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High-performance anion-exchange chromatography using on-line electrolytic eluent generation for the determination of more than 25 intermediates from energy metabolism of mammalian cells in culture

Joachim B. Ritter[a,*], Yvonne Genzel[a], Udo Reichl[a,b]

[a] *Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering, Sandtorstr. 1, 39106 Magdeburg, Germany*
[b] *Otto-von-Guericke-Universität Magdeburg, Lehrstuhl für Bioprozesstechnik, Universitätsplatz 2, 39106 Magdeburg, Germany*

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Abstract

In this work, we present an improved method for the determination of a wide range of intracellular metabolites from mammalian cells by anion-exchange chromatography. The analysis includes the measurement of intermediates from glycolysis and tricarboxylic acid cycle as well as several additional nucleotides and sugar nucleotides. The use of an electrolytic on-line eluent generation device made the method highly convenient, reliable and prone to errors. Due to short delay times of the eluent generator, rapid KOH gradient changes could be applied to improve separation and to speed up elution. Suppressed conductivity and UV in series was used for detection. The detection wavelength of the UV detector was switched from 220 to 260 nm during the elution for a more selective signal depending on the absorption of analytes. Standards from more than 50 metabolites of major cellular pathways were chromatographically tested and compared to chromatograms from extraction samples of Madin–Darby canine kidney (MDCK) and BHK21 cells. A validation for most substances was performed. Detection limits were below the micromolar range and the coefficient of correlation (R^2) was above 0.99 for most analytes. Working ranges were between 0.125–3.875 and 4.5–139.5 μM. Sample pH had a major impact on the quantification of several metabolites, but measurements were robust within a pH range of 6.5–9.0.

Keywords: Anion-exchange chromatography; On-line eluent generation; Metabolism; Tricarboxylic acid cycle; Glycolysis; Organic acids; Nucleotides; Sugar nucleotides; Sugar phosphates; Mammalian cells

1. Introduction

Energy metabolism of mammalian cells in culture and its regulation are still far from being completely understood. Scientists from various fields, including systems biology, mathematical modeling or biotechnology, are focusing on central metabolic pathways today. Standard monitoring methods include the analysis of extracellular metabolites like the waste products lactate and ammonia as well as the substrates glucose and glutamine [1]. Furthermore, amino acids, supplemented to the media, might be consumed or released by the cells; therefore, monitoring of amino acids in culture broth has become a common analytical technique [2].

Additional knowledge about the physiological conditions of cells in culture could be gained by analyzing intracellular metabolite pools. Many different methods have been applied for the analysis of intracellular metabolites, e.g. capillary zone electrophoresis or gas chromatography. In the following the focus will be on methods using liquid chromatography and especially anion-exchange chromatography. In general, two different approaches have been followed. One was the analysis of intracellular nucleotides and the other the investigation of pathway intermediates of glycolysis and tricarboxylic acid cycle. The first approach, the analysis of nucleotides had often the goal to gather information about the influence of nucleotides on the regulation

Abbreviations: cAMP, cyclic adenosine monophosphate; DHAP, dihydroxyacetone phosphate; F1,6bP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; 2PG, D-glycerate 2-phosphate; 3PG, D-glycerate 3-phosphate; R5P, ribose-5-phosphate; S1,7bP, sedoheptulose-1,7-bisphosphate; UDP-GalNAc, uridine diphosphate *N*-acetyl galactosamine; UDP-GlcNAc, uridine diphosphate *N*-acetyl glucosamine

* Corresponding author. Tel.: +49 391 6110 227; fax: +49 391 6110 638.
E-mail address: ritter@mpi-magdeburg.mpg.de (J.B. Ritter).

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of different physiological functions like activation and inhibition of enzymes. Many different organisms and cell lines have been under investigation. A comprehensive discussion on this topic is given in the Handbook of Chromatography [3]. More recent studies were done on, e.g. *Lactococcus lactis* [4], carcinoma cells [5,6], various normal and tumor cell lines [7], and epithelial cell lines [8].

The importance of nucleotides, their concentrations and ratios on regulatory processes in all species has been shown in many publications, e.g. [9–12]. Sugar nucleotides, moreover, may play a crucial role in both metabolism and glycosylation of proteins, especially for mammalian cells [9,10]. In combination with nucleotides, several sugar nucleotides have been detected and quantified in various organisms with different techniques [13–19].

Anion-exchange chromatography with UV detection was first used for the analysis of nucleotides by Cohn [20] and many applications followed, as described in reference [3]. Additionally to nucleotides, sugar nucleotides in insect and mammalian cells were analyzed by Tomiya et al. [18] using both anion-exchange and reversed-phase chromatography combined with UV detection.

The second approach was focused on pathway intermediates of the carbon metabolism. Either sugar phosphates mostly from glycolysis [21–25] or organic acids from the tricarboxylic acid cycle as well as pyruvate and lactate [26–28] were of interest. Sugar phosphates, for example, were measured using an anion-exchange chromatography with pulsed amperometric detection in *Saccharomyces cerevisiae* [22] and *L. lactis* [21]. More metabolites were measured by van Dam et al. [24] for *S. cerevisiae* using anion-exchange chromatography (AEC) connected to an MS detector.

The separation of organic acids from the tricarboxylic acid cycle and inorganic anions can be achieved by ion-exchange chromatography, even though the detection might be difficult compared to nucleotides, because many organic acids show only little or no UV absorption, which is a common detector for HPLC. A less selective detection compared to UV is conductivity. However, especially for gradient elution, suppression of the high background coming from eluents is necessary. The principle of suppressed conductivity detection was first applied by Small et al. [29], who were able to separate and detect a wide range of organic and inorganic ions by elution with sodium phenate using a stripper column. Rocklin et al. [30] separated 36 anions by gradient elution, using NaOH as eluent combined with suppressed conductivity detection. Different liquids, organisms and materials have been analyzed by anion-exchange chromatography combined with suppressed conductivity detection for their organic acid content, including, e.g. fruit juice [31], milk whey [32] and tobacco [33]. An overview of the separation of carboxylic acids by ion-exchange chromatography is given by Hajos and Nagy [34]. Lu et al. [26] analyzed CHO cell culture samples with regard to intracellular organic acids. They were able to measure 13 standard substances on an AS11-HC column with suppressed conductivity detection and a NaOH gradient, containing methanol for better separation of the target substances. Additional peaks appeared in sample runs compared to standards, but no obvious coelution occurred.

