carboxylic acid metabolites from other metabolic pathways (e.g., glycolysis, urea cycle) were successfully separated using background solution with 2,6-pyridinedicarboxylic acid (PDC) as a carrier electrolyte. The optimized method was applied for analysis of carboxylic acid metabolites of *B. subtilis* cell extracts derived from two different carbon sources, glucose and malate. Using CE with electrokinetic injection mode sensitive analysis of the carboxylic acid metabolites on sub-micromolar levels (ppb) was realized.

2. Experimental

2.1. Apparatus

All experiments were performed with a Bio-Focus 3000 capillary electrophoresis system (Bio-Rad, Hercules, CA, USA). For the separation of analytes, fused-silica capillaries, 75 cm (70.6 cm effective length)×75 μm I.D.×365 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) was used. UV detection wavelength was set at 200 nm and the capillary temperature was maintained at 15 °C. The sample compartment was thermostated at 15 °C to prevent sample solvent evaporation from Eppendorf vials. This is very important when dealing with low sample volumes like 20 μl. Samples were injected by both pressure (0.4, 2 or 4 s with 34.5 kPa) and electrokinetic (10 kV in 10 s) mode. Negative polarity was used due to reversed electroosmotic flow caused by the presence of cationic surfactants in the background solution. A constant voltage of −25 kV was applied during the separation run. Prior to first use, a new capillary was pretreated only with the background solution for 20 min. Use of sodium hydroxide, as a flushing medium should be avoided due to low repeatability and loss of analytical performance of the presented method. To ensure repeatability, between each analysis, the capillary was preconditioned with background solution (BGS) for 4 min. Due to the limited buffering capacity of BGS, the buffer in the vials was replaced after every second run. Molar absorptivities were determined using a Shimadzu UV-2450 spectrophotometer (Kyoto, Japan) with 1-cm path length quartz cells.

2.2. Reagents and samples

2,6-Pyridinedicarboxylic acid (PDC), ethylene glycol, citric acid (CA), isocitric acid (IA), 2-oxoglutaric acid (OA), succinic acid (SA), fumaric acid (FA), malic acid (MA), formic acid (FoA), lactic acid (LA) and acetic acid (AcA) were purchased from Wako (Osaka, Japan). Pyruvic acid (PA) and glutamic acid (GA) were from Sigma (St. Louis, MO, USA) and cetyltrimethylammonium bromide (CTAB) was obtained from Tokyo Kasei (Tokyo, Japan). Other reagents were purchased from Kanto Chemical (Tokyo, Japan). All used reagents were of analytical-reagent grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

A sample stock solution of each carboxylic acid (CA, IA, OA, SA, FA, MA, FoA, AcA, LA, PA and GA) was prepared in purified water at a concentration of 3000 ppm and further diluted in water to obtain the appropriate final concentration. Chemical structures of analyzed di- and tricarboxylic acids from TCA cycle are presented on Fig. 1. The optimized background solution (BGS) consisted of 4 m*M* PDC as a strongly UV absorbing carrier electrolyte, 0.2 m*M* CTAB used to reverse EOF and shorten analysis time, together with 10% ethylene glycol and 10% acetonitrile as organic modifiers. The background solution was adjusted to pH 3.5 (with 0.1 *M* NaOH) before addition of organic modifiers. BGS solutions prior to use were sonicated

and filtered through 0.45 μm membrane filters. By spiking standard sample solutions with the known standard the peak identification for each analyte was carried out.

2.3. Bacillus subtilis cell culture

Bacteria *B. 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) consisted of 5 m*M* KH_2PO_4, 10 m*M* $(NH_4)_2SO_4$, 100 m*M* 3-(*N*-morpholino)propanesulfonate (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 $4 \cdot 10^8$ 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 at −80 °C until extraction.