However, the question comes up, if the analysis of intracellular metabolites should be split in groups like organic acids, sugar phosphates or nucleotides, since they are mixed in one pool after extraction. Anion-exchange chromatography provides the opportunity to separate a wide range of negatively charged molecules, thus the analysis of organic acids, sugar phosphates, nucleotides and sugar nucleotides with one measurement should be possible.

Recently, Wittmann et al. [35] reported an adapted method of van Dam et al. [24] analyzing in addition to sugar phosphates five organic acids from the tricarboxylic acid cycle. For further insights into metabolism of *Escherichia coli*, Bhattacharya et al. [36] augmented the analysis to 27 anionic metabolites in the standard solution, including sugar phosphates, organic acids and nucleotides. For separation and detection, an AS11 column with a NaOH gradient, suppressed conductivity and UV detection was used. They could identify 16 metabolites in extraction samples, including adenosine-nucleotides, phosphorylated hexoses and organic acids from tricarboxylic acid cycle. In a similar approach, *S. cerevisiae* extracts were analyzed by Groussac et al. [37]. Here, organic acids, PEP and 6-phosphogluconate could be separated on an AS11 column after supplementing the NaOH gradient with 20% methanol. In addition, they analyzed 12 phosphorylated compounds on a CarboPac PA1 column with PA-detection. However, according to Vogt et al. [38], it is possible to analyze nucleotides and sugar phosphates also on an AS11 column. They investigated myocardial tissue concerning concentrations of metabolites from glycolysis and tricarboxylic acid cycle using a similar method as Bhattacharya et al. [36]. They were able to quantify 25 different metabolites, e.g. sugar phosphates, nucleotides and organic acids in an elution program of 70 min, even though most of the analytes eluted within a time window of approximately 20 min. As described above, anion-exchange chromatography has been applied for the quantification of pools of several intermediates from energy metabolism for yeast and bacteria but also for animal cells. Usually special target metabolites like organic acids or nucleotides have been selected. The goal of our work was to identify and quantify not only certain metabolite classes but the whole range of negatively charged energy metabolism intermediates in cell extracts from mammalian cells in culture. This chromatography should serve as a standard monitoring tool for bioprocesses using animal cell culture and therefore afford only minimum maintenance.

In this work, we present a simple and robust chromatographic method for the quantitative determination of intracellular metabolites for mammalian cells. The procedure is highly convenient and reliable due to an on-line electrolytic eluent generator [39,40] which simply requires water for eluent generation. A separation of more than 30 metabolites from central pathways including organic acids, phosphorylated sugars and nucleotides was accomplished in standard runs. For detection, suppressed conductivity and UV were used in series, which made it possible to distinguish between some coeluting substances. To our knowledge, such complex samples, including organic acids, sugar

phosphates, nucleotides and sugar nucleotides, have not been analyzed by anion-exchange chromatography combined with an on-line electrolytic eluent generation system yet.

2. Experimental

2.1. Chemicals

Chemicals were purchased from Sigma–Aldrich (Munich, Germany) with exception of F1,6bP, acetyl-CoA, succinyl-CoA (Fluka, Buchs, Switzerland) and NaCl, Na_2SO_4, $NaNO_3$, Na_2HPO_4, glutamic acid, HCl, perchloric acid, trichloroacetic acid, formic acid (Merck, Darmstadt, Germany) and MeOH (Roth, Karlsruhe, Germany). S1,7bP was synthesized by an aldolase reaction from E4P and DHAP according to Tsolas [41]. All samples and standards were diluted with 18 MΩ Milli-Q water.

2.2. Cell culture

Adherent Madin–Darby canine kidney (MDCK) cells were cultivated according to Genzel et al. [42]. Cells were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK; No. 841211903) and grown at 37 °C in GMEM (Invitrogen, Karlsruhe, Germany) supplemented with glucose (final concentration 5.5 g/l, Roth), 10% FCS (Invitrogen) and 2 g/l peptone (autoclaved 20% solution, International Diagnostics Group, Lancashire, UK) and 4.0 g/l $NaHCO_3$ (Merck). Cytodex 1 microcarrier (GE Healthcare, Uppsala, Sweden) concentration was 3 g/l. Cells were cultivated in spinner flasks (wv 200–250 ml, Wheaton, Millville, NJ, USA) and a stirring rate of 50 rpm.

BHK cells (BHK21C13-2P, ECACC No. 84111301) were cultivated in the same medium in suspension, additionally supplemented with glutamine (final concentration 4 mM) and cultivated in glass bottles (200 ml, Schott-Duran, Mainz, Germany) with a magnetic stirrer (50 rpm).

Both cell lines are common production cell lines for vaccines [42,43] and the knowledge of intracellular metabolite concentrations might help improving the corresponding production process.

2.3. Sample preparation

MDCK cells on microcarriers (5×10^6–10^7 cells) were allowed to settle in the extraction vessel, supernatant was removed and extraction liquid immediately added. BHK cells ($2–5 \times 10^6$ cells) were centrifuged after sampling for 3 min at $180 \times g$ at 0 °C, similar to Ryll and Wagner [15]. Subsequently, supernatant was removed and extractant added. To avoid changes in metabolite pools, cells were not washed but in one single step quenched and extracted. Samples were placed on ice directly after sampling for all further steps. In the following, samples were vortexed twice for approximately 5 s within 10 min and then centrifuged at 16,400 × *g* for 10 min at 0 °C to remove precipitates. Supernatant was transferred into a 2 ml vial, dried in a stream of air and stored at −80 °C until chromatographic analysis. Prior to measurement, samples were dissolved in 18 MΩ Milli-Q water, centrifuged at 16,400 × *g* for 10 min at 4 °C and the supernatant filtered (0.2 μm, Schleicher & Schuell, Dassel, Germany) into HPLC-vials. Standards were prepared by making a concentrated stock solution in Milli-Q water (between 1 and 100 mM) for each compound (stored at −70 °C). For the standard mixture (concentration between 25 and 800 μM), appropriate volumes of each analyte stock solution were pipetted in one vial, filled up to 50 ml and aliquoted. All samples and standards were stored at −70 °C.

2.4. pH-experiment

Since many extraction procedures include extreme pH values, stability of metabolites was examined in water and PBS at different pH values.

Therefore, the standard mixture was diluted in PBS of five different pH values ranging from 2 to 12 adjusted with 1 M KOH and 1 M HCl. Each pH sample was duplicated and samples were injected eight times within approximately 5 days in a repeating sequence.

Additionally, the standard mixture was diluted in Milli-Q water instead of PBS. Thirteen aliquots of 1 ml were prepared and the pH adjusted with 1 or 0.1 M NaOH for alkaline pH values and 1 or 0.1 M HCl for acidic pH values. A maximum volume of 8 μl was added. Each sample was injected six times. To avoid misinterpretation of the influence of unknown factors on chromatography, the sample sequence was randomized.

2.5. Equipment

All analytical runs were performed using a BioLC Type DX320 (Dionex, Idstein, Germany). The ion chromatographic system consisted of an AS50 autosampler and thermal compartment, an IS25 isocratic pump, an ATC-HC trap column (9 mm × 75 mm), an EG40 eluent generator including an EluGen EGCII-KOH cartridge and a degassing unit, an ATC-3 trap column (4 mm × 35 mm), an ASRS-ULTRA 2 mm self-regenerating suppressor, an ED50 detector in conductivity mode (ECD with typical background conductivity between 0.3 and 0.5 μS) and downstream of the ED50 an AD25 UV detector (UV) with a dead time of 0.18 min. The software Chromeleon 6.60 (Dionex) was used for chromatographic systems control, data acquisition and data analysis. The samples were prepared in standard 1.5 ml glass vials with pre-cut septa.

2.6. Chromatographic conditions

For the chromatographic separation an AG11 guard column (50 mm × 2 mm i.d.) and 2 AS11 analytical columns (250 mm × 2 mm i.d.) (Dionex) in series were used (see Koswig et al. [31]). A flow of 0.35 ml/min was maintained throughout all runs. Suppressor current was set to 70 mA. The thermal compartment was kept at 25 °C; the autosampler at 4 °C and the sample injection volume was 20 μl (sample loop size 25 μl, although the manufacturer recommended a maximum injection volume of 15 μl for this loop size). UV detector wavelength was set

either to 220 or 260 nm; details on the wavelength changes will be explained in Section 3.

3. Results and discussion

3.1. Standards and gradient development

The prerequisite for the quantification of intracellular metabolites was the identification of peaks, which was done in this study by comparison of retention times, UV signal and spiking of standards to samples. Because both composition and concentrations of the metabolites in the sample extracts could not be presented by standards with equal concentrations for all compounds, it was an iterative procedure to select an appropriate standard mixture of metabolites. To identify peaks in extraction samples and to decrease the risk of a coelution of an identified compound with an unknown metabolite, many potentially intracellular intermediates were chromatographically investigated. More than 50 different substances have been tested and most of the larger peaks from the extracts could be assigned as shown in Figs. 3 and 4. Table 1 summarizes the analytes tested. Additionally to the metabolites shown in Table 1, 16 amino acids were tested for the possibility to be analyzed with the used chromatography system; however, they could not be detected.

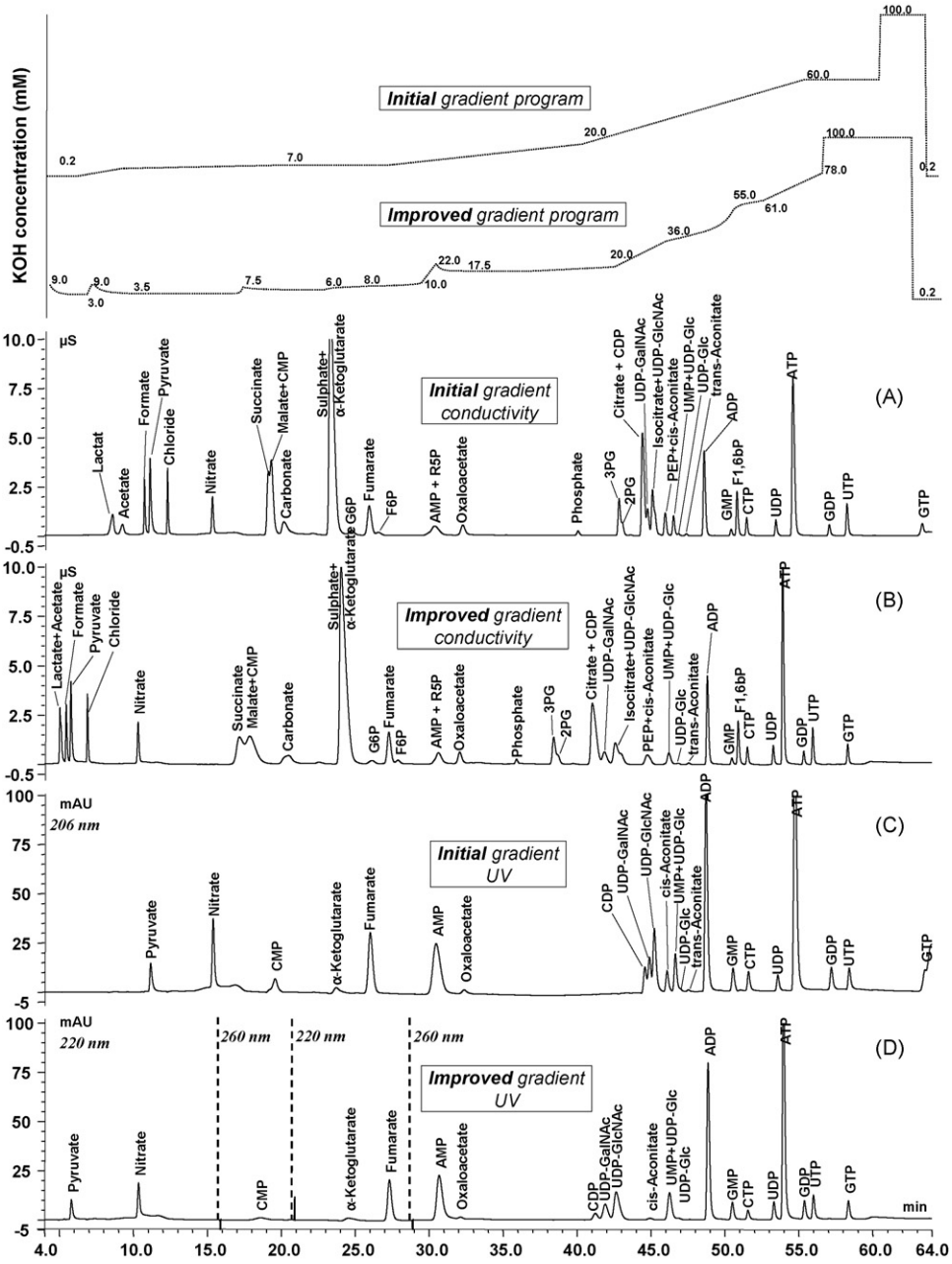

Fig. 1. Gradient programs and corresponding chromatograms of standard mixture (concentration of single components between 3.25 and 104 μM) for initial (A and C) and improved (B and D) gradients. (A and B) Conductivity detection; (C and D) UV detection; black vertical, dashed lines in chromatogram D indicate a wavelength switch to the wavelength on the right of the line and a following autozero of the detector.