2.4. Cell extraction

Cells on the filter were twice extracted with 1 ml of ice-cooled methanol for 20 min. Methanol solutions 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 10 min, the upper layer was withdrawn and centrifuged through a Millipore 5-kDA cutoff filter (Ultrafree-MC, Millipore, MA, USA) 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. Optimization of the separation

Optimum performance of CE analysis by indirect UV detection requires proper selection of carrier electrolyte [8] whose electrophoretic mobility is similar to mobility of analyzed anions. In theory, the carrier electrolyte with mobility matching those of the majority of the analytes would give a better separation and resolution due to peak symmetry. The carrier electrolyte should also possess a high molar absorptivity which is a key parameter influencing the method sensitivity. In this case, the molar absorptivity of PDC (ca. 43 500 l mol^{-1} cm^{-1}) is one of the highest from all available carrier electrolytes and its electrophoretic mobility match closely that of analyzed carboxylic acids [12].

During preliminary experiments the influence of BGS pH on effective mobilities of anions was investigated. A study was made in the BGS consisting of 4 m*M* PDC and 0.2 m*M* CTAB in the pH range from 3.1 to 9.7. The most pronounced changes in electrophoretic mobility of carboxylic acids can be observed in the pH range from 3.1 to 6. This is caused by changes in the ionization states of TCA cycle acids. The highest mobility in pH 3.1 represents 2-oxoglutaric acid ($\mu = 2.25 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) which is in agreement with one of the lowest pK_a value among carboxylic acids studied. The 2-oxoglutarate pK_{a1} value is reported to be 2.47 [18]. This means that in pH about 3.5, one of its carboxylic groups is ionized more than 90% and the remaining carboxylic group exists in non-ionized state. The lowest electrophoretic mobility presents succinic acid ($\mu = 0.37 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) possessing one of the highest pK_a values among tested carboxylic acids. pK_{a1} of succinic acid is about 4.21 [18], which means that in pH 3.5 one of the carboxylic groups has been ionized ca. 20% resulting in relatively slow electrophoretic mobility.

Based on the relationship between the pH of BGS and the electrophoretic mobility of analytes the selection of the optimum pH for separation was determined. The pH changes above 6 have little effect on carboxylic anions mobility, which even with the use of different BGS additives (acetonitrile, ethylene glycol) did not improve resolution of carboxylic acid mixture. The most diversified electrophoretic mobility was found in BGS with a pH below six which is in the range of analytes pK_a. The best resolution was observed when the pH of BGS was lowered to 3.5. This agrees with the principle that better resolution can be achieved when the separation is performed near the pK_a values of analytes [19]. This is especially the case when

separating the two carboxylic acids: citric and isocitric. Both acids have identical molecular mass and very similar values of pK_{a2} and pK_{a3}. Only at pH 3.5 (near pK_{a1} values, 3.13 for citrate and 3.29 for isocitrate [18]) was the separation of analytes by CE possible (Fig. 2).

In BGS of pH 3.5 consisting of 4 m*M* PDC, 0.2 m*M* CTAB with an addition of organic modifiers 10% ethylene glycol and 10% acetonitrile, baseline resolution of eleven carboxylic acids was achieved. Ethylene glycol influences the separation performance by changing the BGS viscosity thus affecting EOF, meanwhile acetonitrile improves selectivity by changing both BGS viscosity and zeta potential. Since the viscosity of the solution drops exponentially with increasing temperature, the electrophoretic mobility increases approximately 2% per one degree. During experiments with a decrease of temperature from 25 to 15 °C the mobility decreased significantly resulting in longer migration time of analytes. Fig. 2 depicts the optimum operation of eleven carboxylic acid metabolites separated using the above-mentioned analytical conditions.

The peak shapes of pyruvate and 2-oxoglutarate are broad and tailing probably due to the higher