Table 1
Summary of separation results for tested analytes (separation with improved gradient, see Table 2)

Analyte	Retention time[a]	S.D.[b]	UV[c] 220	UV[c] 260	Why not in stand.[d]	Coeluting peaks
2-PG[e]	38.80	0.07	n	n		3PG
3-PG[e]	38.60	0.07	n	n		2PG
Acetate[e]	5.15		n	n		Lactate, Hydroxybutyrate
Acetoacetate	5.30		y	?	co	
Acetyl-CoA	fragm		y	y	ns$^+$/nd	
ADP[e]	48.94	0.03	y	y		
AMP[e]	30.94	0.09	y	y		R5P
Asp	11.72		n	n	pq	
ATP[e]	54.01	0.02	y	y		
cAMP	16.25		y	y	nd	
CDP[e]	41.56	0.15	y	y		Citrate
Chloride[e]	6.92	0.01	n	n		
cis-Aconitate[e]	45.15	0.07	y	y		PEP
Citrate[e]	41.34		n	n		CDP
CMP[e]	19.26	0.20	y	y		Malate
CTP[e]	51.62	0.02	y	y		
dATP	53.70		y	y	nd	
dCTP	50.60		y	y	nd	
dGTP	58.00		y	y	nd	
DHAP	fragm		?	?	ns$^+$/nd	
dTTP	55.10		y	y	nd	
E4P	fragm		?	?	ns	
F1,6bP[e]	50.95	0.04	n	n		
F6P[e]	28.18	0.08	n	n		
FAD	fragm		y	y	ns/nd	
Formate[e]	5.50		n	n		
Fumarate[e]	27.52	0.07	y	y		
GAP	fragm		n	n	ns$^+$	
GDP[e]	55.42	0.02	y	y		
Glu	11.05		n	n	pq	
Glucose-6-P[e]	26.51	0.11	n	n		
GMP[e]	50.57	0.02	y	y		PPi
GTP[e]	58.40	0.02	y	y		
Hydroxybutyrate	5.18		n	n	co	Acetate, Lactate
Isocitrate[e]	42.96		n	n		UDP-GlcNAc
α-Ketoglutarate[e]	24.95	0.17	y	n		Sulfate
Lactate[e]	5.09		n	n		Acetate, Hydroxybutryate
Malate[e]	18.43	0.20	y	n		Succinate, CMP
NAD	fragm		y	y	ns$^+$/nd	
NADH	39.00		y	y	nd	
NADP	fragm		y	y	ns$^+$/nd	
NADPH	fragm		y	y	ns/nd	
Nitrate[e]	10.36	0.02	y	n		
Oxaloacetate[e]	32.22	0.06	y	y		
PEP[e]	44.97	0.08	n	n		*cis*-Aconitate
Phosphate	35.84		n	n		
PPi	50.50		n	n		GMP
Pyruvate[e]	5.81	0.01	y	?		
R5P[e]	30.88		n	n		AMP
S1,7bP	51.95		n	n	nd	
Succinate[e]	17.75	0.22	y	n		Malate
Sulfate[e]	24.43	0.21	n	n		α-Ketoglutarate
trans-Aconitate	47.61	0.04	y	y		
TTP	55.20		y	y	nd	
UDP[e]	53.36	0.02	y	y		
UDP-Gal	46.44		y	y	co	UMP, UDP-Glc
UDP-GalNAc[e]	42.26	0.14	y	y		
UDP-Glc[e]	43.01	0.15	y	y		UMP, UDP-Gal
UDP-GlcNAc[e]	46.44		y	y		Isocitrate
UMP[e]	46.44		y	y		UDP-Gal, UDP-Glc
UTP[e]	53.36	0.02	y	y		

[a] Average retention time (min) from validation for all validation runs (56), approximate retention time for not validated peaks (single measurement); fragm: more than one peak detected (fragmentation).

[b] Standard deviation (min) of retention time from validation for all validation runs (56).

[c] UV absorption at corresponding wavelength (nm): (n) no; (y) yes; (?) not tested.

[d] Reason, why not in the standard mixture: (nd) not detected; (ns) not stable under chromatographic conditions ((ns$^+$) not stable under alkaline conditions according to [44]); (co) coelution; (pq) poor quantification compared to other methods [46].

[e] Chemicals contained in the standard mixture.

Several metabolites were not stable under the eluting conditions (high pH values) used for chromatography, resulting in a fragmentation and by that, more than one peak. Bergmeyer [44] reported about instabilities of many of the metabolites under investigation, like NADH, FAD and GAP. In addition to the peak of *cis*-aconitate, a peak for *trans*-aconitate could be found, as it was already reported by Lu et al. [26]. It can be seen in Table 1 that UMP, UDP-Glc and UDP-Gal coeluted at the same retention time. It was not possible to distinguish between UMP and UDP-Gal, due to the exact coelution. Chromatography of UDP-Glc resulted in an additional, small peak, eluting shortly after the main peak. This peak allows the quantification of UDP-Glc separately, compared to UMP.

Lu et al. [26] kept the analysis of their extraction samples from CHO cells on the level of organic acids and did neither include phosphorylated sugars nor nucleotides. Since they did not use an additional detector (like UV) the risk of an undetected coelution of an organic acid with a metabolite not concerned was relatively high. We found that, e.g. UDP-GlcNAc and isocitrate as well as PEP and *cis*-aconitate coeluted. Groussac et al. [37] did not include nucleotides in their standard for the analysis on the AS11 column and thus, coelution of identified metabolites with not considered ones cannot be excluded.

During this work, the gradient was adjusted in several steps. Initial and improved gradient programs and corresponding chromatograms of the standard mixture are shown in Fig. 1.

The gradient was adjusted to the occurrence of peaks in samples empirically. In regions, where usually no peaks or large, not quantified peaks like chloride or phosphate appeared, the slope of the gradient was increased, whereas the separation of coeluting peak pairs was improved by decreasing the gradient in the corresponding regions. To accelerate the elution without deterioration of the separation of some peaks, a short KOH concentration increase was applied followed by a negative slope of the gradient and a subsequent plateau as it can be seen in Fig. 1. In chromatograms A (conductivity detector) and C (UV detector), peaks were eluted with the initial gradient, obtained from Dionex, a modified program from the AS11 column manual. Chromatograms B (conductivity detector) and D (UV detector) were achieved by applying the improved gradient including short KOH concentration peaks followed by a constant concentration plateau. Especially for the peak pair succinate/malate, a fast elution with satisfying separation was possible. The separation of succinate and malate is often discussed and sometimes accomplished by adding methanol to the eluent [26,36–38]. In our work, focus was put on an optimal resolution not only in the standard mixture but also in samples and spiked samples. Thus, the risk of misinterpretation of results because of coelution of known metabolites with not identified metabolites could be limited. Additionally, the cleaning step at the end of the elution program was elongated to assure regeneration of the column. Since columns were stable for our samples for approximately 2500 injections and different gradients were run during that time, usefulness of the longer cleaning step could not be demonstrated so far. Finally selected gradient conditions are summarized in Table 2. The total cycle time from one sample run to the next was 72 min. Due to the wide range of molecules with very different chemical properties, it was not possible to create a shorter chromatography run with a satisfying separation. With the use of an autosampler, the chromatography system can run 24 h a day and 7 days a week making the duration of one run acceptable. Despite the improved gradient, a complete separation of all identified metabolites could not be accomplished. However, by using two detectors it was possible to distinguish between some of the coeluting substances, due to their different absorption spectra for UV light. Many organic acids have a good absorbance around 220 nm in contrast to nucleotides, which in addition absorb selectively at wavelengths of about 260 nm. Using a wavelength of 220 nm at the beginning of the chromatographic run made it possible to detect early eluting substances such as pyruvate and nitrate with the UV detector. As it can be seen in Fig. 1, the first eluting nucleotide in the standard mixture was CMP, basically coeluting with malate. Both peaks could be quantified by changing the wavelength of the detector to 260 nm prior to elution, detecting specifically the nucleotide. Malate was analyzed from the conductivity signal. After detection of CMP, the wavelength was switched back to 220 nm to differentiate between the coeluting peaks sulfate and α-ketoglutarate. Sulfate was not detected in the UV channel. After elution of fumarate, a further wavelength change is performed to be able to detect nucleotides, eluting late, at their specific wavelength of absorbance (260 nm). 0.1 min after each switch of the wavelength an autozero for the detector was performed. Within this 0.1 min, usually a steep increase or decrease of the UV signal could be seen (see Figs. 3 and 4) due to the wavelength change. The first peak after the change was

Table 2
Improved gradient condition (see Fig. 1) for analysis of cell culture samples

Time (min)	KOH concentration (mM)	Curve[a]
0.0	0.2	5
4.0	9.0	7
6.5	3.0	1
6.8	9.0	5
7.0	9.0	5
9.5	3.5	2
16.0	3.5	5
17.0	7.5	9
22.5	6.0	1
25.0	8.0	3
29.0	10.0	7
30.0	22.0	5
32.0	17.5	1
36.5	17.5	5
42.0	20.0	5
45.5	36.0	5
50.0	55.0	7
52.0	61.0	3
56.0	78.0	5
56.1	100	5
62.0	100	5
62.1	0.2	5
68.0	0.2	5

[a] Shapes of gradient curves as defined in pumps operator's manual (Dionex, Idstein, Germany). Curve 5 is linear, whereas concave curves are represented by numbers >5 and convex curves by numbers <5.

AMP, which coeluted with ribose-5-phosphate, but in the UV detection, only AMP absorbed and could thus be quantified. By calculating the corresponding area of AMP for the conductivity channel from the data of the UV channel it was possible to quantify ribose-5-phosphate by subtracting the calculated area of AMP from the measured peak coming from AMP and ribose-5-phosphate in the conductivity channel ($Area_{R5P\_ECD} = Area_{AMP+R5P\_ECD} - Area_{AMP\_ECD\_calc}$; $Area_{AMP\_ECD\_calc} = Area_{AMP\_UV}/area\_ratio_{AMP}$; $area\_ratio_{AMP} = Area_{AMP\_UV}/Area_{AMP\_ECD}$; area_ratio has to be determined for the single component). The same strategy was applied for the coeluting pairs citrate/CDP, isocitrate/UDP-GlcNAc and PEP/*cis*-aconitate. A further advantage using 260 nm was a more stable baseline since many metabolites do absorb at 220 nm but not at 260 nm. In contrast to the improvement of the separation for most of the chromatogram, peaks in the beginning of the chromatogram were less well separated. The reason is that, as shown later, these peaks are mainly hidden behind matrix peaks in extraction samples. Thus, no focus was put on the separation of lactate, formate, acetate and pyruvate, but more on a faster elution program. Furthermore, lactate is usually measured by a bioanalyzer such as YSI model 2700 (Yellow Springs Instruments) or Bioprofile 100 Plus (Nova Biomedical). If interest is especially in early eluting analytes, a different gradient could be applied as, for example, shown by Genzel et al. [45] for the analysis of pyruvate.

3.2. Validation of chromatographic method

To investigate the quality of chromatography, a validation using the standard mixture (see also Table 1) in Milli-Q water was performed. Table 3 summarizes the results. Seven equidistant points were chosen for the calibration and each point was measured for eight independent standard dilutions in random order. As mentioned above, many analytes could only be quantified using one or the other detector channel, either because of no absorption in UV or coelution with a substance not absorbing in UV. Some analytes could theoretically be measured on both channels. The decision which signal had to be used was taken by looking at homogeneity of variances and R^2 values. Same criteria were applied to decide if peak area or height had to be used for calibration. If equal results were obtained, preferably UV detection and area were used. Variances of upper and lower range of quantification were *F*-tested ($P = 99\%$). If the variances were non-homogeneous, weighted regression was applied. Additionally, Mandel's fitting test ($P = 99\%$) was applied to decide about the type of regression (linear or polynomial second order). In cases where variances were non-homogeneous and a polynomial regression should be used according to the Mandel test, the range of quantification was reduced to avoid more complex matrix algorithms. The limit of quantification (=3.3 × standard error of the slope) was for most substances below the micromolar range. The lowest limit of detection was calculated to be 0.014 μM for AMP. The coefficient of correlation (R^2) was in most cases higher than 0.99 indicating a good fit for the regression. Several of the substances measured could certainly be validated for a wider range of concentrations. All nucleotides, for example, having similar properties like ATP, could probably be quantified in the same range as ATP, but due to their low concentrations in extracts from mammalian cells, there was no need for an extension of the range of quantification to higher concentrations. The use of 20 μl injection volume instead of a maximum of 15 μl as recommended by the manufacturer did obviously have no negative influence on the quantification.