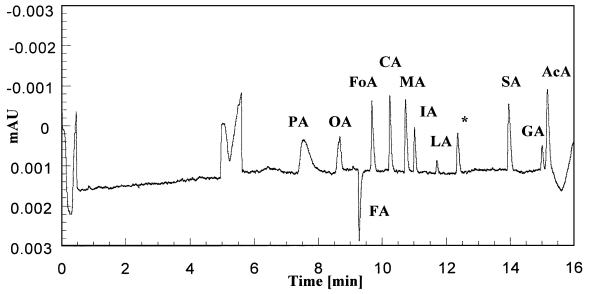

Fig. 2. Indirect UV detection of standard carboxylic acids under the optimized conditions. Experimental conditions: BGS, 4 m*M* PDC, 0.2 m*M* CTAB (pH 3.5), 10% ethylene glycol and 10% acetonitrile; untreated fused-silica capillary, 75 μm I.D., 70.6 cm effective length; electrokinetic injection 10 kV 10 s; detection, 200 nm; voltage −25 kV; temperature 15 °C. Samples: PA, pyruvic acid; OA, 2-oxoglutaric acid; FA, fumaric acid; FoA, formic acid; CA, citric acid; MA, malic acid; IA, isocitric acid; LA, lactic acid; SA, succinic acid; GA, glutamic acid; AcA, acetic acid; *, unidentified peak. The analyzed carboxylic acids were in following concentrations: PA, $1.43\cdot10^{-5}$ *M*; OA, $8.36\cdot10^{-6}$ *M*; FA, $6.04\cdot10^{-6}$ *M*; FoA $2\cdot10^{-6}$ *M*; CA, $2.64\cdot10^{-6}$ *M*; MA, $3.91\cdot10^{-6}$ *M*; IA, $5.27\cdot10^{-6}$ *M*; LA, $3.28\cdot10^{-6}$ *M*; SA, $6.77\cdot10^{-6}$ *M*; GA, $0.9\cdot10^{-6}$ *M*; and AcA $7.31\cdot10^{-6}$ *M*.

effective mobility of pyruvate and 2-oxoglutarate in comparison with the carrier electrolyte. When electrophoretic mobilities of analytes and carrier electrolytes are different, the peak shape of analyte can be affected as a result of electromigration dispersion [20]. Using 200 nm detection wavelengths, fumarate appears as a negative peak. Probably this could be explained by the fact that fumarate in low pH possesses higher absorbance than that of BGS. It is important to note that pK_a values for PDC are 2.16 and 6.92 [12] which means that in pH around 3.5 only one carboxylic group of PDC has been ionized probably affecting the absorptive properties. PDC with both carboxylic groups ionized at a pH above 6 possess significantly higher molecular absorptivity [12] which results in fumarate migrating as a negative peak. Additionally, using BGS at pH 3.5 and with detection at 254 nm wavelengths, fumarate similar to other carboxylic acids appears as a positive peak. However, for the sake of method sensitivity in our studies 200 nm wavelength was used.

3.2. Indirect UV analysis of Bacillus subtilis cell extract

Finally optimized indirect UV method has been applied to the analysis of *B. subtilis* cell extracts from different culture media (Fig. 3). Fig. 3a presents the electropherogram of bacteria *B. subtilis* cell extract from glucose medium. Using CE with indirect UV detection in the cell extract from glucose as a carbon source two carboxylic acids from TCA cycle are detected: citrate and succinate. Four other carboxylic acid metabolites are identified: formate which participates in the urea cycle and in the metabolism pathway of amino groups, lactate and acetate from glycolysis pathway, and glutamate an important intermediate in amino compounds metabolism. The unidentified first major peak in Fig. 3a co-migrated with the peak of 2-oxoglutarate. Fig. 3b presents the electropherogram of bacteria cell extract cultured on malate medium. In comparison to cell extracts from glucose as a carbon source, the cell extract from a malate culture media resulted in the presence of 2-oxoglutarate, fumarate and malate. Malate is in very high concentration which can be explained by the presence of malate in the original culture medium. The fact that bacteria are growing