Bhattacharya et al. [36] also show a table concerning the statistical analysis of chromatography. They found the lowest limit of detection to be at 15 pmol injected (chloride), corresponding to a concentration of 0.3 μM for an injection volume of 50 μl, which is lower than the limit of detection determined in our experiments (0.781 μM) for chloride. For several other metabolites, detection limits were clearly lower in our experiments, e.g. AMP. Moreover, it is difficult to compare limits of detection since it is not clear, how Bhattacharya et al. [36] determined these values. Also Vogt et al. [38] show limits of detection, defined as five times over baseline noise. Several standards have detection limits of 0.2 pmol (2 nM for injection volume of 100 μl), which is about 10 times lower than the lowest limit of detection in our work (AMP: 0.014 μM). But similar to Bhattacharya et al. [36], limits of detection of Vogt et al. [38] are spread over a wide range of concentrations going up to 100 pmol for adenosine nucleotides. Since their determination of limits of detection is different from the method used in our experiments, the value of this comparison is hard to assess.

3.3. Influence of sample pH on quantification

To test stability of metabolites in water and PBS at different pH values, standard mixtures were diluted in both solutions and different pH values adjusted. For most metabolites, no significant degradation could be seen within 4 days at 4 °C neither in PBS nor in Milli-Q water. The main exception was oxaloacetate, which, as expected [26,44], quickly degraded under any condition. Least degradation of oxaloacetate occurred at pH 12, still leading to 20% reduction after approximately 4 days at 4 °C in PBS. Although the stability of metabolites in our measurements could be seen, a dependency of peak area and height on sample pH was observed, independent of the detector used. The signal of many analytes under investigation, including inorganic ions like nitrate or sulfate, showed a clear decline with decreasing pH of the sample. Fig. 2 shows the average peak area of six injections versus pH of six different analytes as an example. ATP exhibited a strong dependence on pH resulting in more than a two-fold decrease in peak area at a pH around 2. At approximately pH 6.5, a maximum value was reached, which did not alter much at higher pH values. Less sensitive to pH was ADP, nitrate and F1,6bP with a constant area value above approximately pH 5. No influence could be seen on the peak area of AMP and succinate. For the other tested nucleotides, a similar pattern was observed. All triphosphates were affected most, followed by the diphosphates, whereas the peak area of monophosphates was almost stable over the tested pH range. Organic acids under examination were hardly influenced by the pH as the example of succinate shows. However, the peak area

Table 3
Validation data of chromatographic method

	Peak[a]	Detector[b]	Var.[c]	Reg.[d]	Limit of detection (μM)	n[e]	Range[f]		R^2[g] (μM)	Concentration (Fig. 1) (μM)
							Low (μM)	High (μM)		
Lactate + acetate	H	ECD	h	p	0.487	7	1.300	40.300	0.996	33.80
Pyruvate	A	ECD	nh	l	0.246	7	1.000	31.000	0.997	26.00
Cl	A	ECD	h	l	0.781	6	3.000	15.500	0.992	13.00
Nitrate	A	ECD	h	l	0.545	6	3.000	15.500	0.996	13.00
Succinate	A	ECD	h	p	0.188	6	3.000	15.500	0.999	13.00
Malate + CMP	A	ECD	h	p	0.149	6	8.250	42.625	0.999	35.75
CMP	A	UV	h	l	0.060	7	0.125	3.875	1.000	3.25
Sulfate + α-ketoglutarate	A	ECD	nh	l	0.827	7	4.500	139.50	0.999	117.00
α-Ketoglutarate	A	UV	h	l	0.623	6	3.000	15.500	0.995	13.00
G6P	A	ECD	h	l	0.221	6	1.050	5.425	0.996	27.30
Fumarate	A	UV	nh	l	0.133	7	0.500	15.500	1.000	13.00
F6P	A	ECD	h	p	0.018	6	1.050	5.425	0.998	4.55
AMP + R5P	A	ECD	h	p	0.185	6	6.000	31.000	0.999	26.00
AMP	A	UV	nh	l	0.014	6	0.750	19.500	0.999	19.50
Oxaloacetate	A	ECD	nh	l	0.100	7	0.500	15.500	0.989	13.00
3PG	A	ECD	nh	l	0.137	7	0.375	11.625	0.999	9.75
2PG	A	ECD	nh	l	0.101	7	0.125	3.875	0.997	3.25
Citrate + CDP	H	ECD	h	p	1.334	6	9.750	50.375	0.999	42.25
CDP	A	UV	nh	l	0.046	6	0.125	3.250	0.999	3.25
UDP-GalNAc	A	UV	nh	l	0.036	7	0.250	7.750	1.000	6.50
Isocitrate + UDP-GlcNAc	A	ECD	nh	l	0.215	7	0.625	19.375	0.999	16.25
UDP-GlcNAc	A	UV	nh	l	0.072	7	0.500	15.500	1.000	13.00
PEP + *cis*-aconitate	A	ECD	nh	l	0.109	7	0.250	7.750	0.999	6.50
cis-Aconitate	A	UV	h	l	0.044	7	0.125	3.875	0.999	3.25
UMP + UDP-Glc	A	UV	nh	l	0.046	7	0.375	11.625	1.000	9.75
UDP-Glc	H	UV	nh	l	0.137	7	0.250	7.750	0.999	6.50
trans-Aconitate	H	ECD	nh	l	0.108	7	0.125	3.875	0.995	3.25
ADP	A	UV	nh	l	0.132	7	1.000	31.000	1.000	26.00
GMP	H	UV	nh	l	0.019	7	0.125	3.875	0.999	3.25
F1,6bP	A	ECD	nh	l	0.143	7	0.375	11.625	0.999	9.75
CTP	H	UV	nh	l	0.038	7	0.125	3.875	0.999	3.25
UDP	H	UV	nh	l	0.055	7	0.125	3.875	0.999	3.25
ATP	A	UV	nh	l	0.249	7	1.250	38.750	1.000	32.50
GDP	H	UV	nh	l	0.045	7	0.125	3.875	0.999	3.25
UTP	A	UV	nh	l	0.043	7	0.250	7.750	1.000	6.50
GTP	A	UV	nh	l	0.026	7	0.125	3.875	1.000	3.25

Twenty microliters injection volume, eight injections of each standard.
[a] Data from peak area (A) or peak height (H).
[b] Detector signal used for validation.
[c] Variance for range of quantification: homogeneous (h) and non-homogeneous (nh).
[d] Regression used according to Mandel's fitting test: linear (l) and polynomial second order (p).
[e] Number of equidistant points for calibration (n).
[f] Range of quantification.
[g] Coefficient of determination.

of fumarate was significantly decreased at pH 2. Below pH 4, also the area values for the tested sugar nucleotides were lower. The large standard deviation at some pH values was traced back to an additional variation of peak area depending on the previous sample's pH. This means, if, by randomization, a sample of pH 6 followed a sample of pH 2, the resulting area was for the pH-sensitive substances clearly decreased. Taken together, many of the tested metabolites revealed a correlation between sample pH and peak area/height, especially for pH values below 4. It did not matter, if conductivity or UV detection was used. Jandik et al. [47] reported for the analysis of amino acids an influence of pH on peak shape, retention time and peak size, but in our case retention times and peak shape of the compounds were not or only slightly changed by pH variation. Furthermore, Jandik et al. [47] reported for some amino acids degradation associated with new peaks. For our experiments, degradation of these substances could be excluded, since no obvious additional peaks occurred in acidic samples and adjusting the pH from acidic to neutral conditions and a second measurement resulted in an increased peak area for all pH-sensitive substances (results not shown). In the following, no further effort was made to explain the phenomenon of the observed dependence of peak area to sample pH. Nevertheless, to assure reliable chromatographic results for the metabolites influenced by pH, sample pH should be above approximately 6.5. In addition it was helpful to keep the pH below a value of 9. Due to high pH values, carbonate dissolved

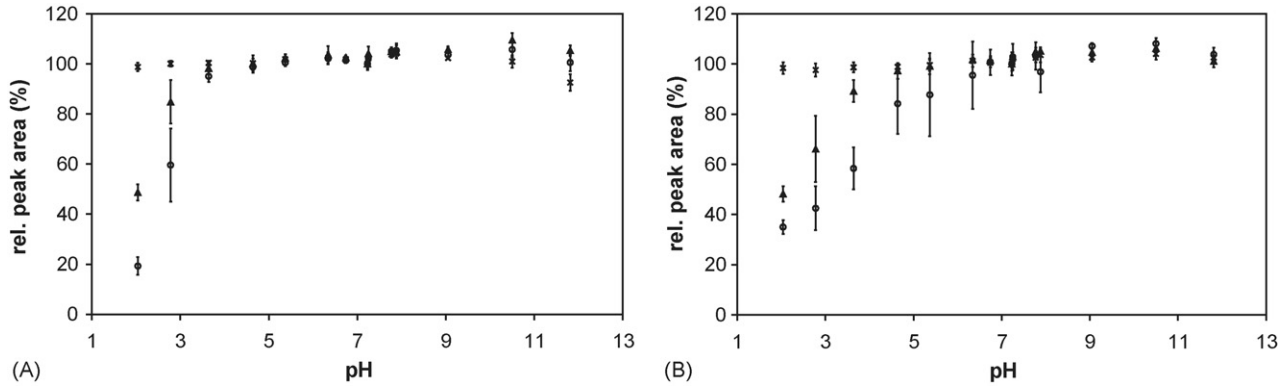

Fig. 2. Effect of sample pH on peak area of selected peaks from standard mixture (concentration of single components between 3.125 and 100 μM); (A) (×) succinate, (▲) nitrate and (◯) F1,6bP (conductivity detection used); (B) (×) AMP, (▲) ADP and (◯) ATP (UV detection used); error bars represent standard deviation of six measurements; relative peak area: area at pH 7.2 set to 100% (calculated from peak area relative to the peak area of the specific component at pH 7.2).

in the sample, leading to an increased peak, which coeluted with succinate, malate and CMP. Another possibility was to adjust sample and standard pH to exactly the same pH. This, however, was difficult for sample volumes after extraction of 600 μl or less.

3.4. Extraction samples

A wide range of different extraction procedures reported in literature were tested in preliminary experiments with both adherent MDCK and BHK suspension cells. Probably the most often applied extraction method for intracellular metabolites in literature uses perchloric acid [6,8,15,26,28]. The major drawback of this method in combination with anion-exchange chromatography is the contamination of samples with perchlorate ions, which cannot be removed completely by neutralisation with, e.g. KOH subsequently from the sample prior to injection and result in a large peak hiding several metabolites in the chromatogram using conductivity detection. Lu et al. [26] also used perchloric acid on an AS11 column, however in atypically low concentrations of 0.3 mM compared to at least 0.1 M used by other experimenters, e.g. [6,15,28]. When testing the procedure from Lu et al. [26] with our samples, no protein was precipitated, which made this method not applicable. The use of trichloroacetic acid as extractant was also not possible due to the large peak in the chromatogram at protein precipitating trichloroacetic acid concentrations. Further methods have been tested including boiling ethanol [18,24], acetonitrile [38] and formic acid [13]. Best results, meaning high concentrations of metabolites, have been achieved using a −70 °C methanol/formic acid mixture for MDCK cells. Highest metabolites levels for BHK cells were obtained by using methanol/tricine mixtures. An adaptation of the extraction method to the cell type used cannot be omitted. Furthermore, optimal extraction procedures for all metabolites are certainly difficult to find and due to the enormous extent of describing these procedures not further discussed here. As an example, Fig. 3 shows chromatograms for both detectors of an extraction of 10^7 MDCK cells.

Clearly, differences to standard runs can be seen (compared to Fig. 1B and D). Large matrix peaks, especially chloride and acetate (from extraction) at the beginning of the chromatogram, cover early eluting organic acids (only partially to be seen; <8 min). Additionally, retention times of some peaks, e.g. succi-

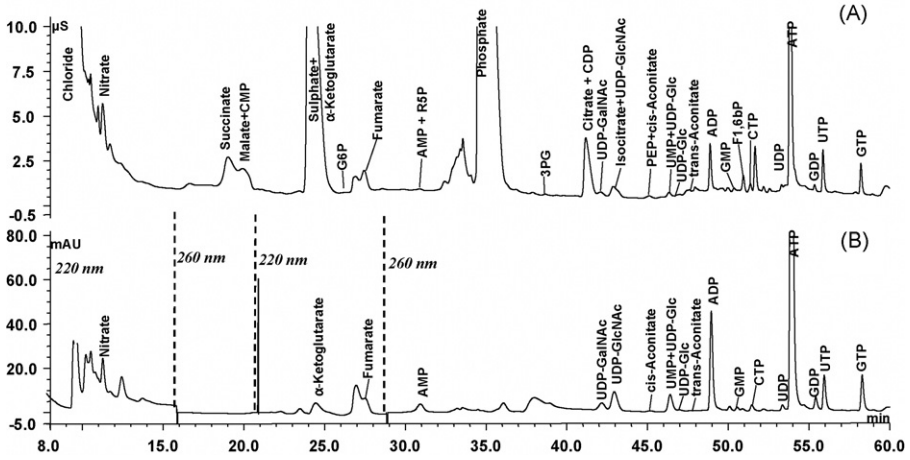

Fig. 3. Chromatogram of an extraction of 10^7 MDCK cells with methanol/formic acid using the improved gradient program. (A) Conductivity detection; (B) UV detection.