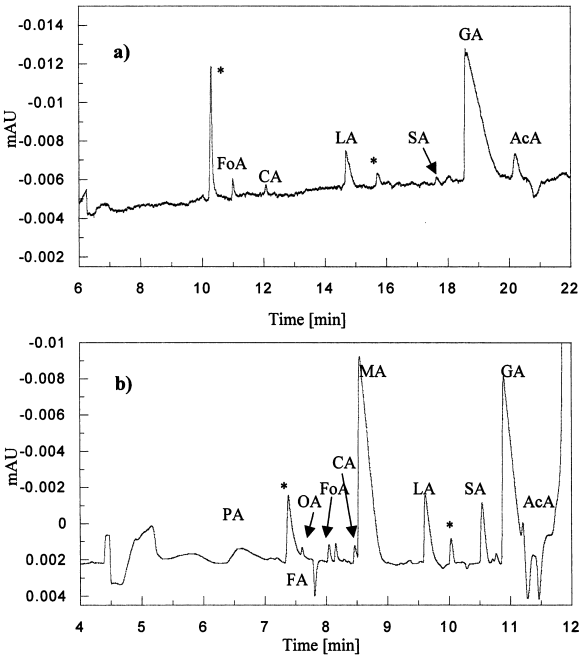

Fig. 3. CE with indirect UV detection of bacteria *Bacillus subtilis* cell extracts from: (a) glucose and (b) malate, as culture medium. Experimental conditions are the same as in Fig. 2 except for applied voltage −30 kV in Fig. 3b. Samples: PA, pyruvic acid; OA, 2-oxoglutaric acid; FA, fumaric acid; FoA, formic acid; CA, citric acid; MA, malic acid; LA, lactic acid; SA, succinic acid; GA, glutamic acid; AcA, acetic acid; *, unidentified peaks. The concentrations corresponding to the peak areas are given in Table 2.

on malate as a carbon source influences the presence of other carboxylic acids from the TCA cycle. The method proves its utility in investigation of effects of environmental conditions (e.g., culture medium or carbon source) on metabolite profiling. It can be of utmost importance if one remembers that bacteria *B. subtilis* are used for the production of several industrial enzymes.

3.3. Reproducibility, linearity, accuracy and limits of detection (LODs)

Usually sample introduction in CE is accomplished by the electrokinetic method or the hydrodynamic method. With the electrokinetic injection the amount of sample injected into the capillary depends on the electric field strength between the electrode and the inlet end of the capillary. In fact, many parameters of sample matrix such as pH, ionic strength, dielectric constant, viscosity, and temperature affect electroosmotic flow and electrophoretic mobilities of ions. Therefore, it was important to verify reproducibility of an established method for further quantitative analysis. Table 1 presents the reproducibility data obtained for migration time, corrected peak area and peak height as reflected by relative standard deviation (RSD). Good reproducibility of migration time indicates proper choice of BGS and analytical conditions. The RSD for the

Table 1
Calibration line equations, limit of detections (LODs) and relative standard deviations (RSDs in %, $n = 6$) obtained for carboxylic acids with indirect UV detection and electrokinetic injection mode

Name	Calibration line		Detection limits ($S/N = 3$)		RSD (%, $n = 6$)		
RSD (%, $n = 6$)	$y = ax + b$	R	LOD (M)	LOD (ppb)	Migration time	Corrected peak area[a]	Peak height
Pyruvic acid	$y = 0.162x - 0.07$	0.996	2.52×10^{-7}	22	0.7	4.7	3.6
2-Oxoglutaric acid	$y = 0.159x + 0.003$	0.997	2.56×10^{-7}	37	1.0	4.9	3.5
Fumaric acid	$y = 0.586x + 0.017$	0.998	2.15×10^{-7}	25	0.7	1.0	5.3
Formic acid	$y = 0.153x + 0.091$	0.994	2.67×10^{-7}	13	0.8	4.7	5.6
Citric acid	$y = 0.331x + 0.04$	0.994	1.23×10^{-7}	24	0.5	4.7	4.2
Malic acid	$y = 0.247x + 0.043$	0.999	1.65×10^{-7}	22	0.7	4.0	4.9
Isocitric acid	$y = 0.710x + 0.034$	0.997	1.77×10^{-7}	34	0.7	5.8	4.4
Lactic acid	$y = 0.068x + 0.239$	0.995	5.96×10^{-7}	54	1.1	3.1	3.6
Succinic acid	$y = 1.127x - 0.456$	0.998	1.12×10^{-7}	13	1.2	6.5	3.0
Glutamic acid	$y = 0.178x + 0.088$	0.994	2.29×10^{-7}	34	1.2	3.9	5.1
Acetic acid	$y = 0.147x + 0.047$	0.998	2.77×10^{-7}	17	1.3	6.8	8.8

[a] Corrected peak area = peak area/migration time.

migration times with six consecutive injections (n = 6) are in the range from 0.5 to 1.3%. The RSDs for the corrected peak areas and peak heights are in the range of 1.0–6.8 and 3.0–8.8%, respectively. Concentration of sample during reproducibility studies was 0.3 ppm and CE analyses were performed with electrokinetic injection (10 kV in 10 s) mode. Electrokinetic injection is generally considered as a less reproducible injection method in terms of electrophoretic parameters than pressurized one.