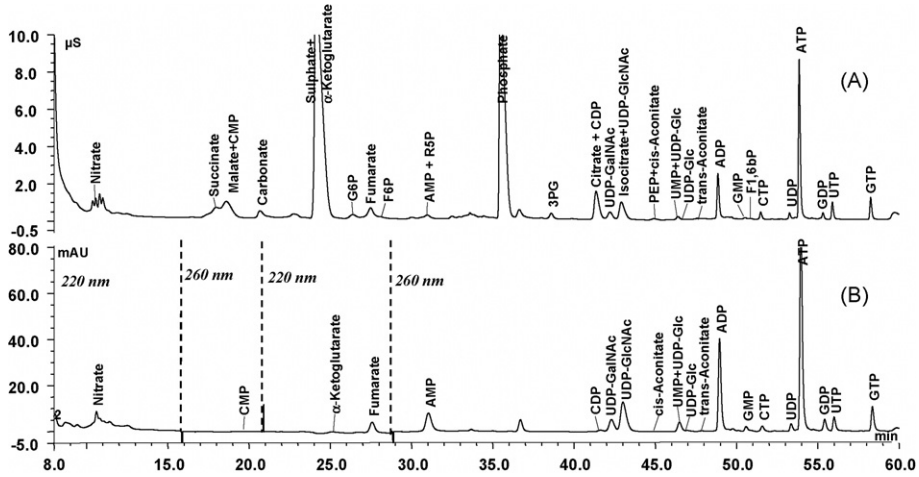

Fig. 4. Chromatogram of an extraction of 3.6×10^6 BHK cells with methanol/tricine using the improved gradient program. (A) Conductivity detection; (B) UV detection.

nate and malate, were shifted, which was also due to the overload of, e.g. the chloride peak. However, most of the peaks in the standard mix could be assigned to peaks occurring in the extraction sample. Fig. 4 shows an example of an extraction chromatogram of 3.6×10^6 BHK cells. Compared to MDCK cells, obvious differences, like larger matrix peaks for MDCK cells or different peaks or peak sizes, could be seen resulting probably also from the different cell numbers extracted. Further possible reasons are on the one hand real differences in metabolism of both cell types, but on the other hand, due to different extraction conditions. When comparing chromatograms of the standard mixture with chromatograms of extraction samples (Figs. 1–3), obviously not all metabolites added to the standard mixture could be found in the extraction samples. Especially, the early eluting organic acids (retention times <8 min) like lactate, pyruvate and acetate were difficult to determine, since both the chloride and formic acid peaks (from extraction of MDCK cells) reveal smaller peaks due to their overload (not shown). One possibility to quantify lactate, which is at high concentrations in the sample, would be to dilute the sample 100-fold with water improving the peak shape of early eluting peaks. Alternatively, an AS11-HC column could be used, as suggested by Lu et al. [26]. However, due to the higher back pressure of such a column (∼2300 psi for a single AS11-HC, data not shown) two serial AS11-HC columns could not be used, resulting in a deteriorated separation of later eluting peaks. Lu et al. [26] needed to supply methanol to the eluent to achieve baseline separation of succinate and malate. This was not possible with our method. Here only one eluent (water) had to be prepared, compared to four with the method of Lu et al. [26]; this might be a reasonable sacrifice. Furthermore, it is difficult to assess the quality of separation of samples including nucleotides and sugar phosphates using the gradient from Lu et al. [26]. The analysis of Tomiya et al. [18] covers a wide range of different nucleotides and sugar nucleotides, but besides the metabolites found in our work, only CMP-Neu5Ac additionally appears in cell extracts of mammalian cells (CHO) at higher concentrations. However, the occurrence of sugar nucleotides certainly varies for different cell lines and clones, also depending on the aim they are cultivated for. Compared to our results, Vogt et al. [38] obtained chromatograms where the majority of the analytes were eluted in a short time window of about 20 min probably complicating peak assignment and quantification. Moreover, they used a detection wavelength of 206 nm resulting in a relatively noisy baseline. Bhattacharya et al. [36] also used a relatively uncomplicated NaOH gradient for the elution of the samples. Similar to our experiments, early eluting peaks were hidden below large matrix peaks. In the later parts of the chromatograms, they could assign more than 15 peaks; however, many peaks could not be identified. Furthermore, due to the high peak density, the question arises if not some of the assigned peaks contain more than one metabolite. Unfortunately, Bhattacharya et al. [36] show no chromatogram of extraction samples recorded with the UV detector at 260 nm, which would give some more qualitative information. In our case, the evaluation of chromatography was clearly easier when the improved gradient was used, because peaks were more widely distributed over the chromatogram. Additionally, the switch of the detection wavelength between 220 and 260 nm during chromatography improved the identification and quantification of especially nucleotides. Groussac et al. [37] used two different anion-exchange chromatography systems to analyze the intracellular contents of organic acids, nucleotides, sugar nucleotides and sugar phosphates in yeast. However, with the method developed in our work, it was possible to measure all these intracellular metabolites simultaneously in one chromatographic run even below micromolar concentrations.

4. Conclusions

A highly convenient and reproducible chromatographic method was developed for the detection of a wide range of intracellular metabolites including tricarboxylic acid cycle as well as glycolytic intermediates and nucleotides. The use of an on-line electrolytic eluent generation system allowed the use of complex gradient programs clearly improving separation. By serial con-

ductivity and UV detection several coeluting peak pairs could be quantified. Changing the wavelength during a chromatographic run made it on the one hand possible to detect organic acids like α-ketoglutarate, pyruvate and fumarate and on the other hand quantify selectively nucleotides. More than 50 metabolites have been tested for their occurrence in extraction samples of mammalian cells, resulting in an optimized standard mixture with appropriate composition and concentrations for identification and quantification. A validation of the chromatographic method was carried out showing that detection limits are below the micromolar range and with most R^2 values higher than 0.99. Quantification of several metabolites was highly affected by sample pH, meaning that low pH values clearly decreased peak area and height for both detectors. However, an optimal pH range (pH 6.5–9.0) for the robust chromatographic determination of metabolites was identified. For the quantification of metabolites in extracts, an additional validation should be performed, adjusted to the procedure of extraction and cell type, since sample matrix effects on chromatography cannot be omitted.

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