The linearity of the presented method was investigated by analyzing standard solutions containing a mixture of carboxylic acids at six known concentrations ranging typically from 50 to 0.5 μM. From a previous report [13] it is known that using CE indirect UV detection, the linearity of calibration lines can be obtained in only two orders of magnitude. In the studied concentration scale of analytes the linearity of calibration lines were sufficiently good and correlation coefficients range from 0.994 to 0.999 (Table 1).

The accuracy was determined by addition of known amounts of arbitrary selected five carboxylic acids (fumarate, citrate, malate, succinate, glutamate) to the cell extract with glucose as a carbon source. After five replicates were obtained, mean recovery were found to be: 95.2% for fumarate, 99.7% for citrate, 102.9% for malate, 97.2% for succinate and 87.4% for glutamate.

Studies of detection limits were investigated using both electrokinetic and pressurized injection modes. Using electrokinetic injection (10 kV in 10 s), larger sensitivity enhancements could be obtained than with pressure injection (4 s). For all carboxylic acids extrapolated LODs with electrokinetic injection (Table 1) were in the range from $1.12 \cdot 10^{-7}$ to $5.96 \cdot 10^{-7}$ M (13–54 ppb) ($S/N = 3$). Using pressure injection LODs for all analytes were found in the range of 10^{-5} M (1–5 ppm). The differences in extrapolated LODs are of two orders of magnitude to the advantage of electrokinetic injection mode. The sensitivity enhancement during electrokinetic injection could be explained by stacking effects. Using electrokinetic injection in a low conductivity sample solution (e.g., water) larger amounts of high mobility ions can be concentrated effectively due to stacking effect [21,22]. In pressure injection mode limitations for concentration of analytes is the amount of sample solutions that could be injected into the capillary. With sufficiently long injection time the analytes become overloaded and instead of increase of sensitivity there is a loss of resolution.

3.4. Estimation of carboxylic acids concentration in bacteria cell extracts

Table 2 presents the data of measured concen-

Table 2
Concentrations of carboxylic acid metabolites in cell extracts and in the single bacterium cell from different culture media (malate and glucose) after CE with indirect UV detection and electrokinetic injection

Carboxylic acid	Concentration of metabolite in malate culture medium (μM)	Concentration of metabolite in bacterium cell from malate culture medium (mM)	Concentration of metabolite in glucose culture medium (μM)	Concentration of metabolite in bacterium cell from glucose culture medium (mM)
Pyruvate	7.88	9.5	nd*	nd
2-Oxoglutarate	2.19	2.6	nd	nd
Fumarate	2.11	2.5	nd	nd
Formate	0.59	0.7	1.25	1.5
Citrate	0.46	0.6	0.49	0.6
Malate	53.93	64.8	nd	nd
Lactate	58.86	70.7	49.62	59.6
Succinate	6.16	7.4	0.71	0.9
Glutamate	56.78	68.2	133.09	160.0
Acetate	5.41	6.5	6.46	7.8

nd–not determined.

trations of carboxylic acids in *B. subtilis* cell extracts. There is a significant difference in numbers of identified acids in bacteria cell extracts from culture media with glucose and malate as a carbon source. The reason for different content in extracts is due to a carbon source. Malate has been a part of the TCA cycle so cell extracts from this culture are more rich in TCA carboxylic acids than one from glucose culture. The carboxylic acids from other than TCA metabolic pathways like glycolysis (pyruvate, lactate, acetate), urea cycle (formate) or metabolism of amino compounds (glutamate) [6] are not significantly affected by the carbon source (malate or glucose) in the culture media. Their quantitative levels are very similar independently from the carbon source with the exception of pyruvate not detected in glucose culture media. Using established indirect UV method and electrokinetic injection six carboxylic acid metabolites were identified and determined including two acids from the TCA cycle (citrate and succinate) in cell extracts from a glucose culture media. The concentration of carboxylic acids were in the range from 0.49 μ*M* for citrate to 133.1 μ*M* for glutamate. In cell extracts from a malate culture media ten carboxylic acids were identified and quantitated including five acids from the TCA cycle. Besides citrate and succinate, which were also present in cell extracts from a glucose culture media, there were also determined 2-oxoglutarate, fumarate and malate. The concentrations of carboxylic acid metabolites in malate culture medium were in range from 0.46 μ*M* for citrate to 58.9 μ*M* for lactate.

Based on the number of bacteria cells per ml of culture medium ($4 \cdot 10^8$ cell/ml) and considering the total volume of the cells in 1 ml medium as 0.83 μl, the concentrations of carboxylic acids per single bacteria cell was estimated (Table 2). The concentration of acids in the single bacterium cell were at m*M* to sub-m*M* range. The highest concentrations in the cells were found for lactic and glutamic acids in both malate and glucose culture medium, 70.7 and 68.2 and 59.6 and 160 m*M* respectively. In malate medium high concentrations in the cells presented also malic acids 64.8 m*M*. Concentrations of other carboxylic acid metabolites in the single bacterium cell from the malate culture medium were in the range from 0.6 to 9.5 m*M* for citrate and pyruvate, respectively. Estimated concentrations of acetate, formate, succinate and citrate in the single bacterium cell from the glucose culture medium were 7.8, 1.5, 0.9, and 0.6 m*M*, respectively.

Due to the nature of electrokinetic injection which introduces compounds based on their electrophoretic mobilities and EOF into the capillary it is impossible to know the exact amount of the injected analytes. Sample matrix differences in ionic strength, pH, viscosity, dielectric constant, temperature as well as variable field strength due to slight changes in the distance between the electrode and the inlet end of capillaries affects the total amounts of injected compounds. This means that, particularly in the case of biological samples, sample matrix effects and competition for current makes quantitative analysis difficult. Although, based on properly obtained calibration lines the relative concentrations of the analytes in the sample solution can be obtained. Good linearity of the calibration lines suggests high reproducibility thus allowing us to conclude on relative concentrations of carboxylic acid metabolites in different culture media. However, one still needs to remember that due to the sample matrix effects measurements of one cell extract content versus another one with electrokinetic injection mode carry a risk of inaccuracy.

4. Conclusion

The established CE method with indirect UV detection proves its utility for analysis of carboxylic acid metabolites from TCA cycle, glycolysis, urea cycle and amino compounds metabolism. Using 2,6-pyridinedicarboxylic acid as a carrier electrolyte (probe) and with electrokinetic injection mode sensitive analysis of TCA metabolites in *B. subtilis* cell extracts was achieved. Extrapolated LODs of carboxylic acid metabolites with electrokinetic injection were between 0.1 and 0.5 m*M*. In conformity to a previous report [13] it was demonstrated that with electrokinetic injection method achieved LODs can be at least two orders of magnitude lower than those after pressure injection. Additionally, low pH (3.5) of the BGS allows for the separation of two citric and isocitric acids which is rather difficult in CZE in higher pHs due to similar ionization states. The optimized method demonstrated acceptable linearity

and reproducibility. RSDs of individual acids corrected peak area and peak height needs more improvement. Average RSDs for eleven analytes of corrected peak areas and peak heights were 4.55 and 4.73%, respectively. An established method founded an application to analysis of cell extracts from *B. subtilis*. The determination of average carboxylic acid metabolites concentrations from about $4 \cdot 10^9$ bacteria cells is of unquestionable informative value. The relative concentrations of carboxylic acid metabolites in the single bacterium cell were estimated at m*M* and sub-m*M* level. Concentrations of intracellular metabolites were determined to be different in cell extracts derived from glucose or malate culture media. The differences in relative concentrations of carboxylic acids were more pronounced in the case of metabolites from the TCA cycle than in the case of metabolites from other biochemical pathways. This observation highlights the role of environmental conditions (e.g., culture medium or carbon source) on intracellular metabolite levels and metabolite profiling. Despite the fact that *B. subtilis* is a bacteria commercially used for the production of several important industrial enzymes, it's metabolome relationships to the genome and the environment are rather poorly studied. Further investigations using CE and sensitive detection schemes on other classes of metabolites present in *B. subtilis* are needed for better understanding of complex cellular processes.

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